Mucosal immune response after the booster dose of the BNT162b2 COVID-19 vaccine

Lorenzo Azzi,[a] Daniela Dalla Gasperina,[a] Giovanni Veronesi,[b] Mariam Shallak,[c] Vittorio Maurino,[d] Andreina Baj,[a] Francesco Gianfagna,[b,e] Pierpaolo Cavallo,[f,g] Francesco Dentali,[a] Lucia Tettamanti,[d] Fabrizio Maggi,[a] Lorenzo Stefano Maffioli,[h] Angelo Tagliabue,[a] Roberto Sergio Accolla,[c] and Greta Forlani[c,*]

[a]Azienda Socio-Sanitaria Territoriale dei Sette Laghi, Department of Medicine and Surgery, University of Insubria, Varese, Italy
[b]Research Centre in Epidemiology and Preventive Medicine (EPIMED), Department of Medicine and Surgery, University of Insubria, Varese, Italy
[c]Laboratory of General Pathology and Immunology "Giovanna Tosi", Department of Medicine and Surgery, University of Insubria, Varese, Italy
[d]Azienda Socio-Sanitaria Territoriale dei Sette Laghi, Department of Biotechnologies and Life Sciences, University of Insubria, Varese, Italy
[e]Mediterranea Cardiocentro, Naples, Italy
[f]Department of Physics, University of Salerno, Fisciano (SA), Italy
[g]Institute for Complex Systems, National Research Council, Rome, Italy
[h]Chief Medical Officer, Azienda Socio-Sanitaria Territoriale dei Sette Laghi, Varese, Italy

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Summary

Background To date, only a few studies reported data regarding the development of mucosal immune response after the BNT162b2-booster vaccination.

Methods Samples of both serum and saliva of 50 healthcare workers were collected at the day of the booster dose (T3) and after two weeks (T4). Anti-S1-protein IgG and IgA antibody titres and the neutralizing antibodies against the Wuhan wild-type Receptor-Binding Domain in both serum and saliva were measured by quantitative and competitive ELISA, respectively. Data were compared with those recorded after the primary vaccination cycle (T2). Neutralizing antibodies against the variants of concern were measured in those individuals with anti-Wuhan neutralizing antibodies in their saliva.

Findings After eight months from the second dose, IgG decreased in both serum ($T2_{GMC}$: 23,838.5 ng/ml; $T3_{GMC}$: 1473.8 ng/ml) and saliva ($T2_{GMC}$: 12.9 ng/ml; $T3_{GMC}$: 0.3 ng/ml). Consistently, serum IgA decreased ($T2_{GMC}$: 48.6 ng/ml; $T3_{GMC}$: 6.4 ng/ml); however, salivary IgA showed a different behaviour and increased ($T2_{GMC}$: 0.06 ng/ml; $T3_{GMC}$: 0.41 ng/ml), indicating a delayed activation of mucosal immunity. The booster elicited higher titres of both IgG and IgA when compared with the primary cycle, in both serum (IgG $T4_{GMC}$: 98,493.9 ng/ml; IgA $T4_{GMC}$: 187.5 ng/ml) and saliva (IgG $T4_{GMC}$: 21.9 ng/ml; IgA $T4_{GMC}$: 0.65 ng/ml). Moreover, the booster re-established the neutralizing activity in the serum of all individuals, not only against the Wuhan wild-type antigen (N = 50; INH: 91.6%) but also against the variants (Delta INH: 91.3%; Delta Plus INH: 89.8%; Omicron BA.1 INH: 85.1%). By contrast, the salivary neutralizing activity was high against the Wuhan antigen in 72% of individuals (N = 36, INH: 62.2%), but decreased against the variants, especially against the Omicron BA.1 variant (Delta N = 27, INH: 43.1%; Delta Plus N = 24, INH: 35.2%; Omicron BA.1 N = 4; INH: 4.7%). This was suggestive for a different behaviour of systemic immunity observed in serum with respect to mucosal immunity described in saliva (Wald chi-square test, 3 df of interaction between variants and sample type = 308.2, $p < 0.0001$).

Interpretation The BNT162b2-booster vaccination elicits a strong systemic immune response but fails in activating an effective mucosal immunity against the Omicron BA.1 variant.

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*Corresponding author. Laboratory of General Pathology and Immunology "Giovanna Tosi", Department of Medicine and Surgery, University of Insubria, Via O. Rossi 9, 21100, Varese, Italy.
E-mail address: greta.forlani@uninsubria.it (G. Forlani).

Keywords: BNT162b2 mRNA vaccine; COVID-19; SARS-CoV-2; Saliva; IgA

Research in context

Evidence before this study

On September 15th, 2022, we searched PubMed website for published peer-reviewed research articles written in English using the search terms "BNT162b2 vaccine", "salivary antibodies", and "mucosal immunity". Several studies have shown that BNT162b2 COVID-19 mRNA vaccine induces neutralizing antibody responses in healthy adults after the primary vaccination cycle and that the booster dose elicits a serologically detectable immune response that is even higher than the one recorded after the first two doses. However, only a few papers, including our previously published study, investigated the activation of a specific oral mucosal immunity after mRNA vaccination. They reported that 1–2 weeks after the primary vaccination cycle vaccinated subjects had S-protein IgG antibodies in their saliva, while IgA were detected in a substantial proportion, but the neutralizing activity in the saliva was low, especially in those individuals that had been not previously exposed to SARS-CoV-2 natural infection. To date, there are only few studies describing the oral mucosal immune response after the BNT162b2-booster vaccination. Assessing the mucosal immune response might provide data about vaccine efficacy against infection and viral spread. This issue is crucial due to the emerging role of the SARS-CoV-2 variants of concern, including the Omicron variant.

Added value of this study

This is one of the few reports assessing the development of anti-S1-protein IgG and IgA antibodies and the neutralizing activity against both the Wuhan wild-type Receptor-Binding Domain and the variants of concern (i.e., Delta, Delta Plus, and Omicron variants) in the saliva of individuals who underwent the BNT162b2-booster vaccination eight months after the completion of the primary vaccination cycle. The follow up of the very same individuals who had participated to our study previously published in this journal allowed us to track closely the development of mucosal immunity from the primary vaccination cycle to the booster dose in a highly homogeneous sample. Our results demonstrate that although salivary IgA titres increase at 8 months from the primary BNT162b2-vaccination cycle, the booster elicits an effective mucosal immune response against the Wuhan-wildtype antigen which is dependent on salivary IgG exuded from the serum rather than salivary IgA, confirming and further extending our previous findings. In addition, the booster dose evokes a strong systemic immune response against the variants of concern, but fails to induce an effective mucosal immune response against them. Of note, the neutralizing activity was dramatically reduced against the Omicron BA.1 variant.

