

Humoral Immunity and Variant Cross-Reactivity Following Asymptomatic and Symptomatic SARS-CoV-2 Infection and Vaccination in Young Adults (The ACE Cohort)

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Abstract

Purpose Asymptomatic SARS-CoV-2 infection was a driving force of the COVID-19 pandemic, acting as a silent transmitter of the virus. The question of whether asymptomatic immunity differs from symptomatic immunity remains, as existing studies fail to recruit ‘well-defined’ asymptomatic individuals. Here, we conducted a longitudinal cohort study to evaluate humoral responses to infection and vaccination in asymptomatic predominantly young adults identified as part of the Asymptomatic COVID-19 in Education [ACE] cohort.

Methods Asymptomatic testing services, located at three UK universities, identified asymptomatic adults who were subsequently recruited with age- and sex-matched symptomatic and uninfected controls. Blood and saliva samples were collected after SARS-CoV-2 Wuhan infection, and again after vaccination. Over 500 participants were recruited, and their IgG and IgA titres were measured in response to 5 Variants of Concern.

Results Pre-vaccination, asymptomatic individuals exhibit significantly lower IgG responses to four of the tested variants; Wuhan, Alpha, Beta, and Delta, compared to symptomatic individuals; these differences diminished post-vaccination. In addition, there were key differences in terms of IgG cross-reactivity, where symptomatic individuals displayed stronger IgG cross-reactivity than asymptomatic and uninfected individuals, alluding to better protection from emerging variants. Individuals without prior natural infection displayed reduced IgG responses compared to symptomatic individuals, to all variants, post-vaccination. Despite their low IgG, salivary IgA levels in uninfected individuals were high, pre-vaccination.

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Conclusion This work demonstrates the requirement of vaccinations to enhance weaker IgG responses but also highlights that vaccines need to be constantly adapted to overcome viral evolution, particularly with more done to improve mucosal delivery options for respiratory viruses to enhance IgA responses.

Keywords Asymptomatic · Symptomatic · SARS-CoV-2 · Vaccination · Antibody · Variants of Concern · IgG · IgA

Introduction

December 2019 saw the emergence of a new coronavirus, SARS-CoV-2, resulting in over 774 million confirmed cases globally, and more than 7 million reported deaths, as of February 2024 [1]. The clinical outcome of infection is highly variable, ranging from completely asymptomatic to severe disease requiring hospitalisation, and, ultimately, death. Approximately 20–44% of SARS-CoV-2 infections were asymptomatic [2–6], with increased rates in younger individuals [6]. As a large proportion of young adults are thought to have transmitted SARS-CoV-2 ‘silently’, and with a viral load comparable to that of symptomatic infection [7], asymptomatic disease was a key contributor to the magnitude of disease transmission during the COVID-19 pandemic.

The immune response to SARS-CoV-2 comprises a humoral and cellular response, with most vaccine candidates designed to target the humoral response, specifically neutralising antibodies, to prevent the ability of SARS-CoV-2 to infect cells [8]. The ability of vaccines to protect against emerging strains of SARS-CoV-2 raises important questions. Indeed, several variants of concern (VoC) were identified throughout the COVID-19 pandemic, including B.1.1.7 (Alpha), B.1.351 (Beta), B.1.617.2 (Delta), P.1 (gamma), and most recently B.1.1.529 (Omicron) [9]. These VoC were reported to have substantial mutations in the spike protein, which mediates the infection of human cells and is the major target of the humoral immune response [10]. Specifically, Omicron had the highest number of alterations from the 2019 isolate, which has led to immune evasion and lack of protection, despite vaccination against previous variants [11].

The immune responses to VoC may differ depending on previous asymptomatic or symptomatic infection, as well as subsequently influencing the efficacy of vaccination. Upon infection, serum IgG, IgA and IgM antibodies are elicited principally against the spike and nucleocapsid proteins [12]. Asymptomatic infection results in the loss of these serum specific-IgG antibodies faster and more frequently than in symptomatic infection [13]. In addition, in symptomatic infection, anti-SARS-CoV-2 serum IgA antibodies better recognize domains in the S1 than those in the serum of asymptomatic patients [14]. Furthermore, SARS-CoV-2 specific IgA and IgG antibody titres in mucosal tissues were higher in symptomatic infection compared to asymptomatic infection [15, 16]. These factors suggest reduced humoral immune efficacy against subsequent infection in asymptomatic individuals.

Previous studies have investigated cellular immunity in asymptomatic individuals [17–21], but of the existing literature investigating humoral immunity in asymptomatic infection, there is a lack of ‘true’ asymptomatic participants, where they are classified with mild symptoms, rather than no symptoms. Thus, this study took advantage of an ongoing asymptomatic screening service developed at three universities in the UK, named the ‘Asymptomatic COVID19 in Education’ Cohort (the ‘ACE’ Cohort). Serological responses to SARS-CoV-2 were measured pre- and post-vaccination in samples from individuals who have no reported history of contracting SARS-CoV-2, those with true asymptomatic infection, and those with symptomatic infection. SARS-CoV-2 secretory IgA (sIgA) responses were also examined to compare mucosal IgA with serum IgG responses. Antibody responses were measured against the Wuhan strain of SARS-CoV-2, as well as responses against the VoC at that time; Alpha, Beta, Delta, and Omicron. Utilising this largely young, healthy demographic with ‘true’ asymptomatic participants, we generate robust data on the humoral immunity to SARS-CoV 2 variants, as well as establish differences in the level of cross-reactivity in uninfected, asymptomatic and symptomatic individuals.

These findings could aid the design of future SARS-CoV-2 management strategies, such as pan-coronavirus therapeutics or vaccines.

Methods

Data and Sample Collection

A multi-centre longitudinal study was conducted across three sites – The University of Nottingham, The University of Cambridge, and Cardiff University. Participants were identified with the University Asymptomatic Testing Services at each site, and individuals were invited to participate through contact by email. Participating individuals were all over 18 years of age, and eligible to receive a SARS-CoV-2 vaccination. All participants provided written informed consent. Potential participants were excluded if they were below 18 years old, ineligible to receive vaccination, unable to provide blood samples, had low English language proficiency (meaning both consenting and survey participation were not possible), were unable to travel for study visits, or were unable to provide informed consent. A baseline questionnaire was administered to participants and included information on demographics, clinical factors (such as previous SARS-CoV-2 infection and tests), and vaccination details. Participants were divided into six groups according to their self-reported positivity, vaccination, and symptomatic status: negative unvaccinated, negative vaccinated, symptomatic unvaccinated, symptomatic vaccinated, asymptomatic unvaccinated, and asymptomatic vaccinated. These self-reported groups were derived from questionnaire answers. All samples were collected from February 2021 to September 2021.

Symptomatic participants were identified by either PCR (89.3%) or antibody tests (10.7%), asymptomatic participants reported no symptoms and were identified by PCR (42.4%), antibody tests (38.4%), or T-cell activation tests (19.2%). All negative participants had no reported symptoms and had never recorded a positive test.

Finger prick blood samples were taken from each participant at each visit using a disposable lancet and a 10 μ L Mitra stick volumetric absorptive microsampling device (Neoteryx), allowing the sample to dry onto the device. Dried samples were eluted in 150 μ L PBS 1% Triton-X100, with two hours of shaking at 400 rpm at room temperature. All samples were frozen at -20°C until thawed for testing. A visual quality control step was included at this stage, excluding any Mitra sticks that were partially filled or otherwise faulty. Participants were invited to return to be sampled longitudinally, before and after each vaccination treatment. At subsequent clinic visits, participants were re-consented, and information gathered regarding interim SARS-CoV-2 infections, and dates of vaccinations.

Participants also provided 2mL of saliva at each visit, which was stored at -20°C . Saliva samples were centrifuged at 1200 rpm for 1 min, and samples with excess debris present were excluded.

