Serological Screening of Immunoglobulin G against SARS-CoV-2 Nucleocapsid and Spike Protein before and after Two Vaccine Doses among Healthcare Workers in Japan

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This study sought to evaluate the effects of two vaccine doses and the extent of SARS-CoV-2 infection among healthcare workers. We measured immunoglobulin G antibody titers against SARS-CoV-2 nucleocapsid and spike protein among healthcare workers at Gunma University Hospital. In March 2021, prior to BNT-162b2 vaccination, two of 771 participants were seropositive for nucleocapsid and spike protein, whereas 768 were seronegative. The remaining one participant was seropositive for nucleocapsid protein but seronegative for spike protein. A total of 769 participants were seropositive for spike protein after two vaccination doses. The two seropositive participants prior to vaccination showed the highest antibody titers after the second vaccination. They were probably infected with SARS-CoV-2 without clinical symptoms before March 2021. Four weeks after the second vaccination, a younger age was associated with higher antibody titers against SARS-CoV-2 spike protein. Thirty-two weeks after the second vaccination, blood samples were collected from 342 of 769 participants. Antibody titers at 32 weeks after the second vaccination significantly decreased compared with those at 4 weeks after the second vaccination among all age groups. The rate of decrease in antibody titers between 4 and 32 weeks after the second vaccination was greater in the female participants. No sex differences were observed in the antibody titers within each age group. BNT-162b2 vaccination thus induced seroconversion in an age-dependent manner. Serological screening could further establish the likelihood of subclinical SARS-CoV-2 infection.

Introduction

The Japanese government implemented two vaccination doses against severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) at the end of 2020. However, as of December 2021, the worldwide spread of SARS-CoV-2 infection has not come under control. The Japanese government thus implemented a third vaccination as booster immunization in December 2021. The Omicron variant of SARS-CoV-2 has raised serious public health concern globally because of its infectivity and transmissibility (National Institute of Infectious Diseases; Disease Control and Prevention Center, National Center for Global Health and Medicine 2022). The high prevalence of fully vaccinated Omicron cases has been reported (Espenhain et al. 2021). The incidences of SARS-CoV-2 infection, hospitalization, and death are higher in unvaccinated than vaccinated individuals, and the incidence rate ratios are related to vaccine

effectiveness (Scobie et al. 2021). The breakthrough SARS-CoV-2 infections among fully vaccinated healthcare workers (HCWs) in 2021 have been correlated with neutralizing antibody titers during the peri-infection period (Bergwerk et al. 2021). Serologic levels among vaccinated individuals decrease over time, and serologic levels have been associated with subsequent infection (Kertes et al. 2022). Decreased susceptibility to vaccine-induced antibodies are caused by the waning of vaccine-induced antibody levels (Levin et al. 2021) and emerging variants such as Delta and Omicron (Lopez Bernal et al. 2021). Therefore, the serological survey is a key strategy in evaluating the extent of SARS-CoV-2 infections in the community, identifying individuals who are potentially "protected" from infection (Sethuraman et al. 2020), and developing a more effective vaccination strategy (Long et al. 2020). Based on public data available on the Gunma Prefecture's official homepage (https://www.pref.gunma.jp/02/d29g_00338.html), from early spring of 2020 to the present, many SARS-CoV-2 infection clusters in medical facilities, nursing homes, and factories in several regions of Gunma Prefecture were under continuous observation. Therefore, serological screening of immunoglobulin G (IgG) against SARS-CoV-2 nucleocapsid protein among HCWs contributes to evaluate the extent of SARS-CoV-2 infection. This study intended to evaluate the effect of two vaccine doses against SARS-CoV-2 and spread of SARS-CoV-2 among HCWs in Gunma University Hospital.