Implications of all the available evidence

Together with the results of our previously published work, this study confirms that the intramuscular administration of the BNT162b2 vaccine elicits a strong systemic immune response, responsible for effective protection against severe outcomes of the disease. However, after the booster, the mucosal immunity against the Wuhan wild-type antigen in saliva relies on salivary IgG exuded from the serum rather than on locally produced secretory IgA. Moreover, the evoked mucosal immunity was insufficient to neutralize the Omicron BA.1 variant at the oral level, thus failing to limit virus entry through this route and, consequently, spreading of the infection. Distinct routes of immunization, such as the nasal or oral, might represent an innovative strategy for the delivery of booster doses of vaccine to increase oral and respiratory immunity against SARS-CoV-2, as, at these sites, the first contact with the virus takes place. Indeed, several reports using *in vivo* animal models indicate that the respiratory delivery (i.e., the nasal or oral route) of the COVID-19 vaccine may induce both systemic and mucosal immunity against the current and future SARS-CoV-2 variants.

Introduction

The authorized vaccines for COVID-19 have been widely used and allowed global health authorities to counter the pandemic and significantly reduce severe and deadly outcomes related to the infection, with a reduction of the adopted measures to limit viral transmission.[1] Both the BNT162b2 (Pfizer-BioNTech) and the 1273-mRNA (Moderna) vaccines, which encode the full-length viral Spike glycoprotein (S) ectodomain of isolated Wuhan-Hu-1 (GenBank accession number, MN908947.3), demonstrated high efficacy in preventing severe cases, hospitalizations, and related deaths.[2] The pivotal phase III clinical trial of the two-dose regimen of the BNT162b2 mRNA vaccine showed 95% efficacy in preventing symptomatic COVID-19, with onset at least 7 days after the second dose.[3] Administration of two mRNA vaccine doses has acted as a primary immunization, inducing a high neutralizing antibodies (NAb) titer, which, however, was not long-lasting. Indeed, several studies showed that vaccine effectiveness against symptomatic SARS-CoV-2 infection in the general population decreased to ~50% 6 months after the second dose.[4] Data from the National Israeli database demonstrated that the risk of infection also increased

considerably 6 months after vaccination.[5] However, although a significative reduction in efficacy over time was observed, this vaccine has proven effective against infection and hospitalization due to the Delta (B.1.617.2) variant[6] and against hospitalization for COVID-19 caused by the Omicron (B.1.1.529) variant.[7] This feature may partly be explained by the important role of cellular immunity in preventing severe outcomes in COVID-19 as a second-level defense against diverse variants.[8,9] Indeed, while a significant decrease was observed for memory B cells and NAb six months after the vaccination, the CD4+ and CD8+ T cell responses against the variants were preserved, including Omicron. This is substantially witnessed by the high rate of preserved functional T cell epitopes in the S-protein of the variants.[10] Waning immunity occurred to a variable extent for all vaccines studied to date, and loss of protection was likely amplified by increased prevalence of both Delta and Omicron variants.[11] With the appearance of these COVID-19 variants of concern (VOC) several countries have introduced COVID-19 vaccine boosters (i.e., third dose) to mitigate the pandemic. The booster dose of the BNT162b2 vaccine, administered 5–6 months after the second dose, reduced the rates of both infection and severe COVID-19 illness in the Israeli population older than 60 years, with a restoration of more than 90% effectiveness against severe COVID-19.[12] On this basis, the regimen of universal boosters 6 months after the second dose was proposed.[13] Globally, the booster was estimated to be 93% effective in preventing COVID-19-related admission to hospital, 92% in preventing severe disease, and 81% in preventing COVID-19-related death.[14] Effectiveness against infection after boosters was higher than 88.8%, supporting current policies in several countries. Moreover, receiving a booster dose of mRNA-based SARS-CoV-2 vaccination 6 months after the second dose restored vaccine effectiveness to higher levels than those seen 1 month after the primary vaccination cycle.[15]

Although several reports have assessed the strong serologically detectable immunogenicity driven by the booster and the generation of specific IgG NAb,[16] there are only few data available regarding the activation and/or boosting of specific mucosal immunity with this regimen. The findings that infection is strongly reduced in vaccinated people suggest that IgG NAb have reached mucosal sites.

The oral mucosa, the respiratory tract, and conjunctival surfaces represent the primary route of entry of SARS-CoV-2, thus COVID-19 vaccines inducing specific immune response at these sites might become a critical tool to limit viral spreading significantly.[17] Mucosal humoral immunity is mainly represented by secretory IgA (sIgA), which play an important role in host defense against respiratory pathogens.[18] Indeed, an effective mucosal immunity induced by the vaccine may preclude SARS-CoV-2 adhesion to target epithelial cells via neutralization of the coronavirus Spike protein, thus preventing not only the severe disease but also virus infection and circulation.

To date, few reports, including our previous study,[19] have shown specific IgG and, to a lesser extent, IgA in the saliva after the first cycle of BNT162b2 mRNA vaccination. These antibodies, however, did not display relevant neutralization activity, particularly in those subjects not previously exposed to natural SARS-CoV-2 infection.[20] Here, we assessed the duration of protection and the restoration of vaccine-driven mucosal immunity by measuring the level of total IgG and IgA antibodies (i.e., Ab) against the S1-protein both in serum and saliva, as well as the specific NAb against SARS-CoV-2 Receptor-Binding Domain (i.e., RBD).

This was performed eight months after the second dose and two weeks after the mRNA BNT162b2 COVID-19 booster. Knowledge about the development of a specific mucosal immunity following the BNT162b2-mRNA booster vaccination will certainly be useful for taking more appropriate decisions relating to the best immunization strategy. This has been particularly emphasized with the emergence of the Omicron BA.1 variant at the end of 2021,[21] and to the consequent pandemic wave associated with the highest peak of contagions recorded in Italy since the first outbreak of COVID-19.[22]

Methods

Study design and participants

We performed an observational cohort study recruiting 50 healthcare workers (HCW) of our hospital (ASST dei Sette Laghi) who underwent the BNT162b2 boosting vaccination by intramuscular injection (i.e., third dose) between October, 2021, and December, 2021 (i.e., eight months after primary vaccination cycle) to assess both the systemic and mucosal antibody response elicited by the vaccine. Of note, the subjects enrolled in this study were the same who voluntarily participated previously to our study on the primary vaccination cycle with the BNT162b2 COVID-19 vaccine recently published in this journal.[19] This was important to obtain a complete description of the mucosal immunity developed in the same individuals through the entire vaccination protocol. Therefore, inclusion criteria were age 18 years or older, being negative for SARS-CoV-2 infection as assessed by Nucleic Acid Amplification Test (NAAT) at the time of recruitment, and be willing to undergo the booster dose injection of the BNT162b2 vaccine. Exclusion criteria were glucocorticosteroid and/or immunosuppressant ongoing therapy, autoimmune disorders, and pregnancy.

