Heliyon 11 (2025) e42171

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Pre-existing IgG antibodies to HCoVs NL63 and OC43 Spike increased during the pandemic and after COVID-19 vaccination

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A B S T R A C T

Preexisting immunity may be associated with increased protection against non-related pathogens such as, SARS-CoV-2. There is little information regarding endemic human coronaviruses (HCOVs) from Pakistan, which experienced a relatively low COVID-19 morbidity and mortality. We investigated antibodies to SARS-CoV-2 and HCoVs NL63 and OC43, comparing sera from prepandemic controls (PPC) period with responses in healthy controls from the pandemic (HC 2021). Further, we investigated the effect of inactivated and mRNA COVID-19 vaccinations on antibody responses to the pandemic and endemic coronaviruses.

We measured IgG antibodies to Spike of SARS-CoV-2, HCoV-NL63 and HCoV-OC43 by ELISA. Serum neutralizing capacity was determined using a SARS-CoV-2 psuedotyped virus assay. Vaccinees were sampled prior to vaccination as well after 6, 12 and 24 weeks after COVID-19 inactivated (Sinovac), or mRNA (BNT162b2) vaccine administration.

PPC sera showed seropositivity of 15 % to SARS-CoV-2, whilst it was 45 % in the HC 2021 group. Five percent of sera showed virus neutralizing activity in PPC whilst it was 50 % in HC 2021. IgG antibodies to Spike of NL63 and OC43 were also present in PPC; anti-NL63 was 2.9-fold, and anti-OC43 was 10.1-fold higher than to anti-SARS-CoV-2 levels. IgG antibodies to Spike SARS-CoV-2 were positively correlated with HCoV-NL63 in HC 2021, indicating recognition of shared conserved epitopes. IgG antibody levels increased during the pandemic; 2.7-fold to HCoV-NL63 and 1.9-fold to HCoV-OC43.

SinoVac and BNT162b2 vaccine induced an increase in IgG antibodies to Spike SARS-CoV-2 as well as HCoV-NL63 and HCoV-OC43.

Our data show that antibodies to spike protein of endemic coronaviruses were present in the prepandemic population. Antibodies to SARS-CoV-2, NL63 and OC43 were all raised during the pandemic and further enhanced after COVID-19 vaccinations. The increase in antibodies to spike of coronaviruses would contribute to protection against SARS-CoV-2.

1. Introduction

The COVID-19 pandemic caused almost 786 million cases up to March 10, 2023 [1]. Pakistan reported an estimated 1.57 million

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https://doi.org/10.1016/j.heliyon.2025.e42171
Received 12 May 2024; Received in revised form 21 January 2025; Accepted 21 January 2025
Available online 22 January 2025

COVID-19 cases and 31,000 deaths (up to April 1, 2023, https://ourworldindata.org/coronavirus-testing). COVID-19 related mortality was lower than 2 % even during the early pandemic period (2020), prior to the introduction of vaccinations [2]. In comparison, case fatality rates (CFR) ranged between 6.2 % in Italy, 3.6 % in Iran, and 0.79 % in South Korea [3,4].

Originally named novel coronavirus 2019, SARS-CoV-2 is a beta-coronavirus which causes respiratory infections ranging from asymptomatic to severe and critical COVID-19 [5]. Protection against SARS-CoV-2 is mediated by both humoral and T-cell immunity, activated after both natural infection and COVID-19 vaccinations [6]. The Spike glycoprotein mediates viral entry by binding ACE-2 receptors on host cells. Spike is highly immunogenic protein and IgG antibodies against it are associated with protection against SARS-CoV-2 infections and have neutralizing potential [7]. Rapid rise in SARS-CoV-2 seropositivity was seen in through serosurveys in Karachi city neighbourhoods during the early pandemic period in 2020, rising from 0.2-0.4 % to 12.8–21.8 % [8]. Further, sera from healthy controls of the pre-pandemic period in Pakistan showed 12 % seroprevalence against whole Spike protein of SARS-CoV-2 whilst by 2021, 50 % of healthy unvaccinated individuals were seropositive [9]. Comparatively, after the first 12 months of the pandemic (by March 2021) the IgG seroprevalence to Spike of SARS-CoV-2 was found to be 17.3 % in Portugal [10]. Population specific differences may be due to many factors including, exposure to endemic viruses.

SARS-CoV-2 has variable sequence homology to endemic human coronaviruses (HCoV) [11]. Seroprevalence of antibodies to Spike proteins of antibodies to alpha coronaviruses HCoV-NL63 and 229E, and beta-coronaviruses HCoV-OC43 and HKU1 have been observed in pre-pandemic sera in many countries [12]. A high degree of structural homology exists between Spike proteins of SARS-CoV-2, HCoV-NL63 and HCoV-OC43 [11]. Cross-reactive antibodies to endemic coronaviruses have been reported to increase after SARS-CoV-2 infection and vaccination [13,14].

COVID-19 vaccinations were introduced at the end of 2020 in the UK, USA and China. mRNA and adenovirus vector COVID-19 vaccines were best studied and used worldwide [15]. However, vaccination strategies differed worldwide based on access and affordability of vaccines. There is limited information on COVID-19 vaccination efficacy from lower-middle income countries (LMIC) [16]. In Pakistan, COVID-19 vaccinations commenced in February 2021 using a phase-wise approach whereby, inactivated vaccines BBIBP-CorV and CoronaVac were first administered and subsequently, replication-deficient adenovector vaccines, and mRNA vaccines became available through the COVAX initiative [17,18].

We wanted to investigate the presence of antibodies against spike of SARS-CoV-2, NL63 and OC43, comparing these in a healthy unvaccinated control group during the pandemic period (HC 2021) as well as in sera from the pre-pandemic period. We also studied the effect of SinoVac inactivated vaccine as well BNT162b2 mRNA vaccinations on IgG antibody levels in the study cohort, measuring longitudinal responses prior to vaccination and after 6, 12 and 24 weeks.

2. Methods

This study was approved by the Ethical Review Committee (ERC) of Aga Khan University (AKU), ERC #2020-5152-11688. It received approval from the Department of Health, Government of Sindh, Pakistan. Participants were recruited at government run adult vaccine centers (AVCs) of district Matiari, Sindh.

2.1. Study subjects

Pre-pandemic sera were those collected from healthy individuals between 2014 and 2018 at AKU, Karachi, Pakistan. These had been banked in an anonymised manner and stored at −80C.

This was a longitudinal observational study carried out between October 2021 to June 2022 (S Fig. 1). All thirty-nine healthy subjects (HC 2021) were first enrolled cohort between October and December 2021. We included adults aged 18–50 years, who were both age and gender matched. We excluded any individual who reported having tested positive for COVID-19 prior to enrolment in the study. None of the individuals had received COVID-19 vaccinations prior to enrolment in the study.

Vaccines were administered at the AVC in district Matiari. Study participants received doses of either CoronaVac (SinoVac, 20 individuals) or BNT162b2 (Pfizer Biontech, 19 individuals) as per intra-dermal injection in the deltoid as per standard guidelines. Each participant received two doses of either vaccine, four weeks apart.

2.2. Specimen collection

Blood samples were taken from the HC 2021 group prior to the first dose of vaccine administration (baseline). Subsequently, blood samples were taken from study subjects at 6, 12 and 24 weeks after the first dose. Serum was isolated from whole blood and stored until tested.

