



## Short communication

# Neutralizing antibody responses in healthcare personnel after three doses of mRNA BNT162b2 vaccine and association with baseline characteristics and past SARS-CoV-2 infection



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## ABSTRACT

**Aim:** To estimate neutralizing antibody (NAb) immunity against SARS-CoV-2 in 739 healthcare personnel (HCP) vaccinated with three doses of BNT162b2 mRNA vaccine.

**Methods:** Serum samples were collected at 3, 6, and 9 months after the second vaccine dose and at 7–55 days after the third dose. Samples were tested for NAb against SARS-CoV-2 receptor binding domain.

**Results:** The mean inhibition rates at 3, 6, and 9 months after the second dose were 86.33%, 73.38%, and 61.18%, and increased to 95.57% after the booster dose. Younger HCP and HCP with past SARS-CoV-2 infection had higher inhibition rates while there was an inverse correlation between NAb levels and comorbidities or tobacco use ( $p$ -values < 0.001). Increased NAb titers were also noticed in women ( $p$ -value = 0.033), especially at the end of the 9-month study period.

**Conclusion:** NAb levels increased considerably after a booster mRNA vaccine dose. Host factors and past SARS-CoV-2 infection influence NAb titers.

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## 1. Introduction

Within less than one year after the declaration of the coronavirus disease 2019 (COVID-19) pandemic, the first mRNA COVID-19 vaccines were authorized for emergency use as a key control measure [1,2]. Yet, humankind continues to experience serious morbidity and mortality [3,4]. According to the World Health Organization, as of 3 July 2022, over 546 million confirmed cases and over 6.2 million deaths have been reported globally [5]. Although it is almost certain that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) will become endemic the next years, there are uncertainties regarding the characteristics of transmission to endemicity in light of the emergence of new virus variants, the duration of immunity after natural infection or vaccination, and the suboptimal COVID-19 vaccination rates almost globally [6–9].

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Neutralization assays have been widely used to study humoral immunity against SARS-CoV-2 [10–12]. Neutralizing antibody (NAb) immunity is elicited post-natural infection or is vaccine-induced and confers cell protection from virus intrusion which is mediated through binding to angiotensin-converting enzyme 2 (ACE2) receptor [10]. There are published data on NAb immunity after three doses of mRNA vaccines [13–17]. We studied NAb responses after three doses of mRNA BNT162b2 vaccine in healthcare personnel (HCP) in Greece. The association of NAb immunity with the characteristics of HCP and a laboratory-confirmed past SARS-CoV-2 infection was also investigated.

## 2. Methods

## 2.1. COVID-19 vaccination campaign

In Greece vaccination of HCP started on January 4, 2021. Two doses of Pfizer-BioNTech BNT162b2 mRNA vaccine are administered three weeks apart. From September 2021 a third (booster) dose was given to HCP.

## 2.2. Study design, data collection, and sera sampling

The study was conducted from April to December 2021 at Red Cross General Hospital, a COVID-19 referral hospital in Athens. HCP who had received three doses of BNT162b2 mRNA vaccine were eligible to participate. The following data were collected using one questionnaire per participant: age, gender, comorbidities, use of immunosuppressive drugs, body mass index (BMI), tobacco smoking, regular exercise, and mild post-vaccination side-effects. The dates of COVID-19 vaccinations and past SARS-CoV-2 infections (if any) were retrieved from the National COVID-19 Vaccination Registry and the National Registry of SARS-CoV-2 Infections, respectively. Participants were invited for serum sampling at 3, 6, and 9 months after the second vaccine dose and at 7–55 days after the third dose. The survey and sera sampling were conducted concomitantly and prospectively.

## 2.3. Detection of total neutralizing antibodies against SARS-CoV-2

Serum samples were tested for NABs against SARS-CoV-2 receptor binding domain (RBD) with GenScript NAb detection kit (GenScript, Piscataway, USA). The GenScript NAb detection kit is a FDA-approved commercial kit with high sensitivity and specificity, and perfectly correlating results with gold standard methods Virus Neutralization Tests (VNT; conventional or pseudovirus-based) and Plaque Reduction Neutralization Tests (PRNT) [18]. Negative neutralization ability was defined as a neutralization rate of < 30% [18]. The inhibition rate of a sample was calculated as follows:  $[1 - \text{optical density (OD) of sample} / \text{OD of negative control}] \times 100$ . OD was measured at 450 nm. Residual serum samples collected before 2018 were used as negative controls.

