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The Breadth of the Neutralizing Antibody Response to Original SARS-CoV-2 Infection is Linked to the Presence of Long COVID Symptoms

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Abstract

Background: The associations between longitudinal dynamics and the breadth of SARS-CoV-2 neutralizing antibody (nAb) response with various Long COVID phenotypes prior to vaccination

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are not known. The capacity of antibodies to cross neutralize a variety of viral variants may be associated with ongoing pathology and persistent symptoms.

Methods: We measured longitudinal neutralizing and cross-neutralizing antibody responses to pre- and post-SARS-CoV-2 Omicron variants in participants infected early in the COVID-19 pandemic, prior to wide-spread rollout of SARS-CoV-2 vaccines. Cross sectional regression models adjusted for clinical covariates and longitudinal mixed-effects models were used to determine the impact of the breadth and rate of decay of neutralizing responses on the development of Long COVID symptoms, as well as Long COVID phenotypes.

Results: We identified several novel relationships between SARS-CoV-2 antibody neutralization and the presence of Long COVID symptoms. Specifically, we show that, although nAb responses to the original, infecting strain of SARS-CoV-2 were not associated with Long COVID in cross-sectional analyses, cross-neutralization ID50 levels to the Omicron BA.5 variant approximately 4 months following acute infection was independently and significantly associated with greater odds of Long COVID and with persistent gastrointestinal and neurological symptoms. Longitudinal modeling demonstrated significant associations in the overall levels and rates of decay of neutralization capacity with Long COVID phenotypes. A higher proportion of participants had antibodies capable of neutralizing Omicron BA.5 compared with BA.1 or XBB.1.5 variants.

Conclusions: Our findings suggest that relationships between various immune responses and Long COVID are likely complex but may involve the breadth of antibody neutralization responses.

Summary:

SARS-CoV-2-specific antibody neutralization of Omicron BA.5 variant approximately 4 months following acute infection with wild-type virus prior to vaccination was independently and significantly associated with greater odds of distinct Long COVID phenotypes.

Keywords

COVID-19; SARS-CoV-2; Neutralizing Antibodies; Long COVID; Post-Acute Sequelae of SARS-CoV-2 infection (PASC)

INTRODUCTION

Many individuals experience post-acute sequelae of SARS-CoV-2 infection, often referred to as Long COVID, that is defined by ongoing symptoms which are often debilitating and affect quality of life [1–3]. The pathology driving Long COVID, however, is poorly understood and likely involves multiple mechanisms [2, 4, 5]. Proposed mechanisms include aberrant autoreactive immune responses, microvascular dysregulation, and reactivation of latent human herpesviruses which may lead to the systemic inflammatory responses now identified in individuals with Long COVID compared to those who fully recovered [6–11]. Furthermore, there is growing evidence that SARS-CoV-2 RNA and proteins are present in the tissues of at least a subset of immunocompetent individuals with Long COVID [12–14]. Although those with persistent symptoms tend to have higher levels of SARS-CoV-2 Spike-specific antibody levels [10, 15–18], we and others have previously demonstrated that Long COVID is associated with adaptive immune dysregulation and exhaustion [15, 18].

SARS-CoV-2 infection leads to rapid development of robust antibody responses, although neutralizing capacity wanes more quickly than total Spike IgG levels over time [17, 19–21]. A higher initial viral burden or persistence of viral antigens may lead to observed dysregulated immune phenotypes and higher antibody levels. However, there is a paucity of information regarding the associations between longitudinal dynamics or the breadth of the neutralizing antibody response with various Long COVID phenotypes with some data showing that weaker antibody responses over time being associated with Long COVID [22].

Recent data suggests that an expanded antibody response against the prior OC43 endemic coronavirus may be associated with Long COVID [23]. This suggests that the breadth of the response to initial infection may play an important role in the development of Long COVID. Given that the rapid emergence of Omicron variants that evade neutralization result from infection from older SARS-CoV-2 strains (*e.g.*, original SARS-CoV-2, Alpha and Delta variants) as well as to COVID-19 vaccines [24, 25], there is strong interest in determining the relationship between the breadth and durability of the initial antibody responses and the presence of Long COVID symptoms. The rapid emergence of novel variants and increased incidence of reinfection also necessitates studies of longitudinal antibody responses following COVID-19 [26].

Here, we measured longitudinal neutralizing antibody responses to pre-Omicron strains and to subsequent Omicron variants in participants infected during the early waves of the COVID-19 pandemic, prior to their receiving SARS-CoV-2 vaccines. Cross sectional regression models adjusted for various clinical covariates and longitudinal mixed effects models were used to determine the impact of the breadth and rate of decay of neutralizing responses on the development of Long COVID symptoms in general, as well as distinct Long COVID symptom phenotypes.

METHODS

Clinical Cohort and Sample Collection.

Participants were enrolled in the University of California, San Francisco (UCSF)-based Long-term Impact of Infection with Novel Coronavirus (LIINC) COVID-19 study (NCT04362150). The cohort design and procedures have been described in detail elsewhere [4]. Briefly, at each visit, participants complete an interviewer-administered questionnaire querying the presence in the preceding 2 days of symptoms that are new since COVID-19 or worsened from pre-COVID baseline, prior to the collection of peripheral blood. This analysis included longitudinal measurements from 184 participants, including plasma samples collected between 1 and 4 months after initial symptom onset. All samples were collected prior to the participant having received any SARS-CoV-2 (Family *Coronaviridae*, genus *Betacoronavirus*, species *SARS-CoV-2*) vaccination and a large majority were collected during the original SARS-CoV-2 wave (timing of sample collections here- maybe first and last date of collection), all prior to Omicron variant emergence. Phenotypic clusters were based on 32 participant-reported symptoms as previously described [4].

