

# Heterogeneity of humoral response patterns in mildly symptomatic, non-hospitalized COVID-19 patients: A one-year longitudinal study

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## Impact Statement

Here, we describe patterns of humoral responses to SARS-CoV-2 in a follow-up study of COVID-19 patients before vaccination. Then, part of the cohort was vaccinated, and part was not, as we continued to follow anti-SARS-CoV-2 antibodies dynamics. We detected a high degree of antibody response's heterogeneity in naïve patients who were infected by SARS-CoV-2. We believe our results and conclusions are relevant and worth publishing in such a prestigious Journal as EBM for two other reasons: first, our cohort is composed of mild COVID-19 patients, who were not hospitalized during their illnesses. This cohort represents more than 80% of all infected people worldwide; nonetheless, most studies on humoral responses to SARS-CoV-2 were conducted in hospitalized patients presenting moderate to severe disease. Second, studies in SARS-CoV-2 naïve patients are nearly impossible nowadays, due to the high global attack rate of the virus, as well as the crescent vaccination levels.

## Abstract

The duration and protectiveness of antibodies against SARS-CoV-2 in infected subjects are still uncertain; nonetheless, anti-S-specific antibodies can contribute to protective immunity against new infections. It has been described that the level of antibodies produced in COVID-19 is related to the severity of symptoms, and the majority of the humoral response studies have been conducted in hospitalized patients who have been, then, followed over time. However, about 80% of SARS-CoV-2 infections in unvaccinated people are mild to asymptomatic, and this percentage reaches more than 95% in vaccinated individuals. Therefore, understanding the long-term dynamics of the antibody responses in this predominant part of the COVID-19-affected population is essential. In this study, we followed a cohort of individuals with mild COVID-19 who did not require hospitalization. We collected blood samples at sequential times after the SARS-CoV-2-positive qRT-PCR result. From 65 recruited patients, 50 had detectable antibodies at screening. Anti-SARS-CoV-2 IgM levels peaked around two weeks post-COVID-19 diagnostics, becoming undetectable after 65 days. IgG levels reached a peak in approximately one month and remained detectable for more than one year. In contrast to the levels of anti-SARS-CoV-2, antibody neutralization potency indexes persisted over time. In this study, humoral responses in mild COVID-19 patients persisted for more than one year. This is an important long-term follow-up study that includes responses from COVID-19 patients before and after vaccination, a scenery that has become increasingly difficult to evaluate due to the growing vaccination of the world human population.

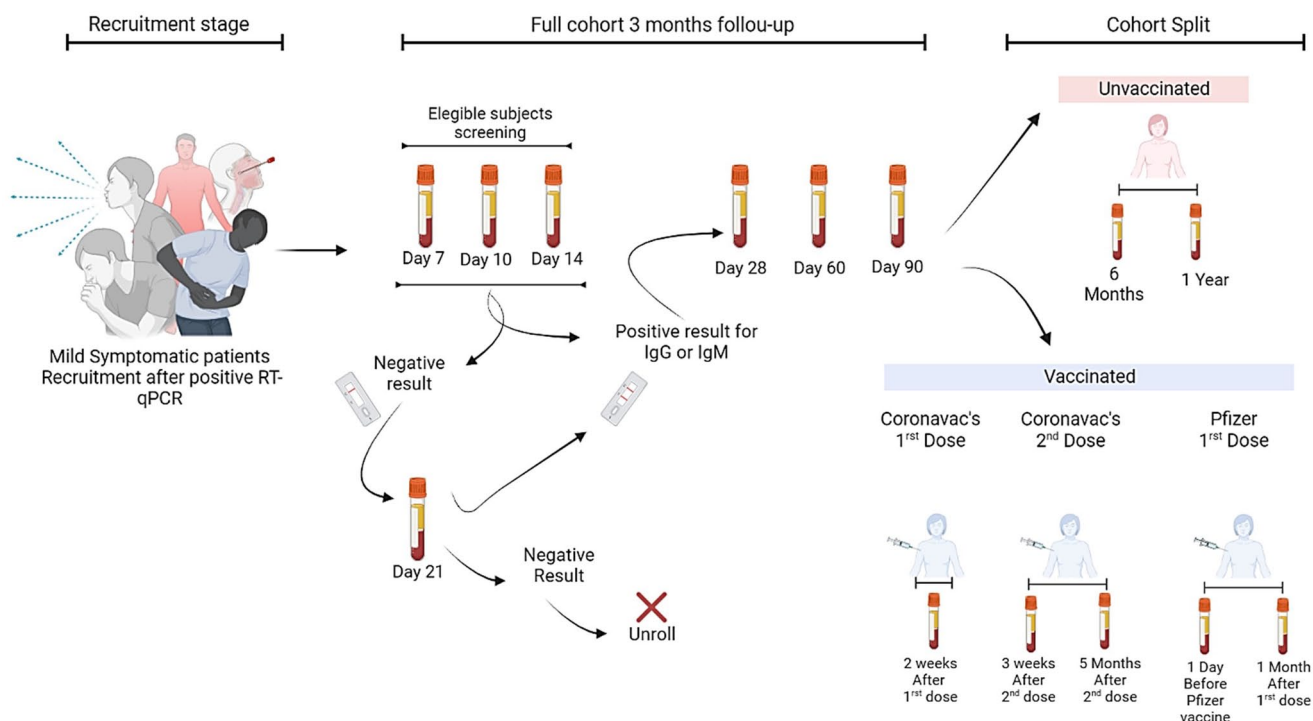
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## Introduction