## 2.4. Diagnosis of SARS-CoV-2 infection

RNA was extracted from nasopharyngeal swabs using the STAR-Mag 96 Kit (Seegene Technologies), followed by real-time PCR using the Allplex SARS-CoV-2 Assay. PCR amplification was run on a CFX96 real-time thermal cycler (Bio-Rad Laboratories) and data were analyzed with the SARS-CoV-2 Viewer (Seegene).

## 2.5. Detection of SARS-CoV-2 variants of concern (VOC) and of interest (VOI)

The following SARS-CoV-2 mutations were detected with Allplex™ SARS-CoV-2 Variants I and II Assay according to manufacturer instructions: E484K, HV69/70 deletion, N501Y (S gene), and mutations K417N, K417T, L452R, W152C (S gene), respectively.

## 2.6. Flow cytometry analysis

Samples of EDTA-anticoagulated peripheral blood were collected from patients and tested within 6 h. CD3<sup>+</sup>/CD4<sup>+</sup>/CD8<sup>+</sup> T cells, CD19<sup>+</sup> B cells, CD3<sup>+</sup>/CD16<sup>+</sup>/CD56<sup>+</sup> NK cells and TCRγδ T cells (cells/μl) were measured via multiple-color cytometry analysis Beckman Coulter.

## 2.7. Statistical analysis

Frequencies and percentages were used for categorical variables. Comparisons were performed by using the two-tailed *t*-test for continuous variables and the chi-square test for categorical variables. *P*-values < 0.05 were considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics 21 software.

## 2.8. Ethical approval

The study protocol was approved by the Scientific Committee of the hospital. Informed consent was obtained from participants. The study was performed in compliance with the national and European regulations.

## 3. Results

A total of 739 HCP who had received three doses of BNT162b2 mRNA vaccine were studied. Table 1 shows their characteristics.

A total of 486 HCP (66.3%) provided a first serum sample at a mean of 95.3 (range: 52–135) days after the second vaccine dose. Of them, 426 HCP (84.0%) had a neutralization level of > 75%, indicating a high protective level, while only 4 (0.8%) had negative neutralization ability. Overall, the mean inhibition rate at first sampling was 86.33%. A second serum sample was collected in 473 HCP at a mean of 184.5 (range: 136–225) days after the second dose. The mean inhibition rate of the second sample was 73.38%, while 14 HCP (3.0%) had no neutralization ability (Fig. 1A). A third serum sample was available in 212 HCP at a mean of 262.9 (range: 226–322) days after the second vaccine dose. The mean inhibition rate in the third measurement was 61.18%, while 22 HCP (10.4%) had no neutralization activity (Fig. 1A).

In order to study the impact of a booster dose on NAb levels, serum samples were collected from 102 HCP at a mean of 21.5 (range: 7–55) days after the third vaccine dose. The NAb levels increased dramatically to 95.58% (Fig. 1A). The neutralization level in the control group did not exceed the limit of 30% (average 12.24%, maximum 24.02%). Subsequently, we focused on individuals

**Table 1**  
Characteristics of tested HCP

Characteristic	N (%)
(n = 739)	
Age, years (n = 714)	
< 40	207 (29.0%)
40–59	435 (60.9%)
> 60	72 (10.1%)
Gender (n = 739)	
male	238 (32.2%)
female	501 (67.8%)
Comorbidities <sup>1</sup> (n = 498)	118 (23.7%)
Immunosuppressive drugs (n = 511)	19 (3.7%)
BMI (n = 467)	
< 20	32 (6.9%)
20–25	188 (40.2%)
> 25–30	167 (35.8%)
> 30	80 (17.1%)
Smoking (n = 510)	
yes	167 (32.7%)
no	343 (67.3%)
Regular physical exercise (n = 513)	
yes	111 (21.6%)
no	402 (78.4%)
SARS-CoV-2 infection <sup>2</sup> (n = 739)	72 (9.7%)
before vaccination	37
before 1st sampling <sup>3</sup>	13
before 2nd sampling <sup>4</sup>	3
before 3rd sampling <sup>5</sup>	19
Mild side effects post-vaccination (n = 510)	269 (52.7%)

n: the number of participants who answered this question; HCP: healthcare personnel; BMI: body mass index; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; RT-PCR: real-time polymerase chain reaction.