PhenoSense SARS CoV-2 nAb Assay.

The measurement of nAb activity using the PhenoSense SARS CoV-2 nAb Assay (Monogram Biosciences, South San Francisco, CA) was performed by generating HIV-1 pseudovirions that express the SARS CoV-2 Spike protein as previously described [20, 27–29]. The pseudovirus is prepared by co-transfecting HEK293 producer cells with an HIV-1 genomic vector that contains a firefly luciferase reporter gene together with a SARS CoV-2 Spike protein expression vector. Neutralizing antibody activity is measured by assessing the inhibition of luciferase activity in HEK293 target cells expressing the ACE2 receptor and TMPRSS2 protease following pre-incubation of the pseudovirions with serial dilutions of the plasma specimen. ID50 values was generated for the original SARS-CoV-2 Spike protein as well as the following variants: Alpha (B.1.1.7), D614G mutant, Delta (B.1.617.2), Omicron BA.1 (B.1.1.529), Omicron BA.5 (B.1.1.529). A subset of samples stratified by negative or ID50s to the BA.5 strain were performed in an expanded pool consisting of pseudoviruses incorporating spike proteins from BA.2, BA.4.6, XBB.1.5 and BQ.1.1.

Statistical analyses.

Antibody data were generated blinded to participant information. Comparisons of ID50 values across comparator groups incorporated non-parametric Mann-Whitney or Freidman tests with Dunn correction for multiple comparisons using Prism v.8 (GraphPad Software) and SPSS v.29 (IBM). Adjusted P-values reported in analyses involving multiple comparisons. For tabular data, two-tailed Fisher's exact testing was performed on categorical data and two-tailed, non-parametric Mann-Whitney testing was performed on continuous variables (SPSS v.29). Spearman Rank Correlation analysis was used to compare T cell, antibody and soluble markers of inflammation (Prism v.8). For longitudinal analyses, linear mixed effects modeling was performed for neutralizing antibody ID50 (log transformed) in R (version 4.0.2) using lme4 package (version 1.1) with time and individual factors (*e.g.,* age, sex, COVID-19 hospitalization, prior history of diabetes, prior autoimmune disease, body mass index >30, Long COVID symptoms) as predictors, and random effects based on participant. Spearman's correlation was performed in R to test for relationships between variant neutralizing antibody ID50 across all time points. Logistic regression models were performed on cross-sectional data including the constant to identify independent associations between model factors and Long COVID outcomes using SPSS v.29. Models included various demographic variables as well as either Log-transformed ID50 values or binary categorical indicators of ID50 values above a specific threshold as determined by sensitivity analyses.

Phage ImmunoPrecipitation Sequencing (PhIP-Seq) to assess participant autoantibody profiles.

To determine whether high neutralization breadth was associated with autoimmunity, we reanalyzed previously described PhIP-Seq data corresponding to certain individuals within this cohort (i.e. those with the top 15% of neutralization ID50s in both the original SARS-CoV-2 and BA.5 variant) which is publicly available at: https://doi.org/10.7272/Q6Z60M99 [30]. Logistic regression machine-learning classifiers were performed using our described methods, which have been previously used to feature-weight autoantibody signal in multiple

autoimmune and COVID-related diseases [30, 31]. Utilizing the Scikit-learn package, logistic regression classifiers were applied to z-scored PhIP-Seq values from individuals with a designated disease category versus the designated control. A liblinear solver was used with L1 regularization. Given varying and often low numbers of cases relative to controls, a three-fold cross-validation was utilized and iterated 10 times.

Study approval.

All participants provided signed written informed consent prior to participation. The UCSF IRB approved the study.

RESULTS

Clinical cohort and participant demographics.

To evaluate neutralizing responses in participants with prior COVID-19 and to assess relationships between these responses and the presence of Long COVID symptoms, we analyzed plasma samples collected from 184 participants with and without Long COVID symptoms across 384 timepoints for up to 4 months following acute infection. Participants had a median of 2 sample time points across all visits (ranging from 1 to 4). In general, participant visits occurred approximately 1 month, 2 months or 4 months following nucleic acid-confirmed SARS-CoV-2 infection. All specimens and symptom reports were timed from the day of initial COVD-19 symptom onset.

All participants were initially infected prior to emergence of the Delta strain (last date of infection was in March of 2021) and all but 7 samples across all time points were collected prior to the end of February 2021 (the time of vaccine availability to the general public in the Unites States). Long COVID was defined broadly as the presence of any symptom new or worsened since acute SARS-CoV-2 infection not clearly attributable to another cause. Table 1 shows the clinical and demographic factors of participants grouped by the presence or absence of any Long COVID symptom. The group with Long COVID was enriched for women (58.4% vs 39.4%, $P<0.05$), those who had been hospitalized during acute COVID-19 (24.8% vs 19.7%, not significant), those with a history of pre-existing autoimmune disease (mainly thyroiditis, 10.6% vs 1.4%, $P<0.05$), persons self-reporting Latinx ethnicity (34.5% vs 19.7%, $P<0.05$), and those with higher body mass index (27.7 vs 26.0 kg/m^2, $P<0.05$) (Table 1).

Breadth of antibody neutralization to the original infecting SARS-CoV-2 variant and subsequent viral variants.