Specific Antibody Response

Separate ELISAs were used for the detection of Nucleocapsid and all variants of Spike-specific antibodies (henceforth collectively referred to as “variants”, “variants of concern”, or “VoC”). In all cases, 384 well Maxi-sorp (NUNC) assay plates were coated with 20 μ L per well of specific antigen (**Supplementary Table 1**) at a concentration of $1\text{ }\mu\text{g mL}^{-1}$ in carbonate-bicarbonate buffer (CBC; Merck). Plates were sealed with foil film and incubated overnight for a minimum of 18 h at 4°C . Plates were then washed 3 times with PBS with 0.05% Tween 20 (PBS-T) using a Biochrom ASYS Atlantis plate washing robot with a 16-channel manifold. Wells were immediately filled with 100 μ L of blocking solution, containing 3% whey powder (w/v) in PBS containing 0.05% Tween 20, 0.05% sodium azide, and 0.01% EDTA and blocked overnight at 4°C . Plates were washed a further 3 times and 20 μ L of diluted serum sample added in duplicate wells. Serum samples were diluted to 1:120 in 3% whey blocking solution. SARS-CoV-2 antibody positive and negative serum controls were obtained from the National Institute of Biological Standards and Controls (NIBSC, UK). Each assay contained a 12-point

standard dilution of NIBSC 20/162 positive standard diluted two-fold from 1:120, two negative controls from the NIBSC assay verification panel, and the QC standard (20/764) all also diluted at 1:120. After incubating for one hour at 21 °C, the plate was washed 3 times, and 20 µL per well was added of gamma chain-specific anti-human IgG HRP conjugate (Sigma, A0170) at a 1:30,000 dilution. This was incubated for 30 min at 21 °C. Following a final three washes with PBS-T, 40 µL One-step Ultra-3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Thermo Fisher Scientific) was added to each well. After incubating for 20 min at 21 °C, 40 µL of 2 N H₂SO₄ was added to each well and absorbance read at 450 and 600 nm using an EPOCH microplate reader (BioTek, UK). Data were presented as either Delta OD (450–600 nm), or a conversion of this into BAU (binding antibody units). All assays were performed on Opentrons OT-2 liquid handling robots. IgA ELISAs were performed using the above method with slight adjustment; samples were diluted 1:15 in 3% whey blocking solution, replacing gamma-chain specific anti-human IgG HRP conjugate with an Alpha-chain specific anti-human IgA HRP conjugate (Sigma, A0295) at a 1:10,000 dilution.

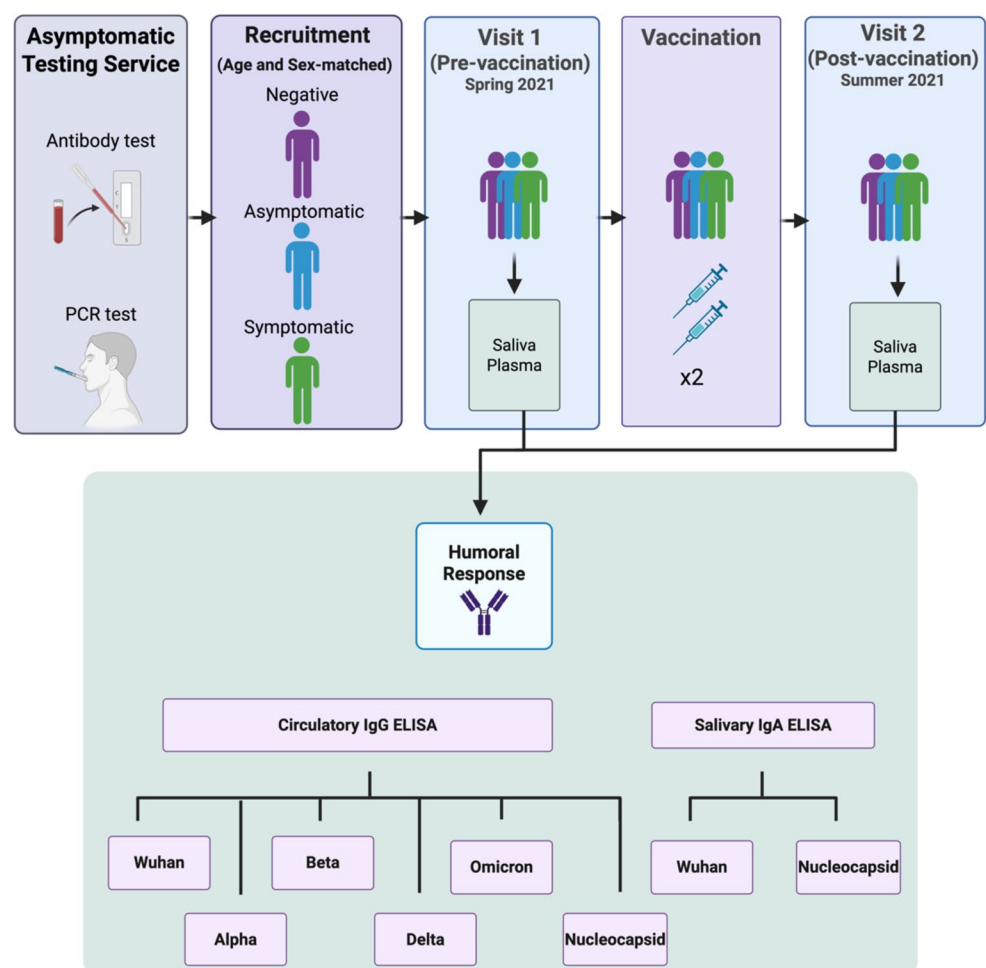
The study design and assays performed are outlined in Fig. 1.

Statistical Analysis

Cohort Response

All data analyses were carried out using R version 4.4.2 [22, 23]. Differences in cohort structure in sex between the six groups were tested with Chi-square tests. To determine whether the sex at birth of a participant impacted

Fig. 1 Study design for the recruitment of participants and analysis of humoral responses to SARS-CoV-2. Asymptomatic adults were identified by the asymptomatic testing service across 3 universities in the UK, utilising PCR, antibody tests, or T-cell activation tests. Asymptomatic participants were then recruited into the study with age and sex-matched Symptomatic and Negative participants. Samples were obtained before and after vaccination, and then variant-specific assays were conducted. Created using BioRender



IgG antibody titres, Mann-Whitney U tests were used, considering each strain of the virus independently. A similar approach was used to determine whether participant age impacted IgG antibody titres, using a Spearman's rho correlation.

Symptomatic Versus Asymptomatic Infection

The difference in antibody titre across variants, symptomatic status, and vaccination status was investigated using generalised linear mixed effects models (GLMMs) with gamma distributed errors. A GLMM was constructed, with vaccination status, symptomatic status, and variant included as fixed factors, and participant ID and sampling location (i.e. Nottingham, Cambridge, or Cardiff) were included as random effects. Sample ID is inherently nested within participant ID, which was reflected in the model fitting procedure. First, saturated models with all main effects, and their two-way interactions were fitted in each case. Terms were removed in a step-wise fashion, testing the effect of deletion using likelihood ratio tests, until only significant terms remained. Testing random effects in generalised linear mixed models is problematic, so the AIC of the saturated GLMM was compared with that of a generalised linear model without the nested random term for sample ID and participant ID to provide an approximate test of the importance of participant [24, 25]. Following GLMM construction, Tukey's pairwise comparisons were carried out to further elucidate differences in immune response among variants of SARS-CoV-2, and unpick differences in significant interaction terms in the constructed model.

In addition, a Kruskal-Wallis test with Dunn's pre-selected multiple comparisons was performed in GraphPad Prism 10.4.1 to compare IgG responses between negative, asymptomatic, and symptomatic individuals. The test was performed independently on pre- and post-vaccination data.

Antibody Cross-Reactivity in Symptomatic and Asymptomatic Individuals

Given vaccinations to SARS-CoV-2 were typically built on the wild-type virus spike protein or its RNA sequence, determining cross-reactivity of spike-specific antibodies is of potential importance. To determine whether combinations of responses to the spike antigen of each possible two-way combination of variants were from the same distribution in multidimensional space (and therefore could be deemed as cross-reactive), 2-dimensional two-sample Peacocks tests were performed. Each combination of responses to variants was tested separately according to the symptomatic status of the participant (symptomatic, asymptomatic, or no infection). These tests were performed using the 'Peacock.test' package in R, and deriving Z-statistics and p-values from the given D-statistic with formulae derived from Peacock [26].

Effect of Infection on IgA Levels

To determine whether sex at birth of a participant impacted on IgA antibody responses to SARS-COV-2, Mann-Whitney U tests were performed between M/F separated sample groups. The impact of age on IgA responses was performed by Spearman's rho correlation. The influence of vaccination status, irrespective of symptomatic status, on IgA levels was performed by Mann-Whitney U tests on relevant sample groups. Correlations between Wuhan-spike and nucleocapsid-specific IgG and IgA antibody titres were assessed separately using Spearman's Rho correlations.