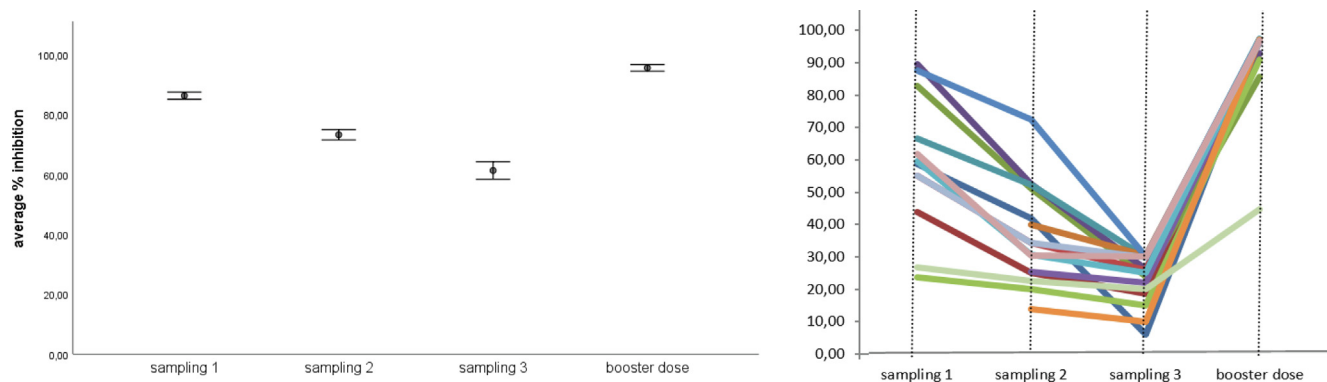
<sup>1</sup> Co-morbidities included diabetes mellitus (26 HCP), autoimmune disorder (44 HCP), and other (57 HCP).

<sup>2</sup> confirmed by RT-PCR.

<sup>3</sup> after 2nd vaccine dose.

<sup>4</sup> after first sampling and before third vaccine dose.

<sup>5</sup> after second sampling and before third vaccine dose.



**Fig. 1.** (A) Mean inhibition rates in HCP by sampling period, (B) inhibition rates in selected individuals that had negative neutralization ability before booster dose. Error Bars 95% CI

Sampling 1: samples collected approximately 3 months after the second vaccine dose (mean: 95.3 days, range: 52–135 days). Sampling 2: samples collected approximately 6 months after the second vaccine dose (mean: 184.6 days, range: 136–225 days). Sampling 3: samples collected approximately 9 months after the second vaccine dose (mean: 262.9 days, range: 226–322 days). The effect of booster dose was estimated approximately 3 weeks after the booster dose (mean 21.5 days, range: 7–55 days) HCP: healthcare personnel; CI: confidence interval

who had no neutralization ability (<30% inhibition rates) before the booster dose. In almost all these cases the inhibition rate exceeded 90%, with the exception of one case, that was further studied (Fig. 1B). At this particular case (54 year old woman with no comorbidities), lymphocyte subpopulations were determined by flow cytometry. A low percentage (16%) and absolute number (255 cells/ $\mu$ l) of CD3<sup>+</sup>/CD4<sup>+</sup> T cells and a high percentage (20.6%) of TCR $\gamma$  $\delta$ T lymphocytes were found.

Table 2 shows the mean inhibition rates according to HCP characteristics. HCP with no history of past SARS-CoV-2 infection noted

significant reduction of mean inhibition rates during the 9-month study period. In contrast, there was no significant reduction of the mean inhibition rate during the 9-month period after the second vaccine dose in HCP with past SARS-CoV-2 infection (mean inhibition rates: 95.41%, 92.29%, and 93.62%, respectively) (Table 2, Fig. 2A).

