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Special Issue Reprint

Advances in COVID-19 Vaccines and Neutralizing Antibody

Edited by
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This is a reprint of articles from the Special Issue published online in the open access journal *Vaccines* (ISSN 2076-393X) (available at: www.mdpi.com/journal/vaccines/special_issues/790JE9VIM3).

For citation purposes, cite each article independently as indicated on the article page online and using the guide below:

Lastname, A.A.; Lastname, B.B. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-7258-2040-5 (Hbk)
ISBN 978-3-7258-2039-9 (PDF)
https://doi.org/10.3390/books978-3-7258-2039-9

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About the Editors

Rishi Jaiswal

I am Rishi Kumar Jaiswal, born in India. In 2017, I successfully completed my Ph.D. in cancer biology at Jawaharlal Nehru University, New Delhi, India, where my research focused on elucidating the role of telomerase in epithelial to mesenchymal transition (EMT). Subsequently, in 2018, I embarked on a new journey as a visiting scientist at Lund University, Sweden, working at the Department of Biology. During my two-year tenure, I led the development of a pioneering method for uncovering 5′ end permutations in yeast. Notably, my contributions were honored with two prestigious research grants from the Royal Society of Sweden.

In 2020, I transitioned to the Department of Cancer Biology at Loyola University Chicago, assuming the position of a research associate faculty. My primary research focus revolved around investigating the protective role of CST (CTC1-STN1-TEN1) in safeguarding stalled replication forks under conditions of perturbed replication. This significant research was published in a Nature Communications in 2023. Subsequently, in January 2024, our laboratory relocated to Rosalind Franklin University of Science and Medicine. Here, my current research endeavors are aimed at understanding how CST facilitates Rad51 recruitment to stalled forks under replication stress conditions.

Looking ahead, my ultimate career aspiration is to secure a faculty position and establish my independent scientific research.

Srijani Basu

I am an Associate Research scientist at Columbia University. Currently, I am studying the role of host-derived bile acids in regulating immune cell function, supported by the Naomi Berrie Foundation Fellowship. My long-term research interests are to fundamentally advance our understanding of human diseases by utilizing innovative basic and translational research on the immune system. Before joining Columbia, I was a postdoc at Weill Cornell Medicine, Cornell University. During my postdoc, I studied the impact of dietary fructose on microbiota composition and experimental colitis. I have received my PhD from the National Institute of Immunology, JNU, and was also the recipient of the prestigious Shyama Prasad Mukherjee Fellowship awarded by CSIR.

Suman Gupta

After completing my Ph.D. from the National Institute of Immunology, New Delhi, I became a postdoctoral scientist in Sutterwala-Cassel lab in Cedars-Sinai Medical center. I am passionate about understanding the intricacies of host-microbial immune axis. During my Ph.D., I studied the implications of host immune response on gut microbiota. Currently, I am studying host-pathogen responses in various infections such as Leptospirosis. Apart from research, I love growing houseplants and listening to true-crime documentaries.

Sneh Lata Gupta

I am a B cell immunologist, with over 10 years of experience. As a proven immunologist, I have successfully led several scientific projects and demonstrated the ability to collaborate across multiple geographies. Currently, I am a Research Specialist at the College of Pharmacy, University of Michigan, Ann Arbor, USA. Prior to this, I worked as a Postdoctoral Researcher at Emory University, Atlanta, GA, and as a Visiting Researcher at Lund University, Sweden. I hold a degree from the National Institute of Immunology, New Delhi, India.

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Article

The Equal Neutralizing Effectiveness of BNT162b2, ChAdOx1 nCoV-19, and Sputnik V Vaccines in the Palestinian Population

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Abstract: Since the beginning of the COVID-19 pandemic, different viral vector-based and mRNA vaccines directed against the SARS-CoV-2 "S" spike glycoprotein have been developed and have shown a good profile in terms of safety and efficacy. Nevertheless, an unbiased comparison of vaccination efficiency, including post-vaccination neutralizing activity, between the different vaccines remains largely unavailable. This study aimed to compare the efficacy of one mRNA (BNT162b2) and two non-replicating adenoviral vector vaccines (ChAdOx1 nCoV-19 and Sputnik V) in a cohort of 1120 vaccinated Palestinian individuals who received vaccines on an availability basis and which displayed a unique diversity of genetic characteristics. We assessed the level of anti-S antibodies and further determined the antibody neutralizing activity in 261 of those individuals vaccinated with BNT162b2a (121), ChAdOx1 (72) or Sputnik V (68). Our results showed no significant difference in the distribution of serum-neutralizing activity or S-antibody serum levels for the three groups of vaccines, proving equivalence in efficacy for the three vaccines under real-life conditions. In addition, none of the eight demographic parameters tested had an influence on vaccination efficacy. Regardless of the vaccine type, the vaccination campaign ultimately played a pivotal role in significantly reducing the morbidity and mortality associated with COVID-19 in Palestine.

Keywords: SARS-CoV-2; vaccination; Palestine; neutralizing antibodies; mRNA vaccine; adenovirus vaccine

Citation: Damour, A.; Faure, M.; Landrein, N.; Ragues, J.; Ardah, N.; Dhaidel, H.; Lafon, M.-E.; Wodrich, H.; Basha, W. The Equal Neutralizing Effectiveness of BNT162b2, ChAdOx1 nCoV-19, and Sputnik V Vaccines in the Palestinian Population. *Vaccines* **2024**, *12*, 493. https://doi.org/10.3390/vaccines12050493

Academic Editor: P. J. Klasse

Received: 29 March 2024
Accepted: 15 April 2024
Published: 3 May 2024

1. Introduction

Since the initial 2019 outbreak of the novel beta coronavirus SARS-CoV-2, the global response has been characterized by the rapid development and deployment of various vaccines to control viral spread and protect individuals from the severe consequences of COVID-19. Alternative vaccine strategies have been developed to provide a safe and effective immune response to protect from severe disease [1]. These strategies include modified mRNA-based vaccines such as BNT162b2 (Pfizer-Biontech, New York, NY, USA/Mainz, Germany) and mRNA-1273 (Moderna, Cambridge, MA, USA) vaccines, non-replicating adenoviral vectors such as the ChAdOx1 nCoV-19 (AstraZenca/University of Oxford, Cambridge, UK/ Oxford, UK), Ad26.Cov2.S (Johnson & Johnson, New Brunswick, NJ, USA), Sputnik V and Sputnik light vaccines (Gamaleya Institute, Moscow, Russia) and inactivated

viral vaccines such as BBIBP-CorV (Sinopharm, Shanghai, China) and CoronaVac (Sinovac, Beijing, China) [2–4]. Among these strategies, viral vector-based and modified mRNA vaccines have shown good profiles in terms of safety and efficacy [5].

The efficacy of these vaccines varied, with reported rates of protection between 70% and 95% in the respective phase III clinical trials [6–12]. The initial efficacy trials did not involve direct comparisons between different vaccines, and the study design and country-specific biased vaccination strategies have made an impartial comparison of vaccination efficiency between the different available vaccines problematic. An important indicator of the development of an immune response in vaccinated individuals is the level of neutralizing antibodies [13]. Such antibodies have been detected in varying levels in individuals who were vaccinated with different vaccines and were shown to be correlated with protection against symptomatic infection [13]. The complex interactions between vaccine types, host genetics and environmental factors may influence the outcomes of vaccination in ways that are not yet fully understood [14].

The vaccination campaign in the Palestinian Occupied Territory (POT) was ambitious, with more than 3.7 million vaccines administered as of 17 October 2023 [15]. These vaccines included, for the most part, BNT162b2, ChAdOx1 Sputnik V and, to a lesser extent, mRNA-1273, BBIBP-CorV and CoronaVac [16]. The variety in vaccine types, each proposed according to availability during the vaccination campaign, as well as the number of vaccinated individuals and the genetic diversity characteristic for the Palestinian population create a fertile ground for investigating the development of neutralizing antibodies by different vaccines and the effect they had on the progression of the disease.

The present study had two main objectives: (1) to assess the antibody response provoked by the main three vaccine types administered to the Palestinian population (BNT162b2, ChAdOx1, Sputnik V) and (2) to compare retrospectively the serum-neutralizing activity produced by each of these three vaccines. Through these objectives, this research aimed to provide comprehensive insights into the effectiveness of the vaccination campaign within the Palestinian population, taking into account several demographic parameters, the use of different vaccine types and the associated neutralizing activity of the elicited antibody response.

2. Materials and Methods

2.1. Study Design and Settings

A cross-sectional study was conducted among Palestinians from the West Bank who received the COVID-19 vaccine prior to 15 May 2022. The inclusion criteria were people aged between 18 and 80 years old who received two doses of the BNT162b2 vaccine or completed the two-shot regimen of the Sputnik V or ChAdOx1 vaccines. Participants over 80 or under 18 years old with autoimmune diseases or immunocompromised conditions who received the Sputnik Light vaccine or received only one shot of the Sputnik V or ChAdOx1 vaccine were excluded from the study.

2.2. Blood Sampling and Data Collection

Approximately 5 mL of venous blood was collected from each participant in a two-month time window between 6 and 8 months following the last vaccine dose, with subsequent separation of serum from the blood followed by storage at -80 °C until required. In conjunction with the blood samples, the participants were required to fill out a questionnaire encompassing diverse demographic and clinical factors.

2.3. Cell Lines

The cell-based neutralization assay was described previously [17]. Briefly, the assay used syncytia formation between two modified human bone osteosarcoma epithelial cell lines (U2OS, ATCC HTB-96, generously provided by M. Piechaczyk, IGMM, Montpellier, France). The cells were genetically labeled with either GFP or mCherry, with GFP expressing U2OS cells expressed the SARS-CoV-2 receptor Angiotensin Converting

Enzyme-2 following lentiviral transduction (ACE2, addgene #145839) and mCherry expressing U2OS cells which expressed all four codon-optimized SARS-CoV-2 structural proteins from the Wuhan strain: nucleoprotein (N, addgene #141391), membrane protein (M, based on addgene #141274), envelope protein (E, based on addgene #141273), and spike surface protein (S, based on addgene #149329). The resulting cell lines U2OS-GFP-ACE2 and U2OS-mCherry-NEMS were cultured in Dulbecco's Eagle Medium (DMEM, Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco), and maintained at 37 °C in a 5% CO_2 humidified atmosphere. Upon reaching confluence, the cells were washed with phosphate-buffered saline (PBS, Gibco) and detached using 0.05% trypsin-EDTA (Gibco).

2.4. Neutralization Assay

U2OS-GFP-ACE2 and U2OS-mCherry-NEMS were combined at a 1:1 (*v:v*) ratio and subsequently seeded at a density of 5×10^4 cells in 150 µL DMEM supplemented with 10% FBS and 1% penicillin-streptomycin per well in 96 well black wall plates (Ibidi, Gräfelfing, Germany). Fifty µL of serum from vaccinated individuals were added to each first well of a row and sequentially diluted in 1:4 steps. The cells were then cultured for 24 h to facilitate the formation of syncytia between the two cell lines in the presence of the serum dilution. The samples from individuals were numbered and blinded prior to the assay. In each assay, non-syncytia-forming controls (U2OS-GFP) were included and treated similarly. After 24 h, each well was captured at a 2.5× magnification using a fully automated CellDiscoverer-7 microscope system (Zeiss, Jena, Germany). Neutralization was assessed by calculating the mean GFP cell surface area using CellprofilerTM software (2.2.1), representing the formed syncytia surface. Ultimately, the serum neutralization titer was determined to be the IC50 representing half-syncytia inhibition based on values obtained from the serial dilution using Prism7.

2.5. SARS-CoV-2 CLIA Assay

All serum samples were anonymized and tested for the total anti-S antibodies using CLIA (SNIBE, Maglumi SARS-CoV-2 antigen). According to the manufacturer's recommendations, samples were considered positive above a cutoff index of 1 AU/mL. All samples with values over 100 AU/mL were diluted and measured as 1/10 or 1/20, allowing extension of the dynamic range of analysis to 2000 AU/mL. For representation, all AUs were converted into the WHO standard *binding antibody units* (BAUs) using the recommended multiplication factor of 4.33 [18].

2.6. Statistical Analysis

The calculation of the required sample size to meet the objectives of the research and ensure sufficient statistical power was based on the equation $n = [\mathrm{DEFF} \times \mathrm{Np}(1 - \mathrm{p})]/[(\mathrm{d}^2/\mathrm{Z}^2_{1-\alpha/2} \times (\mathrm{N} - 1) + \mathrm{p} \times (1 - \mathrm{p})]$. To cover different geographical areas in Palestine, the West Bank was divided into three regions: north, middle, and south. The sample size was calculated for each region. The calculated sample size for each of the West Bank regions was 370. Therefore, the minimum acceptable total sample size was 1110. Multiple data comparison was performed using ordinary one-way ANOVA with a Tukey multiple comparison test. A comparison of two parameters was performed using an unpaired parametric two-tailed *t*-test. To assess the significance of the correlation between two measured variables (e.g., age-IC50), the Pearson correlation coefficient was calculated. Significance levels were denoted as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$, while "ns" indicated non-significance. All raw data points are graphically presented, and statistical analyses were computed using PRISM 7.

2.7. Ethical Approval, Registration and Patient Consent Procedures

All procedures conducted in this study adhered to both federal and institutional ethical guidelines, the 1964 Helsinki Declaration and subsequent amendments or equivalent ethical

standards. Approval for the study was granted by the Institutional Review Board (IRB) committee of An-Najah National University (Reference No. Med Nov.2021/32) and the Palestinian Ministry of Health. Participants were invited to voluntarily take part in the study, and those who chose to participate provided informed consent by signing a consent form. The study's background and objectives were thoroughly explained to the participants to ensure their understanding.

2.8. Data Availability

The authors confirm that the data used for the findings in this study will be made available through the corresponding authors to qualified and interested investigators upon reasonable request.

3. Results

3.1. Demographic Characteristics of the Participants

In this study, the data were collected from 1120 participants. The mean age of the participants was 32.0 ± 14.8 years, and the mean body mass index (BMI) was 24.9 ± 4.6 kg/m^2. Among the participants, 769 individuals (68.7%) were below the age of 40, 540 participants (48.2%) were female, 53 and 537 individuals (4.7% and 47.9%) fell into the underweight or normal weight category, respectively, and 788 participants (70.4%) identified as non-smokers. In terms of vaccine distribution, 727 participants (64.9%) received the Pfizer-BioNTech vaccine, 185 participants (16.5%) were administered the AstraZeneca vaccine, and 208 participants (18.6%) received the Sputnik V vaccine. Additionally, individuals with blood types A and O constituted 75.2% of the participants, with 36.0% and 39.2% representing each blood type, respectively (Table 1).

Table 1. Demographic characteristics of the participants and their correlation with the total antibody titer. Distribution of demographic parameters and the respective levels of SARS-CoV-2 spike (S) specific antibody titer in the analyzed cohort (see text for details).

	Frequency	Percent	Mean of Antibody Titer (AU/mL)	*p* Value
Age				
18–39	769	68.7	285.5	0.591
40–49	119	10.6	294.8	
50 and above	232	20.7	372.0	
Body Mass Index (BMI)				
Underweight	53	4.7	245.4	0.602
Normal	537	47.9	316.7	
Overweight	384	34.3	306.8	
Obese	146	13.0	276.5	
Gender				
Male	580	51.8	332.3	0.105
Female	540	48.2	274.9	
Smoking				
Non-Smoker	788	70.4	313.7	0.087
Current Smoker	332	29.6	283.0	
Blood Group				
A	403	36.0	321.2	0.072
B	190	17.0	322.7	
AB	88	7.9	312.1	
O	439	39.2	280.1	
Type of Vaccine				
Pfizer	727	64.9	322.1	0.126
AstraZeneca	185	16.5	227.1	
Sputnik V	208	18.6	312.8	

3.2. Post-Vaccine SARS-CoV-2 Total Antibodies

Out of 1120 vaccinated participants, only 11 tested negative for SARS-CoV-2 specific S antibodies. The study found no significant differences in the serum levels of specific S antibodies according to vaccine type or other demographic factors, indicating a consistent trend across the participant cohort ($p > 0.05$) (Table 1).

3.3. Serum Neutralization among Individuals Vaccinated with BNT162b2, ChAdOx1 or Sputnik V

The vaccination campaign for the Palestinian population distributed vaccine doses on an availability basis. The mRNA-based vaccine BNT162b2 and the two adenovirus vector-based vaccines ChAdOx1 and Sputnik V were the most applied vaccines. Amongst the cohort of 1120 individuals, we thus randomly selected 261 sera from people who had received at least two doses of a single type of vaccine. Of those, 121 individuals had been vaccinated with BNT162b2a, 72 with ChAdOx1 and 68 with Sputnik V. We then assessed the serum neutralization capacity using our in-house syncytia fusion assay. We determined the syncytia inhibition titer (IC50) for all individuals as a measure of serum neutralization. For the assay, the samples were numbered and analyzed in a double-blind protocol before the sample identity was revealed for further analysis. We first analyzed the distribution of the calculated IC50 values for each vaccine type (Figure 1A). We observed no significant difference in the distribution of IC50 values between the three different vaccine types. Within each vaccinated group, we found individuals with high neutralization and low neutralization activity (Figure 1B). For ethical reasons, no non-vaccinated control group was included in our assay, but we considered IC50 neutralizations values below 25 to be low responders based on our previous application of the assay [17]. Using thresholds, we also did not observe any significant difference in the neutralization efficiency between the three vaccine types, as our analysis was restricted to low responders (IC50 < 25) or high responders (IC50 > 250). The correlation between the level of S-antigen-specific antibodies in the serum and the serum neutralization capacity were analyzed next. Consistent with previous studies showing a decline of such a correlation over time [17,19], we observed a poor overall correlation between the calculated IC50 values and the anti-S antibody levels (Figure 1C). This was even clearer with a pairwise comparison of the S-antibody level (in BAUs) and IC50 neutralization titer per individual (Figure 1D). Interestingly, sera from three vaccinated individuals without detectable S-antibodies (marked with * in Figure 1D) were included in the analysis, and two of them had low but detectable neutralization activity.

3.4. Correlation between Serum Neutralization Efficacy and Demographic Parameters across BNT162b2, ChAdOx1 or Sputnik V Vaccinated Individuals

In each vaccinated group, we observed that part of the vaccinated individuals were low responders concerning the neutralization efficacy. In general, the individual response to vaccination is influenced by many parameters, of which the vaccine type is just one [14]. Other parameters were therefore investigated. For the analysis, the data for all three vaccines were pooled (Figure 2). First, the impact of intrinsic host factors on neutralization response was assessed, especially since several reports indicated a reduced immune response in the elderly [20] as well as a gender-specific better antibody response for several vaccines (summarized in [14]). Our analysis showed neither correlation between neutralization efficacy and age (Figure 2A) or height (Figure 2B) nor differences when comparing the two genders (Figure 2C). There also was no correlation between IC50 and weight (Figure 2D) or BMI (Figure 2E), although a negative correlation between BMI and antibody response was reported for certain vaccine types [21], including for SARS-CoV-2 vaccines [22]. Blood groups, which have been reported to affect the humoral and cellular response following the application of an oral cholera vaccine [23,24], were another intrinsic host factor without influence on the IC50 in our analysis (Figure 2F). We next assessed the environmental and behavioral factors that were collected within the cohort and which may affect vaccine efficacy outcomes due to increased potential exposure to other pathogens [25]. Comparing the individuals living in urban, rural or camp settings did not explain the differences

in the IC50 responses, although the latter sample size was quite small (Figure 2G). Likewise, we did not identify a relation between the smoking status of individuals and their IC50 responses (Figure 2H). Each part of the analysis was also performed individually for each vaccine type and showed no significant differences [26], thus further confirming the observed equivalence of the three vaccines. Taken together, none of the parameters we analyzed were sufficient to explain the difference in the neutralization response within the vaccinated population.

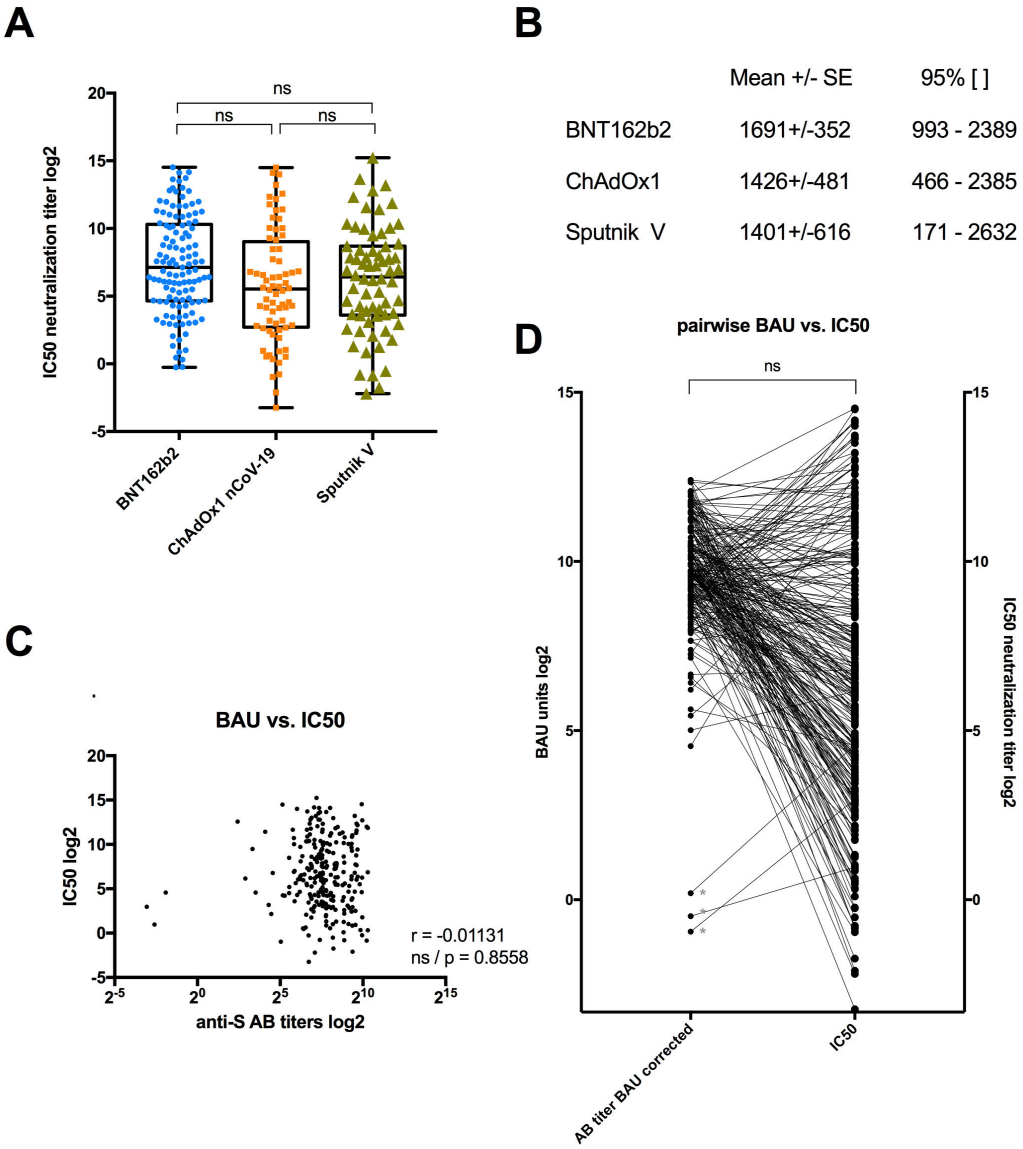

Figure 1. Serum neutralization following vaccination with BNT162b2, ChAdOx1 nCoV-19 or Sputnik V. (**A**) Serum neutralization was determined based on syncytia inhibition (IC50) for vaccinated patients with BNT162b2 (blue, n = 121), with ChadOx1 nCoV-19 (orange, n = 72) or with Sputnik V (green, n = 68). One-way ANOVA with a Tukey multiple comparison test (ns = not significant). (**B**) Mean of IC50 +/− standard error and 95% confidence interval for each type of vaccine tested. (**C**) BAU levels (anti-S Ab titers) and IC50 for each vaccinated patient were normalized and plotted. Pearson correlation coefficient (r) as indicated (ns = not significant). (**D**) Pairwise comparison of BAU levels (anti-S Ab titers) and IC50 for each patient and paired two-tailed t-test (ns = non-significant). Gray * indicates an individual who tested negative for S-antigen.

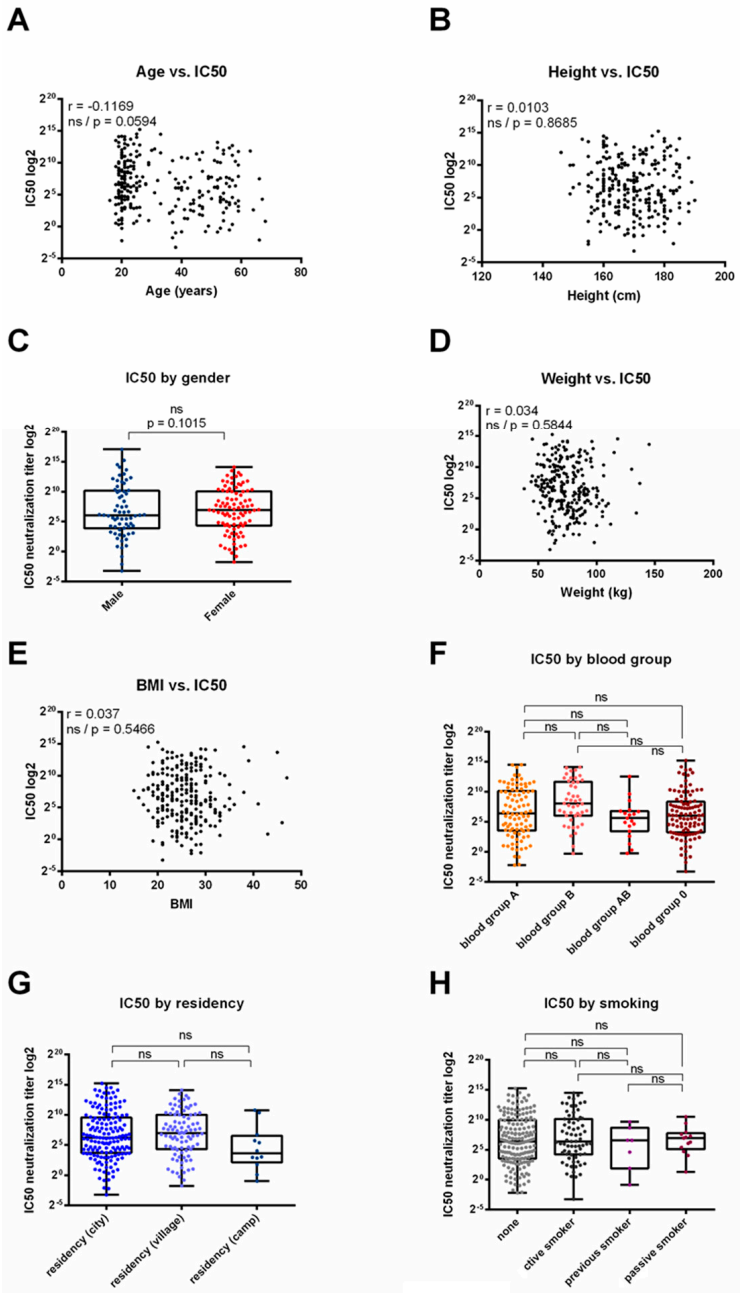

Figure 2. Correlation between serum neutralization and demographic parameters in patients vaccinated with BNT162b2, ChAdOx1 nCoV-19 or Sputnik. (**A**,**B**,**D**,**E**) IC50 and age (**A**), height (**B**), weight (**D**) and BMI (**E**) for each vaccinated patient were normalized and plotted. Pearson correlation coefficient (r) as indicated (ns = not significant). (**C**,**F**,**G**,**H**) Serum neutralization was determined based on syncytia inhibition (IC50) for vaccinated patients according to their gender (**C**), blood group (**F**), residency (**G**) or smoking status (**H**). One-way ANOVA with a Tukey multiple comparison test (ns = not significant).

4. Discussion

Following the outbreak of the COVID-19 health crisis, the Palestinian Ministry of Health and the WHO started a vaccination campaign throughout the POT in the late summer of 2021, with a peak in activity into early 2022. The campaign included multiple vaccines on an availability basis. Due to early availability, the Sputnik V and Sinopharm vaccines were initially administered, while Pfizer-BioNTech and Astra-Zeneca became available later through the COVAX fund, with the final shipment in December 2021 also

including Moderna [16]. Here, we analyzed the effectiveness of the vaccination campaign by determining the elicited anti-S antibody levels in a random cohort of 1120 individuals who received either the Pfizer-BioNTech, AstraZeneca or the Sputnik V vaccine and for whom serum was collected 6–8 months post vaccination (see Table 1). We further determined the IC50 value for the serum neutralization activity in a part of the cohort. Importantly, the use of a diverse range of vaccine types based on availability, a peak period of vaccine application in late 2021 and the collection of serum within 6–8 months following application of the last vaccine dose retroactively created a unique situation to fairly assess the effectiveness of individual vaccines in this single cohort.

Above all, our results demonstrate the success of the Palestinian vaccination campaign, with almost every tested individual showing significant levels of S-antibodies and active serum neutralization activity. However, the cohort only included fully vaccinated individuals and blended out the supply dependence on COVAX and other countries for vaccine donations, which resulted in a delay for the vaccination campaign and a vaccine uptake disparity [16].

Our analysis also demonstrated that neither the level of elicited anti-S antibodies (in BAUs, Figure 1) nor the neutralization capacity of the serum (IC50, Figure 1) was linked to the type of vaccine included in this study. Both the S-antibody levels and serum neutralization activity were distributed over a large range and poorly correlated. Only 11 individuals tested negative for S-antibodies, of which three were tested further for neutralization, with two showing low but measurable serum neutralization activity. This outcome of vaccine equivalence is also supported through meta-data analysis showing that vaccination with several vaccines, including all three vaccines from this study, results in protection from severe COVID-19 disease [27]. There is some inconsistency, with available studies directly comparing vaccine effectiveness between mRNA- and vector-based vaccines revealing somewhat reduced serum neutralization activity in ChAdOx1-vaccinated individuals compared with BNT162b2, especially with variants [28,29]. The reason for this difference could be due to the non-standardized and late collection time of serum post vaccination in our study. Most comparative studies measured the serum effects within days or weeks after vaccination completion [29–31]. This may be an important factor, as when applying the same assay, we recently followed an unrelated cohort receiving mRNA-based vaccination over time, showing that the antibody and neutralization levels declined over time, as well as the number of applied vaccine doses [17]. Indeed, a recent study comparing mRNA- and Ad vector-based vaccines over time showed that mRNA- but not Ad-based vaccines induce a rapid but short-lived peak in S-specific antibodies in the serum which levels out over time [32], potentially dropping to levels with non-significant differences between vaccine groups [32,33]. As a consequence, initial differences in the vaccine response may be less important for the long-term establishment of protective immunity, as shown in our study. We also tested several demographic parameters for their influence on the vaccination response but were unable to find any correlation with the vaccine effectiveness (Figure 2). This result further indicates that individual vaccine responses are multifactorial [14].

One shortcoming of our study is that we did not account for circulating SARS-CoV-2 variants, nor did we measure cellular immunity or attempt MHC characterization in the participants due to the available local setting. Nevertheless, our study is meaningful because it was performed on a large cohort of vaccinated individuals without a biased strategy and shows the success of the vaccination campaign. Notably, it retroactively qualifies the use of more cost-effective vaccines and suggests equivalence between mRNA- and adenovirus vector-based COVID-19 vaccines when applied consistently under field conditions. By highlighting the efficacy of the vaccination program in the POT, this study emphasizes the importance of providing vaccines for all communities, including Palestinians. Moving forward, continued research efforts and collaboration will be essential in ensuring and evaluating the ongoing success of vaccination initiatives and in safeguarding the health and well-being of all communities.

Author Contributions: Conceptualization, H.W., A.D. and W.B.; methodology, H.W., A.D., N.L., N.A. and W.B.; formal analysis, A.D., M.F., N.L., J.R., H.D., H.W. and W.B.; resources, H.D. and W.B.; data curation, H.D., H.W. and W.B.; writing—original draft preparation, H.W., A.D. and W.B.; writing—review and editing, H.W., M.-E.L., A.D. and W.B.; supervision, H.W., M.-E.L. and W.B.; project administration, H.W. and W.B.; funding acquisition, H.W. and W.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Agence universitaire de la Francophonie (AUF) for W.B., grant number "DRMO—6744 (Projet 2818)", with institutional funds to H.W., and the APC was funded by institutional funds to H.W.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (IRB) committee of An-Najah National University (Reference No. Med Nov.2021/32) and the Palestinian Ministry of Health.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The authors confirm that the data used for the findings in this study will be made available through the corresponding authors to qualified and interested investigators upon reasonable request.

Acknowledgments: The microscopy was performed in the Bordeaux Imaging Center, a service unit of the CNRS-INSERM and Bordeaux University and member of the national infrastructure France BioImaging supported by the French National Research Agency (ANR-10-INBS-04). H.W. is an INSERM fellow.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Analysis of the Presence and Levels of IgG Antibodies Directed against the S1 Protein Receptor Binding Domain and the N Protein of SARS-CoV-2 in Patients with Multiple Sclerosis Treated with Immunomodulatory Therapies

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Abstract: The coronavirus 2019 disease (COVID-19) course and serological statuses of patients with relapsing–remitting multiple sclerosis (RRMS), treated with disease-modifying therapies (DMTs) are generally parallel that of the general population. Over the pandemic's course, however, a notable increase in the number of RRMS patients who received vaccination against severe acute respiratory coronavirus 2 (SARS-CoV-2) and those who had COVID-19 (symptomatic and asymptomatic) was reported. This virus and/or vaccination likely influenced DMT-treated RRMS patients' serological statuses regarding the presence of SARS-CoV-2 antibodies and their quantitative expression. This investigation assesses the presence and levels of the antibody directed against the S1 protein receptor binding domain (SRBD) and against the N protein of SARS-CoV-2 in 38 DMT-treated RRMS patients. The findings indicate that people vaccinated against SARS-CoV-2 exhibited significantly higher levels of IgG antibodies against S1-RBD at both assessment points. Patients with a prior history of COVID-19 demonstrated statistically significant increases in anti-N antibodies at visit 1, whereas such statistical significance was not observed at visit 2. DMT-treated RRMS patients generated neutralizing antibodies following vaccination and/or COVID-19 infection. Nevertheless, it is noteworthy that antibody levels more accurately reflect the serological status and exhibit a stronger correlation with vaccination than just the presence of antibodies.

Keywords: COVID-19; SARS-CoV-2; receptor binding domain; spike protein; nucleocapsid protein; multiple sclerosis; disease-modifying therapies; antibodies; vaccines; serology

Citation: Kulikowska, J.; Kapica-Topczewska, K.; Gudowska-Sawczuk, M.; Kulczyńska-Przybik, A.; Bazylewicz, M.; Mirończuk, A.; Czarnowska, A.; Brola, W.; Mroczko, B.; Kochanowicz, J.; et al. Analysis of the Presence and Levels of IgG Antibodies Directed against the S1 Protein Receptor Binding Domain and the N Protein of SARS-CoV-2 in Patients with Multiple Sclerosis Treated with Immunomodulatory Therapies. *Vaccines* **2024**, *12*, 255. https://doi.org/10.3390/vaccines12030255

Academic Editors: Rishi Jaiswal, Srijani Basu, Suman Gupta and Sneh Lata Gupta

Received: 19 January 2024
Revised: 15 February 2024
Accepted: 19 February 2024
Published: 29 February 2024

1. Introduction

Coronavirus 2019 (COVID-19) is a disease caused by the severe respiratory syndrome coronavirus 2 (SARS-CoV-2) virus, which is responsible for the pandemic that started in 2019 [1]. The genetic material of the virus comprises a single-stranded RNA that encodes 16 non-structural proteins and four structural proteins: spike (S), nucleocapsid (N), envelope (E), and membrane (M) [2]. In a clinical context, the pivotal protein is the S protein, which was found to be accountable for facilitating virus entrance into the host cell via binding to the ACE2 (angiotensin-converting enzyme 2) receptor [3]. The spike protein

was selected as a therapeutic target in the design of vaccines against the SARS-CoV-2 virus [4]. In addition, the antibodies against the S protein, which are produced as a result of active immunization (natural infection) and passive immunization (vaccination), are the only ones that have a neutralizing capability, thereby conferring protection against infection or reinfection [5]. It is also known that antibodies directed against the receptor binding domain (RBD) within the S1 subunit (anti-S1RBD antibodies) have the highest neutralizing capacity [6]. Moreover, the S1-RBD subunit exhibits minimal amino acid sequence homology compared to other coronaviruses [4]. The second clinically significant protein of the SARS-CoV-2 virus is the nucleocapsid (N) protein, which is responsible for the replication and transcription of viral RNA [7]. The N protein, as with the S protein, induces a humoral response. However, antibodies directed against the N protein are solely generated after natural infection but not after vaccination [8]. As in most European countries, in Poland, in December 2020, a mass vaccination program against COVID-19 began, initially available to selected risk groups (including healthcare workers). From May 2021, all adult Poles could receive the first dose of the vaccine. Registration for the second dose of the vaccine opened in November 2021. From April 2022, the second booster dose of the vaccine could be administered to people over 80 years of age, and from September 2022, all people over 12 years of age. The first available vaccine was the Comirnaty mRNA vaccine (Pfizer-BioNTech; Marburg, Germany). Subsequently, another mRNA vaccine, Spikevax (Moderna Biotech Spain, S.L, Madrit, Spain), was approved. Other vaccines approved in EU countries (including those used en masse in Poland) were vector vaccines: Vaxzeveria (AstraZeneca; Cambridge, UK) and Janssen Vaccine (Janssen-Cilag International NV; Beerse, Belgium) and a protein vaccine (Nouvaxovid Novavax; Gaithersburg, MD, USA). In May 2021, the Polish Neurological Society published an official position recommending COVID-19 vaccination for patients suffering from multiple sclerosis. Patients treated with beta interferons (INF), glatiramer acetate (GA), teriflunomide (TFN), dimethyl fumarate (DMF), and natalizumab (NTZ) should consider vaccination at every stage of treatment (no change in the therapy schedule is necessary). The guidelines specify groups of patients treated with fingolimod, ocrelizumab, cladribine, and alemtuzumab, where vaccination schemes are proposed depending on the time of DMT administration [9]. Insubsequent stages of the pandemic, the Polish Neurological Society updated its position on additional doses and booster doses [10].

Multiple sclerosis (MS) is a demyelinating autoimmune disease that impacts individuals across various age groups. The disease is predominantly diagnosed during the third decade of life [11]. MS is treated with immunomodulating and immunosuppressive drugs, termed disease-modifying therapies (DMTs). In Poland, 15 DMTs are currently available and financed by the National Health Fund. Nevertheless, the predominant cohort comprises individuals primarily undergoing treatment with dimethyl fumarate (DMF), glatiramer acetate (GA), or beta-interferon (INF) [12]. During the first months of the pandemic, physicians and patients wanted to know whether the treatment and the disease itself would negatively affect the course of SARS-CoV-2 infection [13]. In line with current knowledge, MS patients treated with most of the DMTs were infected by the SARS-CoV-2 infection at similar rates as the rest of society [14]. In addition, the response of this group of patients to vaccination against COVID-19,except for patients treated with anti-CD20 therapies and fingolimod, is normal [15]. At the time of the pandemic and afterward, the number of patients who had received subsequent vaccine doses and those who had come into contact with the SARS-CoV-2 virus increased. This increase probably affected the serological status of this specific group of patients, not only in terms of the mere presence of antibodies against S and N proteins but also the levels of these proteins, which seems to be important for future monitoring of the immunity of patients with MS who are treated with DMTs.

2. Materials and Methods

2.1. Study Group

The study group consisted of patients (n = 38) with relapsing–remitting multiple sclerosis (RRMS) who were treated with selected DMTs: DMF (36.84%; n = 14), GA (26.32%; n = 10), or INF (36.84%; n = 14). All examined patients were diagnosed in accordance with the McDonald criteria 2017 and were under the care of the Department of Neurology, Medical University of Bialystok [16]. Blood samples were collected twice between December 2021 and February 2023 (median 18.33 months) from each patient. During each visit, the following data were collected: (1) patient's age, type and duration of disease-modifying drug used, COVID-19 vaccinations received (number of doses, dates of vaccinations, types of vaccinations), and documented positive result based on the polymerase chain reaction (PCR/COVID-19 antigen test). During both visits, the patients were examined by a neurologist. All individuals signed informed consent to participate in this study.

A total of 57.89% (n = 22) of the study group were women. The average age at the first visit was 44.5 years. The average duration of the disease was 9 years. The average duration of using DMF is 3.4 years (SD $\pm$ 1.4–6.3), GA 5.1 years (SD $\pm$ 1.3–9.1), INF 10 years (SD $\pm$ 1.0–9.2). Among all vaccine doses received by patients, 75% were vaccinated with the Comirnaty vaccine (Pfizer-BioNtech; Marburg, Germany)., 12.50% with the Vaxzeveria vaccines (AstraZeneca; Cambridge, Great Britain, 6.94% with the Spikevax (Moderna Biotech Spain, S.L, Madrit, Spain) and 5.56% with the Janssen Vaccine (Janssen-Cilag International NV; Beerse, Belgium).

The study was approved (approval NAPK.002.230.2020) by the Bioethics Committee at the Medical University of Bialystok, Poland.

The detailed clinical characteristics of the study group are shown in Tables 1 and 2.

Table 1. Characteristics of the study group.

Sex	Female	57.89% (n = 22)
	Male	42.11% (n = 16)
Age (on visit 1)		44,50 (36.25, 48.75) [1]
DMT	DMF	36.84% (n = 14)
	GA	26.32% (n = 10)
	INF	36.84% (n = 14)
Time between visit 1 and 2		18.33 (17.70, 18.84) [1]

[1] Median (Q1, Q3); DMT, dimethyl fumarate; GA, glatiramer acetate; INF, interferon beta.

2.2. Laboratory Tests

An assessment of antibodies against the SARS-CoV-2 virus was conducted: (1)IgG antibodies against the receptor binding domain of S1protein (IgG-S1RBD) and (2) IgG antibodies against N protein (IgG-N). Serum levels of the IgG-S1RBD and IgG-N antibodies were measured by chemiluminescent microparticle immunoassay (CMIA) according to the manufacturer's instructions. The result in the chemiluminescent reaction was assessed as relative light units (RLU) using the automatic Alinity system (Abbott, Chicago, IL, USA) according to the manufacturer's instructions. The level of serum antibodies was directly proportional to the RLU detected by the system optics. The S/C (serum/cut-off) index was determined based on the above relationship. A titer $\geq$ 1.4 (IgG-N) and $\geq$50 (IgG-SRBD) was considered a positive result.

Table 2. Detailed characteristics of study groups.

		Visit 1	Visit 2
Vaccinated [2]		60.52% (n = 23)	71.05% (n = 27)
	One dose	26.09% (n = 6)	3.70% (n = 1)
	Two doses	73.91% (n = 17)	40.74% (n = 11)
	Three doses	0	48.15% (n = 13)
	Four doses	0	7.41% (n = 2)
Time between first dose and visit [months]		1.69 (1.10; 2.48) [1]	27.37(26.24; 28.21) [1]
Time between second dose and visit [months]		1.03 (0.38; 1.53) [1]	18.74 (17.77; 19.66) [1]
Time between third dose and visit [months]		-	11.70 (10.81; 12.42) [1]
Time between fourth dose and visit [months]		-	11.70 (1.81; 12.42)
unvaccinated		39.47% (n = 15)	28.95% (n = 11)
COVID-19"+" [3]		18.42% (n = 7)	23.68% (n = 9)
COVID-19"−" [4]		81.58% (n = 31)	76.32%(n = 29)
Time between COVID-19 and visit [months]		4.93 (4.70; 5.95) [1]	23.56 (12.78; 25.00) [1]

[1] Median (Q1, Q3); [2] Vaccinated—people with multiple sclerosis (PwMS) vaccinated against coronavirus 2019 disease (COVID-19); [3] COVID-19"+"—PwMS with registered positive PCR/antigen test in the past; [4] COVID-19"−"—PwMS with no registered positive PCR/antigen test in the past.

2.3. Statistical Analysis

The statistical analysis was based on a description of groups of patients classified by DMT and survey data (sex, age, COVID-19 status, vaccination status). The significance level of the statistical tests in this analysis was set at $\alpha = 0.05$. The normality of the distributions of the variables was analyzed using the Shapiro–Wilk test. Numerical variables with distributions deviating from the normal distribution were reported as *Mdn* (*Q1*, *Q3*). Examination of differences within a numerical variable with a non-normal distribution between two groups was performed with the Wilcoxon rank sum test and between three or more groups was performed with the Kruskal–Wallis rank sum test. The significance of differences between pairs of groups was tested using Dunn's test. The effects of vaccination or pastCOVID-19 infection over time (visits 1 and 2) on the concentration of SARS-CoV-2 IgG (S-RBD, N) were examined using a linear mixed model. In the case of dichotomous response variables (SARS-CoV-2 IgG positive result for S-RBD or N), a generalized linear model was applied. The magnitude of the effect between categories within an exploratory variable with more than two categories (such as the number of vaccine doses) was estimated by contrast analysis of the estimated marginal means with the Tukey adjustment. Spearman's rank correlation coefficient (rho) was used to measure the strength and direction of association between two variables. Analyses were conducted using the R Statistical language (version 4.1.1; R Core Team, 2021) on Windows 10 x64 (build 19045).

3. Results

3.1. Analysis of Antibodies against S1 Protein

3.1.1. Impact of Vaccination

At visit 1, patients with RRMS who had not been vaccinated against SARS-CoV-2 accounted for 39.47% (n = 15) and vaccinated 60.53% (n = 23). At visit 1, among unvaccinated patients with RRMS, 33.33% (n = 5) had no antibodies against S1-RBD, while 66.67% (n = 10) had positive antibodies. Among vaccinated patients with RRMS, 91.30% (n = 21) showed positive anti-S1RBD IgG antibodies. At visit 2, 28.95% (n = 11) of RRMS patients were unvaccinated, and 71.05% (n = 27) were vaccinated against SARS-CoV-2. Among the unvaccinated patients, 9.09% (n = 1) tested negative for S1RBD antibodies, while 90.91% (n = 10) tested positive. Of the vaccinated patients, 3.70% (n = 1) were S1RBD negative

and 96.30% (n = 26) positive. Statistical analysis showed that percentages of IgGS1RBD results between vaccinated and unvaccinated patients with RRMS were not statistically significant (visit 1: $p = 0.089$; visit 2 $p = 0.501$). However, at visit 1, the number of positives was higher in the vaccinated group than in the unvaccinated group, which was significant at the trend level ($0.050 \leq p < 0.100$). In addition, a significant main effect of the time factor (the odds of getting a positive SARS-CoV-2 anti-S1RBD result at visit 2 was significantly higher (41.227-fold) than at visit 1. Detailed data concerning the presence of anti-S1RBD antibodies are shown in Table 3.

Table 3. Presence of anti-S1RBD IgG antibodies in COVID-19 vaccinated and unvaccinated patients at visits 1 and 2.

	IgG-S1RBD Results	Vaccination against SARS-CoV-2		*p*-Value [1]
		No	Yes	
Visit 1 (n = 38)	Negative	33.33% (n = 5)	8.70% (n = 2)	$p = 0.089$
	Positive	66.67% (n = 10)	91.30% (n = 21)	
Visit 2 (n = 38)	Negative	9.09% (n = 1)	3.70% (n = 1)	$p = 0.501$
	Positive	90.91% (n = 10)	96.30% (n = 26)	

[1] Fisher's exact test.

At visit 1, the mean anti-S1RBD antibody level was 16,863.40 AU/mL among vaccinated patients with RRMS and 197.90 AU/mL among unvaccinated. At visit 2, the mean level of anti-S1-RBD antibodies was 6997.30 AU/mL among vaccinated patients with RRMS and 1342.50 AU/mL among unvaccinated. The statistical analysis showed that vaccination had a statistically significant effect on anti-S1-RBD antibody levels at visit 1 ($p < 0.001$) and visit 2 ($p = 0.038$). Detailed data on the level of anti-S1RBD antibodies are given in Figure 1.

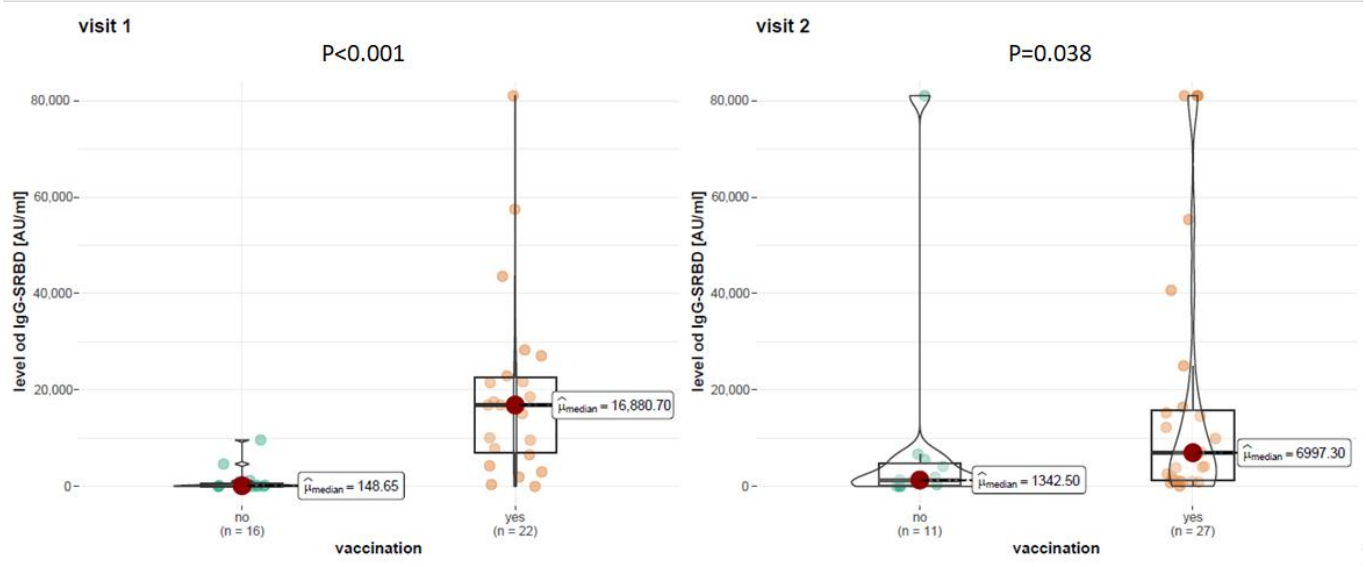

Figure 1. Level of anti-SRBD antibodies in COVID-19 vaccinated and unvaccinated patients at visit 1 and 2.

3.1.2. Impact of COVID-19

A positive antigen test or PCR was a COVID-19 infection indicator. At visit 1, patients withCOVID-19 (+), 100% (n = 7) were positive for anti-S1RBD antibodies. However, among patients withCOVID-19 (−), 77.42% (n = 24) were positive for anti-S1RBD antibodies. At visit 2, amongCOVID-19 (+) patients, 100% (n = 9) tested positive for anti-S1RBD antibodies. However, among COVID-19 (−) patients, 93.10% (n = 27) tested positive for anti-S1RBD

antibodies. Detailed data on the presence of anti-SRBD antibodies are shown in Table 4. The statistical analysis showed that the percentages of anti-S1RBD results between patients with COVID-19 (+) and COVID-19 (−) illness were not statistically significant (visit 1: $p = 0.309$; visit 2 $p = 1.00$).

Table 4. Presence of anti-SRBD IgG antibodies in patients with or without a registered history of COVID-19 at visit 1 and visit 2.

	IgG-SRBD Results	History of COVID-19		*p*-Value [1]
		No	Yes	
Visit 1 $n = 38$	Positive	77.42% ($n = 24$)	100% ($n = 7$)	$p = 0.309$
	Negative	22.58% ($n = 7$)	0.00%	
Visit 2 $n = 38$	Positive	93.10% ($n = 27$)	100% ($n = 9$)	$p = 1.00$
	Negative	6.90% ($n = 2$)	0.00%	

[1] Fisher's exact test.

History of COVID-19 registered positive polymerase chain reaction (PCR)/antigen test in the past. Among the patients with confirmed COVID-19, just one patient was hospitalized due to COVID-19, and the patient received convalescent plasma and steroids.

At visit 1, among COVID-19 (+) patients, the mean level of anti-S1RBD antibodies was 27,086.20 AU/mL, while among COVID-19 (−) patients, it was 1953.90 AU/mL. At visit 2, among COVID-19 (+) patients, the level of anti-S1RBD antibodies was 3886.90 AU/mL, while among COVID-19 (−) patients, it was 4165.20 AU/mL. Statistical analysis showed that COVID-19 survivors had statistically significantly higher levels of anti-S1RBD antibodies at visit 1 ($p = 0.001$) but not at visit 2 ($p = 0.410$). Detailed data on the level of anti-S1RBD antibodies are presented in Figure 2.

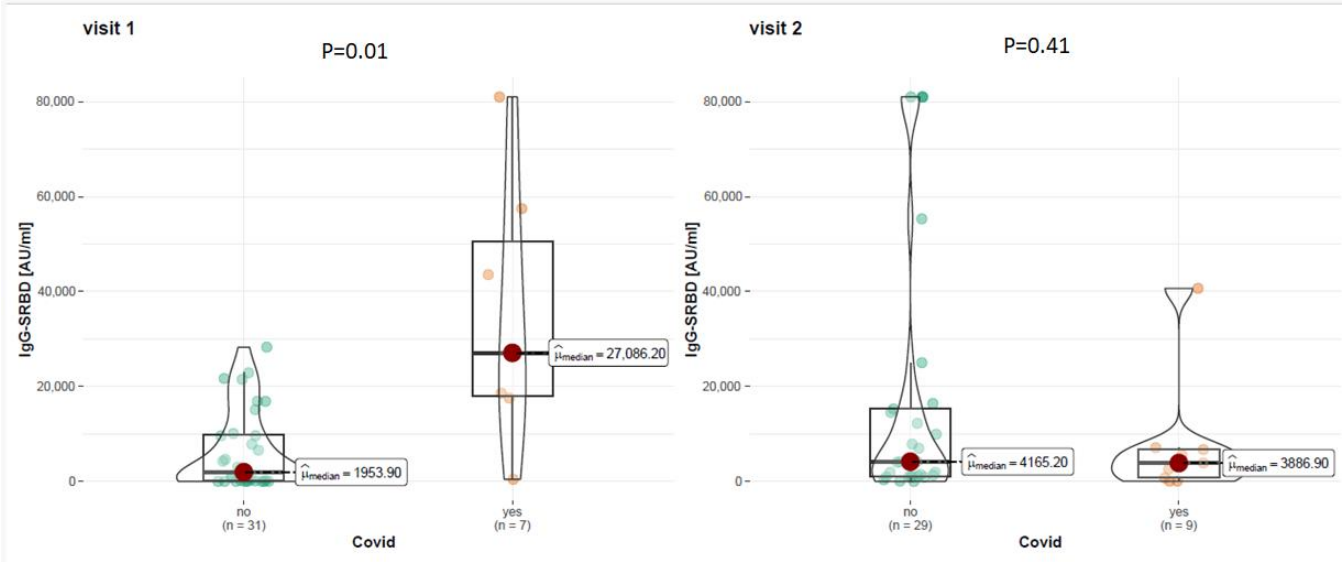

Figure 2. Level of anti-SRBD IgG antibodies in patients with or without registered history of COVID-19 at visit 1 and 2.

3.2. Analysis of Antibodies against N Protein

At Visit 1, 28.57% ($n = 2$) of COVID-19 (+) patients were positive for anti-N antibodies. However, among COVID-19 (−) patients, 9.68% ($n = 3$) were negative for anti-N antibodies.

At visit 2, 66.67% (n = 6) of COVID-19 (+) patients were positive for anti-N antibodies. Among COVID-19 (−) patients, 37.93% (n = 11) tested positive for anti-N protein antibodies. Detailed results of anti-N protein antibodies are shown in Table 5. Statistical analysis showed that the percentages of IgG-N results between patients withCOVID-19 (+) and COVID-19 (−) were not statistically significant (first visit: p = 0.223; second visit p = 0.249).

Table 5. Presence of IgG-N antibodies in patients with or without registered history of COVID-19 at visit 1 and visit 2.

	IgG-N Results	History of COVID-19		*p*-Value [1]
		No	Yes	
Visit 1 n = 38	Positive	9.68% (n = 3)	71.43% (n = 35)	p = 0.223
	Negative	90.32% (n = 28)	28.57% (n = 2)	
Visit 2 n = 38	Positive	37.93% (n = 11)	66.67% (n = 6)	p = 0.249
	Negative	62.07% (n = 18)	33.33% (n = 3)	

[1] Fisher's exact test. History of COVID-19 registered positive PCR/antigen test in the past.

At visit 1, among COVID-19 (+) patients, the mean level of IgG-N was 0.90 AU/mL, while among COVID-19 (−) patients, it was 0.14 AU/mL. At visit 2, amongCOVID-19 (+) patients, the level of anti-N antibodies was 1.99 AU/mL, while for COVID-19 (−) patients, it was 0.79 AU/mL. Detailed data on the level of anti-N antibodies are shown in Figure 3. The statistical analysis showed that COVID-19 (+) patients had statistically significantly higher levels of anti-N antibodies at visit 1 (p = 0.040) but not at visit 2 (p = 0.363). A significant main effect of time was observed, indicating that anti-N levels were significantly higher than at visit 1. Levels of antibodies IgG-S1RBD and IgG-N according to particular DMTs are presented in the Supplementary Materials.

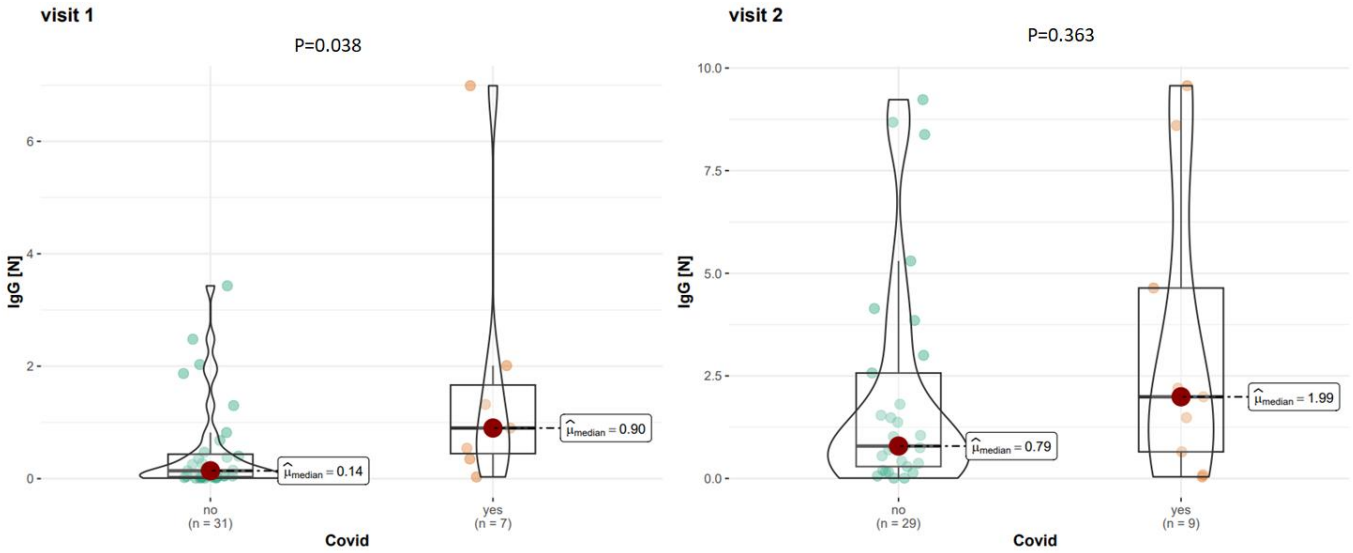

Figure 3. Level of IgG-N antibodies in patients with or without registered history of COVID-19 on visit 1 and 2.