2.3. Spike IgG ELISA assays

Recombinant Spike and RBD proteins were obtained from iBET, Lisbon, Portugal, courtesy of Prof. Paula M. Alves [19,20]. All serum samples were tested at a 1/100 dilution in duplicate using an in-house enzyme-linked absorbent assay (ELISA) [25] and as per the protocol described by Figueiredo-Campos et al. [26]. This assay has been validated in our laboratory [27]. Briefly, SARS-CoV-2 Spike and/or RBD protein were used to coat plates with 50 µl of Spike or RBD protein at a concentration of 2 µg/ml in PBS. Wells were blocked and then incubated with 100 µl serum samples for 2 h. Wells were washed and stained with goat anti-human IgG Fc (HRP) and then developed for colorimetric reading at 450 nm. For assay validation, sera from 45 COVID-19 convalescent cases, drawn

4 weeks after their PCR confirmed diagnosis, were used as positive controls. Sera from 55 healthy individuals from the pre-pandemic period were used as negative controls. The cut-off for positive responses of IgG to Spike and RBD was 0.5 OD450 nm in each case, by calculating the Mean +2SD (OD at 450 nm). IgG positive COVID-19 convalescent sera were pooled and used to run a dilution series on each ELISA plate. The same were used to compare variations between plates and experiments, as a chart of the results of high and low positive sera was maintained for the experiments (Sup Figs. 2–3). Negative sera were also included in each ELISA run. The sensitivity of the Spike ELISA was found to be 100 % (92.1–100, 95 % CI) with a specificity of 100 % (93.5–100, 95 % CI). Sensitivity of the RBD was found to be 91.1 % (78.8–97.5, 95 % CI) with a specificity of 94.6 % (82.4–98, 95 % CI) [18,27].

IgG antibody ELISAs for Spike proteins of HCoV-NL63 and HCoV-OC43 were conducted using protein from SinoBiologicals (catalog number 40604-V08B, and 40607-V08H1 respectively). ELISA optimisations were conducted using 1 ng/μL of Spike to HCoV-NL63 or HCoV-OC43, respectively. COVID-19 positive and negative pooled sera were used for establishing a cut-off for detection. For all the ELISAs conducted, we evaluated inter-plate variation for each Spike target. Variation between highest and lowest values for the positive pooled sera was $\leq$ 2 SD in each case. Sera were tested at 2-fold dilutions starting at 1:50 in the respective ELISA assays. Conditions for development with a HRP conjugated secondary antibody were tested at 1:20,000 and 1:40,000. The positive cut-off value was found to be 0.5 ($OD_{450\ nm}$) for IgG ELISAs to Spike of NL63 and OC43 for the qualitative assays. Details of IgG ELISA optimisation for NL63 and OC43 are shown in Sup Figs. 4–5.

2.4. IgG antibody titrations

Antibody titers to Spike of SARS-CoV-2, HCoV-NL63 and HCoV-OC43 were determined by running ELISA assays under the conditions described above. For titrations, each sera was tested in six sequential 2-fold dilutions starting at 1:50 respectively. Results of the IgG OD values obtained across the titrations for sera were used to plot titration curves for samples tested.

2.5. Diagnostic assay for SARS-CoV-2 (COVID-19) antibodies

SARS-CoV-2 IgG in serum samples was detected using VIDAS® SARS-COV-2 IgG II kit on the VIDAS® (bioMerieux). The assay detects IgG to the SARS-CoV-2 receptor-binding domain (RBD) of the spike protein using the ELFA (Enzyme Linked Fluorescent Assay) technique.

2.6. Pseudovirus neutralizing assay for SARS-CoV-2

The pseudovirus was generated by co-transfection of HEK 293 T cells (BEI Resources, USA) with pLEX-GFP reporter, psPAX2 and pCAGGS Spike and neutralizing assay conducted as described previously [21]. The Spike protein sequenced expressed in the lentivirus vector belonged to the D614G wild-type strain from 2020. For the neutralizing assay, heat-inactivated serum samples were three-fold serially diluted over 6 dilutions, beginning with a 1:30 initial dilution. Dilutions were then incubated with spike pseudotyped lentiviral particles for 1h at 37 °C. The mix was added to a pre-seeded 96 well plate of 293T-ACE2 cells, with a final MOI of 0.2. At 48h post-transduction, the fluorescent signal was measured using the Varioscan Lux multimode microplate reader (Thermofisher scientific, USA). The relative fluorescence units obtained were normalized to those derived from the virus control wells (cells infected in the absence of plasma or serum), after subtraction of the background in the control groups with cells only. The half-maximal neutralization titer (NT50), defined as the reciprocal of the dilution at which infection was decreased by 50 %, was determined using four-parameter nonlinear regression (least squares regression without weighting; constraints: bottom = 0) (GraphPad Prism 10.1).

2.7. Statistical analysis

We used an area under the curve (AUC) analysis to compare the change in IgG antibody titers between study groups who were followed up longitudinally between baseline and post-vaccination timelines. AUC was calculated using the PRISM GraphPad software version 10.1.1. All IgG antibody titers to HCoV-NL63 and HCoV-OC43 HCoVs were log10-transformed to improve the linearity. The geometric mean titers (GMT) and 95 % confidence intervals (95 % CI) were computed as log10-transformed titers. Two-tailed, nonparametric Mann–Whitney *U* test and ANOVA test were performed on numerical data. Spearman's rank correlation analysis

Table 1
Demographics of study subjects.

	Pre-pandemic (PPC) (n = 72)	HC 2021 (n = 39)
Age Mean (± SD)	42.3 ± 14.4	33.0 ± 9.9
Gender (% females)	N = 33 (45 %)	N = 16 (41 %)
Vaccine group	**BNT162b2 (n=19)**	**SinoVac (n=20)**
Date of vaccine administration	**October 2021**	**October 2021**
Age Mean (± SD)	29.7 ± 10.1	36.2 ± 8.9
Gender (% females)	N = 8 (42%)	N = 8 (40%)

compared the antibody response against seasonal HCoV and SARS-CoV-2. The graphs and statistical analyses were conducted using GraphPad Prism (version 10) P-values less than 0.05 were considered statistically significant.

3. Results

3.1. Demographics of the study groups

We included individuals for whom we had banked sera as pre-pandemic controls (PPC) and enrolled healthy controls from 2021 (HC 2021). PPC sera were available from 72 individuals, aged 42.3 (±14.4) years and 45 % of them were females (Table 1). There were 39 individuals in the HC 2021 sampled prior to vaccinations; none had any prior history of any reported COVID-19 infections. The HC 2021 cohort was aged 33.0 (±9.9) years and comprised of 41 % females (Table 1).

3.2. Assessing IgG antibodies to SARS-CoV-2 and HCoVs NL63 and OC43 in pre-pandemic sera

We measured IgG antibodies to SARS-CoV-2 Spike and RBD protein as well as, Spike of HCoV-NL63 and HCoV-OC43 in PPC and HC 2021 groups. Eleven (15.2 %) of the PPC samples were seropositive to SARS-CoV-2 Spike whilst 8.3 % were seropositive to SARS-CoV-2 RBD. Whilst, seropositivity against HCoVs were higher; 59.7 % to HCoV-NL63 and, one hundred percent of samples to HCoV-OC43 (Table 2). In the HC 2021 group, 48.7 % individuals were seropositive against Spike SARS-CoV-2, 33 % to RBD, 92.3 % to HCoV-NL63 and 100 % to HCoV-OC43 Spike (Table 2).

We also compared the IgG antibody levels detected against different spike proteins across both PPC and HC 2021 groups. In the PPC sera, we observed significantly higher IgG levels to Spike HCoV-OC43 as compared with HCoV-NL63 and SARS-CoV-2 ($p = 0.001$, Fig. 1A). With, IgG levels to HCoV-OC43 3.5-fold, and to HCoV-OC43 10.1-fold higher, as compared with SARS-CoV-2. A Spearman's rank correlation analysis revealed an expected positive association between IgG to SARS-CoV-2 Spike and RBD (rho 0.545, $p < 0.0001$**), but none between IgG to Spikes HCoV-NL63, or HCoV-OC43. This suggests that there is cross-reactivity of antibodies between spike proteins of different coronaviruses.