We further investigated the association between NAb levels at 3, 6, and 9 months after the second vaccine dose and baseline characteristics of HCP (Table 2). Women had significantly higher mean inhibition rates than males in all three measurements (p-

**Table 2**

Mean inhibition rates per time sampling<sup>1</sup> and characteristic of HCP

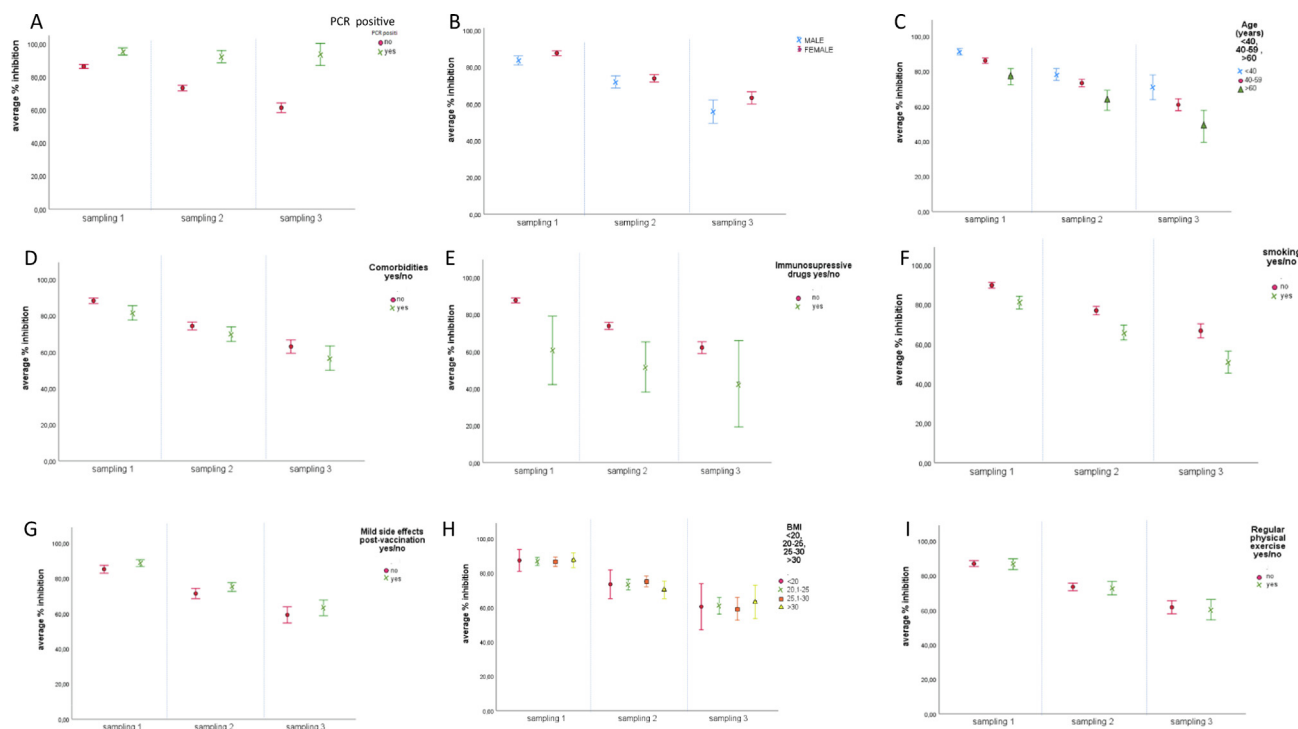
	First sampling		Second sampling		Third sampling		
	n	mean inhibition (%)	n	mean inhibition (%)	n	mean inhibition (%)	p-value
SARS-CoV-2 infection <sup>2</sup>							
no	507	86.33 (13.96)	473	73.38 (19.27)	212	61.18 (21.62)	< 0.001
yes	37	95.41 (6.50)	37	92.29 (10.98)	18	93.62 (13.04)	
Gender							
male	163	83.56 (16.30)	139	72.08 (20.17)	55	55.91 (22.98)	0.033
female	344	87.64 (12.51)	334	73.92 (18.88)	157	63.02 (20.88)	
Age (years)							
<40	133	91.16 (10.96)	115	78.60 (18.29)	39	70.02 (21.54)	< 0.001
40–59	306	85.16 (13.53)	297	73.31 (18.63)	149	60.83 (21.01)	
≥60	55	76.93 (17.20)	53	63.37 (20.80)	21	48.49 (19.99)	
Mild adverse events <sup>3</sup>							
yes	163	88.56 (12.75)	208	75.16 (18.40)	91	63.18 (21.59)	0.023
no	175	85.30 (14.80)	179	71.43 (19.63)	89	59.27 (21.54)	
Comorbidities							
yes	76	81.52 (17.55)	89	69.81 (19.30)	44	56.57 (22.11)	< 0.001
no	255	88.30 (12.41)	291	74.43 (18.94)	134	63.0 (21.17)	
Immunosuppressive drugs							
yes	10	60.61 (25.79)	13	50.81 (22.41)	7	42.56 (20.23)	< 0.001
no	328	87.68 (12.64)	374	73.87 (18.80)	174	62.07 (21.13)	
BMI							
< 20	18	87.29 (12.60)	22	73.46 (18.74)	13	60.43 (22.21)	0.780
20–25	122	86.84 (12.96)	139	73.39 (18.35)	75	60.99 (20.48)	
25.1–30	118	86.44 (14.95)	128	75.32 (17.97)	51	59.14 (23.54)	
> 30	52	87.39 (15.49)	65	70.22 (20.70)	22	63.18 (21.92)	
Smoking							
yes	114	80.93 (17.32)	123	65.82 (21.04)	63	51.06 (21.80)	< 0.001
no	224	89.84 (10.64)	264	76.96 (16.95)	117	66.67 (19.43)	
Regular physical exercise							
yes	74	86.89 (13.12)	75	72.86 (16.71)	47	60.28 (20.13)	0.458
no	266	86.90 (14.12)	312	73.57 (19.58)	134	61.69 (22.10)	