Materials and Methods

Study subjects and study protocol

A two-dose vaccination program against SARS-CoV-2 was started at Gunma University Hospital in March 2021. To evaluate the effects of the vaccine, we measured antibody titers against SARS-CoV-2 spike protein before and after the two vaccine doses (Fig. 1; Protocol Number HS2020-223). A total of 778 out of 2015 HCWs at the Gunma University Hospital, Gunma, Japan, (including physicians, nurses, laboratory medical technologists, X-ray technicians, therapists, pharmacologists, and officers) participated with written informed consent in the study. In March 2021, the immunization of HCWs against SARS-CoV-2 using BNT-162b2 mRNA vaccine (Pfizer, Inc. and BioNTech) was initiated. The schedule of blood sampling and vaccinations is summarized in Fig. 1. Blood samples were collected from 778 participants before the first vaccination. However, seven participants were excluded due to retirement. Therefore, blood sampling was conducted with 771 participants at 4 weeks after the second vaccination. Because an unexpected third booster vaccination was scheduled in December 2021, we incorporated an additional analysis into the study. Blood samples were collected from 342 participants before the third vaccination scheduled in December 2021, specifically at 32 weeks after the second vaccination. We measured antibody titers against SARS-CoV-2 nucleocapsid and spike protein before the first vac-

cination and 32 weeks after the second vaccination. The Gunma University Ethical Review Board for Medical Research Involving Human Subjects approved the study protocol (HS2020-223). All ethical and confidentiality considerations were handled in accordance with the Helsinki Declaration.

Measurement of specific IgG against SARS-CoV-2

IgG antibodies specific to the S1 subunit of the spike protein (anti-spike IgG) were measured using Roche kits (Elecsys® Anti-SARS-CoV-2 S RUO; Roche Diagnostics K.K., Tokyo, Japan). Positive signals were reported at a cut off index ≥ 0.8 U/mL. IgG antibodies specific to the SARS-CoV-2 nucleocapsid protein (anti-nucleocapsid IgG) were measured using Roche kits (Elecsys® Anti-SARS-CoV-2, Roche Diagnostics K.K.). Positive signals were reported at a cut off index ≥ 1.0 U/mL. These immunoassays were performed according to the manufacturer's instructions on a Cobas 8000 e801 module (Roche Diagnostics K.K.). We defined positive or false positive as described following. Both anti-nucleocapsid IgG and anti-spike IgG positive findings indicate infected participants, whereas negative of either is false positive.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 25.0 (Armonk, NY, USA). Because almost all variables were not normally distributed, data were expressed as median values with a 25th-75th percentile range. The distribution of age and antibody titer anti-spike IgG were analyzed using the Dunn test after Kruskal-Wallis test or Mann-Whitney U test to identify statistically significant differences, as appropriate. Statistical significance was set at p value < 0.01. Spearman's correlation analysis was conducted to determine the association between anti-spike IgG antibody titer and age. Differences and correlations were considered significant at p value < 0.01.

Results

In early spring 2021, immunization using BNT162b2 mRNA vaccine for HCWs was implemented in Japan. The serological screening performed before and after two vaccine doses among HCWs of Gunma University Hospital is shown in Fig. 1. Age distribution varied significantly between male and female participants (Fig. 2A). As shown in Fig. 1, 771 out of 778 participants underwent serological screening for anti-spike IgG before and after the two vaccine doses. Among 771 participants, two were positive for anti-spike IgG before the first vaccination (Table 1). Anti-spike IgG antibody titers for the two participants after the second dose were over 9,000 U/mL (increased from 17.0 to 9,367 U/mL and from 99.1 to 10,222 U/mL). Before the first vaccination, the two participants were seropositive for anti-nucleocapsid IgG, as well (95.9 and 2.52 U/mL; Table 1). Therefore, the two participants were probably infected

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│ 778 health care workers registered as a volunteer at March,   │
│ 2021. Protocol number; HS2020-223                             │
└─────────────────────────────────────────────────────────────┘