3.3. Association of IgG levels to SARS-CoV-2 and HCoV-NL63 Spike during the pandemic

Next, we examined the relationship between IgG to Spikes SARS-CoV-2, HCoV-NL63 and HCoV-OC43 in the HC 2021 cohort prior to COVID-19 vaccinations. There was a significant difference between antibody levels to the different coronaviruses, with the highest observed against HCoV-OC43 ($p < 0.0001$, Fig. 1B). Antibodies to HCoV-OC43 were 8.2-fold and those to HCoV-NL63 were 2.4-fold higher than against SARS-CoV-2. In the HC 2021 cohort, there was a positive association between SARS-CoV-2 Spike and RBD antibodies (rho 0.715, $p < 0.0001$) as well as Spike HCoV-NL63 (rho 0.422, $p = 0.007$). Also, there was a positive association between IgG to SARS-CoV-2 RBD and Spike HCoV-NL63 (rho 0.583, $p < 0.0001$). This change in antibody association between SARS-CoV-2 and HCoV-NL63, suggests the expansion of antibodies that recognize both these *Coronaviridae*. IgG antibody levels to HCoV-OC43 were at the (upper) limit of detection therefore we could not conduct correlation analysis with these and we performed titrations to further investigate these.

3.4. Neutralizing activity against SARS-CoV-2 in prepandemic and pandemic healthy controls

To understand the significance of anti-spike IgG antibodies, we investigated their virus neutralization ability against SARS-CoV-2 using a pseudotyped virus assay as described previously [21]. We tested sera randomly selected from PPC and HC 2021 (unvaccinated) cohorts. Of the 16 PPC sera tested, one (6.2 %) displayed low neutralizing activity against SARS-CoV-2 entry into 293T-Ace2 cells (Sup Fig. 7). Of the 18 sera tested from the HC 2021 group, nine individuals (50 %) displayed neutralizing activity against SARS-CoV-2, with NT_{50} values ranging 24 to 227.8 (Sup Fig. 8). The greater proportion of individuals in HC 2021 with neutralizing activity against SARS-CoV-2 is suggestive of asymptomatic SARS-CoV-2 infection in this group (Fig. 1C). Further, we found that all nine HC 2021 group sera with neutralizing capacity were anti-RBD positive, with IgG levels $OD_{450nm} > 0.7$ (data not shown).

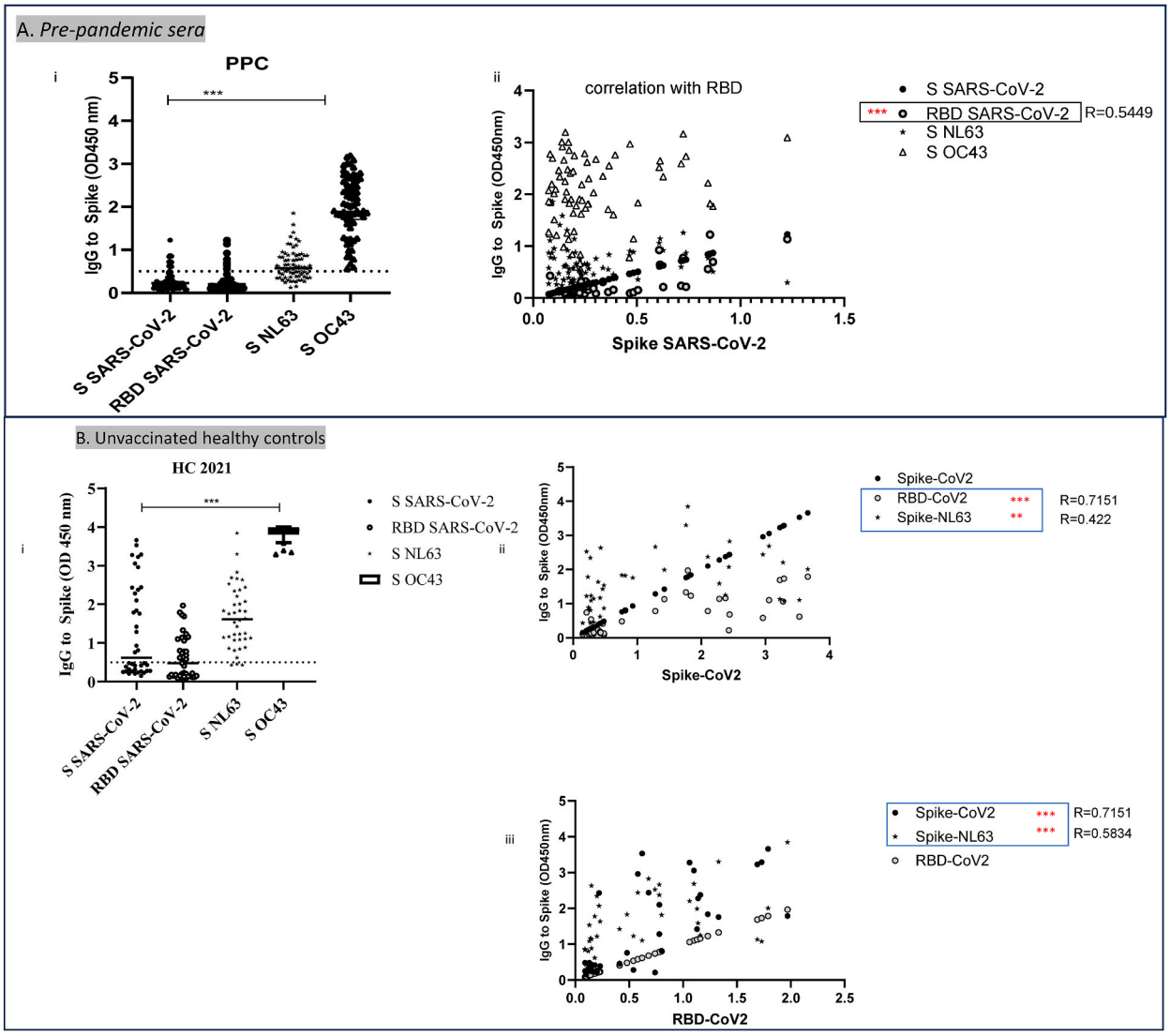
3.5. Determining SARS-CoV-2 infection using a commercial diagnostic assay

We used a COVID-19 VIDAS (Biomerieux) diagnostic assay to test sera from PPC and HC 2021 (baseline) groups. This detects IgG to

Table 2
IgG seropositivity to Spike of SARS-CoV-2, HCoV-NL63 and HCoV-OC43.

Study Cohort		SARS-CoV-2 Spike		SARS-CoV-2 RBD		HCoV-NL63 Spike		HCoV-OC43 Spike	
	Total tested (n)	Positive (n)	%	Positive (n)	%	Positive (n)	%	Positive (n)	%
Pre-pandemic (PPC)	72	11	15.2	6	8.3	43	59.7	72	100
HC 2021	39	19	48.7	13	33	36	92.3	39	100

The table describes the number of samples tested in ELISA assays measuring IgG to spike of SARS-CoV-2, HCoV-NL63 and HCoV-OC43.



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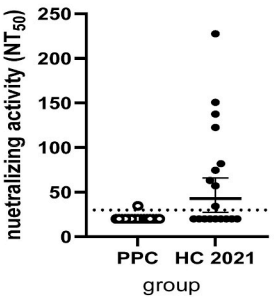

Panel C. SARS-CoV-2 neutralizing titers in sera from PPC and HC 2021 groups. Neutralizing activity against SARS-CoV-2 was determined using a psuedotyped virus assay in sera from 16 individuals from PPC and 18 unvaccinated individuals from HC 2021 (Supplementary figs 7-8). The neutralizing titers for each group are depicted. The horizontal dotted line marks the cut-off for neutralizing titer (NT50=30). Samples with negative titers are plotted with an arbitrary value NT50=20.