4. Discussion

Our study shows the result of the analysis of both the presence and actual levels of antibodies directed against the receptor binding domain of the S1 protein and against

the N protein of the SARS-CoV-2 virus in patients with RRMS who were treated with DMF, GA, or INF. During the first months of the pandemic, it was unknown whether patients undergoing immunomodulatory treatment were at risk of a more severe course of the disease [17]. Current clinical experience shows that SARS-CoV-2 infection can impact on central nervous system, but patients with MS, in most cases, do not suffer more seriously from COVID-19 than the general population [14]. Risk factors for a more severe course, such as male sex, comorbidities, or severe disability, are similar to those in the non-MS group [18–20]. The subsequent months of the pandemic also showed that patients treated with selected and often used DMTs showed an adequate humoral response to vaccinations [21,22]. Moreover, the side effects of vaccinations against SARS-CoV-2 were mild, and the vaccinations themselves were safe in this group of patients [14,23]. During the first period of the pandemic, the serological status of patients was also assessed. It was shown that vaccinations significantly induced the production of neutralizing antibodies in patients treated with DMT, GA, or INF [24]. It is worth noting that it is currently known that in the group treated with anti-CD20 and sphingosine-1-phosphate modulators, the course of the disease may be more severe, and the immune response to vaccinations may be impaired [22]. Over the duration of the pandemic and after it ended, the group of patients who received subsequent doses of the vaccine, in addition to those who had COVID-19 infections, grew. For this reason, the serological status of this group of patients may change, and it seems that in addition to the presence of antibodies, their levels may also be important. Neutralizing IgG antibodies are known to increase from two to eight weeks post-infection, followed by a decline ranging from four to six months with a median time to seronegativity of approximately two years [25].

The literature shows that the most specific and the least likely to cross-react antibodies are those directed against the S1 protein receptor binding domain, so we tested these antibodies in our research [26]. At visit 1, the presence of these antibodies was found in almost 82% of the study group. Analyzing the subgroups at visit 1, we could see that in the vaccinated group, neutralizing antibodies were present in 91.30% of patients with RRMS. At visit 2, in the entire study group, neutralizing antibodies were found in 94.74% of patients, including 96.30% of vaccinated and 90.91% of unvaccinated patients. In the latter group of patients, the presence of neutralizing antibodies is probably due to passive immunization after asymptomatic contact with SARS-CoV-2. The results obtained in our study are similar to previously published studies. A meta-analysis by Gombolay et al. showed that the humoral response after vaccination occurs in 77% of patients with MS compared to 93% of the healthy population [22]. This study also analyzed individual DMTs and found that 96% of those were treated with INF, 95% of those treated with GA, and 99% of those treated with DMF [22]. However, results from the statistical analysis did not show a difference between vaccinated and unvaccinated people, which is probably due to the high prevalence of the SARS-CoV-2 virus in the population of patients with MS. In Poland, the OBSER-CO seroepidemiological study was conducted (IV series of analysis in 2021 and 2022) based on the WHO-Unity protocol: "Population-based age-stratified seroepidemiological investigation protocol for COVID-19 infection" [27]. Comparing the results obtained in our study to the results conducted as part of OBSER-CO in northeastern Poland on a group of patients ofa similar age, we can note that during visit 1, more patients with MS were vaccinated than in the general population (61% vs. 31 -58%). However, during visit 2, these proportions practically equalized (71% vs. 67%). Comparing the seroprevalence (presence of IgG-S antibodies) in the MS population to the general population in northeastern Poland during visit 1, it can be seen that the prevalence of neutralizing antibodies was much higher in the MS group (84.2% vs. 57–73%) [28]. This can be explained by the higher vaccination rate of the studied group of patients, as shown by previous data. During visit 2, within 1.5 years later, the seroprevalence was practically at the same level (94.7% vs. 93.4%). Data from OBSER-CO 2023 have not been published yet.

At visit 1, the number of neutralizing antibody positives was higher in the group vaccinated at the trend level ($p = 0.089$). Many of these patients treated with DMTs had

asymptomatic contact with the virus, which induced the presence of antibodies. In the next step, levels of antibodies directed against the receptor binding domain of the S1 protein were analyzed. A statistical analysis of these data showed that vaccinated patients with RRMS had statistically significantly higher antibody levels at both visits than unvaccinated subjects. It is worth noting that in vaccinated people with multiple sclerosis (PwMS), neutralizing antibody levels were lower at visit 2 (16,863.40 AU versus 6997.30 AU at visit 1). A similar observation was made after analyzing IgG-S1RBD and IgG-N according to particular DMTs. At visit 2, the median time since the last vaccination was longer than at visit 1 (<2 months since the last vaccination at visit1 versus approximately 12–18 months at visit 2). Due to this strong induction of antibody levels, subsequent vaccination doses against COVID-19 are still highly recommended forMS patients. Interestingly, the level of neutralizing antibodies in unvaccinated patients increased at visit 2 (1342.5 AU) compared to visit 1 (197.80 AU) but did not reach the level observed in vaccinated PwMS. However, it should be noted that, currently, the level of neutralizing antibodies that would protect against COVID-19 has not been determined. It is not known whether a higher level clearly means higher protection. Research conducted by Hickey et al. on the general population showed that the levels of antibodies in vaccinated people were significantly higher than in people after infection. Studies show that in addition to antibody levels, avidity was higher in the vaccinated group, which may even better reflect the level of protection against reinfection. For each vaccine, circulating antibody levels decreased one to four months after the second dose [29]. It is worth emphasizing at this point that the protective level of neutralizing antibodies has not yet been determined. In a multicenter study, a group of 2nd and 3rd doses administered to patients with MS similarly caused a decrease in neutralizing antibodies within six months post-vaccination but still remained high compared to unvaccinated subjects. We also analyzed IgG antibodies against the N protein, which are induced only after natural contact with the virus. No significant differences in the percentage of positive results between people with and without previous COVID-19 were found. After analyzing the levels of antibodies, a statistically significantly higher level of antibodies was shown only during the first visit (2021). This seems to be related to less frequent testing of patients forSARS-CoV-2 during the subsequent years of the pandemic (second visit in 2023) and to the greater prevalence of the virus in the population. In addition, a significant factor is also the short duration of antibodies directed against the N protein, namely, less than one year. All these factors make the interpretation of the presence and levels of anti-N protein antibodies difficult and should be closely correlated with the clinical status of the patient. Literature shows that vaccinated people who contracted COVID-19 have higher levels of antibodies compared to people who were only vaccinated or only after natural infection (hybrid immunity) [30]. An interesting observation is that during visit 2, in the subgroup of patients with confirmed COVID-19 in the past, the level of neutralizing antibodies was lower than in the group of patients without documented COVID-19 (3886.9 AU versus 4165.2 AU). In addition to high vaccination rates in the subsequent years of the pandemic, it can be assumed that a significant percentage of the population and patients with MS have already had natural contact with the virus. Our data shows that none of the participants tested positive (PCR or antigen test) for COVID-19 between visits 1 and 2. Moreover, the levels of IgG-N at visit 2 were higher than that in visit 1 in vaccinated and unvaccinated patients and according to particular DMTs. That may indicate that part of the study group probably had SARS-CoV-2 infection and did not decide to test or had asymptomatic infections. Recent studies and literature reviews also point to the importance of assessing not only antibodies but also cell-mediated immunity, which seems to last longer than humoral immunity [31].

Our research has limitations, one of which includes a small study group. The small size probably contributed to the limitations of statistical analyses and did not allow for reliable analysis of particular DMT subgroups. In conclusion, our research shows that, in addition to the presence of antibodies against the S1 protein (RBD), it is important to assess their levels. Patients with RRMS who were vaccinated against SARS-CoV-2 had

significantly higher levels of neutralizing antibodies in subsequent years of the pandemic. The assessment of anti-N antibodies is difficult due to the high seroprevalence of the virus in the population and the short half-life and should be closely correlated with the clinical picture.

5. Conclusions

Our research shows that SARS-CoV-2 vaccinated patients with RRMS treated with DMT, GA, or INF have statistically significantly higher levels of antibodies directed against the receptor binding domain of the S1 protein compared to unvaccinated. This was observed over the course of two years of the pandemic. Levels of neutralizing antibodies seem to better reflect the level of protection against the SARS-CoV-2 virus than their presence alone, but this requires further research. In the presented retrospective study, it was observed that patients treated with the selected DMTs (INF, GA, DMF) were immunocompetent in terms of the production of neutralizing antibodies. In conjunction with the above data and current world literature, recommendations for preventive vaccinations for MS patients are justified. Although a clearly protective level of antibodies has not been currently determined, a higher level potentially provides better protection for patients against disease and reinfection. Further randomized studies are still necessary.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/vaccines12030255/s1, Table S1. IgG-S1RBD and IgG-N levels according to particular DMTs.

Author Contributions: Conceptualization, J.K. (Joanna Kulikowska), B.M., A.K. and K.K.-T.; methodology, J.K. (Joanna Kulikowska), M.G.-S. and B.M.; software, A.M. and M.B.; validation, J.K. (Joanna Kulikowska), A.M., A.K., J.K. (Jan Kochanowicz) and A.C.; formal analysis, A.M, M.B. and J.K. (Joanna Kulikowska); investigation, J.K. (Joanna Kulikowska), A.K.-P. and K.K.-T.; resources, J.K. (Joanna Kulikowska), M.G.-S. and W.B.; data curation, J.K. (Joanna Kulikowska), M.G.-S. and W.B.; writing—original draft preparation, J.K. (Joanna Kulikowska), K.K.-T., A.M., M.B., M.G.-S. and A.K.-P.; writing—review and editing, A.C., A.K., W.B., J.K. (Jan Kochanowicz) and B.M.; visualization, J.K. (Joanna Kulikowska) and A.K.-P.; supervision, J.K. (Jan Kochanowicz) and A.K.; project administration, J.K. (Joanna Kulikowska) and K.K.-T.; funding acquisition, J.K. (Jan Kochanowicz). All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was approved (approval No. APK.002.230.2020) by the Bioethics Committee at the Medical University of Bialystok, Poland.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are contained within the article and supplementary materials.

Conflicts of Interest: The authors declare no conflicts of interest.

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vaccines

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Brief Report

Improving the Antigenicity of SARS-CoV-2 Vaccine Genes by Merging Mutations from Different Variants of Concern

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Abstract: During the COVID-19 pandemic, the early emergence of viral variants repeatedly undermined the effects of vaccination. Our aim here is to explore strategies for improving spike vaccine gene antigenicity by merging mutations from different variants of concern (VOCs) in a single vaccine gene. To this end, newly developed recombinant vaccine genes were designed, cloned into adenoviral vectors, and applied to C57BL/6 mice; then, serum-neutralizing antibodies against the wildtype SARS-CoV-2 strains were determined in neutralization assays. The merger of mutations from different variants of concern (alpha, beta, gamma, and delta) in a single recombinant spike-based vaccine gene provided a substantial improvement in neutralizing immunity to all variants of concern, including the omicron strains. To date, only unmodified spike genes of the original SARS-CoV-2 Wuhan strain (B.1) or dominant variants (BA.1, BA.5, and XBB.1.5) have been used as vaccine genes. The employment of unmodified vaccine genes is afflicted by limited cross-protection among variant strains. In contrast, recombinant vaccine genes that combine mutations from different strains in a single gene hold the potential to broaden and improve immune protection and might help to reduce the need for frequent vaccine adaptations in the future.

Keywords: SARS-CoV-2 vaccine; neutralizing antibodies; mutations; vaccine genes; cross protection

Citation: Herwig, S.; Adler, J.M.; Vladimirova, D.; Trimpert, J.; Sehouli, J.; Cichon, G. Improving the Antigenicity of SARS-CoV-2 Vaccine Genes by Merging Mutations from Different Variants of Concern. *Vaccines* **2024**, *12*, 248. https://doi.org/10.3390/vaccines12030248

Academic Editor: Ralph A. Tripp

Received: 5 January 2024
Revised: 22 February 2024
Accepted: 22 February 2024
Published: 27 February 2024

1. Introduction

On 13 January 2020, only about 2 months after cases of severe pneumonia began to occur frequently in Wuhan, China, the Chinese health authorities identified a novel coronavirus as the cause of an emerging infectious disease and published its sequence data. On 30 January 2020, these spreading infections were classified by the WHO as a global event of concern and finally declared a pandemic on 11 March 2020. Only 11 months later, in December 2020, after the successful completion of phase III clinical trials, the first mRNA-based vaccine from Pfizer/BioNTech was approved for clinical use by health authorities in most countries. Based on the sequence data of the original SARS-CoV-2 Wuhan B.1 strain, the mRNA of the prefusion-stabilized spike protein was used as the virus-specific vaccine gene.

However, as early as May 2020, while the first SARS-CoV-2 vaccine was still being developed, the beta variant appeared in South Africa and, on account of its rapid spread, was classified as a variant of concern (VOC) 8 months later. In September 2020, the so-called alpha variant was isolated in England; in October 2020, the delta variant followed in India; shortly afterwards, in November 2020, the gamma variant was isolated in Brazil; and finally, 1 year later, in November 2021, the first omicron variant was isolated in South Africa.

The emergence of viral variants inevitably raised questions about the efficacy of the vaccine released in December 2020. Neutralization tests using the sera from vaccinated

individuals showed that SARS-CoV-2 vaccines based on the sequence of the original Wuhan B.1 conferred reduced neutralizing activity against the novel variants. Neutralizing antibody titers (NSTs) dropped to 25–50% for the alpha variant, to 10–15% for the beta and gamma variants, and to less than 5% for the omicron BA.1 variant [1–4]. Although the frequency of severe disease progression was further reduced by activation of the cellular immune system, vaccination studies on more than 28,000 vaccinated individuals showed that a lower titer of neutralizing antibodies was clearly associated with an increased risk of symptomatic disease progression [5]. The nearly complete loss of neutralizing activity in the omicron BA.1 variant made adaptations of the vaccine design necessary. BioNTech/Pfizer then delivered a bivalent vaccine in autumn 2022, which, in addition to the spike mRNA of the B.1 strain, also contained the corresponding counterpart of the omicron BA.1 strain. Nevertheless, as early as January and February 2022, two new, rapidly spreading omicron variants (omicron BA.4 and BA.5) were isolated in Africa, which were satisfactorily neutralized neither by a previous infection with omicron BA.1 nor by vaccination with the new bivalent vaccine from BioNTech/Pfizer [6], necessitating re-adaptation of the mRNA vaccines to account for these two omicron variants. Fortunately, the omicron variants were overall characterized by a lower morbidity, despite their high infectivity. Nevertheless, in Germany alone, 19,000 people died with or from COVID-19 in 2023, and a similar number is expected for 2024 (https://corona-pandemieradar.de/de/todesfaelle, accessed on 1 January 2024).

SARS-CoV-2 Spike Protein Binds to the ACE2 Receptor

The primary goal of SARS-CoV-2-specific vaccines is to induce the formation of neutralizing antibodies against the viral spike surface protein, as this protein plays a crucial role in coupling to human host cells. The binding partner of the viral spike protein on human cells is the angiotensin-converting enzyme receptor 2 (ACE2) [7]. The physiologic function of ACE2 is blood pressure regulation and protection against excessive inflammatory reactions by inactivating circulating angiotensin [8]. ACE2 is predominantly expressed in the upper airways, the lungs (alveolar cells), the myocardium, the gastrointestinal tract, endothelia (blood vessels), and the central nervous system, which explains the main symptoms of respiratory tract infections, pneumonia, fever, diarrhea, and loss of smell [9].

Due to the structural similarity of their ACE2, not only humans but also several animal species can become infected with SARS-CoV-2. Primates are particularly at risk, followed by deer and marine mammals (whales and dolphins), but cattle and goats can also be infected. Pets such as dogs and cats are at lower risk, while birds and reptiles are hardly infected [10].

The replication of RNA viruses like SARS-CoV-2 is afflicted by a high number of replication errors that not only change the functional but also antigenic properties of viral proteins and can impair vaccine-mediated immune protection. There was some hope that through repeated boosting, even weaker antigen epitopes might contribute to immune protection and will reduce the need for vaccine adaption [11,12]. However, with the appearance of the first omicron variants and the almost-complete failure of first-generation vaccines to provide sufficient antibody protection, it became evident that adapted vaccines are strongly required [4].

To date, only original spike genes from currently dominant SARS-CoV-2 strains and no recombinant genes have been employed for clinical application.

The aim of the current study is to explore whether the cross-protective properties of vaccine genes against different variants of concern (VOCs) can be improved by merging characteristic mutations from different strains in a single recombinant vaccine gene.

2. Material and Methods

Based on the sequence information for the complete SARS-CoV-2 spike protein of the Wuhan strain B.1 (NCBI reference sequence: NC_045512.2) and the mutation patterns of the alpha, beta, gamma, delta, lambda, and omicron BA.1 variants (https://gisaid.org/,

accessed on 1 January 2024), recombinant genes were designed in silico, and a synthesis was commissioned by GeneArt AG (Thermo Fisher Scientific, Regensburg, Germany). All designed vaccine genes were codon-optimized, and six prefusion-stabilizing mutations were inserted at positions F817P, A892P, A899P, A942P, K985P, and V987P to increase their levels of expression [13]. To facilitate flow cytometric identification and to ensure the integrity of the recombinant gene, an HA-tag (YPYDVPDYA) was added to the 3′ end of each recombinant gene.

2.1. Vaccine Genes

(1) **Wuhan B.1** (1273 aa): original B1 sequence (NC_045512.2) prefusion stabilized by F817P, A892P, A899P, A942P, K986P, V987P.

(2) **AG** (**a**lpha–beta–**g**amma) **gene** (1270 aa): encoding amino acid substitutions/deletions of VOCs alpha, beta, and gamma: L18F, T20N, P26S, HV 69–70 del, D138Y, Y144 del, R190S, K417T, E484K, N501Y, A570D, D614G, H655Y, P681H, T716I, S982A, T1027I, D1118H prefusion stabilized by F817P, A892P, A899P, A942P, K986P, V987P.

(3) **AL** (**a**lpha–beta–gamma–delta–**l**ambda) **gene** (1261 aa): encoding amino acid substitutions/deletions of VOCs alpha, beta, gamma, delta, and lambda: L18f, T19R, T20N, P26S, HV-DEL 69–70, G75V, T76I, D138Y, E156G, FR-DEL 157–158, R190S, D215G, RSYLTPG-DEL 246–252, D253N, K417T, L452R, E484Q, F490S, N501Y, D614G, H655Y, P681R, T716I, T859N, D950N, S982A, T1027I, D1118H, V1176F prefusion stabilized by F817P, A892P, A899P, A942P, K986P, V987P.

(4) **DO** (**d**elta–**o**micron) **gene** (1270 aa): encoding amino acid substitutions/deletions of VOCs delta and omicron BA.1: T19R, T20N, L24 del, A27S, A67V, HV69,70 del, T95I, G142D, del 143–145, Y145 del, E156G, del 157–158, del 211, L212I, Ins 214 EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, L452R, S477N, T478K, E484A, Q493K, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N8 56K, Q954H, N969K, L981F, prefusion stabilized by F817P, A892P, A899P, A942P, K986P, V987P.

2.2. Cloning, Generation, and Purification of Adenoviral Vaccines

The in silico design of the recombinant genes was performed with clone manager basic 9 (Sci Ed software clone manager basic 9, Westminster, CO, USA). The synthesis of the recombinant vaccine genes was performed by GeneArt AG (Thermo Fisher Scientific, Regensburg, Germany). All recombinant constructs were sequenced to check for error-free synthesis. The recombinant genes were first cloned into an adenoviral transfer plasmid (pAd2-CMV-pA-trans) and subsequently inserted into the adenoviral backbone (pAd2-GVL) by homologous recombination. Recombination was performed as a co-transfection of linearized plasmids into *E. coli* BJ5183 (#16398, Addgene, Watertown, MA, USA). The identification of correct clones was performed with a restriction analysis of purified plasmids.

For the generation of the recombinant viruses, adenovirus plasmids were transfected on 293 cells by calcium phosphate/DNA coprecipitation, and subsequent soft-agar overlay (1% Agar Noble (PBS), Carl Roth, Karlsruhe, Germany) was performed. After 8–10 days, the first plaques became visible and were isolated using a micropipette and expanded on the 293 cells (DMEM 10% FCS). The release from the 293 cells and purification of the recombinant viruses were performed using three freeze–thaw cycles and two rounds of cesium chloride density centrifugation, as described elsewhere [14]. The titer viral stocks were determined with a serial dilution assay on 96-well plates. The identity and integrity of cloned SARS-CoV-2 vaccine genes were confirmed after the transfection of recombinant viruses on 293 cells (MOI 10). After 24 h, the transgene expression was explored by immune cytological staining and flow cytometry (MACSQuant Analyzer, Miltenyi Biotec Inc., Bergisch-Gladbach, Germany). The employed antibodies were rabbit polyclonal FITC anti-SARS-CoV-2-spike glycoprotein (Ab01691-23.0, Biozol, Eching, Germany) and FITC anti-HA (130-120-723, 51429 Bergisch-Gladbach, Germany).

2.3. Animals and Vaccination

Animal experiments were conducted in accordance with the European Guidelines for Animal Studies, following approval by both the Institutional Animal Care Committee and the relevant state authority (Landesamt für Gesundheit und Soziales, Berlin, approval number G0017/21). C57BL/6 mice aged 6–8 weeks were procured from Charles River (Sulzfeld, Germany). A total of five groups, each consisting of six animals, were established, and immunization was administered via intramuscular injections of 1×10^9 i.p. Ad2-Wuhan-B.1, Ad2-AG, Ad2-AL, Ad2-DO, and Ad2-lacZ (Ad-mock). Three weeks post immunization, blood samples were collected, and the sera were isolated and stored at −80 °C.

2.4. SARS-CoV-2 Wildtype Viruses

The genetically modified live-attenuated SARS-CoV-2 mutant sCPD9 and SARS-CoV-2 variants B.1 (BetaCoV/Munich/ChVir984/2020; B.1, EPI_ISL_406862), beta (B.1.351; hCoV19/Netherlands/NoordHolland_20159/2021), and delta (B.1.617.2; SARS-CoV-2, Human, 2021, Germany ex India, 20A/452R (B.1.617)) were cultured on Vero E6-TMPRSS2 cells. The omicron BA.1 variant (B.1.1.529.1; hCoV-19/Germany/BE-ChVir26335/2021, EPI_ISL_7019047) was cultured on CaLu-3 cells. Prior to experimental infection, all virus stocks underwent whole-genome sequencing to ensure genetic integrity, particularly at the furin cleavage site. The viral stocks were stored at −80 °C before experimental use.

2.5. Serum Neutralization Tests (SNTs)

The murine serum complement was heat-inactivated for 30 min at 56 °C and then prepared in duplicate as two-fold serial dilutions (ranging from 1:32 to 1:4096) in MEM supplemented with 1% FBS (PAN Biotech, Aidenbach, Germany), 100 IU/mL penicillin G, and 100 mg/mL streptomycin (Carl Roth, Karlsruhe, Germany) in 96-well cell culture plates (Sarstedt, Nümbrecht, Germany). Each serum dilution and corresponding control well received 200 pfu of a SARS-CoV-2 variant, followed by a 1 h incubation at room temperature. The two-fold dilutions were then plated on Vero E6 cells cultured in 96-well plates and incubated at 37 °C under a 5% CO_2 atmosphere for 3 days (for the B.1, alpha, beta, gamma, and delta variants) or 4 days (for the omicron BA.1 and BA.5 variants). Subsequently, the plates were fixed with 4% formaldehyde and stained with 0.75% crystal violet (in an aqueous solution) to assess the cytopathic effects (CPEs). Successful virus neutralization was indicated by the wells showing no evidence of CPE, and the last effective serum dilution was recorded.

3. Results

A single immunization of C57BL6 mice with an adenoviral vector encoding the prefusion-stabilized Wuhan B.1-derived spike gene induced the formation of neutralizing antibodies that differed substantially in their neutralizing properties between SARS-CoV-2 variants of concern. Assuming that the neutralizing capacity of the Wuhan B.1-based vaccine for the original SARS-CoV-2 Wuhan B.1 strain is optimal and can be set to 100%, cross-protection towards the alpha variant reached 75% and that towards the delta variant reached 50%. In the SARS-CoV-2 beta and gamma strains, the neutralizing serum properties of the original vaccine were lower and did not exceed 10% compared to their neutralizing properties against the parental Wuhan B.1 strain. Against the omicron BA.1 and BA.5 strains, the neutralizing serum antibodies were barely detectable (Figure 1a). This response pattern matched well with the results of the human serum neutralization assays using the Wuhan B.1-based vaccine from BioNTech/Pfizer (Wuhan B.1: 100%, alpha variant: 25–50%, beta and gamma variant: 10–15%; omicron BA-1 < 5%) [1–4] and suggests that the results obtained from C57Bl6 mice have a predictive value for clinical applications.

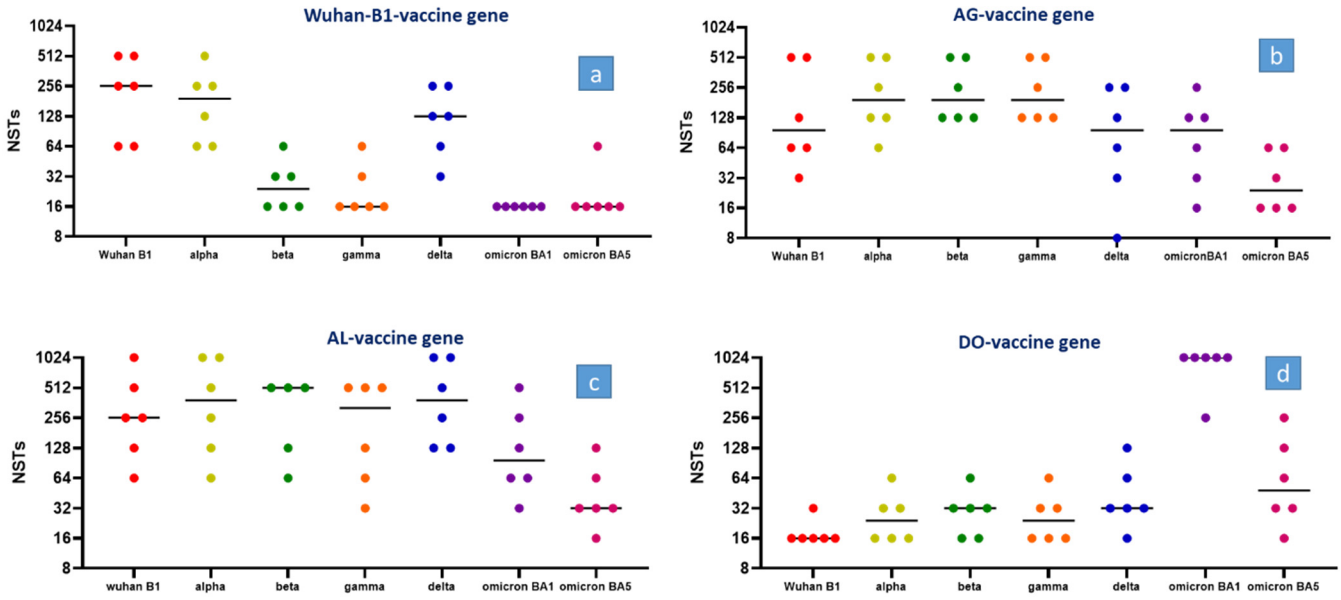

Figure 1. (**a**–**d**) C57BL6 mice (n = 6) were vaccinated with a single intramuscular injection of 1×10^9 i.p. replication-deficient recombinant adenovirus (hAd2) encoding the complete prefusion-stabilized SARS-CoV-2 spike gene of the original Wuhan B.1 strain (**a**) and three recombinant spike genes (**b**–**d**). The AG gene encodes mutations of the SARS-CoV-2 alpha, beta, and gamma strains; the AL gene, mutations of the alpha, beta, gamma, delta, and lambda strains; and the DO gene, mutations of the delta and omicron BA.1 strains. Mice were bled 3 weeks after immunization, and serum neutralization tests were performed, employing wildtype viruses of the Wuhan B.1 strain and the alpha, beta, gamma, delta, and omicron BA.1 and BA.5 variants.

In order to investigate the extent to which the merger of mutations from different variants leads to improved immune protection, mutations from the alpha, beta, and gamma variants were combined to create the AG (**a**lpha–beta–**g**amma) vaccine gene. The immunization of mice with an adenoviral vector that encodes the AG gene improved the neutralizing properties for the beta and gamma variants by almost an order of magnitude and improved immune protection towards the omicron BA.1 and omicron BA.5 strains (Figure 1b).

The AL (**a**lpha–beta–gamma–delta–**l**ambda) gene encodes for mutations of two additional strains and thereby unifies mutations of the alpha, beta, gamma, delta, and lambda variants. The insertion of additional mutations had no negative impact on the vaccine gene performance. Moreover, it increased the neutralizing properties against the delta strains while maintaining moderate reactivity against the omicron BA.1 and BA.5 strains (Figure 1c).

As expected, the DO (**d**elta–**o**micron) gene, which combines mutations of the delta and omicron BA.1 variants, showed excellent neutralizing properties towards the omicron BA.1 variant but failed almost completely against all other VOCs (Figure 1d). Even against the omicron BA.5 strain, the omicron BA.1 vaccine showed only limited cross-protection and did not exceed 10% of the NST levels compared to the titers against the BA.1 strain.

After the immunization of mice with a control adenoviral vector (Ad2-lacZ), no SARS-CoV-2-specific neutralizing antibodies were detected. Therefore, a graphical presentation was omitted.

The spike antigen of the original Wuhan B.1 strain induces high titers of neutralizing antibodies against the original B.1 strain and a fair response against alpha and the delta strain (reduction of 30–50%). Against the beta and gamma strain, the neutralizing properties dropped to less than 10% while the neutralizing antibodies against omicron BA.1 and BA.5 were barely detectable.

The overall performance of the AG gene, which encodes mutations of the alpha, beta, and gamma strains, was substantially better. Except a slight reduction in the neutralizing antibody titers against the B.1 strain, a substantial increase in the neutralizing capacity against beta, gamma, and omicron BA.1 and omicron BA.5 was noticed, while the response towards the alpha strain was preserved.

Employing the AL vaccine gene (encoding the alpha, beta, gamma, delta, and lambda mutations) provides a further increase in the neutralizing activity against the delta strain, while the response to the other strains was mostly comparable to that of the AG gene.

In sharp contrast to these results, the DO gene, which encodes the delta and omicron BA.1 mutations, shows excellent neutralizing properties against omicron BA.1 but mostly failed to neutralize the other strains.

4. Discussion

In this study, we were able to improve the effectiveness of SARS-CoV-2 vaccines against newly emerged virus variants by combining the mutations of different SARS-CoV-2 variants in a single vaccine gene. The recombinant vaccine genes designed in our study combine mutations of the alpha, beta, gamma, delta, and lambda variants and show excellent neutralizing properties towards all older VOCs (alpha–delta). Moreover, they exhibit improved reactivity towards the omicron variants BA.1 and BA.5 compared to the Wuhan B.1-based vaccine.

Interestingly, a comparison of the mutation pattern between the three explored vaccine genes reveals only five matches (Figure 2): T20N, HV69-70del, N501Y, D614G, and H655Y. When projected onto the receptor-binding domain, they share only one mutation, which is N501Y. Despite the little congruence in mutation pattern, the improved antigenicity of the AG and AL genes is evident, which suggests a certain impact on the specific antigenicity of these five mutations. In future vaccine gene designs, it seems less important to consider a large number of mutations than to identify and select only a few of the most relevant ones.

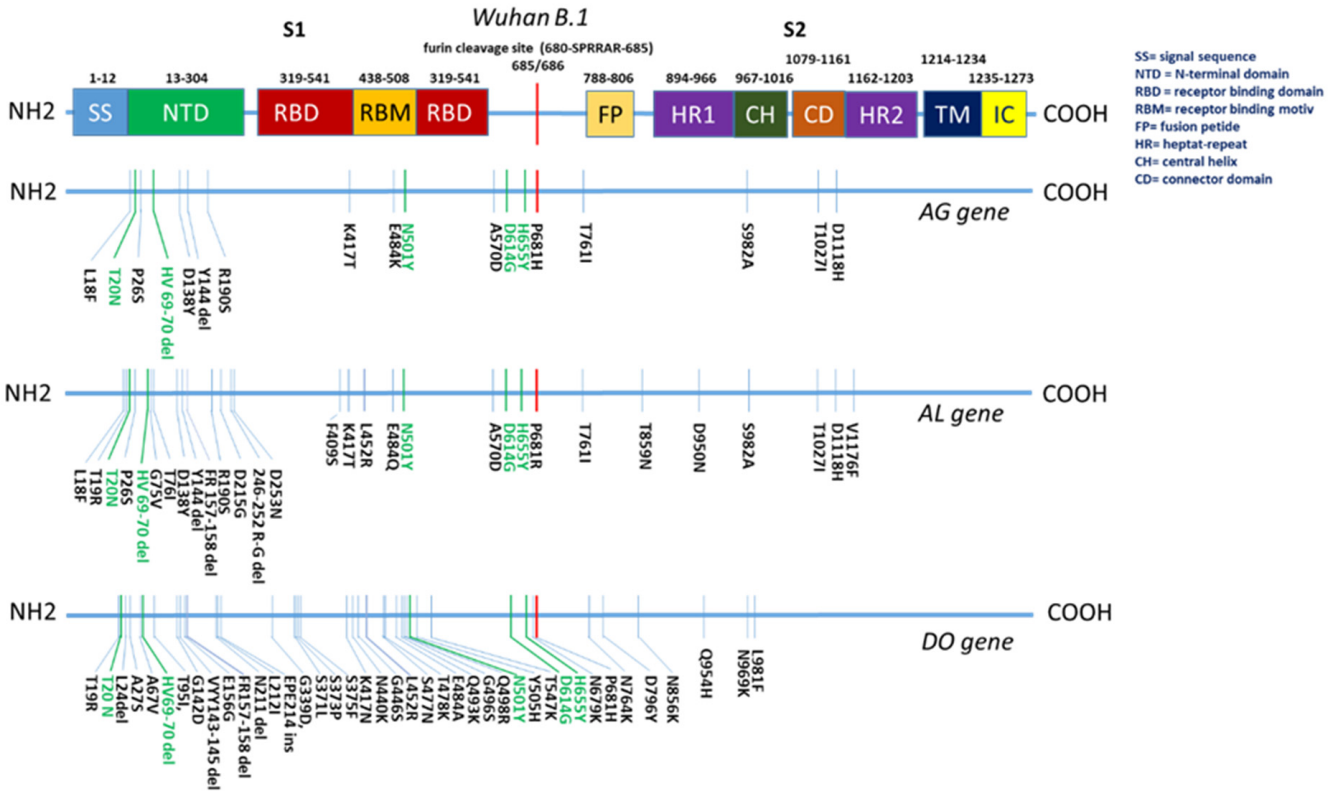

Figure 2. The position of the mutations in the three vaccine genes in the projection onto functional areas of the spike protein. The mutations in light green are present in all three genes.

The most recent SARS-CoV-2 mRNA vaccine, which was approved in autumn 2023, is based on the sequence data of the omicron variant XBB.1.5. Since this variant was isolated for the first time in the USA in October 2022, several omicron variants, namely BQ.1.1 (Cerberus), XBB.1.16 (Arcturus), XBB.2.3 (Acrux), EG5 (Eris), and BA2.86 (Pirola), have emerged and challenged the protective potential of the novel SARS-CoV-2 vaccine.

To date, only original spike genes of dominant variants have been used as vaccine genes (B.1, BA.1, BA.5, and XBB.1.5). These genes regularly provide limited cross-protection among different variants of concern. In particular, spike vaccine genes derived from the omicron strains provide limited neutralizing properties and fail to protect against older VOCs. We therefore hope to initiate a discussion about the clinical employment of recombinant vaccine genes that encode mutations of different strains and that have the potential to broaden immune protection and to hopefully reduce the need for frequent vaccine adaptations in the future.

Author Contributions: Investigations: S.H., J.M.A., D.V. and J.T.; methodology: J.T.; resources: J.S. and G.C.; conceptualization, writing—review and editing: G.C., S.H., J.M.A., D.V. and J.T. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the Berlin Institute of Health at Charité—Universitätsmedizin Berlin (funding number: IA53010372).

Institutional Review Board Statement: All animal experiments were approved by the Landesamt für Gesundheit und Soziales, Berlin (approval number G0017/21).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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vaccines

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Article

Long-Term Immunogenicity and Safety of a Homologous Third Dose Booster Vaccination with TURKOVAC: Phase 2 Clinical Study Findings with 32-Week Post-Booster Follow-Up

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Abstract: Vaccine-induced immunity wanes over time and warrants booster doses. We investigated the long-term (32 weeks) immunogenicity and safety of a third, homologous, open-label booster dose of TURKOVAC, administered 12 weeks after completion of the primary series in a randomized, controlled, double-blind, phase 2 study. Forty-two participants included in the analysis were evaluated for neutralizing antibodies (NAbs) (with microneutralization (MNT_{50}) and focus reduction ($FRNT_{50}$) tests), SARS-CoV-2 S1 RBD (Spike S1 Receptor Binding Domain), and whole SARS-CoV-2 (with ELISA) IgGs on the day of booster injection and at weeks 1, 2, 4, 8, 16, 24, and 32 thereafter. Antibody titers increased significantly from week 1 and remained higher than the pre-booster titers until at least week 4 (week 8 for whole SARS-CoV-2) ($p < 0.05$ for all). Seroconversion (titers $\geq$ 4-fold compared with pre-immune status) persisted 16 weeks (MNT_{50}: 6-fold; $FRNT_{50}$: 5.4-fold) for NAbs and 32 weeks for S1 RBD (7.9-fold) and whole SARS-CoV-2 (9.4-fold) IgGs. Nine participants (20.9%) tested positive for SARS-CoV-2 RT-PCR between weeks 8 and 32 of booster vaccination; none of them were hospitalized or died. These findings suggest that boosting with TURKOVAC can provide effective protection against COVID-19 for at least 8 weeks and reduce the severity of the disease.

Keywords: booster; COVID-19; immunogenicity; inactivated vaccine; neutralizing antibody; S1 RBD; safety; SARS-CoV-2; seroconversion; TURKOVAC

Citation: Sezer, Z.; Pavel, S.T.I.; Inal, A.; Yetiskin, H.; Kaplan, B.; Uygut, M.A.; Aslan, A.F.; Bayram, A.; Mazicioglu, M.; Kalin Unuvar, G.; et al. Long-Term Immunogenicity and Safety of a Homologous Third Dose Booster Vaccination with TURKOVAC: Phase 2 Clinical Study Findings with 32-Week Post-Booster Follow-Up. *Vaccines* **2024**, *12*, 140. https://doi.org/10.3390/vaccines12020140

Academic Editors: Rishi Jaiswal, Srijani Basu, Suman Gupta and Sneh Lata Gupta

Received: 14 January 2024
Accepted: 19 January 2024
Published: 29 January 2024

1. Introduction

Coronavirus disease 2019 (COVID-19) is a highly contagious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which became a pandemic in March 2020, three months after China reported the first case [1]. As of 31 August 2023, the infection had affected more than 770 million people worldwide and caused approximately 7 million deaths [2].

The high transmissibility of SARS-CoV-2 has made vaccination a key pillar of the fight against COVID-19 [3]. Tremendous efforts have been made to develop, manufacture, and distribute safe and effective vaccines against SARS-CoV-2 to reduce the spread and severity of the infection and the associated hospitalizations and deaths [3–6].

Knowledge gained about family *Coronaviridae* during severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) outbreaks, advances in vaccine technology, and collaboration between academia, manufacturers, regulatory agencies, and funding organizations have enabled an accelerated COVID-19 vaccine development process without compromising safety and quality [3,4,7]. Several vaccines became available outside a clinical trial setting within a year after the infection first appeared [4]. As of July 2023, there have been 13 COVID-19 vaccines authorized for emergency use by the World Health Organization (WHO), and hundreds of vaccine candidates are in various stages of development [8].

Türkiye was one of the first countries to initiate research on COVID-19 vaccine development [9]. ERUCoV-VAC, later named TURKOVAC, is an inactivated whole-virion SARS-CoV-2 vaccine developed under the national vaccine development program. Preclinical and interim phase 1 (NCT04691947) and 2 (NCT04824391) trial results of the vaccine have been previously published [10,11]. Based on the immunogenicity and safety findings from these trials, a regimen of two intramuscular (im) injections of TURKOVAC 3 μg administered 28 days apart is recommended for primary immunization [11]. The vaccine has been available in Türkiye since December 2021 with emergency use authorization granted by the Turkish Ministry of Health, and the development program is ongoing [12].

Although COVID-19 is no longer considered a Public Health Emergency of International Concern (PHEIC) [13] as of May 2023, it remains an ongoing health issue due to the emergence of new variants and the waning vaccine-induced immune responses over time. Therefore, booster vaccination has been suggested, especially for at-risk populations, to enhance immunity against SARS-CoV-2 [14].

The Hybrid COV-RAPEL TR Study (NCT04979949) demonstrated that heterologous boosting with TURKOVAC 90 to 270 days after receiving two doses of the CoronaVac vaccine stimulated a significant immune response that persisted up to post-booster Day 84 with acceptable safety and tolerability [15]. However, there was a gap in knowledge about the outcomes of homologous boosting with TURKOVAC. Therefore, we investigated the long-term (32 weeks) immunogenicity, safety, and efficacy of a third, homologous, open-label booster dose of the vaccine in healthy adults administered 12 weeks after completion of the primary series in a randomized, placebo-controlled, double-blind, phase 2 study.

2. Materials and Methods

2.1. Study Design and Participants

In a randomized, double-blind, placebo-controlled, phase 2 immunogenicity and safety trial of the inactivated COVID-19 vaccine TURKOVAC, healthy volunteers <65 years of age were randomly assigned (2:2:1) to receive two intramuscular injections of TURKOVAC 3 μg or 6 μg or a placebo (0.9% saline) 28 days apart. Considering the immunogenicity and safety results for the primary series [11], TURKOVAC 3 μg was selected as the optimal dose to continue the clinical development program, and the study protocol was amended to investigate the immunogenicity and safety of a booster dose of TURKOVAC 3 μg. Subjects who had received two doses of TURKOVAC 3 μg for primary immunization during the study were invited to participate in the booster substudy. Those who gave their consent to receive the booster dose and had had a recent negative reverse transcriptase polymerase

chain reaction (RT PCR) test for SARS-CoV-2 received a third dose of the vaccine 12 weeks after the second dose and were followed up to 32 weeks after the booster injection.

This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee for Clinical Trials of Erciyes University (17 June 2021; 2021/396) and the Turkish Ministry of Health (18 June 2021; E-66175679-514.02.01-463635). Written informed consent was obtained from all subjects involved in the study. The trial is registered on ClinicalTrials.gov (NCT04824391) (10 February 2021).

2.2. Procedures and Outcomes

A microneutralization test (MNT_{50}) and focus reduction neutralization test ($FRNT_{50}$) were performed to measure neutralizing antibodies (NAbs) to wild-type SARS-CoV-2 (hCoV-19/Türkiye/ERAGEM-001/2020 strain, GenBank accession number; MT327745.1 and GISAID; EPI_ISL_424366). IgG responses to SARS-CoV-2 S1 RBD (Spike S1 Receptor Binding Domain) and whole SARS-CoV-2 were evaluated with the Euroimmune anti-SARS CoV-2 IgG enzyme linked immunoassay (ELISA) kit and in-house IgG ELISA (based on purified whole SARS-CoV-2), respectively. Methods of immunogenicity testing were previously reported in detail [11,16,17]. Laboratory investigations for immunogenicity were performed on the day of booster injection (i.e., second dose +12 weeks) and at weeks 1, 2, 4, 8, 16, 24, and 32 thereafter. The geometric mean titers (GMTs) of the antibodies were compared to the pre-booster (second dose +12 weeks) levels. A $\geq$4-fold higher post-booster antibody titer compared to the pre-immune levels served as an immune correlate of protection (ICP) predicting the clinical efficacy of the booster dose.

Adverse event (AE) questioning and laboratory (blood chemistry and hematology) investigations for safety were performed on the same days as the immunogenicity assessments. In addition, daily phone calls were made to collect AEs within the first week of booster injection. AEs were graded as mild (grade 1: requiring no intervention; no impact on activities of daily living (ADL)), moderate (grade 2: requiring minimal, non-invasive intervention; moderate impact on ADL); and severe (grade 3: requiring invasive intervention; major assistance needed for ADL).

2.3. Statistical Analysis

The GraphPad Prism 9.0.1 program was used for statistical analyses and graphical representations of immunogenicity data. Antibody titers were presented as GMTs including 95% confidence interval (CI) and seroconversion rates (number of patients and %). An unpaired t-test was used to compare the antibody titers; Spearman's correlation curves and linear regression analyses were utilized to assess the correlation between MNT_{50} and $FRNT_{50}$ results at pre-determined study time-points.

All volunteers who received a booster dose of TURKOVAC 3 μg constituted the safety population. AEs were descriptively analyzed as number and percentage of events.

A p value < 0.05 was considered statistically significant for all tests.

3. Results

Out of 93 study participants who had received two doses of TURKOVAC 3 μg 28 days apart for primary immunization, 43 (46.2%) agreed to receive a booster dose of the vaccine. The mean age of these subjects was 36.79 $\pm$ 10.20 years (range: 20–57), and 33 of them (76.7%) were men. Their mean body mass index was 25.7 $\pm$ 3.7 (range: 18.3–32.0). Forty-two patients were eligible and included in the analysis.

3.1. Immunogenicity

Table 1 presents the GMTs of NAbs, anti-S1-RBD, and anti-whole SARS-CoV-2 IgG antibodies and the seroconversion rates at baseline (pre-immune) on the day of booster injection (12 weeks after the second dose of primary series; pre-booster) and at weeks 1, 2, 4, 8, 16, 24, and 32 thereafter. The changes in antibody titers over the course of follow-up are shown in Figure 1, including how many times GMTs increased at each time-point compared to pre-immune levels.

Table 1. Pre-immune, pre-booster, and post-booster assessments of antibody titers and seroconversion rates. * Data are % (n/N) [95 %CI]. Seroconversion was defined as fourfold rise over baseline; n = number of participants who achieved seroconversion. N = number of participants included in the immunogenicity analysis; CI = confidence interval.

Antibody Responses	Pre-Immune	2nd Dose + 12 Weeks	3rd Dose + 1 Week	3rd Dose + 2 Weeks	3rd Dose + 4 Weeks	3rd Dose + 8 Weeks	3rd Dose + 16 Weeks	3rd Dose + 24 Weeks	3rd Dose + 32 Weeks
SARS-CoV 2-neutralizing antibodies (MNT_{50}) (GMT-95%CI)	2.0 (2.0–2.0)	10.5 (3.5–23.7)	33.6 (6.7–60.4)	47.9 (21.2–74.5)	44.2 (18.9–69.4)	18.9 (12.7–25.0)	12.0 (5.6–18.3)	5.5 (2.1–8.8)	3.4 (0.1–6.6)
Seroconversion (%) * Seroconverted/tested (*n*) 95%-CI	0.0% 0/43 0.0–0.0	78.5% 33/42 63.1–89.7	97.6% 41/42 87.4–99.9	97.6% 41/42 87.4–99.9	97.6% 41/42 87.4–99.9	91.8% 34/37 78.0–98.3	85.7% 24/28 67.3–95.9	48.1% 13/27 28.6–68.0	30.7% 4/13 9.0–61.4
SARS-CoV 2-neutralizing antibodies ($FRNT_{50}$) (GMT-95%CI)	2.0 (2.0–2.0)	9.1 (3.11–21.3)	30.0 (3.4–56.5)	51.9 (22.8–80.9)	49.5 (20.2–78.7)	21.4 (15.5–27.2)	10.8 (7.4–14.4)	5.3 (2.6–7.9)	3.4 (0.1–6.6)
Seroconversion (%) * Seroconverted/tested (*n*) 95%-CI	0.0% 0/43 0.0–0.0	80.9% 34/42 65.8–91.4	95.2% 40/42 83.8–99.4	97.6% 41/42 87.4–99.9	97.6% 41/42 87.4–99.9	94.5% 35/37 81.8–99.3	85.7% 24/28 67.3–95.9	44.4% 12/27 25.4–64.6	30.7% 4/13 9.0–61.4
Antibody responses to S1-RBD (GMT-95%CI)	45.2 (44.0–47.0)	915.1 (635.5–1194.3)	2893.4 (2347.5–3439.2)	3434.9 (2912.3–3957.4)	3290.6 (2781.8–3799.3)	1801.9 (1245.9–2357.8)	816.5 (336.6–1266.3)	532.9 (243.9–821.7)	359.5 (76.9–887.2)
Seroconversion (%) * Seroconverted/tested (*n*) 95%-CI	0.0% 0/43 0.0–0.0	100% 42/42 91.5–100.0	100% 42/42 91.5–100.0	100% 42/42 91.5–100.0	100% 42/42 91.5–100.0	97.2% 36/37 85.8–99.9	92.8% 26/28 76.5–99.1	85.1% 23/27 66.2–96.1	76.9% 10/13 46.1–94.9
Antibody responses to whole SARS-CoV-2 antigen (GMT-95%CI)	42.6 (41.2–43.9)	486.4 (326.8–645.8)	2067.6 (1754.8–2380.3)	2371.5 (1986.1–2756.9)	2265.0 (1889.0–2641.0)	1230.9 (674.2–1787.5)	1007.9 (406.1–1609.6)	484.3 (272.1–696.4)	400.0 (32.0–946.1)
Seroconversion (%) * Seroconverted/tested (*n*) 95%-CI	0.0% 0/43 0.0–0.0	92.8% 39/42 80.5–98.5	100% 42/42 91.5–100.0	100% 42/42 91.5–100.0	97.6% 41/42 87.4–99.9	97.2% 36/37 85.8–99.9	96.4% 27/28 81.6–99.9	74.0% 20/27 53.7–88.8	61.5% 8/13 31.5–86.1

At 12 weeks after the second vaccination, before the booster shot, NAb seroconversion persisted in approximately 80% of subjects (Table 1), with 5.2-fold and 4.5-fold higher NAb GMTs in MNT_{50} and $FRNT_{50}$ assays compared to the pre-immune levels, respectively (Figure 1A,B). Significant increases in NAb GMTs occurred from 1 week after booster vaccination compared to pre-booster levels, peaking at week 2 and persisting until week 4 ($p < 0.05$ for all). The NAb titers then showed a gradual decline and became comparable to pre-booster levels at weeks 8, 16, 24, and 32. However, they remained $\geq$4-fold higher than at pre-immune status at weeks 8 and 16 after the booster shot. The seroconversion rates for NAbs were below 50% and their GMTs were four times lower than the pre-immune levels at weeks 24 and 32 (Table 1 and Figure 1A,B). The results of MNT_{50} and $FRNT_{50}$ assays were very strongly correlated at all assessment time-points and showed a perfect correlation at week 32 (r = 1; $p = 0.001$) (Figure 2).

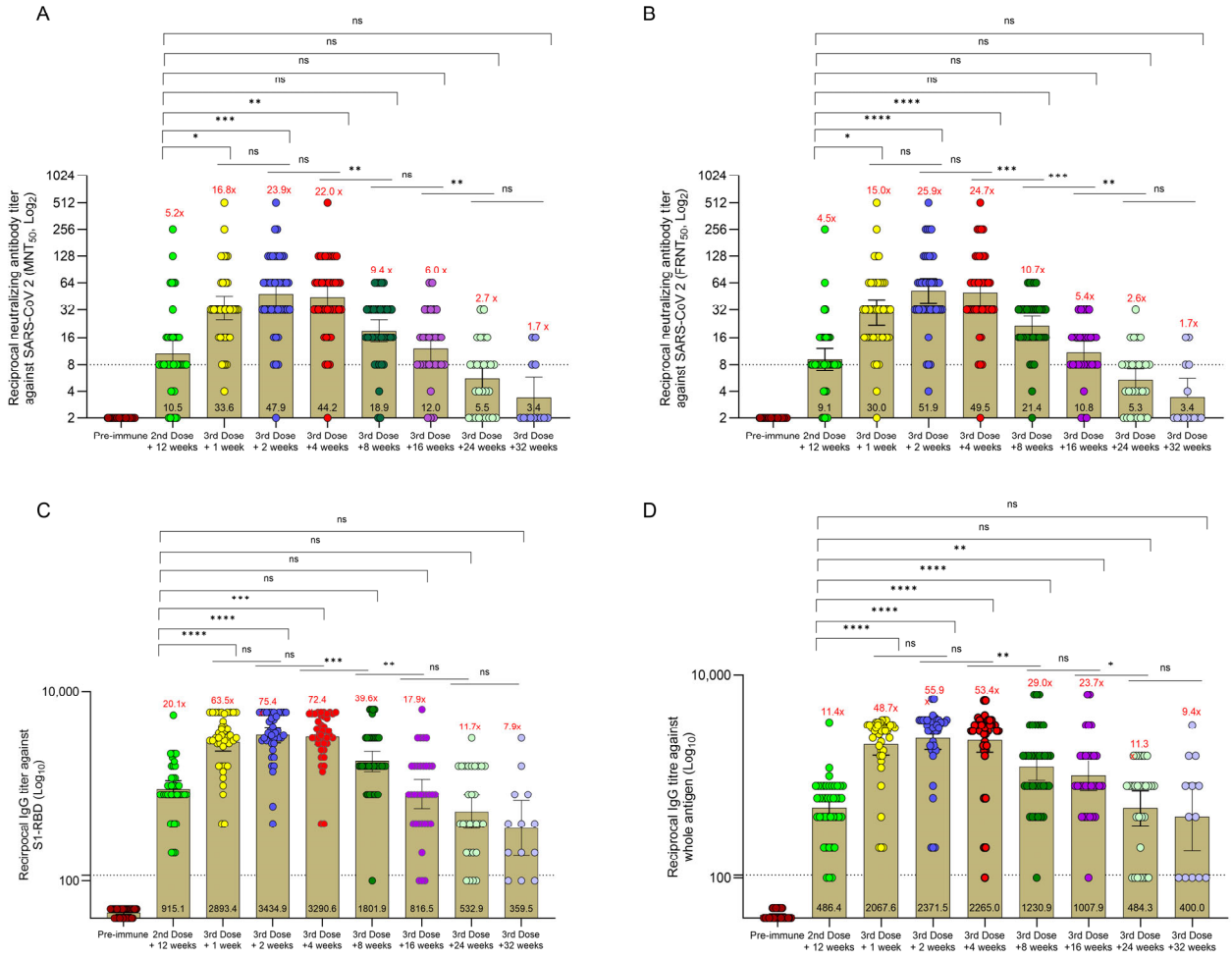

Figure 1. Comparison of antibody titers across the study assessment time-points. (**A**) shows the neutralizing antibody titer in the MNT_{50} (micro-neutralization test) assay. (**B**) shows the neutralizing antibody titer in the $FRNT_{50}$ (focus reduction neutralization test) assay. (**C**) shows the IgG titer against S1-RBD. (**D**) shows the IgG titer against the whole SARS-CoV 2 antigen. The values inside the bars represent geometric mean titers (GMTs), and the values above the bars (shown in red) show how many times GMT values increased versus the pre-immune levels. The dotted line represents the threshold value for the experiments. The unpaired t-test was used to determine the statistically significant differences between groups. $p < 0.05$ indicates statistically significant differences, with ns indicating nonsignificant; * < 0.05, ** < 0.005, *** < 0.0005 and **** <0.0001.

As presented in Table 1, the seroconversion rates for anti-S1-RBD and anti-SARS-CoV-2 IgG antibodies on the day of booster injection were 100% and 92.8%, respectively. The GMTs of both antibodies significantly increased, and all subjects achieved seroconversion at week 1 after the booster injection ($p < 0.0001$ for both) with 63.5-fold and 48.7-fold-higher GMTs for anti-S1-RBD and anti-whole SARS-CoV-2 IgGs compared to the pre-immune values, respectively. IgG antibody titers peaked 2 weeks after the booster shot and gradually declined in subsequent visits. The anti-S1-RBD IgG GMTs at weeks 8, 16, 24, and 32 after the third injection were comparable to the pre-booster level but remained $\geq$4 fold higher than the titer at the pre-immune state at all these time-points. The anti-whole SARS-CoV-2 IgG GMT also peaked 2 weeks after the booster dose administration. Unlike the anti-S1-RBD IgG, the GMTs of anti-whole SARS-CoV-2 IgG at weeks 8 and 16 were significantly higher than the pre-booster level ($p < 0.0001$ and $p < 0.005$, respectively). At week 32 after the booster dose, the GMTs of anti-S1-RBD and anti-whole SARS-CoV-2 IgG antibodies were 7.9-fold and 9.4-fold higher than the pre-immune levels, respectively (Figure 1C,D). The

percentage of seroconverted patients was 76.9% for anti-S1-RBD IgG and 61.5% for the anti-whole SARS-CoV-2 IgG at this time-point.