Using the PhenoSense assay (Monogram Biosciences), we measured the inhibitory plasma dilutions at which 50% neutralization occurred (ID50) using pseudoviruses expressing SARS-CoV-2 Spike protein from the original strain (with which a majority of our participants were infected) and the following subsequent variants: Alpha (B.1.1.7), D614G mutant, Delta (B.1.617.2), Omicron BA.1 (B.1.1.529), and Omicron BA.5 (B.1.1.529). Overall, neutralization levels were highly variable between participants. Antibody neutralization responses were consistently highest to the original, infecting SARS-CoV-2 strain along with the Alpha and Delta variants, with levels declining between 1 and 4 months

following acute infection (Figure 1A–C). Although very low levels of cross-neutralization were observed with the Omicron BA.1 variant, a higher proportion of participants had antibodies able to neutralize Omicron BA.5 up to four months following initial presentation (the last study time point). Across all time points, 12.5% of neutralizing titers were below the level of assay positivity to the original SARS-CoV-2 pseudovirus and 78.4% and 67.4% below the level of positivity for the BA.1 and BA.5 variant, respectively. Correlations of neutralization capacity to each SARS-CoV-2 variant are shown in Supplementary Figure 1.

We tested a subset of 16 participants including samples chosen with either high or low Omicron BA.5 responses across all timepoints on an expanded panel of Omicron sub variants to better understand the full breadth of cross-neutralization responses between original and recent or current circulating strains as shown in Figure 1D. The expanded neutralization panel included BA.4.6, BQ.1.1 and XBB.1.5. Similar neutralization titers were observed between BA.5, BA.4.6, and BQ1.1, whereas XBB.1.5 responses most closely resembled BA.1 responses, which were overall low or negative across participants. BA.2 responses were more evenly distributed across a range of neutralization ID50s compared to the other Omicron sub variants.

Decay of variant-specific SARS-CoV-2 antibody neutralization by clinical phenotype.

Leveraging mixed effects modeling approaches, we analyzed neutralizing responses over time by clinical and demographic characteristics, including age, sex, hospitalization during acute infection, body mass index (BMI), and a pre-existing history of diabetes mellitus for the most clinically relevant variants in our study population (original SARS-CoV-2, Delta and Omicron sub variants; Figure 2). Overall, antibody neutralization ID50 decreased over time for all strains ($p<0.01$) with the exception of the Omicron BA.1 variant, ($p=0.16$) for which initial levels were substantially lower than to the other variants. When stratifying by age greater than 50 years, we identified no difference in antibody neutralization to all variants tested across all time points in the mixed effects models. In contrast, male sex was associated with higher viral neutralization for original SARS-CoV-2, Delta, and Omicron BA.5 variants, but not for Omicron BA.1, across all time points. These observed sex-based differences were similar between original and Delta variants, each approximately 0.41 and 0.33 $\log_{10}$ higher, respectively, observed in males compared to females recovering from COVID-19.

For those hospitalized during acute infection, neutralization levels were significantly higher across all strains with the exception of BA.1. The magnitude of the difference in responses for those hospitalized versus not hospitalized was diminished with those strains with lower overall responses: 1.18 and 0.94 $\log_{10}$ higher responses for the original virus and Delta variant versus 0.33 and 0.28 $\log_{10}$ higher for omicron BA.1 and BA.5 responses, respectively. Individuals with pre-existing diabetes also had differential neutralization across variants, with higher initial levels and longitudinal levels over all time points, and showing a more rapid decline over time compared to those without diabetes. The magnitude of wild-type SARS-CoV-2 responses for those with a BMI >30 was higher across all time points for the original and delta viral strains (All $P<0.001$) and declined more rapidly for the original Delta and Omicron BA.5 variants (All $P>.012$; Supplemental Figure 2). In contrast,

neutralization ID50s from participants with a pre-existing history of diabetes mellitus were overall lower across all time points for the original SARS-CoV-2 (All P <0.05) and ID50s to the original strain and Delta variant declined less rapidly (P<0.05).

Breadth of the neutralizing antibody responses is associated with increased odds of Long COVID.

In order to determine whether neutralization capacity was related to Long COVID, we performed logistic regression modeling with either the presence of any Long COVID symptom at a given sample time point or with specific Long COVID symptom phenotype (neurocognitive, cardiopulmonary, gastrointestinal, musculoskeletal and fatigue) at the three main collection time points: 1 month (N=69; median 33 days), 2 months (N=115; median 59 days) and 4 months (N=119; median 120 days) following acute infection. Data from only one time point per participant within each time period was included to avoid oversampling of specific individuals. Specifically, the sample time closest to 30 days within a 21–45 day window, 60 days within a 56–75 day window, and 120 days within a 100–150 day window were included. Factors included in the first model (Figure 3A) included the neutralizing antibody ID50 (continuous variable), prior hospitalization during acute COVID-19, female sex, and age greater than 50 years of age. Overall, there were no significant differences between neutralization ID50 to any strain and the presence of Long COVID in general or any specific Long COVID phenotype at 1 and 2 months following acute infection (all P>0.05). As shown in Figure 3A, the neutralization ID50 of the original SARS-CoV-2 (the infecting strain in this study population), as well as Alpha and Delta variants, were not significantly associated with the presence of any Long COVID symptom or specific Long COVID phenotype approximately 4 months after acute infection. However, cross-neutralization ID50s to Omicron BA.5 were significantly and positively associated with neurocognitive and gastrointestinal symptoms (*i.e.* higher odds of having symptoms within these phenotypes). There were no significant associations between BA.5 neutralization ID50 and fatigue and cardiopulmonary symptoms or as shown in Supplementary Figure 3). Regression analyses including ID50s to both wild-type SARS-CoV-2 and Omicron BA.3 were also performed as in Supplementary Figure 4A. Including ID50s to both wild type and BA.5 strains led to similar results, with cross-neutralization to Omicron BA.5 being significantly associated with having any Long COVID symptom and neurocognitive symptoms, whereas original SARS-CoV-2 ID50 were not significantly associated with Long COVID or any symptoms cluster.