Data were collected on the day of the booster (T3) and two weeks after the booster (T4), and were compared to those previously published at two weeks after the completion of the primary vaccination cycle (T2).

Blood samples were collected into a sterile 5 ml container with gel separator, and then centrifuged at 3,000 g for 5 min to obtain the serum fraction. Saliva was self-collected by spitting under the supervision of a trained provider, as previously described (Supplementary Appendix).[23]

To verify whether the recruited subjects had been exposed to SARS-CoV-2 infection during the interval between the primary vaccination cycle and the booster dose, anti-nucleocapside (N) chemiluminescent microparticle immunoassay (CMIA) testing was performed on serum samples collected at T3 (Supplementary Appendix, Alinity^{TM}, Abbott, cat. 06R90).

The following clinical data were recorded: age, sex, the exposure to SARS-CoV-2 infection in the period between T2 and T3 and/or after the booster shot until 31st January, 2022.

Ethics

The clinical protocol for sample and data collection was approved by the Institutional Ethics Committee (Comitato Etico dell'Insubria, n° 165/2020).

Antibody measurement

A commercial enzyme-linked immunosorbent assay (ELISA) specific for S1 protein of SARS-CoV-2 was used to measure both IgG and IgA Ab titres in serum and salivary samples according to the manufacturer's instructions (Anti-nCoV19 S1 IgG/IgA HS Immunospark, Pomezia, Italy, cat. ELVI021G and ELVI021A). Serum samples were analyzed at 1:100, 1:1,000, and 1:10,000 dilution at T3, while they were diluted at 1:1,000, 1:10,000, and 1:30,000 at T4. Salivary samples were analyzed at 1:1, 1:3, and 1:6 dilution at T3, and they were diluted at 1:3, 1:9, and 1:27 at T4. The results were expressed as ng/ml; the lower threshold established by the manufacturer was 0.625 ng/ml for the IgG Ab subtype and 0.0625 ng/ml for the IgA Ab subtype (Supplementary Appendix), as previously described.[19]

Furthermore, the presence of anti-RBD NAb in serum and saliva was assessed by competitive ELISA, following the manufacturer's instructions (cPASS™ SARS-CoV-2 Neutralization Antibody Detection Kit, GenScript, cat. L00847-C), as previously described.[19] Briefly, 10 µl of serum were diluted in 90 µl of sample dilution buffer, while the saliva was used undiluted. Positive and negative serum controls provided within the kit were used as reference for the serum NAb, while known positive and negative salivary samples were used as reference for the salivary NAb. The optical density (OD) average of the negative controls was used to calculate the percentage of inhibition according to the formula: (1 – OD value of the sample/OD value of negative control) × 100%. According to the manufacturer's instructions, a cut-off value of 30% was used to discriminate between the presence and absence of NAb. In the case of positivity, the percentage of inhibitory activity (i.e., INH) was also assessed.

Finally, in order to verify the efficacy of the mucosal immune response against the VOC, the serum and the salivary samples of those individuals who showed NAb in their saliva against the wild-type antigen at T4 (i.e., Wuhan) were also tested against the RBD of the Delta variant (lineage B.1.617.2; L452R, T478K, Genscript, cat. Z03614), the Delta Plus variant (lineage B.1.617.2.1; K417N, L452R, T478K, Genscript, cat. Z03690), and the Omicron BA.1 variant (lineage B.1.1.529; G339D, S371L, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, Genscript, cat. Z03730).

Statistical analysis

To account for their skewed distributions, at each visit time, we calculated sample medians and interquartile range (IQR) for serum and salivary IgG and IgA, in the overall sample and according to SARS-CoV-2 exposure prior to the first vaccination cycle (i.e., previous SARS-CoV-2 infection).

To estimate time trends in serum and salivary IgG and IgA, we used repeated-measure regression models, with time visit as independent variable. Again, we modelled log-transformed IgG and IgA values, and reported the geometric mean concentrations (GMC). We used an unstructured variance-covariance matrix, to allow a flexible intra-individual correlation between IgG and IgA at different times and robust standard error estimates (empirical GEE). At T3 and T4, differences in logarithmic IgG and IgA baseline at T2 with 95% confidence intervals (i.e., 95% CI) were estimated using SAS macro NLEstimate.[24] We reported the Pearson correlation coefficients between log-transformed serum and salivary IgG and IgA Ab at each time, as well as between salivary IgG and IgA Ab at T4.

Individuals were categorized according to the presence of NAb in saliva at T4 (Wuhan RBD). We summarized the distribution of serum and salivary IgG and IgA levels in the two groups using sample medians and IQR, and tested the null hypothesis of homogeneity in distributions using Wilcoxon rank tests. Finally, we tested whether the prevalence of NAb varied by sample type (i.e., serum or saliva) and virus variance (i.e., Wuhan wildtype, Delta, Delta Plus, and Omicron BA.1 strains), using a regression model with repeated-measure (unstructured variance-covariance matrix) and including an interaction term between sample and variant (3 degrees of freedom, Wald chi-square test).

Statistical analyses were carried out using the SAS Software (9.4 release), and pictures were drawn using R (3.6.3 version).

Role of funders
The study funders had no role in study design, data collection, data analyses, interpretation, or writing this report.

Results

Participants
Our previous findings clearly demonstrated that BNT162b2 mRNA vaccination induced a strong systemic response, but a weak mucosal response characterized by SARS-CoV-2 specific IgG and IgA in both serum and saliva.[19] Consistently, high levels of NAb were detected in serum, but only in saliva of the subjects previously exposed to SARS-CoV-2 natural infection. In order to evaluate the duration of the vaccine efficacy after the second BNT162b2 mRNA dose and the triggering of specific SARS-CoV-2 immunoglobulins both in serum and saliva after the booster, fifty HCW who had participated in our previous study were enrolled, and their total IgG and IgA were measured in serum and saliva fluids eight months after the second dose (T3) and two weeks after the booster (T4). Ten subjects of our previous report did not participate in the study because either they moved to different geographic regions or for personal decision to postpone the boosting dose (i.e., response rate 83.3%). There was not statistical difference between participants and dropouts as regards to age ($p = 0.54$), sex ($p = 0.78$), and the serological status before the primary vaccination cycle ($p = 0.86$) (Table S1).