Results

The Effect of Participant Demographics on IgG Levels

An overview of participant demographics is shown in Table 1 and in Supplementary Fig. 1. Cambridge, Cardiff, and Nottingham, respectively, contributed 208, 117, and 205 participants to the study, with each of these cohorts having a similar distribution of age and sex. In Cambridge, 57.21% of participants were female, with an age range of 18–62 and a median age of 27. In Cardiff, 69.23% of the participants were female, with an age range of 18–64 and a median age of 29. Of the Nottingham participants, 63.02% were female, the age range was 18–61, with a median of 22. Participants were split into cohorts according to their symptomatic status (symptomatic, asymptomatic, or negative/no known natural infection) and their vaccination status (unvaccinated or vaccinated), with various iterations of these groupings used for analyses. Based on sample collection dates, all asymptomatic and symptomatic participants were infected with the Wuhan or Alpha variant.

There were no significant differences in sex between groups (Chi-squared; $\chi^2_{(df)} = 7.259_{(5)}$, $p = 0.2021$), or in average IgG antibody response between males and females across any variants tested (Fig. 2a). Each of the six groups (negative unvaccinated, negative vaccinated, asymptomatic unvaccinated, asymptomatic vaccinated, symptomatic unvaccinated, and symptomatic vaccinated) had a similar median age and range, and where age had a significant effect on antibody titre (Fig. 2b), the magnitude of the association (R value) was very weak to negligible.

The Effect of Symptomatic Status on IgG Levels

The effect of the presence of symptoms during SARS-CoV-2 infection on IgG responses to each VoC was first examined pre-vaccination. The differences in IgG responses between negative (uninfected), asymptomatic, and symptomatic individuals for each VoC, as well as in response to nucleocapsid are presented in Fig. 3. Pre-vaccination, there were significantly higher IgG levels in symptomatic compared to asymptomatic individuals, in response to Wuhan (Fig. 3A), Alpha (Fig. 3B), and Delta (Fig. 3D), but not Beta (Fig. 3C), Omicron (Fig. 3E) or Nucleocapsid (Fig. 3F). There were also minimal antibody responses to all variants in the negative group pre-vaccination, with significantly lower IgG levels for each VoC and Nucleocapsid, compared to both the asymptomatic and symptomatic groups, reaffirming the negative group is truly negative.

Table 1 Demographics of participants. The sex, age, and ethnicity of the 530 participants in the ACE cohort (see also Supplementary Fig. 1a and 1b). Ethnicity is divided into White and Other Ethnicities as a large majority of the ACE cohort self-identified as “White: British, Irish, Other”

		Negative		Asymptomatic		Symptomatic		Total
		Unvaccinated	Vaccinated	Unvaccinated	Vaccinated	Unvaccinated	Vaccinated	
Sex (%)	Male	57 (35%)	31 (28%)	30 (36%)	22 (49%)	24 (43%)	25 (35%)	189 (36%)
	Female	107 (65%)	78 (72%)	54 (64%)	23 (51%)	32 (57%)	47 (65%)	341 (64%)
Age	Median	29	25	22	24	21	25	-
	Range	18–64	18–62	18–60	18–59	18–52	19–63	-
Ethnicity	White	97	73	61	38	41	48	358
	Other	14	5	19	7	8	18	71
	Unknown	53	31	4	0	7	6	101
Total N (%)		164 (31%)	109 (20.5%)	84 (16%)	45 (8.5%)	56 (10.5%)	72 (13.5%)	530

The Effect of Vaccination on IgG Levels

Next, the effect of vaccination status on IgG antibody responses between groups was examined. Vaccination status increased spike-specific IgG antibody responses to each SARS-CoV-2 variant in all groups (Fig. 4A). However, the effect of vaccination on antibody titre differs according to variant, as shown by a significant two-way interaction in present between variant and vaccination for all VoC, but as expected, not for nucleocapsid (Fig. 4B and Supplementary Tables 2 and 3). Effect sizes are similar between Wuhan and Delta, and Alpha and Beta, and whilst significant, the effect of vaccination is slightly lower on the average specific Omicron response.

Interestingly, IgG levels to all SARS-CoV-2 spike variants and nucleocapsid were significantly higher in the vaccinated symptomatic group compared to the vaccinated negative group. In addition, there were significantly higher Wuhan- and Delta- and nucleocapsid-specific IgG in the vaccinated asymptomatic group compared to the vaccinated negative group (Fig. 4Bi, 4Biv and 4Bvi), but not for Alpha, Beta or Omicron specific IgG levels (Fig. 4Bii, 4Biii, and 4Bv).

Importantly, for this analysis, the number of days post-vaccination a sample was received was noted. In negative, symptomatic, and asymptomatic groups, the distribution of time between vaccine and sample is similar. In negative individuals, the median number of days between receiving a vaccine and sample collection was 105, with a minimum of 13 and a maximum of 356. In asymptomatic individuals, there was a median of 125, and a range of 17–314 days. In symptomatic individuals there was a range of 32–380 days, with a median number of days between vaccination and second sample collection of 155.

IgG Cross-reactivity

After establishing that specific SARS-CoV-2 variants have an effect on IgG binding titres, differences between combinations of variants were assessed (Table 2, Supplementary Fig. 2). The average specific antibody response to the Wuhan variant is not significantly different from the specific Alpha or Delta responses, but does significantly differ from Beta, Omicron, and nucleocapsid responses. As expected, the Alpha response is on average the same as Wuhan and Beta (i.e. those closest in evolutionary trajectory), but different from all other variants. Beta responses are significantly different from all other variants, except Alpha. Delta responses are different from all other variants, except Wuhan. Nucleocapsid and Omicron responses are different from all comparisons, except for that with one another. Overall, IgG cross-reactivity was greatest among earlier, evolutionarily related variants (Wuhan, Alpha, and Delta), as IgG binding responses were most similar within these variants, whereas Beta and Omicron showed broader divergence; Beta remained similar to Alpha, whereas Omicron was distinct from all spike variants and only comparable to nucleocapsid response.

After establishing variant cross-reactivity at a population level, differences in cross-reactivity pre- and post-vaccination within each symptomatic group was analysed by comparing patterns of spike-specific IgG responses across pairs of variants. Significant differences were observed in all pairwise comparisons of spike-specific IgG responses across the five VoC between symptomatic and asymptomatic individuals, as well as between symptomatic and uninfected individuals. This indicates that stronger cross-reactive IgG responses were mounted by symptomatic individuals (i.e. a symptomatic individual with a strong IgG response to the Wuhan variant is also likely to have a strong response to the Alpha and Beta variants), but this is not true of asymptomatic or uninfected individuals. In contrast, no significant differences were detected between asymptomatic and uninfected individuals in any of the 10 pairwise comparisons (Fig. 5; Table 3), suggesting that similar population-level antibody responses to each variant were exhibited by these two groups. Each test in Table 3 compares the overall pattern of paired IgG responses between two symptomatic groups