Female sex was positively and significantly associated with an increased odds of Long COVID in models including both the original infecting virus and cross-neutralization to the Omicron BA.5 variant. Hospitalization for acute COVID-19 was only significantly associated with Long COVID in the model incorporating the original SARS-CoV-2 strain pseudovirus. A pre-existing history of diabetes mellitus or autoimmune disease were included in subsequent regression models but were not significantly associated with Long COVID outcomes and did not influence significance of other factors.

To further test the relationship between cross-neutralization of BA.5 pseudoviruses with the development of Long COVID symptoms, we performed binary logistic regression

including only the top 15% of neutralization ID50s in both the original SARS-CoV-2 and BA.5 variant based on results from a sensitivity analysis to evaluate the influence of the samples with robust cross-neutralization of BA.5 as shown in Figure 3B. Consistent with the above analyses, there were no significant associations between the top neutralization responders to the original virus and Long COVID or Long COVID phenotype, whereas presence of robust cross-neutralization to Omicron BA.5 was significantly associated with a higher odds of any Long COVID symptom and neurocognitive and gastrointestinal Long COVID phenotypes. We repeated regression analysis with original wild-type and Omicron BA.5 in the same model and results were similar with high cross-neutralization to BA.5 being significantly associated with any Long COVID symptom and neurocognitive and gastrointestinal symptoms clusters (Supplementary Figure 4B).

Decay of SARS-CoV-2-specific antibody neutralization over time by Long COVID phenotype.

Finally, we assessed neutralizing antibody responses against the original infecting SARS-CoV-2 strain by individual Long COVID phenotype (*i.e.* non mutually exclusive symptom cluster) compared to those without any Long COVID symptoms or those with or without symptoms but not within the specific Long COVID symptom cluster (Figure 4A & B, respectively). Overall, differences in levels across all time points or changes over time were similar, with high interpatient variation in neutralization ID50s observed. Nonetheless, we found that those with gastrointestinal and cardiopulmonary symptoms had 0.27 and 0.43 $\log_{10}$ higher neutralization ID50 compared to those without any Long COVID symptom across all data points over time (P=0.04 and <0.001, respectively; Figure 4A). Decay in neutralization ID50 was faster (*i.e.* more negative slope in mixed linear effects model) in participants with cardiopulmonary and musculoskeletal symptoms compared to those without any symptoms (P=0.01 and 0.047, respectively). Compared to those with or without persistent symptoms, but no symptoms in the specified phenotype cluster, those with cardiopulmonary or musculoskeletal symptoms were overall higher across all time points (both P<0.001), and those with musculoskeletal, cardiopulmonary and neurocognitive symptoms declined more rapidly than those without those specific symptoms (all $P < 0.05$; Figure 4B).

Absence of autoimmune signature distinctive of participants with a high breadth of neutralization antibody responses across SARS-CoV-2 variants.

We previously utilized PhIP-Seq technology to identify an autoreactive signature distinctive of prior SARS-CoV-2 infection within our LIINC cohort. However, the autoreactivities contained within this signature were similarly present in individual with and without Long COVID symptoms [30]. To determine whether participants with a high breadth of SARS-CoV-2 cross-variant neutralization as defined above and in Figure 3B had a distinctive autoantibody signature, we reanalyzed these PhIP-Seq data for this particular study population. No distinctive autoimmune signatures could be identified in those with a high breadth of neutralization of original SARS-CoV-2 and Omicron BA.1 or BA.5 variants with average Receiver Operating Characteristic (ROC) area under the curve (AUC) ranging from 0.37–0.52 (Supplemental Figure 5).

DISCUSSION

In this longitudinal study of a well-characterized cohort of people recovering from COVID-19 during the early waves of the pandemic prior to emergence of Delta and subsequent variants and availability of vaccines, we identified several novel relationships between SARS-CoV-2 antibody neutralization and the presence of Long COVID symptoms. First, we show that, although neutralizing antibody responses to the original, infecting strain of SARS-CoV-2 were not associated with Long COVID in cross-sectional analyses, cross-neutralization ID50 levels to the Omicron BA.5 variant approximately 4 months following acute infection were independently and significantly associated with greater odds of Long COVID in general and specifically with persistent gastrointestinal and neurological symptoms. It is possible that these gastrointestinal symptoms are related to autonomic nervous system dysfunction in addition to direct tissue inflammation or damage, and defining Long COVID phenotypes is always challenging given the often diverse and overlapping nature of clinical presentations. Nonetheless, these data suggest that a broad antibody response to subsequent viral variants may predict emergence of various Long COVID symptoms. Supporting this finding, a recent preprint suggested that people with Long COVID may have an expanded antibody response against the prior OC43 endemic Coronavirus [23]. The researchers also demonstrated more avid IgM responses and inflammatory OC43 S2-specific Fc-receptor binding responses but weaker Fc-receptor binding to SARS-CoV-2 [23]. Whether or not the association between breadth of response and Long COVID is due to or related to processes such as autoreactive antibody formation warrants further investigation.