The mean age of the study participants was 41.8 ± 10.4 years (range, 26–62 years) and 34 subjects (i.e., 68%) were female. Eight individuals were infected with SARS-CoV-2 before undergoing the primary vaccination cycle (i.e., seropositive subjects, SP) and 42 subjects were never infected (i.e., seronegative subjects, SN). All the recruited subjects were not infected with SARS-CoV-2 in the period between the primary vaccination cycle and the injection of the booster. Indeed, all individuals did not show serum anti-N Ab at T3, except a SP subject, renowned for having been infected before the first vaccination cycle, who showed a weak positivity. Two to four months after the vaccine booster, 8 SN subjects were infected with SARS-CoV-2 as assessed by NAAT, and none developed severe disease or required hospitalization, reinforcing the efficacy of the booster in preventing the severity of the disease. Of note, these cases of infection were diagnosed during the peak of the fourth wave of the pandemic in Italy, when the Omicron BA.1 variant superseded the other circulating variants.

BNT162b2-vaccine booster induced antibody response in both serum and saliva
IgG and IgA Ab titers in both serum and saliva were measured at the day of the booster (T3) and two weeks after the booster (T4), and they were compared to those previously reported two weeks after the second dose (T2, in this case set as the baseline). All the serum and salivary samples of the recruited subjects were collected and analyzed; thus, we did not have missing data. We observed that in the serum IgG dramatically dropped at eight months from vaccination ($T2_{GMC}$: 23,838.5 ng/ml; $T3_{GMC}$: 1473.8 ng/ml) (Fig. 1A, Table 1). The inferential statistical analysis (i.e., Δ and CI values) corresponding to all the data presented are specified in Table 1. A similar scenario was observed for serum IgA ($T2_{GMC}$: 48.6 ng/ml; $T3_{GMC}$: 6.4 ng/ml) (Fig. 1B, Table 1). The booster immunization elicited a significant increase of humoral immune response, with high levels of both serum IgG ($T4_{GMC}$: 98,493.9 ng/ml) (Fig. 1A, Table 1) and serum IgA ($T4_{GMC}$: 187.5 ng/ml) (Fig. 1B, Table 1). Of note, the titres of both IgG and IgA in the serum of vaccinated individuals at T4 were much higher than those found at T2 (Serum IgG $T4_{GMC}/T2_{GMC}$ 4.13; Serum IgA $T4_{GMC}/T2_{GMC}$ 3.86) (Table 1). At variance to previous observations showing a maximal level of serum IgG after the first dose in previously infected subjects (i.e., seropositive subjects, SP), no difference in the titers of Ab at T4 between naïve subjects (i.e., seronegative subjects, SN) and SP was observed (Serum IgG $p = 0.65$; Serum IgA $p = 0.30$).

To further characterize the mucosal immune response elicited by the BNT162b2 booster vaccination, we measured both S1-specific IgG and IgA titres in saliva. At T2 all vaccinated subjects showed the presence of salivary IgG, as previously described,[19] but at T3 a significant decline of humoral immunity was observed, and only 28 subjects (i.e., 56%) maintained specific IgG in the saliva. The reduction of salivary IgG titre from T2 to T3 ($T2_{GMC}$: 12.9 ng/ml; $T3_{GMC}$: 0.3 ng/ml) was similar to that observed for serum IgG, supporting the hypothesis that IgG are of serum origin. Consistently, after the booster, almost all individuals (i.e., 48/50) showed a significant increase in salivary IgG ($T4_{GMC}$: 21.9 ng/ml), with a mean titer higher than that measured at T2, albeit to a lesser extent than in serum (Salivary IgG $T4_{GMC}/T2_{GMC}$ 1.7) (Fig. 1C, Table 1).

By contrast, this was not true for the SARS-CoV-2 specific IgA response in saliva. At T2 only 50% of recruited individuals showed salivary IgA, and they were mainly exuded from the serum, as previously described.[19] However, differently from what was observed for IgG, at T3 the IgA titers were higher compared to those measured at T2, and SARS-CoV-2 specific salivary IgA were found in almost all subjects (i.e., 48/50 subjects) ($T2_{GMC}$: 0.06 ng/ml; $T3_{GMC}$: 0.41 ng/ml) (Fig. 1D, Table 1). The booster vaccination increased the amount of IgA in saliva ($T4_{GMC}$: 0.65 ng/ml) at levels higher compared to those at T2. Furthermore, the increasing rate of the salivary IgA was significantly higher than that observed for salivary IgG (Salivary IgA $T4_{GMC}/T2_{GMC}$ 10.8). As stated above for