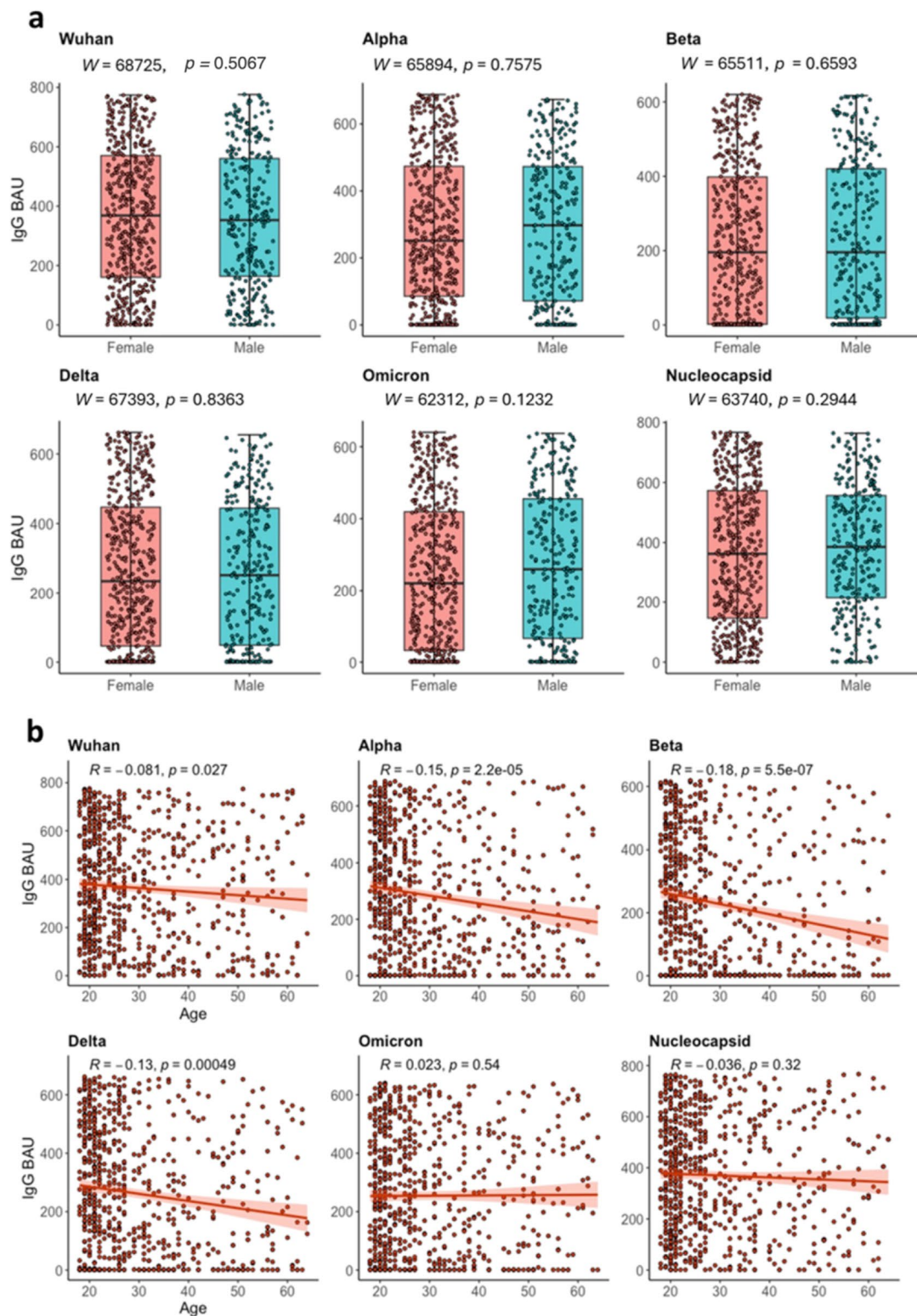


Fig. 2 IgG Antibody response against six focal variants of SARS-CoV-2. **(a)** Differences in IgG titres (BAU – binding antibody units) between biological sex at birth across all six SARS-CoV-2 strains. Total $N=530$ (341 female, 189 male). Participants who did not wish to share their sex at birth were excluded from analyses. Mann-Whitney U tests were performed for statistical analyses. **(b)** Correlations between IgG titres (BAU) and age across all six focal SARS-CoV-2 variants. Total $N=530$. Spearman's rho correlations were performed for statistical analyses. Shaded area around regression line represents the 95% confidence interval in each case

as shown in Fig. 5, treating each panel as a single distribution of points. For example, the Symptomatic–Asymptomatic Wuhan: Alpha comparison evaluates whether the joint distribution of responses to these two variants differs between symptomatic and asymptomatic individuals, that is, whether the relationship between IgG responses to Wuhan and Alpha varies between the two groups. Together, these results suggest that the presence of symptoms in a SARS-CoV-2 infection is a key factor in the cross-reactivity of IgG response, with asymptomatic infection providing limited added benefit over no infection in terms of immune response to variants of concern.

The Effect of Infection on Mucosal IgA levels

Like the IgG responses, there was no significant association of age with SARS-CoV-2 specific salivary IgA levels for any of the participant groups (**Supplementary Table 4**). Interestingly, IgA levels across the negative, asymptomatic and symptomatic groups were similar, with a spread of antibody responses to both Wuhan spike (Fig. 6A) and nucleocapsid (Fig. 6B) detected, and this did not change following vaccination. Furthermore, IgA spike and nucleocapsid responses were significantly correlated in all groups, regardless of vaccination status (Fig. 6C).

We then examined if there was a link between serum IgG and mucosal IgA levels (Fig. 7). As mentioned previously, in the negative group, there were no IgG nucleocapsid responses, but, importantly, there were IgA nucleocapsid responses, and these antibody levels were not affected by vaccination (Fig. 7i.a.). The same is true for Wuhan spike responses in the negative group, but following vaccination, as expected, Spike IgG levels increased (Fig. 7ii.a.). The responses to both Wuhan spike and Wuhan nucleocapsid IgG and IgA were the same in both the asymptomatic and symptomatic groups (Fig. 7i.b/c and ii. b/c). Interestingly, there is no correlation between mucosal IgA and serum IgG in either specific Wuhan spike or nucleocapsid antibody responses in all groups.

- (i) IgA nucleocapsid reactivity (x-axis) vs. Ig nucleocapsid reactivity (y-axis) of unvaccinated individuals (open circle) and vaccinated individuals (closed circle) for negative (a), asymptomatic (b) and symptomatic (c) groups. Correlation coefficients (r) and p values were calculated using Spearman's rho correlation and are presented below with the corresponding sample number (n). (a) Unvaccinated: $r=0.4768$, $p<0.5975$, $n=125$; Vaccinated: $r=0.1425$, $p<0.3137$, $n=52$. (b) Unvaccinated: $r=0.0668$, $p<0.5998$, $n=64$; Vaccinated: $r=0.0607$, $p<0.6721$, $n=51$. (c) Unvaccinated: $r=0.0926$, $p<0.5596$, $n=42$; Vaccinated: $r=0.0927$, $p<0.3987$, $n=85$.
- (ii) IgA spike reactivity (x-axis) vs. IgG spike reactivity (y-axis) of unvaccinated individuals (open circle) and vaccinated individuals (closed circle) for negative (a), asymptomatic (b) and symptomatic (c) groups. Correlation coefficients (r) and p values were calculated using Spearman's rho correlation and are presented below with the corresponding sample number (n). (a) Unvaccinated: $r=0.6300$, $p<0.4818$, $n=125$; Vaccinated: $r=0.0154$, $p<0.9139$, $n=52$. (b) Unvaccinated: $r=0.0370$, $p<0.0.7716$, $n=64$; Vaccinated: $r=0.0640$, $p<0.6559$, $n=51$. (c) Unvaccinated: $r=0.1987$, $p<0.2071$, $n=42$; Vaccinated: $r=0.0915$, $p<0.4050$, $n=85$.

Discussion

Asymptomatic infection was a silent transmitter of the COVID-19 pandemic, but existing humoral immunity studies often used a cohort lacking a 'true' asymptomatic diagnosis. Thus, this large, well-defined, cohort study recruited predominantly young, asymptomatic adults via an established asymptomatic testing service across 3 UK centres, with age/sex-matched symptomatic and uninfected controls, to investigate SARS-CoV-2 humoral immunity. Our robust analysis of serum IgG and mucosal IgA responses highlights key differences in humoral