┌──────────────────────┐                    ┌──────────────────────────────┐
│ First blood sampling  │ ────────────────▶  │ Measurement of immunoglobulin │
└──────────────────────┘                    │ G against nucleocapsid and    │
         │ Within 1 week                     │ spike protein of SARS-CoV-2   │
         ▼                                   └──────────────────────────────┘
┌──────────────────────────────────────┐
│ First vaccination (BNT-162b2 mRNA      │
│ vaccine)                               │
└──────────────────────────────────────┘
         │ 3 weeks later
         ▼
┌──────────────────────────┐            ┌──────────────────────────┐
│ Second vaccination        │ ─────────▶ │ 7 participants were       │
│ (BNT-162b2 mRNA vaccine)  │            │ excluded from this study  │
└──────────────────────────┘            │ due to retirement.        │
         │ 4 weeks later                 └──────────────────────────┘
         ▼
┌──────────────────────────────────┐    ┌──────────────────────────────┐
│ 771 participants. Second blood     │──▶ │ Measurement of immunoglobulin │
│ sampling                           │    │ G against spike protein of    │
└──────────────────────────────────┘    │ SARS-CoV-2                    │
         │ 28 weeks later                └──────────────────────────────┘
         ▼
┌──────────────────────────────────┐    ┌──────────────────────────────┐
│ 342 participants. Third blood      │──▶ │ Measurement of immunoglobulin │
│ sampling                           │    │ G against nucleocapsid and    │
└──────────────────────────────────┘    │ spike protein of SARS-CoV-2   │
         │ Within 1 week                 └──────────────────────────────┘
         ▼
┌──────────────────────────────────┐
│ Third vaccination (BNT-162b2 mRNA  │
│ vaccine)                           │
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(32 weeks spans from Second vaccination to Third blood sampling)
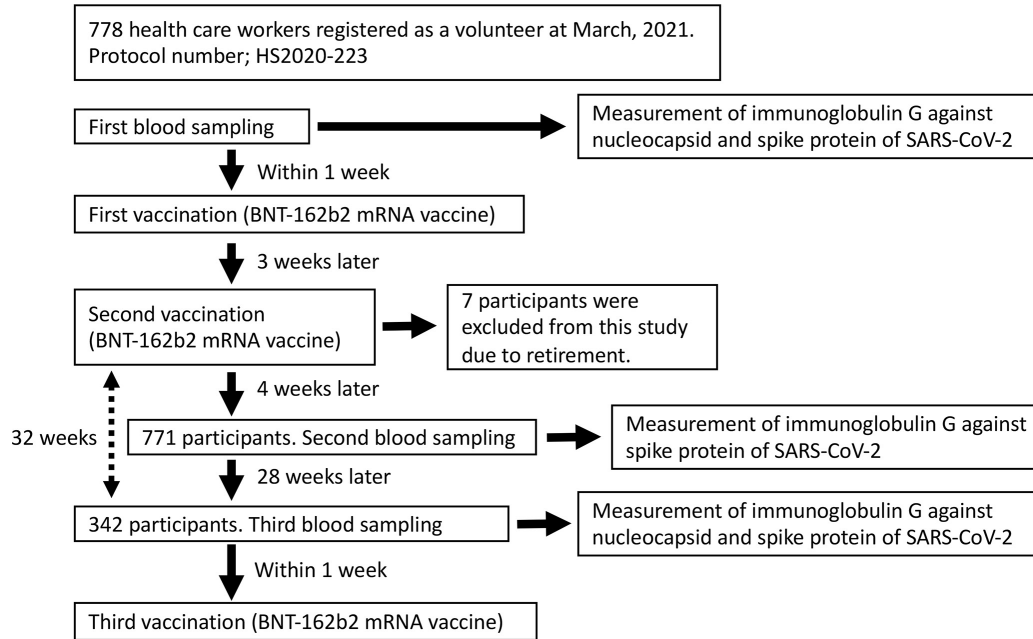
Fig. 1. The schedule of blood sampling and vaccinations in healthcare workers at the Gunma University Hospital.
In March 2021, 778 healthcare workers were measured for IgG against SARS-CoV-2 before and after two doses of BNT-162b2 mRNA vaccine (Protocol Number HS220-223). Thirty-two weeks after the second vaccination, 342 out of 778 participants were recruited to evaluate immunoglobulin G antibody titers against SARS-CoV-2.
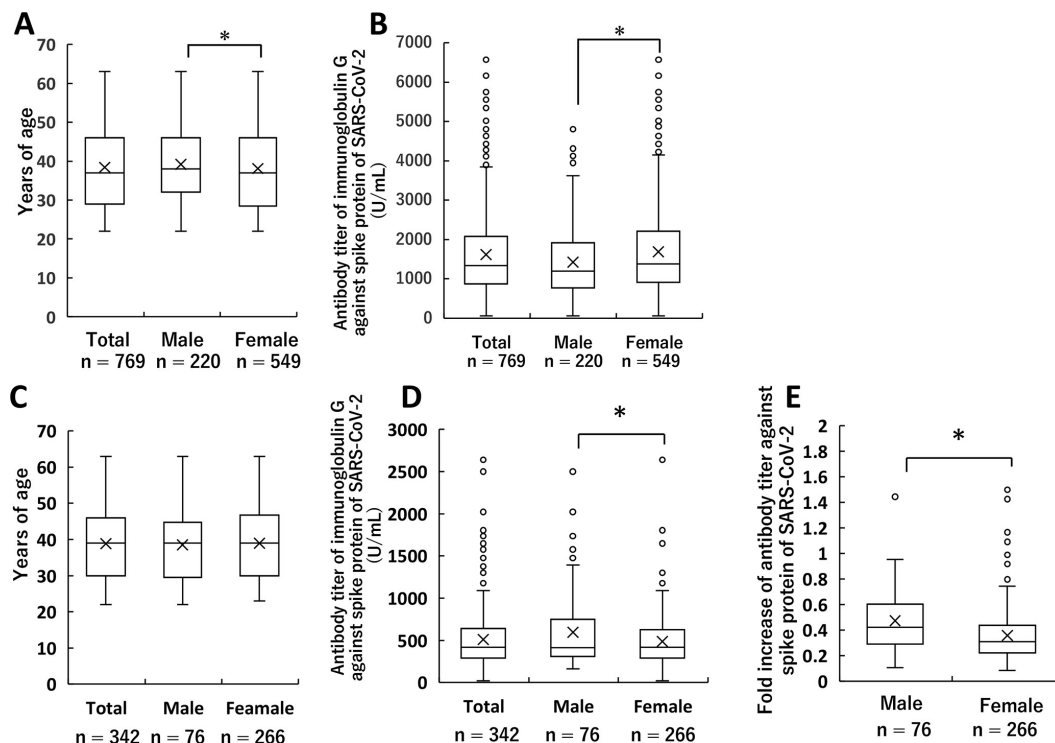