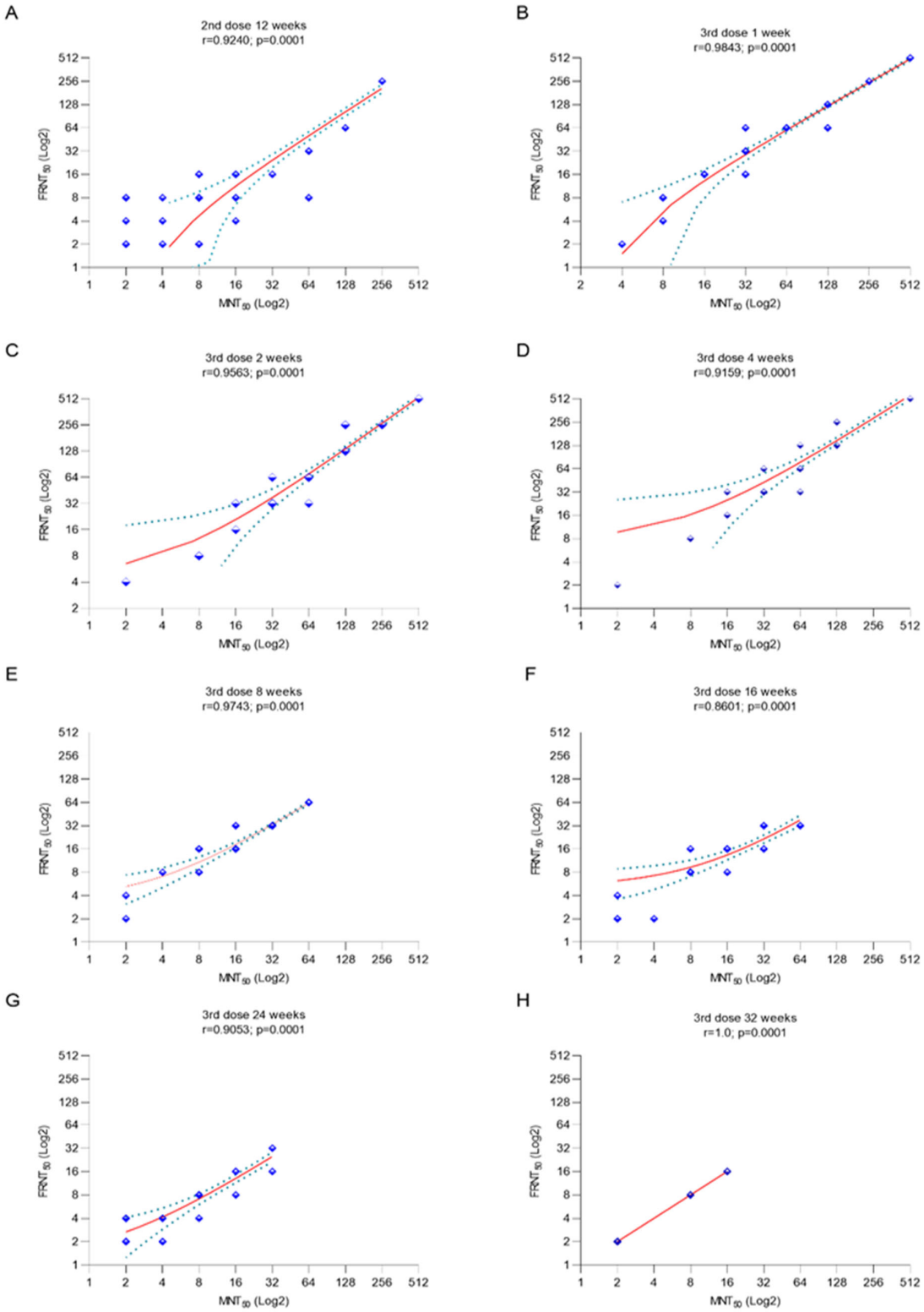

Figure 2. Correlation between MNT_{50} and $FRNT_{50}$ results. (**B**–**H**). Correlation between MNT_{50} and $FRNT_{50}$ at post-booster 1, 2, 4, 8, 16, 24, and 32 weeks. r: correlation coefficient $p < 0.05$ indicates statistical significance. ——— Regression line ····· Error bar. (**A**). Correlation between MNT_{50} and $FRNT_{50}$ at 2nd dose, 12 weeks.

3.2. Safety

Table 2 provides a summary of the 46 AEs experienced during the post-booster 32 weeks. None of these events were severe. Almost two thirds of the events (63.3%; 30 AEs in 19 participants) occurred after the 8th week of booster shot. Headache (n = 7; 43.8%) was the most common AE experienced within the initial 8 weeks that followed the booster injection.

Table 2. Adverse events experienced after the booster injection.

Type of Event	Time from the Booster Dose		Overall *n* (%)
	0–8 Weeks *n* (%)	9–32 Weeks *n* (%)	
Positive SARS-CoV-2 RT PCR test	-	9 (30)	9 (19.6)
Headache	7 (43.8)	2 (6.7)	9 (19.6)
Weakness	1 (6.3)	4 (13.3)	5 (10.9)
Runny nose	1 (6.3)	3 (10)	4 (8.7)
Joint pain	1 (6.3)	3 (10)	4 (8.7)
Sore throat	1 (6.3)	2 (6.7)	3 (6.5)
Toothache	2 (12.5)	-	2 (4.3)
Backpain	-	2 (6.7)	2 (4.3)
Chills	1 (6.3)	1 (3.3)	2 (4.3)
Nosebleed	1 (6.3)	-	1 (2.2)
Cough	-	1 (3.3)	1 (2.2)
Anosmia	-	1 (3.3)	1 (2.2)
Shoulder pain	-	1 (3.3)	1 (2.2)
Tibia fracture	-	1 (3.3)	1 (2.2)
Cat scratching	1 (6.3)	-	1 (2.2)
Total	16 (100)	30 (100)	46 (100)

n—number of events; a RT PCR—reverse transcriptase polymerase chain reaction; SARS-CoV-2—severe acute respiratory syndrome coronavirus 2.

None of the study participants had a laboratory-confirmed SARS-CoV-2 infection within 8 weeks of booster injection. In total, nine participants were tested positive for COVID-19 by SARS-CoV-2 RT-PCR after the eight week of booster vaccination. Among these cases, three were diagnosed between weeks 8 and 16, with neutralization titers ranging from 1/8 to 1/16. The remaining six cases were detected beyond week 16, also with neutralization titers ranging from 1/8 to 1/16, except for one case which had a negative neutralization titer. None of the infected patients had a severe disease requiring hospital admission. There were no deaths associated with COVID-19.

Eleven subjects (26.2%) had an overall 15 abnormal laboratory test results requiring repeat testing within the same period; abnormal blood glucose levels (n = 9) in six subjects (14.3%) were the most common laboratory abnormalities, followed by abnormal white blood cell counts (n = 3) in three subjects (7.1%) and abnormal blood urea nitrogen levels in two subjects (4.8%).

4. Discussion

We found that a homologous booster shot with TURKOVAC, administered 12 weeks after the completion of primary immunization against SARS-CoV-2, elicited rapid and robust immune responses with acceptable safety and tolerability in healthy adults <65 years of age. Overall, the results of this study are consistent with those of the previously published studies that investigated the immunogenicity and safety of homologous boosting with inactivated vaccines against SARS-CoV-2 [18–26].

Previous studies on inactivated COVID-19 vaccines have demonstrated that the humoral immune responses elicited by a two-dose primary immunization gradually diminished over time, typically remaining detectable for up to 6 months following the second dose [15,18–28]. Ates et al. conducted an investigation to assess the long-term immuno-

genicity of the TURKOVAC and CoronaVac vaccines when administered as booster doses subsequent to the second dose of primary vaccination with CoronaVac. Their findings revealed a slight decline in antibody positivity on Day 84 compared to Day 28; however, there was no statistically significant difference observed between the two vaccine groups in terms of antibody response [18]. The study conducted by Zeng et al. investigated the immune persistence and efficacy of CoronaVac, a two-dose COVID-19 vaccine, in individuals aged 18 years and older. The results indicate that after a period of six months, the levels of neutralizing antibodies induced by the two-dose regimen of CoronaVac declined to low concentrations. However, the administration of a third dose, eight months after the second dose, led to a significant enhancement in the immune response, with neutralizing antibody levels increasing three-fold to five-fold. This study also demonstrated the safety of the third dose, as no adverse events were reported, and the reactogenicity of the vaccine was comparable to that of the placebo. Notably, regardless of age group, a high seropositivity rate ranging from 98% to 100% was achieved after the administration of the third dose. These findings suggest that the third dose of CoronaVac, given at an interval of eight months after the second dose, substantially augments neutralizing antibody levels, potentially conferring longer-lasting immunity and a heightened level of protection compared to the standard two-dose schedule [27]. AI et al. conducted a study to evaluate the immunogenicity and safety of a third homologous BBIBP-CorV booster vaccination administered four to eight months after the initial two doses. The results demonstrated that the third dose of BBIBP-CorV was well tolerated and highly immunogenic in healthy adults aged 18–59 years. This study presented additional evidence demonstrating the effectiveness of a third dose in generating strong humoral and cell-mediated immune responses, specifically targeting variants of concern (VOCs). The administration of a third dose of BBIBP-CorV vaccine effectively stimulated and promptly elevated the humoral immune response by enhancing antibody levels. Moreover, the third dose demonstrated both safety and efficacy in eliciting robust humoral and cell-mediated immune responses. These findings provide support for the potential adoption of a third homologous BBIBP-CorV booster vaccination approach to enhancing and extending protection against COVID-19 [28].

The administration of a third dose, utilizing different vaccine platforms in addition to inactivated vaccines, has been shown to rapidly enhance the immune response and maintain its effectiveness for an extended period. The safety and immunogenicity evaluation of a booster dose of the BNT162b2 vaccine, given 7 to 9 months after the initial two-dose series, indicates that a third dose has the potential to extend the duration of protection and further strengthen the breadth of defense against COVID-19. These findings emphasize the scientific rationale and importance of administering a third dose to optimize and sustain immune protection, especially in the face of emerging variants and the ongoing need for long-lasting immunity in the fight against the COVID-19 pandemic [29]. Flaxman et al. investigated the immune responses to ChAdOx1 nCoV-19 following a second dose with an extended interval between the first and second dose, as well as after a third dose with an extended interval between the second and third doses. Notably, they found that prolonging the interval between the first two doses to 44–45 weeks resulted in higher antibody titers after the second dose compared to a shortened interval. Moreover, administering a third dose 28–38 weeks after the primary series led to antibody titers surpassing those observed after a second dose with a shortened interval. Importantly, the reactogenicity was lower after the second or third dose compared to the first dose [30].

In our study, the GMTs of NAbs and ELISA-detected SARS CoV-2-specific IgGs were above the seropositivity thresholds for the relevant assays on the day of booster administration, i.e., 12 weeks after completing the primary series, and the seroconversion rates were approximately 80% for NAbs and exceeded 90% for IgGs. Although these findings suggest that a substantial group of participants might have had the potential to remain seropositive for longer periods of time after primary immunization, we do not know what the impact of delaying the booster administration would be as we only tested the 12-week boosting schedule.

Protection against SARS-CoV-2 infection and a reduction in disease severity in affected individuals are complex processes in which both the humoral and cellular components are involved [25,31–38]. Various humoral markers, including anti-spike protein/anti-RBD IgG and IgA and NAbs, have been suggested as potential surrogate markers of SARS-CoV-2 vaccine efficacy, but there are no established protective thresholds or ranges for these antibodies [34–38]. In this study, a booster dose of TURKOVAC increased the seroconversion rate of NAbs to >95% and those of anti-SARS-CoV-2 S1 RBD IgG to 100% as early as 1 week after the injection, and >90% of the subjects remained seropositive for both antibodies for at least 8 weeks after the vaccination. None of the participants had a PCR-confirmed SARS-CoV-2 infection during this period. It is noteworthy to mention that two thirds of the confirmed cases of infection occurred after the sixteenth week of booster administration, when the GMTs of NAbs fell below six times the pre-immune levels and there were no hospitalizations or deaths due to COVID-19 throughout the 32-week study period despite the declining antibody GMTs over time. Although this study was not designed to determine an ICP, our findings suggest that the NAbs may be a potential correlate of protection at least against laboratory-confirmed SARS-CoV-2 infection for TURKOVAC. The GMTs of IgGs, which remained above the lower limit of seroconversion throughout the study period, might be explained by the persistence of specific immune memory cells allowing for antibody production following exposure to the relevant antigens. Overall, our findings show the clinical efficacy of boosting with TURKOVAC in preventing SARS-CoV-2 infection and reducing COVID-19 severity and are complementary to those from previous studies of various inactivated vaccines which reported low rates of infection, pneumonia, hospitalization, and death associated with SARS-CoV-2 infection after the administration of a booster dose [39–46].

The current study did not reveal any new concerns regarding TURKOVAC safety. All AEs were mild to moderate in severity and resolved within a few days. In contrast to other inactivated COVID-19 vaccine studies [19–21,23,28,29,39], including those of TURKOVAC [11,15,18,32], none of the participants in this study reported pain at the injection site after receiving a booster injection. This may be because we collected AEs through spontaneous reporting, unlike previously published TURKOVAC studies where safety assessments included both solicited and unsolicited data collection and pain at injection site was the most reported local reaction.

To our knowledge, this is the first paper to report the outcomes in volunteers who were boosted with homologous TURKOVAC vaccine. The strengths of this study are the long follow-up period extending up to 32 weeks after the booster dose and the assessment of immunogenicity with both NAbs and SARS-specific IgGs. This provides valuable information about the long-term immunogenicity and efficacy of a booster dose of TURKOVAC.

The following limitations should be considered when interpreting the results. This was a small-sized, single-arm study which included healthy adults aged <65 years and investigated the immunogenicity and safety of a single boosting scheme. In addition, this study only evaluated the antibody responses against wild-type SARS-CoV-2 and did not include cellular immune response assessments.

One of the limitations of our study is that we lack information about the specific variants or lineages with which the nine volunteers were infected, despite their positive rt-PCR results during the study. However, it is worth noting that a study conducted in Türkiye between April 2021 and February 2022 analyzed 492 SARS-CoV-2 strains. Out of these, 64% were identified as variants, while 16% were classified as the wild type. During this period, seven different lineages and a sublineage were reported among the variant sequences. Initially, the Alpha variant was dominant, followed by the Beta, Delta, Eta, and Lota variants. However, by September 2021, the Delta variant became the dominant variant in Türkiye. In December 2021, the Omicron variant was reported for the first time, and by February 2022 it overtook the Delta variant [47]. According to these results, it can be speculated that the Alpha variant was initially dominant during the study, followed by the

Delta variant, and in the final stages of the study, the Omicron variant was detected for the first time.

Ongoing studies are actively investigating the vaccine's efficacy against variants of concern (VoCs) and evaluating cellular immune responses, with these studies currently in the process of being prepared for submission.

5. Conclusions

The administration of a third homologous booster dose of TURKOVAC, an inactivated whole-virion SARS-CoV-2 vaccine, 12 weeks after the completion of primary immunization can safely provide effective protection against SARS-CoV-2 infection and reduce the severity of COVID-19 by inducing strong humoral immune responses which persist at least 8 weeks in healthy adults under 65 years of age. Future research and real-life data on the immunogenicity, efficacy, or effectiveness of various boosting regimens against the variants of concern in study populations, including those who are vulnerable to SARS-CoV-2 infection, will help optimize the immunization strategy for TURKOVAC.

6. Patents

Aykut Ozdarendeli, Shaikh Terkis Islam Pavel, Hazel Yetiskin, Muhammet Ali Uygut, and Gunsu Aydin are the named inventors on patent applications covering inactivated COVID-19 vaccine development.

Author Contributions: Conceptualization, Methodology, Data analysis, Investigation, Writing—Original draft, Writing—review and editing, A.O. and Z.S.; Methodology, Data analysis, Writing—Original draft, S.T.I.P.; Methodology, Data analysis, Writing—Original draft A.I., H.Y., B.K. and M.A.U.; Methodology, Data analysis, A.F.A., A.B., M.M., G.K.U., Z.T.Y., G.A. and R.K.K.; original draft, Writing—review and editing, I.A. and A.K. All authors have read and agreed to the published version of the manuscript.

Funding: The study was funded by the Health Institutes of Türkiye (TUSEB) (with grant ID: 11484) and Erciyes University (with grant ID: 9644 and with grant ID: 11418).

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki. Studies were approved by the Ethics Committee for Clinical Trials of Erciyes University and Turkish Ministry of Health (2020/548, 28 October 2020, for phase 1 and 2021/74, 1 February 2021, for phase 2). Both trials are registered at ClinicalTrials.gov (phase 1, NCT04691947 and phase 2, NCT04824391).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The Health Institutes of Türkiye (TUSEB) and Erciyes University provided the funding this study; approved the final protocols and final manuscript, and made the decision to submit for publication but had no role in data collection, data analysis, data interpretation, or writing the report. Ideal CRO (Ankara, Türkiye) acted as the contract research organization representing TUSEB and contributed to correspondence between investigators, the ethics committee, and the Ministry of Health; monitoring, site management, storage, and distribution of the consumables; developing electronic case report forms, data management, statistical analyses, and overall project management. Kocak Pharma provided the investigational products.

Conflicts of Interest: Aykut Ozdarendeli, Shaikh Terkis Islam Pavel, Hazel Yetiskin, Muhammet Ali Uygut, and Gunsu Aydin are the named inventors on patent applications covering inactivated COVID-19 vaccine development. All remaining authors declare no competing interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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vaccines

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Article

A SARS-CoV-2 Vaccine Designed for Manufacturability Results in Unexpected Potency and Non-Waning Humoral Response

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Abstract: The rapid development of several highly efficacious SARS-CoV-2 vaccines was an unprecedented scientific achievement that saved millions of lives. However, now that SARS-CoV-2 is transitioning to the endemic stage, there exists an unmet need for new vaccines that provide durable immunity and protection against variants and can be more easily manufactured and distributed. Here, we describe a novel protein component vaccine candidate, MT-001, based on a fragment of the SARS-CoV-2 spike protein that encompasses the receptor binding domain (RBD). Mice and hamsters immunized with a prime-boost regimen of MT-001 demonstrated extremely high anti-spike IgG titers, and remarkably this humoral response did not appreciably wane for up to 12 months following vaccination. Further, virus neutralization titers, including titers against variants such as Delta and Omicron BA.1, remained high without the requirement for subsequent boosting. MT-001 was designed for manufacturability and ease of distribution, and we demonstrate that these attributes are not inconsistent with a highly immunogenic vaccine that confers durable and broad immunity to SARS-CoV-2 and its emerging variants. These properties suggest MT-001 could be a valuable new addition to the toolbox of SARS-CoV-2 vaccines and other interventions to prevent infection and curtail additional morbidity and mortality from the ongoing worldwide pandemic.

Keywords: SARS-CoV-2; COVID-19; vaccine; durable immunity; emerging variants; protection

Citation: Campbell, E.; Dobkin, J.; Osorio, L.J.; Kolloli, A.; Ramasamy, S.; Kumar, R.; Sant'Angelo, D.B.; Subbian, S.; Denzin, L.K.; Anderson, S. A SARS-CoV-2 Vaccine Designed for Manufacturability Results in Unexpected Potency and Non-Waning Humoral Response. *Vaccines* **2023**, *11*, 832. https://doi.org/10.3390/vaccines11040832

Academic Editors: Rishi Jaiswal, Srijani Basu, Suman Gupta and Sneh Lata Gupta

Received: 11 March 2023
Revised: 3 April 2023
Accepted: 7 April 2023
Published: 12 April 2023

1. Introduction

More than three years have elapsed since the first cases of SARS-CoV-2 infection were reported in humans. Rapid transmission and continued evolution of the virus have led to a pandemic that persists to the present day. The first approved SARS-CoV-2 vaccines were remarkably effective against the ancestral strain, with multiple clinical trials demonstrating vaccine effectiveness at preventing severe disease of over 90% [1,2]. However, waning immunity and the emergence of new variants, many of which possess some degree of immune escape [3,4], has necessitated boosters and spurred the development of variant-specific and

pan-coronavirus vaccines. Further, despite the availability of approved vaccines, accessibility has been problematic outside of the developed world, and hesitancy towards vaccines and new vaccine technologies have slowed vaccination rates everywhere. Finally, efficacious vaccines and strategies for members of the population who are immunocompromised remain a significant scientific and medical challenge.

Continued research and development of novel vaccines, adjuvants, and immunization strategies to combat these weaknesses remain a high priority [5,6]. The WHO Target Product Profiles for COVID-19 Vaccines was revised in April 2022 to reflect this need and described several desired characteristics for the next generation of vaccine candidates. Notable among these are the durability of protection, broader protection against emerging variants, and ease of manufacture and distribution. No current vaccine meets all of these criteria. Booster doses have been shown to enable protection against some emerging variants, but with the rapid waning of effectiveness and continued vaccine hesitancy [7,8] it is not clear whether current booster administration paradigms will comprise a sustainable strategy, even with variant-specific modifications to current vaccines [9,10].

Among the earliest vaccines approved in the US and EU were two mRNA vaccines from Pfizer/BioNTech (BNT162b2) and Moderna (mRNA-1273), and two viral-vectored vaccines from Janssen/J&J (Ad26.COV2.S) and Oxford/AstraZeneca (ADZ1222). The mRNA vaccines BNT162b2 and mRNA-1273 elicit extremely high antibody titers [11], but studies have shown that the humoral immunity fades relatively quickly [12], prompting many countries to recommend a third booster dose and, presently, even a fourth or fifth booster in some cases [13]. Unfortunately, even with multiple boosts, protection against SARS-CoV-2 variants remains modest [14]. Conversely, the viral-vectored vaccines Ad26.COV2.S and AZD1222 elicit lower initial antibody responses [15], but protection seems to be more durable as immunological readouts remain relatively constant over time [12,16]. Perhaps most unexpectedly, and in stark contrast to the waning antibody titers observed for the mRNA vaccines, both the magnitude and breadth of the humoral immune response appear to increase with time after vaccination with Ad26.COV2.S [17,18]. The mechanisms mediating this non-waning behavior are unclear, but it may be due, at least in part, to differences in the kinetics of antigen presentation. The mRNA vaccines have been shown to produce a large bolus of short-lived spike protein [19], whereas the viral-vectored vaccines may provide modest, yet sustained, antigen levels over a more extended period [20].

The choice of immunogen remains an open question as well. Whereas most approved vaccines use the full-length SARS-CoV-2 spike protein as an immunogen, a strong argument can be made for a vaccine based on a smaller fragment of the spike protein encompassing the receptor binding domain (RBD). RBD-based SARS-CoV-2 vaccines have been shown to elicit a higher fraction of neutralizing antibodies (nAbs) than vaccines based on the full-length spike protein, likely due to the entire immune response being directed toward the RBD [21,22]. A high neutralizing titer is desirable, as numerous studies have shown that nAb levels strongly correlate with protection [23–25]. A comprehensive review of the potential advantages of RBD-based vaccines has been presented [26]. Despite the many potential benefits, existing RBD vaccine candidates have often suffered from relatively poor expression and/or reduced immunogenicity. Previous efforts to design RBD constructs have, at times, attempted to trim the domain down to the "minimal expressible unit" containing the receptor binding motif (RBM), either by inspection or based upon homology to constructs used for other coronavirus RBDs [27–33]. These approaches often truncate a significant portion of the local "context" of protein structure surrounding the RBM, which might negatively impact protein folding and stability. Several such constructs have been designed with key glycosylation sites knocked out, disulfides removed, or stabilizing mutations made within the structure in order to rescue protein expression [27,28,30]. However, such changes may lead to an immunogen 3D structure that differs from the native conformation of the target viral protein antigen. This could potentially negatively impact antigenicity and thus the utility of the vaccine.

Bearing these considerations in mind, we designed a novel protein component vaccine based on the RBD and RBD-adjacent sequences of the SARS-CoV-2 spike protein. By focusing the immune response on the region of the spike protein, where the bulk of the epitopes for neutralizing (including broadly neutralizing) antibodies reside [34,35], we aimed to enable high potency. We also sought to design a recombinant immunogen that would be stable, highly soluble, capable of expression at high levels, and amenable to streamlined purification protocols. We reasoned that this would endow the vaccine with relatively uncomplicated manufacturing and distribution requirements that would facilitate its adoption on a global scale.

We show here that with a 2-dose prime-boost regimen in BALB/cJ mice, the resultant vaccine, MT-001, exhibited peak anti-spike IgG ELISA titers comparable to those reported in studies with mRNA vaccines from Pfizer/BioNTech (BNT162b2) and Moderna (mRNA-1273) at similar doses in the same animal model [36,37]. When adjuvanted with both aluminum hydroxide (Alhydrogel) and the TLR-9 CpG agonist ODN1826, the MT-001 vaccine in BALB/cJ mice showed a balanced Th1/Th2 response as well as peak anti-spike RBD IgG midpoint ELISA titers on the order of 10^6 GMT. Syrian golden hamsters vaccinated with MT-001 adjuvanted with alum plus CpG exhibited undetectable levels of SARS-CoV-2 in lung tissue four days after intranasal challenge with SARS-CoV-2/US-WA1. We also observed that anti-spike IgG ELISA titers in sera from vaccinated mice were durable, with EC_{50}s in the range of 10^5–10^6 up to 12 months post-vaccination. Furthermore, the results showed a meaningful breadth of the response, with significant neutralization titers against the Omicron BA.1 variant at this time point.

Combined, these attributes make MT-001 a compelling candidate for further research and development as a next-generation COVID-19 vaccine. MT-001 (or variant-updated versions thereof) could be particularly valuable as an annual booster to augment immunity in individuals with diverse histories of vaccination, SARS-CoV-2 infection, and/or predispositions resulting in an immunocompromised state.

2. Materials and Methods

2.1. Design and Expression of MT-001

The sequence of the ancestral SARS-CoV-2 Wu-1 strain spike protein (YP_009724390.1) was analyzed using publicly available bioinformatics tools for calculating structural, biophysical, and biochemical properties of potential constructs. Access to such tools can be found on the DisMeta server [https://montelionelab.chem.rpi.edu/ (accessed 19 September 2022)]. Shown in Figure S1 is an example output from DisMeta for residues 300–600 of the spike protein. The results of these analyses were used to parse the sequence to yield a final expression construct designed to encompass the annotated receptor binding domain (residues 319–541), but with the construct N- and C-termini extended to include additional spike protein structural elements flanking the RBD domain that might promote proper domain folding and improved stability. The resulting RBD construct, MT-001, corresponded to residues 316–594 of spike fused to a C-terminal C-tag. The MT-001 construct was codon-optimized and expressed via a secretion vector in HEK293 cells (ATUM, Inc., Newark, CA, USA), and purified in a single affinity chromatography step using the CaptureSelect C-tagXL system (ThermoFisher Scientific, Waltham, MA, USA) [38,39]. The final purified yield was >160 mg from 1 L suspension culture. The purified protein was >96% monomeric with an apparent molecular weight of 39.4 kDa (calculated 31.6 kDa) by HPLC-SEC and had an apparent purity of >99% by capillary electrophoresis (Figure S2). Solubility was determined to be >10 mg/mL in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4). Aliquots were formulated in PBS with 10% glycerol as a cryoprotectant and stored at −80 °C until use.

2.2. Immunization of BALB/cJ Mice

Cohorts of 5–10 female, 8–10 week old BALB/cJ mice (The Jackson Laboratory, Bar Harbor, ME, USA) were immunized by injection into the gastrocnemius muscle with the

indicated amount of MT-001 adjuvanted in 500 μg Alhydrogel® (InvivoGen, San Diego, CA, USA) in a final volume of 50 μL. Mice were boosted 21 days later with an injection of the same MT-001/Alhydrogel dose. Where indicated, 20 μg of CpG-ODN1826 (InvivoGen, San Diego, CA, USA) was added to the MT-001/Alhydrogel mix immediately before immunization. Pre-immune sera were collected 3 days prior to the initial immunization, and immune sera were collected after immunizations, as indicated in each figure.

2.3. RBD-Binding ELISA

RBD-specific IgG antibody levels were assessed using a novel sandwich ELISA (Figure S5). This assay was developed to maintain 3D conformational epitopes of RBD and prevent the loss of epitopes that may be denatured by direct adsorption of protein to plastic. Plates were coated with 1 μg/mL streptavidin (Sigma-Aldrich, St. Louis, MO, USA) diluted in PBS and incubated at 4 °C overnight. The next day, plates were washed three times with 0.1% TWEEN-20 in PBS (PBS-T), blocked with PBS-T containing 3% BSA for 1 h at room temperature, and incubated at 4 °C overnight with 1 μg/mL biotinylated-camelid α-C-Tag-specific antibody (ThermoFisher Scientific) in PBS-T. Plates were washed, incubated for 1 h at room temperature with 5 μg/mL MT-001 (containing the C-tag) in PBS-T, washed and incubated at 4 °C overnight with serially diluted mouse serum in blocking buffer. Antibody levels specific to Delta variant RBD were assessed in mouse sera by direct ELISA: Plates were coated with 1 μg/mL Delta variant RBD (Leinco Technologies, Inc., Fenton, MO, USA) or MT-001 diluted in PBS and incubated at 4 °C overnight. Plates were washed, blocked, and incubated with serially diluted mouse serum in blocking buffer at 4 °C overnight, as described. To quantify total IgG levels in the ELISAs, plates were washed and incubated for 1 h at room temperature with goat anti-mouse IgG horseradish peroxidase (HRP) (Jackson ImmunoResearch Labs, West Grove, PA, USA): IgG1 and IgG2a/b levels were quantified using goat anti-mouse IgG1 HRP (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) and goat anti-mouse IgG2a HRP (Southern Biotechnology Associates, Inc., 1080-05) with anti-mouse IgG2b HRP (Southern Biotechnology Associates, Inc.), respectively. ELISAs with hamster sera utilized goat anti-Syrian hamster IgG HRP (Jackson ImmunoResearch). All HRP-conjugated secondary antibodies were diluted 1:5000 in PBS-T. Finally, plates were washed, and 1-Step Ultra TMB-ELISA substrate solution (ThermoFisher Scientific) was added to each well to detect HRP activity. Development was halted by adding 1M sulfuric acid and absorbance at 450 nm was assessed using a SpectraMax i3 microplate reader (Molecular Devices, San Jose, CA, USA). Background values were recorded from wells containing block solution only and subtracted from the raw OD_{450} values. For ELISAs with mouse serum, a standard curve was derived using a titrated anti-RBD mouse monoclonal antibody (Sino Biological, Wayne, PA, USA) included on each plate as a technical control to monitor plate-to-plate variability. Data were analyzed in GraphPad Prism (GraphPad Software, Boston, MA, USA) using a sigmoidal four-parameter logistic (4PL) fit, and ELISA half-maximal titers were defined as the reciprocal serum dilution that yielded 50% maximal absorbance. ELISAs were repeated at least three times for each mouse or hamster serum sample, and the data represent average half-maximal titers for each set of replicates. Independent confirmation of the precision and accuracy of our indirect "sandwich" RBD ELISA method was obtained by submitting a test panel of mouse sera for analysis by Nexelis (Laval, Quebec, CA, USA) using a clinically validated SARS-CoV-2 anti-spike IgG ELISA assay [https://nexelis.com/our-expertise/infectious-diseases/vaccine/sars-cov-2/ (accessed 10 August 2021)]. Replicate serum samples assayed by the anti-RBD IgG ELISA at Rutgers (above) and, in parallel, an optimized automated anti-spike IgG ELISA at Nexelis yielded highly concordant results with quantitatively similar titers (Figure S4).

2.4. Propagation of SARS-CoV-2

Vero E6 cells (ATCC, Manassas, VA, USA) were propagated in DMEM (Sigma-Aldrich) containing L-glutamine and 10% FBS (Sigma-Aldrich) to 80% confluency in multiple T75 flasks (Corning Inc., Corning, NY, USA), and harvested by gentle dissociation of the

monolayer with Accutase Cell Detachment Solution following the instructions of the manufacturer (ThermoFisher Scientific). Pooled cells were washed twice in sterile PBS (pH 7.2) and checked for viability by the Trypan Blue (ThermoFisher Scientific) exclusion method. Cells were seeded into T75 flasks to ~80% confluency in DMEM containing 10% FBS, and after 18 h, the spent media was decanted, and the cells were washed with sterile PBS (pH 7.2). To determine the viral titer, the original stock vial of SARS-CoV-2/USA-WA1/2020 strain (BEI Resources, Manassas, VA, USA), obtained as lysate of infected cells, was diluted in DMEM containing 2% FBS and used for infection as we described previously (40). Briefly, about ~8×10^6 Vero cells in a T75 flask were infected with 1 mL of virus suspension and incubated at 37 °C for 1 h, followed by replenishing cells with 10 mL of DMEM containing 2% FBS. The cell culture supernatant containing the virus was harvested at 72 h post-infection by centrifugation, followed by filtration using a 0.4-micron filter (Millipore-Sigma, Burlington, MA, USA). Aliquots of virus-containing media (inoculum) were stored at −80 °C until ready to use. The infectious virus particles in the inoculum were quantitated by plaque assay (see below).

2.5. Virus Inoculum Titration

Virus infectivity and inoculum titer were quantitated by plaque assay using Vero E6 cells. Briefly, 4×10^5 Vero cells/well were seeded onto a six-well cell culture plate (Corning) in DMEM supplemented with L-glutamine and 10% FBS. At 18 h post-seeding, the cells were washed with sterile PBS (pH 7.2), and 400 µL of 10-fold dilutions of the virus inoculum, prepared in serum-free DMEM, was added to each well and incubated at 37 °C with gentle rocking of plates every 15 min for 1 h. Then, the virus inoculum was carefully removed, and the infected cells were overlayed with 4 mL/well of 1.6% agarose prepared in DMEM with 4% FBS. The plates were allowed to solidify at room temperature for ~15 min and transferred to a 37 °C incubator with 5% CO_2. At 3 days post-infection, the plates were fixed with 10% buffered formalin (VWR, Radnor, PA, USA) for 30 min and washed with sterile PBS (pH 7.2). The agar plugs were gently removed, and the cells were stained with 0.2% crystal violet in 20% ethanol (VWR) for 10 min. The wells were washed with sterile water and dried, and the clear plaques were counted and presented as the number of plaque-forming units (PFU) of the virus per gram or ml of tissue or lysate.

2.6. Hamster Infection Studies

Five-to-six-week-old male golden Syrian hamsters (*Mesocricetus auratus*) were procured from Envigo (Indianapolis, IN, USA) and housed in animal biosafety level-2 containment (BSL2) for a week to acclimate. One group of hamsters (n = 8) was vaccinated with adjuvanted MT-001, and another group of hamsters (n = 6), injected with PBS plus Alhydrogel, served as the control. The MT-001 vaccine or PBS was mixed with Alhydrogel and incubated for 5 min with gentle rocking. Then, the MT-001/Alhydrogel and the PBS/Alhydrogel mixtures were supplemented with CpG-ODN1826 immediately prior to injection. Each hamster was injected intramuscularly, in the flank, with 50 µL of the respective RBD or control vaccines containing 10 µg of MT-001 (or an equal volume of PBS), 500 µg of Alhydrogel, and 100 µg of ODN1826. The hamsters were administered a second dose of MT-001 or PBS control 21 days after the primary dose. Blood from all animals was collected on the day of vaccination (day 0; pre-bleed) and at 14, 21, 28, 35, and 42 days post-vaccination. On day 42, post-primary vaccination, all animals were challenged with SARS-CoV-2/USA-WA1/2020 strain (BEI Resources) at 10^5 PFU/hamster in 40 µL through intranasal instillation (20 µL/nostril) as we reported previously [40]. Hamsters were weighed every day following infection and euthanized on day 4 post-infection. Necropsy was performed, and blood and lungs were collected under aseptic conditions.

2.7. Lung Viral Load Assessment

Lung homogenates were prepared using a 0.3 mg (~40% total lung weight) portion of lung tissues in a screw cap vial containing 1 mL of DMEM media and 0.3 mL (w/v)

of 1 mm Zirconia/silica beads (MP Biomedicals, Irvine, CA, USA). Tissues were lysed by using a FastPrep homogenizer (MP Biomedicals). The homogenates were centrifuged, and the supernatant was filtered through a 0.45-micron filter (Millipore-Sigma), diluted in serum-free DMEM, and 400 µL was used to infect Vero E6 cell monolayers in the six-well plates for a virus plaque assay.

2.8. Determination of Viral Load by Quantitative PCR

Total RNA was extracted from the lungs using TRIzol reagent (ThermoFisher Scientific) and purified by RNeasy mini columns (Qiagen Sciences Inc., Germantown, MD, USA). The eluted RNA was subjected to complementary DNA synthesis using a High-Capacity cDNA Reverse Transcription Kit as per the suggested protocol (ThermoFisher Scientific). Quantitative PCR (qPCR) was performed as described by Ramasamy et al. [40] using SARS-CoV-2 N gene-specific primers (SARS-CoV-2_N-F1: GTGATGCTGCTCTTGCTTTG and SARS-CoV-2_N-R1: GTGACAGTTTGGCCTTGTTG) (IDT, Coralville, IA, USA) and Power SYBR Green PCR MasterMix as per the manufacturer's protocol (ThermoFisher Scientific). The SARS-CoV-2 N gene-specific primers were used to amplify a 97 bp product by conventional PCR and this was purified by the Qiagen gel extraction kit (Qiagen). The purified N gene PCR products were used in real-time PCR to prepare a standard curve. Viral copy numbers in the lung samples were determined from the standard curve.

2.9. Virus Neutralization Assay

The SARS-CoV-2 neutralization assay was performed using the standard protocol described by Ravichandran et al., 2020 [41]. Briefly, 100 $TCID_{50}$ of SARS-CoV-2 isolate USA-WA1/2020 or Omicron variant (B.1.1.529) was added to a two-fold dilution series of serum samples in DMEM containing 10% fetal bovine serum. The serum-virus mixtures were incubated at 37 °C for 1 h. Meanwhile, a single-cell suspension of Vero E6 cells was prepared in DMEM containing 10% fetal bovine serum at 1.4×10^4 cells in 20 µL/well in white 96-well flat-bottom Nunc MicroWell plates (ThermoFisher Scientific). Following incubation, 100 µL of the serum-virus mixture was added to each well. Additional wells omitting either the serum samples or the virus were included as controls. The plates were gently rocked for the uniform distribution of cells and then incubated for 72 h at 37 °C with 5% CO_2. Plates were equilibrated to room temperature for 30 min, after which 50 µL of CellTitre Glo reagent (Promega, Madison, WI, USA) was added to each well, and the plates were gently rocked for 2 min and incubated at room temperature for 10 min. The luminescence from the wells was measured using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT, USA). The luminescence from blank wells containing 120 µL DMEM with 10% fetal bovine serum and 50 µL CellTitre Glo reagent was recorded as baseline values. The 50% neutralization titer (NT_{50}) was calculated using Graph Pad Prism from a sigmoidal four-parameter logistic (4PL) fit the luminescence data using the geometric means of the positive and negative controls to bind the top and bottom of the curve.

2.10. Histopathology

The formalin-fixed hamster lung portions were embedded in paraffin and sectioned following standard protocol, as we reported previously [40]. The hematoxylin-eosin-stained lung sections were analyzed using the EVOS FL cell imaging system (ThermoFisher Scientific). The pulmonary inflammation was scored according to the severity as follows: 0—no cellular infiltration and intact alveoli, 1—mild cellular infiltration with one or two foci and intact alveoli, 2—prominent multifocal cellular infiltration with no visible alveoli, 3—significant cellular infiltration involving a larger area of the lung with no visible alveoli, and 4—highest cellular infiltration involving extensive area of the lung with no visible alveoli.

3. Results

3.1. Antigen Construct Design Impacts Both the Manufacturability and Immunogenicity of a Protein Component Vaccine

Upon the publication of the ancestral SARS-CoV-2 Wu-1 strain DNA sequence in early 2020 [42], we applied antigen expression construct design principles established in the lab, aiming to create a well-folded and soluble spike RBD antigen based on a fragment of the S1 subunit. The design of the construct is critical when parsing a multi-domain protein into smaller expressible subunits [43]. The lab previously provided over 1000 unique human antigens to the *NIH Common Fund Protein Capture Reagents Program* for renewable antibody generation [43,44]. Central to this effort was a bioinformatics toolbox, developed by the Northeast Structural Genomics Consortium, for parsing multi-domain proteins into subdomains that could be expressed recombinantly [45]. These tools, involving meta-analyses of protein amino acid sequences using various protein structure prediction methods, have been used successfully to design and optimize thousands of protein constructs for NMR and crystallization studies [46] as well as antigens for antibody discovery [43]. In all cases, domain boundaries and other sequence features were given special weight, so as not to truncate constructs within ordered regions required for proper folding or presentation of conformational epitopes [28–30].

We reasoned that an immunogen designed to preserve domain structure would enhance expression yields and promote optimal manufacturability. By combining bioinformatics predictions from DisMeta [45] with protein homology models and sequence alignments to known structures, we identified clear domain boundaries that separated the RBD region from the surrounding N-terminal and C-terminal regions of the spike protein. The resulting fragment, consisting of residues 316–594 of the full-length SARS-CoV-2 spike protein, encoded a 279 amino acid polypeptide with two complex subdomains containing non-contiguous N-terminal and C-terminal residues distal to the RBD ACE2 binding region (Figure 1; PDB IDs: 7BYR, 7KNE). In addition to the so-called CD1, RBM (receptor binding motif), and CD2 regions (Figure 1), this fragment also included the region immediately C-terminal to the RBD, previously termed C-terminal domain 1 (CTD1), and a portion of the so-called "N-terminal domain" of S1 in SARS-CoV [47]. A short, four-residue "C-tag" [-EPEA] was appended to the C-terminus of the fragment to facilitate efficient purification from cell culture [38], and the resulting construct was termed MT-001. No linkers or protease cleavage sites were included in order to minimize the number of non-native residues in the expressed protein. As the N- and C-termini of the construct are predicted to be located on the face of the protein opposite the RBM (Figure 1), it was thought to be unlikely that the short C-tag would sterically hinder desired antibody interactions. The C-tag also provided a convenient site-specific handle for immobilization when used in downstream assays (see ELISA in Methods). Finally, the immunogenicity of the C-tag has been investigated, and no significant anti-C-tag antibody responses have been observed [39]. Transient expression of MT-001 with a mammalian cell secretion vector (ATUM, Inc.) in HEK293 suspension culture resulted in high titers of MT-001, as described in Methods. The purified protein was nearly all monomeric, with an apparent molecular weight of 39.4 kDa, consistent with what would be expected for a glycosylated protein (calculated unglycosylated MW = 31.6 kDa) (Figure S2).

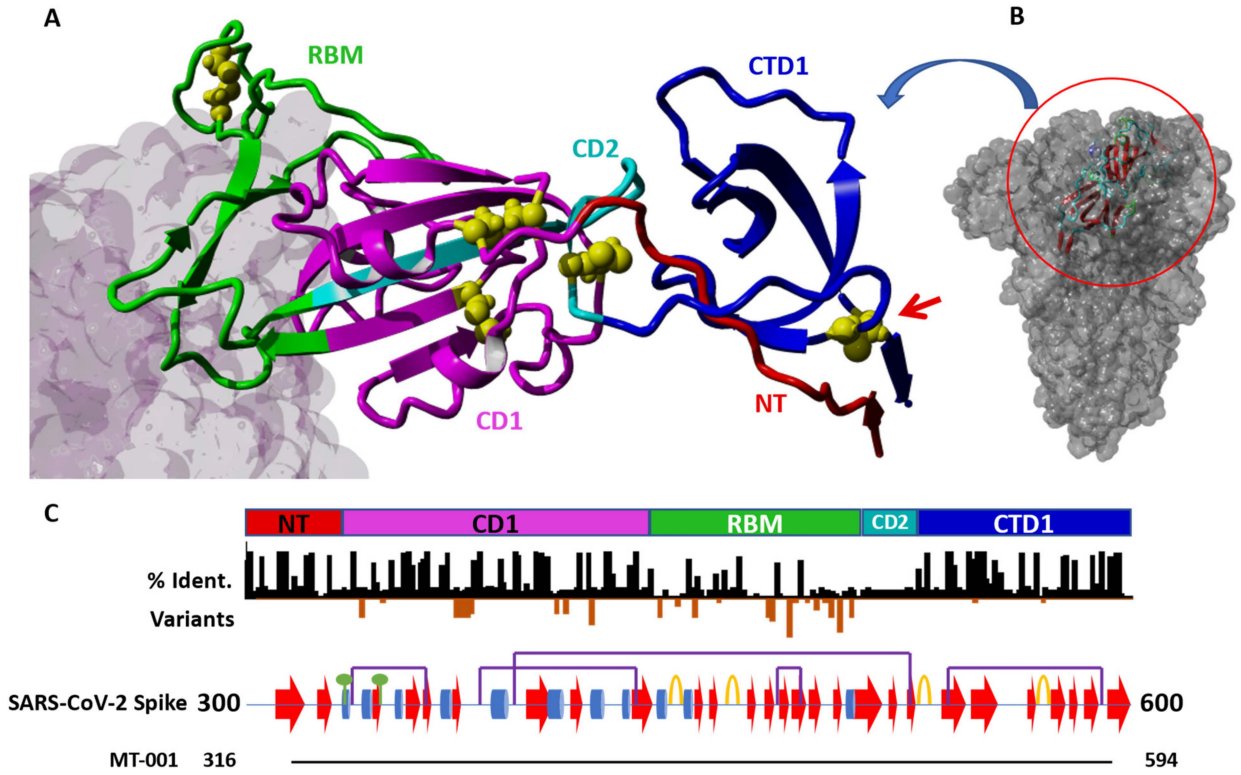

Figure 1. Detail of the SARS-CoV-2 spike protein in the region 300–600 and MT-001 construct design. (**A**) Structure of MT-001 construct derived from PDB IDs 7BYR and 7KNE. The RBD construct is color-coded by annotated blocks of amino acid sequence ("regions", see panel C and [45]). Cysteines are shown as yellow balls. The cell surface target of the RBM, ACE2 (from 7KNE), is shown as a gray molecular surface (left). (**B**) The MT-001 construct (ribbon) shown in the context of the full-length spike trimer (space-filling model). (**C**) Schematic of the regions shown in (A). Top: Color-coded region key for the MT-001 construct in (**A**). NT: N-terminal region (residues 316–332, red); CD1: "core domain 1" region (333–436, magenta); RBM: receptor binding motif (437–508, green); CD2: "core domain 2" region (509–527, cyan); CTD1: "C-terminal domain 1" region (528–594, blue). The 538–590 disulfide bond that stabilizes CTD1 is indicated by a red arrow. Middle: Black bars—Sequence identity per residue between SARS-CoV-2 spike and representative members of the coronavirus superfamily, demonstrating highly conserved regions N- and C-terminal to the RBM (Table S1). Orange bars—Sites of and frequency of mutations in characterized SARS-CoV-2 variants [3]. Lower: Schematic showing the secondary structure and post-translational modifications in the region from residues 300–600 in the SARS-CoV-2 spike protein. Alpha helices are shown as blue cylinders, beta sheets as red arrows, and turns as orange loops. Disulfide bonds are denoted with purple bridges, and N-linked glycosylation sites are denoted with green circles. Bottom: Alignment of the MT-001 construct with the visualized region.

3.2. MT-001 Induces a Potent and Durable Anti-SARS-CoV-2 RBD Immune Response in BALB/cJ Mice

To explore the immunization dose-response characteristics and measure the durability of elicited antibody levels, an experiment was performed in which two cohorts of five 8- to 10-week-old female BALB/cJ mice were immunized with 1 μg, 3 μg, or 15 μg of MT-001. The MT-001 immunogen was formulated with 500 μg Alhydrogel (alum) and administered as two intramuscular (IM) injections at a 3-week interval (Figure 2A). Sera were collected at 5, 29, and 52 weeks following the primary immunization (Figure 2A). The highest RBD-specific IgG half-maximal geometric mean titers (GMTs) at each time point were observed with the 3 μg and 15 μg doses of MT-001 ($EC_{50} > 10^5$, Figure 2B). MT-001 at the 3 μg dose exhibited half-maximal ELISA GMTs comparable in potency to reported 2-shot

prime/boost immunization results with approved mRNA vaccines and protein component vaccines assayed in the same BALB/cJ mouse system [36,37,48,49]. Most notably, there was no significant waning of the MT-001-induced specific anti-RBD antibody levels in the animals between 5 weeks and 52 weeks post-immunization (Figure 2). This is in contrast to the mRNA- and most other protein component-based vaccines where protective antibody levels typically wane with a half-life of approximately two months [50,51].

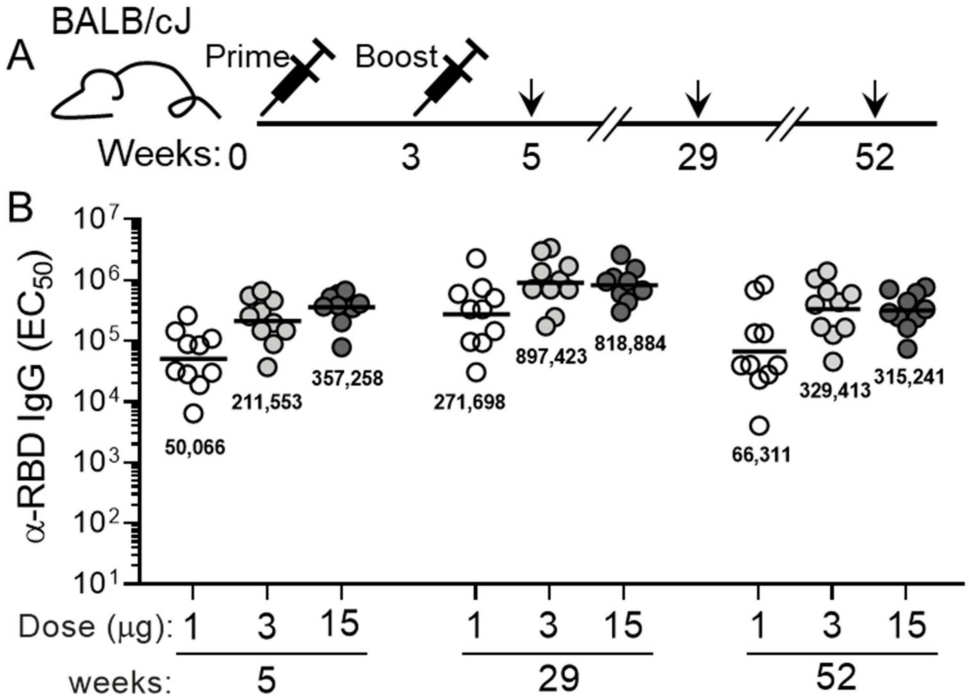

Figure 2. Dose-response and durability of anti-RBD serum IgG levels in mice vaccinated intramuscularly with Alhydrogel-adjuvanted MT-001. (**A**) Immunization and bleeding schedule. Prime and secondary immunizations of the animals were at weeks 0 and 3, respectively; bleeds were performed on week 5, week 29, and week 52 to provide sera for analyses. Primary and secondary immunizations were with the same amount of MT-001 antigen per animal—1 μg, 3 μg, or 15 μg—for each group of 10 mice. (**B**) Midpoint (EC_{50}) geometric mean anti-RBD IgG ELISA titers for each dosage group at each time point. GMTs are indicated numerically below each cluster of points.

3.3. Addition of a TLR-9 Agonist CPG ODN1826 to the MT-001 Vaccine Mixture Further Increases Antibody Titers and Promotes a More Balanced Immune Response

Since alum-based adjuvants such as Alhydrogel promote a type 2 inflammatory response [52], we next tested if the addition of a TLR-9 agonist, CpG ODN1826, would promote a more balanced immune response. Mice immunized with 3 μg MT-001 formulated with 500 μg Alhydrogel and 20 μg CpG ODN1826 exhibited significantly increased RBD-specific IgG binding titers compared to mice immunized with MT-001 and Alhydrogel alone by 5 weeks post-primary immunization (ELISA GMTs $\approx 2 \times 10^6$ for mice where CpG ODN1826 was included vs. $\approx 3.5 \times 10^5$ when omitted), and the enhanced response persisted through 47 weeks (Figure 3B). In addition to significantly higher levels of RBD-specific IgG1 antibodies (Figure 3C), these mice had robust RBD-specific IgG2a/b titers (Figure 3D). Thus, the average IgG1:IgG2a/b ratio in mice adjuvanted with both Alhydrogel and CpG ODN1826 was significantly increased (Figure 3E), indicative of a more balanced Th1/Th2 response, which may strengthen the protective efficacy of MT-001.

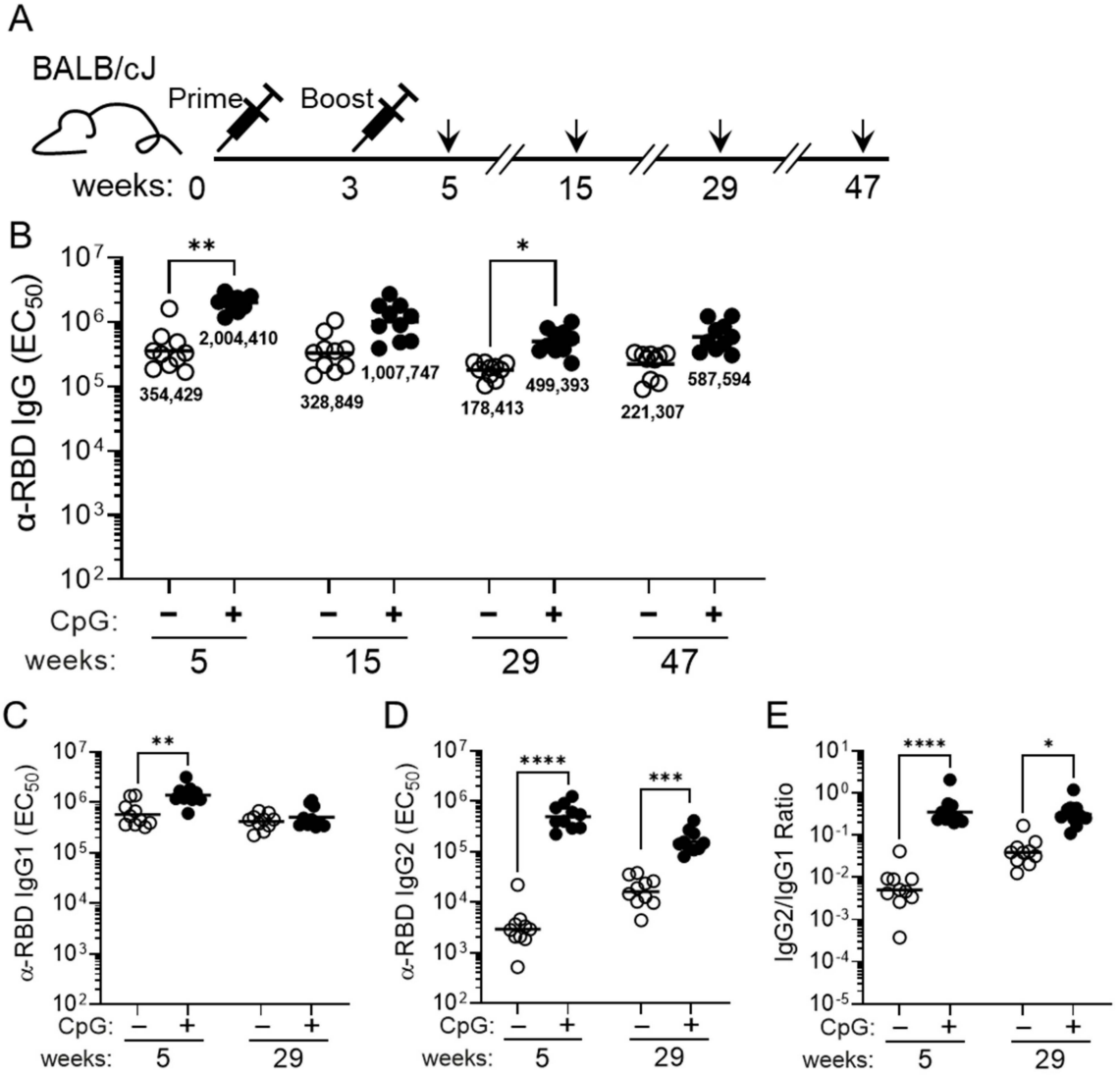

Figure 3. Immunization of mice with 3 μg of MT-001 antigen in Alhydrogel with or without the TLR-9 agonist co-adjuvant, CpG ODN1826. (**A**) Schematic illustrating the MT-001 prime-boost regimen and bleeding schedule for 8–10-week-old female BALB/cJ mice (n = 10). (**B**) RBD-specific IgG binding titers were assessed in mice immunized with 3 μg MT-001 adjuvanted with 500 μg Alhydrogel only (−, open circles) or with 500 μg Alhydrogel plus 20 μg CpG ODN1826 (+, closed circles). Binding antibody responses are displayed at 5, 15, 29, and 47 weeks post-primary immunization. The balanced Th1/Th2 response resulting from the addition of CpG ODN1826 is evidenced by increased RBD-specific IgG1 (**C**) and IgG2 (**D**) antibody titers. This corresponded to an increased ratio of IgG2 to IgG1 antibody levels in CpG ODN1826-adjuvanted animals (**E**). Each circle (open or solid) represents the half-maximal titer for each serum sample averaged across at least three independent ELISAs. Horizontal bars indicate geometric mean titers per dose. p values reflect unpaired t tests between groups (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

3.4. MT-001 Protects Syrian Golden Hamsters in a SARS-CoV-2 Pulmonary Challenge Model

Syrian hamsters are an accepted in vivo model for human SARS-CoV-2 infection as they mimic many of the characteristics of human COVID-19 [53]. Therefore, we next tested the protective efficacy of MT-001 against SARS-CoV-2 infection in vivo. Hamsters were immunized with 10 μg MT-001 adjuvanted with 500 μg Alhydrogel and 100 μg CpG ODN1826 and boosted with the same dose 3 weeks later (Figure 4A). Control animals were only mock vaccinated with the adjuvant (Alhydrogel plus CpG ODN1826). The MT-001 vaccinated hamsters had robust RBD-specific IgG titers after six weeks post-primary

immunization ($EC_{50} \approx 10^5$, Figure 4B). To determine if the antibody response was protective, the vaccinated and control hamster cohorts were challenged intranasally with 10^5 PFU of SARS-CoV-2 (USA-WA1 strain) after six weeks post-primary immunization and monitored for 4 days before analysis. The level of infectious SARS-CoV-2 in lung homogenates from MT-001 vaccinated hamsters was undetectable by plaque assay even at the lowest dilution (1/10) of the sample used (Figure 4E). Therefore, the viral load per gram of lungs in these animals was calculated based on the lower limit of detection of the assay. In the control group of mock-vaccinated hamsters, infectious virus plaques were detected even at a $1{:}10^6$ dilution of lung homogenates. Thus, the viral load per gram of lung tissue was significantly lower (less than or equal to 10^3 PFU/g) in MT-001 vaccinated hamsters than in mock-vaccinated hamsters (10^9 PFU/g) (Figure 4C). Likewise, N gene copy numbers as determined by qPCR were on average 1000-fold lower in MT-001 vaccinated hamsters compared to hamsters that received adjuvant alone (Figure 4D). While weight loss and lung pathology are usually associated with SARS-CoV-2 infection in hamsters, at the viral dose used there were no significant differences between MT-001 vaccinated and mock-vaccinated animals with respect to these two parameters up to 4 days post-infection when the hamsters were sacrificed (Figure S3A–C). However, a significant reduction in viral burden was observed in hamsters vaccinated with MT-001 compared to those that received adjuvant alone. Compared to the uninfected group, hamsters in both the MT-001 vaccinated and adjuvant-vaccinated groups showed a significantly higher degree of lung inflammation, marked with infiltration of immune cells into the interstitial space that resulted in the partial collapse of alveoli at 4 days post-infection (Figure S3B,C). It should be noted that previous SARS-CoV-2 challenge studies have indicated that the lung pathology between vaccinated and mock-vaccinated hamsters does not begin to differ until four to six days post-challenge [54,55]. However, despite the difference in lung viral load, no striking differences were noticed in the lung disease pathology (Figure S3C) or physiological aspects (body temperature and general locomotor skills) between MT-001 vaccinated and mock-vaccinated animals. Collectively, these studies show that vaccination with MT-001 protected hamsters from SARS-CoV-2 infection.