Interestingly, we observed that a higher proportion of people infected early during the pandemic had antibodies capable of neutralizing Omicron BA.5 compared with BA.1, to which very few participants demonstrated any cross-neutralization response. The reason for this is not known, but several amino acid mutations in the receptor binding domain (RBD) of SARS-CoV-2 Spike protein in the BA.1 strain reverted to wild-type in the BA.5, such as G446S, Q493R and G496R [19, 32]. The BA.5 variant also had reversion of amino acid mutations or insertions in non RBD areas of Spike to pre-BA.1 variants (*e.g.* L981F, ins214EPE, T95I) [19, 32]. In the sub-analysis of samples from 16 participants stratified by the highest and lowest BA.5 neutralization titers, we also observed consistently higher cross-neutralization with BA.2, BA.4.6 and BQ.1.1 sub variants. In contrast, lower neutralization ID50s to XBB.1.5 pseudovirus were observed, similar to BA.1 neutralization. Whether or not there are any clinical implications of increased cross-neutralization in those infected early in the pandemic with subsequent Omicron variants is not known.

We also evaluated the longitudinal relationships between antibody neutralization responses, various clinical factors, and Long COVID phenotypes. The mixed effects models allowed us to determine differences between these variables across all data points over time (including multiple time points for each individual) and changes (*i.e.* decay) over time. These analyses revealed relationships between neutralization responses that were not observed in the cross-sectional analyses, such as significantly higher SARS-CoV-2 neutralizing responses to the original infection SARS-CoV-2 across all time points for those with gastrointestinal and cardiopulmonary Long COVID symptoms. The reasons for these initially higher and

broader antibody levels are not known, but could reflect more extensive tissue dissemination or higher viral tissue-burden during acute and convalescent infection. Regardless of the overall higher antibody levels, a faster decay of neutralizing ID50 was observed for these phenotypic clusters, in addition to those with musculoskeletal symptoms. Of note, neutralization titers decay more rapidly on a whole than common epitope antibody responses which have been associated with various Long COVID symptoms in longitudinal analyses [16, 17, 20], highlighting importance of temporal immune dynamics in the study of this condition. Together, these and the cross-sectional data suggest that an overall higher neutralization response that wanes more quickly, or ones that remain broad over time, are associated with Long COVID.

Whereas the causes of these dynamics are unknown, one possibility is that persistent SARS-CoV-2 antigen presentation in tissues, which has been proposed as a potential mechanism of Long COVID, may lead to overall higher antibody neutralization over time, and potentially to a broader response to subsequent variants. Recent studies have suggested that infection with Omicron strains lead to decreased risks of developing Long COVID [33, 34] and this may be due to less initial viral dissemination across deeper tissues. Whereas a broader initial antibody response to BA.5 from ancestral variant infection as observed in this study may protect people from more severe subsequent re-infection, it is also associated with developing Long COVID. These potential processes may not mutually exclusive. Further investigation of how post-Omicron variants or re-infection with other strains modulate development of Long COVID or subsequent sever disease are warranted.

While is possible that a broader antibody response to SARS-CoV-2 could be associated with increased autoreactive antibodies, we did not find evidence to suggest this occurs with PhIP-Seq detected autoreactivities. This same PhIP-Seq library has been used successfully to identify autoimmune signatures in post-COVID sequelae such as MIS-C [31] but has known limitations in detecting conformational antigens, and therefore cannot exclude the possibility that autoantibodies are differentially present in this cohort. While speculative, our findings suggest that relationships between various immune responses and Long COVID are likely complex, and different approaches to data analyses may yield different, but complementary information.

Strengths of this study include the use of highly characterized samples from the pre-vaccine and pre-Omicron era, before reinfections became common. This allowed for a more straightforward analysis of neutralization dynamics in the absence of these complex confounding factors. In addition, both those with and without Long COVID were recruited and assessed in an identical manner, addressing potential biases that might occur when comparison groups are derived from different cohorts as has been common in studies of Long COVID. As in similar analyses where we have been able to evaluate mechanisms according to distinct Long COVID phenotypes [9], we leveraged our high degree of symptom characterization to analyze different case definitions of Long COVID. This approach is informative, especially since the case definition remains controversial and it is possible that different phenotypes are driven by different mechanisms. Limitations of the study include a lack of participants infected with more recent variants, preventing us from extending our observations into more recent waves of the pandemic. The widespread but

inconsistent rollout of vaccination and multiple re-infections in more recent months makes studying the association between immune responses and Long COVID more difficult. The cohort was a convenience sample, and although this allows for valid inferences regarding Long COVID biology comparing people with and without the phenotype of interest within the cohort, extrapolation to all individuals with prior COVID-19 must be done cautiously. The neutralization assay used Spike protein pseudoviruses rather than intact, replication-competent virus, but these pseudovirus assays have been shown to have comparable results in several studies [27, 29, 35–37]. Nonetheless, we believe that these results suggest at least one potential contributor to Long COVID, although more work will be necessary to validate these observations in other cohorts, including those derived from later waves of the pandemic in the setting of vaccination or reinfection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement:

The data that support the findings of this study are available upon request.