Fig. 2. Distribution of age and antibody titer among 769 healthcare workers at the Gunma University Hospital.
(A) Box beard graph of age distribution of all 769 participants (220 male and 549 female participants). (B) Box beard graph of immunoglobulin G antibody titer against the SARS-CoV-2 spike protein among 769 healthcare workers. (C) Box beard graph of age distribution in 342 participants (76 male and 266 female participants) recruited 32 weeks after the second vaccination. (D) Box beard graph of antibody titer against SARS-CoV-2 spike protein among 342 participants (76 male and 266 female participants). (E) Box beard graph of the fold increase of antibody titer against SARS-CoV-2 spike protein between 4 weeks and 32 weeks after the second vaccination among 76 male and 266 female participants. $*p < 0.01$.

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with SARS-CoV-2 before the first vaccination dose in March 2021. Both were asymptomatic and were thus unaware that they were infected with SARS-CoV-2.

Subsequent studies were conducted with data from 769 participants negative for anti-spike IgG before the first vaccination. All 769 participants showed seroconversion from negative to positive for anti-spike IgG after the second vaccination (Table 1). The maximum anti-spike IgG antibody titer at 4 weeks after the second vaccination was 6,571 U/mL in a female participant in her 40s, and the minimum was 57 U/mL in a female participant in her 20s. The anti-spike IgG antibody titer at 4 weeks after the second vaccination was negatively correlated with age in the total population ($r = -0.191$, $p < 0.001$) and male ($r = -0.222$, $p < 0.001$) and female subgroups ($r = -0.172$, $p < 0.001$). The anti-spike IgG antibody titers of participants in their 20s were significantly higher than those in their 40s, 50s, and 60s, whereas no significant difference was detected between participants in their 20s and 30s (Fig. 3A). No significant difference in anti-spike IgG antibody titer was observed between the age groups in the male population (Fig. 3B). However, in the female population, the anti-spike IgG antibody titers of participants in their 20s were significantly higher than those in their 40s, 50s, and 60s, whereas no significant difference was detected between female participants in their 20s and 30s (Fig. 3C). No sex differences were observed in the anti-spike IgG antibody titers within each age group. On the other hand, the anti-spike IgG antibody titers were significantly lower in the male participants (Fig. 2B).