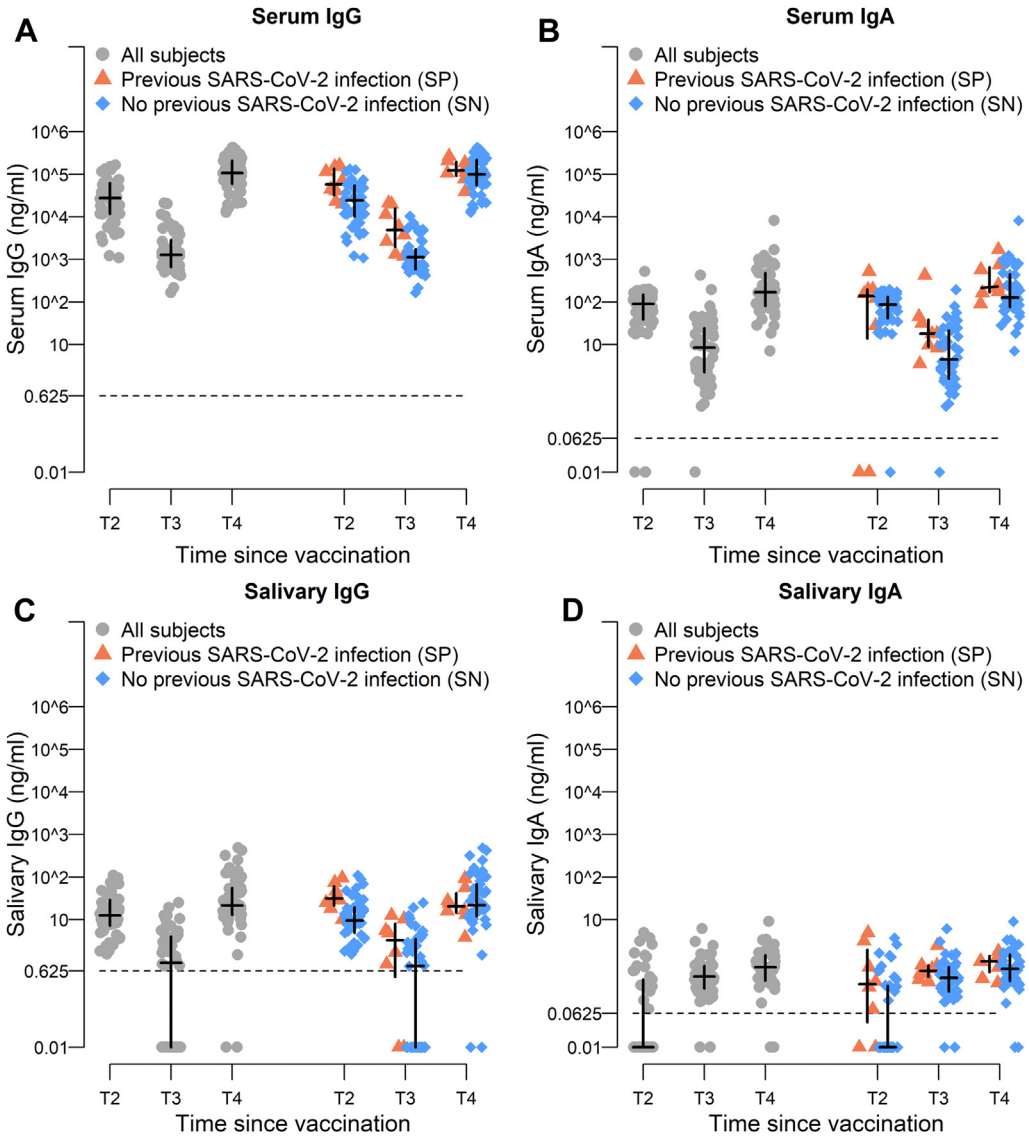

Fig. 1: Distribution of serum (panels A and B) and salivary (panels C and D) IgG and IgA Ab, at different times, for all the recruited individuals and according to exposure to SARS-CoV-2 infection before the primary vaccination cycle. In each group, the horizontal line represents the sample median, while the vertical line the interquartile range. T2 = 14 days after the second dose; T3 = day of the booster dose; T4 = 14 days after the booster dose. Statistical analysis was reported in Table 1.

serum Ab, we could not detect any differences in the immune response in the saliva between SP and SN subjects (Salivary IgG p = 0.97; Salivary IgA p = 0.63), supporting the notion that the booster triggers a specific and effective humoral immune response, regardless of previous natural infection (Fig. 1, compared orange and blue dots).

As shown in Fig. 2A, at T2 and after the booster (T4) we were able to detect IgG in saliva when its serum titer was higher than 10^3 ng/ml. These data strongly support the idea that IgG from the serum could permeate the saliva in a concentration-dependent way (Table S2). By contrast, the presence of IgA in saliva was not correlated to their titer in the serum, especially at T3 (Fig. 2B). These data suggest that salivary IgA at T3 were locally produced and not exuded from serum, indicating thus a delayed activation of local mucosal immunity after the primary vaccination cycle. The booster dose increased both serum and salivary IgA within two weeks, but the rapid increase of salivary IgA titres at T4 probably relied on transudation from serum, as observed during the primary vaccination cycle (Table S2).[19]

Variable, time	Mean[a]	Δ from T2 (IC 95%)	Δ from T3 (IC 95%)
Serum			
IgG, ng/ml[b]			
T2	23,838.5	–	–
T3	1473.8	−22,364.6 (−30,259; −14,470.3)	–
T4	98,493.9	74,655.4 (51,481.4; 97,829.3)	97,020 (71,696.3; 122,343.8)
IgA, ng/ml[b]			
T2	48.6	–	–
T3	6.4	−42.1 (−71.9; −12.4)	–
T4	187.5	138.9 (70.5; 207.4)	181.1 (115.7; 246.5)
Saliva			
IgG, ng/ml[b]			
T2	12.9	–	–
T3	0.3	−12.6 (−16.5; −8.7)	–
T4	21.9	9 (−1.7; 19.8)	21.6 (9.8; 33.4)
IgA, ng/ml[b]			
T2	0.06	–	–
T3	0.41	0.3 (0.2; 0.5)	–
T4	0.65	0.6 (0.4; 0.8)	0.2 (0; 0.4)

[a]Geometric mean concentration. [b]Change in geometric mean concentration, modelled through a log-linear regression model for repeated measures with unstructured variance-covariance matrix.

Table 1: **Time trends for IgG and IgA antibodies in both serum and saliva. N = 50 subjects.**