(caption on next page)

Fig. 1. Baseline IgG antibody levels in pre-pandemic sera of healthy individuals to Spike proteins of SARS-CoV-2 and HCoVs in healthy individuals from the pre-pandemic and pandemic periods. Panel A. *Pre pandemic sera*. ELISA assays were carried on sera at a dilution 1:100 with each of the antigens, to determine the intensity of antibody binding (OD 490 nm). IgG antibodies to the three Spike antigens of SARS COV-2, NL63 and OC43, were assessed in PPC (n = 72); X axis indicates the antigens tested and Y axis shows the OD. i. IgG antibodies are plotted as scatter plots for each group with geometric means indicated by a horizontal line. Significant differences among groups were assessed using ANOVA test, '***', $p < 0.0001$. ii. IgG intensity shown as a scatter comparing Spike SARS-CoV-2 IgG antibodies to different Spike proteins respectively (SARS-CoV-2 RBD, Spike NL63 and OC43). X axis indicates Spike SARS COV-2 as the primary antigen and Y axis shows tensity of binding as OD 490 nm. Significance of correlation between IgG binding to different antigens was determined using Spearman's rank correlation analysis shown on the right ('***', rho 0.5449). Panel B. *Healthy individuals (HC) during 2021*. Sera from 39 unvaccinated healthy controls obtained during 2021 were tested for IgG to Spike and RBD of SARS-COV-2, and Spike of NL63 and OC43, respectively. Sera were tested at a dilution of 1/100. All other parameters are the same as Panel A ii. Panel C. SARS-CoV-2 neutralizing titers in sera from PPC and HC 2021 groups. Neutralizing activity against SARS-CoV-2 was determined using a psuedotyped virus assay in sera from 16 individuals from PPC and 18 unvaccinated individuals from HC 2021 (Supplementary Figs. 7–8). The horizontal dotted line marks the cut-off for neutralizing titer ($NT_{50} = 30$). Samples with negative titers are plotted with an arbitrary value $NT_{50} = 20$.

SARS-CoV-2 RBD (S1 region) unlike the Spike ELISA which includes S1 and S2 regions. We included both SARS-CoV-2 Spike seropositive and seronegative sera; 15 from HC 2021 comprising eight seropositive and seven seronegative individuals. From the PPC group we included six seropositive and two seronegative sera – all eight samples tested negative on the diagnostic assay. Seven of the eight (89 %) Spike positive samples from the HC 2021 group were identified as COVID-19 positive by the diagnostic assay, whilst all seven seronegative sera were confirmed negative (Table 3). One low seropositive Spike seropositive HC 2021 sera (median OD 0.78) was found to be negative on the VIDAS assay, likely suggesting cross-reactive antibodies in the former assay. A positive control COVID-19 convalescent case tested positive both on Spike ELISA and VIDAS assays. These data show that the ELISA to Spike detects a broader range of epitopes than the RBD region thus, differential spike seropositivity was observed between pre-pandemic and pandemic samples. This shows that the Spike IgG ELISA is not specific for SARS-CoV-2.

3.6. IgG titers to Spike SARS-CoV-2 and OC43 increased during the pandemic

IgG antibody titrations were conducted to further investigate anti- HCoV-NL63 and HCoV-OC43 as high levels (>2.0 OD_{450nm}) were observed in PPC and HC 2021 groups. We randomly selected a subset of sera from each cohort, 16 from PPC and 15 sera from HC 2021 and determined IgG titers to Spike of SARS-CoV-2, HCoV-NL63 and HCoV-OC43 (Fig. 2 panel A–B). IgG titers against each spike were compared between PPC and HC 2021 groups using an AUC analysis. Median AUC levels indicated a significant increase (by 1.8-fold) in IgG antibodies to SARS-CoV-2 between pre-pandemic and pandemic cohorts ($p = 0.04$, Fig. 2C). IgG titers to HCoV-NL63 showed an increasing trend between the PPC and HC 2021 groups ($p = 0.08$), but this was not significantly different. Of note, IgG titers to HCoV-OC43 significantly increased (3.7-fold) in HC 2021, $p = 0.002$. Overall, these data confirm that IgG antibodies specific to SARS-CoV-2 and HCoVs increased during the pandemic.

3.7. IgG antibodies to SARS-CoV-2 increase after SinoVac and BNT162b2 vaccination

We investigated how vaccination affected antibodies to SARS-CoV-2 by longitudinal testing of sera from individuals in the HC 2021 cohort, comparing IgG prior to vaccination and after 6, 12 and 24 weeks of receiving the first dose of vaccination. We studied responses to inactivated SinoVac (CoronaVac) and mRNA vaccinations. SinoVac administration in 20 individuals was followed up in a time-series after first dose of vaccination. We found an increase in anti-Spike IgG antibody levels compared with baseline; 8.1-fold after 6 weeks, 5.9-fold by 12 weeks and 4.8-fold by 24 weeks after (Fig. 3A, $p < 0.0001$). To further confirm this, we measured IgG antibody titers in the time-wise series of sera collected from nine individuals. Anti-Spike titers are shown at baseline (Fig. 3B), 6 weeks (Figs. 3C), 12 weeks (Figs. 3D) and 24 weeks (Fig. 3E) after SinoVac vaccination. The increase in anti- Spike SARS-CoV-2 is also shown through an AUC of longitudinal titers in the vaccinees ($p = 0.005$, Fig. 3F).

Table 3
Comparison of anti-spike ELISA and VIDAS SARS-CoV-2 IgG assay.

Study cohort	Samples based on anti-Spike SARS-CoV-2 positivity	Anti-Spike ELISA	Result on VIDAS IgG RBD ELFA assay
		IgG (OD 450 nm, median)	Positive samples n (%)
PPC (n = 8)	seropositive (n = 6)	0.78	0 (0)
	seronegative (n = 2)	0.10	0 (0)
HC 2021 (n = 15)	seropositive (n = 9)	2.30	8 (88)
	seronegative (n = 7)	0.24	0 (0)
COVID-19 convalescent sera(n = 1)	seropositive (n = 1)	2.27	1 (100)
	–		–

Sera from pre-pandemic controls (PPC, n = 8), HC2021 (n = 15) and COVID-19 convalescent case (n = 1) were used to measure SARS-CoV-2 IgG to spike using an ELISA assay and to RBD using VIDAS® SARS-COV-2 IgG II (BIOMÉRIEUX, France) kit based on ELFA (Enzyme Linked Fluorescent Assay) technique. Data is shown as the number of positive results (%) out of the total. The cut-off value of positive result used by assay is equivalent to Index ≥1.00.