Thirty-two weeks after the second vaccination, blood samples were collected from 342 of 769 HCWs, the latter of whom had been vaccinated for the third time after blood sampling. Of the 342, 76 were male and 266 were female. The maximum anti-spike IgG antibody titer at 32 weeks after the second vaccination was 2,641 U/mL (increased from 1,852 to 2,641 U/mL) in a female participant in her 20s, and the minimum was 21.3 U/mL (decreased from 93.0 to 21.3 U/mL) in a female participant in her 40s. No significant difference in age distribution was observed between the male and female participants (Fig. 2C). The anti-spike IgG antibody titers of 76 male HCWs were significantly higher than those of the 266 female HCWs (Fig. 2D). The rate of decrease in anti-spike IgG antibody titer between 4 weeks and 32 weeks after the second vaccination was greater among the female participants (Fig. 2E). In each age group, the anti-spike IgG antibody titer at 32 weeks after the second vaccination was significantly lower than those at 4 weeks (Fig. 4). Among the 342 participants, the anti-spike IgG antibody titers of participants in their 20s were significantly higher than those in their 40s, 50s, and 60s, whereas no significant difference was detected between those in their 20s and 30s (Fig. 4A). No significant difference in anti-spike IgG antibody titer was observed across the age groups among the 76 male participants out of the 342 (Fig. 4B). Among the 266 female participants out of the 342, the anti-spike IgG antibody titer of participants in their 20s were significantly higher than those in their 40s, 50s, and 60s, whereas no significant difference was found between those in their 20s and 30s (Fig. 4C). No sex differences were observed in anti-spike IgG antibody titers within each age group. On the other hand, 32 weeks after the second vaccination, anti-spike IgG antibody titer increased in three HCWs; however, all three HCWs were seronegative

Table 1. Prevalence of immunoglobulin G against SARS-CoV-2 nucleocapsid and spike protein among healthcare workers.

		Immunoglobulin G against SARS-CoV-2			
		Anti-nucleocapsid protein		Anti-spike protein	
	Age	Positive (number)	Negative (number)	Positive (number)	Negative (number)
Spring 2021 Before the first vaccination					
Total (n = 771)	37 (29-46)	3	768	2	769
Male (n = 222)	38 (32-46)	2	220	2	220
Female (n = 549)	37 (29-46)	1	548	0	549
4 weeks after the second vaccination					
Total (n = 771)	37 (29-46)	Not tested		771	0
Male (n = 222)	38 (32-46)	Not tested		222	0
Female (n = 549)	37 (29-46)	Not tested		549	0
32 weeks after the second vaccination					
Total (n = 342)	39 (30-46)	1	341	342	0
Male (n = 76)	39 (29-44)	0	76	76	0
Female (n = 266)	39 (30-46)	1	265	266	0

The age is expressed as median values with a 25th-75th percentile range.
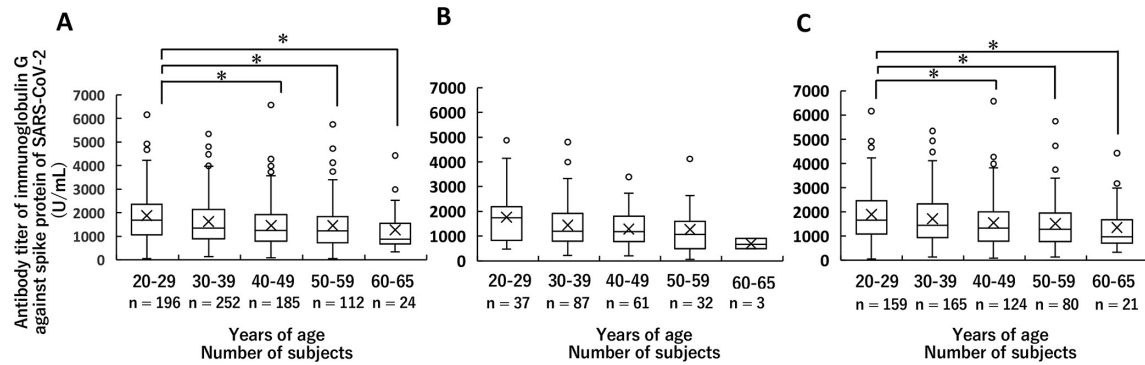
Fig. 3. Distribution of immunoglobulin G against SARS-CoV-2 spike protein among 769 healthcare workers.
(A) Distribution of immunoglobulin G against SARS-CoV-2 spike protein 4 weeks after the second vaccination among 769 healthcare workers classified by age (20s, 30s, 40s, 50s, and 60s). (B) Distribution of immunoglobulin G against SARS-CoV-2 spike protein among 220 male healthcare workers classified by age (20s, 30s, 40s, 50s, and 60s). (C) Distribution of immunoglobulin G against SARS-CoV-2 spike protein among 549 female healthcare workers classified by age (20s, 30s, 40s, 50s, and 60s). *$p < 0.01$.