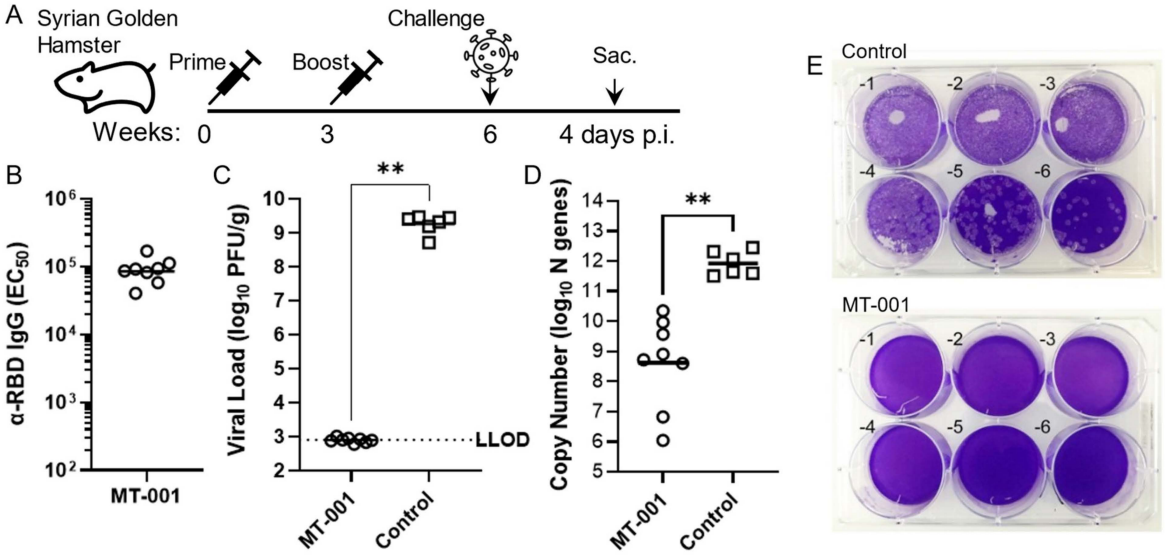

Figure 4. SARS-CoV-2 challenge of hamsters vaccinated with MT-001 adjuvanted with Alhydrogel and CpG ODN1826. (**A**) MT-001 prime-boost regimen and SARS-CoV-2 challenge schedule for Syrian golden hamsters. (**B**) Midpoint hamster RBD-specific IgG ELISA GMTs. (**C**) Lung viral load in hamsters vaccinated with 10 μg of MT-001 co-adjuvanted with 500 μg Alhydrogel + 100 μg CpG ODN1826, or mock-vaccinated with adjuvants alone, and infected with 10^5 PFU of SARS-CoV-2 six weeks after the primary immunization (top). Individual data points and mean +/− S.D for MT-001 with adjuvants (open circles; $n = 8$) or adjuvants alone (open squares; $n = 6$) is shown. (**D**) Viral RNA

copy numbers in the lungs of hamsters vaccinated with MT-001 or adjuvants alone four days after intranasal infection with SARS-CoV-2. Data were analyzed by non-parametric Mann–Whitney test (** $p < 0.01$). (**E**). Representative plates from lung viral load assessment. Lung homogenates from adjuvant-only (control) or MT-001-immunized (MT-001) hamsters were prepared 4 days post-infection and used to infect Vero E6 cells. No plaques are observed with the lung homogenates from MT-001 immunized hamsters even at a 1:10 dilution, while plaques are visible from the lungs of control animals even at a 1:1,000,000 dilution.

3.5. Immunization with MT-001 Produces a Broad Antibody Response Capable of Recognizing and Neutralizing Emergent Variants including Delta and Omicron

We then asked if the response in mice vaccinated with MT-001 resulted in antibodies that were reactive to emerging variant SARS-CoV-2 strains. We first compared serum anti-RBD IgG ELISA titers directed against the ancestral SARS-CoV-2 Wu-1 ("WT") strain to IgG ELISA titers from the same sera with the SARS-CoV-2 Delta variant RBD. Sera from mice vaccinated with MT-001 adjuvanted with Alhydrogel + CpG ODN1826 showed less than a 4-fold decrease in titer with Delta RBD when compared to the titers obtained for WT (Wu-1/US-WA1) RBD (GMTs: 194,082 vs. 739,163, respectively; Figure 5A). Vaccination without the inclusion of the CpG ODN1826 co-adjuvant, however, resulted in a more than 20-fold decrease in the Ab binding titer to the Delta RBD as compared to WT RBD (ELISA GMTs: 10,237 vs. 223,688, respectively). This indicated that the magnitude and breadth of the cross-reactive antibody response to variants elicited by MT-001 were enhanced by the inclusion of CpG ODN1826.

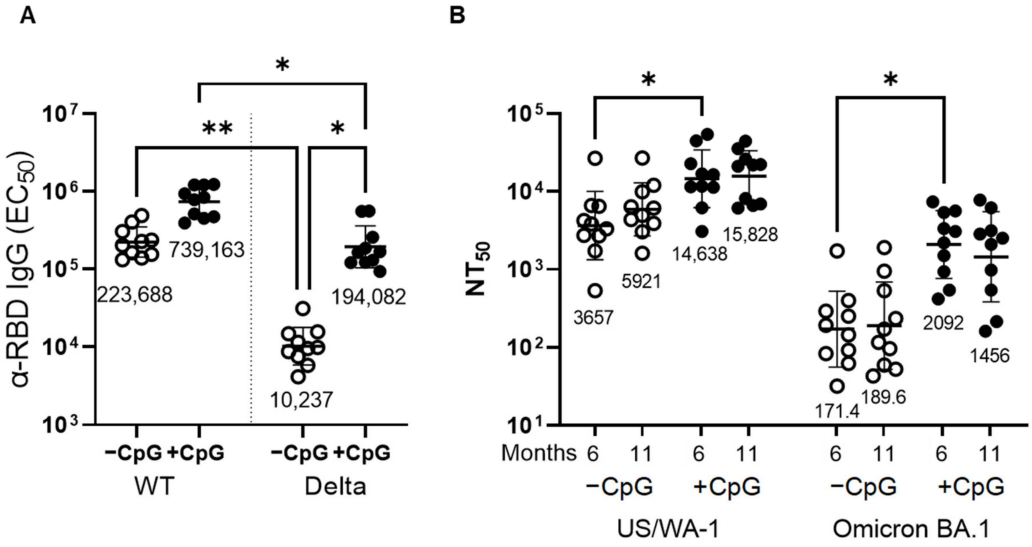

Figure 5. SARS-CoV-2 variant neutralization. BALB/cJ mice (n = 10 per group) were immunized twice at a three-week interval with 3 μg MT-001 and 500 μg Alhydrogel, with or without 20 μg ODN1826 (CpG), as indicated. Mice were bled at 29 and 47 weeks post-primary immunization (see Figure 3A), and sera were assayed for antibody binding and neutralization. (**A**) Anti-RBD midpoint ELISA titers at 29 weeks post-primary immunization were determined using the Wu-1 RBD (WT) or the Delta variant RBD (Delta) as a target. (**B**) Mouse serum-virus neutralization titers at approximately six months (29 weeks) and eleven months (47 weeks) post-primary immunization with MT-001 + Alhydrogel without (−CpG) or with (+CpG) ODN1826 were determined using SARS-CoV-2 USA-WA1/2020 (US/WA-1) or SARS-CoV-2 isolate hCoV-19/USA/MD-HP20874/2021 (Omicron BA.1) as described in Methods. Geometric mean titers are as indicated. Asterisks indicate statistical significance as determined by a two-tailed Kruskal–Wallis test with subsequent Dunn's multiple-comparisons test (* $p < 0.05$; ** $p < 0.01$).

We next tested if the enhanced recognition of the Delta variant RBD by sera from mice vaccinated with MT-001 adjuvanted with Alhydrogel + CpG ODN1826 correlated with an enhanced ability to neutralize the Omicron BA.1 SARS-CoV-2 variant. In a live virus in vitro neutralization assay, sera from mice immunized with MT-001 adjuvanted with Alhydrogel + CpG ODN1826 had an Omicron BA.1 virus neutralization titer (NT_{50}) of 2092 at six months post-boost and 1456 at 11 months post-boost (Figure 5B). When CpG ODN1826 was not included, the NT_{50}s were reduced to 171 at six months post-boost and 190 at 11 months post-boost. Strikingly, the Omicron BA.1 neutralization titers obtained with MT-001 adjuvanted with Alhydrogel + CpG ODN1826 were comparable to neutralizing titers reported in BALB/c mice immunized twice with a variant-matched vaccine, mRNA-1273.529 [56]. These data show that MT-001 was efficacious in generating significant nAb responses against emergent variants, despite being based on the ancestral SARS-CoV-2 sequence, and that these nAb responses were durable for at least 11 months.

4. Discussion

Designing an expression construct that incorporates a fragment of the SARS-CoV-2 spike protein, encompassing both the spike ACE2 receptor binding motif (RBM) as well as surrounding sequences that provide the local structural context, is not a straightforward task [30]. Ideally, the design should result in good expression yields of a relatively "well-behaved" (i.e., stable, well-folded, and soluble) gene product while maximizing antigen immunogenicity and preserving conserved regions that might serve as targets for broadly neutralizing antibodies. Our MT-001 RBD expression construct (Figure 1), which includes the spike protein RBM (residues 437–508, green) together with upstream and downstream regions (residues 316–436, red and magenta, and residues 509–594, cyan and blue), encodes a section of the spike protein with an extended polypeptide architecture that appears to be composed of three distinct domain-like regions (Figure 1). The term "domain-like" in this sense refers to compact, structurally contiguous subdomain regions of the protein that may have distinct structural and/or functional roles. For example, ACE2 receptor binding is carried out by the domain-like RBM [57]. The extended three-subdomain structure is tethered at its N- and C-termini by residues S316 and G594 in close proximity, forming the ends of a short antiparallel beta-sheet (Figure 1). At several points within some of the subdomains, residues that are relatively distant from the primary sequence form significant interactions in the secondary and tertiary structure. For example, in the central subdomain domain (Figure 1, magenta), which consists of a twisted five-stranded antiparallel beta-sheet composed mostly of CD1 amino acids, the center beta strand (Figure 1, cyan) comes from the CD2 region C-terminal to the RBM. Additionally, the CTD1 subdomain (the region from 528 to 594, Figure 1, blue) in our construct forms a well-defined structure, stabilized by the 538–590 disulfide bond (Figure 1B, red arrow), and packs against the N-terminal region ("NT", 316–332) of the MT-001 spike fragment (Figure 1, red).

These interactions likely play an important role in the proper folding of the RBD; shorter constructs, involving truncations that result in the loss of these interactions, might partially destabilize the native structure, and could even introduce non-native conformational epitopes. This domain architecture suggests that the spike S1 region represented by our RBD construct, spanning amino acids 316–594, may have evolved via two consecutive nested domain insertion events [58,59]. The resultant 316–594 region of the spike protein may have then undergone selection as a coherent structural and functional unit involved in the conformational transition between the "RBD-down" and "RBD-up" states of the prefusion spike trimer [60]. Thus, for an RBD-centric immunogen in a SARS-CoV-2 vaccine, the MT-001 construct is arguably close to the optimal choice. Furthermore, structural analysis of the full-length spike protein shows that CTD1 may act as a relay between the RBD and the fusion-peptide proximal region (FPPR) domains to trigger fusion upon receptor binding [61]. The proposed relay function of CTD1 suggests that some antibodies targeting this region might interfere with viral entry and thus have SARS-CoV-2 neutralization activity. Since this region is relatively conserved among sarbecoviruses and contains few

mutations found in SARS-CoV-2 variants of concern (VOCs), it may be able to elicit broadly neutralizing antibodies (bnAbs) ([62,63]; Figure 1C).

The properties that make an antigen well-suited for expression and purification may also translate into improved immunogenicity in the context of vaccines. By providing an "extended" RBD-containing SARS-CoV-2 spike protein construct that is stable and well-folded without requiring non-native modifications to the sequence, the immune response can be focused on a critical region of the spike protein containing many neutralizing epitopes [22,63,64]. This strategy may, in addition, minimize decoy or immunodominant epitopes, steric hindrance, or possibly immune suppressive components of the full-length protein. Outbreaks of SARS-CoV and MERS-CoV earlier this century, combined with the periodic emergence of new SARS-CoV-2 variants of concern and the constant threat of future coronavirus pandemics, motivate the development of a broadly protective pan-coronavirus vaccine. Within the spike protein, the RBM shares low sequence homology across the coronavirus family, which is expected given the numerous hosts and range of cellular receptors targeted. However, some regions flanking the RBM are relatively highly conserved, especially within the CTD1 subdomain (Figure 1 and Table S1). For an S1 sub-fragment RBD-based vaccine [26], the inclusion of the CTD1 subdomain should present additional conserved B-cell and T-cell epitopes which may provide broader pan-variant and pan-coronavirus responses compared to RBD constructs where this region has been truncated. Indeed, a recent study involving a hierarchical Bayesian regression model trained on more than 6 million SARS-CoV-2 genome sequences predicted that even for *future*, yet-to-emerge variants of concern (VOCs), mutations in the CTD1 subdomain were likely to be relatively rare due to their negative contributions to overall viral fitness [65].

The fusion of purification tags and other non-native sequences must also be considered when designing a vaccine construct. MT-001 employs the C-tag, a short four-residue (-Glu-Pro-Glu-Ala) tag incorporated at the C-terminus of the construct, to enable efficient purification and site-specific immobilization for use in downstream assays [66]. This provides several advantages over other commonly used purification tags. Due to its size, the C-tag would be expected to have minimal effect on protein expression and solubility, and tag cleavage may not be required. Studies have found the C-tag itself to be minimally immunogenic, and it has been used successfully in GMP processes for vaccine manufacturing [39]. Purification step-yields are high after only a single tag-specific affinity chromatography step and, unlike RBD constructs incorporating fusions to non-viral scaffolding moieties [30,32], nearly 100% of the expressed protein consists of the target SARS-CoV-2 antigen (Figure S2). The C-tag allows for indirect solid phase immobilization of the antigen (e.g., in microtiter plate wells) and, with the tag being located on the opposite side of the antigen from the receptor binding motif, allows for an unimpeded display of the native 3D antigen structure and efficient capture of antibodies recognizing discontinuous conformational B-cell epitopes of the RBD.

Our animal experiments have shown that immunization with MT-001 markedly enhanced the production of IgG antibodies specific to SARS-CoV-2 spike proteins, with levels comparable to the most effective vaccines characterized in the literature to date [36,37,48]. In mice, following a two-dose immunization with as little as 1 μg MT-001 adjuvanted with Alhydrogel, high anti-RBD (Figure 2) and anti-spike IgG (Figure S4) titers were observed. These were associated with the increased production of neutralizing antibodies against both pseudovirus (unpublished results) and infectious virus (Figure 5). Moreover, as demonstrated in two independent experiments, these immune responses were remarkably durable, with minimal waning in antibody titers observed between 5 weeks and one-year post-primary immunization (Figures 2 and 3). This is in stark contrast to the widely used mRNA vaccines, where antibody titers decay significantly after 6 months [67,68]. Considering the lack of durability observed for most COVID-19 vaccines to date, results from our long-term in vivo studies further differentiate MT-001 from other immunization strategies directed against SARS-CoV-2 (See Figure 1 of [69]). Durable immunity conferred by vaccines has been attributed to the generation of long-lived plasma cells

(LLPCs) residing in bone marrow, which in some cases can express and secrete protective, pathogen-specific antibodies for decades [70,71]. It is possible that MT-001 is unusually capable, especially compared to other SARS-CoV-2 vaccines [71], in eliciting high levels of spike protein-specific LLPCs.

In the hamster model of pulmonary SARS-CoV-2 infection, vaccination with MT-001 protected the animals by significantly reducing the lung viral burden. However, the body weight loss and pulmonary pathology of SARS-CoV-2 infection were comparable between adjuvant-only vaccinated and MT-001 vaccinated hamsters. This observation indicates that disease pathology is not directly proportional to the lung viral load and that vaccinations with an immunogenic adjuvant may not have direct effects in reducing pulmonary pathology. Nonetheless, our observation is consistent with other hamster SARS-CoV-2 challenge studies in the literature, where the lung pathology between vaccinated and mock-vaccinated hamsters does not begin to differ until four to six days post-challenge [54,55]. Further studies will be required to address the mechanistic basis of the immune response elicited by MT-001 at the cellular level.

Alum-based adjuvants such as Alhydrogel are known to elicit a type 2 inflammatory response [72], which may not be ideal for inducing protective immunity against pathogens [73]. Previously, CpG-containing oligonucleotides have been shown to induce a type 1 response by acting as Toll-like receptor 9 (TLR9) agonists, providing a more balanced Th1/Th2 response when used in conjunction with alum adjuvants [28,52,74]. Mice immunized with a dose of 3 μg MT-001 adjuvanted with Alhydrogel and the TLR9 agonist CpG ODN1826 exhibited significantly higher anti-RBD IgG titers at 5 weeks post-primary immunization compared to mice immunized with 3 μg MT-001 adjuvanted with Alhydrogel alone. This increased titer appeared to be primarily due to a two-order-of-magnitude increase in the anti-RBD IgG2a/b titers in CpG adjuvanted mice, which resulted in a more balanced IgG2a/b to IgG1 ratio. This is consistent with data reported for another RBD-based protein vaccine utilizing a different CpG oligonucleotide and alum as adjuvants [74]. The significantly increased IgG2a/b titer associated with the CpG adjuvant persisted for at least 29 weeks post-primary immunization and was correlated with increased neutralization titers against both the Wu-1 strain and variants of SARS-CoV-2 (Figures 3 and 5; additionally, see below).

CpG ODN1826, when used as a co-adjuvant with Alhydrogel, has previously been shown to enhance peak immunogenicity in mice and hamsters with RBD-based SARS-CoV-2 vaccines [74]. We have shown here that, in addition to enhancing the potency and Th1/Th2 balance of the immune response elicited by MT-001 (Figure 3), the inclusion of CpG ODN1826 also enhanced the antibody response to emerging variants (Figure 5). This could have been due to a direct enhancement of the breadth of the response (see below) or simply due to a mass action effect where the levels of pre-existing anti-variant antibodies were elevated above a threshold concentration in the serum where they were rendered detectable by the assays used. Further work will be required to determine the details underlying CpG augmentation of the immune response in this system. In addition, unlike the waning of immunity against variants seen with spike-based mRNA vaccines, a two-dose regimen of MT-001 elicits a diverse and protective antibody response that persists for at least eleven months. In one recently described experiment, where BALB/c mice were pre-immunized with two doses of the mRNA vaccine BNT162b2 and boosted on day 104 with the same vaccine, the *peak* post-boost neutralization titer against Omicron BA.1 was reported to be 2075 GMT (BioNTech Innovation Series Presentation, 29 June 2022). Here, we report a similar Omicron BA.1 virus neutralization titer (GMT 2092) in MT-001-immunized BALB/c mice at six months post-immunization without an additional booster dose (Figure 5). In the absence of an additional booster, mRNA-vaccinated BALB/c mice typically show virtually no detectable variant neutralization titers at a comparable time interval post-immunization [55].

The increased breadth of the immune response observed when the MT-001 immunogen is adjuvanted with both alum and CpG ODN1826 comports with data showing that TLR-9

agonists activate the innate immune system by signaling through IRF7 while also directly stimulating B cells and dendritic cells [73]. This is consistent with the view that adjuvants such as TLR agonists (perhaps necessarily in the presence of a co-adjuvant such as alum) promote B-cell maturation in germinal centers, leading to higher affinity and broader antibody repertoires [73]. It has further been suggested that imprinting by innate signals during vaccination, dependent on the type and structure of the immunogen, the adjuvant(s), and the mode of delivery, among other variables, may drive the durability of the immune response by promoting the creation of long-lived plasma cells in bone marrow [69]. In translational studies aimed at developing a human vaccine, caution must be exercised when interpreting murine results with TLR-9 agonists. Compared to mice, humans and other primates express TLR-9 in a more limited subset of immune cell types, chiefly plasmacytoid dendritic cells and B cells [75]. However, it is reassuring that, for at least one other RBD-based SARS-CoV-2 vaccine ("RBD-I53-50"), a careful comparison of results with alum plus a TLR-9 agonist (CpG-1018) in both mice (strain C57BL/6) and NHPs (rhesus macaques) has been published [76–78]. It is noteworthy that for several key immunological metrics, including peak neutralizing antibody titers against the parent SARS-CoV-2 strain, neutralizing antibody titers against variants, CD4 T-cell responses, Th1 cytokine responses, and protection in a virus challenge assay, comparable responses were observed in both mice and nonhuman primates for the RBD-I53-50 vaccine co-adjuvanted with alum and CpG-1018. This concordance is encouraging and suggests that the results presented here for MT-001 will have predictive value in translational preclinical and clinical studies.

Regarding the translational relevance of the preclinical animal data presented here to future expectations for a human vaccine, particularly with respect to the durability of the immune response, attention should be paid to recent Phase 2 clinical data presented for the Corbevax vaccine [79]. Corbevax, like MT-001, incorporates an RBD-based immunogen, although the construct used to express the antigen for Corbevax, compared to the MT-001 design, is truncated at both the N- and C-termini (332–549) and modified to remove an unpaired cysteine (C538A) [27,28]. Corbevax is also produced in yeast cells rather than in animal cells as is MT-001. However, like MT-001, Corbevax is co-adjuvanted with alum and a CpG TLR-9 agonist (CpG ODN1826 in mice; CpG1018 in humans). In BALB/c mouse studies, Corbevax, when adjuvanted with alum alone, exhibits only modest IgG titers and pseudovirus neutralization titers [80]; hence, clinical studies with this vaccine have focused exclusively on formulations incorporating both the alum and the CpG adjuvants. Recently published Phase 2 studies of Corbevax have shown that it, like MT-001, exhibits remarkable durability up to 12 months post-vaccination [79]. Another notable SARS-CoV-2 vaccine for comparison purposes is the SCB-2019 vaccine developed by Clover Biopharmaceuticals. The SCB-2019 immunogen is the full-length spike protein ectodomain (based on residues 1–1211 of the ancestral Wuhan-HU-1 strain), trimerized via a proprietary C-terminal tag derived from human collagen [49]. Like Corbevax, SCB-2019 is adjuvanted with alum and CpG1018. When used to immunize female BALB/c mice at a 3 μg dose with a simple prime/boost regimen three weeks apart, SCB-2019 exhibited excellent persistence of the antibody broad neutralization titers after 140 days ([81], Figure 4C). However, compared to durable MT-001 live virus neutralization titers after 6 months of approximately 2000 GMT against Omicron BA.1.1.529 (Figure 5B, this work), the SCB-2019-immunized mice, without a third dose, exhibited Omicron BA.1.1.529 pseudovirus neutralization titers of <100 GMT) at all the later time points (Table 2 ["No 3rd dose boost Control"] and Figure 4C in reference [81]). We have also successfully expressed, in good yield and at high purity, additional variants using the same basic expression construct design and protocols used for MT-001. For example, a recombinant antigen containing the 17 point mutations found in Omicron BA.4/5 spike protein in the region corresponding to residues 316–594 of the parental strain was expressed and purified (unpublished results). This demonstrates that it should be feasible to re-design and produce updated annual booster vaccines with our system, creating new immunogens as needed that reflect the sequence information concerning recently emerged SARS-CoV-2 variants.

Taken together, the above results strongly suggest that the vaccine durability results presented here for MT-001 in mice will translate to humans. Moreover, our results show, at least for the MT-001 construct studied, that the aluminum hydroxide adjuvant alone, without the CpG TLR-9 agonist co-adjuvant, is sufficient to endow the vaccine humoral immune response with the property of high durability (Figures 2 and 3). Interestingly, the Omicron neutralizing antibody titers elicited by MT-001 are significantly higher (>20-fold) than those elicited by SCB-2019, even though the spike fragment sequence of MT-001 is entirely contained within the sequence of the SCB-2019 spike ectodomain sequence. The higher anti-Omicron titers observed in mice for MT-001 vs. SCB-2019 might be due to differences in the respective neutralization protocols (e.g., live virus assays for MT-001 vs. pseudovirus assays for SCB-2019). Alternatively, MT-001 may display to the immune system cryptic B-cell epitopes that, when buried in the 3D structure of the full-length trimeric spike ectodomain holoprotein, are effectively unavailable for neutralizing antibody elicitation.

Immunization with the RBD-based MT-001 construct focuses the immune response on the RBD domain, which has been demonstrated to elicit a significantly higher proportion of neutralizing antibodies compared to immunization with the full-length spike protein [26]. This distinction may be especially important in the context of boosting immunity with a variant-matched vaccine after prior vaccination or infection, as mutations arising in the RBD are often associated with immune escape. A variant-matched vaccine based on the full-length spike possesses a high degree of similarity to ancestral strains and existing vaccines. Boosting with such a vaccine has been shown to drive an immune response to conserved regions among the variant and ancestral strains previously imprinted by vaccination or infection, leading to only modest anti-variant antibody titers [82]. Conversely, a variant-matched vaccine based on MT-001 would not contain many of these shared ancestral spike epitopes, and the resulting response to the variant RBD region would be expected to induce a much stronger variant-specific neutralizing response. Combined with the durable immunity shown here, a variant-matched vaccine based on the MT-001 construct may be ideal for use as an annual booster designed to provide continuing protection against future SARS-CoV-2 infections.

5. Conclusions

MT-001 was designed from inception for improved manufacturability using construct design techniques refined during the operation of a high-throughput human protein production pipeline [83,84]. High-yield streamlined GMP manufacturing using standard protocols and existing infrastructure widely available in the biotech and pharmaceutical industry (e.g., 2000 L bioreactors, production-scale protein purification systems, know-how, and associated ancillary equipment) should facilitate large-scale, cost-effective production of MT-001. The ability to neutralize emerging variants, combined with MT-001's potent and durable immunogenicity, its favorable biophysical properties, and reduced logistical requirements for widespread distribution, make it an attractive candidate for further development on a global scale. Since the virus emerged in late 2019 the SARS-CoV-2 pandemic has enveloped the entire world and, as the virus continues to evolve and new variants emerge, medical countermeasures are still playing catch-up. Vaccines such as MT-001 could be in the vanguard of a future toolkit of impactful new vaccines and therapies that offer the promise of a globally coherent solution.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/vaccines11040832/s1, Figure S1: Example DisMeta Output; Figure S2: Expression and Purification of MT-001; Figure S3: Hamster challenge data; Figure S4: External validation; Figure S5: Sandwich ELISA format; Table S1: Structural alignment of the coronavirus superfamily.

Author Contributions: Conceptualization, E.C., D.B.S., S.S., L.K.D. and S.A.; Methodology, E.C., J.D., D.B.S., S.S., L.K.D. and S.A.; Software, E.C.; Validation, E.C., J.D., A.K. and S.R.; Formal Analysis, E.C., J.D., S.S., L.K.D. and S.A.; Investigation, E.C., J.D., L.J.O., A.K., S.R., R.K. and L.K.D.; Resources, E.C.,

J.D., L.J.O., A.K., S.R., S.S. and L.K.D.; Data Curation, E.C., J.D., S.S. and L.K.D.; Writing—Original Draft Preparation, E.C., J.D., S.S., L.K.D. and S.A.; Writing—Review and Editing, E.C., J.D., D.B.S., S.S., L.K.D. and S.A.; Visualization, E.C., J.D., S.S. and L.K.D.; Supervision, D.B.S., S.S., L.K.D. and S.A.; Project Administration, E.C., S.S., L.K.D. and S.A.; Funding Acquisition, D.B.S., S.S., L.K.D. and S.A. All authors have read and agreed to the published version of the manuscript.

Funding: Funding for this project was provided in part by grants from the Rutgers University Center for COVID-19 Response and Pandemic Preparedness (CCRP2) to S.A (# CCRP2 Anderson 2020) and S.S (#302211), and the New Jersey Health Foundation (#PC 111-21), and by Macrotope, Inc. (Princeton, NJ, USA).

Institutional Review Board Statement: All mouse experiments were performed under a Rutgers University Institutional Animal Care and Use Committee-approved protocol (IACUC Protocol number: PROTO99990006). All hamster experiments were performed in the ABSL3 facilities, following the ethical policies and protocols approved by the Rutgers University Institutional Animal Care and Use Committee (IACUC Approval no. PROTO202000103), which is consistent with the policies of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), the American Veterinary Medical Association (AVMA), the Center for Disease Control (CDC) and the United States Department of Agriculture (USDA).

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

Acknowledgments: We thank the Rutgers Center for Advanced Biotechnology and Medicine, the Rutgers-RWJMS Child Health Institute of New Jersey, and the Public Health Research Institute, Rutgers-New Jersey Medical School, for institutional support. In-kind support for third-party testing of sera at Nexelis by the Coalition for Epidemic Preparedness Innovations (CEPI) is also gratefully acknowledged. The SARS-Related Coronavirus 2, Isolate USA-WA1/2020, NR-52281 was deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH.

Conflicts of Interest: Authors EC and SA are co-founders and shareholders of Macrotope, Inc. and are named as inventors on patent applications describing MT-001 filed by Rutgers University. The remaining authors declare no competing interests.

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SARS-CoV-2-Neutralizing Antibody Response and Correlation of Two Serological Assays with Microneutralization

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Abstract: SARS-CoV-2 has caused a huge pandemic affecting millions of people and resulting innumerous deaths. A better understanding of the correlation between binding antibodies and neutralizing antibodies is necessary to address protective immunity post-infection or vaccination. Here, we investigate the humoral immune response and the seroprevalence of neutralizing antibodies following vaccination with adenovirus-based vector in 177 serum samples. A Microneutralization (MN) assay was used as a reference method to assess whether neutralizing antibody titers correlated with a positive signal in two commercially available serological tests:a rapid lateral flow immune-chromatographic assay (LFIA) and an enzyme-linked Fluorescence Assay (ELFA). Neutralizing antibodies were detected in most serum samples (84%). COVID-19 convalescent individuals showed high antibody titers and significant neutralizing activity. Spearman correlation coefficients between the serological and neutralization results ranged from 0.8 to 0.9, suggesting a moderate to strong correlation between commercial immunoassays test results (LFIA and ELFA) and virus neutralization.

Keywords: SARS-CoV-2; serology; neutralizing antibody; COVID-19; vaccination

Citation: Souiri, A.; Lemriss, S.; El Maliki, B.; Falahi, H.; El Fahime, E.; El Kabbaj, S. SARS-CoV-2-Neutralizing Antibody Response and Correlation of Two Serological Assays with Microneutralization. *Vaccines* **2023**, *11*, 590. https://doi.org/10.3390/vaccines11030590

Academic Editors: Rishi Jaiswal, Srijani Basu, Suman Gupta and Sneh Lata Gupta

Received: 6 January 2023
Revised: 17 February 2023
Accepted: 21 February 2023
Published: 3 March 2023

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) appeared in late 2019 in China and causes COVID-19 [1]. This is a potentially fatal infection with severe immunopathology in the respiratory system [2]. The virus has since spread across the world inducing more than 6.8 million deaths [3] and creating a significant burden on healthcare infrastructures and global economies. Natural SARS-CoV-2 infection generates an antibody response targeting nucleocapsid (N) and spike (S) proteins, including the receptor-binding domain (RBD) of the S protein. Before the introduction of SARS-CoV-2 vaccines, a serological test could be used to identify past infection by detecting any of the SARS-CoV-2 viral protein antibodies. The majority of the available vaccines introduce genetic information in the form of a nucleic acid-encoding SARS-CoV-2 spike protein into host cells. The generated spike protein can then induce binding antibodies to the spike protein and neutralizing antibodies (NAbs). Vaccinated individuals with no history of infection can only test positive for the vaccine protein targets [4]. Otherwise, not all binding antibodies can neutralize the virus because they recognize antigenic determinants that are not involved in the virus entry. Therefore, the detection of neutralizing antibodies is of major significance, since they block attachment of the S protein RBD to the cell surface receptor angiotensin-converting enzyme 2 (ACE2), preventing viral entry and replication [5].

There is considerable interest in identifying SARS-CoV-2 NAbs for measuring immune status and assessing vaccine responses. The neutralizing assay is regarded as the gold

standard method to measure functional NAbs [6], although it is quite cumbersome, time-consuming and has not been standardized. Little is known about the relationship between SARS-CoV-2 immune response and NAb responses. A few studies have reported that anti-SARS-CoV-2 NAb titers could have some relation with anti-RBD IgG and IgM antibody levels. Anti-SARS-CoV-2 IgM antibodies reach a peak within 3 weeks and then start to decrease rapidly, while IgG antibodies remain elevated for a long time. Moreover, the correlation between anti-N IgG antibody levels and NAb titers exhibit inconsistence [7].

Given the important penetration of serological rapid tests for the detection of specific anti-SARS-CoV-2 antibodies, mostly immunochromatographic and other automatized assays in Morocco and that have received marketing authorization by the Ministry of Public Health, it is necessary to study their serologic diagnostic accuracy and their performance in detecting vaccination-associated anti-SARS-CoV-2 Ab seroconversion in healthy and convalescent individuals.

In Morocco, the national immunization program that began in January 2021 gives priority to those on the front lines, such as medical staff, national authorities, security forces and those involved in the national education system, as well as the elderly and people vulnerable to the virus [8]. Health service workers including laboratory staff may come into contact with patients with COVID-19. It is important to note that the seroprevalence of anti-SARS-CoV-2 antibodies in health facilities may give an overview of the effectiveness of prevention and control measures.

This paper aims to study the seroprevalence of neutralizing activity and the concordance between two commercial SARS-CoV-2 antibody detection tests, which are not designed to specifically detect neutralizing antibodies, and the microneutralization assay using 177 sera from healthy and convalescent laboratory employees after vaccination campaign. This comparison was made at qualitative and quantitative levels.

2. Materials and Methods

2.1. Specimen Collection

The total number of laboratory workers participating in this study was 177, comprising 138 PCR-negative individuals and 39 recovered COVID-19 patients. The convalescent participants were diagnosed as COVID-19 positive during late 2020 and the first quarter of 2021. All PCR results, negative and positive, were recorded by routine testing conducted in the same period for symptomatic individuals and contacts. All participants received two doses of the ChAdOx1 nCoV-19 (AstraZeneca, Oxford) vaccine. Serum samples were collected during April and May 2021, approximately 2 months after the last dose of the vaccination administered during the vaccinationcampaigns launched on 29 January 2021 and 10 February 2021, according to the participants' ages. All participants were negative in PCR during the serum collection. Laboratory personnel were invited to participate and were informed about the purpose of the study.

The participants gave oral informed consent and were informed that the study results would not influence any clinical decisions about their specific case.

Blood samples were taken by trained healthcare personnel. The sera were processed by centrifuging at 3000$\times$ *g* for 10 min at room temperature, and were used to assess antibodies against SARS-CoV-2 usingthree different methods: microneutralization (MN) assay and two commercial assays, namely, COVID-PRESTO® (AAZ-LMB, Boulogne-Billancourt, France) a rapid lateral-flow immunochromatographic assay (LFIA), and VIDAS® SARS-CoV-2 IgG (9COG), an automated enzyme-linked fluorescent assay (ELFA) performed in VIDAS instrument (Biomérieux, Marcy-l'Etoile, France).

2.2. Rapid Lateral Flow Immune-Chromatographic Assay (LFIA)

The sera were screened for the presence of anti-SARS-CoV-2 antibodies using a rapid lateral-flow immunochromatographic assay (LFIA), COVID-PRESTO® (AAZ-LMB, France), targeting immunoglobulin-M (IgM) and immunoglobulin-G (IgG) anti-S and anti-N antibodies. The assay results were provided within 10 min and positive results were pho-

tographed. Scores from 0 to 4 were attributed to each band of IgG according to line intensity: no visible line (negative), faint line, faint band, weak band and clear band respectively.

The band intensity was read by two independent operators who were trained to score the intensity from the pictures of each value (Figure 1). As previously described, this scoring was performed for research purposes to capture semi-quantitative data about the rapid test readout and the reproducibility of subjective interpretation, considering that these are the major analytical factors that affect test performance [9].

Visual result:					
Band intensity:	No visible line	Faint line	Faint band	Weak band	Clear band
Score:	0	1	2	3	4

Figure 1. Test line scoring of the qualitative detection of SARS-CoV-2 IgG with COVID-PRESTO®(AAZ) based on intensity of lines.

2.3. Enzyme-Linked Fluorescence Assay (ELFA)

The VIDAS® SARS-CoV-2 IgG (9COG) (ref.423834) assay is a semi-automated qualitative assay run on the Vidas instrument (bioMérieux, Marcy-l'Étoile, France), using the ELFA (enzyme-linked fluorescent assay) principle to detect IgG specific to N and S proteins of SARS-CoV-2.

An index value (i) corresponds to the division of relative fluorescence values (RFV) by the RFV of the provided standard. The assay is considered negative when $i < 1.00$ and positive when $i \geq 1.00$. Assay sensitivity is 96.6% at ≥16 days after positive rRT-PCR confirmation [10].

2.4. Micro-Neutralization (MN) Assay

SARS-CoV-2 was isolated from a positive nasopharyngeal swab during August 2020 and propagated in Vero cells (ATCC® CCL-81™), using complete DMEM supplemented with 1% FBS. Then, 250 µL of the clinical specimen was used to inoculate a 25 cm^2 cell culture flask. Infectivity was checked with SARS-CoV-2-specific RT-PCR through the reduction of Ct values in the culture supernatant. The virus stock was titrated in 96-well culture plates of Vero cells using 1 log serial dilutions (1 to 11 log) to obtain a 50% tissue culture infective dose (TCID50). Cultures were observed daily using inverted microscope within 3 days for the presence of the cytopathic effect (CPE). The viral titer was expressed in TCID50/mL and calculated using the Spearman and Kärber method.

A day before the neutralization assay, each well was seeded with 20,000 cells, to obtain a 70–80% sub-confluent monolayer after 24 h. The MN assay was performed as previously reported by Grzelak et al. [11]. Briefly, after heat-inactivation, the serum samples were mixed with equal volumes of 100 TCID 50 of SARS-CoV-2 at 2-fold serial dilutions starting from 1:10. The serum-virus mix was incubated for 1 h at 37 °C with 5% CO_2. After incubation, 100 µL of the mixture at each dilution was passed in duplicate to a 96-well cell plate containing a 70–80% confluent Vero monolayer. A virus back-titration was performed with culture medium replacing the serum to assess the input virus dose. After 3 days of incubation, the plates were inspected under an inverted microscope for CPE. The endpoints of each serum are reported as a serum neutralization titer, which corresponds to

the reciprocal of the highest serum dilution that neutralizes the infectious virus using the Spearman and Kärber method as modified by Finney [12]. Samples with a neutralization titer ≥10 were considered positive.

All of the steps manipulating the SARS-CoV-2 and infected cell cultures were carried out at the biosafety level 3 laboratory of the Department of Biosafety PCL3, Laboratory of Research and Medical Analysis, Gendarmerie Royale, Rabat, MA.

2.5. Statistics

A comparison of the commercial assay results with the gold standard microneutralization assay was made to assess their performance in detecting NAbs. For sensitivity, calculations were only carried out with microneutralisation positive samples. Negative samples were used to assess specificity and cross-reactivity.

Figures including plotting and receiver operating characteristic (ROC) curves were drawn with Prism (Version 9, GraphPad, San Diego, CA, USA). The convalescent group was considered as such when the serum sample did not pass 14 days after a laboratory-confirmed COVID-19 diagnosis.

p-value < 0.05 was considered statistically significant.

3. Results

3.1. Neutralizing Antibodies to SARS-CoV-2

The serum samples obtained from 177 laboratory workers, including 39 diagnosed as COVID-19 positive during late 2020 and the first quarter of 2021, were assessed using a cell-based virus neutralization test (Figure 2). Neutralizing antibodies (NAb) against SARS-CoV-2 were detected in 149 (84%) of the total number of sera.

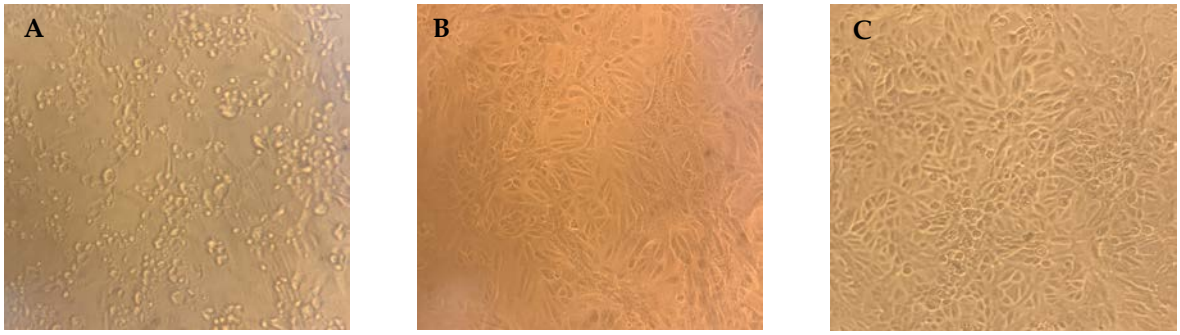

Figure 2. Viral cytopathic effects (CPE) of SARS-CoV-2 on Vero cells and neutralizing antibody activity. (**A**) Non-protective serum showing lysed cells due to viral replication (absence of neutralizing antibodies); (**B**) protective serum at low dilution of 1:10, showing inhibition of CPE by specific neutralizing antibodies; (**C**) highest serum dilution of 1:320 that protected cells from CPE taken as the neutralizing antibody titer.

The NAb titers were highly variable and ranged between 10 and 640, with a mean ± SD of 213 ± 187 (median, 160; IQR, 80–320) for convalescent, and a mean ± SD of 61 ± 114 (median, 20; IQR 10–40,) for PCR-negative individuals.

Titers of 10 to 40 were categorized as low titers, 80 to 160 as moderate, and ≥320 as high titers. The distribution of the measured neutralization titer is different between the two studied groups after vaccination (PCR-negative individuals and recovered COVID-19 patients) (Figure 3).

The vast majority of the convalescent individuals had moderate-to-high titers of neutralizing antibodies: 11 (28.2%) for 160 and 11 (28.2%) for ≥320 titers (Figure 3 left). Neutralizing antibodies were undetectable in only one convalescent individual(<10).

Vaccinated PCR-negative individuals presented low titers (Figure 3 right). Of 138, the majority (49%) had a titer ranging between 10 and 40, and 14 (10%) between 40 and 80. A total of 27 (19.5%) had no neutralizing antibodyresponse.

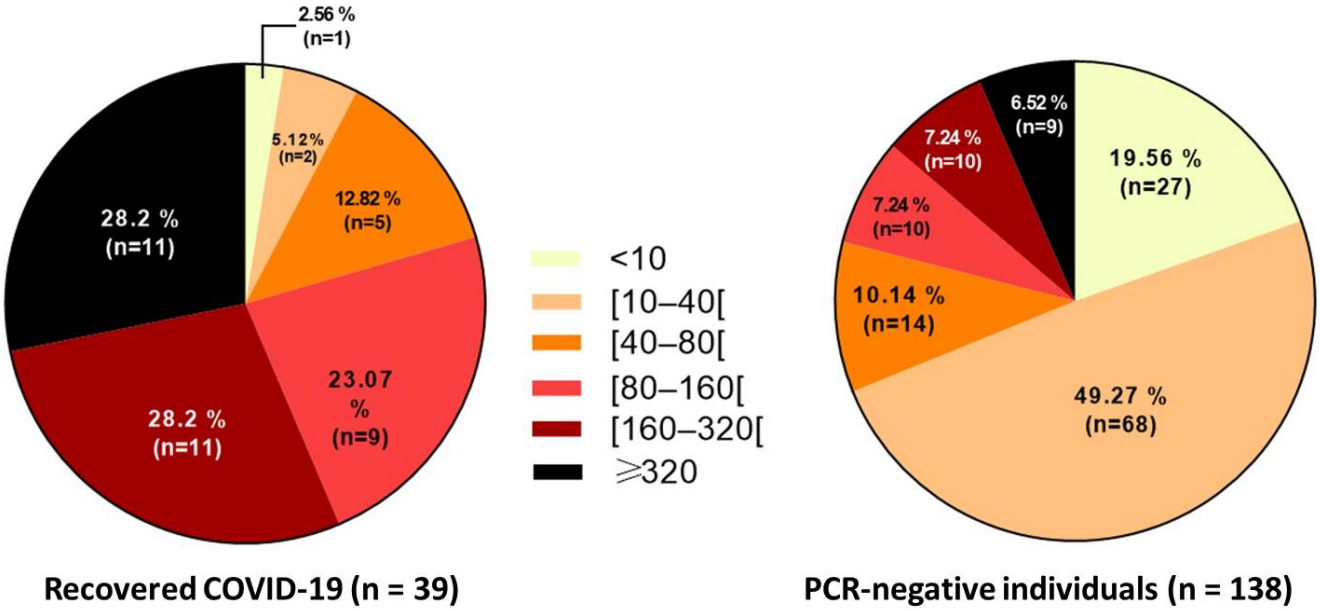

Figure 3. Distribution of neutralizing antibody titers among recovered COVID-19 patients (**left**) and PCR-negative individuals (**right**) after vaccination. The titer values are indicated by a gradient; lighter colors toward darker colors, to indicate the level of neutralizing activities between individuals.

The small number (n = 9) (Figure 3 right) of PCR-negative individuals showing a titer ≥320 may have contracted the disease, but their PCR was negative or they were not diagnosed during the infection. They may have felt some of the symptoms of COVID-19 (headache, loss of smell and taste).

3.2. Qualitative Serology

In total, 177 samples were examined in parallel comparing SARS-CoV-2 neutralization assay and both rapid LFIA and the automated ELFA mentioned above.

In the rapid LFIA, 149 samples were positive and 28 were negative. The same totals were found in the MN test. In addition, 146 samples were determined to be positive in both cases, and 25 were found to be negative by both assays, resulting in a consensus for 96.61% of the samples. Three samples that were negative in the rapid LFIA were positive in the MN test, and three other samples that were positive in the rapid LFIA were negative in the MN test.

The positive concordance rate of the rapid LFIA was 97.9%, compared with the MN test, while the negative concordance rate was 89.28% (Table 1).

Table 1. Determination of the concordance of rapid LFIA for the detection of anti-SARS-CoV-2 IgG antibodies to microneutralization assay for the detection of neutralizing antibodies.

Rapid LFIA COVID-PRESTO® (AAZ)	Microneutralization Test		
	Positive	**Negative**	**Total**
Positive	146	3	149
Negative	3	25	28
Total	149	28	177

We set a titer of 10 as a limit of detection in the neutralization assay. The absence of a colored band in the test region is a negative result in the rapid LFIA test.

False negative LFIA results were obtained in 1.6% of the patient sera, mainly containing low levels of neutralizing antibodies.

In automated ELFA VIDAS®, 168 samples were found to be positive and nine were negative. In total, 148 samples were determined to be positive by both MN and VIDAS®

assays, and eight were negative in both assays, which represented a consensus for 88.13% of the samples. The positive concordance (PC) rate of the automated ELFA in comparison with the MN test was 99.32%, while the negative concordance rate (NC) was 28.57%. False positive results were obtained in 20 patient seradue to the presence of other anti-SARS-CoV-2 non-neutralizing antibodies, namely, anti-nucleocapsid and anti-spike proteins (Table 2).

Table 2. Determination of the concordance of VIDAS® SARS-CoV-2 IgG for the detection of anti-SARS-CoV-2 IgG antibodies and microneutralization assay for the detection of neutralizing antibodies.

ELFA VIDAS®SARS-CoV-2 IgG	Microneutralization Test		
	Positive	**Negative**	**Total**
Positive	148	20	168
Negative	1	8	9
Total	149	28	177

We set a titer 10 as a limit of detection in neutralization assay, and an index = 1 as a limit of detection of IgG in VIDAS® SARS-CoV-2 test.

In summary, the qualitative results showed that the sensitivity of the rapid LFIA COVID-PRESTO® (AAZ) was 97.98% (95% confidence interval [CI] 96–100) and the specificity was 89.28% (95% CI 77100). For the VIDAS® SARS-CoV-2 IgG, the sensitivity was 99.33% (95% CI 98100) and the specificity was 28.57% (95% CI 360) when compared to the MN assay as reference method. In both antibody tests, the seropositive specimens revealed a quite good to moderate correlation.

3.3. Quantitative Serology

A comparison between the neutralizing antibody titers range in the MN assay and the IgG antibody index levels in the VIDAS® SARS-CoV-2 IgG as well as the score values assigned to the rapid lateral flow was made to explore their correlation. The quantitative results of both commercial methods (177 samples from 177 patients) were plotted against the reciprocal neutralizing titer (Figures 4 and 5).

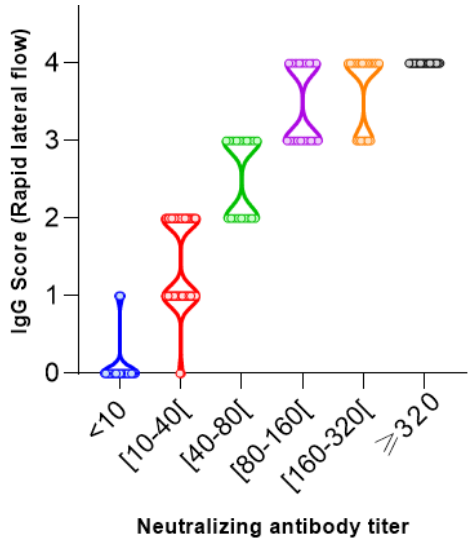

Figure 4. SARS-CoV-2 neutralizing antibody titers correlate with SARS-CoV-2 rapid lateral flow (AAZ®) (Spearman correlation coefficients ρ = 0.9341 and *p*-value < 0.0001).

The two-dimensional distribution diagrams (Figures 4 and 5) show a moderate-to-high correlation with low dispersions of the antibody values within the SN titers. Additionally, median antibody levels increased with increasing neutralizing activity.

Correlation coefficient is employed to describe the strength and direction of the linear association between the neutralizing activity and two SARS-CoV-2 tests. Although both assays showed a positive correlation with neutralizing activity, the strongest (ρ = 0.9341) was found for the rapid lateral flow (AAZ®) (Figure 4). The VIDAS SARS-CoV-2 IgG assay (Figure 5) showed a moderate positive correlation (ρ = 0.8995).

In participants with a negative virus neutralization test (<10), the antibody levels vary remarkably in the VIDAS SARS-CoV-2 IgG assay (Figure 5). The absence of neutralization is accompanied by a low antibody level ranging from an index of 1.01 to 3.75 (data not shown). However, we cannot establish a threshold above which neutralization activity is clearly present, due to the overlap of positive and negative values of serum neutralization.

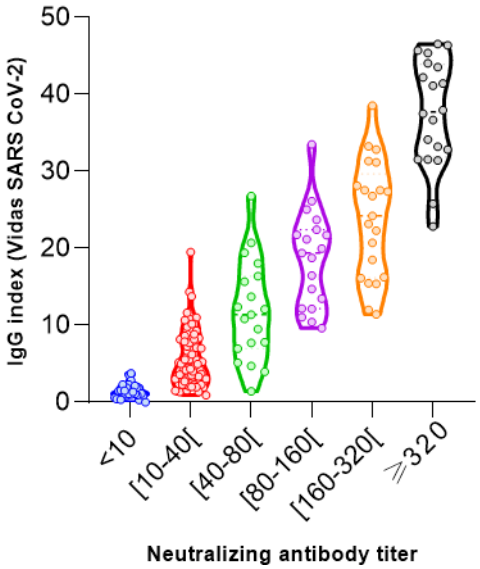

Figure 5. SARS-CoV-2 neutralizing antibody titers correlate with SARS-CoV-2 IgG (VIDAS®) (Spearman correlation coefficients ρ = 0.8995 and *p*-value < 0.0001).

In the case of the rapid lateral flow (AAZ®), only 3 of 28 samples with a positive score (score = 1) were not expected to neutralize.

3.4. Receiver Operating Characteristics Analysis (ROC)

Finally, receiver operating characteristics (ROC) curves were generated to assess the performance of each serological assay to detect the presence of neutralizing antibodies (NT > 10) (Figure 6).

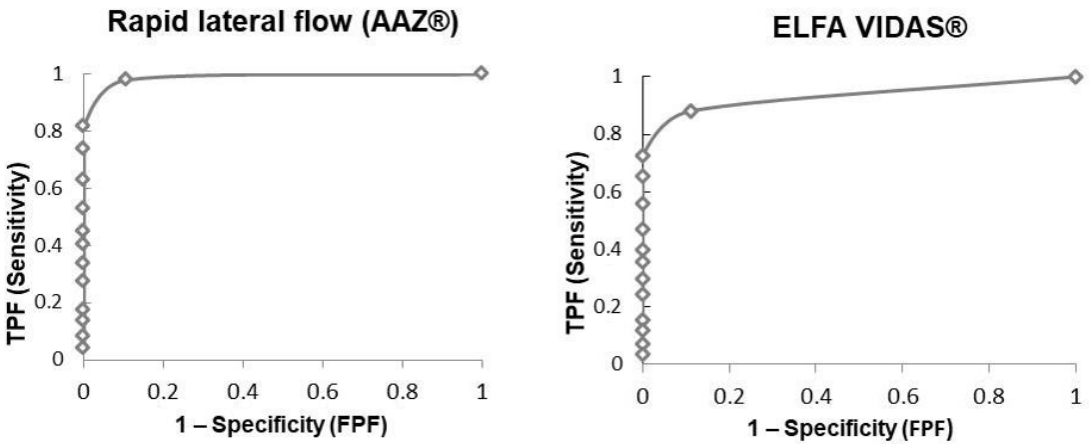

Figure 6. Receiver operating characteristics (ROC) curve analysis for two immunoassays compared to MN assay (titer > 10). TFP: true positive fraction. FPF: false positive fraction.

The areas under the curve (AUC) were 0.97 for the rapid lateral flow (AAZ®) and 0.88 for the ELFA VIDAS®, which means excellent performances for both immunoassays, but a better performance in estimating the presence of neutralizing antibodies for the first method.

4. Discussion

In the first part of the study, the neutralization activity was investigated for 177 serum samples of convalescent and PCR-negative individuals, with both groups immunized with adenovirus-based vaccines. Serostatus data were not available before vaccination, only PCR results were used to distinguish between the convalescents and healthy individuals. The percentage of vaccinated individuals with a positive seroneutralization result was more meaningfully important in the convalescent group than in the PCR-negative group (97.5% vs. 80.5%). We conclude that most of the convalescent individuals have moderate-to-high titers of neutralizing antibodies in comparison to the PCR-negative individuals after 6 to 8 weeks of their second dose. Studies have shown that the NAb response peaks at 3–5 weeks after infection and degrades over 8 monthsfollowing infection. The long-term responses of NAb titers, especially after AstraZeneca vaccination was investigated, demonstrating a possible influence of genderandage. Lim et al. found that NAb titers among the elderly population start to decrease at 8 weeks, and at 16 weeks after the second inoculation [13].

Further studies are needed to monitor post-vaccination immune responses beyond two months and after the third dose to determine the duration of vaccine effectiveness represented by neutralizing activity in particular against emerging variants of SARS-CoV-2. Indeed, some studies have provided assurance of a protective immune response after booster vaccination against SARS-CoV-2 variants [14].

Overall, our results showed that the COVID-19 vaccine improves the level of neutralizing antibodies, and significantly boosts those in individuals naturally infected compared with those with no previous SARS-CoV-2 infection. A previous study showed that the immunity provided by two doses of ChAdOx1 (AstraZeneca/Oxford) is somewhat weaker and declines faster than mRNA vaccines. However, with the combination of infection-induced immunity and vaccine-induced immunity called "hybrid immunity", neutralizing antibody titers and the extent of SARS-CoV-2 variant recognition are significantly higher in previously infected individuals receiving at least one dose of a COVID-19 vaccine. Moreover, hybrid immunity from vaccination and subsequent infection also results in equally robust immune responses [15]. Indeed, it has been well established that SARS-CoV-2 infection significantly elicits the neutralizing antibody response before or after vaccination in comparison with two doses of vaccine alone [16] and the infection alone delivers temporary protection from COVID-19 [17], confirming the importance of vaccination, regardless of infection history.

Rapid LFIA COVID-PRESTO® (AAZ) and VIDAS® were compared with the microneutralization assay for the qualitative detection of antibodies against SARS-CoV-2. A correlation was found between anti-SARS-CoV-2 IgG response between both methods and neutralizing activities.

In the sensitivity test, both assays demonstrated excellent sensitivity greater than 97%. VIDAS® SARS-CoV-2 IgG showed slightly higher sensitivity than rapid LFIA COVID-PRESTO® (AAZ) when compared to the MN assay. However, the specificity was lower meaning that these assays generated false positive results due to the detection of non-neutralizing antibodies.

In previous studies, the RBD protein provides lower sensitivity and higher specificity than the N protein. A correlation was found between anti-RBD IgG response and neutralizing activities [8].

Considering that N-based serological tests are more sensitive than S protein, while RBD-based serological tests are more specific [18], a better composition of RBD and N

protein in serological tests can improve both sensitivity and specificity for forecasting NAb activity.

Moreover, it was recently demonstrated that the VIDAS SARS-CoV-2 test was able to detect virus neutralizing antibodies with perfect concordance (Cohen's kappa coefficient of 0.9) between the IgG performed in VIDAS and the MN test [19].

Furthermore, the performance of COVID-PRESTO® (AAZ) was evaluated in a clinical study for its specificity and sensitivity compared to a test of reference (RT-PCR) [20]. However, there are no previously published results regarding the correlation with neutralizing activity. Our results showed that rapid LFIA COVID-PRESTO® (AAZ) has a higher specificity (89.28%) to detect NAbs with a reduced number (3/28) of false positive results in comparison with ELFA VIDAS®, whose false positives reached 20/28 due to thedetection of non-neutralizing antibodies.

Our results demonstrate a strong positive correlation between the gold standard MN assay and the quantitative results of both immunoassays (LFIA and ELFA) with Spearman's ρ values ranging from 0.8 to 0.9. The strongest positive correlation was found for the rapid LFIA COVID-PRESTO® (AAZ) assay, with an area under the ROC curve of 0.97, confirming that the rapid test is as an efficient tool to assess neutralizing activity as the MN test.

Previous studies showed that the positivity threshold reported in the instructions for using commercial anti-SARS-CoV-2 serology assays is not a threshold for correlating with neutralization. In order to correlate perfectly with seroneutralization, higher titers of antibodies are needed although this depends on the diversity of the response for each individual [21,22].

Gillot et al. attempted to adapt the cut-offs of some serological assays to improve the capacity of NAbs detection for these assays. However, it was difficult to deal with the loss of specificity or sensitivity to increase other parameters [23].