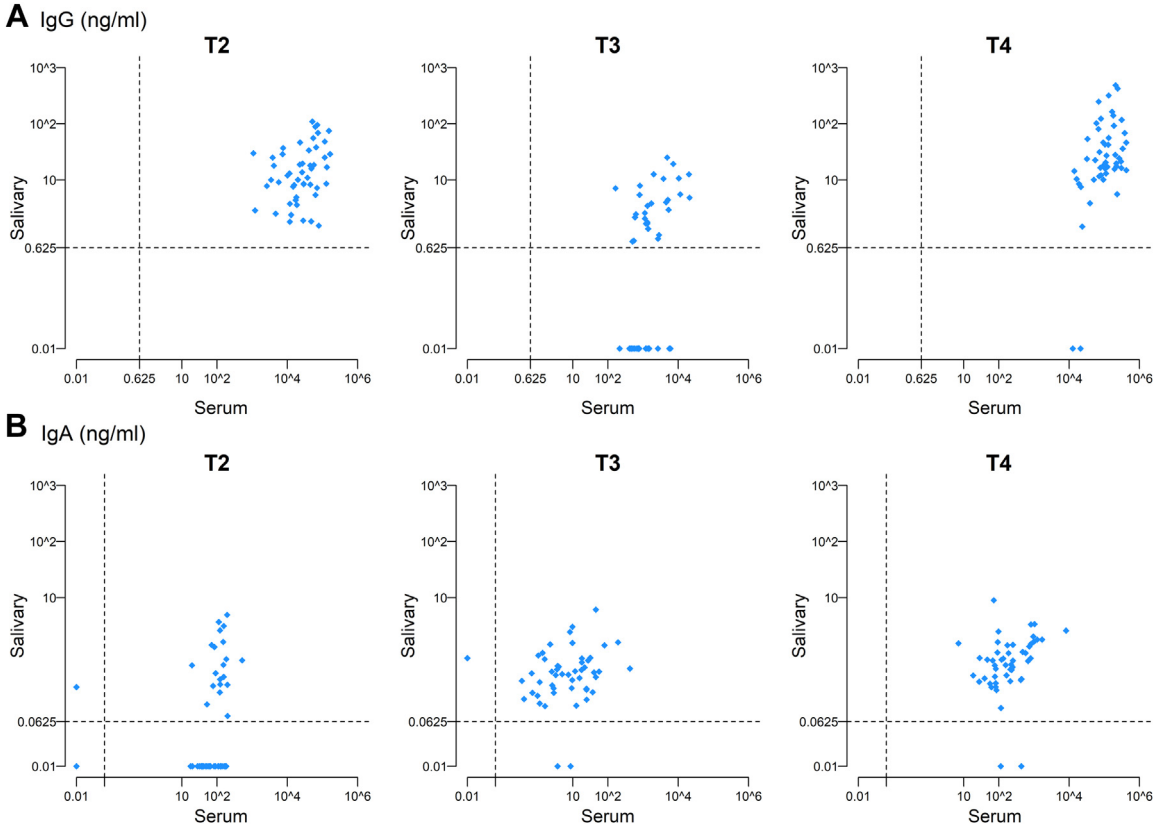

Fig. 2: Scatter-plot for salivary and serum IgG (panel A) and IgA (panel B) at different times. T2 = 14 days after the second dose; T3 = day of the booster dose; T4 = 14 days after the booster dose. Correlations between serum and salivary IgG Ab titres: T2: r = 0.295, p = 0.04; T3: r = 0.442, p = 0.0013; T4: r = 0.521, p = 0.0001. Correlations between serum and salivary IgA Ab titres: T2: r = 0.211, p = 0.15; T3: r = 0.172, p = 0.23; T4: r = 0.261, p = 0.07.

BNT162b2 vaccine booster elicits neutralizing activity in saliva against Delta and Delta Plus but not against Omicron BA.1 SARS-CoV-2 variants

Two doses of BNT162b2 vaccine elicit high levels of protection from symptomatic disease, but this wanes over time. The BNT162b2 booster immunization can restore effectiveness to more than 90%, eliciting a strong systemic neutralizing activity but does not significantly reduce viral spreading. Our previous findings demonstrated that the primary vaccination cycle elicited NAb in serum but not in saliva, suggesting that the lack of a sterilizing mucosal immunity might contribute to the circulation of the virus. To evaluate whether the limited capacity of the booster dose in counteracting the circulation of the virus might also depend on the lack of NAb at the mucosal sites, we analyzed the neutralization efficiency of the BNT162b2 booster vaccination against wild-type SARS-CoV-2 (i.e., Wuhan) both in serum and in saliva at T4.

By using a competitive ELISA assay, we previously demonstrated that at T2 all the 50 recruited subjects showed NAb in their serum (N: 50/50; INH: 87.2 ÷ 93.0%; $T2_m$ 91.7 ± 0.9%) (Table S3). However, at eight months after the administration of the second dose (T3), the level of inhibitory activity was strongly reduced (N: 47/50; INH: 32.8 ÷ 90.8%; $T3_m$ 68.2 ± 15.2%) (Table S3), confirming the waning of humoral immunity over time. The following booster immunization strongly restored INH in all the subjects at levels comparable to those measured after the second dose (INH: 90.2 ÷ 92.9%; $T4_m$ 91.6 ± 0.8%) (Table S3).

By contrast, in saliva only 28% (14/50) of the recruited subjects showed NAb at T2 (n: 14/50; INH: 30.3 ÷ 72.7; $T2_m$ 55.0 ± 14.0%), indicating a poor activation of oral mucosal immunity, as previously reported[19] (Table S3). At T3, the neutralization in saliva decreased. Indeed, only 6 subjects had NAb in the saliva and the INH was low (n: 6/50; INH: 30.3 ÷ 48.3%; $T3_m$ 37.7 ± 6.5%) (Table S3). Interestingly, the booster vaccination significantly elicited neutralizing activity in saliva. We detected NAb in 72% of the enrolled subjects (36/50) with higher levels of INH compared to those measured at T2 (INH: 32.1 ÷ 87.2%; $T4_m$ 62.2 ± 17.1%) (Table S3).

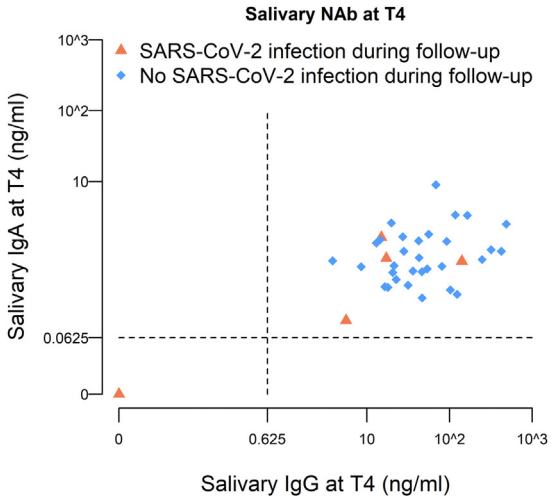

Fig. 3: Scatter-plot for salivary IgG ad IgA among individuals with positive salivary NAb (N = 36) at T4, according to exposure to SARS-CoV-2 during the follow-up after the booster dose. Spearman r: 0.523, p < 0.0001.

As shown in Fig. 3, in these 36 individuals the booster dose induced an increased amount of both salivary IgG and IgA at T4 mainly due to exudation from serum, as stated above (Spearman r = 0.523, p < 0.0001). However, the induction of neutralizing activity by BNT162b2 vaccine booster at T4 was mainly related to the presence of salivary IgG (NAb-negative subjects: salivary IgG median 11.6 ng/ml, IQR 10.1–16.3; NAb-positive subjects: salivary IgG median 39.3 ng/ml, IQR 17.6–97.6, p = 0.002) rather than of salivary IgA (NAb-negative subjects: salivary IgA median 0.6 ng/ml, IQR 0.4–1.1; NAb-positive subjects: salivary IgA median 0.8 ng/ml, IQR 0.5–1.5, p = 1.0) (Table S4).

This scenario was also reported in our previous study at the end of primary vaccination cycle (Fig. S1).[19]

Finally, we analyzed whether the neutralization efficiency of the BNT162b2 vaccine booster against Delta, Delta Plus, and Omicron BA.1 variants of concern was similar in serum and saliva in the subjects in which we found positivity for specific NAb against Wuhan wild-type SARS-CoV-2 in saliva (i.e., 36/50). In the repeated measure model, the interaction between the four viral strains and the two samples was statistically significant (Wald chi-square test with 3 df of interaction between VOC and sample type = 308.2, p < 0.0001), suggesting a different behaviour across the samples.