for anti-nucleocapsid IgG throughout this study.

A total of 341 out of 342 HCWs were seronegative for both anti-nucleocapsid IgG and anti-spike IgG before the first vaccination. The remaining one HCW was seropositive for anti-nucleocapsid IgG (2.84 U/mL before the first vaccination and 3.66 U/mL 32 weeks after the second vaccination) but seronegative for anti-spike IgG (0.4 U/mL) before the first vaccination.

Discussion

This study demonstrated the efficacy of BNT-162b2 mRNA vaccine and the usefulness of serological screening of IgG against SARS-CoV-2 to check for asymptomatic SARS-CoV-2 infection. Additionally, sex differences in immune response to two doses of the BNT-162b2 vaccine were observed.

All participants showed seroconversion against SARS-CoV-2 after two doses. Among all 769 participants, two doses of BNT-162b2 induced anti-spike IgG production in an age-dependent manner. Thus, the younger the age, the higher the antibody titer. These results are supported by a recent report indicating an age-dependent humoral response to the BNT-162b2 mRNA vaccine (Terpos et al. 2021).

The effectiveness of two doses of the BNT-162b2 vaccine was also demonstrated. The reduction in viral transmissibility after two doses have been reported (Harris et al. 2021; Levine-Tiefenbrun et al. 2021). Furthermore, the viral load was substantially reduced for infections occurring 12-37 days after the first dose (Levine-Tiefenbrun et al. 2021). Among patients with coronavirus disease-2019 (COVID-19), those who had been vaccinated were likely to be less severely symptomatic (Bergwerk et al. 2021; Harris et al. 2021; Levine-Tiefenbrun et al. 2021; Scobie et al. 2021). Despite these positive effects, breakthrough infections in fully vaccinated HCWs occurred (Bergwerk et al. 2021). The vaccine protected against symptomatic disease but not against infection and that secondary infections could be prevented (Bergwerk et al. 2021). Higher peri-infection neutralizing antibody titers were associated with lower infectivity (Bergwerk et al. 2021). Thus, low titers of neutralizing antibody and anti-spike IgG may serve as markers of breakthrough infection (Bergwerk et al. 2021). However, only a limited number of facilities can measure neutralizing antibodies. The anti-spike IgG concentrations as measured with the Roche assay correlate well with SARS-CoV-2 neutralization activities (L'Huillier et al. 2021). In the present study, five HCWs showed low anti-spike IgG antibody titers (< 100 U/mL) after two doses. These HCWs may thus require additional vaccination. Anti-spike IgG antibody titers were found to decrease in a time-dependent manner (Kertes et al. 2022). Consistent with this report, anti-spike IgG antibody titers among HCWs was significantly lower at 32 weeks than those at 4 weeks after the second vaccination. These facts thereby support booster vaccination and indicate that serological anti-spike IgG surveys after two doses can help develop a more effective vaccination strategy.

In accordance with a previous study (Terpos et al. 2021), anti-spike IgG antibody titers 4 weeks after the second vaccination was significantly higher among female participants. This could be explained by the differences in age distribution between the male and female participants. On the other hand, anti-spike IgG antibody titers 32 weeks after the second vaccination decreased significantly among female participants. In the additional analysis involving 342 of 771 participants in December 2021, no significant difference between male and female participants was observed. The rate of decrease in anti-spike IgG antibody titer from 4 weeks to 32 weeks after the second vaccination was higher in female participants. These results could be partially explained by differences in immune response between men and women.