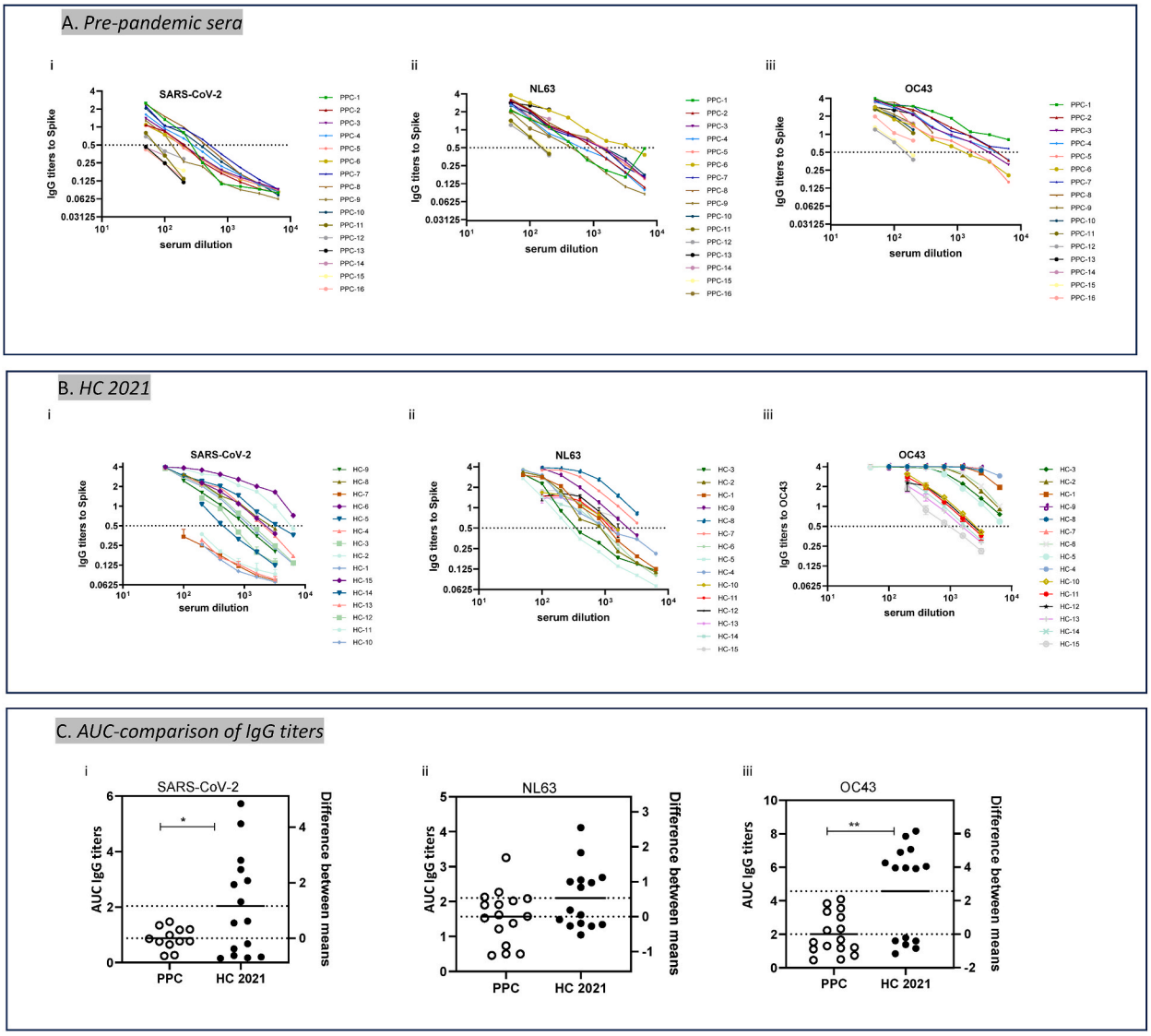

Fig. 2. IgG titers of sera from PPC and HC 2021 show an increase in antibodies to SARS-CoV-2 and OC43.
Panel A. Graphs show IgG titers in sera of 16 individuals from the PPC group tested against Spike of (i) SARS-CoV-2, (ii) NL63 and (iii) OC43. Panel B. Graphs show IgG titers in sera of 15 individuals from the HC 2021 cohort (prior to vaccination) tested against Spike of (i) SARS-CoV-2, (ii) NL63 and (iii) OC43. Sera were run at 2-fold dilutions starting at 1:50 in the respective ELISA assays (X-axis). The line graphs depict results of each individual as coded by colour and ID. Panel C. The IgG titers against (i) SARS-CoV-2, (ii) HC0V-NL63 and (iii) HCoV-OC43, determined for PPC and HC 2021 groups were compared using an under the curve (AUC) analysis. Graphs depict mean differences between AUC using the Mann-Whitney *U* test. Statistical analysis between groups is indicated by '*', $p < 0.05$, '**', $p < 0.001$.

Next we investigated the impact of BNT162b2 vaccination on IgG antibodies to SARS-CoV-2. We ran SARS-CoV-2 Spike antibody titrations in the sera of 19 vaccinees comparing results at baseline (week 0) with those after 6, 12 and 24 weeks of the first dose. A significant increase in SARS-CoV-2 IgG levels ($p < 0.0001$) were seen, with a 7.2-fold increase by 6 weeks, after which there was a decrease in antibody levels although the levels at 12 and 24 weeks remained raised (Fig. 4A). To further examine this, we ran IgG titers in longitudinally collected sera of six of the vaccinees; at baseline, 6, 12 and 24 weeks after vaccination (Fig. 4B–E). An AUC analysis of IgG titers compared with baseline levels showed; 2.6-fold increase by 6 weeks, 3.4-fold increase at 12 weeks and 3.2-fold increase by 24 weeks respectively (Fig. 4F).

3.8. IgG antibodies to HCoV-NL63 increase after SinoVac and BNT162b2 vaccination

The effect of vaccination on antibody levels to HCoV-NL63 was investigated in the HC 2021 cohort by determining IgG titers to HCoV-NL63 after SinoVac and BNT162b2 vaccinations. In the SinoVac cohort, anti-HCoV-NL63 titers compared at baseline, then 6, 12