n: number of samples; HCP: healthcare personnel; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; BMI: body mass index.

<sup>1</sup> after the 2nd vaccine dose

<sup>2</sup> 92 samples collected from 72 HCP with SARS-CoV-2 infection

<sup>3</sup> after vaccination



**Fig. 2.** Mean inhibition rates per time sampling<sup>1</sup> and: (A) SARS-CoV2 infection: ● no past SARS-CoV-2 infection and × past SARS-CoV-2 infection; (B) gender: × male and ● female; (C) age: × < 40 years old, ● 40–59 years old and ▲ > 60 years old; (D) comorbidities: ● no, × yes; (E) use of immunosuppressive drugs: ● no, × yes; (F) tobacco use: ● no, × yes; (G) mild adverse events post vaccination: ● no, × yes; (H) BMI: ● < 20, × 20.1–25, ■ 25.1–30 and ▲ > 30; (I) regular physical exercise: ● no, × yes.

**Sampling 1:** samples collected approximately 3 months after the second vaccine dose (mean: 95.3 days, range: 52–135 days). **Sampling 2:** samples collected approximately 6 months after the second vaccine dose (mean: 184.6 days, range: 136–225 days). **Sampling 3:** samples collected approximately 9 months after the second vaccine dose (mean: 262.9 days, range: 226–322 days). Error Bars 95% CI SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; BMI: body mass index; CI: confidence interval

value = 0.033), mainly in the third measurement ( $p$ -value < 0.001) (Fig. 2B). Stratification of NAb levels by age showed a negative correlation with age. In particular, HCP < 40 years and HCP 40–59 years old had significantly higher inhibition rates at 3, 6, and 9 months after the second vaccine dose compared to HCP ≥ 60 years old ( $p$ -value < 0.001) (Fig. 2C). On the other hand, there was a significant negative correlation between NAb levels and the presence of comorbidities, immunosuppressive drugs or regular tobacco smoking ( $p$ -value < 0.001 for all comparisons) (Fig. 2D, 2F). Furthermore, HCP with mild adverse events post-vaccination had significantly higher mean inhibition rates compared with HCP without mild adverse events ( $p$ -value = 0.023) (Fig. 2G). There was no significant association between regular physical exercise or BMI and NAb levels.

Among 72 HCP with a history of past SARS-CoV-2 infection, 35 were infected after COVID-19 vaccination, including 10 within 90 days post-vaccination, 3 within 91–180 days post-vaccination, and 22 > 180 days post-vaccination. All tested SARS-CoV-2 samples were identified as B.1.1.7 or B.1.617 variants. According to our results, high levels of NAb did not prevent SARS-CoV-2 infection, which is in accordance with the fact that very high levels of NAb were detected few days before SARS-CoV-2 infection in several cases. For example, there was one HCP with SARS-CoV-2 infection 14 days after NAb measurement estimated at 91.7%, and another case of infection 12 days after NAb measurement estimated at 93.5%. None of these cases required hospitalization.