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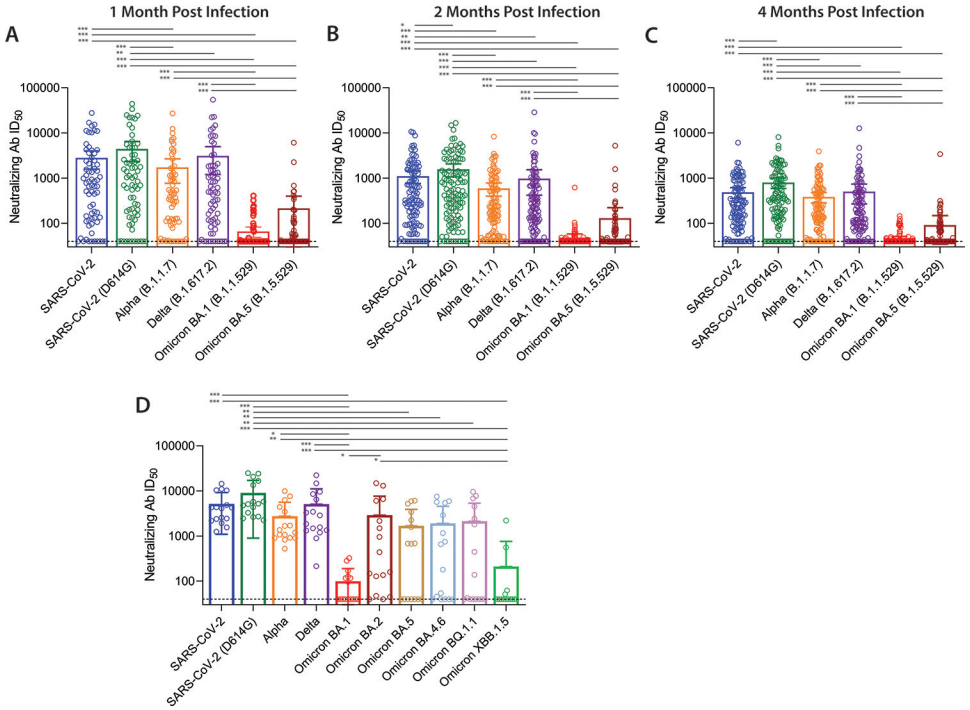

Figure 1. SARS-CoV-2 neutralization to the original infecting strain and cross-neutralization to subsequent viral variants.

Ex vivo antibody neutralization of the original SARS-CoV-2 virus and subsequent variants approximately 1 month (**A**, N=69), 2 months (**B,** N=115), and 4 months (**C,** N=119) following acute infection for all participants. Subgroup analysis of cross-neutralization in an expanded Omicron sub variant panel (N=16) in a subset of participants that had samples across all timepoints with either high or negative neutralization to BA.5 (**D**). Most samples collected prior to SARS-CoV-2 vaccination and prior to the emergence of Delta and Omicron variants. Bars represent mean antibody (Ab) infectious dose 50% (ID50) values and 95% confidence intervals (* P<0.05, ** P<0.01, ***P<0.001 by two-sided Freidman test adjusted for multiple comparisons).