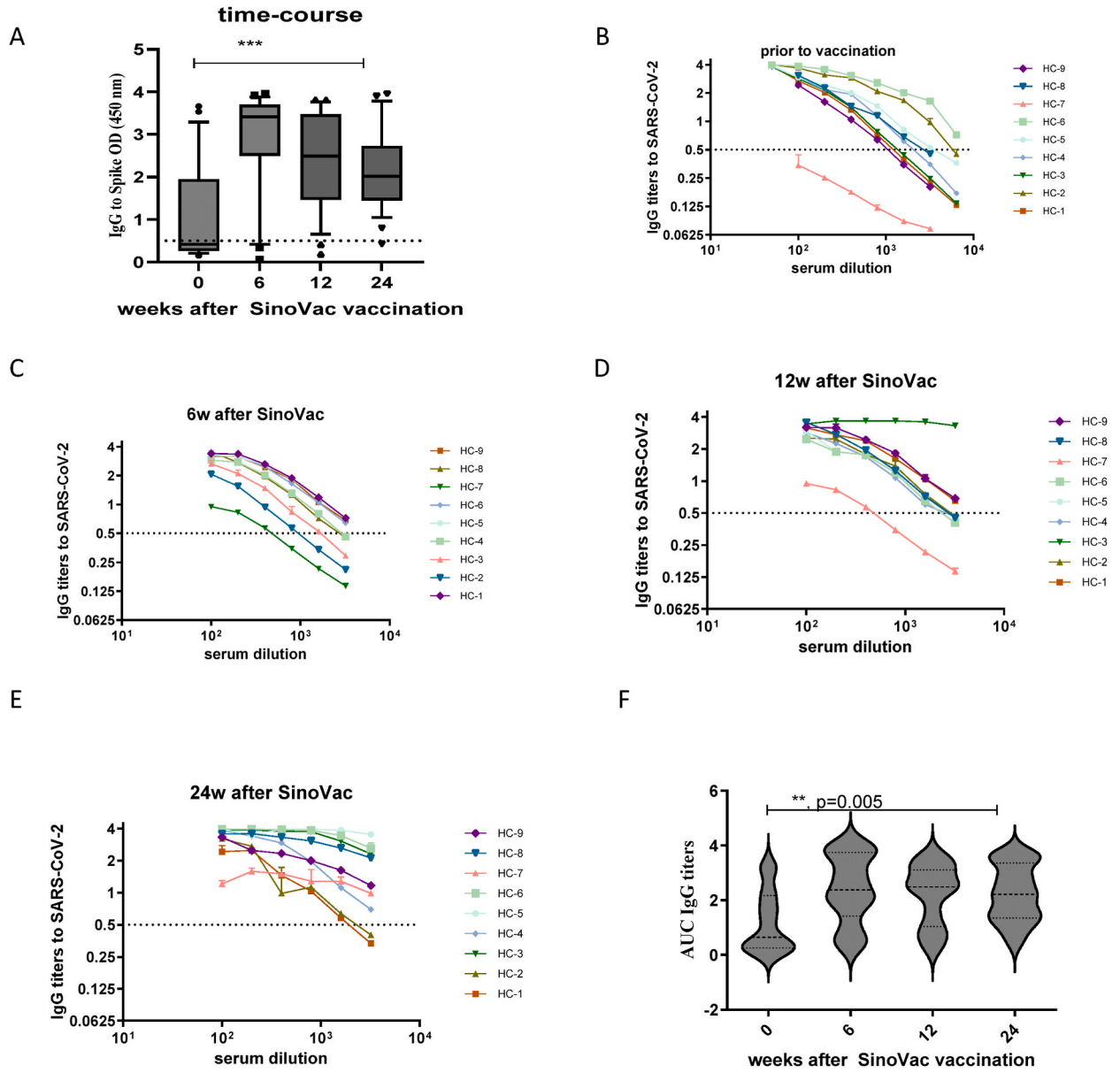

Fig. 3. IgG titers of sera from PPC and HC 2021 show an increase in antibodies to SARS-CoV-2 and OC43. Panel A. Graphs show IgG titers in sera of 16 individuals from the PPC group tested against Spike of (i) SARS-CoV-2, (ii) NL63 and (iii) OC43. Panel B. Graphs show IgG titers in sera of 15 individuals from the HC 2021 cohort (prior to vaccination) tested against Spike of (i) SARS-CoV-2, (ii) NL63 and (iii) OC43. Sera were run at 2-fold dilutions starting at 1:50 in the respective ELISA assays (X-axis). The line graphs depict results of each individual as coded by colour and ID. Panel C. The IgG titers against (i) SARS-CoV-2, (ii) HC0V-NL63 and (iii) HCoV-OC43, determined for PPC and HC 2021 groups were compared using an under the curve (AUC) analysis. Graphs depict mean differences between AUC using the Mann-Whitney *U* test. Statistical analysis between groups is indicated by '*', $p < 0.05$, '**', $p < 0.001$.

and 24 weeks after the first dose of SinoVac (Sup Fig. 9A–D) were found to be increased. This was evident by AUC analysis that showed IgG titers to HCoV-NL63 increased 2.2-fold by 6 weeks, 2.5-fold by 12 weeks and 2.8-fold by 24 weeks after vaccination (Fig. 5A, $p < 0.0001$).

The effect of BNT162b2 vaccination on anti-HCoV-NL63 was also studied, comparing antibodies to in the same individuals prior to vaccination and then longitudinally until 24 weeks afterwards (Sup Fig. 10A–D). An AUC analysis of time-wise IgG titers showed that antibody levels increased by −1.3 fold (6 weeks), −1.7-fold (12 weeks) and 0.9-fold (24 weeks) after vaccination (Fig. 5B, $p = 0.0003$).

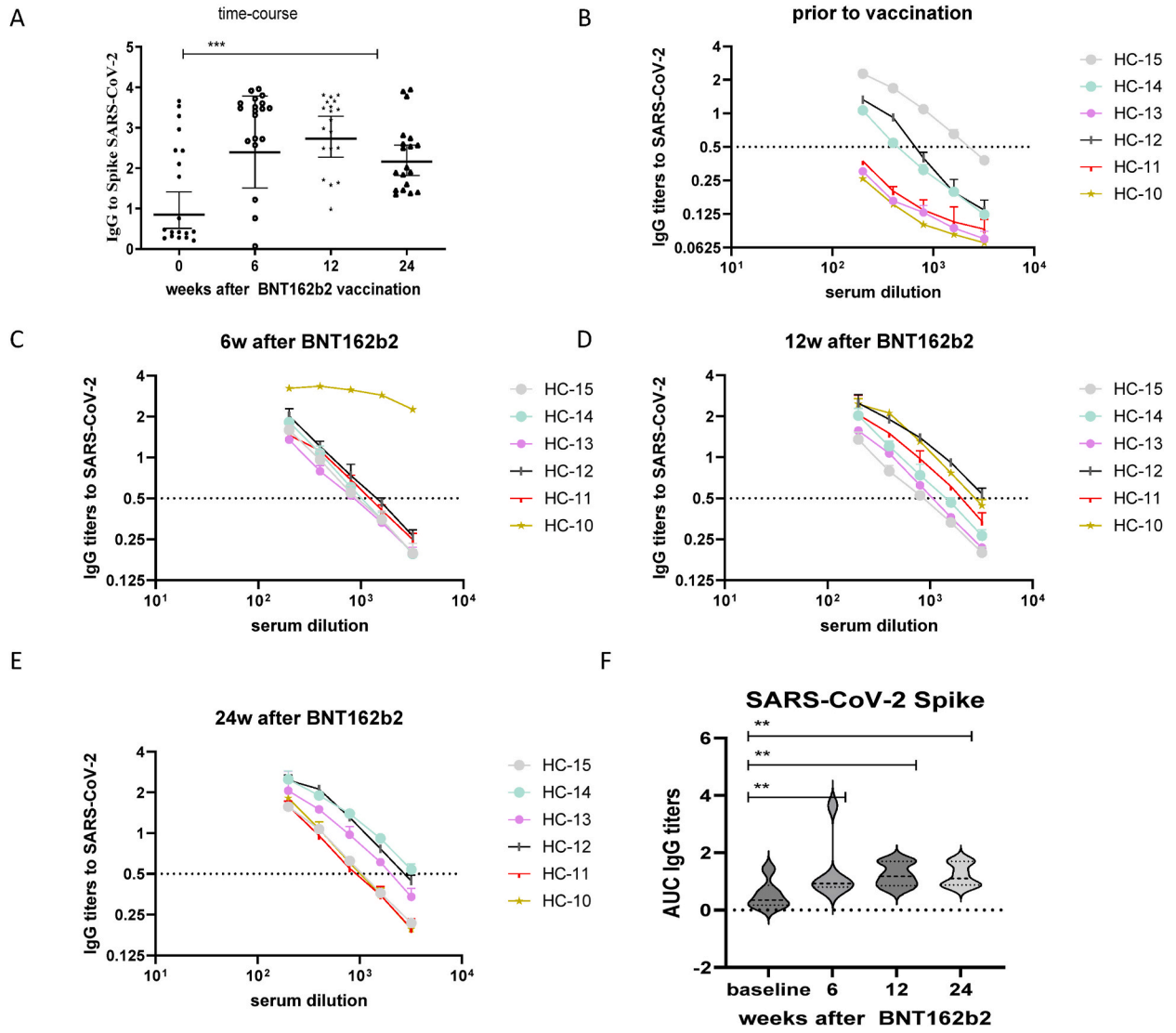

Fig. 4. Increase in IgG antibodies to SARS-CoV-2 Spike after SinoVac vaccination. A, IgG to Spike SARS-CoV-2 was measured in 20 individuals from HC 2021 at 0 (prior to vaccination), after 6, 12 and 24 weeks after first dose of SinoVac. Sera were tested at 1/100 serum dilution. Graphs show IgG titrations on sera of nine individuals (from A) tested at (B) 0 weeks, (C) 6, (D) 12 and (E)24 weeks. F, AUC analysis of IgG titers compared between 0, 6, 12 and 24 weeks. The line graphs depict results of each individual as coded by colour and ID. Statistical analysis between groups was run using the ANOVA, '*', $p \leq 0.05$.