In particular, when using LFIA, it is necessary to establish a band interpretation system for each laboratory, along with observer training to allow more objective results. We also anticipate that such a serological binding method will play a critical role in SARS-CoV-2 antibody testing and become a convenient routine neutralizing antibody test.

This work establishes the effectiveness of vaccination against a strain that circulated in 2020–2021 and its ability to neutralize the virus. Although the study did not investigate the humoral response to novel variants, other papers have been able to demonstrate vaccine efficacy against SARS-CoV2 mutations, showing that spike-binding and neutralizing activity was maintained and remained unaffected by viral genome variations [24].

Our findings demonstrate a strong positive correlation among SARS-CoV-2 IgG antibody titers in both binding antibody assays (LFIA and ELFA) and neutralizing activity. The strongest positive correlation to neutralizing activity was found for the rapid LFIA. Although this method has only been designed for the qualitative analysis of SARS-CoV-2 antibodies so far, it provides the rapid detection of neutralizing antibodies with high specificity and sensitivity, and thus possesses advantages over conventional microneutralization, which involves the manipulation of the live virus, as well as being a low-cost, equipment-free and on-site test. To the best of our knowledge, no other study has been published that rapid LFIA COVID-PRESTO® (AAZ) assay correlates with the neutralizing antibody response against SARS-CoV-2.

Author Contributions: Conceptualization, A.S., S.L., H.F. and S.E.K.; methodology, A.S., S.L., B.E.M. and H.F.; software, A.S.; validation, A.S., S.L., H.F. and S.E.K.; formal analysis, A.S. and S.L.; investigation, A.S., S.L. and H.F., resources, B.E.M. and S.E.K.; data curation, A.S., S.L. and H.F.; writing—original draft preparation, A.S. and S.L., writing—review and editing, A.S., S.L., B.E.M., E.E.F., H.F. and S.E.K. visualization, A.S., S.L., B.E.M. and H.F.; supervision, S.E.K.; project administration, S.E.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study protocol was in accordance with the Declaration of Helsinki and was approved by the Medical Ethical Committee of Hospital Cheikh Zaid Foundation, Rabat, Morocco (approval number CEFCZ/PR/2021/Ennibi-26, delivered on 2 November 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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vaccines

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Review

Children's SARS-CoV-2 Infection and Their Vaccination

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Abstract: SARS-CoV-2, a novel coronavirus, causes respiratory tract infections and other complications in affected individuals, and has resulted in numerous deaths worldwide. The unprecedented pace of its transmission worldwide, and the resultant heavy burden on healthcare systems everywhere, prompted efforts to have effective therapeutic strategies and vaccination candidates available to the global population. While aged and immunocompromised individuals form a high-risk group for COVID-19 and have severe disease outcome, the rate of infections among children has also increased with the emergence of the Omicron variant. In addition, recent reports of threatening SARS-CoV-2-associated complications in children have brought to the forefront an urgent necessity for vaccination. In this article, we discuss the current scenario of SARS-CoV-2 infections in children with a special focus on the differences in their immune system response as compared to adults. Further, we describe the various available COVID-19 vaccines, including the recent bivalent vaccines for children, in detail, intending to increase willingness for their acceptance.

Keywords: SARS-CoV-2; MIS-C; Omicron; EUA

Citation: Gupta, S.L.; Tyagi, R.; Dhar, A.; Oswal, N.; Khandelwal, A.; Jaiswal, R.K. Children's SARS-CoV-2 Infection and Their Vaccination. *Vaccines* **2023**, *11*, 418. https://doi.org/10.3390/vaccines11020418

Academic Editor: Pedro Plans-Rubió
Received: 23 December 2022
Revised: 8 February 2023
Accepted: 10 February 2023
Published: 12 February 2023

1. Introduction

SARS-CoV-2 has displayed a very high rate of spread since December 2019, resulting in an enormous global death toll [1–4]. A total of 754 million confirmed cases and 6.81 million deaths due to SARS-CoV-2 infections have been reported until now, as per the World Health Organization (WHO) epidemiological report (https://covid19.who.int/, accessed on 30 November 2022). The emergence of new variants of SARS-CoV-2 such as Alpha, Beta, Delta, and the most recent Omicron variant and subvariants have posed a challenge for the healthcare system due to the high transmissibility [5–7], contributing to the need to design effective treatment measures. According to published data, elderly individuals and those with co-morbidities have shown a higher incidence of fatality, and therefore are at a higher risk due to the virus infection [8,9]. On the other hand, SARS-CoV-2 infections in children have mostly been reported to be mild, with a low fatality rate [10–13].

A major protective measure taken to reduce COVID-19 infections in children has been the closure of schools and play areas, but these measures have drawbacks and cannot be implemented long-term [14]. Despite the early notion of a low number of cases in children, an increase in the number of infections and hospitalizations due to Delta and the subsequent Omicron variants of SARS-CoV-2 has been observed [15]. In addition, children can form a point of contact with COVID-19 infection for their family members, which might constitute individuals in the high-risk group. The vaccination drive against COVID-19 in adults has helped in controlling disease transmission and severity to a large extent [16]. Therefore, it is also necessary to extend this protection to children by promoting their vaccination. Vaccination against many life-threatening infections is commonly administered to children

during the first few years after birth, and including COVID-19 vaccination might be a significant step.

A majority of the available literature deals with SARS-CoV-2 infection in adults and their vaccination, though limited research has been carried out in cases of children. This is largely explained by preliminary results which showed that children are either asymptomatic or have mild symptoms after SARS-CoV-2 infection. However, it does not rule out the importance of vaccination among children to achieve herd immunity and risk of getting exposed with the continuous emergence of newer SARS-CoV-2 variants. Vaccination of children requires parental consent, and vaccine hesitancy among parents exists due to limited literature availability, fewer numbers of or lack of available vaccines, lack of FDA approved bivalent vaccine for kids aged 5 years or less, and the available vaccines being under the Emergency Use Authorization (EUA) category and not fully licensed for vaccination in children in the public domain as compared to adults. Infants younger than 6 months are dependent on maternal antibody transfer, as no vaccine is available for them. Therefore, an infant will not receive protective antibodies unless the mother is vaccinated or possesses antibodies against SARS-CoV-2 after an infection. Additionally, cases of Multisystem Inflammatory Syndrome in children (MIS-C) have also been reported, though the exact causative mechanism has not yet been understood, which has hindered the development of a universal treatment for MIS-C. Children also differ from adults in terms of innate, adaptive, and mucosal immune responses. Hence, the immune response to an infection or vaccination in adults is different from that observed in children. All these challenges make children a special cohort and highlight the need for an increase in the availability of literature in the public domain. This review encourages parents to get their children vaccinated, and the researchers and policy makers to assess pediatric vaccination safety, immunogenicity, and efficacy data and facilitate its proper dispersal. This review also reflects the importance of finding new alternatives of vaccine availability, their dose, route of administration, etc., that might differ from adults in the future.

COVID-19 is an infectious disease that is transmitted mainly via respiratory droplets. The SARS-CoV-2 virus has also been reported to be sustained on surfaces, and hence maintaining social distancing, use of masks, and hand sanitizers were recommended to prevent community transmission [17–21]. In a family setting, there were reported cases of family transmission of SARS-CoV-2 infection and related disease symptoms. A case study conducted in China, including 14 families and nine children, reported that adults in these families were SARS-CoV-2 PCR positive and had moderate to severe symptoms, and all nine children were also positive but had mild symptoms (3) or were asymptomatic (6) compared to adults. Similarly, other reports also showed that children can be potential carriers of this disease and become infected via family transmission [22–24]. In children, severe disease did not positively correlate with viral load [25]. This suggests that even asymptomatic children, or those with mild symptoms, could have higher viral titers and serve as a potential carrier for community disease transmission and the emergence of SARS-CoV-2 viral variants.

Newborn infants can develop SARS-CoV-2 infection, and around 2–3% of maternal to fetal vertical transfer (intrauterine transfer) of infection (lower risk of incidence) has been reported [26–29]. Infants receive maternal antibodies (vertical transmission) from their mother from the placenta and during the lactation period via breast milk [30–32]. A recent study suggested that antibodies are transferred from vaccinated or SARS-CoV-2-infected mothers to their babies via placental transfer. The transferred antibody titers sharply decrease from birth until 6 months of age [33–37], as shown in Figure 1. Pregnant women vaccinated with the mRNA vaccine show better antibody-mediated function and Fc receptor binding than the adenovirus-based vaccine. Among the three trimesters during pregnancy, vaccination in the first or third trimester gives a better immune response than vaccination in the second trimester. The transfer efficiency of antibodies from mother to fetus is also higher in the first and second trimesters compared to the third trimester when tested in both maternal and umbilical cord blood sites [38]. Maternal antibodies

are also transferred via breastfeeding during lactation [39]. Children have been shown to have a durable immune response after COVID-19 infection. As reported, children showed long-term Receptor Binding Domain (RBD) binding antibody responses to SARS-CoV-2 infection in various age groups up to 10 months post-COVID-19 mild infection [40]. It was observed that very young kids, such as children <5 years of age, who were affected by severe acute COVID-19, and later hospitalized, had a greater reduction in neutralizing antibodies to SARS-CoV-2 variants as compared to children >5 years of age. This report also confirmed that convalescent COVID-19 and MIS-C affected pediatric cohorts showed higher neutralization titers as compared to acutely infected COVID-19 populations [41]. Studies have shown that kids younger than 5 years of age had a higher incidence rate during the emergence of the Omicron variant in comparison to the Delta variant. This suggested that younger kids can transmit the Omicron variant infection at a higher rate, but have less severe clinical outcomes. Among these kids, those belonging to the age group of 0–2 years had a higher monthly incidence rate of COVID-19 as compared to those of 3–4 years of age during the emergence of the Omicron variant [42–44].

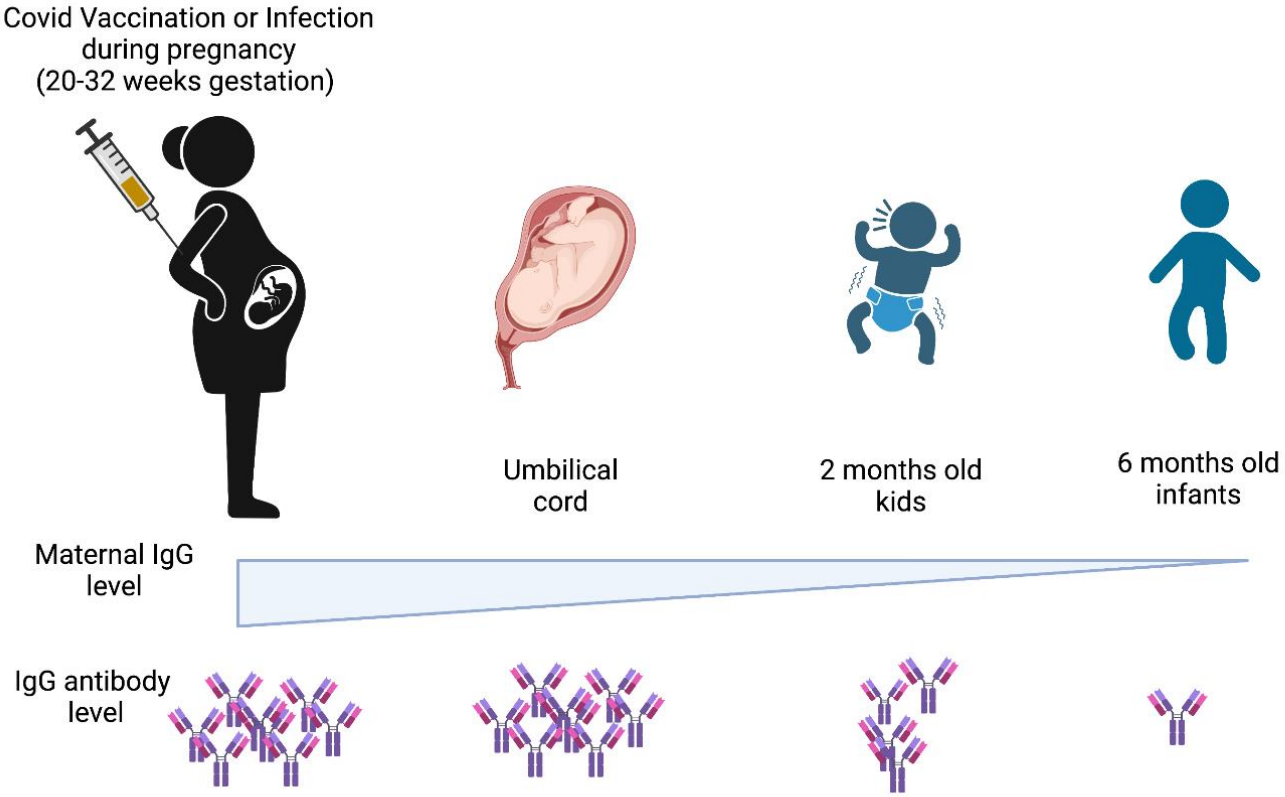

Figure 1. Levels of maternal IgG antibody transferred from mother to infant wanes during 0–6 months after birth. Maternal anti-SARS-CoV-2 IgG antibodies acquired either by natural infection or vaccination are transferred to the fetus via placenta. The level of maternal antibodies decreases with the infant's age and lasts up to 6 months. SARS-CoV-2 infection or COVID-19 vaccination during 20–32 weeks of gestation generated the highest antibody titer in pregnant women. This gradually declines when tested in umbilical cord samples at the time of birth, and then further declines at 2 months after birth as observed in infant serum samples and reaches a minimal amount at the age of 6 months, as represented in Figure 1.

Various studies have explained that the reasons children have a milder infection are strong innate responses due to higher levels of IFN-gamma and IL-17A, and lower levels of TNF-alpha and IL-6 in serum. SARS-CoV-2 infected children also have a lower adaptive immune response as shown by lower memory T cells, Fc gamma receptor levels, lesser

ADCP (antibody-dependent cellular phagocytosis) reactions, and reductions in neutralizing antibody responses than adults [45]. The innate immune response in the nasal mucosa of children is also stronger and more vigorous than adults. Nasopharyngeal swabs from COVID-19-infected children and adults were compared; it was found that children displayed higher gene expression related to IFN and NLRP3 inflammasome signaling [46,47]. Children also showed higher basal level gene expression of pattern recognition receptors (PRRs), such as MDA5 and RIG-1, in innate immune cells in upper respiratory airways than adults, which explains the robust anti-viral innate immune response in children compared to adults [48]. The adaptive immune system of children is naïve compared to that of adults. As a result, they have a higher frequency of naïve T cells, especially cytotoxic T cells and NK cells, and less clonal expansion in the T cell repertoire. Together, these factors possibly contribute to the absence of an exacerbated immune reaction and hyperinflammation post-SARS-CoV-2 infection in children [49]. Children tend to have higher clonotype diversity and enriched naïve B cell and T cell populations as compared to adults. On the other hand, adults have a higher frequency of systemic IFN-induced immune cells such as B cells, T cells, NK cells, and monocytes which induce IFN in blood, as well as enrichment of cytotoxic immune cells [50]. One in vitro assay assessing SARS-CoV-2 specific IFN-gamma producing T cells showed that they are higher in adults compared to children, in cases of mild to moderate SARS-CoV-2 infection [51]. Children also developed a stronger spike specific B cell mediated antibody response, as well as T cell response, which was also persistent and durable (more than 6 months), as compared to adults (faster decay), post SARS-CoV-2 infection [52,53]. Both acute and memory CD4+ T cells and CD8+ T cell response is lower in children than adults [54].

There are also other factors contributing to lower susceptibility to SARS-CoV-2 infection in children. The first factor is the lower intensity of exposure to SARS-CoV-2, since the family dynamics established during the pandemic were intended to protect the children. The second factor is cross reactivity, since children tend to have a higher frequency of recurrent and concurrent viral exposure as well as vaccinations. These repeated and multiple viral infections and various early-stage vaccinations lead to an ongoing state of activation of the innate immune system [55,56]. Similarly, children have a higher exposure to helminth infections than adults, which causes modulation of host inflammatory components [57]. Besides these factors, several other immunological factors are different in cases of adults than in children. The first is that adults have an alteration in their endothelial function and coagulation [58]. The second is the difference in enzymatic density and affinity on respiratory mucosal epithelial cells. These enzymes are angiotensin converting enzyme (ACE2) and transmembrane protease, serine 2 (TMPRSS2) [59]. In addition, adults have a higher immune senescence and chronic inflammation rate than children, and a higher prevalence of comorbidities and underlying chronic conditions [60]. In summary, all these differences in innate and adaptive immune responses reflect why children show a distinct immune response post-SARS-CoV-2 infection than adults.

2. Vaccine Hesitancy in Parents to Have Their Children Vaccinated

A major challenge in containing the spread of the SARS-CoV-2 virus is to have most of the population vaccinated, potentially leading to lower disease severity and transmission [61]. While various countries have taken measures to promote vaccination, such as the provision of free vaccines, the percentage of people taking the vaccines in developed countries is not 100%. As reported in latest data on 11 February 2023 69.4% world population has received one dose of vaccine but only 26.4% in low income countries got their one dose. (https://ourworldindata.org/covid-vaccinations, accessed on 30 November 2022) [62]. This indicates a certain degree of hesitancy among the adult population regarding the risks of taking the vaccine, and this is largely expected to affect the vaccination rate in children. Several studies have attempted to understand the factors that affect parents' decision to get their children vaccinated based on surveys. One study conducted among parents of adolescent children of 16+ years old showed that the most common reasons for hesitancy

among parents for vaccinating their children were concerns about long-term side effects and possible negative effects [63]. Another study tried to review the reasons affecting vaccine acceptance among low-income group parents with children in the age group of newborns to 17 years old. In the study, some parents described their unwillingness to vaccinate their children due to milder forms of the disease occurring in the children. Other factors also noted as reasons for hesitancy in the study include possible negative side effects of the vaccine, lack of knowledge about the long-term effects, and the speed of the vaccine development [64]. Furthermore, a survey-based study also found that the vaccine hesitancy was also influenced by the gender of the parents, being higher in females, their economic status being higher among low-income groups, and their political beliefs. This study also noted the primary concern of parents was the safety of the vaccine in comparison to its effectiveness [65]. Another factor influencing the decision of vaccination of children is the fear of vaccines originating from abroad. In addition, this study also noted that the anxiety of parents about negative vaccine side-effects and lack of knowledge about vaccine effectiveness were the most common reasons for hesitancy in taking the vaccine [66].

Many parents rely on online sources of information, which very often are biased and foster disbelief in the parents about the risks of vaccinating their children [67,68]. To overcome these challenges, better spread of correct information through online channels, and promotion of vaccine uptake by medical practitioners and government agencies are important measures. Also, vaccination against other diseases in children has rarely shown long-term negative effects, suggesting that COVID-19 vaccines have a very low risk factor when considered long term [69].

3. Multi-Inflammatory Syndrome in Children

In children, COVID-19 infections are mild or asymptomatic and mostly self-limiting at the time of acute infection. Post 3–4 weeks from COVID-19 infection, some children develop multiorgan hyperinflammatory syndrome, known as MIS-C. It is a rare disease condition that afflicts both children (MIS-C) and adults (MIS-A). In April 2020, a few cases of MIS-C were reported in Europe. It is a systemic disorder affecting multiple organs. Fever, gastrointestinal, abdominal pain are key diagnostic symptoms of this disorder. In addition to these symptoms, cardiac disease is the most common comorbidity associated with MIS-C, along with respiratory and neurological disorders. It should also be understood that while disease severity in children has been reported to be mild, a serious form of the disease, i.e., MIS-C, has also been reported [70,71]. The most frequent symptoms observed in this syndrome are depicted in Figure 2. Due to the similarity of MIS patient symptoms with septic shock, toxic shock syndrome (TSS) and Kawasaki disease (KD), the initial treatment prescribed to treat MIS-C was similar to TSS and KD, but later MIS-C was found to be associated with COVID-19 disease [72,73]. The majority of MIS-C cases require hospitalization and intensive care unit (ICU) admission. Despite severe symptoms, mortality rate was as low as 1.9%. It has been reported that MIS-C occurrence dominates in males and black ethnic groups [74]. MIS-C patients suffer from a hyperinflammatory condition, which might occur due to an increase in the amount of activated immune cells such as neutrophils, monocytes, DCs, NK cells, B and T cells, and flares of cytokines and chemokines that cause vascular patrolling of these active cells to organs, and in some cases a higher amount of autoantibodies cumulatively affected multiple organs [75–77]. One interesting observation was that Omicron infected children developed lesser MIS-C, especially post vaccination, or in cases of reinfection as compared to the Delta variant, although the MIS-C phenotype was mostly similar. This may be due to the modulation of immune system by vaccination or previous infection to cause lesser hyperinflammation. Thus, children in the Omicron wave had a lesser risk of MIS-C development than in the earlier Delta or Alpha variant waves [78,79]. The exact mechanism and early disease biomarkers for MISC-C are still undetermined, and the paradox of complex hyperinflammation remains largely unsolved.

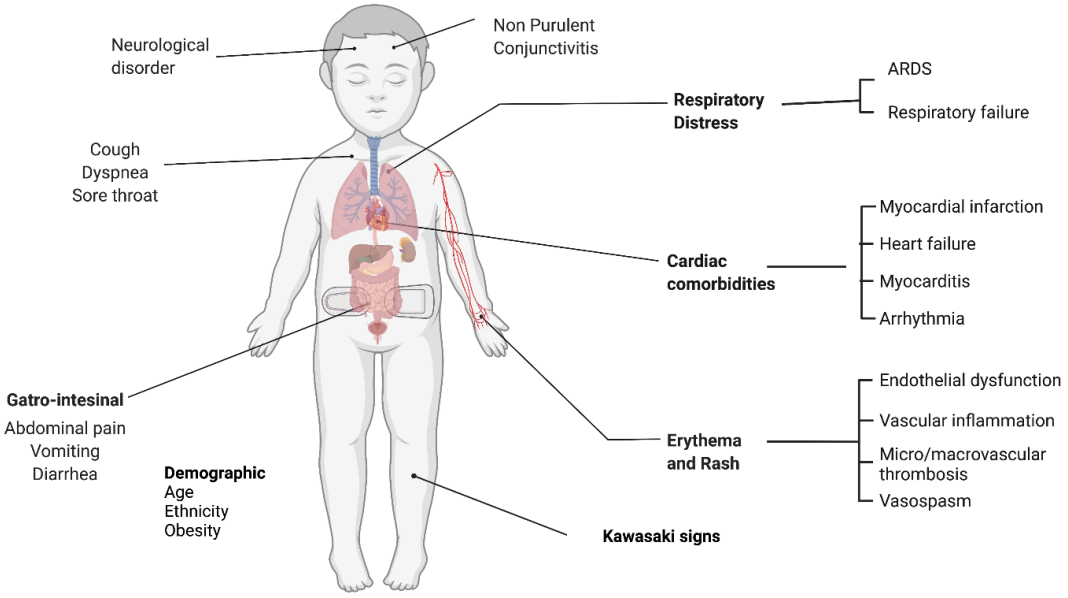

Figure 2. Organs affected in MIS-C immunopathological disorder in children. This disorder affects multiple organs and shows a systemic immune response. It mainly causes respiratory distress, cardiac comorbidities, gastrointestinal upset, and vascular dysfunction. This disorder is associated with demographic features such as various age form, ethnicity, and obesity level.

4. Status of Vaccination in Children

Safe, immunogenic, and effective vaccines for children of all ages are currently needed, and only a few are approved for children. As per the WHO report on 10 January 2023, there are a total of 176 vaccines in clinical trials and 199 in pre-clinical development for adults, with very limited availability for children (https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines, accessed on 30 November 2022). As per the WHO dashboard on 31 January 2023, more than 13 billion vaccine doses have been administered (https://covid19.who.int/, accessed on 30 November 2022). To fill the gap in vaccine coverage and prevent community transmission, the FDA approved the Pfizer vaccine for adolescents aged 12–17 years and children in the age group of 5–11 years under EUA. As per CDC guidelines, children above 5 years of age are eligible for full vaccination. Clinical trials are still ongoing to fully approve Pfizer and other alternative vaccines. As per CDC data, in the USA, 1.55 million children of more than 5 years of age have received at least one dose of vaccine since 18 June 2022. Recent COVID-19 vaccination status in USA according to CDC is shown in Table 1. The percentages of the populations in the USA and other countries that has received the primary series of vaccination, including children, are listed in Table 2. The CDC recommends that everyone 6 months and older get a COVID-19 vaccine, and booster for 5 years and older, as shown in Table 3. In adolescents, the Pfizer vaccine dose is equivalent to adults as 30 mg and given in two doses 21 days apart. However, in children, a 10 mg dose is administered to avoid the adverse side effects of using a higher dose. A clinical trial using Pfizer's 10 mg dose vaccine in children aged 5–11 years has shown it to be safe, immunogenic, and with equivalent neutralization titers as seen in adolescents with an efficacy around 90% [80]. Both mRNA vaccines' description is shown in Table 3. Three doses of the Moderna vaccine for the primary series of vaccination are recommended for immunocompromised individuals. If administered in two doses, it is given four weeks apart (28 days), and in the case of three doses, there should be a gap of a minimum of one month. The Pfizer-BioNTech vaccine was first approved in December 2020 and later fully licensed on 23 August 2021, while the Moderna vaccine was first approved in December 2020 and fully licensed in January 2022. These vaccines are administered via an intramuscular injection into the deltoid muscle. Recently, Pfizer BioNTech's and Moderna's bivalent vaccines also received

approval under EUA for use in children above 5 years of age to receive their single dose as a booster. These bivalent boosters consist of Wuhan spike mRNA and BA.5 spike mRNA in a 1:1 ratio. These bivalent vaccines provide higher or equivalent neutralizing antibody titers against the Omicron subvariant [81–83]. So far, no bivalent vaccine is available for kids aged 6 months to 4 years. A summary of the currently available bivalent vaccines is provided in Table 4. Children responded in a similar manner or better post-vaccination than adults. Both antibody binding and neutralizing antibody responses to SARS-CoV-2 in both very young children and adolescents exceeded those in adults [84,85]. Besides these two mRNA-based vaccines, Sinovac-CoronaVac and BBIBP-CorV, which are based on the inactivated virus, were approved in China for children aged 3–17 years [86,87]. Similarly, Covaxin (BB152), which is also based on an inactivated virus strategy and developed by Bharat Biotech, India, was approved in India under the EUA category [88,89]. Alternative vaccines which are under clinical trials include the Adenovirus-based vaccine Ad5-nCoV in China for 6–17 years old children, and the DNA vaccine ZyCoV-D in India for children aged 12–17 years [90]. mRNA vaccination has been reported to be minimally or moderately affected by variants of concern (VOCs), such as Alpha (B.1.17), Delta (B.1.617.2), and Beta (B.1.351) variants. However, the recent emergence of Omicron caused serious concerns about vaccine efficacy and neutralization potential. A study was conducted in fully vaccinated children between the ages of 5–11 years and 12–17 years to evaluate vaccine efficacy during the Omicron variant from December 2021 to January 2022. They observed that vaccine efficacy was reduced by 66–51% in adolescents (12–17) and 68–12% among children aged 5–11 years, which shows that children in the age group of 5–11 years were more affected by the loss of vaccine efficacy than adolescents [91]. Similarly, another report showed that neutralization by post-vaccination serum from children was reduced against VOCs such as Alpha, Beta, Gamma, and Delta, and the most reduction was seen in the Omicron variant [41].

Table 1. COVID-19 Vaccination status in the United States as per CDC records who have completed their primary vaccination series.

Fully Vaccinated People	Percent of US Population
Total	68.7%
Population $\geq$ 5 Years of Age	72.8%
Population $\geq$ 12 Years of Age	76.9%
Population $\geq$ 18 Years of Age	78.5%
Population $\geq$ 65 Years of Age	93.7%

Table 2. COVID-19 Vaccinations status in the global context as per WHO records who have completed their primary vaccination series (https://covid19.who.int/table, accessed on 30 November 2022).

Fully Vaccinated People	Per 100 Persons
World	64.51
USA	68.42
India	68.94
France	78.9
Germany	76.37
Brazil	79.52
Japan	81.54
Republic of Korea	87.17
Italy	82.95
The United Kingdom	74.59
Russian Federation	53.6

Table 3. COVID-19 vaccine doses and status for different age groups.

Pfizer-BioNTech Vaccine Authorized Age	**Dose**	**Usage Status**
16 years and older (Comirnaty brand name)	2 dose primary series (30 μg/dose)	Fully licensed
12–16 years old	2 dose primary series. (30 μg/dose)	Fully approved
5–11 years old	2 dose primary series. (10 μg/dose)	Under EUA and not fully approved
6 months- 4 years old	3 dose primary series (3 μg/dose)	Under EUA and not fully approved
Moderna vaccine authorized age		
17 years and older (Spikevax brand name)	2 dose primary series (100 μg/dose)	Fully licensed
12–16 years old	2 dose primary series (100 μg/dose)	Under EUA
6–11 years old	2 dose primary series (50 μg/dose)	Under EUA
Novovax vaccine authorized age		
12 years and older	2 dose primary series (0.5 mL/dose)	Under EUA
Coronavac-Sinovac and BBIBP-CorV (Sinopharm)		
3–17 years	2 doses (0.5 mL/dose)	Approved by China officials [92–94]
Ad5-nCoV (CanSino)		
6–17 years		Phase 2b clinical trial in China [95]
Covavax from Novavax company		
12–17 years	10 doses (0.5 mL/dose)	
6 months to 11 years	2 doses of 5 μg	https://clinicaltrials.gov/ct2/show/NCT05468736, accessed on 30 November 2022
Covaxin (BB152) by Bharat Biotech		
12–17 Years		Approved by Indian Officials
2–18		Phase 2–3 clinical trial in India [96]
Corbevax	2 doses (0.5 mL/dose)	[97]
5–17-year-old		
ZyCoV-D (Zydus Cadila)		Phase 3 clinical trial in India [98]
12–17 years old		
ChAdOx1 nCov-19 (AZD1222)		Phase 2 clinical trial in UK
6–17 years old	2 doses (5×10^{10}) viral particle	[99]

Table 4. Bivalent Vaccines approved for children so far are listed below.

Pfizer-BioNTech Bivalent Vaccine Authorized Age	Dose	Usage Status
16 years and older	1 dose (30 μg/dose)	Under EUA
12–16 years old	1 dose (30 μg/dose)	Under EUA
5–11 years old		Under EUA
6 months–4 years old		Not approved yet
Moderna vaccine bivalent authorized age		
12 years and older	1 dose (50 μg/dose)	Under EUA

5. Conclusions

The primary aim of this review is to provide the reader with a detailed picture of the current COVID-19 infections in children and highlight various issues that play a role in the decision process for the vaccination of children. Despite the high percentage of efficacy observed in the case of mRNA vaccines, and large range of protection they offer against various variants of SARS-CoV-2, the vaccination rate among children is still low. Children's immune system is still at a naïve stage of development, and several differences exist in the immune response of children vs. adults during COVID-19 infections. The role of children in the spread of COVID-19 was initially less appreciated, as the overall infection rate in them was low, and they often remained asymptomatic or with milder symptoms. However, in the case of the Omicron variant, the number of cases among children was seen to increase. Additionally, COVID-19 associated multisystem inflammation cases have been reported in children (MIS-C), and a study conducted in the Danish population reported a reduced occurrence of MIS-C in a cohort of vaccinated children [78]. All these factors have made it imperative for governments in various countries to ensure absolute vaccine coverage for the different age groups of children in their population. This is more important, as children can act as potential carriers of the virus and contribute to the transmission to high-risk people within their families. Their infections may go unnoticed otherwise, due to mild symptoms.

The growing need to increase the rate of vaccination in children depends, to a large extent, on the approval of their parents. While misinformation about vaccination has hindered the acceptance of COVID-19 vaccines among parents, a shortage of studies on the possible long-term side effects on children's health also contributes to the challenge. In addition, other important factors affecting vaccine coverage in children are the availability of vaccines in different geographic areas, and the effect of existing endemic infectious disease on the immune response to vaccines, e.g., helminth immunomodulation in sub-Saharan Africa. The spread of relevant and correct information by healthcare practitioners and national agencies about vaccines will help more people accept and realize the benefits of childhood vaccination and help control the transmission of COVID-19 to a large extent.

Author Contributions: Conceptualization, S.L.G., R.T., A.D., N.O. and A.K. writing original draft preparation, review, and editing, S.L.G. and R.K.J. supervision, R.K.J. and S.L.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study does not conduct on animal or human so no ethical approval is required. Further, no institutional review board permission is needed to write this article.

Informed Consent Statement: Not applicable as no experiments on human are involved.

Data Availability Statement: All authors are ready to share the information which we have provided in this article.

Conflicts of Interest: The authors declare no conflict of interest.

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Lack of Evidence on Association between Iron Deficiency and COVID-19 Vaccine-Induced Neutralizing Humoral Immunity

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Abstract: Iron is a crucial micronutrient for immunity induction in response to infections and vaccinations. This study aimed to investigate the effect of iron deficiency on COVID-19-vaccine-induced humoral immunity. We investigated the effectiveness of COVID-19 vaccines (BNT162b2, mRNA-1273, and ChAdOx nCov-2019) in iron-deficient individuals (n = 63) and provide a side-by-side comparison to healthy controls (n = 67). The presence of anti-SARS-CoV-2 spike (S) and anti-nucleocapsid (NP) IgG were assessed using in-house S- and NP-based ELISA followed by serum neutralization test (SNT). High concordance between S-based ELISA and SNT results was observed. The prevalence of neutralizing antibodies was 95.24% (60/63) in the study group and 95.52% (64/67) in the controls with no significant difference. The presence/absence of past infection, period since vaccination, vaccine type, and being iron-deficient or having iron-deficiency anemia did not exert any significant effect on the prevalence or titer of anti-SARS-CoV-2 neutralizing antibodies. NP-based ELISA identified individuals unaware of exposure to SARS-CoV-2. Moreover, absence of anti-NP IgG was noted in participants who were previously diagnosed with COVID-19 suggesting the unpredictability of after-infection immunity. To sum up, this study demonstrated an initial lack of evidence on the association between iron deficiency and the effectiveness of COVID-19-vaccine-induced neutralizing humoral immunity. Similar studies with larger sample size remain necessary to obtain comprehensive conclusions about the effect or lack of effect of iron on COVID-19-vaccine effectiveness.

Keywords: COVID-19 vaccine; iron deficiency; SARS-CoV-2; humoral immunity; ELISA

Citation: Faizo, A.A.; Bawazir, A.A.; Almashjary, M.N.; Hassan, A.M.; Qashqari, F.S.; Barefah, A.S.; El-Kafrawy, S.A.; Alandijany, T.A.; Azhar, E.I. Lack of Evidence on Association between Iron Deficiency and COVID-19 Vaccine-Induced Neutralizing Humoral Immunity. *Vaccines* **2023**, *11*, 327. https://doi.org/10.3390/vaccines11020327

Academic Editors: Rishi Jaiswal, Srijani Basu, Suman Gupta and Sneh Lata Gupta

Received: 21 December 2022
Revised: 19 January 2023
Accepted: 26 January 2023
Published: 1 February 2023

1. Introduction

Iron deficiency (ID) is a global health problem that affects nearly two billion people [1], and iron deficiency anemia (IDA) is considered the topmost leading cause of anemia worldwide [2]. The prevalence of ID and IDA is generally higher in women of childbearing age, preschool children, and individuals with low socioeconomic status [1,3]. The underdeveloped regions have a five-time higher prevalence rates of ID and IDA compared to the developed nations [4]. This detrimental situation might have been worsened in recent years due to COVID-19 impact on food security [5].

Iron is an important nutrient for the development of efficient immune response to infections and vaccinations [6–9]. The mechanisms underlying reduced effectiveness of host immunity under iron-deprived state is not fully understood. However, iron is required

for monocyte/macrophage differentiation and antimicrobial activity (e.g., the nicotinamide adenine dinucleotide phosphate hydrogen(NADPH)-dependent oxidative burst) [10]. Iron is also necessary for lymphocyte proliferation and differentiation as well as cytokines production [11,12]. Under the iron-deprived state, the immune system undergoes different alterations such as reduction in the number of CD4+ and CD8+ lymphocytes, and the levels of inflammatory cytokines [10–12]. Hypoferremia in animal models substantially decreased the effectiveness of immune response mediated by the effector and memory cells following vaccinations such as tetanus toxoid and *Mycoplasma hyopneumoniae* [7,13]. An independent study demonstrated suboptimal protective immunity induction following receiving diphtheria, pertussis, and pneumococcal (DPT) vaccines in children with ID compared to those with normal iron levels [14]. On the other hand, simultaneous administration of iron-fortified micronutrient powders with measles vaccine enhanced the antibody avidity and seroconversion of the vaccine recipients [14]. Another investigation conducted on humans found that some individuals possess defective B- and T-cells due to a rare mutation that interferes with cellular iron uptake [15].

In COVID-19 patients, hypoferremia and low transferrin saturation were marked as predictors for severe illness (e.g., hospitalization and admission to intensive care unit) [16–20]. It is not entirely clear how hypoferremia may worsen the outcome of COVID-19. Inefficient cellular oxygen sensing, impaired response to hypoxia, and reduction of lymphocyte count and activities are potential mechanisms underlining severe COVID-19 illness in individuals with hypoferremia [16–20]. With the introduction of COVID-19 vaccines, studies on vaccine safety and effectiveness are required to assess the actual level of protection afforded by immunization [21,22]. One of the recommendations raised by experts is to "correct the iron deficiency before administration of the COVID-19 vaccine" [23]. This is largely because of previous reports highlighting the necessity of iron for host immunity. However, there is lack of sufficient evidence about COVID-19 vaccine effectiveness per se in vaccine recipients with ID or IDA profile. Hence, we aimed in this study to investigate the effectiveness of COVID-19 vaccines in inducing protective humoral immunity in ID and IDA individuals and provided a direct side-by-side comparison with healthy controls.

2. Materials and Methods

2.1. Study Population and Demographic Data

The study was approved by the Biomedical Research Ethics Committee of Umm Al Qura University (protocol code HAPO-02-K-012-2021-09-747 and date of approval 7 September 2021). We invited students and staff at the College of Medicine, Umm Al-Qura University in Makkah and the faculty of applied medical sciences, King Abdulziz University in Jeddah through announcements and personal invitations to eligible candidates. Informed consent was obtained from all subjects involved in the study. A total number of 130 (65 female and 65 male) participants were enrolled in the study. The subjects' demographic data were obtained through questions provided along with the consent forms. The demographic data included age, gender, and comorbidities, and awareness and dates of previous COVID-19 infections, as well as the COVID-19 vaccine profile. All participants had had double shots (either homologous or heterologous) of COVID-19 vaccines belonging to BNT162b2 by Pfizer-BioNTech, mRNA-1273 by Moderna, or ChAdOx nCov-2019 by AstraZeneca.

2.2. Hematological and Biochemistry Testing

Venous blood samples were drawn from all participants in plain tubes and ethylenediamine tetra-acetic acid dipotassium salt (EDTA-2K) tubes. Plain tubes were centrifuged at 3500 rpm for 5 min, after which ferritin level was determined by the Alinity system (Abbott Laboratories, IL, USA) that utilizes chemiluminescent microparticle immunoassay (CMIA). The EDTA-2K tubes were used to assess the hemoglobin level through complete blood

count (CBC) utilizing the modern automated hematology analyzer (Sysmex Corporation, Kobe, Japan).

2.3. Serological Assays

Sera were subsequently subjected to in-house immunoassays: S-based and NP-based indirect ELISA, and serum-neutralization (SN) assay to assess the presence and activity of anti-SARS-CoV-2 antibodies. The detailed protocols for these immunoassays were previously described. The cut-off optical density values for S-based and NP at 450 nm were 0.27 and 0.17, respectively, while SN titers of $\geq$1:20 were considered positive. Our local clinical isolate of SARS-CoV-2 (SARS-CoV-2/human/SAU/85791C/2020, gene bank accession number: MT630432) was utilized in SN assay.

2.4. Statistical Analysis

All statistical analysis and graphing was done using GraphPad prism 9 software (GraphPad Software, La Jolla, CA, USA). Mann–Whitney U test, Kruskal–Wallis test and Fisher's exact test were done as appropriate with p value of $\leq$0.05 considered statistically significant.

3. Results

A total number of 130 participants were recruited in this study. The participants belonged to both genders, with a mean age of 21.9 years. All participants had received two homologous or heterologous doses of COVID-19 vaccines belonging to BNT162b2 by Pfizer-BioNTech, mRNA-1273 by Moderna, or ChAdOx nCov-2019 by AstraZeneca. The participants were divided into a control group and a study group based on their hemoglobin and ferritin levels, where a hemoglobin level of less than 13 g/dL for men and 12 g/dL for women coinciding with a ferritin level below 30 ng/mL indicated IDA, while a normal hemoglobin occurring with a ferritin level below 30 was considered as an insufficient iron store or iron ID. The cut-off of ferritin < 30 ng/mL was used because it was shown to exhibit high sensitivity and specificity (92% and 98%, respectively), to diagnose ID [24–26]. ID (n = 41) and IDA (n = 22) together comprised the entire study group (n = 63), while the control group comprised all other subjects with normal hemoglobin and ferritin levels (n = 67). Figure 1 shows the difference between the hemoglobin and ferritin levels for the controls and study group. The characteristics of the control and study groups are summarized in Table 1. All data shown on the table were extracted from the questionnaire provided to the participants at the time of recruitment with the exception of hemoglobin and ferritin levels that were determined as described in the Materials and Methods section. Statistical analysis revealed no significant difference between the control and study groups in most variables including age, BMI, previous diagnosis with COVID-19, and vaccination profile. On the other hand, hemoglobin and ferritin levels were significantly lower in the study group compared to the controls (Table 1 and Figure 1A). This remained true whether data of ID and IDA individuals were combined or separated with further significant reduction detected among IDA participants (Figure 1B).

To investigate COVID-19-vaccine effectiveness in producing anti-S IgGs, an S-based in-house ELISA was performed for all samples. In all 130 samples, anti-S IgG was identified in 126 (96.92%) samples with OD_{450} values > 0.27. The prevalence rates of anti-S IgG for the study and control groups were 98.41% (62/63) and 95.52% (64/67), respectively, with no significant difference observed (p = 0.61, 95% CI = 0.02612 to 2.377). To exclude any possible interference caused by previous COVID-19 infections, an NP-based in-house ELISA was performed because all types of vaccines received by the participants specifically mount anti-S antibodies. Anti-NP IgG was detected in 32/130 sample (24.62%) with OD_{450} values > 0.17. The prevalence rate of anti-NP IgG for the study group was 20.63% (13/63) and for the control was 28.36% (19/67), showing non-significant difference (p = 0.31, 95% CI = 0.6609 to 3.467). Our findings remained consistent whether data from ID and IDA participants were combined or separated (Figure 2 right and left panels, respectively).

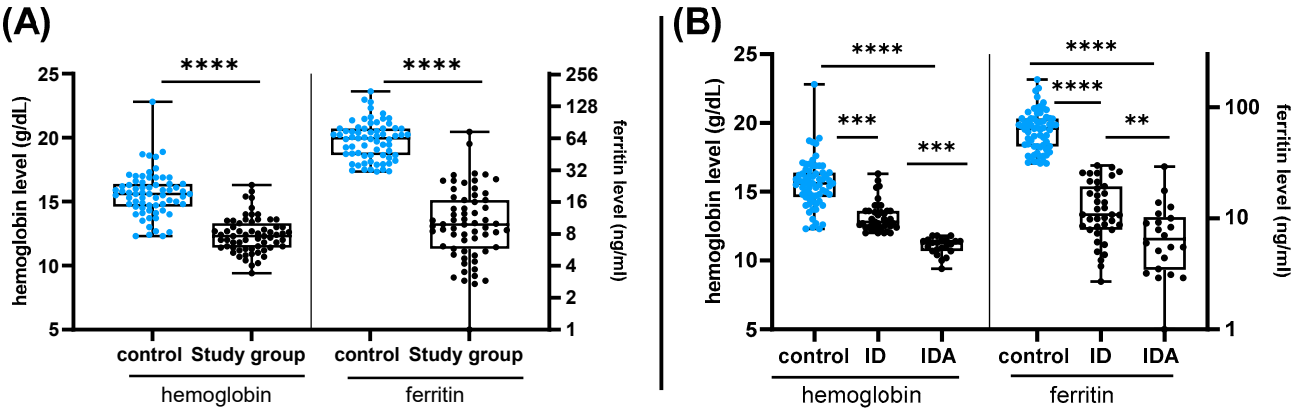

Figure 1. The levels of hemoglobin and ferritin among control and study group. (**A**) Hemoglobin levels (g/dL) and ferritin levels (ng/mL) for control group and study group (ID and IDA combined). (**B**) Hemoglobin and ferritin levels for control group and study group (separated as ID and IDA). Boxes represents 25th to 75th percentile range, black line demonstrates the median, and whiskers show minimum and maximum values. p values were calculated by Mann–Whitney U test and Kruskal–Wallis test, as appropriate. ** = p value < 0.01, ***, = p value < 0.001, **** = p value < 0.0001.

Table 1. Characteristics of control (normal hemoglobin and ferritin) and study group (ID, IDA).

Variables		Control (n = 67)		Study Group (n = 63)		p Value
Hemoglobin (g/dl)		15.6 ± 1.74		12.39 ± 1.38		<0.0001
Ferritin (ng/mL)		66.3 ± 29.13		13.3 ± 12.16		<0.0001
Age		21.42 ± 2.48		22.47 ± 6.2		0.854
Body mass index (BMI)		25.54 ± 6.65		23.13 ± 5.29		0.0998
Gender	Male	60	89.55%	5	7.94%	<0.0001
	Female	7	10.45%	58	92.06%	
Previously diagnosed with COVID-19 by RT-PCR	Yes	12	17.91%	14	22.22%	0.6617
	No	55	82.09%	49	77.78%	
Vaccination	Homologous	53	79.10%	55	87.30%	0.2477
	Pfizer	48	71.64%	53	84.13%	
	AstraZeneca	5	7.46%	2	3.17%	
	Heterologous	14	20.90%	8	12.70%	
	Pfizer/AstraZeneca	14	20.90%	7	11.11%	
	AstraZeneca/Moderna	0	0.00%	1	1.59%	
Days since vaccination	<180	46	68.66%	48	76.19%	0.4332
	>180	21	31.34%	15	23.81%	

Further analysis performed on IgG NP findings revealed that 62.50% (20/32) did not belong to participants who reported previous COVID-19 infection but rather, belonged to those who did not report any previous infection (Figure 3A,B). Interestingly, 53.83% (14/26) of those who reported a previous infection tested negative for IgG NP. These findings indicate that many participants with positive IgG NP were unaware of a possible COVID-19 infection acquisition prior the time of the study. In addition, the noticed variability with IgG NP results despite the time after infection suggests the unpredictability of after-infection immunity (Figure 3C).

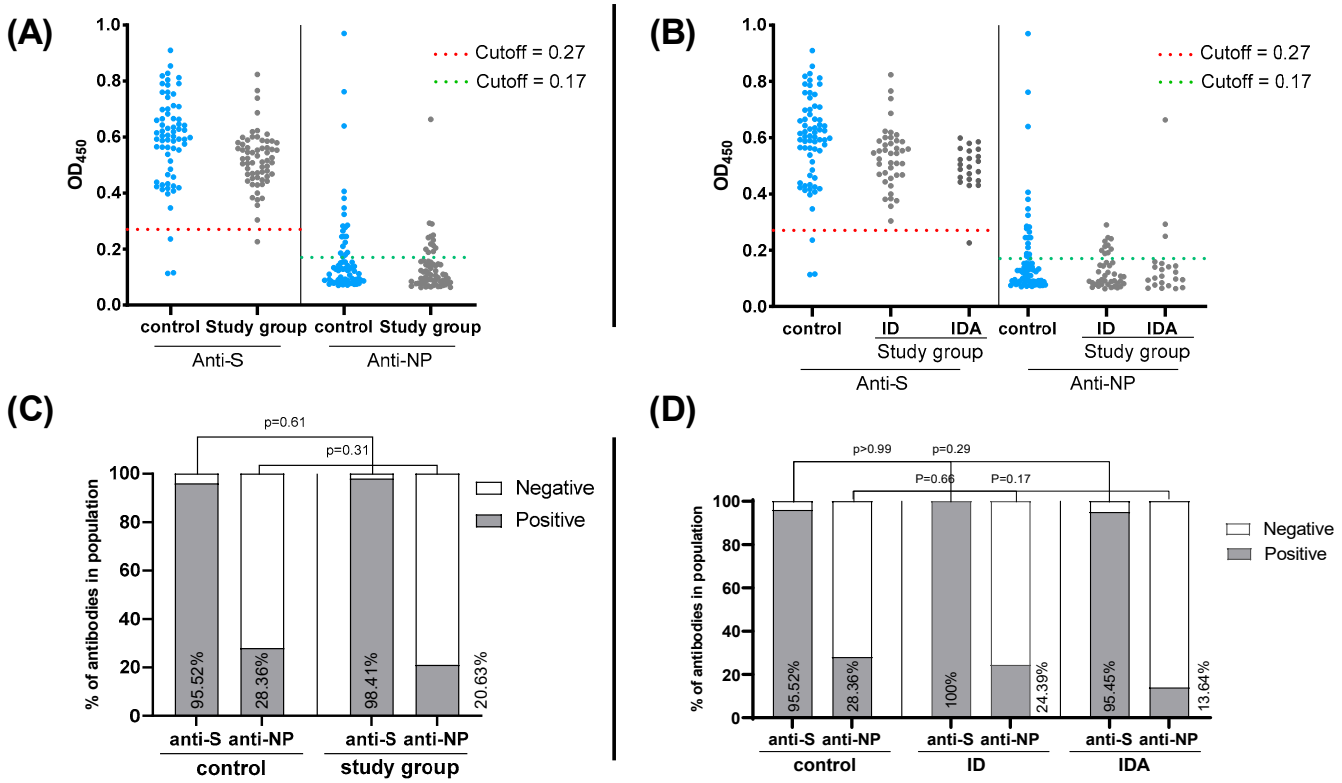

Figure 2. Optical density (OD) values and the prevalence of anti- SARS-CoV-2 S and NP IgGs among control group (normal hemoglobin and ferritin) and study group (ID, IDA). (**A**,**B**) Optical density values at 450 nm (OD450) as obtained from S- and NP-based ELISAs for control and study group: (**A**) ID and IDA combined and (**B**) ID and IDA separated. Dashed red lines represent the cut-off value for S-ELISA (OD450 = 0.27) and dashed green lines represent NP-ELISA cut-off value (OD450 = 0.17). (**C**,**D**) The prevalence (%) of anti-S and anti-NP in control and study group: (**C**) ID and IDA combined and (**D**) ID and IDA separated. *p* values were calculated by Fisher's exact test, *p* value < 0.05 is considered significant.

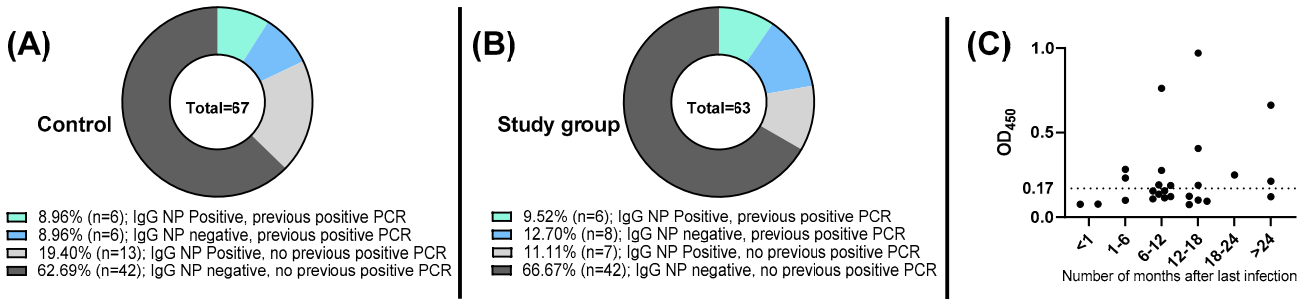

Figure 3. Analysis of anti-SARS-CoV-2 NP IgG in control group (normal hemoglobin and ferritin) and study group (ID, IDA). (**A**) Prevalence of IgG NP among control group. (**B**) Prevalence of IgG NP among study group. (**C**) OD450 readings of NP-based ELISA across different time periods among participants who had reported previous positive COVID-19 PCR result in the entire population. Dashed black line represents NP-based ELISA cut-off (OD450 = 0.17).

When SN was employed for further confirmation and assessment of neutralizing capacity of the antibodies produced by vaccines, the results obtained corresponded well with ELISA since 95.24% (60/63) of the study group and 95.52% (64/67) of the control group tested positive ($p > 0.99$, 95% CI = 0.2416 to 4.706). When IgG NP sero-positive

samples were excluded, a negligible change was observed, as shown in Figure 4A. Where 94% (47/50) and 95.83% (46/48) of the study group and control, respectively, remained positive expressing no significant difference. We further assessed whether iron deficiency may affect the titer of neutralizing antibodies, and we did not identify any significant difference between the study group and controls that persisted even after anti-NP sero-positive samples were excluded (Figure 4A,B). Similar findings were obtained when the study group was subdivided into ID and IDA (Figure 4C,D). Moreover, we did not find a statistically significant correlation between hemoglobin and ferritin levels with serum neutralization titer (Figure 4E).

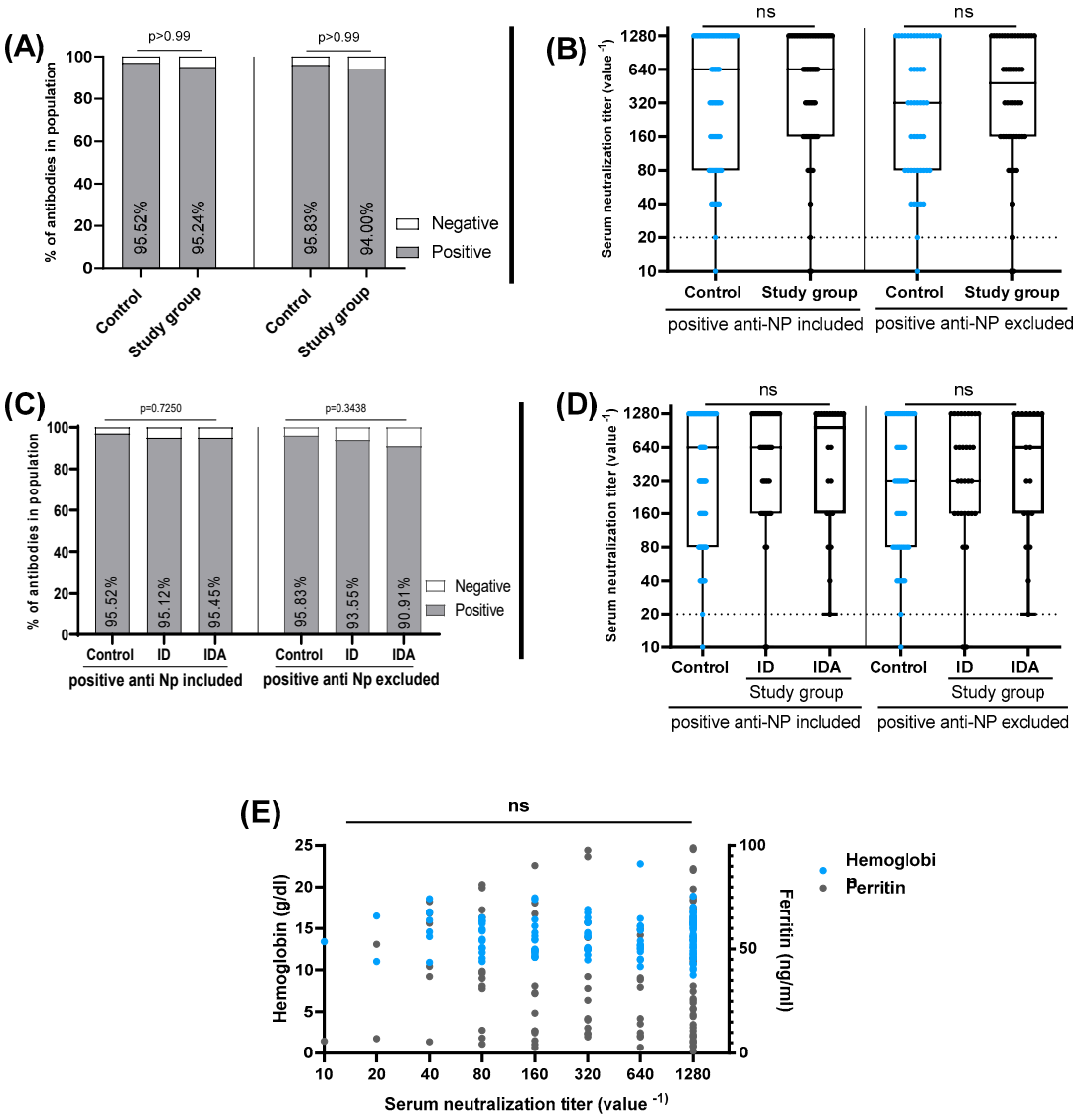

Figure 4. The prevalence of COVID-19 neutralizing antibodies in the control group (normal hemoglobin and ferritin) and study group (ID, IDA). (**A**) The prevalence (%) of neutralizing antibodies in control group and study group (ID and IDA combined) when anti-NP sero-positive cases were included (left) and excluded (right). *p* values were calculated by Fisher's exact test. (**B**) Comparison of neutralizing antibody titers among control group and study group (ID and IDA combined) when anti-NP sero-positive cases were included (left) and excluded (right). (**C**) The prevalence (%) of neutralizing antibodies in control group and study group (ID and IDA separated) when anti-NP sero-positive cases were included (left) and excluded (right). *p* values were calculated by Fisher's exact test. (**D**) Comparison of neutralizing antibody titers among control group and study group (separated as ID and IDA) when anti-NP sero-positive cases were included (left) and excluded (right). Dashed

black line indicates the cut-off value (SN titer ≥ 1:20 were positive). Boxes represents 25th to 75th percentile range, black line demonstrate the median, and whiskers show minimum and maximum values. The horizontal lines represent the medians. *p* values were calculated by Mann–Whitney U test and Kruskal–Wallis test, as appropriate. ns = *p* value > 0.05. (**E**) Spearman correlation between hemoglobin and ferritin levels and serum neutralization titer. ns = *p* value < 0.05.

Finally, when the scale was adjusted for gender (Figure 5A,B), type of vaccine administered (homologous or heterologous) (Figure 5C,D), or number of days since vaccination (Figure 5E,F), and while excluding anti-NP positive samples, no significant differences were observed. Again, combining or separating ID and IDA in the study group did not affect our findings.