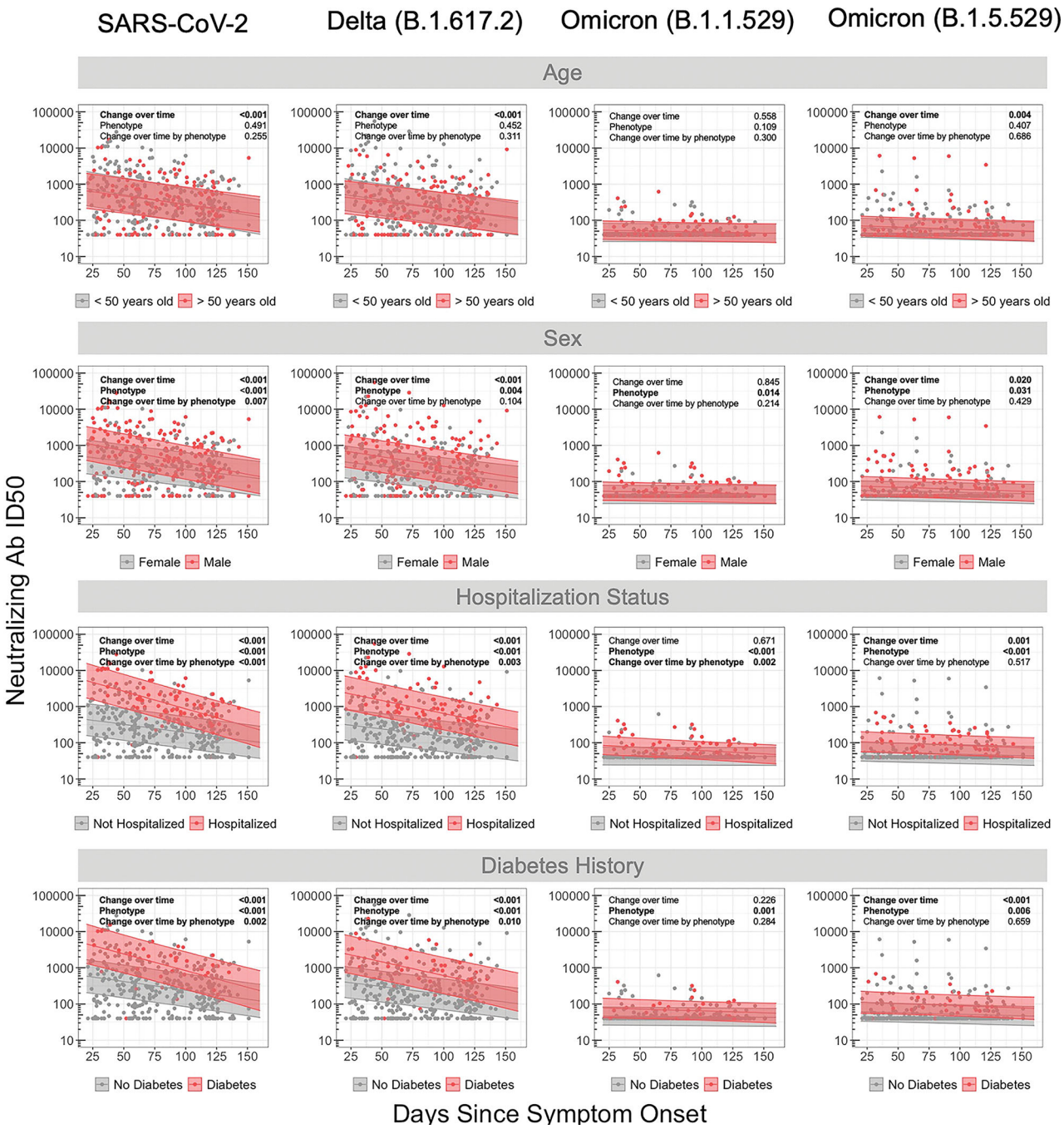

Figure 2. Longitudinal analysis of antibody neutralization by demographics, clinical factors and SARS-CoV-2 variant.

Mixed-linear regression model with four covariates (*i.e.* age, sex, hospitalization status, and prior diabetes history) for different variants: SARS-CoV-2, B.1.617.2, B.1.1.529 and BA.4/5. P-values denote if a significant difference was observed for change in antibody neutralization over time (Change over time), between subgroup (Phenotype, e.g., no diabetes versus diabetes), and difference in change over time by subgroup (Change over time by phenotype, *e.g.* difference in slope of decline in antibody levels dependent on diabetes

versus no diabetes). Shaded region represents 95% confidence intervals around the median line.

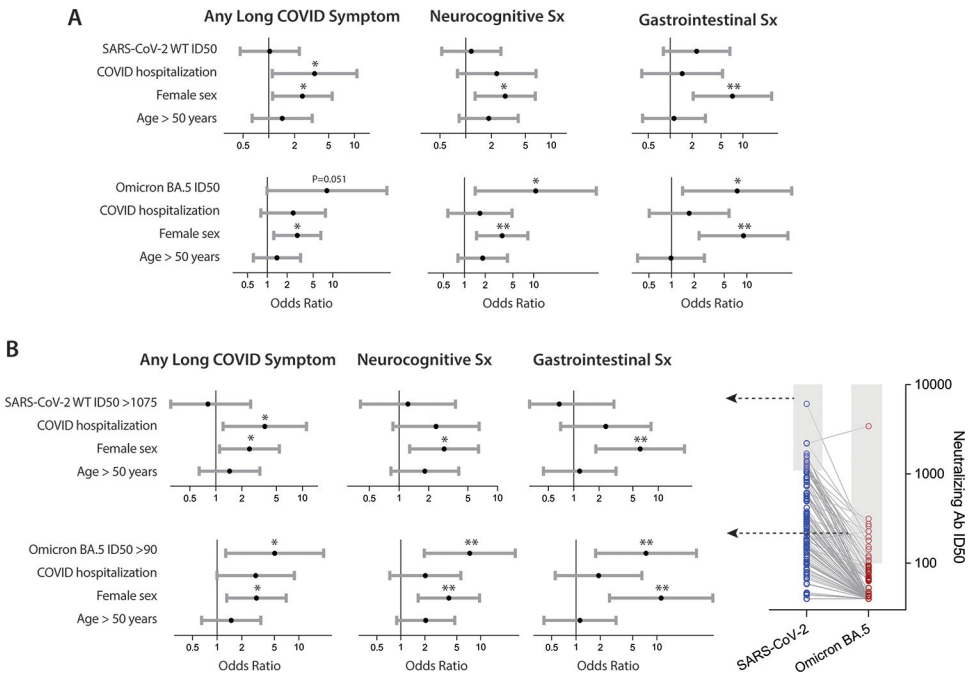

Figure 3. Association between SARS-CoV-2 neutralization, hospitalization during acute infection and demographic factors and the odds of experiencing Long COVID approximately four months following acute COVID-19.

The top panel shows odds ratios (points) and 95% confidence intervals (bars) for each variable included in logistic regression models using continuous neutralization ID50 values for assays using the original and Omicron BA.5 pseudoviruses (**A**). The bottom panel shows odds ratios and 95% confidence intervals of developing Long COVID or specific Long COVID phenotypes for logistic regression models incorporating a binary variable indicating if a sample had a neutralization ID50 in the top 15% of the cohort to either original SARS-CoV02 or Omicron BA.5 pseudovirus (**B**). * P<0.05, ** P<0.01 from covariate adjusted logistic regression.