3.9. BNT162b2 induces an increase in IgG antibodies to HCoV-OC43

The effect of inactivated and mRNA vaccinations on anti-HCoV-OC43 levels was then determined. As HCoV-OC43 IgG levels were found to be homogenous high in individuals and thus not informative, we ran IgG titrations to study the antibody dynamics after vaccination. We compared vaccine-induced responses in nine individuals from HC 2021, testing sera at 0, 6, 12 and 24 weeks after the first dose (Sup Fig. 11A–D). Evaluation of AUC of HCoV-OC43 IgG titers did not reveal any increase after SinoVac vaccination in the cohort tested (Fig. 6A).

IgG titers to HCoV-OC43 increased after BNT162b2 vaccination, compared with baseline (median values); 1.8- fold by 6 weeks, 2.2-fold by 12 weeks and 2-fold by 24 weeks (Sup Fig. 12A–D). This is shown through an AUC analysis of anti- HCoV-OC43 titers (Fig. 6B, $p < 0.0001$).

Overall, these data show that SinoVac vaccination induced IgG antibodies to Spike proteins of SARS-CoV-2 and HCoV-NL63. Whilst, BNT162b2 vaccination induced antibodies to Spike proteins of SARS-CoV-2, HCoV-NL63 as well as HCoV-OC43.

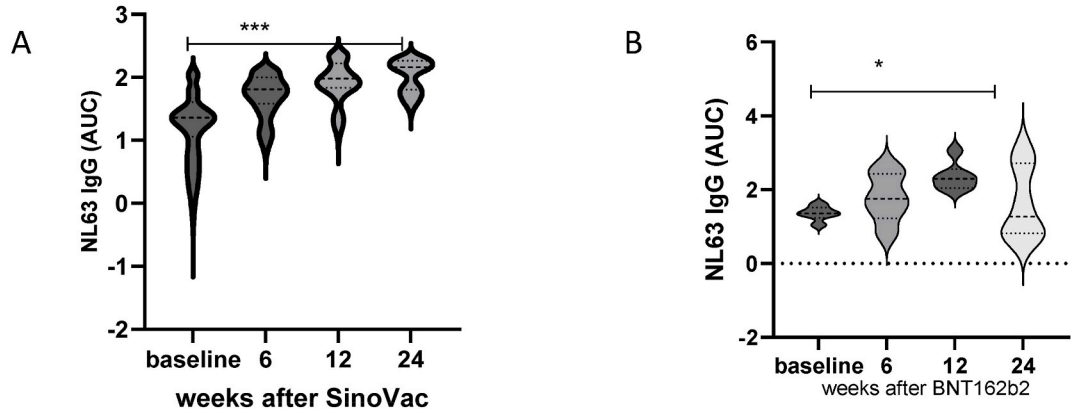

Fig. 5. Increase in IgG antibodies to HCoV-NL63 Spike after vaccinations. The figure shows titrations for IgG to Spike NL63 in individuals (HC 2021) vaccinated and tested sera at baseline (0), 6, 12 and 24 weeks. A, SinoVac vaccination in nine individuals. B, BNT162b2 vaccination in six individuals. The graph shows AUC of IgG titers compared between different time intervals. Statistical analysis between groups was run using the ANOVA, '***', $p < 0.0001$, '*', $p < 0.05$.

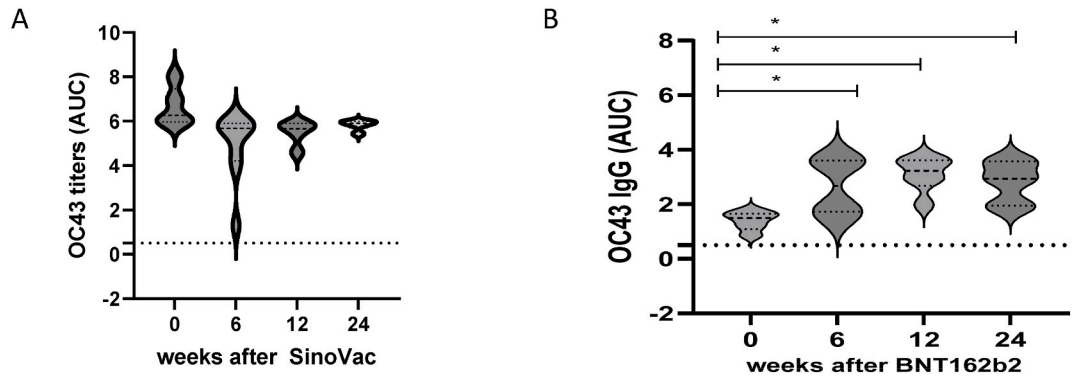

Fig. 6. Increase in IgG antibodies to HCoV-OC43 Spike after vaccinations. The figure shows titrations for IgG to Spike NL63 in individuals (HC 2021) vaccinated and tested sera at baseline (0), 6, 12 and 24 weeks. A, SinoVac vaccination induced IgG levels in nine individuals. B, BNT162b2 vaccination induced IgG titers in six individuals. The graph shows AUC of IgG titers compared between different time intervals. Statistical analysis between groups was run using the ANOVA, '***', $p < 0.0001$, '*', $p < 0.05$.

4. Discussion

Our data shows that antibody responses to endemic coronaviruses were present prior to the COVID-19 pandemic, and that antibodies to OC43 and NL63 expand after COVID-19 vaccinations in addition to those to SARS-CoV-2. We compared sera from pre-pandemic and pandemic periods from Pakistan. Furthermore, we determined the effect of both inactivated SinoVac and mRNA BNT162b2 types COVID-19 vaccinations on the expansion of spike antibodies.

Whilst 15 % of pre-pandemic sera were seropositive to Spike and 8 % to RBD of SARS-CoV-2, only one sera showed any SARS-CoV-2 neutralizing capacity. Suggesting that the antibody reactivity was likely non-specific. Importantly, such IgG antibodies to SARS-CoV-2 as well as virus neutralizing ability were increased in the HC 2021 cohort prior to COVID-19 vaccinations. The observation of low-level neutralizing activity in pre-pandemic sera (5.6 %) also fits with earlier reports from our population [22], as does increased IgG seropositivity to SARS-CoV-2 Spike and RBD in healthy controls during [9]. IgG antibodies to RBD are associated with neutralizing activity to SARS-CoV-2 [23,24] and its immune protection against disease [25]. Our study subjects were from a rural region in the Sindh province, from which low rates of COVID-19 had been reported through the pandemic. However, there may have been under-reporting of COVID-19 infections. It is likely though that COVID-19 cases were under-reported, due to the limited testing available and resource constraints [8]. At the peak of the pandemic in 2020, the maximum number of tests conducted daily was about 70,000 in a population of 220 million [13]. The high rate of neutralizing ability in sera in the HC 2021 group (50 %) indicates the high likelihood of asymptomatic infection in individuals during the pandemic. Further, sera with neutralizing activity were also positive for COVID-19 antibodies using a commercial diagnostic assay confirming SARS-CoV-2 infection. This likely reflect high transmission and infection rates in the local population, associated with high rates of SARS-CoV-2 antibody seropositivity in high density areas [26].