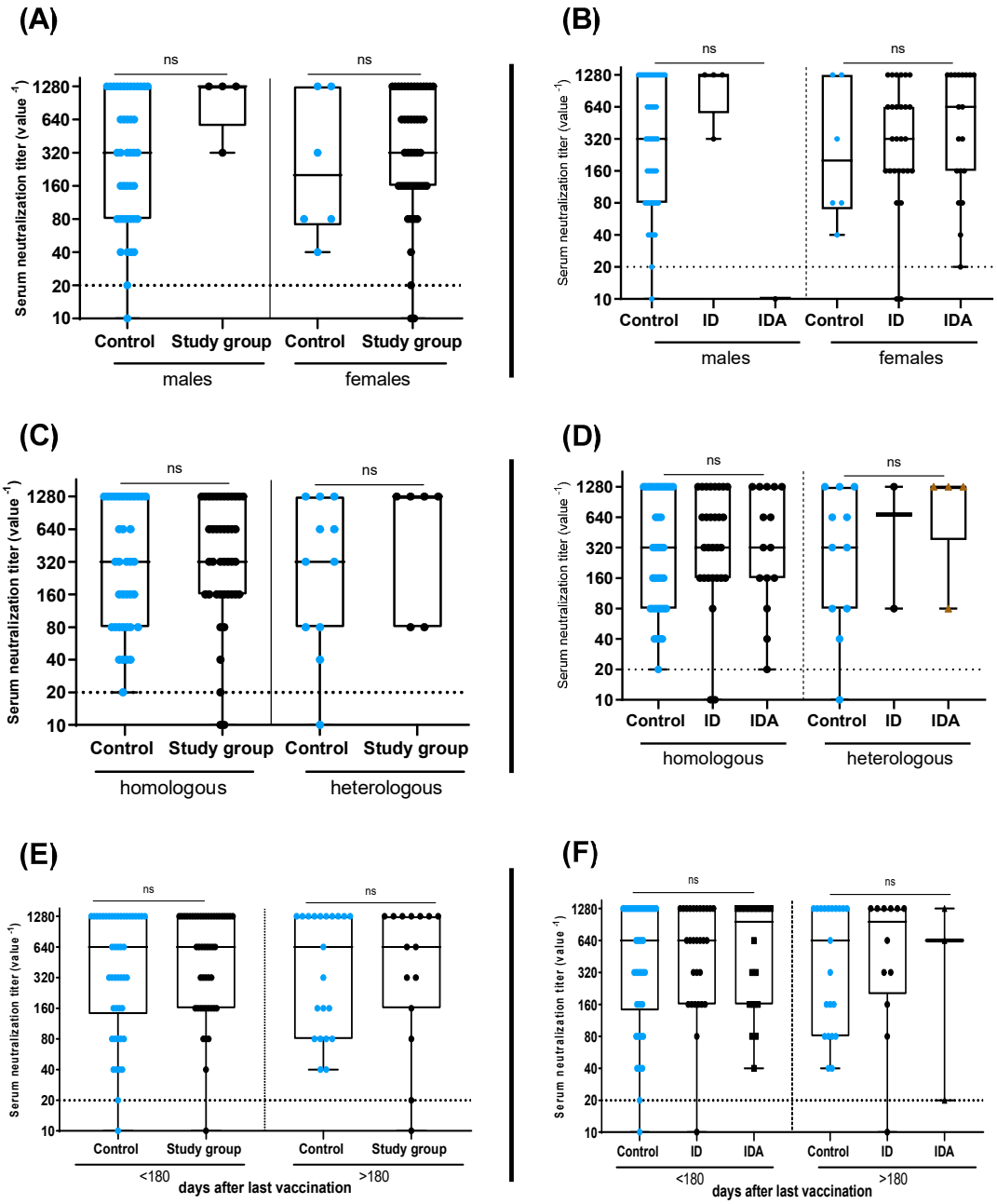

Figure 5. The effect of different variables on the neutralization titer among control group (normal

hemoglobin and ferritin) and study group (right panel: ID and IDA combined; left panel: ID and IDA separated). Neutralizing antibody titers related to gender (**A**,**B**), type of vaccine received (**C**,**D**), and period since vaccination (**E**,**F**). Boxes represents 25th to 75th percentile range, black line demonstrate the median, and whiskers show minimum and maximum values. Mann–Whitney U test and Kruskal–Wallis test were used as required. ns indicates non-significant.

4. Discussion

ID is among the most frequent micronutrient insufficiency, impacting billions globally [27]. Iron is required for efficient induction of host immunity following immunization by infections or vaccinations [14,28–30]. Iron is known to be necessary for the differentiation of monocytes and macrophages as well as for some antimicrobial activities [10]. Additionally, the generation of cytokines, lymphocyte differentiation, and proliferation require iron [11,12]. Recently, lower hemoglobin and ferritin levels were strongly linked to severe outcomes of COVID-19, which placed a great burden on the vaccines to resolve that problem [16–20]. Yet, lower ferritin and hemoglobin levels have also been suggested to pose challenges for some vaccines effectiveness (e.g., Rubella, DPT, *Haemophilus influenzae* type b (Hib), *Streptococcus pneumoniae* serotype 1 (PS1)) [7,13,14,27]. Although a recommendation to correct iron level before receiving COVID-19 vaccination was raised, the picture with regards to the effect of iron level on COVID-19 vaccines effectiveness is still vague. A study has introduced a ferritin-based COVID-19 vaccine to mice to assess whether ferritin can boost vaccines-induced antibodies. Impressively, their vaccine induced high titers of efficient neutralizing antibodies that lasted for more than seven months compared to a control group that received ferritin lacking equimolar vaccine [31]. To our knowledge, there is a lack of reports about COVID-19 vaccines effectiveness specifically targeting individuals with ID and IDA. Hence, this study to our knowledge is considered the first to remove the fog upon that issue.

In this study, the prevalence of anti-S and anti-NP antibodies were assessed using our lab-developed S and NP based ELISAs and their neutralizing capacity was assessed using SN assay [32–34]. Our primary target population was iron deficient individuals and we recruited healthy individual as a control group, from both genders. All study participants have received two doses of COVID-19 vaccines either homologous or heterologous (BNT162b2 by Pfizer-BioNTech, mRNA-1273 by Moderna, and ChAdOx nCov-2019 by AstraZeneca). The S-based ELISA results identified anti-S IgG in nearly all subjects -apart from four- regardless of their ferritin or hemoglobin status. This indicates that the formerly mentioned vaccines have successfully induced anti-S antibodies in both ID and IDA patients and healthy controls. The four subjects who tested negative for anti-S IgG, three of them belonged to the control group and the other one was among the study group (ID and IDA). Two of the three subjects were smokers and the third had asthma, while the one in study group was IDA with no other comorbidities. Harnessing SN to evaluate the vaccines-induced antibodies neutralization capacity was a core milestone in this study. As it is considered the gold standard method to measure antibodies titers and their neutralization activities [35]. SN results showed almost exact resemblance to S-based ELISA results. No significant difference was observed between controls and study group, male and females, and those who received homologous or heterologous vaccines as seen in similar studies [36,37]. Further, we have analyzed the data of ID and IDA separately given the fact that they represent two distinct clinical conditions. Our data remained intact and no significance difference was observed when the study group was separated into ID and IDA groups. This indicates that COVID-19 vaccines are highly effective in inducing the production of highly efficient neutralizing antibodies in humans notwithstanding lower hemoglobin or ferritin levels. Two recent studies conducted on hemodialysis patients identified a positive correlation between hemoglobin and ferritin levels and anti-S antibody titer [38,39]. This may suggest that their finding might have been influenced by other factors. In addition, the presence of anti-SARS-CoV-2 antibodies rather than neutralization activity was considered in these studies [38,39]. However, the findings of our study may not be utterly conclusive when considering the small sample size. Another limitation of this

study was the distribution of male and female among control and study groups. Most of the study group were female compared to dominance of male in the control group, which is expected taking into consideration that iron deficiency is more prevalent in females due to several factors (e.g., menstruation, pregnancy, and malnutrition) [40]. Although controversial, others and we have previously shown that gender does not affect COVD-19 vaccine-induced humoral immunity, which suggest that gender suboptimal distribution may have a negligible or no effect on our study findings [41–43]. Yet, similar studies with larger sample sizes and more appropriate gender distribution remain necessary to draw comprehensive conclusions about the effect of Iron on COVID-19 vaccine effectiveness. It is not until then that one can advocate for or against the recommendation of correcting the iron level prior to vaccination.

When NP-based ELISA results were analyzed, 53.83% (14/26) of the participants who reported previous COVID-19 infection tested negative for anti-NP IgG regardless to the duration between infection and sample collection. These data suggest that after infection natural immunity may wane which was observed in previous studies [44–47]. Furthermore, we were able to detect anti-NP IgG in 46.17% (12/26) of participants who reported previous COVID-19 infection even after more than two years from the last infection. These findings propose that the durability of infection-induced antibodies is to some context distinct from person to person. However, upon a second look at other positive NP-based ELISA results, a notable bigger proportion (62.50%, 20/32) of the participants belonged to those who did not report a previous infection; highlighting the participants' lack of awareness over a possible SARS-CoV-2 infection prior to sample collection [46–49]. This unawareness of previous infection can be explained, at least in part, by the circulation of rapidly transmitting less virulent variants of SARS-CoV-2, or vaccine-mediated reduction of disease severity.

5. Conclusions

Low iron levels have always been implicated in abnormal immune responses. Hence, ID and IDA conveyed concerns for COVID-19-vaccine effectiveness. This study demonstrated that COVID-19 vaccines successfully induced neutralizing-antibody production in individuals with ID and IDA to similar levels observed in healthy controls. Further analyses indicate that the incidence of past infection, type of COVID-19 vaccine, or period since last vaccination did not significantly affect the presence or titer of the vaccine-mediated neutralizing anti-SARS-CoV-2 antibodies. This study was limited by the sample size and gender bias, which necessitates similar studies with larger sample size and more appropriate gender distribution to confirm our findings.

Author Contributions: Conceptualization, A.A.F., M.N.A. and T.A.A.; data curation, A.A.F., A.A.B. and T.A.A.; formal analysis, A.A.F., A.A.B. and T.A.A.; funding acquisition, A.A.F. and T.A.A.; investigation, A.A.F., A.A.B., A.M.H., F.S.Q., A.S.B. and T.A.A.; methodology, A.A.F., A.A.B., M.N.A., A.M.H., A.S.B. and T.A.A.; project administration, T.A.A. and E.I.A.; resources, M.N.A., F.S.Q., T.A.A. and E.I.A.; software, A.A.F., A.A.B. and T.A.A.; supervision, A.A.F. and T.A.A.; validation, A.A.B., S.A.E.-K., T.A.A. and E.I.A.; visualization, A.A.B. and T.A.A.; writing—original draft, A.A.F., A.A.B., S.A.E.-K. and T.A.A.; writing—review and editing, A.A.F., A.A.B., M.N.A., A.M.H., F.S.Q., A.S.B., S.A.E.-K., T.A.A. and E.I.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research work was funded by the Institutional Fund Projects under grant No. (IFPIP:272-290-1443). The authors gratefully acknowledge technical and financial support provided by the Ministry of Education and King Abdulaziz University, DSR, Jeddah, Saudi Arabia.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Research Ethics Committee (REC) at Umm Al Qura University (protocol code HAPO-02-K-012-2021-09-747 and date of approval 7 September 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data sharing not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

Anti-SARS-CoV-2 IgM Secondary Response Was Suppressed by Preexisting Immunity in Vaccinees: A Prospective, Longitudinal Cohort Study over 456 Days

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Abstract: To obtain more insight into IgM in anti-SARS-CoV-2 immunity a prospective cohort study was carried out in 32 volunteers to longitudinally profile the kinetics of the anti-SARS-CoV-2 IgM response induced by administration of a three-dose inactivated SARS-CoV-2 vaccine regimen at 19 serial time points over 456 days. The first and second doses were considered primary immunization, while the third dose was considered secondary immunization. IgM antibodies showed a low secondary response that was different from the other three antibodies (neutralizing, total, and IgG antibodies). There were 31.25% (10/32) (95% CI, 14.30–48.20%) of participants who never achieved a positive IgM antibody conversion over 456 days after vaccination. The seropositivity rate of IgM antibodies was 68.75% (22/32) (95% CI, 51.80–85.70%) after primary immunization. Unexpectedly, after secondary immunization the seropositivity response rate was only 9.38% (3/32) (95% CI, 1.30–20.10%), which was much lower than that after primary immunization ($p = 0.000$). Spearman's correlation analysis indicated a poor correlation of IgM antibodies with the other three antibodies. IgM response in vaccinees was completely different from the response patterns of neutralizing, total, and IgG antibodies following both the primary immunization and the secondary immunization and was suppressed by pre-existing immunity induced by primary immunization.

Keywords: COVID-19; anti-SARS-CoV-2 IgM; immunity; secondary response; vaccination

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Citation: Xu, Q.-Y.; Xie, L.; Zheng, X.-Q.; Liang, X.-M.; Jia, Z.-J.; Liu, Y.-Y.; Liang, X.-Y.; Liu, L.-L.; Yang, T.-C.; Lin, L.-R. Anti-SARS-CoV-2 IgM Secondary Response Was Suppressed by Preexisting Immunity in Vaccinees: A Prospective, Longitudinal Cohort Study over 456 Days. *Vaccines* **2023**, *11*, 188. https://doi.org/10.3390/vaccines11010188

Academic Editors: Rishi Jaiswal, Srijani Basu, Suman Gupta and Sneh Lata Gupta

Received: 4 December 2022
Revised: 10 January 2023
Accepted: 13 January 2023
Published: 16 January 2023

1. Introduction

Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has turned into a global epidemic and presented tremendous healthcare concerns. According to data reported by the World Health Organization on 07 December 2022, the confirmed cases were 642,379,243, and approximately 6,624,118 cases had died from COVID-19. Currently, the pandemic of COVID-19 is as yet a worldwide challenge as there are continually genetic mutations in the SARS-CoV-2 genome, and variations in its protein S are increasingly reported [1]. For controlling the COVID-19 pandemic, vaccination may be the most efficient strategy. According to data reported by the World Health Organization on 4 December 2022, approximately 12,998,974,878 doses of vaccines for COVID-19 have been inoculated globally (https://covid19.who.int/ (accessed on 4 December 2022). However, the vaccines for COVID-19 have to remain effective against severe COVID-19 infections and deaths caused by COVID-19, including those caused by the delta variant. The massive number of breakthrough infections caused by viral diversification and waning immunity warrants a new vaccine or a third vaccine dose. To combat the ongoing resurgence of the COVID-19 epidemic, the US Food and Drug Administration

authorized use of a third dose of booster for all adults after completion of primary vaccination with approved COVID-19 vaccines [2]. This step seems essential because preliminary studies indicated that administration of three doses of the Pfizer–BioNtech mRNA vaccine can neutralize the omicron variant of the virus with an approximately 40-fold decline in the viral titer, but administration of two doses is less effective [3]. Our previous study also indicated that one or more booster vaccine doses are warranted because of waning immunity and a massive number of breakthrough infections caused by viral diversification [4]. A booster dose was shown to be extremely helpful in the fight against COVID-19 and accompanying severe illness and death [5]. According to data from the Centers for Disease Control and Prevention, unvaccinated adults have a nearly six-fold increased risk of testing positive for COVID-19 and a fourteen-fold increased risk of dying from the virus than individuals who had been vaccinated [6]. Therefore, continuous monitoring of the protective effect of COVID-19 vaccines against more virulent variants is critical.

SARS-CoV-2 infections can activate a strong response of B-cells. Within days, the specific antibodies immunoglobulin M (IgM), IgG, and IgA are detectable in patients. At first, the antibodies bind to the virus' external spike glycoprotein and internal nucleocapsid proteins, then the antibodies block the binding of SARS-CoV-2 to the host cell's surface receptor angiotensin-converting enzyme 2 (ACE2) effectively [7]. The specific antibody response to SARS-CoV-2 inoculation is still under detailed investigation, and a comparison between protective immunity to SARS-CoV-2 infection in COVID-19 patients and in vaccines is urgently required for guiding decisions from public health employees and guiding information about vaccine management [8]. SARS-CoV-2 antigen-specific antibody response is observed as a measure of protective immunity following the administration of SARS-CoV-2 vaccination [9]. The current vaccines for SARS-CoV-2 induce a robust specific IgG antibody response, which has been the subject of investigation [10]. However, limited data are available on the development and maintenance of vaccine-elicited specific IgM antibody responses. IgM antibodies are generally believed to respond early during viral infections and are expected to neutralize a broader range of viral strains than related IgG antibodies [11,12]. Our previous research demonstrated that the IgM seropositivity rate was only 59.02% two weeks after primary immunization [4]. Notably, it was also reported that a proportion of patients never developed an IgM antibody response [13–15]. The reason for the difference in IgM response patterns remains unclear. To obtain more insight into IgM in vaccine-elicited immunity, especially over longer periods of time after three doses of vaccination, we performed a prospective cohort study to longitudinally profile the dynamic response of anti-SARS-CoV-2 IgM antibodies at 19 serial time points over 456 days following serial inactivated CoronaVac vaccination. Additionally, the IgM antibody response patterns in vaccinees after primary immunization and secondary immunization were evaluated. Furthermore, correlation of IgM with three other subsets of anti-SARS-CoV-2 antibodies was analyzed.

2. Materials and Methods

2.1. Study Design and Participants

The 32 participants from Xiamen Boson Biotech Co., Ltd., Xiamen, China, were the same as those in a previous study from our research team [16]. On 24 January 2021, all participants received the first dose (0.5 mL/dose) of the inactivated CoronaVac vaccine (Sinovac Life Sciences, Beijing, China), the second dose 28 days later (on 21 February 2021), and the third dose 276 d later (on 27 October 2021). In this study, the first and second doses were considered primary immunization, while the third dose was considered secondary immunization. Anti-SARS-CoV-2 IgM antibodies (against the spike protein, IgM), anti-SARS-CoV-2 IgG antibodies (against the spike protein, IgG), anti-SARS-CoV-2 neutralizing antibodies (against the receptor-binding domain (RBD), neutralizing antibody) and anti-RBD total antibodies (against the RBD, total antibody) were serially determined to evaluate responses and durations every 7 d for 28 d following each dose, with 6 more visits (102 d, 132 d, and 248 d after the second dose and 61 d, 92 d, and 180 d after the third dose).

This research (#xmzsyyky2021196) was authorized by the Institutional Ethics Committee of Zhongshan Hospital of Xiamen University, China. This research complied with the Declaration of Helsinki guidelines and national legislation. Written informed consent was provided by all participants.

2.2. Laboratory Assays

Around 3 mL of venous blood from all participants who had fasted for no less than 8 h was collected in procoagulant tubes. The blood samples were centrifuged for 10 min with 3000× *g*, and the serum on the upper layer of blood was examined for four subsets of anti-SARS-CoV-2 antibodies within 6 h. The four subsets of anti-SARS-CoV-2 antibodies were examined utilizing the reagent that matched measured with an Autolumo A2000 plus system (Anto Biological Pharmacy Enterprise Co., Ltd., Zhengzhou, China), which employs a chemiluminescence microparticle immunoassay as its basis for functioning. Detection experiments were conducted based on the manufacturer's instructions and a previous study [4]. The result of the chemiluminescent reaction was evaluated in relative light units (RLU). IgM and IgG antibodies were measured using the S/CO (RLU of samples/cut-off) value, with S/CO ≥1.00 deemed positive and <1.00 deemed negative. A one-step competitive method was used to detected neutralizing antibodies. Specific anti-SARS-CoV-2 neutralizing antibodies in blood samples bound to a horseradish peroxidase-labeled RBD antigen, which then neutralized the binding of ACE2 that had been coated on the microparticles and the RBD antigen. The horseradish peroxidase-labeled RBD antigen not neutralized by specific anti-SARS-CoV-2 neutralizing antibodies formed a complex with ACE2 on the microparticles. The RLU was inversely proportional to the amount of specific anti-SARS-CoV-2 neutralizing antibodies in the sample. The neutralizing antibodies were calibrated, calibration was within range of the First World Health Organization International Standard (NIBSC20/136), and were recorded in international units (IU)/mL [4]. Based on 50% protection from infection with SARS-CoV-2, ≥54.00 IU/mL was regarded as positive, and <54.00 IU/mL was defined as negative [9]. Arbitrary units (AU)/mL were used for total antibody concentration, <8.00 AU/mL was defined as negative, and ≥8.00 AU/mL was defined as positive.

2.3. Statistical Analysis

Statistical analyses were conducted using IBM SPSS statistics version 25 (SPSS, Inc., Chicago, IL, USA). After performing the Shapiro–Wilk normality test to assess the normality of distribution, Spearman's correlation analysis was employed to calculate the correlation coefficient of anti-SARS-CoV-2 antibodies. The *r* values of correlation of the results were categorized as extreme (0.91–1.0), strong (0.71–0.9), moderate (0.41–0.7), weak, or poor (0–0.4). An antibody heatmap was generated using the Pheatmap package with default parameters using R version 3.6.3. The McNemar test was used to compare paired positive conversion. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Characteristics of Participants

As shown in Table 1, a total of 32 participants who provided blood samples at 19 serial time points over 456 days were included in this study, with 24 women (75%; median age 34 years) and 8 men (25%; median age 36 years). The age distribution was not different between men and women ($p = 0.287$). During the pre- and post-vaccination sampling periods, all participants had no history of COVID-19 infection. All participants were of Han nationality. At the time of vaccination, none of the participants had any of the following symptoms: cough, fever, sore throat, fatigue, diarrhea, runny nose, shortness of breath, muscle aches, or loss of or change in sense of smell and taste.

Table 1. Clinical Characteristics of 32 participants to whom three doses of CoronaVac Vaccine were administered.

Characteristics	Value
Gender	
Female, N, (%)	24 (75%)
Male, N, (%)	8 (25%)
Age	
Female age (IQR), years	34 (31–40)
Male age (IQR), years	36 (31–42) [a]
Race	
Han, N, (%)	32 (100%)
Vaccination schedule	
Days between the first dose and second dose (days)	28
Days between the second dose and third dose (days)	248
SARS-CoV-2 infection	
Before vaccination, N, (%)	0 (0%)
After vaccination, N, (%)	0 (0%)
Presenting COVID-19 symptoms:	
Cough, N, (%)	0 (0%)
Fever, N, (%)	0 (0%)
Sore throat, N, (%)	0 (0%)
Fatigue, N, (%)	0 (0%)
Diarrhea, N, (%)	0 (0%)
Runny nose, N, (%)	0 (0%)
Shortness of breath, N, (%)	0 (0%)
Muscle aches, N, (%)	0 (0%)
Loss of or change to a sense of smell and taste, N, (%)	0 (0%)

[a] $p = 0.287$, compared with female age; N, number; IQR, interquartile range.

3.2. Anti-SARS-CoV-2 Antibody Responss to the Vaccines

For all thirty-two participants we successfully performed analyses of four subsets of anti-SARS-CoV-2 antibodies (neutralizing, total, IgG, and IgM antibodies) at 19 serial time points within 456 days following vaccination, and then profiled the kinetics of the antibodies. Heatmaps were used to reflect trends in the four subsets of antibodies for individuals based on vaccination dose and time (Figure 1A–D). After the first dose, all four subsets of anti-SARS-CoV-2 antibodies had a minimal response (Figure 1A–D), and the seropositivity rate for all subsets of antibodies was extremely low (Figure 1E). Encouragingly, after receiving the second dose (42 days), neutralizing, total, and IgG antibodies produced a strong response (Figure 1A–D), and the rate of seropositivity significantly increased and reached 100% (32/32). Then, their peaks were maintained for approximately 2 months before they began to decline gradually. In comparison to the second dose of vaccination, the levels of those three antibodies (neutralizing, total, and IgG antibodies) again increased significantly after the third dose and lasted longer, for up to 6 months (Figure 1A–C). In contrast, IgM antibodies increased to a peak of only 59.38% (19/32) and rapidly decreased (Figure 1E) after the second dose (42 days). Unexpectedly, IgM antibodies exhibited only a minimal response of 0.08 (0.03–0.20) S/CO, and the seropositivity rate was only 9.38% (3/32) after the third dose, which was much lower than that after the second dose (Figure 1D,E). The results indicated that IgM antibodies showed a low secondary response, which was different from the other three antibodies, neutralizing, total, and IgG antibodies.

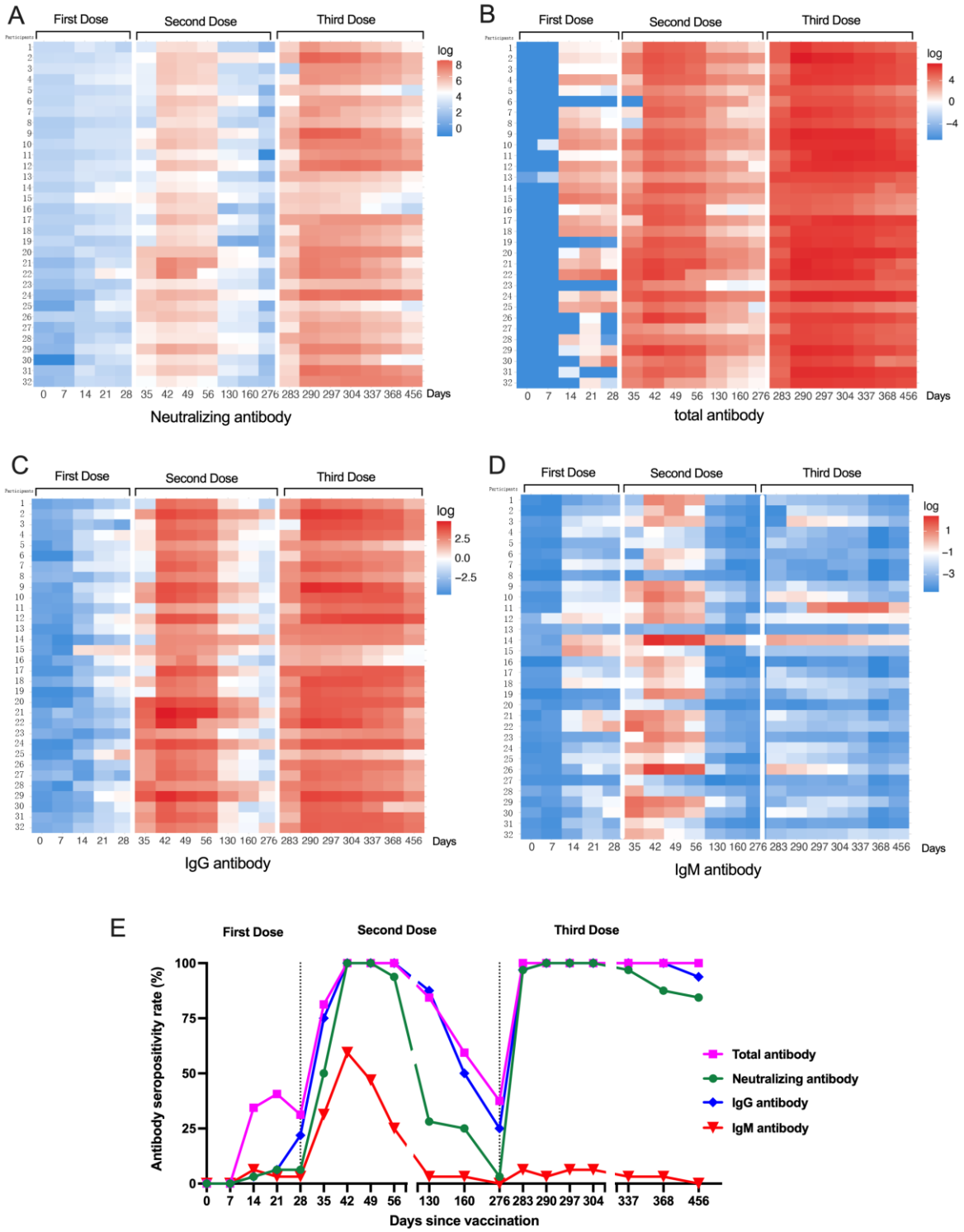

Figure 1. Anti-SARS-CoV-2 antibody response to the vaccines for 32 participants. Heatmaps of the kinetics of anti-SARS-CoV-2 neutralizing antibodies (**A**), anti-SARS-CoV-2 total antibodies (**B**), anti-SARS-CoV-2 IgG antibodies (**C**), and anti-SARS-CoV-2 IgM antibodies (**D**) induced by a three-dose regimen of vaccination. (**E**) Seropositivity rate for the four subsets of anti-SARS-CoV-2 antibodies.

3.3. Positive Conversion of Anti-SARS-CoV-2 IgM in the Secondary Immunization

To better evaluate the response of anti-SARS-CoV-2 IgM antibodies after each of the three doses, positive conversion was longitudinally investigated. There were 10 (31.25% (10/32) (95% CI, 14.30–48.20%)) participants who never achieved positive conversion after

vaccination during the three-dose regimen. Their IgM response was minimal after the third dose. Notably, 19 (86.36% (19/22) (95% CI, 70.80–100.00%)) of the participants who had achieved positive conversion after the second dose did not exhibit positive conversion again after the third dose (Figure 2A). According to serial immunization, the seropositivity response rate was 68.75% (22/32) (95% CI, 51.80–85.70%) after primary immunization. Unexpectedly, after secondary immunization, the seropositivity response rate was only 9.38% (3/32) (95% CI, 1.30–20.10%), which was much lower than the seropositivity response rate after primary immunization (p = 0.000) (Figure 2B), indicating that the secondary response was suppressed by primary immunization.

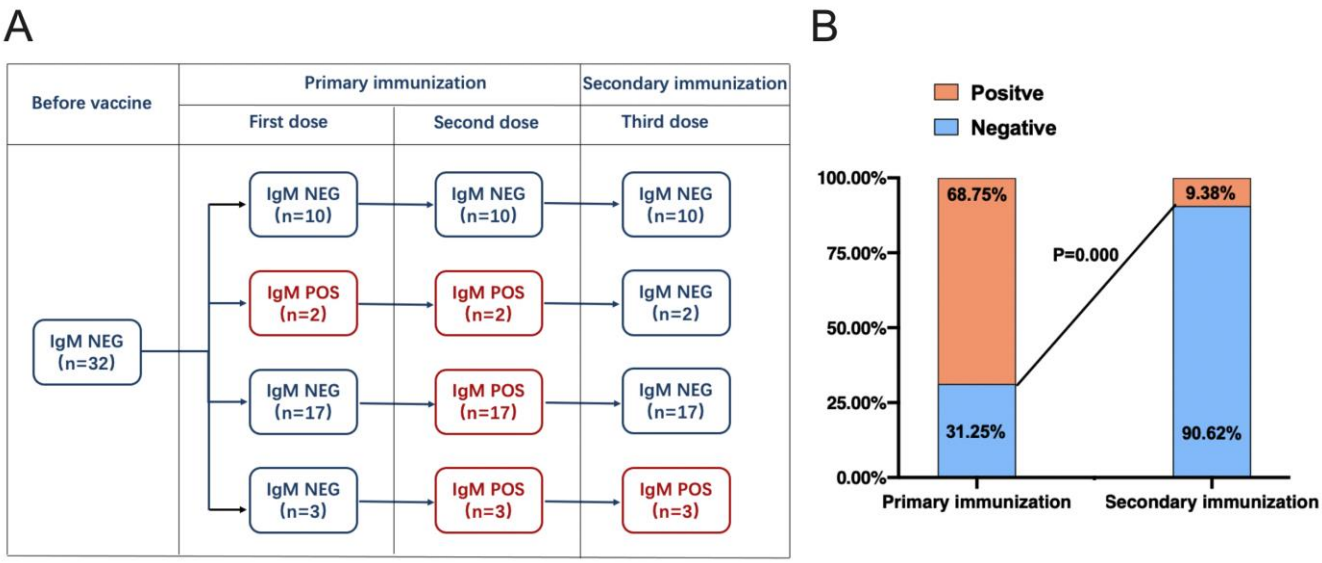

Figure 2. Positive conversion of IgM in primary immunization and secondary immunization. (**A**) Longitudinal positive conversion of anti-SARS-CoV-2 IgM antibodies. (**B**) Positive conversion rates of anti-SARS-CoV-2 IgM antibodies in primary immunization and secondary immunization. The McNemar test was used to compare paired positive conversion between primary immunization and secondary immunization. Abbreviations: NEG: negative, POS: positive.

3.4. Correlation between Anti-SARS-CoV-2 IgM Levels and the Other Three Antibodies

The above results indicated that anti-SARS-CoV-2 IgM antibodies showed a low secondary response which was different from the other three antibodies, neutralizing, total, and IgG antibodies. Spearman's correlation analysis was further conducted to analyze correlations between the four anti-SARS-CoV-2 antibodies. There was a strong correlation between anti-SARS-CoV-2 neutralizing and anti-SARS-CoV-2 total antibodies (r = 0.88; $p < 0.001$), and between anti-SARS-CoV-2 neutralizing and anti-SARS-CoV-2 IgG antibodies (r = 0.71; $p < 0.001$). A moderate correlation was also observed between anti-SARS-CoV-2 total and anti-SARS-CoV-2 IgG antibodies (r = 0.66; $p < 0.001$). However, anti-SARS-CoV-2 IgM antibodies showed a poor correlation with the other three antibodies, neutralizing, total, and IgG antibodies (r = −0.05~0.15; $p > 0.05$). In general, IgM response was different from those of the other three antibodies after prolonged inoculation (Figure 3).

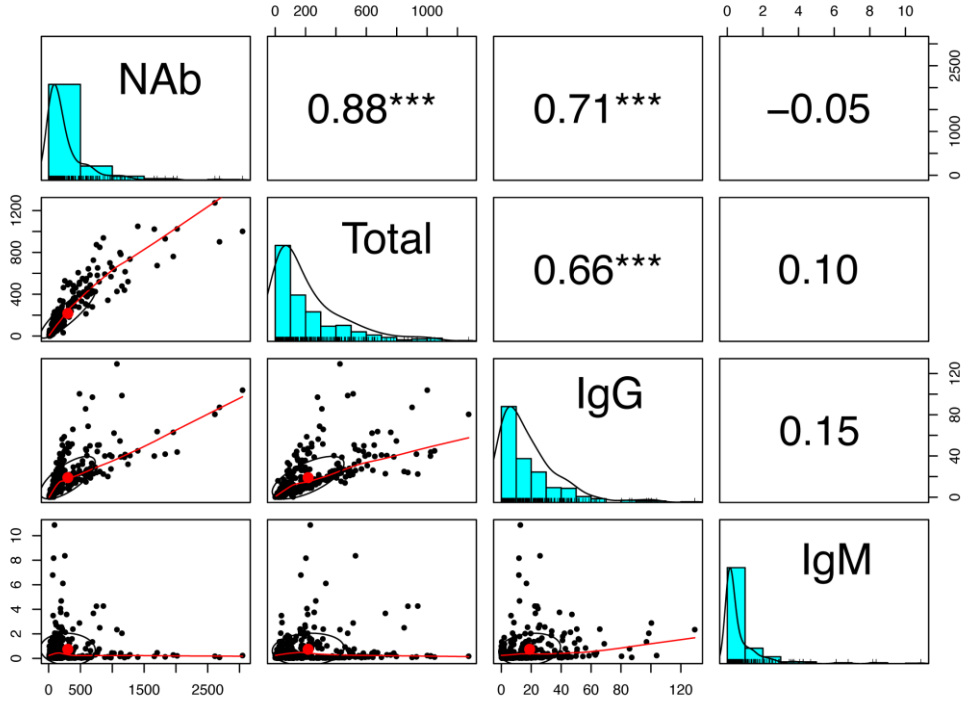

Figure 3. Correlation between anti-SARS-CoV-2 IgM levels and the other three antibodies. Each dot represents the antibodies in one individual at each time point (n = 608). ***, significant at the 0.001 probability level. Abbreviations: NAb: anti-SARS-CoV-2 neutralizing antibodies; Total: anti-SARS-CoV-2 total antibodies; IgG: anti-SARS-CoV-2 IgG antibodies; IgM: anti-SARS-CoV-2 IgM antibodies.

4. Discussion

The presence of SARS-CoV-2 changed the way of our lives, having a significant impact on the balance of social life and public health. SARS-CoV-2 is a novel and exceptionally infectious respiratory pathogenic virus that has in practically no time spread across the globe. The pathogenic virus utilizes a protein called spike and its related RBD to interact with ACE2 in host cells. Interaction between viral Spike/RBD and ACE2 on the cell surface is the main fundamental stage in SARS-CoV-2 infections [17]. One of the most effective approaches to significantly lower severe disease and death caused by SARS-CoV-2 infection is vaccination [18]. The program for COVID-19 vaccinations in China was initiated at the end of December 2020, with the vaccine administered in two doses in an interval of 28 days. At the end of September 2021, the National Health Commission of the People's Republic of China approved the administration of a booster dose of the vaccine at least 6 months after the second dose for persons aged 18 years and older. As per the World Health Organization, immunization is considered a secure, effective, and simple means for protecting the population against the risk of developing severe illness or contracting infectious diseases [19]. One of the principal reasons for administration of the vaccine is utilizing the host immune system to produce specific antibodies for developing resistance against the pathogen, which could be effective against repeated infections or recurrent infections caused by the same pathogen [2]. A crucial parameter in assessing the effects of vaccination and SARS-CoV-2 infection is the level of anti-SARS-CoV-2 antibodies, which facilitates decision-making for subsequent illness prevention and control, as well as vaccine strategy formulation. To date, much research has been performed on neutralizing antibodies, total antibodies, and IgG antibodies after vaccination or natural infection [20,21], and less is known about the characteristics of anti-SARS-CoV-2 IgM antibody response patterns. In this study, we provided a detailed assessment of the kinetics of anti-SARS-CoV-2 IgM during a three-dose schedule of inactivated vaccine administration, with measurements performed at 19 serial time points over 456 days, and detected that anti-

SARS-CoV-2 IgM antibodies showed a low response, which was different from the other three antibodies (neutralizing, total, and IgG antibodies). The results were supported by the response of IgM antibodies exhibiting a lower seroconversion peak (59.38% (19/32) after primary immunization and only 9.38% (3/32) following secondary immunization), while the seropositivity rate of neutralizing, total and IgG antibodies peaked at 100% (32/32) after primary immunization or secondary immunization, indicating that IgM antibodies were almost unresponsive after secondary immunization.

Specific antibodies assume a critical part in protective immunity against SARS-CoV-2 infections. A longitudinal understanding of the dynamic changes in antibodies produced by the humoral response after immunization will act as basis for the improvement of successful inoculation and detection strategies. In our study, 32 participants all received three doses of the vaccine and their serum antibodies were successfully measured at 19 serial time points within 456 days following vaccination. The seropositivity rate for four subsets of antibodies was extremely low in the 28 days following receipt of the first vaccine dose. The neutralizing, total, and IgG antibodies produced a strong response, and the rate of seropositivity significantly increased and reached 100% after receiving the second dose, and their peaks were maintained for approximately 2 months before they began to decline gradually. Furthermore, the levels of those three antibodies (neutralizing, total, and IgG antibodies) increased significantly after the third dose and lasted longer, for up to 6 months. The third dose of the vaccine could increase antibody levels which lasted for a longer time [16].

Anti-SARS-CoV-2 neutralizing antibodies against the RBD of the spike protein inhibit the binding of the ACE2 receptor, thereby blocking virus entry into human cells and consequently exerting an antiviral effect. Level neutralization is considered an important predictor of vaccine efficacy [22]. A couple of studies utilized immunoassays to detect neutralizing antibodies for evaluating the values of neutralization in persons who were either COVID-19 patients or vaccinees [23]. The results of neutralizing antibodies were also utilized for evaluating the efficacy of SARS-CoV-2 vaccines [24]. In our study, anti-SARS-CoV-2 total antibodies and anti-SARS-CoV-2 IgG antibodies were both strongly correlated with neutralizing antibodies. For some policies or commercial factors, anti-SARS-CoV-2 total antibodies and anti-SARS-CoV-2 IgG antibodies may be an alternative method for neutralizing antibody detection. Further comparison of the alternation of anti-SARS-CoV-2 total antibodies or anti-SARS-CoV-2 IgG antibodies with neutralizing antibodies is required in future studies. However, we observed that anti-SARS-CoV-2 IgM antibodies showed a poor correlation with the other three antibodies, neutralizing, total, and IgG antibodies. The result also confirmed that IgM response was different from those of the other three antibodies after prolonged inoculation.

The clinical effects of vaccine-induced immunity in protection from infection and severe disease necessitate immediate investigation. To date, detailed monitoring of the adaptive immune response to vaccines can be used as a measure of protective immunity against infection with SARS-CoV-2. IgM antibodies are produced early in the humoral immune reaction against viral infections and provide fast protective immunity. Then, following maturation and isotype class switching, memory IgG antibodies with increased affinity are produced. In our study, 31.25% of the participants never developed IgM during the three-dose vaccination regime. According to serial immunization, the IgM seropositivity rate was 68.75% after primary immunization, 86.36% of positive conversion participants did not exhibit positive conversion again after the third dose, and only 9.38% (3/32) after secondary immunization, which was much lower than that after primary immunization. Moreover, the three individuals with positive IgM after the secondary immunization had already developed positive IgM after the primary immunization. None of the individuals negative after the primary immunization developed IgM antibodies after secondary immunization, and the IgM secondary response to the vaccine was suppressed by pre-existing immunity. A low IgM or negative IgM response has been reported in COVID-19 patients and in vaccines [13–15,25]. Alessandra Ruggiero et al. proposed that

these noncanonical responses may indicate pre-existing immunity to cross-reactive human coronaviruses [8]. In addition, a significant pairwise correlation was observed among neutralizing, total, and IgG antibodies; however, IgM antibodies showed poor correlation with the other three antibodies. In short, IgM response was different from those of the other three antibodies after prolonged inoculation, which needs further research on the specific mechanism.

To the best of our knowledge, this is the first research to report that the IgM response to COVID-19 vaccines is suppressed by pre-existing immunity. However, the limitations of the study should be considered. Firstly, only 32 uninfected individuals were enrolled, which is a relatively small sample size. Secondly, we only studied IgM developed in response to an inactivated vaccine, without any data about IgM developed after mRNA vaccination. A comparison of the IgM developed in response to an inactivated vaccine with different types of vaccine regimens will be one of our future studies. Thirdly, effective vaccines must elicit a diverse repertoire of antibodies (humoral immunity) and CD8+ T-cell responses (cellular immunity). Unfortunately, the immune cell response was not evaluated in this study. Fourthly, we only performed a prospective cohort study to longitudinally profile the dynamic response of anti-SARS-CoV-2 IgM antibodies following vaccination and detected that the IgM response was different from that of the other three antibodies after prolonged inoculation. It would be better to compare IgM against different pathogens, e.g., IgM response to SARS-CoV-2 and influenza viruses, to confirm the protection by IgM induced by the vaccine. Comparison of IgM against different pathogens for exploring IgM-mediated protective mechanisms would be an interesting aspect that we should focus on in the future. Finally, laboratory data of the participants, such as the level of fasting plasma glucose, glycated hemoglobin(A1c), triglyceride, or cholesterol may be correlated with IgM kinetics, it is a pity that those laboratory data were not evaluated in this study.

5. Conclusions

In conclusion, our results indicated that the anti-SARS-CoV-2 IgM response to vaccines was completely different from the response patterns of neutralizing, total, and IgG antibodies following both the primary immunization and the secondary immunization and was suppressed by pre-existing immunity. These findings may contribute to our understanding of the characteristics of anti-SARS-CoV-2 immunity and to the characterization of IgM responses to infection with other pathogens or vaccination.

Author Contributions: Research idea, study design, and writing: T.-C.Y., L.-R.L., Q.-Y.X., L.X., X.-Q.Z. and X.-M.L.; data acquisition: Z.-J.J., Y.-Y.L. and X.-Y.L.; data analysis/interpretation: T.-C.Y., L.-R.L., Q.-Y.X., L.X., X.-Q.Z. and X.-M.L.; statistical analysis: L.-L.L. and Q.-Y.X.; supervision or mentorship: T.-C.Y. and L.-R.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Natural Science Foundation of China [grant numbers 82272370, 82271387, 82172331, 81973104, 81972028, and 81971147], the Key Projects for Science and Technology Program of Fujian Province, China (grant numbers 2021J02055, 2022J02055, 2020D017, and 2020J011208), and the project for Xiamen Medical and Health Guidance (grant number 3502Z20214ZD1037). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by Institutional Ethics Committee of Zhongshan Hospital of Xiamen University, China (#xmzsyyky2021196l).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated during the current study are available from the corresponding author on reasonable request.

Acknowledgments: The authors thank all the contributors to this work. We thank Xiamen Boson Biotech Co., Ltd., Fujian, China, for participant recruitment and sample collection.

Conflicts of Interest: The authors declare no conflict of interest.

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vaccines

MDPI

Editorial

An Assessment of the Bivalent Vaccine as a Second Booster for COVID-19

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In the USA, two monovalent COVID-19 mRNA vaccines are primarily used for vaccination. One is BNT162b2 (Pfizer-BioNTech, Comirnaty) and the other is mRNA-1273 (Moderna, Spikevax); they are administered in two doses and used in the primary series of the vaccine, as well as being the single dose in their first booster [1]. Both mRNA vaccines were initially approved under emergency use authorization (EUA) and recently received full licensing by the Food and Drug Administration (FDA). Both of the mRNA monovalent vaccines are based on the Wuhan strain (ancestral) spike mRNA. In their primary vaccination series, mRNA-1273 was administered in two doses of 100 µg, while BNT162b2 was administered at 30 µg. Both the vaccines' efficacies were more than 90%, with a durability of 5–6 months after administration [2,3]. The first booster was recommended following the continuing emergence of the SARS-CoV-2 variant after six months of their primary vaccination series. The mRNA-1273 booster was administered as a 50 µg dose (half the initial dose) due to safety concerns, and BNT162b2 was administered as 30 µg. However, the mRNA vaccine effectiveness against the more recent Omicron subvariant BA.4/BA.5 was jeopardized even after getting the first booster [4–6]. In the USA, to identify and track novel SARS-CoV-2 variants, the Centers for Disease Control and Prevention (CDC) uses genomic surveillance data, and it was suggested that on 26 November 2022 that the previous Omicron subvariant BA.5 was quickly displaced by dominant BQ.1, BQ1.1., and BF.7 subvariants (https://covid.cdc.gov/covid-data-tracker/#variant-proportions, accessed on 27 December 2022), thus demonstrating the rapid appearance of new mutations in the SARS-CoV-2 virus. As the immune response through neutralizing antibodies induced by vaccination declines over time and new Omicron subvariants emerge with more effective viral fitness and stronger immunity escape from anti-spike antibody responses, the need for variant-customized updated COVID-19 vaccine boosters is increasingly imminent. It is challenging to predict future COVID-19 vaccines and maintain antibody neutralization potential against circulating variants. Considering this, Omicron-containing bivalent vaccine provides an alternative modern approach to minimizing COVID-19-related hospitalizations and deaths. There are many bivalent vaccines under clinical trial, such as mRNA-1273.211, mRNA-1273.617.2, mRNA-1273.213, mRNA-1273.529, and mRNA-1273.214 (clintrials.gov NCT04927065). The FDA authorized Moderna and Pfizer-BioNTech bivalent COVID-19 vaccines for use as a booster dose in the 5-year old or above age group. The Modena and Pfizer-BioNTech bivalent booster containing a 1:1 ratio of Wuhan strain spike mRNA and omicron strain BA.4/BA.5 is recommended two months after their completion of the primary series or first booster vaccination under the EUA category [1]. The Pfizer-BioNTech bivalent vaccine consists of BNT162b2 and BNT162b2 Omi (30 µg) components that include 15 µg ancestral (Wuhan) spike mRNA and 15 µg BA.1 spike mRNA. A 50 µg bivalent vaccine mRNA-1273.214 (consists of 25 µg each of ancestral Wuhan spike and Omicron BA.1 spike mRNAs) was compared with the previously authorized 50 µg mRNA-1273 for their

check for updates

Citation: Gupta, S.L.; Jaiswal, R.K. An Assessment of the Bivalent Vaccine as a Second Booster for COVID-19. *Vaccines* **2023**, *11*, 79. https://doi.org/10.3390/vaccines11010079

Received: 12 December 2022
Accepted: 28 December 2022
Published: 29 December 2022

second booster dose administration. The objectives phase 2/3 clinical trial aimed to assess the safety, reactogenicity, and immunogenicity of bivalent vaccine mRNA-1273.214 with monovalent mRNA-1273 28 days after their second booster dose. The bivalent omicron-containing mRNA-1273.214 vaccine had an equivalent safety and reactogenicity profile to the monovalent mRNA-1273 booster vaccine. Furthermore, the mRNA-1273.214 vaccine was better at eliciting a neutralizing antibody response against the Omicron BA.1, BA.4, and BA.5 variants, especially if the person had previous infection exposure status [7]. Another study from a group with a similar study also reported that a bivalent vaccine in their phase 2/3 trial, containing an equal amount of the South African Beta (B.1.351) variant, is safe, immunogenic, and has longer antibody durability [8]. One study measured the neutralization titer using a live virus neutralization test and found that the bivalent vaccine containing BA.5 Omicron spike variant illustrated a broadened and improved neutralization potential against the BA.2.75.2 and BQ.1.1 omicron subvariant [9]. Mice data (pre-clinical study) have also suggested that bivalent SARS-CoV-2 mRNA vaccines enhance the breadth of neutralization titers and protection against the BA.5 Omicron variant. This piece of work evaluated the immunogenicity and protective efficacy of two bivalent vaccines recently authorized for use in Europe and the USA, which contained two mRNAs encoding Wuhan-1 and either BA.1 (mRNA-1273.214) or BA.4/5 (mRNA-1273.222) spike proteins. When administered to K18-hACE2 transgenic mice as a booster seven months after the primary vaccination series with mRNA-1273, the bivalent vaccines induced greater breadth and magnitude of neutralizing antibodies than a monovalent mRNA 1273 booster [10]. Another bivalent vaccine comprising Omicron BA.2 and Delta bivalent LNP-mRNA demonstrated a robust antibody response not only for BA.2 but also for BA.2.12.1, BA.2.75, and BA.5 Omicron subvariants. Similarly, the BNT162b2 bivalent BA.4/5 COVID-19 vaccine also showed higher neutralization titers against BA.4.6, BA.2.75.2, BQ.1.1, and XBB.1 subvariant when tested in participants aged >55 years [11]. These data cumulatively support the decision to use these bivalent vaccines for their second booster [12]. On the other hand, two recent independent studies also suggested that antibody neutralization titers against omicron BA.4/BA.5 were similar after getting a bivalent mRNA vaccine booster to the monovalent vaccine [13,14]. One study indicated that when administering a bivalent vaccine (BNT162b2mRNA Wuhan/Omicron BA.4-5 vaccine), healthcare workers observed more adverse side effects than with the monovalent vaccine (BNT162b2mRNA Wuhan). These reports made everyone mindful of the future decision of taking updated bivalent vaccines and raised red flags concerning safety and reactogenicity [15]. Limitations of all the above-mentioned studies include the trial not being randomized, the sample size being too small, the variant sequences causing COVID-19 not being determined, and follow-up studies being scarce, which ultimately means we do not have information about antibody durability and efficacy post-bivalent vaccine administration. None of the trials were designed to evaluate the effectiveness of the bivalent vaccine. Cases are observed when SARS-CoV-2 infection occurred, even after getting a second booster from both monovalent and bivalent vaccines. No bivalent booster has been authorized for younger kids from 6 months to 4 years of age. This single dose of bivalent vaccine is only approved as a booster and not for the primary vaccination series.

In conclusion, from the above-mentioned results, predicted data from future modeling suggested that getting the boosters out as soon as possible could save millions of lives if the world experienced another winter surge. Hence, the FDA authorized the BA.5 bivalent vaccines ahead of their phase 3 clinical trial results to provide quick access to the public domain based on BA.1 data. The primary purpose of the rapid rollout of bivalent vaccines is to reduce severe COVID-19 disease and related hospitalization, especially in the case of an immunocompromised person. However, we still need to carefully assess its safety and immunogenicity profile. Despite this, the question looking to the future remains around bivalent vaccine immunogenicity and effectiveness against the currently dominating omicron variants, such as BQ.1 and BQ1.1. and BF.7 subvariants.

Author Contributions: Conceptualization, R.K.J. and S.L.G.; writing—original draft preparation, R.K.J. and S.L.G.; writing—review and editing, R.K.J., S.L.G. supervision, R.K.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

Long-Term Dynamic Humoral Response to SARS-CoV-2 mRNA Vaccines in Patients on Peritoneal Dialysis

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Abstract: Introduction. Patients on peritoneal dialysis (PD) present an impaired humoral response against SARS-CoV-2, at least after the initial vaccination and booster dose. Until now, the effect of a fourth dose has not been established. The aim of the present study is to evaluate the long-term dynamics of the humoral response of PD patients to multiple doses of SARS-CoV-2 vaccines, focusing on the effect of the fourth dose. Methods. This is an analysis of the prospective and multicentric SENCOVAC study. We included patients on PD without additional immunosuppression that had received at least 3 SARS-CoV-2 mRNA vaccine doses. We evaluated anti-spike antibody titers after the initial vaccination, third and fourth doses, using prespecified fixed assessments (i.e., baseline, 28 days, 3, 6, and 12 months after completing the initial vaccine schedule). Breakthrough infections were also collected. Results. We included 164 patients on PD (69% males, 62 ± 13 years old). In patients who had received only two doses, the rates of positive humoral response progressively decreased from 96% at 28 days to 80% at 6 months, as did with anti-spike antibody titers. At 6 months, 102 (62%) patients had received the third vaccine dose. Patients with the third dose had higher rates of positive humoral response ($p = 0.01$) and higher anti-spike antibody titers ($p < 0.001$) at 6 months than those with only 2 doses. At 12 months, the whole cohort had received 3 vaccine doses, and 44 (27%) patients had an additional fourth dose. The fourth dose was not associated to higher rates of positive humoral response (100 vs. 97%, $p = 0.466$) or to statistically significant differences in anti-spike antibody titers as compared to three doses ($p = 0.371$) at 12 months. Prior antibody titers were the only predictor for subsequent higher anti-spike antibody titer (B 0.53 [95%CI 0.27–0.78], $p < 0.001$). The 2 (1.2%) patients that developed COVID-19 during follow-up had mild disease. Conclusions. PD presents an acceptable humoral response with three doses of SARS-CoV-2 vaccines that improve the progressive loss of anti-spike antibody titers following two vaccine doses.

Keywords: SARS-CoV-2; COVID-19; humoral response; anti-spike antibodies; peritoneal dialysis; chronic kidney disease; booster

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Citation: Quiroga, B.; Soler, M.J.; Ortiz, A.; Gansevoort, R.T.; Leyva, A.; Rojas, J.; de Sequera, P., on behalf of SENCOVAC Collaborative Network. Long-Term Dynamic Humoral Response to SARS-CoV-2 mRNA Vaccines in Patients on Peritoneal Dialysis. *Vaccines* **2022**, *10*, 1738. https://doi.org/10.3390/vaccines10101738

Academic Editors: Rishi Jaiswal, Srijani Basu, Suman Gupta and Sneh Lata Gupta

Received: 12 September 2022
Accepted: 17 October 2022
Published: 18 October 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Patients with chronic kidney disease (CKD) are at high-risk for complications due to coronavirus disease-19 (COVID-19) [1]. Among the CKD spectrum, kidney transplant recipients and dialysis patients present inherent characteristics that make them more vulnerable to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and

condition a worse prognosis [2,3]. Peritoneal dialysis (PD) is a home dialysis therapy that has been one of the preferred techniques for patients requiring renal replacement therapy (RRT), especially for incident patients, during COVID-19 pandemic [4]. In contrast, patients on in-center hemodialysis have presented higher rates of COVID-19 as they are required to attend a healthcare center 3 to 6 times per week and to use healthcare transportation; which may be shared, both factors potentially linked to SARS-CoV-2 transmission [5]. Beyond the first waves of the pandemic, in which mortality rates were high, vaccination and new less lethal variants have changed the paradigm and prognosis of COVID-19 [6–10].

In patients on PD, SARS-CoV-2 vaccination has demonstrated to be safe and to promote early and strong seroconversion at least with the initial schedule (i.e., two doses of mRNA-based vaccines [BNT162b2, mRNA-1273] or ChAdOx1-S or one dose of Ad26.COV.2) [6,11]. However, anti-spike antibody titers in PD decrease over time, as is the case for other RRT patients, potentially leading to suboptimal protection against SARS-CoV-2 [12,13]. In this regard, a booster (or third) dose was considered early for these vulnerable patients. Although evidence on the effectivity of the third dose on patients in PD is scarce, some studies have suggested a robust humoral response rise [14,15]. More importantly, a booster dose has been demonstrated to seroconvert patients that had previously lost their humoral immunity against SARS-CoV-2 [16,17]. Some countries have provided a fourth vaccine dose to some groups of vulnerable or elderly patients. However, we are not aware of assessments of the effects of the fourth dose in patients on PD.

The aim of the present study is to evaluate the long-term humoral response to SARS-CoV-2 mRNA vaccines in patients on PD, focusing on the anti-spike antibody titers after the third and fourth doses.

2. Materials and Methods

2.1. Design

This is an analysis of the SENOVAC study. SENCOVAC is a multicentric, observational and prospective study that aimed to evaluate the safety and humoral response of SARS-CoV-2 vaccines across the CKD spectrum (kidney transplant recipients, patients on hemodialysis, PD and with advanced CKD). In Spain, the choice of the specific type of vaccines in the initial schedule (i.e., BNT162b2 [Pfizer-BioNTech®], mRNA-1273 [Moderna®], ChAdOx1-S [AstraZeneca®] or Ad26.COV.2 [Janssen®]) was made by the national health authority and not by SENCOVAC investigators. In addition, mRNA-based vaccines (i.e., BNT162b2 [Pfizer-BioNTech®], mRNA-1273 [Moderna®]) were used for the third and fourth doses, and again, the specific vaccine used was determined by the Spanish health authority.

2.2. Population

For the present analysis, we included patients on PD that had received at least 3 doses of mRNA-based vaccines. During the follow-up, the humoral response (anti-SARS-Cov-2 spike antibodies) was determined at baseline (before vaccination), 28 days, 3 months, 6 months, and 12 months after completing the initial vaccination schedule of 2 mRNA vaccine doses. We excluded patients with solid organ transplantation, active neoplasia, primary immunodeficiencies, human immunodeficiency virus, and patients who had received immunosuppressive treatment within 6 months before vaccination. Between the 6-month and 12-month assessments, some patients received a fourth dose of vaccine, following health authority policy that was independent of SENCOVAC researchers. For the present analysis, we divided the cohort into two groups depending on the vaccination status (three or four doses) at 12 months. This permitted us to compare humoral responses in patients on PD with three or four doses of SARS-CoV-2 vaccines. In addition, at month 6, some patients had received the third (or booster) dose, allowing us to compare patients with only the initial complete vaccination to patients with the initial complete vaccination plus the third dose at this time point.

2.3. Objectives and Outcomes

The primary objective was to evaluate the humoral response during the 1-year follow-up after SARS-Cov-2 vaccination in patients on PD and the factors associated with the humoral response. Secondary objectives included the analysis of the impact of third and fourth doses on anti-spike antibody titers and the registration of breakthrough COVID-19.