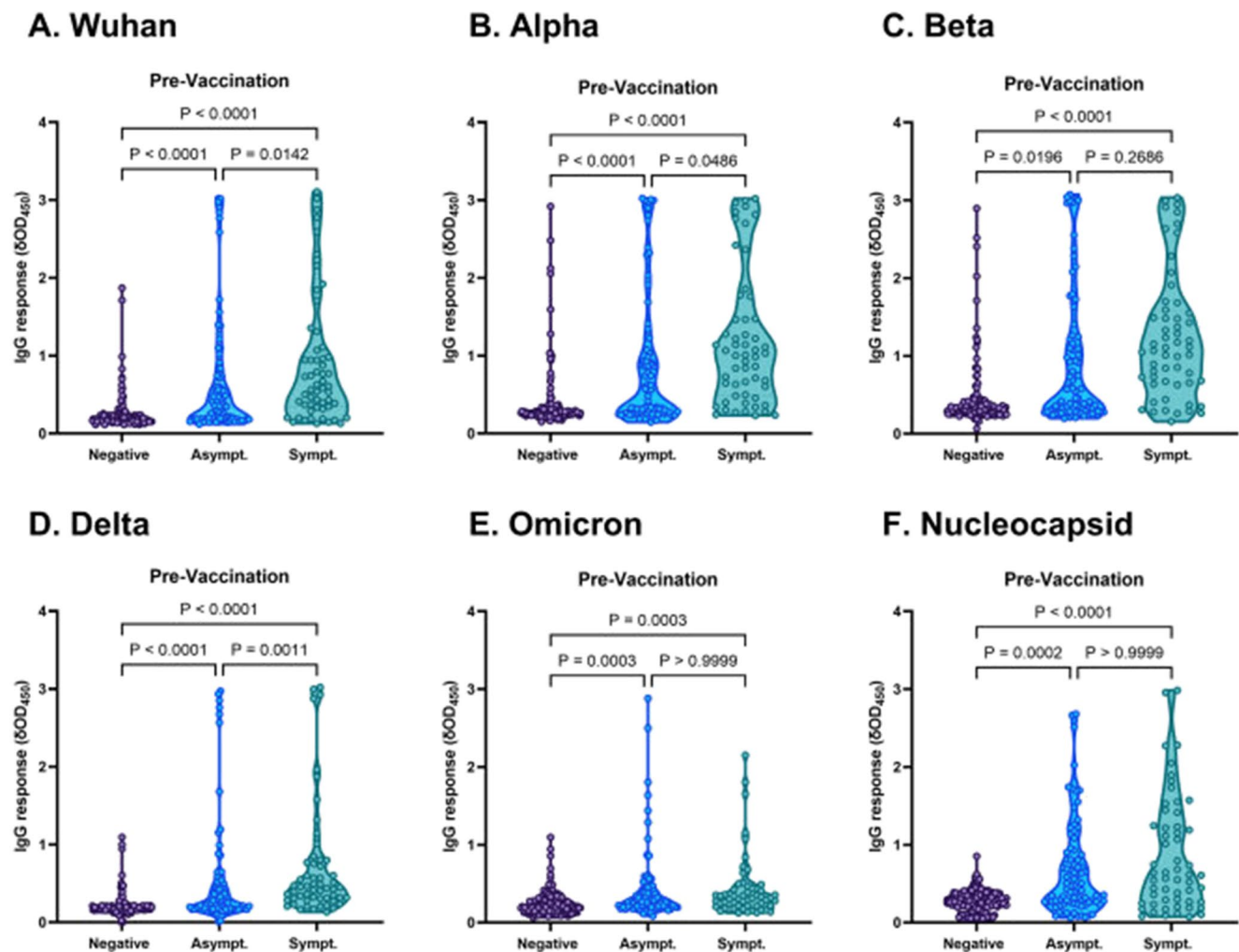


Fig. 3 Differences in IgG between negative, asymptomatic, and symptomatic individuals to each VoC. Violin plots of IgG levels according to symptomatic status, pre-vaccination, in response to (A) Wuhan, (B) Alpha, (C) Beta, (D) Delta, (E) Omicron, and (F) Nucleocapsid. A Kruskal-Wallis test with Dunn's pre-selected multiple comparisons was conducted for all pre-vaccination data as well as post-vaccination data. Total $N=304$

immunity based on the presence of symptoms during previous SARS-CoV-2 infection, and the importance of vaccination to diminish these differences.

Importantly, our study includes robust groupings. We present both a true negative population, as determined by negative IgG values before vaccination in the uninfected group, compared to positive spike and nucleocapsid IgG values in the asymptomatic and symptomatic groups, as well as true asymptomatic individuals, who were identified through our asymptomatic testing services to present with no symptoms. In terms of age and sex influences, we found age and sex of participants did not influence IgG antibody titre, irrespective of viral strain. This contrasts with studies concerning elderly cohorts, where age did influence IgG titres [27], but is not unexpected in this predominantly young cohort.

The relationship between asymptomatic infection and the development of humoral immunity, particularly in young adults, remains unclear. In this study, we show that unvaccinated young adults who experienced asymptomatic infection exhibited weaker IgG responses to most variants of concern (VoC) compared to symptomatic young adults, consistent with previous research [28]. However, these differences disappeared after vaccination, suggesting that current vaccinations are equally effective at inducing IgG responses in both asymptomatic and

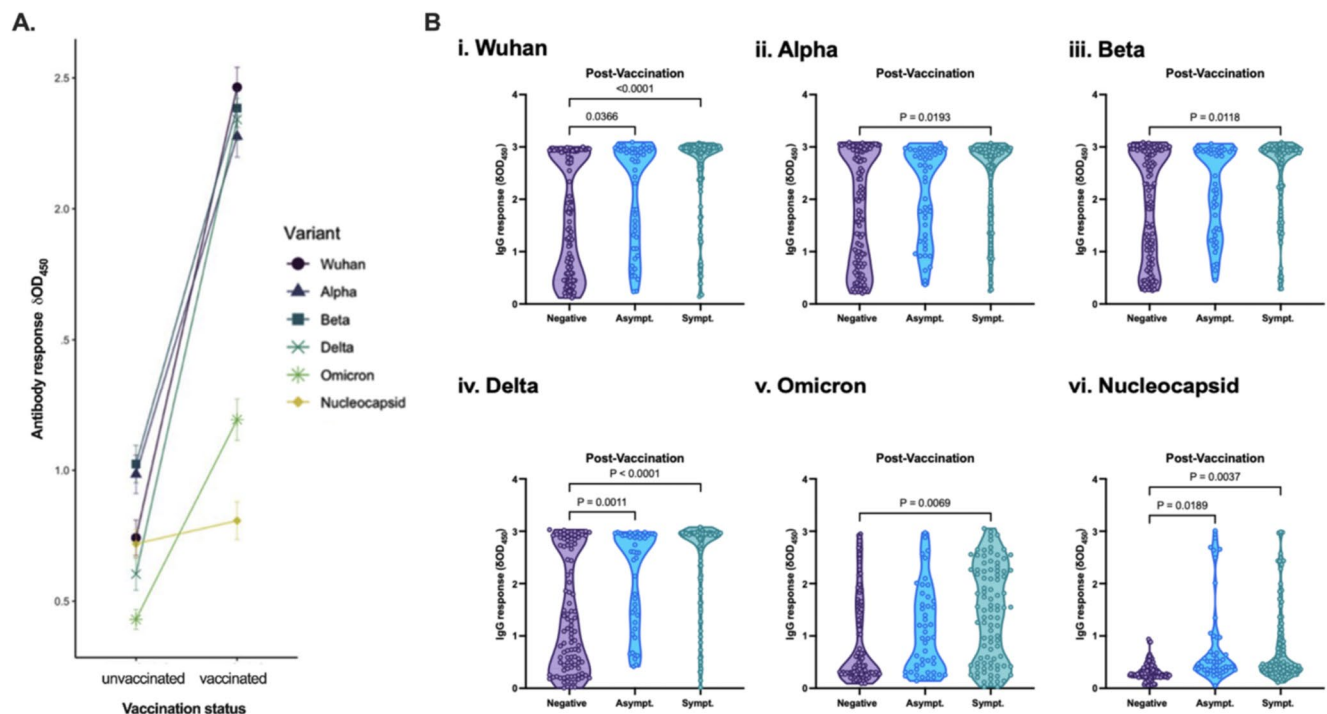


Fig. 4 Effect of vaccination status on IgG levels in SARS-CoV-2 positive participants of the ACE cohort. **(A)** The two-way interaction in the Generalised linear mixed effects model (GLMM) between SARS-CoV-2 variant and vaccination status. $\delta OD_{[450]}$ refers to the OD_{450} results, with an OD_{580} subtracted from the results. **(B)** Violin plots of variant-specific antibody responses according to vaccination and symptomatic status. Figure 4A $N=530$, Fig. 4B $N=226$

Table 2 Results of post-hoc pairwise Tukey tests, comparing average IgG antibody response for all ACE cohort members between each of the SARS-CoV-2 variants. Pairs with significant differences are highlighted in bold. The estimate refers to the difference between the average responses of two variants, the SE refers to the uncertainty in this estimated difference, and the z-ratio indicates how large the difference is relative to this uncertainty. Larger values of Z provide stronger evidence of a true difference between variants after accounting for multiple comparisons. Negative values of estimate and Z occur when the average antibody response for the second variant in the comparison is higher than that of the first variant. These results are presented graphically in Supplementary Fig. 2.)