Through the ELISAs to Spike of SARS-CoV-2, HCoV-NL63 and HCoV-OC43 we observed that antibodies to HCoVs were highly present in pre-pandemic sera. The lower level of IgG to SARS-CoV-2 Spike in the pre-pandemic group are likely due to lower affinity of

cross-reactive antibodies to HCoV Spike, especially the RBD region [27]. Whilst it is likely that the assay was not very specific, it is evidence of the preponderance of antibodies to different coronaviridae. The presence of antibodies to HCoV-NL63 and HCoV-OC43 in pre-pandemic sera is in line with reports regarding endemic coronaviruses in other countries. Cross-reactive immunity has been reported in pre-pandemic sera from Central and West African Populations [28], Sub-Saharan Africa [29], Kenya [30], Italy [31] and Turkey [32], amongst other countries. The absence of correlation in pre-pandemic sera between antibodies to SARS-CoV-2 and those to HCoV-NL63 and HCoV-OC43 is in contrast to data from the USA, which showed sera from children and young adults to have anti-Spike and anti-RBD antibodies that correlated with HCoV-OC43 antibody titers [12]. Similarly, anti-HCoV-OC43 antibodies found in Finnish children correlated significantly with the level of cross-reactive anti-SARS-CoV-2 antibodies present [33]. However, our data agrees with other reports on pre-COVID-19 antibodies which showed an absence of correlation between anti-Spike HCoV-OC43 and SARS-CoV-2 [34]. Such differences may be suggestive of differences in circulating endemic HCoV in different regions. Bioinformatics based analysis of Spike proteins of SARS-CoV-2 shows homology ranging between the wild-type strain to delta variant against HCoV-NL63 (31.4–31.7 %) and HCoV-OC43 (42.7–44.8 %) [11]. The differential homologies found between HCoVs and SARS-CoV-2 strains from wild-type to variants of concern are relevant. At the time of this study in 2021, Pakistan had experienced several COVID-19 waves and the delta variant was predominant [35].

The increase in IgG antibodies to HCoV-NL63 in pandemic controls, as well as its correlation with antibodies to SARS-CoV-2 indicates recognition of similar epitopes. Cross-reactive antibodies present in circulation likely precede the higher affinity antibodies produced by plasma cells from germinal centers as a consequence of antibody maturation [36]. Such antibodies are associated with an expansion of B cells after SARS-CoV-2 infection [37]. Previous reports indicate that prior antibodies to the alpha coronavirus HCoV-NL63 are associated with increased immunity to SARS-CoV-2 leading to a greater chance of asymptomatic infection [38].

In both pre-pandemic and pandemic controls, the magnitude of IgG antibodies to HCoV-OC43 Spike was greater than to SARS-CoV-2 and HCoV-NL63. Previous reports showed an increase in antibodies to Spike HCoV-OC43 in individuals' convalescent after COVID-19, indicating activation of cross-reactive immunological memory to beta-coronavirus Spike after SARS-CoV-2 infection [12,39,40]. Another report proposes that recent infection with HCoV-OC43 may be associated with protection against SARS-CoV-2 infection [39]. Further, a broadly cross-reactive antibody has been shown to have a neutralizing potential that can protect against sarbecovirus challenge in mice [41].

COVID-19 vaccinations in Pakistan were administered in a phase-wise approach focusing first on older aged individuals and health care workers then, younger age groups. The vaccinations were available based on initiatives from China, the COVAX initiative and other international efforts, with limited imports. Hence, we had a mixed-vaccine population and it was important to understand the comparative effectiveness of the different COVID-19 vaccines administered.

We observed an increase in antibodies to SARS-CoV-2 after vaccination with both inactivated and mRNA vaccines. This is as expected after COVID-19 vaccinations and shown elsewhere [23,42]. Of note, both in our earlier report [43] and in our current study, we did not observe any waning of anti-Spike antibodies after vaccination for up to 24 weeks after vaccination. This is in contrast with earlier reports of waning of IgG antibodies to Spike SARS-CoV-2 with time [44]. Globally, such data may vary depending on circulating viruses as well as the type of vaccine used. When individuals from the pandemic cohort were vaccinated, there was an increase in SARS-CoV-2, HCoV-NL63 and HCoV-OC43 antibodies. This rise in NL63 IgG levels was observed both in the group vaccinated with inactivated SinoVac and mRNA BNT162b2 vaccines. However, after SinoVac vaccination did not observe the enhancement of anti-OC43, likely due to the high basal levels observed prior to vaccination. In a study from China by Hu *et* al. showed an increase in HCoV-OC43 and HCoV-NL63 antibodies after inactivated vaccine (BBIBP-CorV) administration, with greater increase in SARS-CoV-2 antibodies in those who had pre-existing HCoV-OC43 titers [45].

Our observation that mRNA vaccination resulted in an increase in anti-spike antibodies to HCoV-NL63 and HCoV-OC43 fits with previous reports. Asamoah-Boaheng *et* al. showed that COVID-19 mRNA vaccines induced antibodies to HCoVs and also that higher baseline HCoV-NL63 titers were associated with an enhanced vaccine induced antibody response [46]. Overall, our data fits with earlier reports that show that the post-COVID-19 population has high prevalence of cross reactive antibodies to spikes from all Orthocoronavirinae genera, including β-coronaviruses, α-coronaviruses and pandemic variants [14], indicating recognition of conserved epitopes shared across spike proteins of the different genera. It was observed that post -BNT162b2 vaccination, there are cross reactive antibodies to OC43, MERS but not 229E [47]. This may be due to variations in B cell epitopes in different HCoVs resulting differential recognition by IgG antibodies.

A limitation of our study is that we do not have prior knowledge of circulating HCoVs in the population studied. Unfortunately, due to technical limitations we could perform neutralizing testing on all samples. We have used AUC analysis where IgG titers were available. Further, we show consistency between the trends observed for Median and AUC comparisons. Importantly, the study samples selected were representative of a population that received vaccinations and this age group was chosen as the under 50 years to avoid any confounders due to a higher age; as observed previously that inactivated vaccine induced antibody responses were lower in those aged 50 years [43]. Also, we could not conduct assays to test serum neutralizing activity against endemic coronaviruses. However, the current learning through new generation of antibodies is that they can generate a broad spectrum of activity against different coronaviruses, bat and human [48]. In the case of SARS-CoV-2 breakthrough infections it has been shown that vaccination intervals affect cross-neutralizing activity of antibodies against different variants [49]. It would be important to conduct further studies to investigate the functional implications of such antibodies in conferring protection against viral infections.

In conclusion, we observed IgG antibodies present in prepandemic sera which recognised spikes of NL63 and OC43 likely bound SARS-CoV-2 through cross-reactive antibodies. Antibodies were found to expand through the pandemic likely due to both natural infection and COVID-19 vaccinations. As preexisting antibodies lead to early expansion of virus specific responses, circulating coronaviruses likely affect the magnitude of responses from natural infection and COVID-19 vaccines.

CRediT authorship contribution statement

Zahra Hasan: Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Kiran Iqbal Masood:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis. **Marc Veldhoen:** Writing – review & editing, Methodology, Investigation. **Shama Qaiser:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Marta Alenquer:** Writing – review & editing, Resources, Methodology. **Mishgan Akhtar:** Writing – original draft, Data curation. **Sadaf Balouch:** Methodology, Data curation. **Junaid Iqbal:** Supervision, Resources, Investigation. **Yaqub Wassan:** Writing – review & editing, Project administration, Methodology. **Shahneel Hussain:** Methodology, Investigation, Data curation. **Khalid Feroz:** Software, Methodology. **Sajid Muhammad:** Project administration, Methodology. **Atif Habib:** Resources, Project administration, Methodology. **Akbar Kanji:** Methodology, Investigation, Data curation. **Erum Khan:** Resources. **Afsar Ali Mian:** Resources, Methodology. **Rabia Hussain:** Writing – review & editing, Methodology, Formal analysis. **Maria Joao Amorim:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **Zulfiqar A. Bhutta:** Writing – review & editing, Resources, Methodology.

Data availability

The data will be made available upon request upon acceptance of this article.

Funding

This study was supported by the Provosts Academic Priorities Fund, Aga Khan University, Pakistan.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Zahra Hasan reports financial support was provided by The Aga Khan University Medical College Pakistan. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Paula M. Alves from iBET, Portugal for providing SARS-CoV-2 recombinant proteins.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e42171.

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