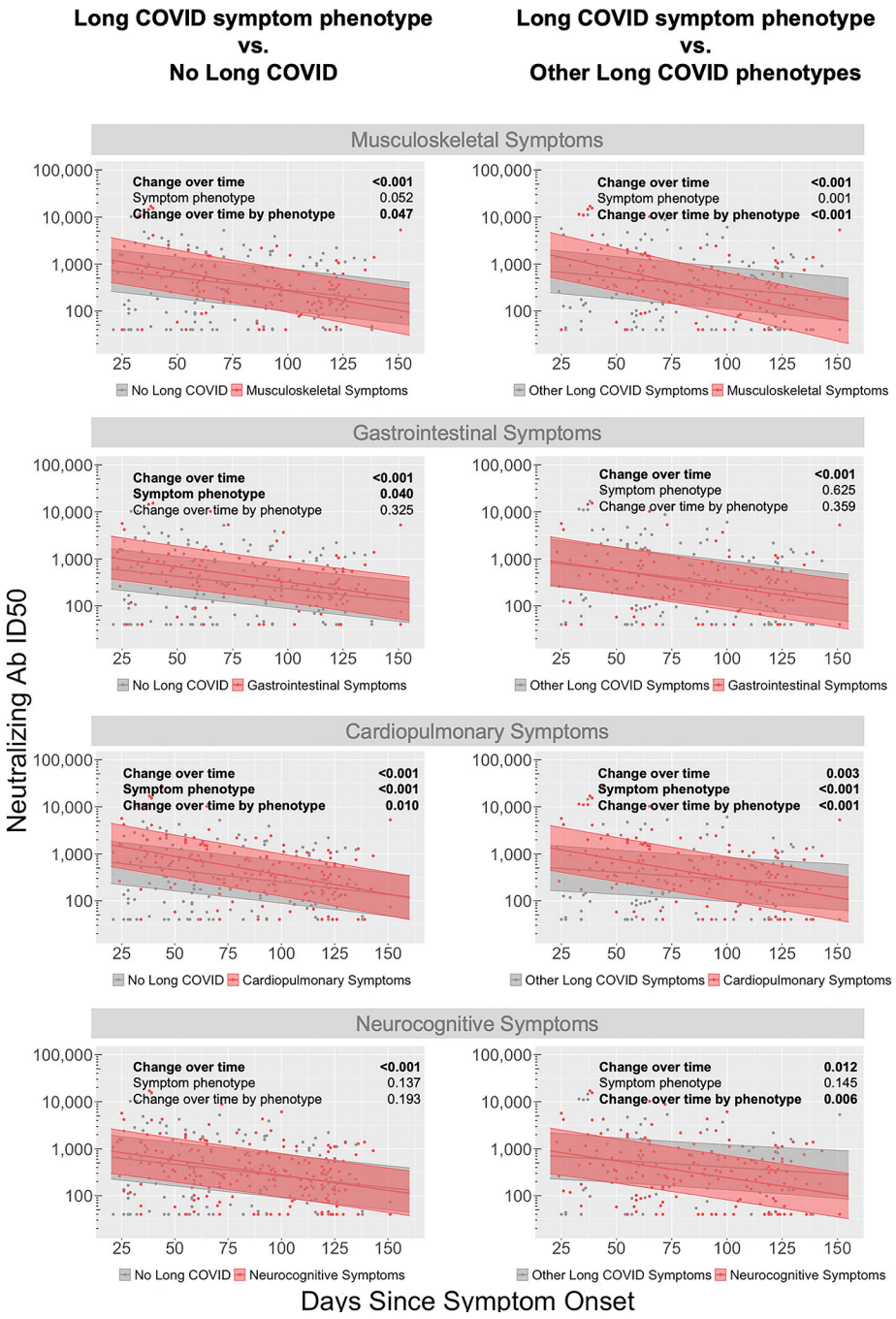

Figure 4. Differential decay of SARS-CoV-2-specific neutralizing antibody responses among Long COVID Symptom Phenotypes.

Longitudinal decay of antibody responses compared between participants with Long COVID Symptom Phenotype (e.g. musculoskeletal, gastrointestinal, cardiopulmonary, neurocognitive) **a)** versus those without Long COVID (left panels) or **b)** versus those with other Long COVID Symptom Phenotypes (right panels). Each panel includes p-values for: the decay across all participants ("change over time"), differences in antibody neutralization for those with Long COVID phenotype across all timepoints (Symptom Phenotype), and

differences in change over time with a given Long COVID phenotype (Change over time by phenotype). Shaded region represents 95% confidence intervals around the median line.

Table 1.

Participant demographics, comorbidities, and clinical presentation in participants with and without Long COVID

	All Participants	No Long COVID	Long COVID[a]
N	184	71	113
Female Sex	94 (51.1%)	28 (39.4%)	66 (58.4%) *
Age (median, IQR)	44 (34, 53)	43 (33, 53)	45 (36, 54)
COVID-19 Hospitalization	42 (22.8%)	14 (19.7%)	28 (24.8%)
Pre-Existing Medical Condition			
Diabetes Mellitus	20 (10.9%)	11 (15.5%)	9 (8.0%)
Autoimmune Disease	13 (7.1%)	1 (1.4%)	12 (10.6%) *
Race/Ethnicity			
Latinx	53 (28.8%)	14 (19.7%)	39 (34.5%) *
White	100 (54.3%)	40 (56.3%)	60 (53.1%)
Black/African American	4 (2.2%)	3 (4.2%)	1 (0.9%)
Asian	22 (12.0%)	13 (18.3%)	9 (8.0%)
American Indian/Native Alaskan	1 (0.5%)	0 (0%)	1 (0.9%)
Pacific Islander/Native Hawaiian	3 (1.6%)	0 (0%)	3 (4.2%)
Body Mass Index[b]	26.7 (23.4, 31.2)	26.0 (23.1, 28.8)	27.7 (23.6, 32.9) *

COVID-19 = coronavirus disease 2019; IQR = interquartile range

[a] Long COVID as defined as any persistent symptom new since COVID-19 onset at least 3 months following acute infection.

[b] n = 178 excluding missing values

* P < 0.05 by two-sided Fisher's exact test for categorical data or by two-sided Mann-Whitney U test for continuous data.