Specifically, in these 36 individuals, the INH means in serum were 91.6% (Wuhan wild-type), 91.3% (Delta variant), 89.8% (Delta Plus variant), and 85.1% (Omicron BA.1 variant) (Fig. 4, Table S5). Therefore, all the subjects showed NAb against the VOC in the serum, and only a mild decrease of the INH against the Omicron BA.1 variant was observed. At variance, in saliva we found 75% (N = 27/36) and 66.7% (N = 24/36) of subjects with NAb against Delta and Delta Plus variants, respectively, and the neutralization efficiency against Delta (INH: 43.1%) and Delta Plus (INH: 35.2%) variants was lower compared to wild-type virus. More importantly, we observed a dramatic decrease in neutralizing activity against the Omicron BA.1 variant (INH: 4.7%) (Fig. 4, Table S5) accompanied by a marked reduction in the number of subjects with Omicron BA.1-specific NAb in saliva (11.1%, N = 4/36).

Of note, seven of the vaccinees who were infected after the booster dose did not show NAb against Omicron BA.1 variant in their saliva, while the INH detected

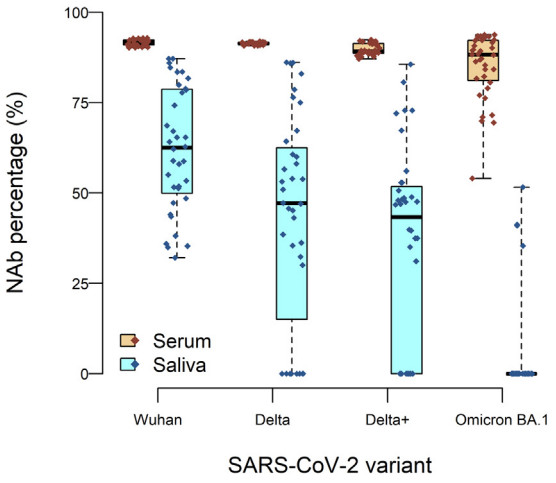

Fig. 4: Serum (orange) and salivary (light blue) NAb percentage distribution according to SARS-CoV-2 variant, among individuals showing salivary NAb against the Wuhan antigen at T4 (N = 36).

against the Wuhan wildtype antigen was lower when compared to the other individuals, even though statistical analysis was not performed due to the very limited sample size. Thus, the importance of a booster dose of vaccine is clear, due to the greater neutralization efficiency in the serum against the main VOC; however, even with three doses of the vaccine, neutralization against the Omicron BA.1 variant significantly decayed in saliva, this may partially explain the rapid circulation of this virus variant.

Discussion

The success of the COVID-19 vaccination campaign relies on the long-standing effectiveness of the approved vaccines in preventing severe disease, hospitalizations, and related deaths.[25,26]

Although many reports have assessed the efficacy of the booster dose of the BNT162b2 COVID-19 vaccine in preventing severe outcomes related to COVID-19, and the generation of vaccine-induced IgG NAb in the serum of subjects who received the booster, there are only few published data on the induction of a specific immune response in the mucosa, which represents the primary route of entry and infection by SARS-CoV-2.[27,28] Indeed, the airways are the most important entry sites for pandemic pathogens, and local secretion of airway Ab, mostly represented by IgA subclass, has the potential to generate truly sterilizing immunity by directly neutralizing pathogens before they are able to infect epithelial cells or invade tissues. Our study previously published in this journal[19] indicated that the BNT162b2 vaccine evokes a potent systemic immunity against the virus, but only a weak mucosal immunity, especially in individuals that had not been previously exposed to natural infection (i.e., SN). These results were also confirmed by other reports.[29,30] Indeed, we previously analyzed the serum and salivary samples of 60 HCW who underwent a complete primary vaccination cycle with the BNT162b2 vaccine and found that the low mucosal immunity is mainly represented by salivary IgG exuded from the serum rather than locally produced sIgA. Consistently, we could rarely detect the presence of NAb in the saliva of SN individuals (i.e., 18%) at two weeks from the injection of the second dose (T2).

In the current study, we evaluated the persistence of specific SARS-CoV-2 Ab in serum and saliva, by analyzing IgG and IgA levels at eight months from the completion of primary vaccination cycle (T3) in 83.3% of the subjects (50/60) enrolled in our previous study. We also assessed the efficacy of the booster, by measuring the Ab titres two weeks after the injection (T4) of the vaccine. In line with other reports,[31] at T3 we could observe a waning of the vaccine-induced systemic immunity, with a significant decline of anti-S1 IgG Ab in the serum. This result was accompanied by a predictable decrease of IgG titers in the saliva, as in this compartment IgG Ab were mostly the exudate of serum IgG.[32] In contrast, the IgA showed a different behavior in the two compartments: indeed, serum IgA declined similarly to serum IgG, while salivary IgA showed a five-fold increase between T2 and T3. Furthermore, salivary IgA were detected in almost all recruited subjects (i.e., 48/50) at T4, while in only 50% of the individuals we found salivary IgA at T2. These data suggest a delayed activation of the mucosal immune system after the primary vaccination cycle, with the consequent local production of sIgA.[33,34] However, this increase of salivary IgA was not correlated with the generation of specific salivary NAb, as these were represented mostly by IgG. Very few data are available on the difference of circulating, antibody producing B cells and their mucosal counterpart in the saliva. Resident B cells in salivary glands and oral mucosa can be a primary source of antigen-specific IgA production with respect to circulating B cells homing preferentially in other lymphoid organs, such as the spleen. Indeed, B cells in salivary glands and oral mucosa show immunoglobulin rearrangements qualitatively distinct from those of spleen B cells,[35] and possibly generate also from a different involvement of specific subpopulations of T helper cells driving the isotype switch toward IgA.[36] However, this increase of salivary IgA was not correlated with the generation of specific salivary NAb, as these were represented mostly by IgG.

As far as memory B cells responsible of anti-Spike glycoprotein ectodomain Ab production, they are indeed present in vaccinated subjects in consistent amounts and readily triggerable by further contact with antigen.[37] Results suggest, however, that the mode of immunization greatly favors the generation of IgG-switched memory B cells, including B cells producing NAb, as compared to other isotype-specific B cells. As

outlined above, IgA-switched B cells are present, but apparently, they are not skewed toward the production of NAb [preprint].[38] Beside the above described distinct homing of B cells, either mucosal or spleen, the distinct specificity of IgA antibodies may be the mirror of the two alternative functions of mucosal IgA aimed at maintaining not only the healthy balance between the host and the invading pathogens, but also at generating a sterilizing immunity in the upper airways. Indeed, IgA protect against infection by covering some pathogens to avoid invasion and inflammatory responses, thus also favouring the retention of beneficial, but pro-inflammatory, commensal strains; at the other side, this unusual IgA "opsonization" will also hinder strong inflammatory antigen-specific immune response against bacteria and viruses, promoting the formation of inhibitory environment associated to the increase of regulatory T cells.[39] The progressive decline of serum Ab at T3, including NAb, would further suggests that memory B cells against SARS-CoV-2 may be indeed relatively few or require frequent antigen challenge to maintain elevated synthesis of specific Ab. In fact, the booster immunization elicited a strong increase in the titres of both IgG and IgA Ab in the serum; these findings are consistent with the above hypothesis and with other findings reported in the literature, confirming that the third dose of the BNT162b2 vaccine boosts the immunity acquired with the first two doses.[40]