#### 4. Discussion

In order to study the humoral immunogenicity of the mRNA BNT162b2 COVID-19 vaccine, we estimated the NAb levels in

serum samples of 739 HCP collected at 3, 6, and 9 months after the second vaccine dose and at 7–55 days after the booster vaccine dose. Breakthrough infections in HCP after full COVID-19 vaccination may jeopardize healthcare services [19]. Although there are published data on NAb levels after a third mRNA vaccine dose [13–17], further studies are needed to define the magnitude and duration of immunity among HCP after a booster dose.

According to others [8,9], we found that four out of five vaccinated HCP had strong evidence of a high protective level 3 months after the second mRNA vaccine dose (86.33% inhibition). Nonetheless, in our series, NAb titers gradually waned and ended to 73.38% and 61.18% 6 and 9 months post-vaccination, respectively. A study which was conducted to assess the impact of several SARS-CoV-2 variants on antibodies elicited by mRNA vaccine showed that immune responses waned but were still detectable 6 months post-vaccination in most individuals, along with functional B cells and induction of long-term humoral and cellular immunity [20].

In our series, NAb levels increased almost uniformly to > 90% inhibition rate after a booster dose. Similarly, a mean inhibition rate of 95.9% was detected one month after a third (booster) dose administered to HCP nine months post-full vaccination with the mRNA BNT162b2 vaccine [14]. Another recently published study among 328 HCP showed that a BNT162b2 booster dose increased antibody levels 4-fold compared to the second dose, inducing cross-protective neutralizing antibodies against several SARS-CoV-2 variants [16]. Robust humoral responses were also noted after a BNT162b2 third dose, superior to those recorded after the second dose, among immunocompetent and immunocompromised individuals [15], as well as among persons > 60 years old [17].

Another finding of our study is the significant association between host factors and NAb response after vaccination. In our



series, women and younger HCP had significantly higher inhibitory rates throughout the 9-month study period after the second vaccine dose. Recent evidence indicates an association between age, gender, host factors, and post-vaccination immune responses after COVID-19 vaccination [21]. In this latter study, significantly greater antibody titers after a single dose of mRNA vaccine have been detected in younger and normal-weight individuals [21]. Similarly, weaker immune responses have been induced after a booster dose in older HCP (55–65 years old) compared to 35–54 years age-group [16]. In our series we found no significant association between NAb levels and BMI. However, there was a significant inverse association between NAb levels, comorbidities, and tobacco smoking. Studies show evidence of host-associated variations in immune responses to other vaccines, including influenza and measles-mumps-rubella vaccines [22,23]. It is well established that vaccine-induced immunity against influenza gradually wanes over time and this may particularly affect immunocompromised individuals and the elderly [21].

We also investigated the role of past SARS-CoV-2 infection and found that a history of laboratory-confirmed infection was associated with sustained high NAb immune responses in all three measurements after the second vaccine dose. High levels of anti-RBD IgG and NAb titers have been also detected after only one dose of mRNA vaccine among most vaccinated persons recovered from COVID-19 [12].

A limitation of the current study is the fact that four serum samples were not available in all participants. Another limitation is that there were no samples available beyond 55 days after the booster dose. The role of repeated, thought undiagnosed, natural exposure to SARS-CoV-2 which may boost HCP immunity should also be considered. A clear strength is the prospective and concomitant collection of serum samples and data from a large number of HCP, which gave us the opportunity to explore the role of host characteristics. Finally, data about vaccinations and past SARS-CoV-2 infections were retrieved from the national registries.

In conclusion, our study offers insight on NAb immune responses after three doses of mRNA vaccine. In addition, our study provides information to understand the association between host factors, past SARS-CoV-2 infection, and immunogenicity after mRNA vaccination. Higher NAb titers after two doses of mRNA vaccine were detected over a 9-month period in women, younger individuals, no smokers, and individuals with past SARS-CoV-2 infection. A booster vaccine dose was associated with high NAb levels. Further studies are needed to explore the duration of immune responses after a third vaccine dose.

## Declaration of Competing Interest

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

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