Seropositivity for anti-nucleocapsid IgG reflects natural SARS-CoV-2 infection. Three HCWs were seropositive or anti-nucleocapsid IgG before the first vaccination. All three HCWs exhibited no symptoms of COVID-19 through-
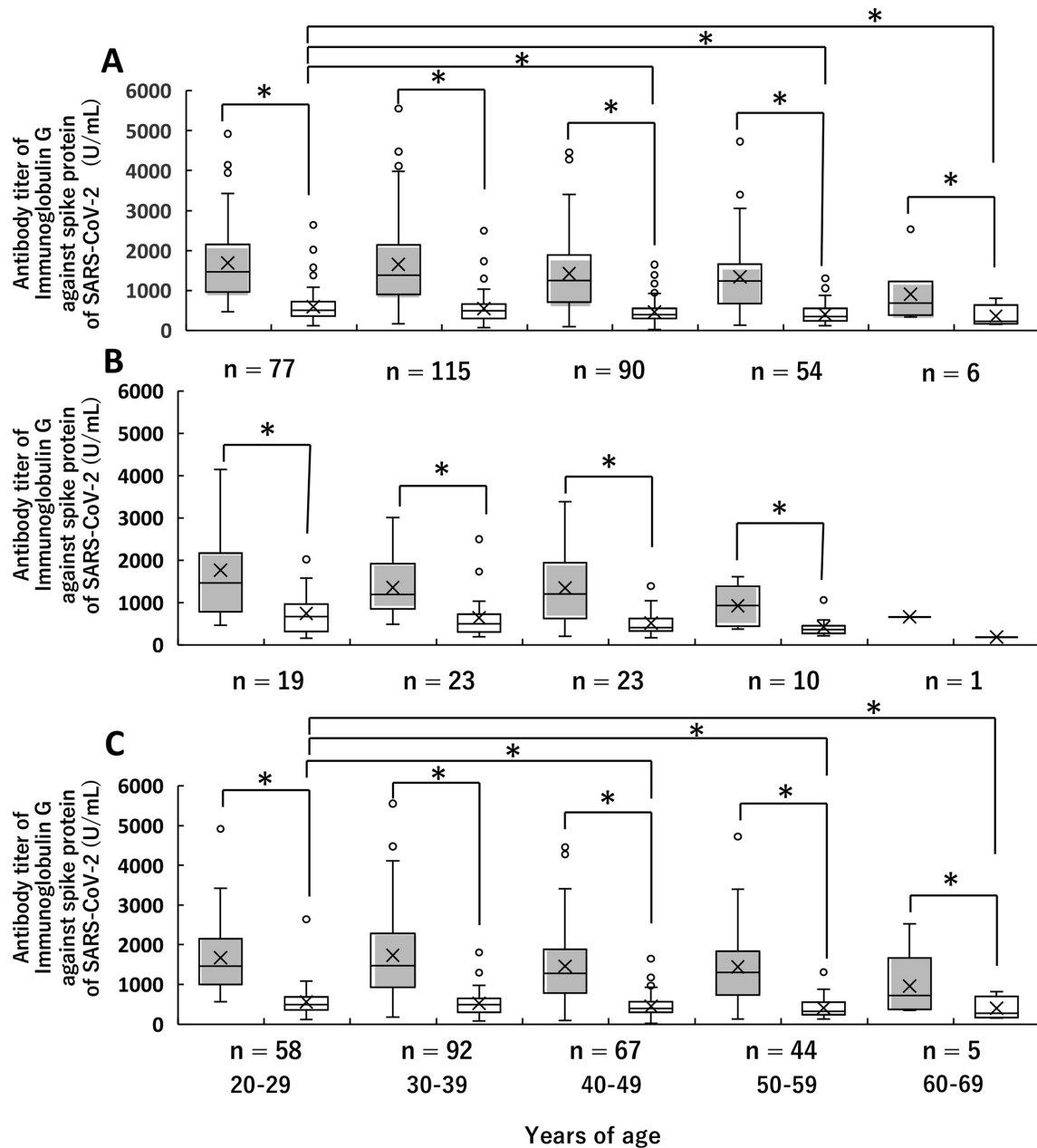
Fig. 4. Distribution of immunoglobulin G antibody titer against SARS-CoV-2 spike protein at 4 weeks and 32 weeks after the second vaccination among healthcare workers.