Although we were not able to discriminate between IgA exuded from the serum and sIgA, it is highly probable that the rapid increase in salivary IgA after the booster vaccination was mainly due the transudation of serum IgA, similarly to what we observed in our previous report.[19]

As mentioned above, the increase in Ab titers correlated with a parallel increase of NAb in the serum, with all individuals showing high levels of INH similar to those observed after the first two doses. The increase of neutralizing activity was more prominent in the saliva, where 72% (i.e., 36/50) of recruited subjects had NAb, with INH significantly higher than that recorded at T2 and T3. Of note, a similar result was observed in the SP subjects after the second dose in our previous study, since these individuals have been further exposed to the viral antigens during the infection. The development of neutralizing activity in the saliva after the booster vaccine was statistically associated with the increase of salivary IgG and not of salivary IgA, confirming that this vaccination protocol does not elicit a specific mucosal immune response at the oral level, at least not in the immediate post-vaccination period.

Of importance, however, was the finding that at the serum level vaccine boosting resulted in NAb not only against the original strain of SARS-CoV-2 (i.e., Wuhan) but also against the most representative strains that characterized the third and the fourth pandemic waves in Italy, e.g., the Delta, Delta Plus, and Omicron BA.1 variants. Indeed, the neutralizing activity of Ab did not decrease when tested with the VOC antigens, showing that the BNT162b2 COVID-19 vaccine evokes a systemic immune response effective not only against the original strain (i.e., INH 91.6%), but also against the Delta variant (i.e., INH 91.3%) and the Delta Plus variant (i.e., INH 89.8%).[41] In addition, there was only a slight decrease of the INH of serum samples against the Omicron BA.1 variant (i.e., INH 85.1%). These data are consistent with those reported in other studies, and underline the importance of BNT162b2 vaccine in eliciting a strong systemic immune response that can protect from the severe outcomes of the disease even when the infection is caused by a VOC.[42]

At variance, although the booster induces an effective salivary neutralizing activity against the Wuhan antigen (i.e., INH 62.2%) in 36 of the recruited subjects, the number of individuals with salivary NAb decreased against the Delta variant (i.e., INH 43.1%, N = 27) and the Delta Plus variant (i.e., INH 35.2%, N = 24). What actually worsens this scenario is that the neutralizing activity was completely absent in almost all the individuals when tested against the Omicron BA.1 variant (i.e., INH 4.7%, N = 4). These findings strongly sustain the notion that the BNT162b2 COVID-19 vaccine may elicit a mucosal immune protection against the wild-type antigen, even though it mainly depends on the transudation of serum IgG into the salivary compartment, but it fails in providing immune protection against the infection caused by the VOC. More importantly, it seems completely inefficient against the Omicron BA.1 variant at the oral level. These results could partially explain the role of the Omicron BA.1 variant during the fourth wave of the pandemic in Italy.[43] This VOC is known for its high contagiousness, responsible for the peak of infections recorded between December, 2021 and February, 2022 in Italy,[44] the highest peak recorded since the onset of the pandemic in 2020. Thus, the absence of protection at the mucosal level after the three-dose protocol correlates with the ability of the Omicron BA.1 variant to infect the subjects who recently underwent the booster vaccination. Although the Omicron BA.1 variant is associated with a less severe disease, the related pandemic wave was characterized by severe outcomes in those individuals who refused the vaccination, thus lacking a strong systemic immune response.[45]

Although this study presents some limitations (i.e., the recruitment limited to healthcare workers in a hospital setting, the sample size small due for the high cost of the laboratory procedure), nevertheless it provided unprecedented data on mucosal immunity developed by the same individuals after each dose of the BNT162b2 COVID-19 vaccine, without missing data. Importantly, precise hypotheses were formulated before undergoing analysis, avoiding thus the multiple testing problem.

In conclusion, the results reported in this study provide new insights to the role of the mucosal immune response in the effectiveness of the BNT162b2 vaccine in blocking infection, and a concern on defining optimal strategies of anti-SARS-CoV-2 vaccination. The third dose of the BNT162b2 COVID-19 vaccine, administered by intramuscular injection, drastically increases both total and NAb concentration in the serum and salivary compartments. Nevertheless, the increase of IgA in the saliva was no paralleled by a corresponding increase in virus-neutralizing activity.

Within this frame, reconsidering the vaccination strategy to prevent not only the severe disease but the viral infection (i.e., the so-called 'sterilizing immunity') should represent the goal for the generation of second-line COVID-19 vaccines aimed at reinforcing the mucosal immune response.[46] Several reports using *in vivo* animal models indicate that the respiratory delivery (i.e., the nasal or oral route) of the COVID-19 vaccine may provide both systemic and mucosal immunity against current and future VOC.[47]

These findings suggest that the administration of a mucosal vaccine in addition to the approved intramuscular protocol could provide the sterilizing immunity required to efficiently block viral circulation and the emergence of new VOC efficiently.[48,49]

The requirement of re-scheduling of the booster vaccine delivery route is further suggested by the preliminary data on the fourth dose administration of BNT162b2 mRNA vaccine in Israel showing a reinforcement of protection against severe outcomes, but only a short-lived defense against the infection.[50]

Contributors

Conceptualization: L. Azzi, G. Forlani. Data curation: L. Azzi, D. Dalla Gasperina, M. Shallak, A. Baj, V. Maurino, G. Forlani. Investigation: L. Azzi, D. Dalla Gasperina, M. Shallak, V. Maurino, P. Cavallo, G. Forlani. Formal analysis: G. Veronesi. Visualization: G. Veronesi. Methodology: D. Dalla Gasperina, F. Gianfagna, F. Dentali. Validation: F. Maggi, G. Forlani. Supervision: D. Dalla Gasperina, F. Maggi, L. Tettamanti, R.S. Accolla. Project administration: L. Azzi, F. Dentali, L.S. Maffioli, G. Forlani. Resources: A. Tagliabue, L.S. Maffioli. Funding acquisition: F. Gianfagna, A. Tagliabue. Writing – original draft: L. Azzi, G. Veronesi, G. Forlani. Writing – review and editing: D. Dalla Gasperina, R.S. Accolla.

Data sharing statement

Raw data and individual level de-identified trial data and protocol will be shared on request for researchers after obtaining a data use agreement through the Azienda Socio-Sanitaria Territoriale dei Sette Laghi Hospital Direction.

Declaration of interests

None to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2022.104435.

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