Contrast	estimate	SE	Z ratio	p-value	
Wuhan	Alpha	-0.1252	0.0483	-2.5937	0.0987
Wuhan	Beta	-0.1894	0.0483	-3.9241	0.0012
Wuhan	Delta	0.1295	0.0483	2.6829	0.0786
Wuhan	Omicron	0.5540	0.0491	11.2855	9.33E-15
Wuhan	Nucleocapsid	0.4785	0.0495	9.6569	4.33E-14
Alpha	Beta	-0.0642	0.0482	-1.3334	0.7664
Alpha	Delta	0.2547	0.0483	5.2674	2.06E-06
Alpha	Omicron	0.6792	0.0490	13.8511	<0.0001
Alpha	Nucleocapsid	0.6037	0.0494	12.2096	<0.0001
Beta	Delta	0.3189	0.0484	6.5941	6.42E-10
Beta	Omicron	0.7434	0.0490	15.1612	<0.0001
Beta	Nucleocapsid	0.6679	0.0494	13.5079	<0.0001
Delta	Omicron	0.4245	0.0491	8.6467	5.08E-14
Delta	Nucleocapsid	0.3490	0.0495	7.0433	2.82E-11
Omicron	Nucleocapsid	-0.0755	0.0499	-1.5139	0.6553

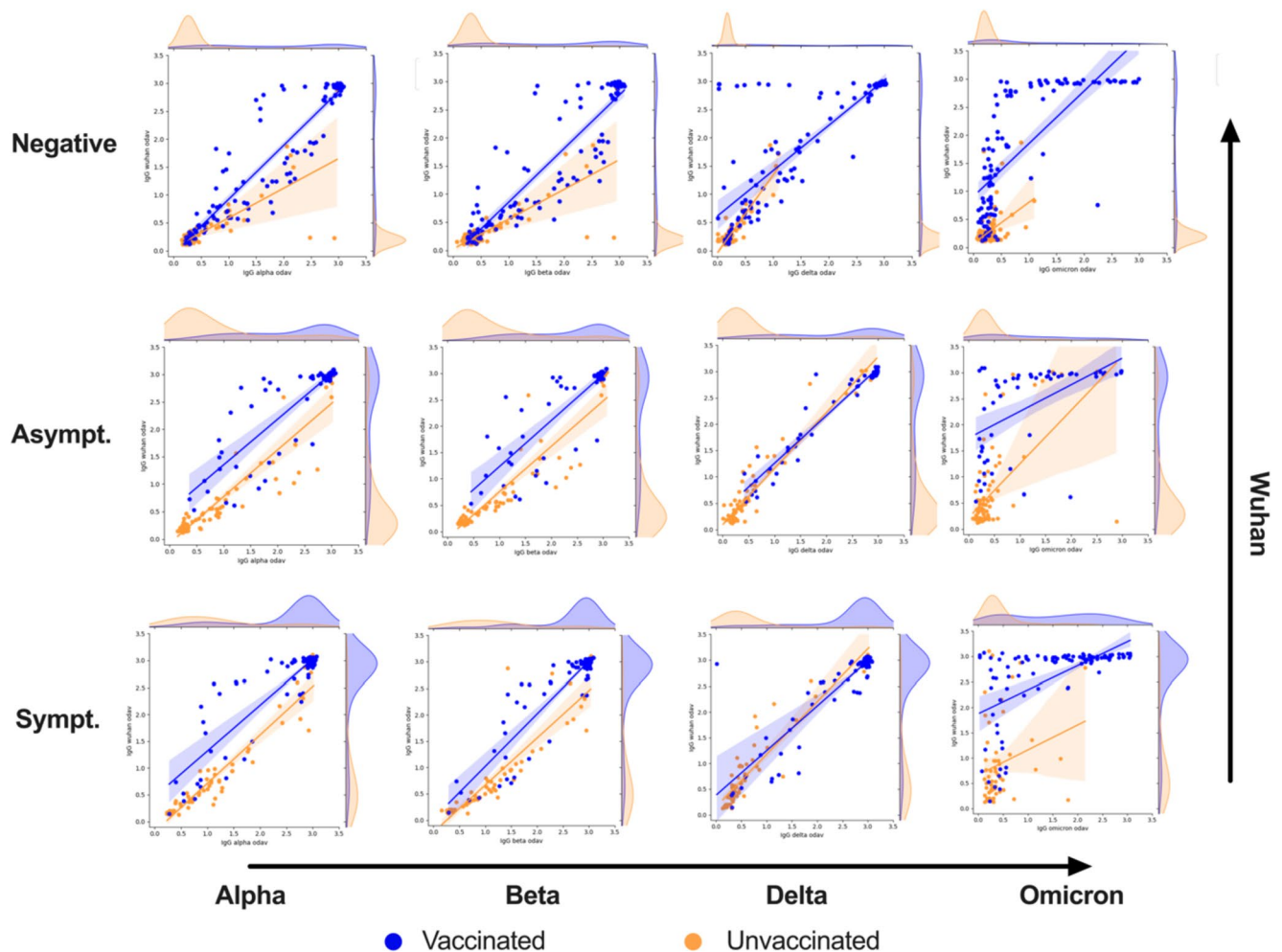


Fig. 5 Pairwise cross-reactive spike-specific IgG antibody responses against Alpha, Beta, Delta, and Omicron variants in three symptomatic status groups: Negative, Asymptomatic (Asympt.), and Symptomatic (Sympt.) in vaccinated and unvaccinated individuals. Blue dots represent vaccinated individuals, and orange dots represent unvaccinated individuals. Shaded regions around regression lines represent 95% confidence intervals. Each panel represents the full two-dimensional distribution of paired IgG responses for a given variant pair within a symptomatic group; these distributions form the basis of the statistical comparisons reported in Table 3. Only comparisons between Wuhan spike-specific responses and each of the other variants of concern are graphically represented, statistical comparisons for all cross-reactive responses are shown in Table 3. $N=530$

symptomatic individuals. In individuals with no prior infection, vaccination also significantly increased IgG levels, but not to the same extent as individuals with previous infection. Thus, ‘hybrid immunity’ acquired by previous infection and vaccination provides better protection against re-infection, compared to vaccination alone. This is in accordance with other studies, which show vaccination alone provides low-to-moderate protection, due to waning protection after each dose, whereas hybrid immunity provides the most robust protection [29].

We also observed differences in cross-reactive IgG responses between the three groups. Cross-reactivity to the Wuhan and Alpha variants were similar, in both symptomatic and asymptomatic groups, indicating comparable population-level responses to these strains. Conversely, among individuals with no known natural infection, the IgG responses to Wuhan and Alpha differed. Across all groups, the IgG responses to Wuhan and Delta variants were comparable, with no significant differences in response distribution. However, the

Table 3 Results of 2-dimensional two samples Peacock tests, comparing distributions of IgG response ($\delta OD_{[450]}$) of individual sample responses to combinations of VoC specific spike proteins of SARS-CoV-2. For each variant pair, the test compares the full two-dimensional distribution of paired IgG responses between symptomatic status groups, corresponding to the distributions visualised in Fig. 5 (i.e. each row in the table is a direct comparison of the distribution shape of points between panels in Fig. 5). Each symptomatic status comparison is analysed separately. Significant comparisons ($p < 0.05$) are shown in italics

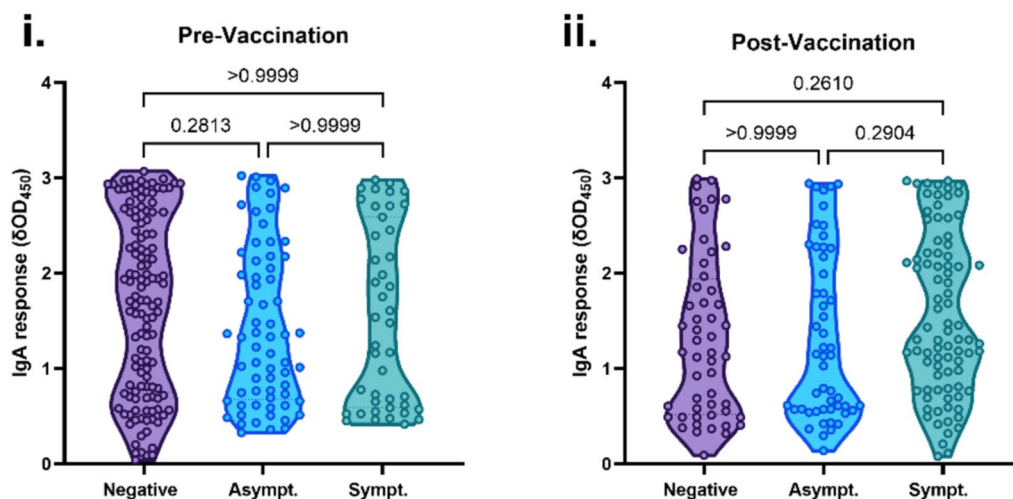
		Symptomatic - asymptomatic		Symptomatic – no infection		Asymptomatic – no infection	
		D statistic	p-value	D statistic	p-value	D statistic	V
Wuhan	Alpha	<i>2.3029</i>	<i>0.0030</i>	<i>2.6738</i>	<i>0.0002</i>	1.1998	0.7511
Wuhan	Beta	<i>2.3524</i>	<i>0.0021</i>	<i>2.4942</i>	<i>0.0007</i>	1.1778	0.7979
Wuhan	Delta	<i>2.2664</i>	<i>0.0039</i>	<i>2.7337</i>	<i>0.00009</i>	1.1798	0.7980
Wuhan	Omicron	<i>2.5689</i>	<i>0.0004</i>	<i>2.5387</i>	<i>0.0005</i>	1.1315	0.9008
Alpha	Beta	<i>2.3851</i>	<i>0.0016</i>	<i>2.5533</i>	<i>0.0004</i>	1.1290	0.9064
Alpha	Delta	<i>2.3396</i>	<i>0.0023</i>	<i>2.7337</i>	<i>0.00009</i>	1.2485	0.6522
Alpha	Omicron	<i>2.6481</i>	<i>0.0002</i>	<i>2.6246</i>	<i>0.0002</i>	1.2729	0.6055
Beta	Delta	<i>2.4538</i>	<i>0.0009</i>	<i>2.7337</i>	<i>0.00009</i>	1.1119	0.9457
Beta	Omicron	<i>2.6747</i>	<i>0.0002</i>	<i>2.6888</i>	<i>0.0001</i>	1.2485	0.6522
Delta	Omicron	<i>2.5813</i>	<i>0.0003</i>	<i>2.7935</i>	<i>0.00005</i>	1.3217	0.5183