2.4. Variables

At baseline, epidemiological (age and sex) data, etiology of CKD, and dialysis modality were registered. At baseline, 28 days, 3, 6, and 12 months after completing the initial vaccination, serum samples were obtained and sent to a central laboratory where anti-spike antibodies were measured. Anti-spike antibodies were tested by a CE-marked quantitative chemiluminescence immunoassay (CLIA, COVID-19 Spike Quantitative Virclia® IgG Monotest, Vircell SL, Granada, Spain), with a sensitivity and specificity of 96% and 100%, respectively, that detects IgG antibodies against the SARS-CoV-2 spike protein. This assay was calibrated against the First WHO International Standard for anti-SARS-CoV-2 human immunoglobulin (NIBSC code: 20/136), and results were expressed as IU/mL. According to performance studies by the manufacturer, titers $\leq$32 IU/mL were considered negative, between 32 and 36 equivocal and >36 IU/mL positive, reflecting the presence of anti-spike IgG antibodies resulting from previous infection or vaccination. The highest titer that was measurable was 10,000 UI/mL. Thus, a titer of 10,000 UI/mL means 10,000 UI/mL or higher. During the 1-year follow-up, breakthrough SARS-CoV-2 infections were registered. The definition of infection required a positive rt-PCR or antigen test.

2.5. Statistics

Data is expressed as median (interquartile range) or percentage depending on the type of variables. We used the Fisher test and Mann–Whitney test for comparing categorical and continuous variables, respectively. Linear regression analysis was performed to assess variables associated to 12-month anti-spike antibody titers in adjusted models, including confounders. SPSS version 26.0 (IBM Corp., Armonk, NY, USA) was used for statistics and GraphPad Prism version 9.02 (GraphPad Holdings, LLC, CA 92037, USA) for plotting.

3. Results

3.1. Baseline Characteristics

We included 164 patients on PD (69% males, 62 $\pm$ 13 years old). Eighty-five patients (52%) were on continuous ambulatory peritoneal dialysis (CAPD) and 79 (48%) on automated peritoneal dialysis. Regarding the etiology of CKD, 44 (27%) had a glomerular disease, 31 (19%) diabetic kidney disease, 28 (17%) nephroangiosclerosis, 24 (15%) unknown disease, 18 (11%) autosomal polycystic kidney disease, 10 (6%) interstitial nephritis, and 9 (5%) others (Supplementary Table S1).

3.2. Humoral Response after the Initial Vaccination Schedule

Initial vaccination was performed using mRNA-based vaccines, 123 (75%) patients received mRNA-1273, and 41 (25%) received BNT162b2.

Humoral response varied during the follow up. At baseline, 15 (15%) of the 102 tested patients presented positive humoral responses because of previous SARS-CoV-2 infection. Twenty-eight days after complete vaccination, 138 (96%) of the 143 tested patients presented positive humoral responses (Figure 1). Among the 81 patients without a humoral response at baseline, 78 (96%) seroconverted after vaccination. Anti-spike antibodies were significantly higher one month after vaccination than at baseline (4 [2–7] UI/mL vs. 2109 [775–5982] UI/mL, $p < 0.001$) (Figure 2).

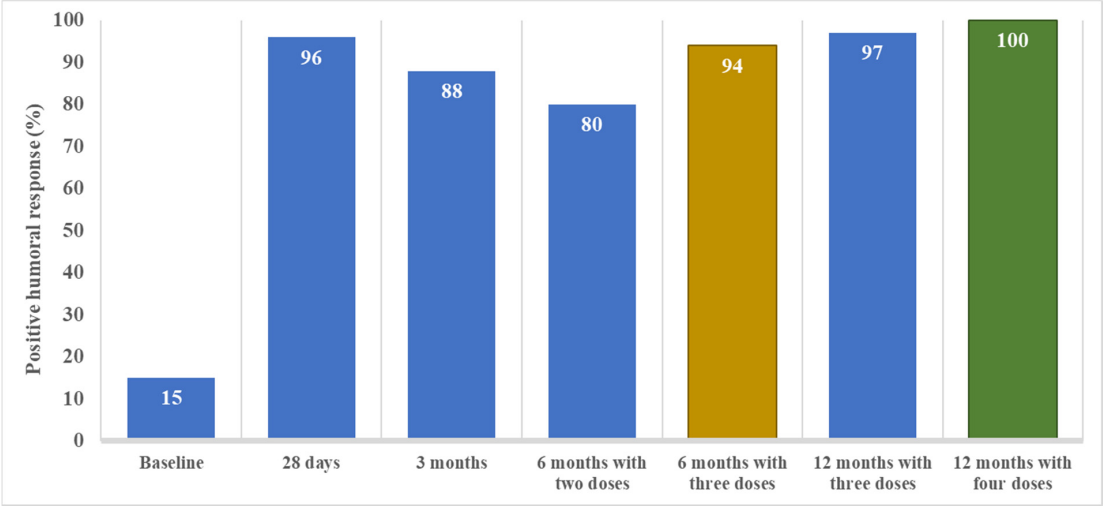

Figure 1. The positive humoral response over time from baseline (prior to SARS-CoV-2 vaccination) to 12 months after completing the initial vaccine schedule (i.e., 2 doses of mRNA vaccine) in CKD patients on peritoneal dialysis. A positive humoral response was defined as anti-spike antibody titers above 36 UI/mL. Note that samples obtained at 6 and at 12 months following the initial vaccine schedule of two doses of mRNA-based vaccines are divided into patients that had received 2 (just the initial schedule) or 3 doses (initial schedule plus one booster dose) at the 6-month timepoint or 3 or 4 doses (12-month timepoint).

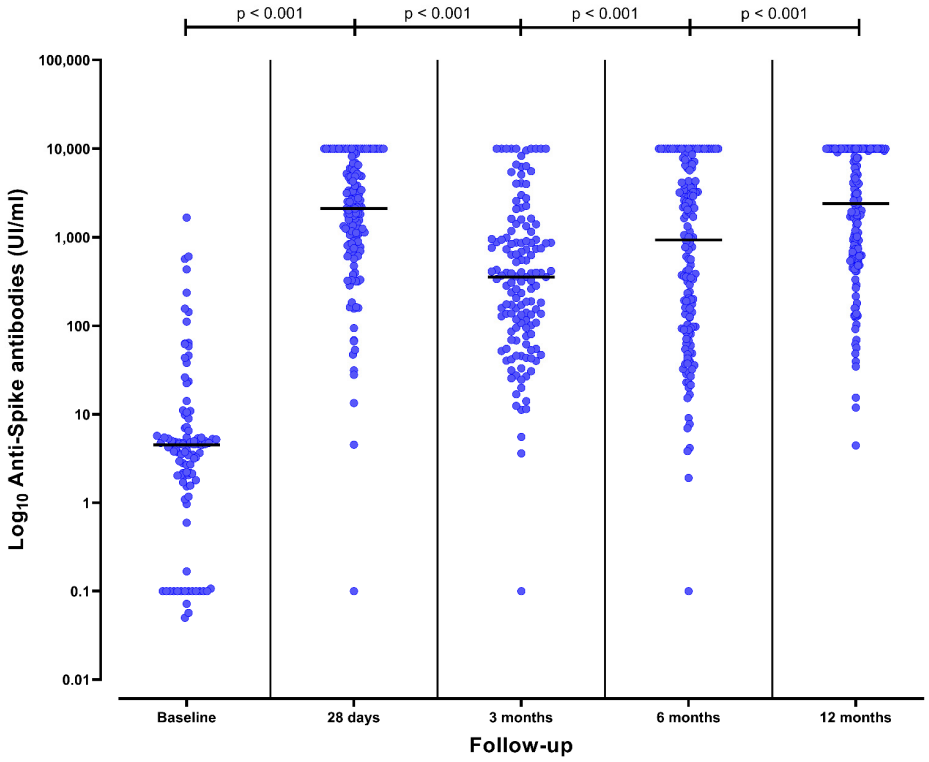

Figure 2. Anti-spike antibody titers over time from baseline (prior to SARS-CoV-2 vaccination) to 12 months after completing the initial vaccine schedule (i.e., 2 doses of mRNA vaccine) in CKD patients on peritoneal dialysis. The black line represents the median value of each strata.

Three months after vaccination, 123 (88%) of the 139 tested patients presented a positive humoral response (Figure 1). Among the 123 patients with previous positive humoral response, 10 (8%) had a negative humoral response at 3 months and one (1%)

had an equivocal response. Anti-spike antibodies significantly decreased at 3 months in comparison to one-month titers (356 [95–992] UI/mL vs. 2109 [775–5982] UI/mL, $p < 0.001$) (Figure 2).

In the absence of a booster (third dose), a positive humoral response was present at 6 months in 43 (80%) of the 54 patients who at this timepoint had only received the initial two vaccine doses ($p = 0.040$ vs. 3 months). The anti-spike antibodies significantly decreased at 6 months in comparison to 3-month titers (100 [37–365] UI/mL vs. 356 [95–992] UI/mL, $p < 0.001$). During the initial 6-months of follow-up, patients with the absence of a booster presented a decreased in anti-spike antibodies (p for trend < 0.001).

3.3. Humoral Response after the Third Dose at 6 Months

Among the 164 patients that received the third dose, 133 (81%) received mRNA-1273 and 31 (19%) BNT162b2.

At 6 months, 139 (89%) of the 157 tested patients presented a positive humoral response (Figure 1). At this time point, 102 (62%) patients had received the third dose. Positive humoral response at 6 months was achieved more frequently in patients that had received the third dose than in a dose with only two doses (96/102 vs. 43/54, $p = 0.01$). Sixteen patients (10%) and 2 patients (1%) presented a negative and uncertain humoral response, respectively, at 6 months. Among negative patients, 12/16 (75%) had not received the third vaccine dose before the 6-month assessment. In contrast, the two patients with uncertain humoral response had received the third dose.

Anti-spike antibody titers significantly increased at 6 months in comparison to 3 months (931 [96–6035] UI/mL vs. 356 [95–992] UI/mL, $p < 0.001$) (Figure 2). Patients that had received a third dose had higher anti-spike antibody titers at 6 months than patients with only two doses (2499 [564–8657] UI/mL vs. 100 [37–365] UI/mL, $p < 0.001$) (Figure 3). The median time elapsed between the third dose and the 6-month assessment was 32 (20–51) days.

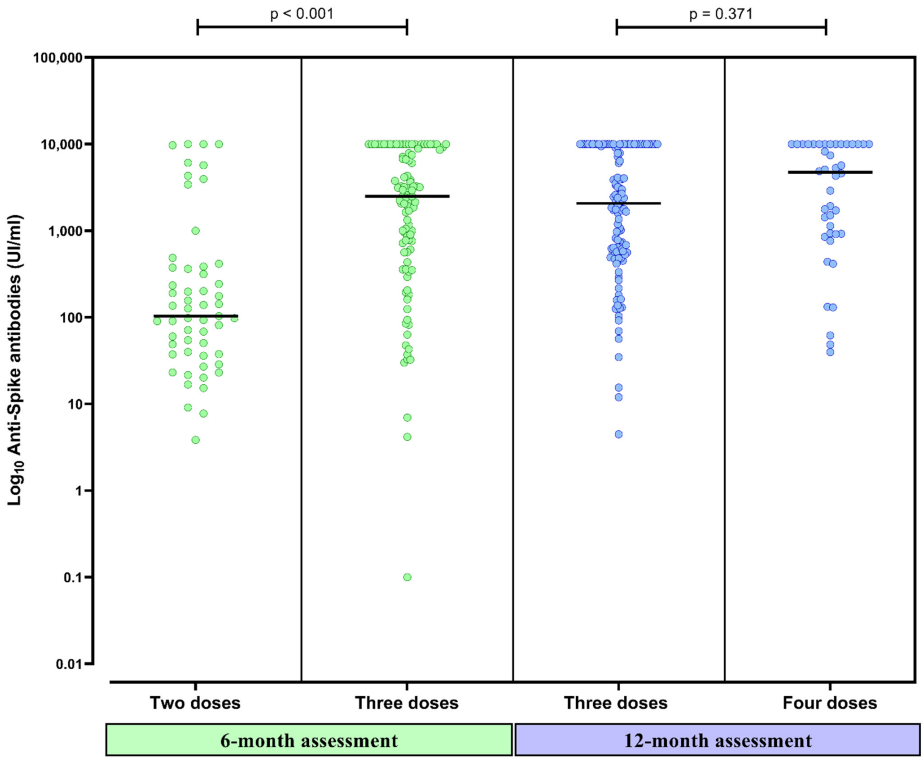

Figure 3. Anti-spike antibody titers 6 months after completing the initial vaccine schedule in CKD patients on peritoneal dialysis who had received two and three doses of SARS-CoV-2 vaccines and titers 12 months after completing the initial vaccine schedule in patients with three and four doses. The black line represents the median value of each strata.

3.4. Humoral Response after the Fourth Dose at 6 Months

Forty-four patients (27%) had received a fourth dose (31 [70%] mRNA-1273 and 23 [30%] BNT162b2). At 12 months, 160 (98%) of the 164 tested patients presented a positive humoral response (Figure 1). Having received the fourth dose did not significantly increase the already high rate of positive humoral response (44/44 vs. 114/118, $p = 0.466$). Three patients presented a negative humoral response and one presented an uncertain response. None of them had received the fourth dose. Anti-spike antibodies significantly increased at 12 months in comparison to 6-month titers (2391 [614–10,000] UI/mL vs. 931 [96–6035] UI/mL, $p < 0.001$) (Figure 2). Patients that had received the fourth dose had higher anti-spike antibody titers in comparison to those having received three doses, although these differences were not statistically significative (4933 [919–10,000] UI/mL vs. 2065 [560–10,000] UI/mL, $p = 0.371$) (Figure 3). The median time elapsed between the fourth dose and the 12-month assessment was 64 (27–80) days.

3.5. Factors Associated with Stronger Humoral Response at 12 Months

In a model adjusted for age, sex, type of mRNA vaccine, and having received the fourth dose, anti-spike antibody titers at 6 months were the only independent factor associated with a stronger humoral response at 12 months (Table 1). There was a lack of association between anti-spike antibody titers and the etiology of CKD, PD technique, or treatment with renin–angiotensin–aldosterone system inhibitors and erythropoietic stimulant agents.

Table 1. Multivariate analysis using adjusted linear regression for factors associated with higher anti-spike antibody titers at 12 months.

	B (95%CI)	*p*
Age (years)	44 (−52, 141)	0.913
Gender (male)	1726 (−519, 3972)	0.129
mRNA-based vaccine (mRNA-1273)	−914 (−3054, 1225)	0.394
Fourth dose (yes)	2086 (−386, 4560)	0.096
Anti-Spike antibody titers at 6 months (per UI/mL)	0.530 (0.274, 0.786)	<0.001

3.6. Breakthrough Infections

Only two patients (1.2%) presented a SARS-CoV-2 infection during follow-up. The clinical presentation was mild. One patient had not received the third vaccine dose at the time of COVID-19 and the other developed COVID-19 before the fourth dose (Table 2). Both had last known anti-spike antibody titers below 5000 UI/mL.

Table 2. Characteristics of patients that developed COVID-19 during follow-up.

Patient	Timing of COVID-19	Initial SARS-CoV-2 Vaccine	Third SARS-CoV-2 Vaccine Dose	Last Anti-Spike Titers Prior to COVID-19 (mUI/L)	Infection Severity	Anti-Spike Titers Post-COVID-19 (mUI/L)
1	Before 3rd dose	BNT162b2	–	1065	Mild symptoms without admission	6599
2	Before 4th dose	mRNA-1273	mRNA-1273	3135	Mild symptoms without admission	10,000

4. Discussion

The key finding of this analysis of the SENCOVAC study is that patients on PD achieve high rates of positive anti-spike humoral response after the third dose of mRNA-based SARS-CoV-2 vaccines that reverse the progressive decrease of anti-spike antibodies over time observed following two doses. A fourth dose may further increase the humoral response in some patients and result in higher rates of patients with high antibody titers. Patients on PD belong to the especially vulnerable group of kidney failure patients; thus the objective of vaccination against SARS-CoV-2 should include not only achieving a positive humoral response but also high and long-lasting anti-spike antibody titers, as this has been related to lower rates of breakthrough infections and better prognosis [18].

Although there is scarce information on the effectivity of a fourth SARS-CoV-2 vaccine dose in patients on PD, real-world data, including hemodialysis patients, shows a positive effect on the humoral response, even against the Omicron variant [19]. In this regard, repetitive immunological hits (i.e., breakthrough infections and multiple vaccine doses) improve protection against the most recent SARS-CoV-2 variants that now predominate [20]. From the epidemiological point of view, it is prudent to estimate the lower efficacy of first-generation vaccines against novel and future SARS-CoV-2 variants that may more negatively impact vulnerable patients such as CKD patients on RRT [21]. To address this enhanced risk of infection and complications, a multi-vaccine strategy may reduce the rates of suboptimal responses, especially in immunosuppressed patients [21]. However, a recent study of a hemodialysis cohort showed that booster doses were beneficial in virus-naive patients but not in SARS-CoV-2–recovered patients, at least in terms of cellular immunity [22]. Therefore, efforts should be directed at maintaining an optimal immune response, especially in patients who have not been infected or with lower antibody titers, as neutralizing antibody titers are related to higher protection [18]. In this regard, there is insufficient information regarding the optimal cut-off point for anti-spike antibody titers that provide optimal protection against COVID-19 and against severe complicated COVID-19. In our study, only two infections were detected, and both were mild and did not need hospital admission. Both the immune response to vaccines and the successful limitation of exposure to the virus may have contributed to this observation [23].

The progressive decrease in antibody titers over time after each vaccination dose is a cause for concern. Previous reports have shown that 3 to 6 months after initial vaccination and booster doses, significant anti-spike titer decline is observed in dialysis patients [12,24]. At present, individualizing additional dose prescriptions is probably the best strategy to obtain an optimal immune response, avoiding the risk of hyperstimulation and immune exhaustion [25,26]. Vulnerable patients with lower antibody titers or negative humoral response are the highest risk group. Some factors have been proposed to predict an early decline in antibody titers or a lower immediate humoral response. In agreement with our data, the previous humoral response is probably the better predictor of response to successive booster vaccine doses. Additionally, factors such as immunosuppressive drugs or conditions, obesity, older age, absence of previous or breakthrough COVID-19 or lower vaccine dose (number of doses or type of vaccine) can predict a suboptimal immunological response in CKD patients on RRT [6,8–10,27]. Thus, these factors should be considered when assessing the risks of not receiving early additional booster vaccine doses.

Our study presents some limitations to be acknowledged. First, the inherent bias of observational studies. However, performing a randomized clinical trial with a placebo group is ethically unacceptable. In addition, the evidence provided by our study can be extrapolated to clinical practice as it is based on real-world data, and both the timing and type of vaccines were determined by a stakeholder external to the study, the health authorities. Second, the low rate of breakthrough infections does not allow an assessment of the efficiency of SARS-CoV-2 vaccines in PD. This is probably due to the sample size but also due to the dynamic changes of the pandemic (new variants, vaccine protocols) that have attenuated its effects. Indeed, some asymptomatic cases can go unnoticed, as periodic SARS-CoV-2 screening was not performed in ambulatory patients such as those on PD.

This may have underestimated the infection rate, but it is unlikely to have missed severe COVID-19. Third, the observational design of the study may have resulted in missing data.

In conclusion, patients on PD present an acceptable humoral response with three doses of SARS-CoV-2 vaccines that improve on the natural history of progressive loss of anti-spike antibody titers following two vaccine doses. Adding a fourth vaccine dose did not significantly improve the already high positive humoral response rate. However, there was some suggestion of a stronger immune response in terms of anti-spike antibody titers, and all patients that received the fourth dose developed a positive humoral response. Thus, assessment of anti-spike antibody titers may identify vulnerable patients, such as PD patients, that may derive the most benefit from further booster vaccine doses, leading to individualized booster prescriptions according to immunological background and risk for complications.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/vaccines10101738/s1, Table S1: Baseline characteristics of the participants included in each humoral response assessment.

Author Contributions: Research idea and study design: B.Q., M.J.S., A.O., R.T.G. and P.d.S.; data acquisition: B.Q., M.J.S., A.O., P.d.S. and SENCOVAC collaborative group; data analysis/interpretation: B.Q., M.J.S., A.O., A.L., J.R. and P.d.S.; statistical analysis: B.Q., M.J.S., A.O. and P.d.S.; supervision or mentorship: B.Q., M.J.S., A.O., R.T.G. and P.d.S. All authors have read and agreed to the published version of the manuscript.

Funding: The present project has been supported by Diaverum, Vifor Pharma, Vircell, Fundación Renal Iñigo Álvarez de Toledo, and ISCIII FEDER funds RICORS2040 (RD21/0005).

Institutional Review Board Statement: The study was approved by the Ethical Committee of Fundación Instituto de Investigación Sanitaria de la Fundación Jiménez Díaz (February 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data available on request due to restrictions.

Acknowledgments: We thank all the involved centers and healthcare workers, especially the nurse team, for their implication in this project. In addition, we want to thank the support of the Sociedad Española de Enfermería Nefrológica (SEDEN), Organización Nacional de Trasplantes (ONT), and Sociedad Española de Trasplante (SET).

Conflicts of Interest: B. Quiroga has received honoraria for conferences, consulting fees, and advisory boards from Vifor-Pharma, Astellas, Amgen, Bial, Ferrer, Novartis, AstraZeneca, Sandoz, Laboratorios Bial, Esteve, Sanofi-Genzyme, Otsuka. M.J. Soler reports honorarium for conferences, consulting fees, and advisory boards from Astra Zeneca, NovoNordsik, Esteve, Vifor, Bayer, Mundipharma, Ingelheim Lilly, Jansen, ICU Medical, and Boehringer. A. Ortiz has received consultancy or speaker fees or travel support from Astellas, Astrazeneca, Amicus, Amgen, Fresenius Medical Care, Bayer, Sanofi-Genzyme, Menarini, Kyowa Kirin, Alexion, Otsuka and Vifor Fresenius Medical Care Renal Pharma and is Director of the Catedra Mundipharma-UAM of diabetic kidney disease and the Catedra Astrazeneca-UAM of chronic kidney disease and electrolytes. A. Leyva, J. Rojas, R. T. Gansevoort do not present conflict of interests. P. de Sequera reports honorarium for conferences, consulting fees, and advisory boards from Amgen, Astellas, Astra Zeneca, Baxter, Braun, Fresenius Medical Care, GlaxoSmithKline, Nipro, Otsuka, Sandoz, Nipro, Vifor-Pharma. She is the present president of the Spanish Society of Nephrology (S.E.N.).

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Relationship between Humoral Response in COVID-19 and Seasonal Influenza Vaccination

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Abstract: There is evidence that vaccination against seasonal influenza can improve innate immune responses to COVID-19 and decrease disease severity. However, less is known about whether it could also impact the humoral immunity in SARS-CoV-2 infected patients. The present study aimed to compare the SARS-CoV-2 specific humoral responses (IgG antibodies against nucleocapsid; anti-N, receptor binding domain; anti-RBD, subunit S2; anti-S2, and envelope protein; anti-E) between non-hospitalized, COVID-19 unvaccinated, and mild COVID-19 convalescent patients who were and were not vaccinated against influenza during the 2019/2020 epidemic season (n = 489 and n = 292, respectively). The influenza-vaccinated group had significantly higher frequency and titers of anti-N antibodies (75 vs. 66%; mean 559 vs. 520 U/mL) and anti-RBD antibodies (85 vs. 76%; mean 580 vs. 540 U/mL). The prevalence and concentrations of anti-S2 and anti-E antibodies did not differ between groups (40–43%; mean 370–375 U/mL and 1.4–1.7%; mean 261–294 U/mL) and were significantly lower compared to those of anti-RBD and anti-N. In both groups, age, comorbidities, and gender did not affect the prevalence and concentrations of studied antibodies. The results indicate that influenza vaccination can improve serum antibody levels produced in response to SARS-CoV-2 infection.

Keywords: heterologous protection; trained immunity; adaptive immunity; immunology; SARS-CoV-2; pandemic

Citation: Poniedziałek, B.; Hallmann, E.; Sikora, D.; Szymański, K.; Kondratiuk, K.; Żurawski, J.; Rzymski, P.; Brydak, L. Relationship between Humoral Response in COVID-19 and Seasonal Influenza Vaccination. *Vaccines* **2022**, *10*, 1621. https://doi.org/10.3390/vaccines10101621

Academic Editors: Rishi Jaiswal, Srijani Basu, Suman Gupta and Sneh Lata Gupta

Received: 5 September 2022
Accepted: 25 September 2022
Published: 27 September 2022

1. Introduction

A broad range of factors can affect the host immune response to viral infection, including the pathogen's immunogenicity, the disease's clinical course, human age, sex, and health status [1–3]. During the pandemic of coronavirus disease 2019 (COVID-19), increasing attention has been given to the cross-protective effects of different vaccinations. As demonstrated by selected epidemiological studies, individuals vaccinated against influenza had lower odds of SARS-CoV-2 infection, hospitalization, need for mechanical ventilation, and death due to COVID-19 [4–6]. The data also demonstrate that the bacillus Calmette−Guérin (BCG) vaccine against tuberculosis can confer protection against other infectious diseases, including influenza staphylococci and yellow fever [7–9]. This phenomenon has been attributed to the so-called "trained immunity", a process of epigenetic reprogramming of transcriptional pathways induced by infections and vaccinations that ultimately allows the innate immune system to exhibit adaptive characteristics [10,11].

However, there is also initial evidence that previous vaccinations against other respiratory diseases could improve the humoral response to the COVID-19 vaccine. In one study, individuals receiving concomitant influenza and pneumococcal or only influenza

vaccination revealed significantly increased micro-neutralization titers after administration of the BNT162b2 vaccine (BioNTech/Pfizer, Germany, Mainz/New York, NY, USA) compared to those not vaccinated against influenza/pneumococcal disease [12]. Another study recently confirmed this finding, demonstrating higher titers of antibodies against the SARS-CoV-2 receptor binding domain following BNT162b2 vaccination in healthcare workers who previously received the seasonal influenza vaccine [13]. The exact molecular mechanisms behind this effect are yet to be elucidated.

The first investigations of the humoral response to hemagglutinins of the influenza virus during the COVID-19 pandemic [14] provided the passage for further studies evaluating whether vaccination against seasonal influenza could also impact the humoral immunity in SARS-CoV-2 infected patients is less known. Therefore, the present study aimed to compare the SARS-CoV-2 specific humoral responses between non-hospitalized, COVID-19 unvaccinated, and mild COVID-19 convalescent patients who were and were not previously vaccinated against influenza during the 2019/2020 epidemic season. To this end, the prevalence and concentrations of four IgG antibodies specific to SARS-CoV-2 were evaluated in both groups.

2. Materials and Methods

2.1. Patients and Serum Samples

All serum samples were purchased in 2020 from the Regional Blood Donation and Blood Treatment Centers in Poland from units located in 8 voivodeships in the following cities: Białystok, Warsaw, Radom, Racibórz, Kalisz, Bydgoszcz, Łódź, Szczecin, and Wrocław. All samples were collected between September and December 2020 from SARS-CoV-2 infected patients (confirmed by RT-PCR) 1 month (+/− 2 weeks) after the resolution of symptoms/end of the isolation period. This period was dominated by infections with Nextstrain clades 20A, 20B, and 20C [15], which did not reveal major differences in clinical outcomes [16,17]. In total, we purchased 659 serum samples from individuals vaccinated against influenza during 2019/2020 epidemic season and 659 serum samples from unvaccinated persons. All influenza-vaccinated individuals received the vaccine in the recommended period between September and December 2019, approximately one year prior to infection with SARS-CoV-2. The patient's age, gender, comorbidities (present or not), and COVID-19 severity were collected for all samples. The frozen samples were transported frozen to the Department of Influenza Research, National Influenza Centre in National Institute of Public Health—National Research Institute. The research project was approved by the Bioethical Committee of the Institute of Public Health—National Research Institute (approval no. 4/2020; date of approval: 6 August 2020) and the Bioethics Committee at Poznan University of Medical Sciences (approval no. 429/22; date of approval: 11 May 2022). Considering that severity of SARS-CoV-2 infection can significantly influence the humoral responses [18,19], individuals who underwent mild COVID-19, not requiring hospitalization, were selected for this analysis. In total, 781 sera samples were analyzed, with 292 originating from individuals not vaccinated against influenza and 489 from those vaccinated in the 2019/2020 epidemic season. As the samples originated from 2020, all individuals were not vaccinated against COVID-19.

2.2. Determination of Anti-SARS-CoV-2-Specific IgG Antibodies

The collected serum samples were tested using the CE-IVD certified Microblot-Array COVID-19 IgG assay (TestLine Clinical Diagnostics, Brno, Czech Republic) for the presence and titer of the specific SARS-CoV-2 IgG antibodies against the receptor binding domain of the spike protein (anti-RBD), S2 subunit of the spike protein (anti-S2), nucleocapsid protein (anti-N), and envelope protein (anti-E). In this assay, recombinant and purified native antigens are immobilized on specific spots of nitrocellulose membrane fixed at the bottom of the microplate well [20]. The concentrations for all four antibodies were given as U/mL and interpreted as positive if above 210 U/mL.

2.3. Statistical Analyses

Data were analyzed with Statistica v.13.3 (StatSoft Inc., Tulsa, OK, USA). Because no assumption of Gaussian distribution was met (Shapiro–Wilk's test, $p < 0.05$), a non-parametric Mann–Whitney U test was employed to compare groups vaccinated and unvaccinated against influenza. Comparison of titers of different antibodies was performed with Kruskal–Wallis ANOVA using Dunn's test as a post hoc method. Spearman's rank coefficient was used to assess the relationship between concentrations of different antibodies. The prevalence of antibodies in influenza vaccinated and unvaccinated were compared with Pearson's χ^2 test. When $p < 0.05$, differences were deemed statistically significant.

3. Results

3.1. Demographic Characteristics

Serum samples collected from 781 mild COVID-19 convalescent patients were analyzed, among whom 62.6% were vaccinated against influenza in the 2019/2020 infection season. Groups of patients vaccinated and unvaccinated against influenza did not differ in age and gender, but the former was represented by a higher frequency of comorbidities (Table 1).

Table 1. The demographic characteristics of the studied groups of COVID-19 convalescent patients.

Parameter	Unvaccinated against Influenza (n = 292)	Vaccinated against Influenza (n = 489)	p-Value
Age (years), mean ± SD	35.8 ± 8.5	37.0 ± 10.3	>0.05
≥50 years, % (n)	5.1 (15)	11.9 (58)	0.002
Women/men, % (n)	17.1 (50)/82.9 (242)	23.3 (114)/76.7 (375)	>0.05
Comorbidities, % (n)	1.7 (5)	5.1 (25)	0.02

3.2. Prevalence of SARS-CoV-2-Specific IgG Antibodies

The prevalence of anti-N, anti-RBD, anti-S2, and anti-E IgG antibodies in the studied cohort was 71.3, 81.6, 41.7, and 1.5%, respectively (Table 2). In general, 12.7% had undetectable levels of any of the considered antibodies, 15.7% tested positive for one, 35.7% for two, 34.4% for three, and 1.4% for all four. Group vaccinated against influenza in the 2019/2020 season revealed a higher prevalence of anti-N (by 8.8%) and anti-RBD (by 8.4%) antibodies compared to those who did not receive such vaccination (Table 2). In both groups, the prevalence of any antibody was not differentiated by age ≥ 50 years, comorbidities ($p > 0.05$ in all cases, Pearson's χ^2 test), or between women and men ($p > 0.05$ in all cases, Mann–Whitney U test).

Table 2. The frequencies (%) of IgG antibodies against SARS-CoV-2 nucleocapsid protein (anti-N), receptor binding domain of spike protein (anti-RBD), subunit S2 of spike protein (anti-S2), and envelope protein (anti-E) in mild COVID-19 convalescent individuals not vaccinated and vaccinated against seasonal influenza. The p-value refers to difference between these groups examined with Pearson's χ^2 test.

IgG Antibodies	Unvaccinated against Influenza (n = 292)	Vaccinated against Influenza (n = 489)	p-Value	Total (n = 781)
anti-N	65.8	74.6	0.008	71.3
anti-RBD	76.7	85.1	0.001	81.6
anti-S2	39.7	42.9	>0.05	41.7
anti-E	1.7	1.4	>0.05	1.5

3.3. Titers of SARS-CoV-2-Specific IgG Antibodies

Generally, the serum concentrations of anti-N, anti-RBD, anti-S2, and anti-E IgG antibodies (mean ± SD) in all studied patients who tested positive for their presence were 545.8 ± 212.6, 566.0 ± 217.7, 373.2 ± 165.3, and 280.3 ± 78.8 U/mL, respectively. Group vaccinated against seasonal influenza revealed significantly higher concentrations of anti-N and anti-RBD antibodies than those who did not receive the influenza vaccine; the difference in means was 39.5 (7.6%) and 40.0 (7.4%) U/mL, respectively (Figure 1). Within both subgroups, titers of anti-N and anti-RBD antibodies were higher than that of anti-S2 and anti-E (Figure 1).

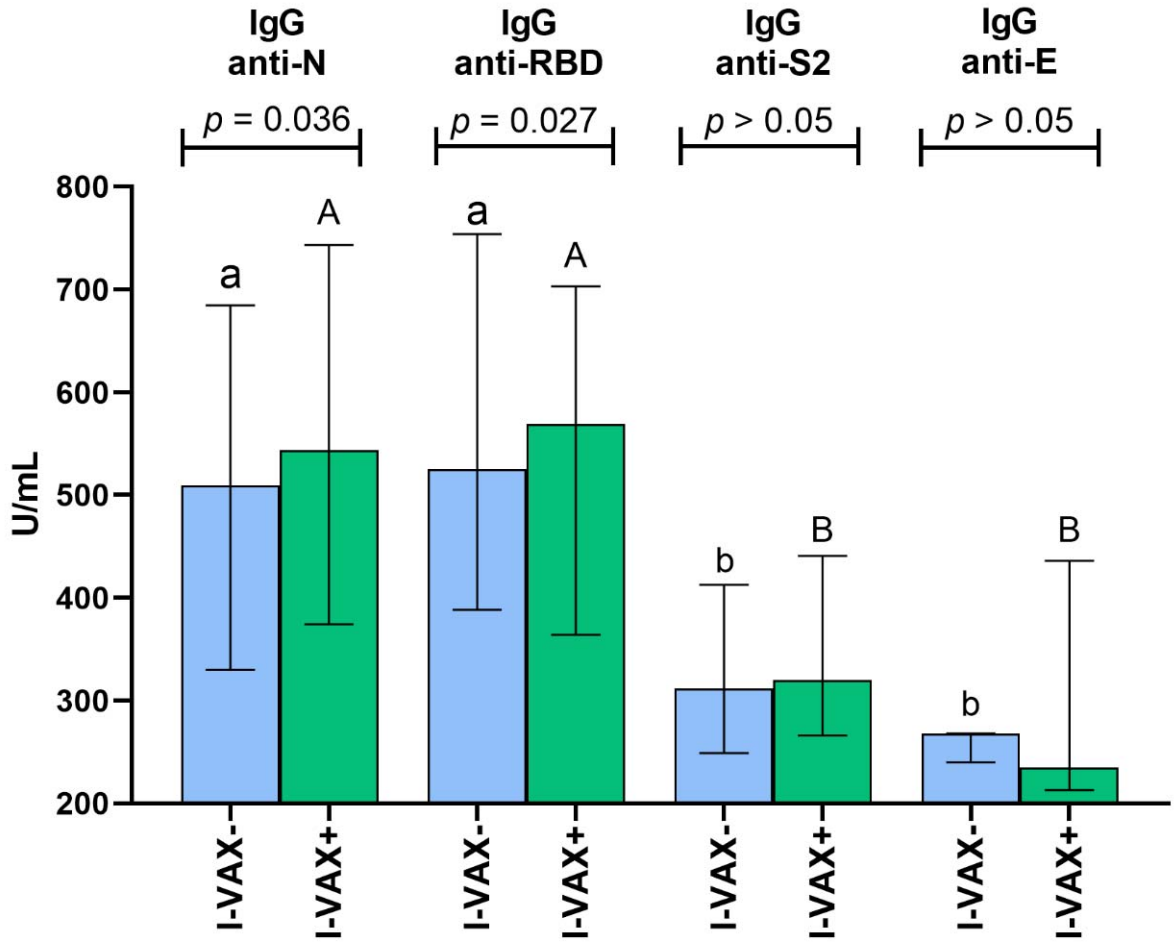

Figure 1. Serum titers (median and interquartile range) of IgG antibodies against SARS-CoV-2 nucleocapsid protein (anti-N), receptor binding domain of spike protein (anti-RBD), subunit S2 of spike protein (anti-S2), and envelope protein (anti-E) in mild COVID-19 convalescent individuals not vaccinated (I-VAX-) and vaccinated (I-VAX+) against seasonal influenza. The *p*-value refers to the difference between these groups examined with the Mann–Whitney U test. Different small letters (a, b) above bars indicate a significant difference between antibody concentrations within the I-VAX- group, while different capital letters (A, B) indicate it within the I-VAX+ group (Kruskal–Wallis ANOVA with Dunn's post hoc test).

In both groups, serum concentration of any antibody was not differentiated by age ≥ 50 years or comorbidities and did not differ between women and men ($p > 0.05$ in all cases, Mann–Whitney U test). The serum concentrations of anti-N were significantly correlated with anti-RBD and anti-S2 titers in both groups. Additionally, in individuals vaccinated against seasonal influenza, anti-RBD and anti-S2 concentrations were positively associated (Table 3).

Table 3. Relationship (given as Spearman's rank correlation coefficient) between serum concentrations of IgG antibodies against SARS-CoV-2 nucleocapsid protein (anti-N), receptor binding domain of spike protein (anti-RBD), subunit S2 of spike protein (anti-S2), and envelope protein (anti-E) in mild COVID-19 convalescent individuals not vaccinated and vaccinated against seasonal influenza.

IgG Antibodies	Unvaccinated against Influenza (n = 292)				Vaccinated against Influenza (n = 489)			
	anti-N	**anti-RBD**	**anti-S2**	**anti-E**	**anti-N**	**anti-RBD**	**anti-S2**	**anti-E**
anti-N	-	0.56 **$p < 0.05$**	0.24 **$p < 0.05$**	0.15 $p > 0.05$	-	0.38 **$p < 0.05$**	0.21 **$p < 0.05$**	0.14 $p > 0.05$
anti-RBD	-	-	0.19 $p > 0.05$	0.67 $p > 0.05$	-	-	0.38 **$p < 0.05$**	0.32 $p > 0.05$
anti-S2	-	-	-	0.32 $p > 0.05$	-	-	-	0.04 $p > 0.05$
anti-E	-	-	-	-	-	-	-	-

4. Discussion

The present study demonstrated some beneficial relationship between seasonal influenza vaccination and humoral response in SARS-CoV-2 infection. Individuals who received the influenza vaccine during the 2019/2020 epidemic season revealed higher frequency and titers of anti-N and anti-RBD IgG antibodies. The increased levels of these antibodies can translate into better protection against reinfection or exert neutralization effects if the virus still replicates in tissues [21]. Although age, gender, and comorbidities were previously observed as potential factors influencing humoral responses in COVID-19 [22–25], this was not the case in the present cohort of patients who underwent mild disease. These findings add to the body of knowledge on the positive effects of influenza vaccination in COVID-19 [4–6,26,27].

Our results suggest that influenza vaccination may increase the strength of the adaptive response to other viral infections. Although the mechanisms behind this phenomenon are not known, it can be speculated that vaccination positively affects the production of interleukin-4 by T-helper 2 cells, leading to better clonal expansion of B cells and/or interleukin-5 and interleukin-6, which contribute to later phases of B-cell activation by driving their differentiation and supporting antibody production [28]. Moreover, it is suggested that influenza vaccination may induce innate immune training in myeloid cells by altering cytokine production through epigenetic changes [29–31]. It is plausible that such trained myeloid cells may also support humoral responses during SARS-CoV-2 infection. Further investigations are required to understand better the exact nature of immunological events in play and their role in the cross-protective effects of influenza vaccination against heterologous infection.

Compared to anti-RBD IgG antibodies, anti-N were less prevalent in the studied cohort (by 10.3%), as well as in subsets of individuals vaccinated (by 10.5%) and unvaccinated (by 10.9%) against seasonal influenza. This is in line with other studies, which also reported a lower prevalence of anti-N IgG antibodies compared to anti-RBD [32,33]. This is due to the different dynamics of these antibodies, from which anti-N are detected earlier and have a significantly lower half-life [33,34]. Moreover, a lower prevalence of anti-N antibodies is likely also due to the location of nucleocapsid protein inside the lipid bilayer envelope, which can blunt its recognition by immune cells [35,36]. In turn, less than 50% of analyzed serum samples were positive for anti-S2 IgG antibodies. Experimental vaccine research revealed that the S2 subunit of SARS-CoV-2 S protein, which has distinct domains involved in mediating viral fusion of viral envelope, can be similarly immunogenic as S1, which contains RBD and the N-terminal domain [37]. However, these observations relate to the immunogenicity comparison of different subunit vaccine candidates, whereas in the case of the virion, S2 is much less accessible for immune cell recognition and contains

a lower number of predicted epitopes than S1 [38]. Similarly to our observations, other studies also reported a low prevalence of anti-S2 IgG antibodies. For example, an Italian serological study found that the prevalence of anti-S2 IgG antibodies in SARS-CoV-2 infected patients was 42% compared to 87% for anti-S1 and 93% for anti-RBD [39]. Notably, the S2 subunit is more conserved among coronaviruses than S1 [40], while anti-S2 antibodies can harbor Fc-dependent effect function [41] and reveal pan-betacoronavirus neutralization potencies [42–44]. Therefore, their presence can enhance the host's antiviral humoral immunity. In our study, the prevalence of anti-S2 Igg antibodies in individuals vaccinated against influenza was only slightly and statistically insignificantly higher compared to unvaccinated patients (by 3.2%), while serum concentrations in both groups were similar. However, in the former subset of subjects, the anti-S2 titers were positively correlated with those of anti-RBD. Although the exact nature of this relationship remains unclear, it may suggest that vaccination against influenza could enhance the simultaneous recognition of S2 and RBD in some individuals.

We also found that influenza vaccination was not associated with a more frequent presence or higher serum levels of anti-E IgG antibodies. Moreover, these antibodies were very rare in the studied cohort, and their concentration was significantly lower than that of anti-N and anti-RBD. Other serological research also observed a very low or zero prevalence of anti-E IgG antibodies [20,45]. The envelope protein is the smallest structural protein of SARS-CoV-2 (length 75 amino acids) and has a low protrusion of its ectodomains that could be recognized as epitopes [35,46]. Although it is abundantly expressed inside the infected cell, only a small portion is incorporated into the virion envelope [47,48].

Our study has some limitations. Firstly, serum samples were collected before the emergence of SARS-CoV-2 variants of concern, such as Alpha, Delta, and Omicron, which may differ in clinical severity and antigenicity [17,49]. Secondly, due to the unavailability of data, the study did not include some patient characteristics, which may also influence humoral responses, e.g., body mass index, specific comorbidities, or the use of medications (prior to and during the SARS-CoV-2 infection). However, one should note that the studied individuals underwent mild COVID-19 and did not require hospitalization. Thus, it is unlikely they were ordered any specific anti-SARS-CoV-2 treatment that could affect humoral responses (e.g., glucocorticoid), as such treatment was not recommended at the time of our study (September–December 2020), while specific anti-SARS-CoV-2 medications were not available [50,51]. Further research is required to understand whether influenza vaccination could be associated with modified humoral response in asymptomatic and severe SARS-CoV-2 infections. Moreover, it is unknown whether influenza vaccination could also be associated with the response of other immunoglobulin classes that play an important role in SARS-CoV-2 infection, i.e., IgM and IgA [52]. The potential association between repeated influenza vaccination with humoral responses in COVID-19 also remains to be investigated since some data show that it may blunt immune reactions and lead to a decline in the effectiveness of influenza vaccines (although this phenomenon remains controversial, while the underlying mechanism is not clear) [53,54]. One should also bear in mind that our study did not investigate the function of anti-SARS-CoV-2 antibodies. Therefore, whether higher antibody concentrations found for influenza-vaccinated individuals would translate into better virus neutralization requires further research. However, it was demonstrated that the presence of antibodies, such as IgG anti-N, the prevalence of which was higher in individuals vaccinated against influenza, was associated with a substantially reduced risk of reinfection [55,56]. Last but not least, adaptive cellular immunity that underpins protection against severe disease [57] was not a subject of this study.

5. Conclusions

This study showed better anti-N and anti-RBD antibody response to SARS-CoV-2 infection in individuals vaccinated against seasonal influenza than in those who did not receive such vaccination. Further research is required to understand the mechanisms

underlying this phenomenon. Nevertheless, the results add to accumulating evidence on the broadly beneficial effects of influenza vaccination in COVID-19.

Author Contributions: Conceptualization, P.R. and L.B.; methodology, B.P., J.Ż. and P.R.; investigation, B.P., E.H., D.S., K.S., K.K., P.R. and L.B.; resources, B.P., J.Ż., P.R. and L.B.; writing—original draft preparation, P.R.; writing—review and editing, B.P. and E.H.; supervision, P.R. and L.B.; funding acquisition, B.P. and L.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Institute of Public Health NIH—National Research Institute, grant number 1BIBW/2022.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Bioethical Committee of the Institute of Public Health—National Research Institute (protocol code 4/2020, 6 August 2020) and the Bioethics Committee at the Poznan University of Medical Sciences (protocol code 429/22, 11 May 2022).

Informed Consent Statement: Patient consent was waived because serum samples for research were purchased from Regional Blood Donor Centers in Poland.

Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Therapeutic Role of Neutralizing Antibody for the Treatment against SARS-CoV-2 and Its Emerging Variants: A Clinical and Pre-Clinical Perspective

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Abstract: Since early 2020, the entire world has been facing a disastrous outbreak of the SARS-CoV-2 virus, with massive reporting of death and infections per day. Medical practitioners adopted certain measures such as convalescent plasma therapy, antibody treatment, and injecting vaccines to eradicate the pandemic. In this review, we have primarily focused on the neutralizing antibodies presently under pre-clinical and clinical trials, focusing on their structures, binding affinity, mechanism of neutralization, and advantages over other therapeutics. We have also enlisted all the nAbs against SARS-CoV-2 and its emerging variants in different phases of clinical trials (phase-1, phase-II, and phase-III). The efficacy of administering antibody cocktails over the normal antibodies and their efficacy for the mutant variants of the SARS-CoV-2 virus in minimizing viral virulence is discussed. The potent neutralizing antibodies have eliminated many of the common problems posed by several other therapeutics. A common mechanism of the antibodies and their relevant sources have also been listed in this review.

Keywords: neutralizing antibody; SARS-CoV-2; pre-clinical trials; clinical trials

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Citation: Bhattacharya, M.; Chatterjee, S.; Mallik, B.; Sharma, A.R.; Chakraborty, C. Therapeutic Role of Neutralizing Antibody for the Treatment against SARS-CoV-2 and Its Emerging Variants: A Clinical and Pre-Clinical Perspective. *Vaccines* **2022**, *10*, 1612. https://doi.org/10.3390/vaccines10101612

Academic Editors: Rishi Jaiswal, Srijani Basu, Suman Gupta and Sneh Lata Gupta

Received: 8 August 2022
Accepted: 21 September 2022
Published: 26 September 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

The current pandemic of Coronavirus disease (COVID-19) started in the Wuhan province of China in December 2019. During infection, the SARS-CoV-2 virus triggers various immune cascades. Effective, balanced immune components are required to control the pathogenesis of COVID-19 [1–3]. The neutralizing antibodies (nAb's) have shown defense against infected or vaccinated individuals, and can be used as a promising therapeutics component in humans [4]. The nAb is a class of antibodies that neutralize the invading cells of the disease-causing pathogens, thus providing immunity. Such antibodies might be triggered by the use of vaccines or an earlier infection, which are retained inside the body for a longer time than the therapeutic ones. Therefore, neutralizing antibodies are employed for treating several critical pathogenic infections due to their enhanced specificity [5,6].

Before or after viral infection, nAbs can be transferred passively to patients to treat COVID-19 [6]. It has also been proven to be very effective for patients with clinically mild symptoms in the early onset of disease [7]. One of these nAb's sources is the B cell, isolated from the convalescent plasma donors. An elucidative screening of these antibodies has shown that they can hinder viral entry and prevent SARS-CoV-2 infection [8–10]. Some of

these nAbs can be derived from humanized mice or convalescent patients and follow the same action mechanism [11]. Antibody development events from the smallpox vaccination were a breakthrough, and this event has shown a new direction for the treatment of COVID-19 as an anti-SARS-CoV-2 mAb (Figure 1). Events that led to the development of antibodies are shown in Table 1. nABs have an effective therapeutic role in preventing SARS-CoV-2 infection. Considering the antigenic part of the spike (S)-protein, the nAbs are developed, which can be specifically bound to the RBD of S-glycoprotein [12–14]. The nAbs development depends not only on the structure but also on the alteration of the protein conformation. The nAb, with accurate structure and conformation, invades the host cells, and it is important for the functionality of the nAbs against the infection of the virus [6,15].

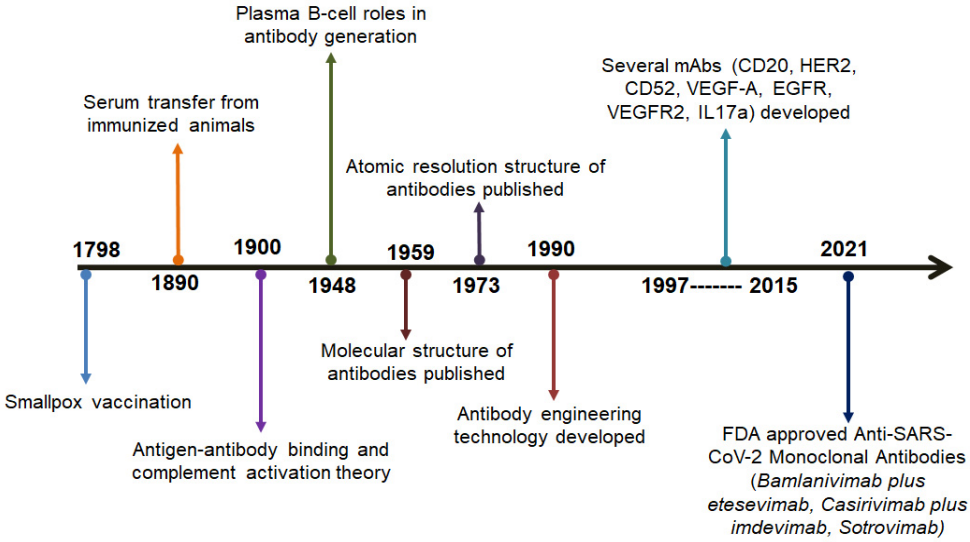

Figure 1. Timelines show different breakthroughs in antibody development.

Table 1. Events that led to the development of antibodies.

Sl. No.	Year	Scientists Involved	Progress in the Development of Antibodies	Reference
1.	1798	Edward Jenner	The breakthrough of the smallpox vaccine	[16]
2.	1890	Emil von Behring and Shibasabura Kitasato	The transfer of serum to cure diphtheria taken from immunized animal	[17]
3.	1900	Paul Ehrlich	The advancements of several concepts such as antigen-antibody binding, side-chain theory, and complement activation	[18]
4.	1948	Astrid Fagraeus	Elucidated the importance of B cells	[19]
5.	1959	Gerald Edelman and Rodney R. Porter	The publication of the molecular tructures of various antibodies	[20]
6.	1973	D Inbar, J Hochman, and D Givol	The publication of the molecular tructures of the antibodies fragment	[21]
7.	1990	A Plückthun	Antibody engineering	[22]
8.	1997–2015	-	Development of various antibodies such as CD20, HER2, CD52, VEGF-A, EGFR, VEGFR2, and IL17A	[23,24]
9.	2021	-	Development of anti-SARS-CoV-2 antibodies such as Bamlanivimab plus etesevimab, Casirivimab plus imdevimab, and Sotrovimab	[25]

For the SARS-CoV-2 infection, a few nAbs are highly specific to the S-glycoprotein and prevent the binding of S-glycoprotein to the RBD-ACE2 complex in the host cell. A group of

scientists isolated the B cells from the infected individuals and initiated the preparation of different types of nAbs, currently in the pre-clinical and clinical phases (P2C-1F11, BD-368-2, P2B-2F6, COV2-2196, COV2-2130, etc.) [6,26,27]. A model shows where the SARS-CoV-2 spike glycoprotein (S-protein) ectodomain is bound to two copies of domain-swapped natural antibody 2G12 (Figure 2). Likewise, SARS-CoV-2 S-protein in trimeric form also makes a complex with the human nAb (C002) Fab fragment (Figure 3). Many of the nAb isolated from human beings had proven to be effective in treating the SARS-CoV-2 infection in several animals, namely the transgenic mice, hamsters, etc. The nAb (S2M11) Fab part bind with the adjacent receptor-binding domains of S-glycoprotein present in a closed conformation (Figure 4). Another nAbs, named Vh–Fc ab8, targets the spike RBD, which is also very effective in treating the infection [17–19]. However, researchers developed some nAbs with a different target site other than the spike RBD. They also targeted other regions besides RBD in the S-protein, and are currently entering the pre-clinical stages [6,28]. It has been noted that nAbs targeting the spike RBD are more efficient than the nAb targeting the other regions of S-protein (other than RBD). At the same time, scientists have shown, through electron microscopy, that the nAb can also bind to the NTD of S-protein. One such example is mAb (4A8), which binds with the S-protein NTD part (Figure 5). Therefore, the NTD might be a potent target for therapeutic mAbs against the COVID-19. This target specificity of the spike RBD has not only proved to be effective for the wild-type strain but also for several emerging mutant variants. They have very minimal immune escape property [13,29]. Liu et al. stated that RBD is a highly conserved region, and researchers should develop more nAb targeting RBD to treat the infection [30].

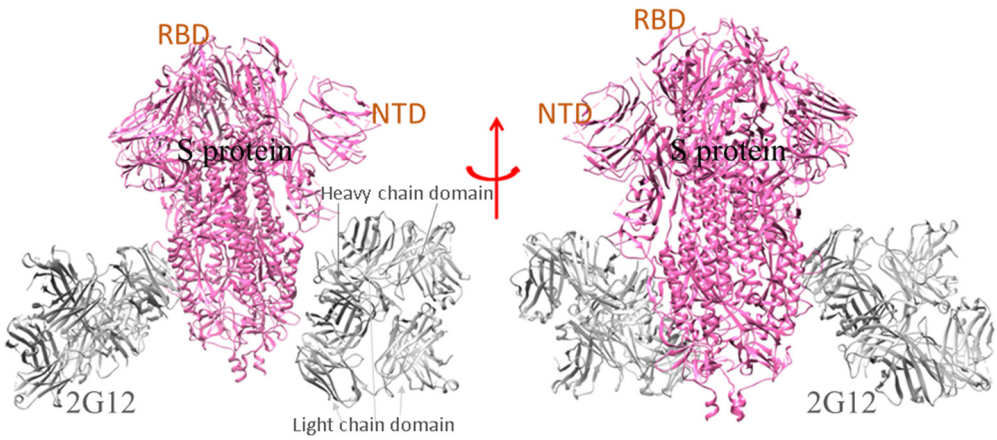

Figure 2. The ribbon model shows the SARS-CoV-2 2P S-protein ectodomain bound to two copies of domain-swapped antibody 2G12 [PDB id: 7L06]. The heavy chain domain of Ab(2G12) interacts with the NTD part of the S-protein.

It has been noted that various nAbs were employed to treat SARS-CoV infection previously, and these nAbs are also used for neutralizing the SARS-CoV-2 infection. However, some of the nAbs of the SARS-CoV virus fail to target the spike RBD region of the SARS-CoV-2 and are unable to neutralize the future viral infection [6]. Some mutational modifications in the SARS-CoV-2 variants may affect the viral infectivity, and also, the mutation-related S-protein configuration change might alter the nAbs binding affinity. On the other hand, it was also recorded that the protein modifications can make them efficient in targeting the conserved epitopes of spike RBD which could enhance the neutralizing capacity of nAbs [31–33]. The researcher also reported that the nAbs inhibit the interaction of spike protein with the ACE2 receptor preventing membrane fusion [34]. It is also noted that some of the RBD targeting nAb or the non-RBD targeting ones are incompatible with preventing spike protein interaction against the ACE2 receptor. These nAbs exhibit the viral neutralizing capacity and bind to other S-glycoprotein regions, preventing the entry of the SARS-CoV-2 virus [35]. Several scientists analyzed the detailed phenomena of incapability

of nAb to prevent viral entry. They found that these nAbs interact with the Fcγ receptor and lead to the antibody-dependent enhancement (ADE) with the target cells. This ADE formation subsequently leads to the release of cytokines such as IL-6 [36]. However, there are no reports of ADE formation in patients with SARS-CoV-2 infection till today [6].

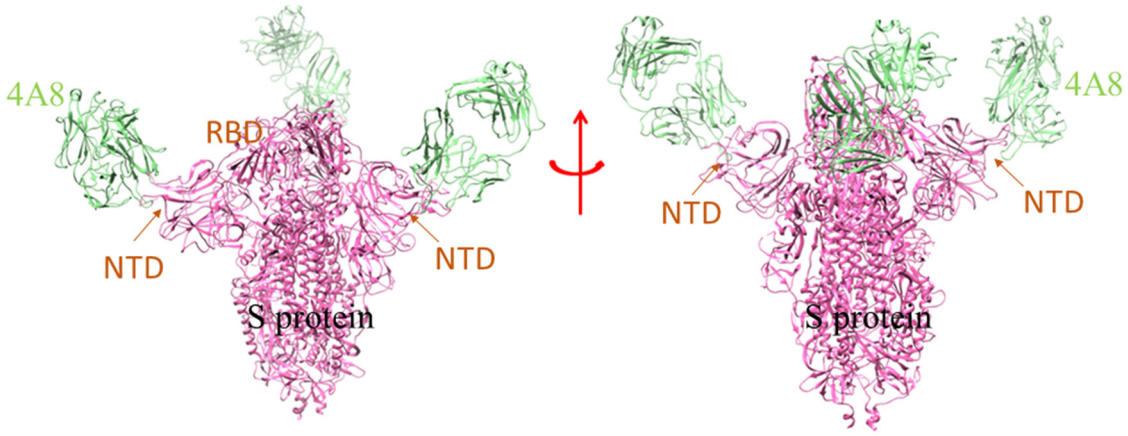

Figure 3. The model demonstrates the binding of human mAb (4A8) to the NTD of S-protein of SARS-CoV-2 [PDB id: 7C2L]. The chains of the mAb unit interact with the different NTD of the S-protein trimeric sub-unit domain.