(A) Distribution of immunoglobulin G antibody titer against SARS-CoV-2 spike protein at 4 weeks and 32 weeks after the second vaccination among 342 healthcare workers classified by age (20s, 30s, 40s, 50s, and 60s). (B) Distribution of immunoglobulin G antibody titer against SARS-CoV-2 spike protein at 4 weeks and 32 weeks after the second vaccination among 76 male healthcare workers classified by age. (C) Distribution of immunoglobulin G antibody titer against SARS-CoV-2 spike protein at 4 weeks and 32 weeks after the second vaccination among 266 female healthcare workers classified by age. Gray box: antibody titer against SARS-CoV-2 spike protein at 4 weeks after the second vaccination, white box: antibody titer against SARS-CoV-2 spike protein at 32 weeks after the second vaccination. *$p <$ 0.01.

out this study. Two of the three HCWs were seropositive for anti-spike IgG before the first vaccination. Therefore, these two HCWs may have had a history of SARS-CoV-2 infection, as indicated by serological screening, prior to the first vaccination. Since both of them were asymptomatic, they were not aware that they were infected with SARS-CoV-2. A total of 771 of the 2015 Gunma University

Hospital staff were enrolled in this study. Therefore, it is probable that there were over 6 asymptomatic SARS-CoV-2 infected persons. As demonstrated in other recent reports (Favresse et al. 2021; Manisty et al. 2021), anti-spike IgG antibody titers of the two HCWs were high ($> 9,000$ U/mL) after two vaccine doses. A single dose of BNT-162b2 vaccine was found sufficient in boosting antibody titers in pre-

viously infected individuals (Favresse et al. 2021). Thus, serological screening for anti-nucleocapsid IgG can help evaluate the extent of asymptomatic SARS-CoV-2 infection and develop a more effective vaccination strategy. Although nosocomial SARS-CoV-2 infections did not occur in the Gunma University Hospital until January 2022, serological screening for SARS-CoV-2 suggests the likelihood of subclinical infections. Therefore, measurement of anti-nucleocapsid IgG titer helps to alert HCWs to SARS-CoV-2 infection.

One HCW was seropositive for anti-nucleocapsid IgG while seronegative for anti-spike IgG. Such discordant results with the Roche assay kit have been previously reported (Mueller 2021). Thus, the HCW may not have been infected with SARS-CoV-2. Furthermore, a single measurement of anti-nucleocapsid IgG may be an insufficient indicator of natural SARS-CoV-2 infection.

Incidence rate ratios for hospitalization and death changed relatively little after the SARS-CoV-2 B.1.617.2 (Delta) variant reached predominance, suggesting high, continued vaccine effectiveness against severe COVID-19 (Keehner et al. 2021). A booster dose of the BNT162b2 vaccine affected the severity of SARS-CoV-2 Delta variant infection (Bar-On et al. 2021). Monitoring COVID-19 incidence by vaccination status might provide early signals of potential changes in vaccine effectiveness that can be confirmed through robust controlled studies (Keehner et al. 2021). In California, the dramatic change in vaccine effectiveness from June to July was likely due to both the emergence of the Delta variant and waning immunity over time, compounded by the end of masking requirements (Brown et al. 2021). These findings support continued implementation of prevention strategies, including masking in indoor public settings regardless of vaccination status (Brown et al. 2021). At the Gunma University Hospital, all HCWs wear masks and wash hands as appropriate. These facts partially contributed that no nosocomial SARS-CoV-2 infections have occurred, despite the subclinical SARS-CoV-2 infections occurred among HCWs. We must carefully continue infection control and prevention.

This study has some limitations. First, this is a single-center study that included only Japanese participants. Second, the population size was small. Third, neutralizing antibodies were not measured, although the commercial assay kits we employed in this study were closely correlated with neutralizing antibody titer (L'Huillier et al. 2021).

In conclusion, this study showed that two doses of BNT-162b2 vaccine-induced seroconversion in all of 769 recipients and that serological screening of anti-nucleocapsid IgG could reveal subclinical SARS-CoV-2 infection among HCWs in medical facilities. Both vaccination and serological screening contribute to infection control and prevention against COVID-19.

Conflict of Interest
The authors declare no conflict of interest.

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