Wuhan spike response was consistently stronger than responses to the Beta and Omicron variants. Notably, in both the asymptomatic and no known infection groups, a substantial proportion of individuals showed poor or negligible IgG responses to the Omicron spike protein. Strikingly, when comparing the cross-reactivity of pairs of VoC between the three groups, symptomatic young adults had significantly stronger cross-reactivity compared to asymptomatic and uninfected participants. This eludes to better protection against future variants when individuals exhibit symptoms during infection. Indeed, symptomatic infections generally lead to stronger immune responses because they tend to trigger a more robust activation of the immune system, possibly due to higher viral load and stronger immune activation in individuals who show symptoms. This is then likely to lead to a broader immune memory [17], providing better cross-reactivity to new variants or strains of SARS-CoV-2.

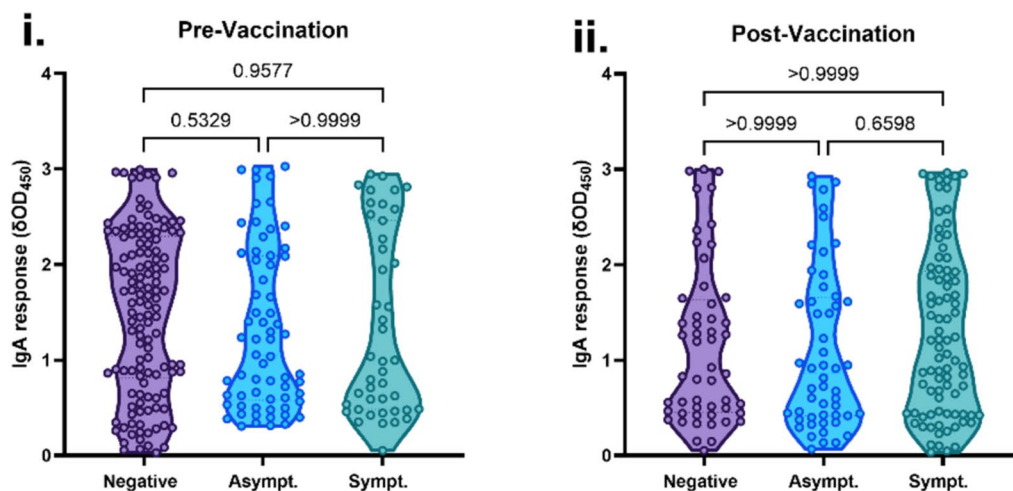
Interestingly, cross-reactivity between the spike protein of each of the SARS-CoV-2 VoC does not seem to correspond to the number of mutations present on the protein. Here, we have demonstrated that the Wuhan and Delta strains appear to have high levels of cross-reactivity with one another, in that the cohort-wide density distributions do not differ. This is perhaps counterintuitive according to the number of mutations present on the spike protein itself. The Alpha variant has a total of 9 mutations versus the Wuhan strain, the Beta variant has 10. The Delta variant has 17 mutations, and Omicron has 39. It should be noted, however, that none of the VoC, with the exception of Omicron, has a known impact on vaccine effectiveness. This result does agree with other research, where two doses of the AZ vaccine had 93.7% effectiveness in Delta infected patients, versus 88.0% effectiveness in patients infected with the Alpha strain [30, 31].

Furthermore, the significance of the two-way interaction between variant and vaccination indicating that vaccination affects antibody titre differently according to variant is perhaps unsurprising, due to the nature of the vaccine itself. The vaccine is built around the spike protein, so it is expected that one or more vaccinations would not have an impact on the nucleocapsid-specific antibody response of participants. The potential significance of antibody cross-reactivity is crucial due to the challenge of existing immune evasion by emerging SARS-CoV-2 variants. Indeed, existing research has demonstrated SARS-CoV-2 protection is influenced by host immunity and viral evolution, with transmissibility driving pre-Omicron variants, but immune escape dominating post-Omicron variants [32]. This highlights the requirement for regular vaccine modifications with evolving variants.

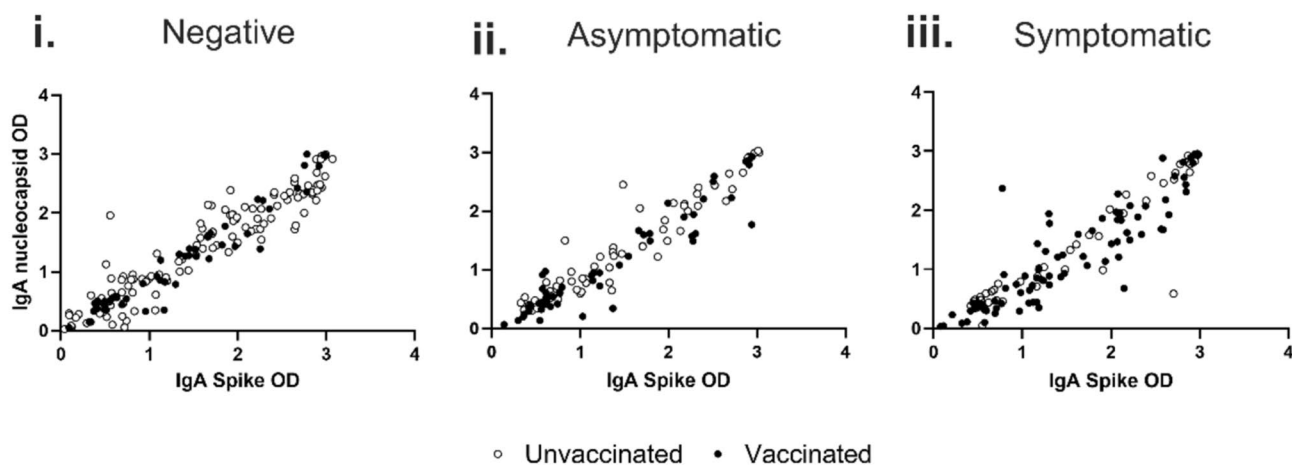
A) Wuhan-IgA



B) Nucleocapsid-IgA



C) Wuhan vs Nucleocapsid IgA



◀ **Fig. 6** Serum salivary IgA reactivity to Wuhan Spike and nucleocapsid proteins within the participant groups. (A) Violin plots of serum IgA levels according to symptomatic status in response to Wuhan, pre-vaccination (i) and post-vaccination (ii). (B) Violin plots of serum IgA levels according to symptomatic status in response to nucleocapsid, pre-vaccination (i) and post-vaccination (ii). A Kruskal-Wallis test with Dunn's multiple comparisons was conducted. (C) A comparison of IgA spike (x-axis) vs. IgA nucleocapsid (y-axis) reactivity of unvaccinated individuals (open circle) and vaccinated individuals (closed circle) for negative (a), asymptomatic (b) and symptomatic (c) groups. Correlation coefficients (r) and p values were calculated using Spearman's rho correlation and are presented below with the corresponding sample number (n). (a - Negative) Unvaccinated: $r=0.9355$, $p<0.0001$, $n=126$; Vaccinated: $r=0.9400$, $p<0.0001$, $n=52$. (b - Asymptomatic) Unvaccinated: $r=0.9498$, $p<0.0001$, $n=65$; Vaccinated: $r=0.9092$, $p<0.0001$, $n=52$. (c - Symptomatic) Unvaccinated: $r=0.9245$, $p<0.0001$, $n=43$; Vaccinated: $r=0.9038$, $p<0.0001$, $n=85$

Salivary IgA reactivity is detectable to both SARS-CoV-2 spike and nucleocapsid across the participant groups examined. This IgA response showed no correlation with age or with serum IgG responses across the participant groups, the latter being consistent with previous reports [14, 33].