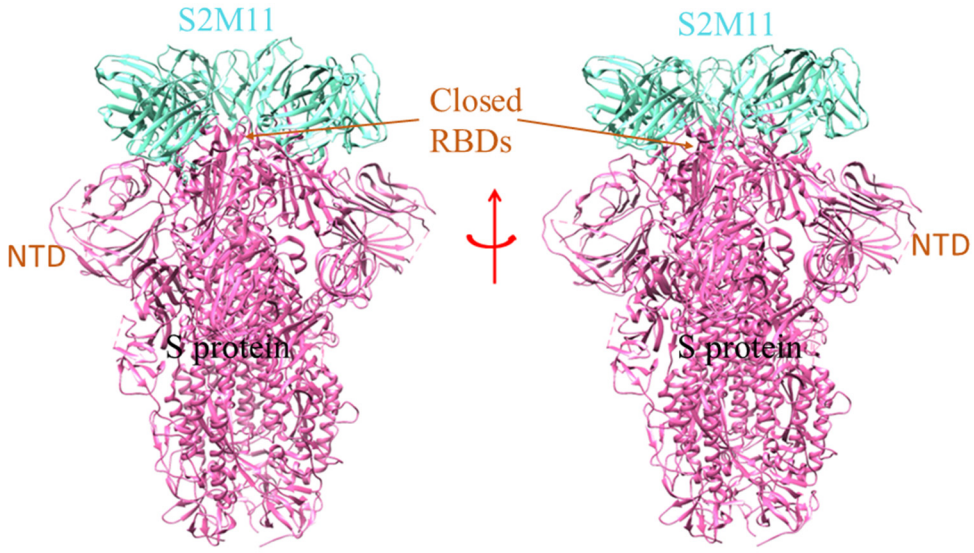

Figure 4. The model shows the human nAb (S2M11) binding with the adjacent part of RBD in closed conformation of S-protein [PDB id: 7K43]. The different domain of SARS-CoV-2 S-protein is marked, where the RBD region interacts with the single unit of nAb (S2M11).

Given the therapeutic potential of nAbs in viral protection, here, we summarize the nAbs presently under pre-clinical and clinical trials for COVID-19 treatment with special attention to their structures, binding affinity, mechanism of neutralization, and advantages over other therapeutics. Subsequently, we also highlighted the efficacy of administering antibody cocktails over the normal ones and their efficacy against the significant mutant variants of the SARS-CoV-2 virus. The specific role of potent single domains antibodies is also discussed for therapeutics. A collective mechanism of the neutralizing antibodies and their sources are also listed in this review.

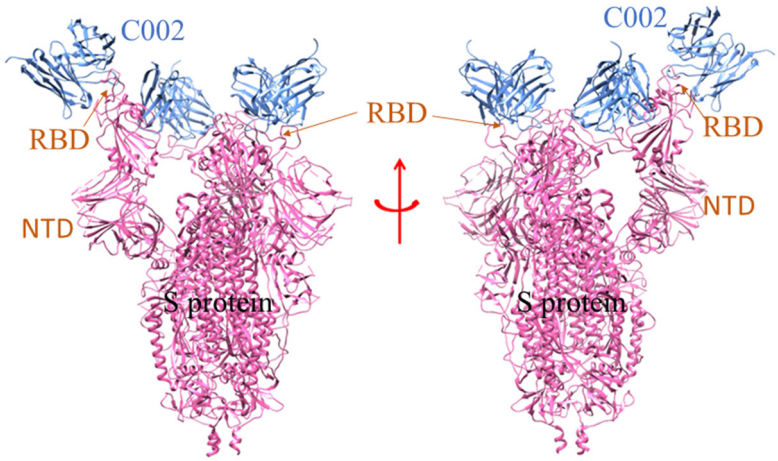

Figure 5. The structure shows the SARS-CoV-2 S 6P trimer in a complex with human nAb (C002) Fab fragment [PDB id: 7K8T]. The nAb (C002) Fab fragment partially interacts with the RBD and NTD regions of the S-protein of SARS-CoV-2.

2. Structure of a Neutralizing Antibody

One of the common features of all the nAbs is the CDRH3 region (complementarity-determining region 3) in the heavy chain. The CDRH3 region comprises of a few gene segments with unique amino acid residues. The three genes present are V (variable), D (diversity), and J (joining). Several studies highlighted that the antibodies interact with the antigens and elicit the immune response, which solely depends on the CDRH3 region [37]. The researcher also studied the CDRH3 region of the antibodies released from the B cells elicited by the spike glycoprotein of SARS-CoV-2. They did not find any significant variation in the length of the CDRH3 region in antibodies compared to the normal population. However, they found that the average length of these isolated CDRH3 regions was nearly 20 amino acids long [37].

The nAbs isolated from the convalescent plasma donors are specific to the RBD epitopes. However, some of these nAbs might target overlapping epitopes [38]. These antibodies protect the virus from interacting with the ACE2 receptor preventing viral infection. Like other antibodies, the nAbs employed specifically for preventing the SARS-CoV-2 infection also comprise two chains: heavy and light. The heavy chain is segmented into smaller regions which are encoded by the VH3-53 or VH3-66 genes. In addition, it comprises three complementary determining regions of the heavy chain (CDRH), namely CDRH1, CDRH2, and CDRH3. The CDRH3 region is generally shorter in length than the other two regions [37]. One in vitro study isolated the SARS-CoV-2 nAb and found that it possesses a similar target and comprises VH3-30 genes in the CDRH3 region [37]. According to the structural and functional attributes, Barnes et al. have categorized the nAbs into four types which are:

i. VH3-53 encoded gene that blocks the host ACE2 only in the ‘up’ conformation of the RBD. They exhibit a shorter CDRH3 region (Figure 6a).

ii. Another class of ACE2 blocking antibody is functional in both (up and down) RBD conformations and can even contact adjacent RBDs (Figure 6b).

iii. An additional class of nAb that hinders viral entry by occupying the outer surface of the ACE2; functional in both the up and down conformation of the RBD (Figure 6c).

iv. The fourth class does not interact with the ACE2 receptor and is functional in the ‘up’ conformation of the RBD (Figure 6d) [37].

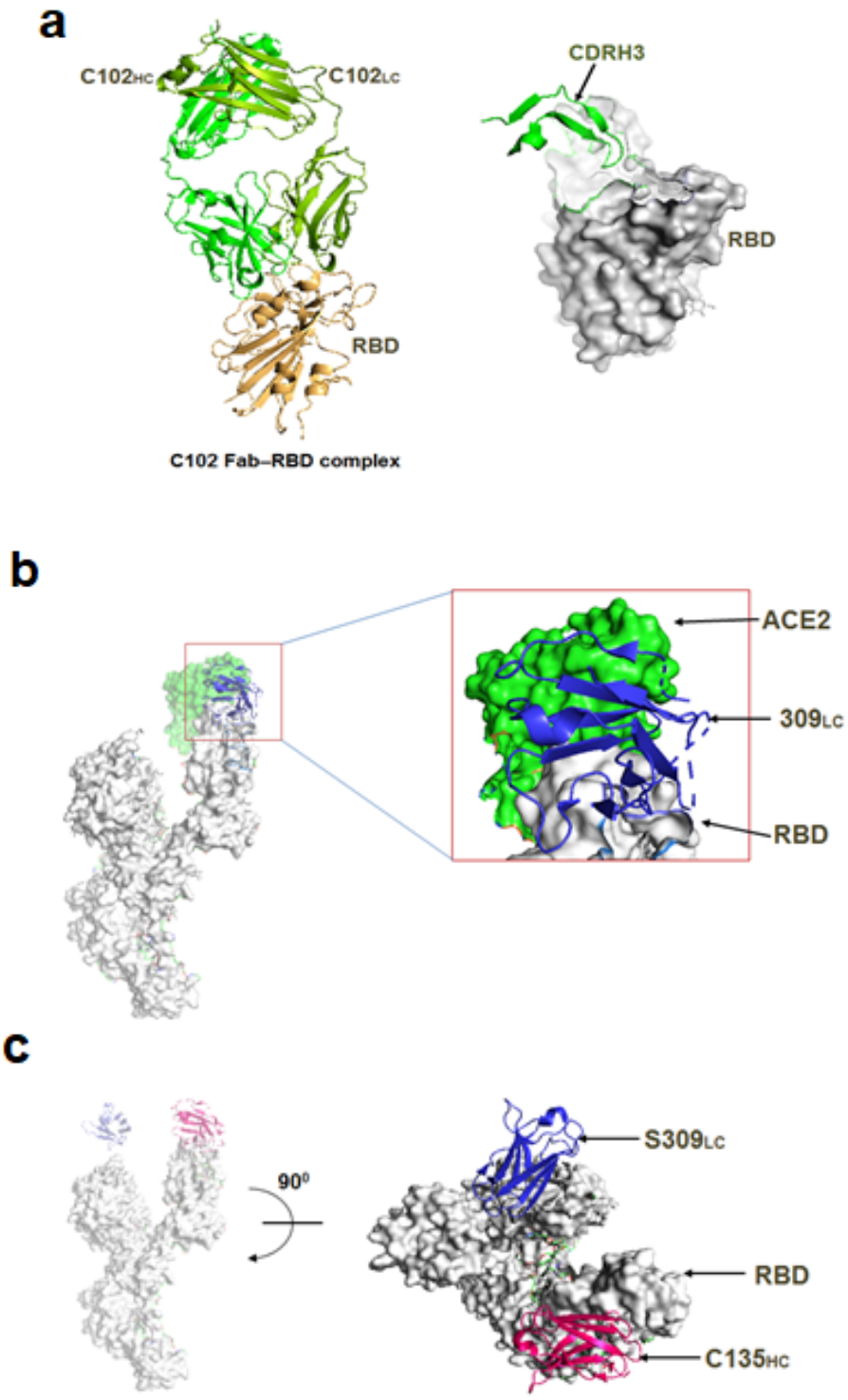

Figure 6. *Cont*.

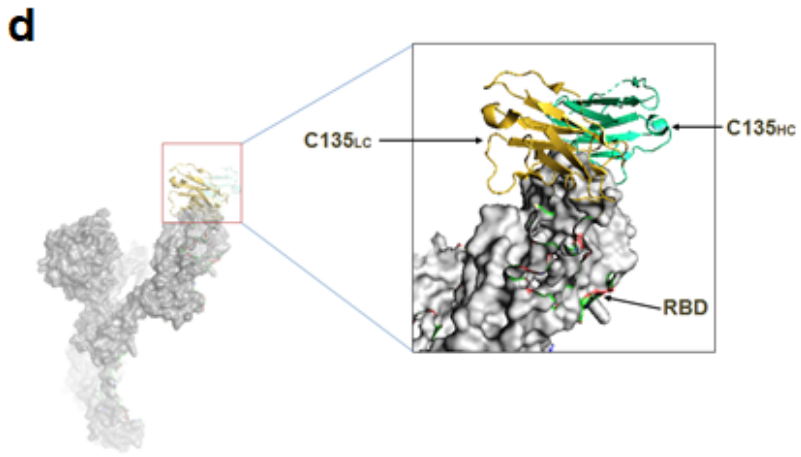

Figure 6. Different types of nAb based on structural and functional attributes. (**a**) Class I nAb (CDRH3) binds with open RBD (C102 Fab-RBD complex). (**b**) Class II nAb S309 bind with both ACE2 and RBD (309LC-ACE2 RBD complex). (**c**) Class III nAb C135 bind distinct RBD part (C135HC and S309LC with RBD). (**d**) Class IV nAb C135 bind with only up conformation of RBD ($C135_{HC}$ and $C135_{LC}$ with RBD). [PDB id: 6WPS, 7K8M].

3. Types of mAbs

Remarkably, the beneficial antibodies being cloned in a laboratory need not come from humans but can be from definite animals also. Accordingly, mAbs are of four extensive types.

i. Murine: made from mouse proteins; names of drugs based on this end in -omab.

ii. Chimeric: a combination of mouse and human proteins; names of drugs based on this end in -ximab.

iii. Humanized: here, small doses of mouse proteins are attached to human proteins; names of drugs based on this end in -zumab.

iv. Human: these are fully human proteins; names of drugs based on this end in -umab.

3.1. mAb in Treatment of COVID-19

The mAbs have been used to combat MERS, SARS, and other important infections caused by the corona family of viruses in the last decade. Since the COVID-19 pandemic broke out in early 2020, there has been an accelerated drive to use mAbs to fight the virus. The FDA performed a significant role in approving and regulating the use of mAbs to treat COVID-19. Moreover, it was in charge of the guidelines on which mAb-based drugs should be used.

Therefore, in the last two years, a number of drugs are already in use, such as Bamlanivimab and etesevimab, which are used when there is mild to moderate infection by SARS-CoV-2 infection. Casirivimab and imdevimab are used when there is mild to moderate infection and the patient is at risk of developing a severe infection, but the person does not need oxygen therapy. In some previously infected people with the SARS-CoV-2 virus, the immune system goes into overdrive, releasing bursts of proteins known as cytokine storms. While these may or may not fight this virus effectively, they definitely cause severe inflammation in the body, which can be life-threatening. In such a scenario, the antigens from these cytokines must also be suppressed. This is undertaken by a definite category of mAbs called anti-interleukin-6 receptor mABs. Levilimab and Tocilizumab (which are widely present) are examples of such drugs.

Anti-CD6 mAbs are similar to anti-interleukin-6 receptor mAbs, but the biochemical mechanism is slightly different. Itolizumab is an example of such a drug type.

3.2. Mechanism of Action of SARS-CoV-2 nAb

The main target of most of the SARS-CoV-2 nAb is the spike glycoprotein which is responsible for triggering a strong host immune response due to its high antigenicity [38–40]. Among the reported nAbs, more than 90% can bind with the RBD and block the viral interaction with the host ACE2. According to Jin D et al., the binding of the nAb to the ACE2 receptor can be subdivided into two regions, namely a/b. The binding affinity of the nAbs to these two sites is expected to inhibit a potent neutralizing effect in hindering the binding of the spike protein. Moreover, these antibodies are unable to bind with the RBD in the same manner. The variable binding of these antibodies helps segregate them [41]. Therapeutic monoclonal antibodies can interact with one or more epitopes of the RBD region [42]. This variation of neutralization in the case of nAb also varies with respect to diverse interaction sites of antigens. For instance, the mAb named S2H13 possesses neutralizing activity by identifying the conformation of the spike protein. However, it has been noted that the EY6A and ACE2 neutralization have different action mechanisms. It has been revealed that during neutralization, EY6A binds to the lower portions of the b region of ACE2 and thus is unable to interact with S1 and S2 junctions of S-glycoprotein. Therefore, these event makes the epitope incapable of binding with the ACE2 receptor (Figure 7). Usually, the nAbs that target a single epitope alter the conformation of the spike glycoprotein (in a down conformation). It helps to make it inaccessible to interact with the ACE2 receptor [43,44]. One of the major reasons enabling the neutralization of the SARS-CoV-2 virus is a creation of a stearic obstacle due to the orientation of the nAbs. This obstacle makes the spike protein incapable of interacting with the host receptor. S2A4 has a very strong viral neutralizing capacity compared to the other nAb's. After binding with the spike RBD, this antibody causes the shedding of the S1 subunit, hindering the access to bind with the ACE2 receptor. Some nAbs use three spike epitopes to restrain the interaction with the ACE2 receptor. These antibodies surround the RBD in many ways. They bind to the edges and tip of the RBD and use either the heavy chain or light chain to interact with the viral epitopes [41].

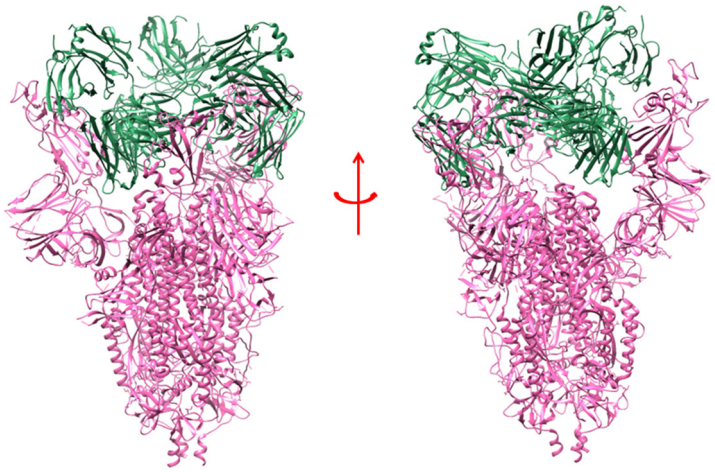

Figure 7. The SARS-CoV-2 S-protein in a complex with a nAb EY6A Fab [PDB id: 6ZDH].

Here, in the earlier section, we have discussed that the nAbs target the RBD and NTD (N-terminal domain). The exact mechanism of NTD attachment is not vividly elucidated to date. However, the structural analysis of the spike protein showed that these nAbs bind with the NTD and alter the RBD conformation (down conformation). This phenomenon creates a stearic obstacle coinciding with the antibody and its binding to the ACE2 receptor. Consequently, the spike protein is unable to interact with the ACE2 receptor due to nAb-ACE2 complex [31].

4. Advantages of nAb over Vaccines and Convalescent Plasma Therapy

During the pandemic, one of the alarming situations prevailing throughout the world is the evolving SARS-CoV-2 variants and their strategy to immune escape. In this regard, the application of nAbs might be more effective than vaccines. The administration of two or more antibodies (antibody cocktails) together has proven too efficient in the case of evolving variants [45]. Moreover, a more efficient approach must be taken to develop vaccines against the evolving variants to eradicate the pandemic.

Researchers aim to establish the COVID-19 treatment using several nAbs by replacing convalescent plasma therapy (CPT). The primary reason behind replacing CPT with nAbs is the elimination of some blood diseases which are generally the side-effects of CPT. The use of the nAbs helps in the faster development of epitope-specific antibodies. Moreover, a proper dosage of these nAbs forms a high titer of antibodies within a very short time compared to CPT. The high efficacy of nAbs has also proven to have superior results in cases of COVID-19 and certain other disease outbreaks [11].

It has been noted that the FDA approved the CPT to treat hospitalized COVID-19 patients; however, more accurate results are awaited from the undergoing clinical trials. Patients undergoing plasma transfusion should not be comorbid and should not have any chance of antibody-dependent enhancement (ADE). In this regard, nAbs are highly effective against the CPT treatment [46]. We have listed different nAbs in different phases of clinical trials (Table 2).

Table 2. Different neutralizing antibodies which are in clinical trials.

Sl. No.	nAb	Trial No.	Status	Recruitment	No. of Participants	Sponsor	Country	Allocation	Remarks
1.	JS016	NCT04441918	Phase II	Recruiting	40	Shanghai Junshi Bioscience Co., Ltd.	China	Randomized	A randomized, placebo-controlled study reporting its safety, pharmacokinetics, and immunogenicity administered in healthy subjects.
2.	LY3832479	NCT04441931	Phase II	Completed	26	Eli Lilly and Company	United States	Randomized	A randomized, placebo-controlled study reporting its safety, tolerability, and pharmacokinetics of the mAb in healthy adult volunteers.
3.	LY-CoV016	NCT04427501	Phase II	Active but not recruiting	3290	Eli Lilly and Company	United States	Randomized	A randomized, placebo-controlled study reporting the tolerability, efficiency, and safety profile of the antibody in COVID-19 patients with mild to moderate symptoms.
4.	TY027	NCT04429529	Phase III	Completed	32	Tychan Pte Ltd.	Singapore	Randomized	A randomized, placebo-controlled, time-lagged study conducted in healthy subjects.
		NCT04649515		Recruiting	1305			Randomized	A randomised, placebo controlled study of TY027 aimed for treating COVID-19 patients.
5.	BRII-196	NCT04479631	Phase III	Completed	16	Brii Biosciences Limited	China	Randomized	A randomized, placebo-controlled study of BRIL-196 monoclonal antibodies reporting its safety, tolerability, and pharmacokinetics.
6.	BRII-198	NCT04479644	Phase III	Completed	17	Brii Biosciences Limited	China	Randomized	A randomized, placebo-controlled study of BRIL-196 monoclonal antibodies reporting its safety, tolerability, and pharmacokinetics.
7.	CT-P63	NCT05017168	Phase I pending	Not yet recruiting	24	Celltrion	Poland	Randomized	A randomized, placebo-controlled study reporting the tolerability, efficiency, and safety profile of the antibody in COVID-19 patients with mild to moderate symptoms.
8.	XVR011	NCT04884295	Phase I	Recruiting	279	ExeVir Bio BV	Belgium and Italy	Randomized	A randomized, placebo-controlled study reporting the tolerability, efficiency, and safety profile of the antibody in COVID-19 patients with mild to moderate symptoms.

Table 2. *Cont.*

Sl. No.	nAb	Trial No.	Status	Recruitment	No. of Participants	Sponsor	Country	Allocation	Remarks
9.	ABBV-47D11	NCT04644120	Phase I	Completed	25	AbbVie	United States	Randomized	A randomized, placebo-controlled study of ABBV-47D11 and ABBV-2B04 monoclonal antibodies reporting its safety, pharmacodynamics, and pharmacokinetics.
10.	HFB30132A	NCT04590430	Phase I	Active but not recruiting	24	HiFiBiO Therapeutics	United States	Randomized	A randomized, placebo-controlled study reporting its safety, tolerability, and pharmacokinetics of the mAb in healthy adult volunteers.
11.	ADM03820	NCT04592549	Phase I	Recruiting	40	Ology Bioservices	United States	Randomized	A randomized, placebo-controlled study reporting its safety, pharmacokinetics, and immunogenicity.
12.	DXP604	NCT04669262	Phase I	Completed	25	BeiGene	Australia	Randomized	A randomized, placebo-controlled study reporting its safety, pharmacokinetics, and immunogenicity in healthy volunteers.
13.	HLX70	NCT04561076	Phase I	Not yet	24	Hengenix Biotech Inc	United States	Randomized	A randomized, placebo-controlled study reporting its safety and pharmacokinetics.
14.	COR-101	NCT04674566	Phase I and Phase II	Recruiting	45	Corat Therapeutics Gmbh	Germany	Randomized	A randomized, placebo-controlled study reporting its safety, tolerability, and pharmacokinetics and immunogenicity of COR-101 in hospitalized COVID patients.
15.	VIR-7832	NCT04746183	Phase I and Phase II	Recruiting	600	University of Liverpool	United Kingdom	Randomized	A randomized, placebo-controlled trial aimed to evaluate the efficacy of the drug in treating COVID-19 patients.
16.	LY-CoV1404, LY3853113	NCT04634409	Phase II	Active but not recruiting	1782	Eli Lilly and Company	United States	Randomized	A randomized, placebo-controlled study reporting the tolerability, efficiency, and safety profile of the antibody in COVID-19 patients with mild to moderate symptoms.
17.	COVI-AMG (STI-2020)	NCT04734860	Phase II	Recruiting	500	Sorrento Therapeutics, Inc.	United States	Randomized	A randomized, placebo-controlled study aimed to evaluate the safety and efficacy of the nAb in patients having mild COVID-19 symptoms.

Table 2. *Cont.*

Sl. No.	nAb	Trial No.	Status	Recruitment	No. of Participants	Sponsor	Country	Allocation	Remarks
18.	DXP593	NCT04532294	Phase II	Completed	18	BeiGene	Australia	Randomized	A randomized, placebo-controlled study reporting its safety, pharmacokinetics, and immunogenicity in healthy volunteers.
		NCT04551898			181		United States		A randomized, placebo-controlled study highlighting the neutralizing efficiency of the BGBDXP593 mAb in COVID-19 patients having mild and moderate symptoms.
19.	MW33	NCT04533048	Phase II	Completed	42	Mabwell (Shanghai) Bioscience Co., Ltd.	China	Randomized	A clinical study to evaluate the safety, pharmacokinetics, and immunogenicity of MW33 in normal, healthy volunteers.
		NCT04627584		Recruiting	150				A randomized, placebo-controlled study reporting the efficiency and safety profile of the antibody in COVID-19 patients with mild to moderate symptoms.
20.	MAD0004J08	NCT04932850	Phase II and Phase III	Active but not recruiting	30	Toscana Life Sciences Sviluppo s.r.l.	Italy	Randomized	A randomized study to evaluate the safety, pharmacokinetics, and immunogenicity of MAD0004J08 in normal, healthy volunteers.
		NCT04952805		Recruiting	800				A randomized, placebo-controlled study aimed to evaluate the safety and efficacy profile of the antibody in adult COVID-19 volunteers who were asymptomatic, or had moderately severe symptoms.
21.	C144-LS and C-135-LS	NCT04700163	Phase II	Active but not recruiting	23	Rockefeller University	United States	Randomized	A randomized study to evaluate the safety, pharmacokinetics of two antibodies in normal, healthy volunteers.

Table 2. *Cont.*

Sl. No.	nAb	Trial No.	Status	Recruitment	No. of Participants	Sponsor	Country	Allocation	Remarks
22.	SCTA01	NCT04483375	Phase II and Phase III	Completed	33	Sinocelltech Ltd.	China	Randomized	A randomized, placebo-controlled study reporting its safety, tolerability, and pharmacokinetics of SCTA01 in healthy adult volunteers.
		NCT04644185		Recruiting	795		United States		A randomized, placebo-controlled study employed for examining the efficiency of SCTA01 in COVID-19 affected subjects having severe symptoms.
23.	ADG20	NCT04805671	Phase II and Phase III	Recruiting	1084	Adagio Therapeutics, Inc.	Germany, Greece, Brazil, Argentina, Poland, Ukraine	Randomized	A randomized, placebo-controlled study reporting the efficacy of the ADG20 mAb in healthcare workers having mild to moderate symptoms.
		NCT04859517			6412		United States		A randomized, placebo controlled trial for evaluating the safety profile of the antibody in preventing SARS-CoV-2 infection.
24.	AZD7442 (AZD8895 + AZD1061)	NCT04507256	Phase III	Active but not recruiting	60	AstraZeneca	United Kingdom	Randomized	A randomized, placebo-controlled study reporting its safety, tolerability, and pharmacokinetics of AZD7442 in healthy adult volunteers.
		NCT04625725			5197		United States		A randomized, placebo-controlled study employed for evaluating the efficiency of AZD7442 in subjects who are not yet encountered by the SARS-CoV-2 virus.
		NCT04625972			1121		United States		A randomized, placebo-controlled study employed for evaluating the efficiency of AZD7442 in subjects who are already infected by the SARS-CoV-2 virus.

Table 2. *Cont.*

Sl. No.	nAb	Trial No.	Status	Recruitment	No. of Participants	Sponsor	Country	Allocation	Remarks
25.	CT-P59	NCT04525079	EUA	Recruiting	32	Celltrion	Republic of Korea	Randomized	A randomized, placebo-controlled study reporting its safety and pharmacokinetics of CT-P59 in healthy volunteers.
		NCT04593641		Active but not recruiting	18				A randomized, Double-placebo-controlled, study reporting the viral nature, safety, and tolerability of the nAb in patients having mild symptoms.
		NCT04602000		Recruiting	1020				A randomized, placebo-controlled study employed for examining the efficiency of CT-P59 in COVID-19 affected subjects having severe symptoms.
26.	VIR-7831	NCT04545060	EUA	Completed	1057	Vir Biotechnology, Inc.	United States	Randomized	A randomized, placebo-controlled study employed for evaluating the safety and efficiency of the nAb for treating COVID-19 patients who did not require any hospital support.
27.	REGN-COV2	NCT04425629	EUA	Recruiting	6420	Regeneron Pharmaceuticals	United States	Randomized	A master protocol study reporting the safety and efficacy of the anti-spike mAbs in healthcare works affected with SARS-CoV-2 virus.
		NCT04426695		Completed	2252				A master protocol study reporting the safety and efficacy of the anti-spike mAbs in COVID-19 positive patients who required hospital support.
		NCT04452318		Active but not recruiting	3750				A randomized, placebo-controlled study evaluating the efficacy and the safety of the mAb in the household contacts to prevent SARS-CoV-2 infection.

Table 2. *Cont.*

Sl. No.	nAb	Trial No.	Status	Recruitment	No. of Participants	Sponsor	Country	Allocation	Remarks
28.	LY-CoV555 (LY3819253); combination of LY-CoV555 with LY-CoV016 (LY3832479)	NCT04411628	Phase I	Completed	24	Eli Lilly and Company	United States	Randomized	A randomized, placebo-controlled study reporting its safety, tolerability, and pharmacokinetics of LY3819253 in hospitalized COVID-19 patients.
		NCT04427501	Phase II	Recruiting	3290				A randomized, placebo-controlled study highlighting the neutralizing efficiency of the mAb in COVID-19 patients having mild and moderate symptoms.
		NCT04497987	Phase III	Completed	1374				A randomized, placebo-controlled trial highlighting the safety and efficacy of the mAb alone and in combination to evaluate the immune response in nursing staffs to prevent SARS-CoV-2 infection.
		NCT04501978	Phase III	Recruiting	10,000	University of Minnesota			A randomized, blinded controlled trial reporting the safety and efficacy of the COVID-19 positive patients who required hospital support.
		NCT04518410	Phase II and Phase III	Recruiting	8797	National Institute of Allergy and Infectious Diseases (NIAID)			A randomized study evaluating the efficacy of LY3819253 in COVID-19 patients who did not require hospital support.
29.	Anti-SARS-CoV-2 mAb	NCT04748588	Phase IV	Recruiting	648	University of Calgary	Canada	Randomized	A final trial aiming to evaluate the efficacy and safety of the antibody in the nosocomial COVID-19 patients in Canada.

Table 2. *Cont.*

Sl. No.	nAb	Trial No.	Status	Recruitment	No. of Participants	Sponsor	Country	Allocation	Remarks
30.	BI 767551	NCT04822701	Phase II and Phase III	Active but not recruiting	5	Boehringer Ingelheim	United States	Randomized	A randomized, placebo-controlled study reporting the tolerability, efficiency, and safety profile of the antibody in COVID-19 patients with mild to moderate symptoms.
31.	SAB-185	NCT04469179	Phase I	Active but not recruiting	21	SAb Biotherapeutics, Inc.	United States	Randomized	A randomized study evaluating the efficacy of SAB-185 in COVID-19 patients who did not require hospital support.
32.	Bamlanivimab	NCT04796402	Phase IV	Active but not recruiting	576	Fraser Health	Canada	Randomized	A Phase IV study implicated for the emergency use of Bamlanivimab during the pandemic.

5. Different nAbs Employed for Treating SARS-CoV-2 That Are in Clinical Trials

We have listed different nAbs that are currently in clinical trials (Table 2). The detailed account of these nAbs is also discussed in the following sections.

5.1. JS016

Etesevimab (also known as JS016) is a neutralizing monoclonal antibody possessing certain replacements in the amino acid residue (L234A, L235A) in the Fc region, which prevents the interaction of the S glycoprotein with the ACE2 receptor. This mAb also aims to prevent host–cell invasion and viral replication. It belongs to the class of IgG1 isotype and the LALA mutation in the Fc region that prevents various properties such as antibody-dependent cellular cytotoxicity and antibody-dependent enhancement. It mitigates the activation of macrophages to diminish the excessive cytokine storm observable in severely affected COVID-19 patients, proving the effectiveness of the antibody. An in vitro study conducted on a macaque model reported satisfactory results of JS016 in preventing SARS-CoV-2 infection [47].

5.2. MW33

The MW33 is a nAb of the type IgG1κ, highlighting several essential features for preventing COVID-19 disease. The rhesus monkey was the animal model where this particular antibody was administered. The MW33 antibody targets the spike RBD, but the conventional cytochrome P450 enzymes do not mediate its expulsion from the body. Instead, it is carried out by some non-specific proteolytic enzymes. The Phase I clinical trials of the MW33 have shown some decrement in the biochemical parameters. Later stages of the clinical trials will be performed to conclude more about the MW33 antibody to assess its safety, tolerability, and other important profiles [48].

5.3. CT-P59

The CT-P59 is a nAb obtained from patients who had convalescent plasma therapy for treating the SARS-CoV-2 infection. This antibody hinders the interaction of the spike RBD with the host ACE2 receptor, and this antibody inhibits the viral replication capacity, thus, reducing the viral load. The Phase I trial was conducted in two stages to establish the safety, tolerability, and pharmacokinetic profile of the CT-P59 antibody in healthy volunteers as well as patients with mild symptoms. It is also proved to be very efficient against the evolving variants. For example, the administration of CT-P59 proved to reduce the viral load in the respiratory tract (upper and lower) against the Beta variant. Further trials are also in the process that will elucidate more about the safety, tolerability, and pharmacokinetics profiling of the antibody [49].

5.4. REGEN-COV

According to the information obtained from the reports of the three clinical trial phases, REGEN-COV proved to be an efficient nAb for COVID-19 disease. The first two phases indicate that the administration of REGEN-COV has potentially lowered the rate of hospitalizations for COVID patients. In addition, it also hinders viral replication capacity, leading to a lower viral load. REGEN-COV was able to lower the rate of mortality as well as intensive care support, and it reduced the symptoms caused by the SARS-CoV-2 infection. The REGEN-COV also proved to be a very efficient mAb for several emerging SARS-CoV-2 variants, namely the Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Delta (B.1.617.2) variants. In late 2020, due to the increased efficacy, REGEN-COV also achieved emergency approval from the FDA for administration in the SARS-CoV-2-infected patients with mild and moderate symptoms who did not require hospitalization support [50].

5.5. LY3819253/LY-CoV555

The administration of LY3819253 nAb was mainly on COVID-19 patients whose symptoms ranged from mild to moderate. The trial report indicated a reduction in the patients' viral load administered with the antibody compared to the placebo. Moreover, the safety profile analysis for the antibody is very convincing, suggesting it to be an efficient treatment method for COVID patients in an emergency. Even it proved its superiority in the various zones having high-risk patients. The administration also reduced the rate of hospitalization, in turn reducing mortality. In addition, the administration of LY3819253 showed no adverse effects other than diarrhea and vomiting in a few volunteers. It also possesses special features responsible for viral clearance within a very short time [51].

5.6. VIR-7831

The VIR-7831 is similar to the S309 antibody isolated from the patients who recovered from SARS-CoV-2 infection. S309 has also shown quite a good result in neutralizing the SARS-CoV-2 virus. VIR-7831 is being modified to enhance its ability to recognize the SARS-CoV-2 virus. The main aim behind engineering VIR-7831 is to make it capable of recruiting cytotoxic T cells, killing the virally infected cells effectively. In addition, antibody engineering will strengthen the safety, tolerability, and pharmacokinetic profile of VIR-7831. However, this antibody administration results in certain adverse outcomes, notably the formation of anti-drug antibodies against VIR-7831. Further trials are still undergoing and are expected to overcome the flaws, and therefore, VIR-7831 will be an effective treatment against treating the pandemic [52].

5.7. BGB DXP593

The exact mechanism that enables the BGB-DXP593 antibody to inhibit the SARS-CoV-2 virus entry into the host cell is not completely known. However, by analyzing the structural similarity of the SARS-CoV and SARS-CoV-2 researchers expect that the antibody employed for neutralizing SARS-CoV could be potentially be useful in treating the SARS-CoV-2 infection. This antibody possesses a complementary region named CDR3H, which targets the spike RBD. BGB-DXP593 mainly inhibits viral entry by CDR3H. Currently, the Phase 2 trial of this antibody is elucidating more about its efficiency and safety profile in preventing SARS-CoV-2 infection. This is mainly applied to COVID-19 patients having mild to moderate symptoms [52].

5.8. SCTA01

Like Etesevimab, SCTA01 also possesses LALA modification in the Fc region and hinders the interaction between the spike protein and the ACE2 receptor. It is also known as HB27, and is a member of the IgG1 antibody isotype. It shares some common functionality with the Bamlanivimab. The amino acid residue (LALA) mutation is responsible for eliminating the antibody-dependent cellular cytotoxicity and antibody-dependent enhancement properties. The pre-clinical reports of SCTA01 elucidated the safety and antiviral characteristic of this mAb. All the potential effects seen in the volunteers were mild and did not require additional support to cure them. The in vitro study of this nAb on mice and rhesus monkeys also highlighted its ability to lower the viral load [53].

5.9. DZIF-10c

The DZIF-10c is one of the most potent antibodies employed for treating the SARS-CoV-2 infection. The evolving mutations in several VOCs and VOIs are proven to impart immune escape properties. Studies have shown that DZIF-10c effectively neutralizes the virus in 16 prevalent mutations. The neutralizing inability was only observed in the case of the K444Q mutation. DZIF-10c possesses a much greater antiviral characteristic than the other antibodies, suggesting a more efficient neutralization. This antibody's safety and pharmacokinetic profiling highlighted its use for clinical purposes. In addition, the extended half-life and greater loads of neutralization titers make it more suitable for clinical

usage. It is completely capable of neutralizing the SARS-CoV-2 infection by the alpha variant (B.1.1.7), and it also plays a pivotal role in neutralizing the infection by the beta variant (B.1.351) [54].

5.10. SAB-185

According to the Phase I clinical trial reports, SAB-185 has shown a convincing safety and tolerability profile for future use. It is a very potent, full-human polyclonal antibody capable of neutralizing most of the evolving mutations. The common immune escape property of S477N, D614G, N501Y, and E484K are eliminated by the administration of SAB-185. This antibody is isolated from the specially engineered bovines by hyperimmunization. This polyclonal antibody can recognize a series of epitopes in the spike antigen. As a result, single-point mutations cannot alter their neutralizing capacity. This antibody has provided a potent neutralization for SARS-CoV-2 and many viruses, namely Ebola, Haantan, MERS-CoV, etc. [55].

5.11. COR-101

COR-101, also known as STE90-C11, targets the ACE2-RBD complex, affecting viral entry. The neutralizing effect of this antibody notably brings no difference in the case of the mutations in the RBD. These antibodies share some common features with the human germline genes (VH3-66 family). The selection of COR-101 is better for SARS-CoV RBD because it does alter its conformation, and there is no problem of any stearic clash. COR-101 also interacts with the CB6 and B38 epitopes of RBD, such as the other antibodies. COR-101 has successfully treated patients with mild to moderate infection symptoms. Moreover, another advantage of COR-101 is its interaction with the 473 to 476 amino acid residues, a harbor for many evolving mutations. It has shown a greater tolerance for most of the evolving variants such as Kappa, Delta, etc. [56].

5.12. Bamlanivimab and Etesevimab

This antibody cocktail comprises two mAbs, Bamlanivimab and Etesevimab resulting in the spike protein binding with the Fc fragment of the two and making it more efficient. A comparison of the effects of these nAbs with the placebo group after 3 to 11 days significantly reduced the viral load, and a minimal amount of people infected with COVID-19 required hospitalization support. A report from the Phase III clinical trial also indicated the efficiency of this antibody cocktail in high-risk groups of people. This nAb cocktail reduced the hospitalization and death rate by up to 70% compared to the placebo group [11].

6. nAbs Employed for Treating SARS-CoV-2 and Are in Pre-Clinical Trial

The numbers of nAbs against SARS-CoV-2 infection in the pre-clinical trial are listed in Table 3.

Table 3. Different neutralizing antibodies which are in the pre-clinical trial.

Sl. No.	nAb	International Nonpropreitary Name (INN)	Source	Type
1.	LY-CoV555	Bamlanivimab	Human B cells	mAb human IgG1
2.	JS016	Etesevimab + Bamlanivimab	Human B cells	mAb human, combination of 2 mAb
3.	LY-CoV016	Etesevimab + Bamlanivimab	Human B cells	mAb human, combination of 2 mAb
4.	LY3832479	Etesevimab + Bamlanivimab	Human B cells	mAb human, combination of 2 mAb
5.	REGN-COV2	Casirivimab + Imdevimab	Convalescent sources and immunization	mAb human

Table 3. *Cont.*

Sl. No.	nAb	International Nonpropreitary Name (INN)	Source	Type
6.	TY027	-	-	mAb
7.	BRII-196	-	Human B cells	mAb human
8.	BRII-198	-	Human B cells	mAb human
9.	CT-P59	Regdanvimab	Human B cells	mAb human
10.	SCTA01	-	-	mAb humanized
11.	SAB-185	-	Immunization	Polyclonal recombinant human Ab
12.	MW33	-	-	mAb human
13.	AZD7442	Tixagevimab + Cilgavimab	Human B cells	mAb human
14.	VIR-7831	Sotrovimab	Human B cells	mAb human
15.	DXP-593	-	Human B cells	mAb
16.	Anti-SARS-CoV-2 mAb	-	-	mAb, chicken IgY
17.	ABBV-47D11	-	Immunization	mAb human IgG1
18.	DXP604	-	Human B cells	mAb
19.	COVI-AMG (STI-2020)	-	In vitro libraries	mAb human
20.	C144-LS and C-135-LS	-	-	Mixture of 2 mAb
21.	ADG20	-	Human B cells	mAb human
22.	COR-101	-	In vitro libraries and human B cells	mAb human

6.1. AR-712

The antibody cocktail is a way of treating the present VOCs and VOIs. AR-712 is a cocktail that efficiently neutralizes the dominating Delta variant. This mAb cocktail, developed by Aridis Pharmaceuticals, was self-administered to COVID-19 patients and did not require any hospitalization. This cocktail was identified from the convalescent plasma of the SARS-CoV-2 infected patients. It consists of two IgGs isolated from the B-cells of these patients [57].

6.2. IMM-BCP-01

The IMM-BCP-01, developed by Immunome Inc., is an antibody cocktail with great potential in neutralizing the Delta variant of the SARS-CoV-2. According to the reports published in late July 2021, this nAb will enter the first phase of the clinical trial. It is a cocktail functional with three mAbs and targets nearly three non-overlapping epitopes of this virus. It will also collaborate with the US FDA to submit an Investigational New Drug Report before entering the clinical trial [57].

6.3. SPKM001

An anti-SARS-CoV-2 nAb developed by SpikImm and Institut Pasteur is the SPKM001. It is expected to enter the clinical trial in Europe, Brazil, and North America by early 2022. It has effectively neutralized most VOCs and VOIs such as Alpha, Delta, Beta, Gamma, and Delta Plus. It has a strong binding affinity towards the RBD of SARS-CoV-2, thus hindering the interaction of these variants with the host receptors [57].

7. New Emerging SARS-CoV-2 Variants and Possible Therapeutic Interventions

With the advent of time, several new mutations have accumulated in the SARS-CoV-2 viral genome. These mutations have altered the characteristics of the virus in terms of

transmissibility and infectivity. On this basis, the WHO and CDC categorized emerging variants as VOC and the VOI. Several mutations in these variants confer the ability to escape the mAbs and vaccines [58,59].

7.1. B.1.1.7 (Alpha)

This variant was first detected in the UK. Several mAbs, including antibody cocktails, have been found to be very potent in neutralizing this variant. For instance, the COVOX-222 has an efficient adequate neutralizing system. It interferes with the amino acid substitution at the 417 positions and neutralizes this variant efficiently [59]. Due to the presence of the N501Y mutation, the interaction with ACE2 and RBD is strengthened, facilitating increased neutralization. Another unique antibody cocktail, LYCoV-555 (Bamlanivimab + Etesevimab), has a very strong binding affinity with the RBD in both possible conformations and is not afflicted by the substitution of Y501. According to Jiejie Geng and his colleagues, CD147 is very active in blocking viral entry into the host cells [60]. CD147 has a neutralization efficiency nearly equal to 69% at a certain concentration. It also prevents the building of cytokine storms in individuals infected with the virus. A combination of casirivimab and imdevimab has shown efficient neutralization efficiency in the case of the Alpha variant. This combination attaches to both sides of the RBD of the S-glycoprotein [60,61].

7.2. B.1.351 (Beta)

This lineage isolated from South Africa possesses several missense mutations and deletions. The neutralizing mAb MG1141A has been extremely proficient in neutralizing the B.1.351 variant. In addition to neutralization, MG1141A also plays a pivotal role in viral clearance by utilizing the immune cells' property to undergo phagocytosis [62]. As previously stated, CD147 functions to prevent the entry of the Beta variant. In the case of B.1.351, the neutralization efficiency is nearly 75%. Another antibody cocktail, Tixagevimab and Cilgavimab, is very potent in dominating the B.1.351 variant [63]. It can significantly identify the non-conserved epitopes residing in the RBD of the S-glycoprotein and inhibit the viral entry into the host cells.

7.3. P.1 (Gamma)

The first evidence of the Gamma variant was made in Manaus, Brazil. It consists of several mutations, making it inevitable that the available antibodies will neutralize it. Many antibodies neutralizing the alpha and beta variants have also efficiently blocked P.1 [60]. The mAbs such as COVOX-222 and COVOX-253 follow a common mechanism of neutralization, i.e., they interact with the ACE2 receptor, making it difficult to bind with the RBD of the spike protein. However, casirivimab, a potent nAb, is inefficient at blocking the Gamma variant. It needs to be combined with imdevimab to accelerate the neutralization efficiency [64]. The most common antibody that can neutralize most of the evolving SARS-CoV-2 variants is CD147 or meplazumab. In the case of P.1, its neutralization efficiency stands at nearly 50% [59].

7.4. B.1.617.2 (Delta)

This VOC, which dominated the second wave of the COVID-19 pandemic, was isolated from India. It differs from the other VOCs to a greater extent, possessing a single mutation (D614G) common with the others. The exclusive mutations L452R and T478K make this variant extremely contagious with more virulence. According to the data highlighted in Table 4, it is evident that the commonly used antibodies for Alpha and Beta variants have shown significant neutralization efficacy for Delta. The mechanism of action of these neutralizing antibodies is also the same in this case, as discussed earlier.

7.5. B.1.1.529 (Omicron)

According to Zeng et al., the Omicron variant confers a wider ability to escape antibodies compared to the other variants [65]. The structural modeling and sequence-based study also stated that the improved binding affinity of Omicron S-protein with the hACE2 receptor caused increased virulence [66]. The Omicron variant has more mutations than any other previously reported SARS-CoV-2 variant. It possesses 50 mutations, out of which 32 pertain to the spike protein, which is the target site for most vaccines to neutralize the virus. Many mutations are novel and not found in the previous viral variants. Specifically, the variant is characterized by 30 amino acid changes, three small deletions, and one small insertion in the spike protein compared with the original virus, of which 15 are located in the receptor-binding domain (residues 319–541) [67]. The nAbs Sotrovimab, Paxlovid, and molnupiravir have shown efficiency in the case of this variant. Due to a large number of residing mutations, the Omicron variant is highly resistant to antibody cocktails [68–70]. Many resistant mutations residing in the spike protein of the Omicron variant are responsible for lowering the Ab titers elicited by the vaccination [71]. Rather, a single antibody is more efficient in combating the SARS-CoV-2 infection. Sotrovimab is extremely efficient in binding to the conserved antigenic epitopes rather than the non-overlapping ones. However, the most efficient antibody that can combat the Omicron variant is molnupiravir. Upon entering the host cell, molnupiravir interferes with the viral replication of the B.1.1.529 variant, a unique property that is possessed by an antibody [72]. Thus, molnupiravir can neutralize this variant to a greater extent.

Table 4. Emerging variants of SARS-CoV-2 and nAb, which are in the pre-clinical and clinical stage.

Sl. No.	Name of the Variant	Effective nAb against the SARS-CoV-2 Variants	Reference
1.	B.1.1.7 (Alpha)	CD147 (Meplazumab), COVOX-222, COVOX-253, A23-58.1, MG1141A, Sotrovimab, Casirivimab + Imdevimab, Bamlanivimab + Etesevimab, Tixagevimab + Cilgavimeb	[59–61]
2.	B.1.351 (Beta)	CD147 (Meplazumab), MG1141A, Casirivimab + Imdevimab, Sotrovimab, Tixagevimab + Cilgavimeb	[59,60,62]
3.	P.1 (Gamma)	CD147 (Meplazumab), COVOX-222, COVOX-253, A23-58.1, Sotrovimab, Casirivimab + Imdevimab, MG1141A, Tixagevimab + Cilgavimeb	[59,60,62]
4.	B.1.617.2 (Delta)	CD147 (Meplazumab), A23-58.1, Sotrovimab, Casirivimab + Imdevimab, Bamlanivimab + Etesevimab, Tixagevimab + Cilgavimeb	[60,62,67]
5.	B.1.1.529 (Omicron)	Sotrovimab, Paxlovid, molnupiravir	[73,74]

8. Heavy Chain Antibodies (HCAbs) against SARS-CoV-2

Heavy chain antibodies (HCAbs) are specialized active antibody fragments that are not associated with the light chains, and their VH (variable heavy) regions are also functional as a part of a single unit (Figure 8(A1,A2)) [75]. The VH regions serve as perfect building blocks for several antibody-based treatments as they permit the addition of molecules in sequence to construct multispecific antibodies. The HCAbs possess a unique paratope that interacts with the variable domain of the heavy chain without involving any light chain domains (Figure 8B). These classes of antibodies originated from the camelid species and were found to be extremely proficient in treating COVID-19. They are more immunogenic than conventional ones and possess some unique physical properties that help in the larger production of these antibodies. These HCAbs, however, have a lower affinity for binding with the antigenic epitope and are easily excreted by the kidney. The HCAbs (especially nanobodies) can even be used to detect the presence of the SARS-CoV-2 virus (Figure 8(C1–C3)). These nanobodies are capable of being inhaled by the patients, and, thus, can be used to prevent viral replication in the lungs. Mostly, the RBD of the spike protein is a potential target of HCAbs [76,77].

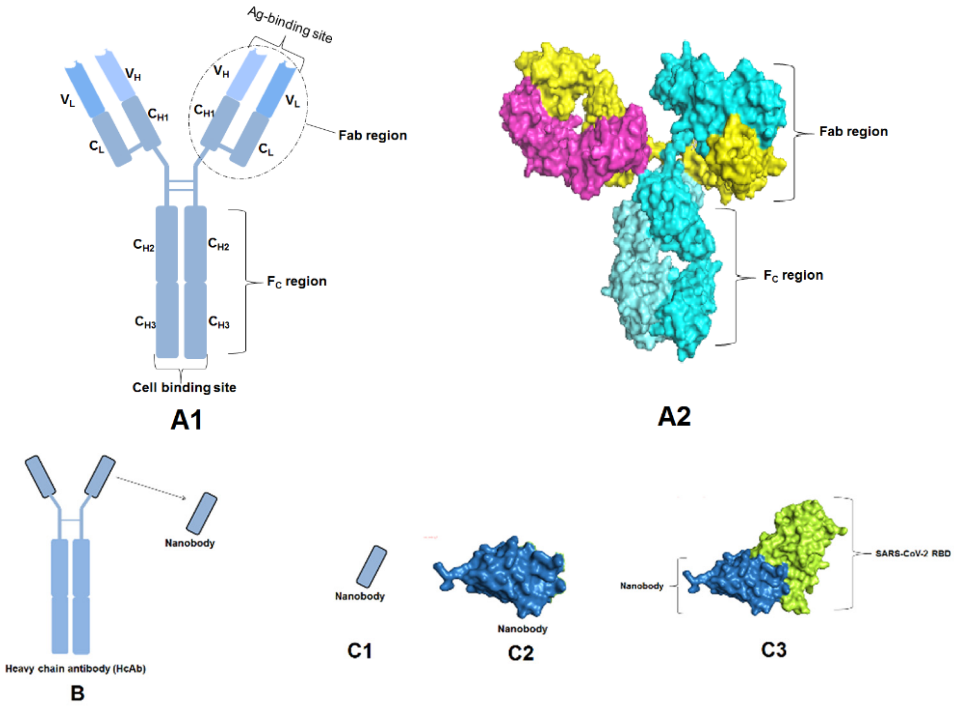

Figure 8. Structure of heavy chain antibodies and its parts. (**A1**) Schematic structure depicted different parts. (**A2**) Surface structure shows Fc and Fab region. (**B**) Heavy chain antibodies along with the nanobody. (**C**) Nanobody complex with the SARS-CoV-2 RBD.

9. Single Domain Antibody against SARS-CoV-2

To establish an efficient therapeutic against the SARS-CoV-2, heavy chain single domain antibodies (sdAb) have shown promising results. Experiments suggest that the competitive binding of these sdAbs plays a pivotal role in hindering the interaction between the hACE2 receptor and the viral RBD [75,78]. Moreover, fusing the IgG1 Fc with these sdAbs accelerates their neutralizing efficiency to a greater extent. These antibodies, also called nanobodies, have the antigen-binding capacity to a greater extent, making them an effective tool for designing therapeutics to eradicate this global outbreak. These sdAbs are cost-effective and comparatively more stable than nAbs [75]. Considering the present scenario of COVID-19 treatment with the administration of certain vaccines and antibody therapy, the development of the sdAbs will be more susceptible to treating the infection. The administration of these antibody cocktails is believed to give a more durable protective response than the current therapeutics [78]. According to several pieces of research, sdAbs are extremely efficient in targeting the epitopes in the RBD of the SARS-CoV-2 variants [75]. These epitopes, in turn, are responsible for the extremities caused to human health upon the viral entry. The extraordinary feature of these antibodies makes them compatible with being used as a particle delivery system. sdAbs can be delivered into the lungs through nasal delivery as well as the gastrointestinal tract to prevent the interaction of the virus with the ACE2 receptor [79,80]. Studies also suggest that IgA is a better neutralizing tool than IgG; thus, the fusion of IgA with the sdAb's will be extremely efficient in serving as a diagnostic tool to eradicate the pandemic.

10. Conclusions

An efficient strategy to combat this pandemic is the administration of nAbs. The FDA has approved several mAbs for use against SARS-CoV-2. A brief timeline depicting the development of FDA approval for the mAbs against SARS-CoV-2 with their mechanism of neutralization is shown in the Figure 9.

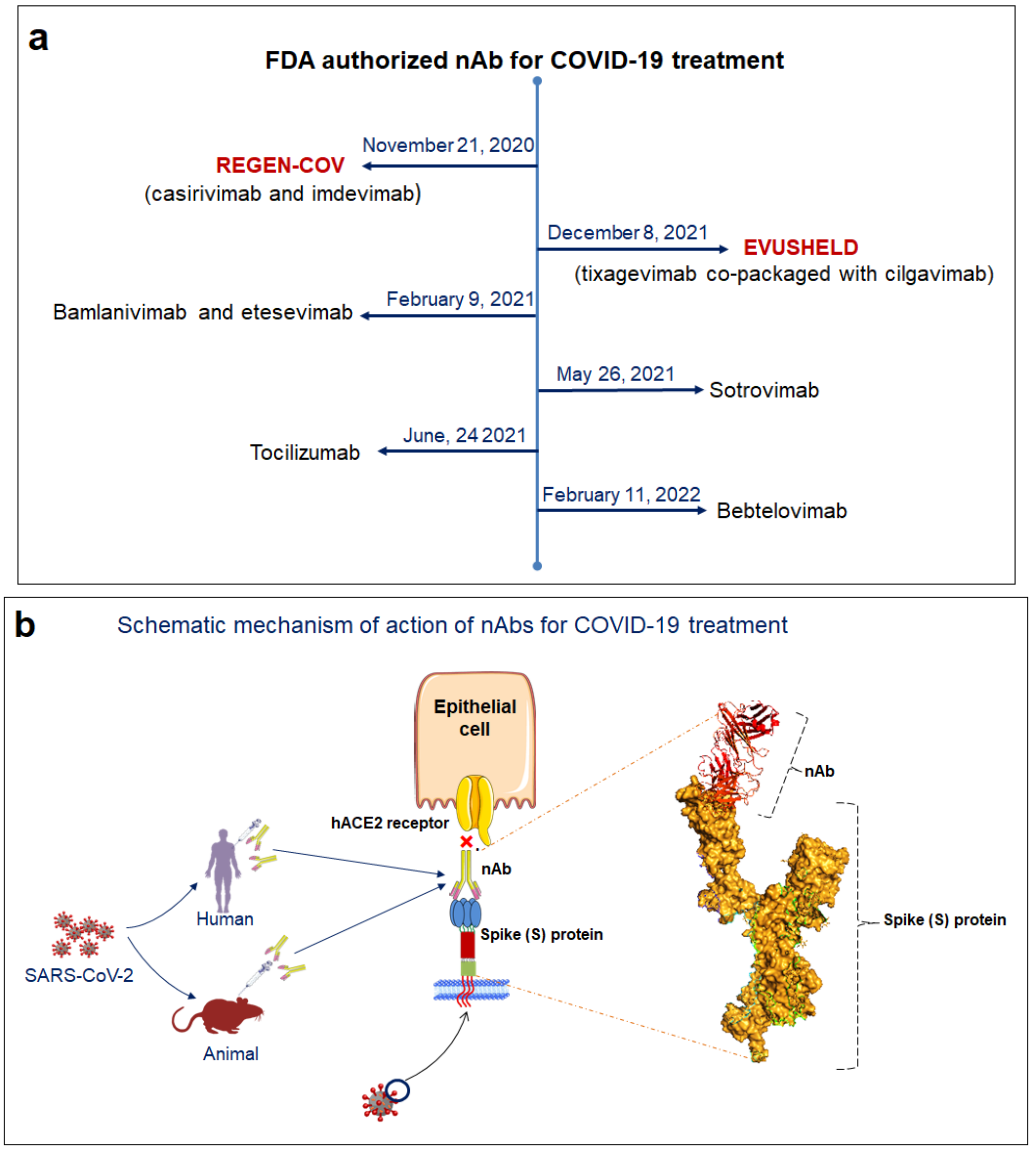

Figure 9. FDA approved mAbs against SARS-CoV-2 and their mode of action. (**a**) Timeline depicting the development of FDA approval for the mAbs against SARS-CoV-2. (**b**) The schematic diagram illustrated the mechanism of action of nAbs. It shows the neutralization of SARS-CoV-2 spike protein.

These nAbs have given promising results in minimizing the virulence of the SARS-CoV-2 virus. Several antibody treatments administered by following an appropriate dosage are considered prophylactic measures for treating severe patients. Before the emergence of vaccines, this therapeutic strategy provided a bit of relief to the world in controlling the havoc. Moreover, the administration of combined antibodies (known as the antibody cocktail) has been extraordinarily efficient for the evolving mutants, reducing the chance of escaping the immune system. Several subsets of nAbs isolated from SARS-CoV and MERS-CoV patients had also shown viral neutralization capacity in SARS-CoV-2 patients. Studies also highlight that the administration of these antibodies at an early stage will be more helpful for the population in preventing COVID-19. In turn, high-throughput engineering strategies can be used to construct more neutralizing antibodies with a very high binding affinity, thereby providing great relief for the entire world.

Author Contributions: M.B.: Validation, Formal analysis, Investigation, Figure development, Writing—Original Draft. S.C.: Data curation, Resources, Investigation, Writing—Original Draft. B.M.: Validation; Figure development. A.R.S.: Investigation, Formal analysis, Validation, Writing—

Review & Editing, Fund acquisition. C.C.: Conceptualization, Methodology, Project administration, Writing—Review & editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: This study was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (NRF—2020R1C1C1008694).

Conflicts of Interest: The authors declare no competing interests.

Abbreviations

nAb	Neutralizing Antibody
mAb	Monoclonal Antibody
RBD	Receptor binding domain
ACE2	Angiotensin-converting enzyme 2
S-protein	Spike glycoprotein
NTD	N-terminal domain
CPT	Convalescent plasma therapy
ADE	Antibody-dependent enhancement
HCAbs	Heavy chain antibodies
IL-6	Interleukin 6
CDRH	Complementarity-determining regions of heavy-chain
FDA	Food & Drug Administration
IgA	Immunoglobulin A
MERS-CoV	Middle East respiratory syndrome coronavirus
VH	Variable domain heavy chain
VOI	Variant of Interest
VOC	Variant of Concern

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ISBN 978-3-7258-2039-9