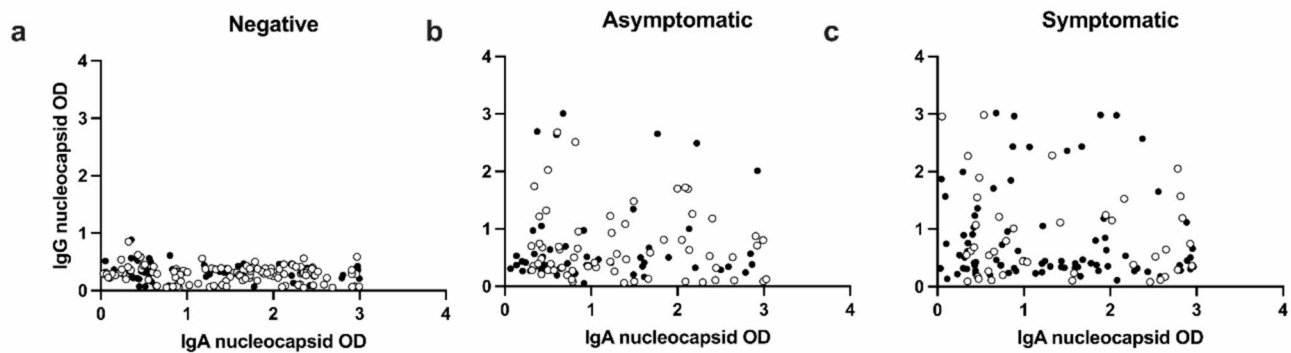
Comparing unvaccinated and vaccinated participants in the symptomatic and asymptomatic participant groups indicated no significant differences in salivary IgA levels. However, significant differences were seen in the SARS-CoV-2 negative participants when comparing unvaccinated and vaccinated individuals. When considering responses to both Wuhan Spike protein and nucleocapsid, this significance is associated with a median decrease in salivary IgA levels in the vaccinated participants, relative to the unvaccinated. Vaccination has been reported to suppress mucosal IgA responses relative to IgG responses, but breakthrough infection can restimulate the mucosal IgA response [34, 35].

SARS-CoV-2 reactive and virus-neutralising IgA responses in pre-pandemic and uninfected individuals have previously been reported in African and Japanese populations [36–38]. The study of Tsukinoki et al. [38] indicated a 52.7% percent SARS-CoV-2 reactive salivary IgA positivity rate in uninfected individuals. This was attributed to cross-reactive ‘natural’ IgA responses developed against the seasonal coronaviruses. Furthermore, in a 2022 study examining the protection of Swedish healthcare workers against infection with SARS-CoV-2, not contracting the infection was associated with female Sex, the presence of neutralising IgA, and absence of specific IgG responses. This was found in 10% of the study participants [39]. Our present study supports this viewpoint, that high SARS-CoV-2-reactive IgA and low SARS-CoV-2-reactive IgG are associated with uninfected individuals.

There are several hypotheses, which are not mutually exclusive, put forward to explain levels of salivary IgA reactivity in non-infected individuals. Firstly, that strong responses to seasonal coronaviruses (HCoV: 229E, OC43, NL63 and HKU1) elicit antibodies providing a variable cross-reactive response to SARS-CoV-2 within a population [38]. Secondly, that strong and rapid innate mucosal response to SARS-CoV-2 promotes local mucosal IgA production and avoidance of detectable viral infection. Strong IgA responses have been observed in children at high risk of exposure to the SARS-CoV-2 virus (i.e. family members are infected) [40]. In addition, it has been reported that rapid mucosal responses may be enhanced by prior infection, for example with rhinovirus, which raises interferon-stimulated gene expression, thus activating enhanced innate protection [35]. Overall, this work, supported by previous research [41, 42], suggests the efficiency of SARS-CoV-2 vaccines may be enhanced by targeting IgA responses.

In summary, unvaccinated asymptomatic young adults exhibit weaker IgG responses compared to their symptomatic counterparts, to all tested variants, but these differences dissipate post-vaccination, while individuals without prior natural infection still display weaker humoral immunity. Although, these individuals without prior infection had high SARS-CoV-2 specific IgA titres, pre-vaccination, supporting the notion that a strong and rapid innate mucosal response to SARS-CoV-2 may promote local mucosal IgA production and avoidance of detectable viral infection. Furthermore, the IgG cross-reactivity between combinations of each VoC was inferior in asymptomatic and uninfected individuals compared to symptomatic individuals, suggesting better protection from emerging variants when symptomatic. This work demonstrates the requirement of vaccinations to enhance

i. Wuhan Nucleocapsid Responses



ii. Wuhan Spike Responses

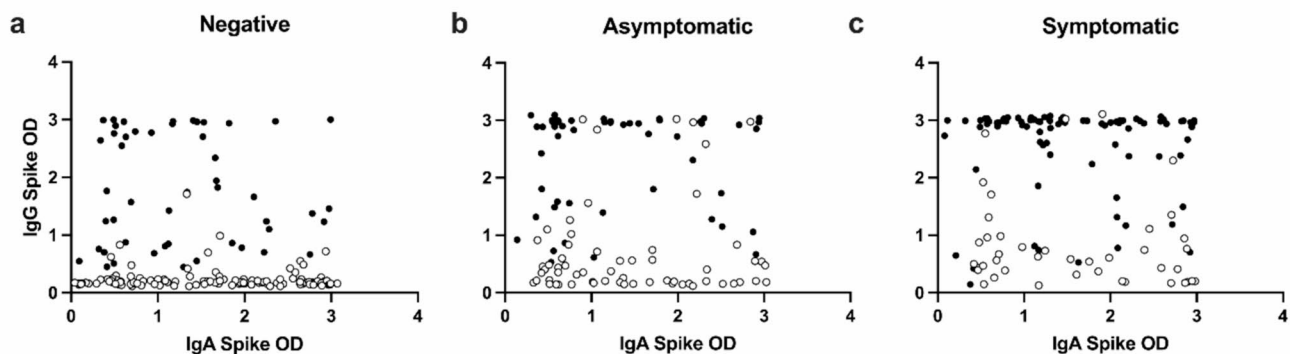


Fig. 7 A comparison of Serum IgG and salivary IgA reactivity to Wuhan spike and nucleocapsid

the weaker IgG responses seen in asymptomatic infection, but also highlights that vaccines need to be constantly adapted to overcome viral evolution, particularly with more done to improve mucosal delivery options for respiratory viruses, such as SARS-CoV-2 and its variants.

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Author contributions GH, NG, DT, LB, GS, and MB recruited patients and processed blood for sera isolation, and processed saliva samples. Mpe, Mpo, MM, BK, and MW processed blood for sera isolation and processed saliva samples. SL, MS, LC, SB, TR, KS, MS, Aga, and MM all performed venipuncture and processed blood for sera isolation, and ran T cell/

serology assays to determine SARS-CoV-2 positivity. TH-C prepared all reagents. HJ performed antibody titre assays. HJ, GH and PJT performed the analysis. HJ and GH wrote the manuscript and prepared figures with comments from LF, MW, AGo, PJT, BK, MPe, and MPo. LF, MW, and AGo designed the study, acquired the funding, and were site-specific project coordinators, and LF managed the project.

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Data Availability All data required to evaluate the conclusions in the paper are present in the manuscript or its appendix. Further information on the study protocol or de-identified datasets generated and analyzed within this publication are available from the corresponding authors on reasonable request.

Declarations

Ethics Approval Ethics approval for the study was granted by the University of Nottingham Medical School Ethics Committee (FMHS 96–0920), Cambridge COVID-19 NIHR BioResource joint Consent Form (Research Ethics Committee (NRES REC Ref 17/EE/0025), and Cardiff University School of Medical Research Ethics Committee (SMREC 21/01).

Consent to Participate All participants provided written informed consent.

Competing Interest MJS is a founder of and holds equity in ImmunoServ Ltd. AG is a founder of ImmunoServ Ltd. All other authors declare no competing interests.

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