Since declared a pandemic public health emergency in March 2020, the coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2 infections has caused approximately 6.4 million deaths in almost three years.<sup>1</sup> This disease can vary

from severe illness to asymptomatic infection,<sup>2</sup> but most affected patients do not develop severe disease and do not need hospitalization.<sup>3</sup> In most cases, individuals with positive RT-qPCR diagnostic develop specific antibodies against the surface Spike (S) glycoprotein and nucleocapsid (N) within one to two weeks post infection,<sup>4</sup> and meanwhile,



**Figure 1.** Study design and follow-up of participants. The blood sampling chronogram is divided into three stages: recruitment of COVID-19 mildly affected patients, screening within a follow-up time of three months, and the division of the cohort between vaccinated and unvaccinated individuals. Created with BioRender.com.

a percentage ranging between 10% and 20% show undetectable specific antibodies.<sup>5</sup> Understanding the dynamics of antibodies produced against SARS-CoV-2 proteins is important both to diagnose past infections in seroprevalence and/or surveillance studies and to verify protection against future infections.

The duration and neutralizing ability of antibodies are still subject to debate, especially after mild infections. It has been demonstrated that critically ill patients usually display hallmarks of extrafollicular B cell activation and produce high levels of low-potency neutralizing antibodies.<sup>6,7</sup> Nonetheless, about 80% of all SARS-CoV-2 infections are mild to asymptomatic,<sup>3,8,9</sup> and understanding the dynamics of anti-SARS-CoV-2 antibody responses of this dominant portion of COVID-19 affected population is extremely relevant to define public health strategies or even in terms of predictions about the future of COVID-19 amid us. Here, we investigated the antibody dynamics in mild COVID-19 patients over a period of one year after the onset of disease. The evaluated population included non-vaccinated and vaccinated individuals, and results showed important differences in these two subpopulations. Nonetheless, overall, our follow-up study indicates that anti-SARS-CoV-2 antibodies are long-lasting.

## Material and methods

### Ethics and recruitment

Sixty-five participants were recruited with the following inclusion criteria: positive qRT-PCR result for SARS-CoV-2 or inconclusive qRT-PCR result and a reagent result in the rapid DPP COVID-19 IgM/IgG Bio-Manguinhos test. Exclusion criteria were negative qRT-PCR result and no detectable

antibodies until the fourth blood collection (see Figure 1). Demographic information, medical history, and COVID-19 symptoms were obtained by filling out electronic forms. This study included subjects who did not require hospitalization. The study was approved by the Ethics Committee of the Federal University of Minas Gerais (UFMG) (CAAE: 1686320.0.0000.5149). The subjects signed the free and informed consent form (TCLE) to enroll in the study.

### Sample collection methodology and chronogram

At first, individuals had blood samples collected at four sequential times using the RT-qPCR result positive as a set point: T1 (day 7), T2 (day 10), T3 (day 14) to T4 (day 29 if there were detectable specific antibodies if not, T4 took place one week after T3). Subjects with undetectable specific antibodies until T4 were unenrolled from the study. The others had two more blood collections: T5 with 60 days and T6 with 92 days. After that point the cohort was split into two groups: those who remained unvaccinated, with two more blood draws taking place, T7 and T8 (six months and one year after RT-qPCR result); and those who received either Coronavac (inactivated) or Pfizer (mRNA) vaccines. These individuals were subjected to five more collections: T7, T8, T9, T10, and T11 (two weeks after the first dose, three weeks, five and eight months after the second Coronavac vaccine dose) and one month after a Pfizer vaccine booster dose. The complete study design is shown in Figure 1.

### DPP/ELISA

Serum samples were characterized by the rapid test DPP COVID-19 IgM/IgG Bio-Manguinhos (Lot 204EXVD01Z) following the manufacturer's instructions and by enzyme-linked

immunosorbent assay (ELISA) for detection of IgM-type antibodies using a combination of N (300 ng) and S (200 ng) proteins. For the detection of IgG-type antibodies, an ELISA containing N (400 ng) and S (400 ng) proteins was used as described.<sup>10</sup>

### Plaque reduction neutralization assay

Serum samples were tested at Biosafety level 3 facilities, in duplicate, days apart, as previously described.<sup>11</sup> The PRNT<sub>50</sub> was defined as the highest sample dilution that showed 50% reduction in number of plaques formed compared with positive control, which consisted in the number of plaques in wells inoculated with SARS-CoV-2 alone.

### Statistical analysis

We used standard descriptive statistics analysis to summarize data. The ELISA index was calculated as OD/cutoff.<sup>12</sup> For PRNT<sub>50</sub> analysis, median for each sample was calculated as a mean of two duplicates. Sera dilutions were transformed in Log(X) and a nonlinear regression (curve fit) was made. The neutralizing potency index (NPI) was calculated as the PRNT<sub>50</sub>/ELISA index ratio where NPI < 100 are considered low neutralization potency index and > 100 are considered high NPI as previously described.<sup>13</sup> Statistical significance between groups medians was determined by *t*-test. All data were analyzed using GraphPad Prism Software (version 8.0.1).

## Results

### Cohort description

Of the 65 subjects with SARS-CoV-2 infection confirmed by RT-qPCR, only 50 had antibody responses detected during the screening process. The cohort consisted of 58% women (29/50) and 42% men (21/50). The average age was 35.9 years (from 21 to 58). Ethnicity information was not collected on this cohort. Forty-two percent (21/50) were health care workers. Seventy-five percent had no declared comorbidities, and the others declared comorbidities that included, not exclusively, obesity, arterial hypertension, pulmonary disease, diabetes mellitus, thyroid changes, and cardiopathy. The most frequent COVID-19 reported symptoms were anosmia (80%), fatigue (77%), headache (61%), and running nose (52%). The duration of the symptoms varied considerably, but 8–14 days were the most frequent (36% of the cohort) (see Supplementary Material 1).

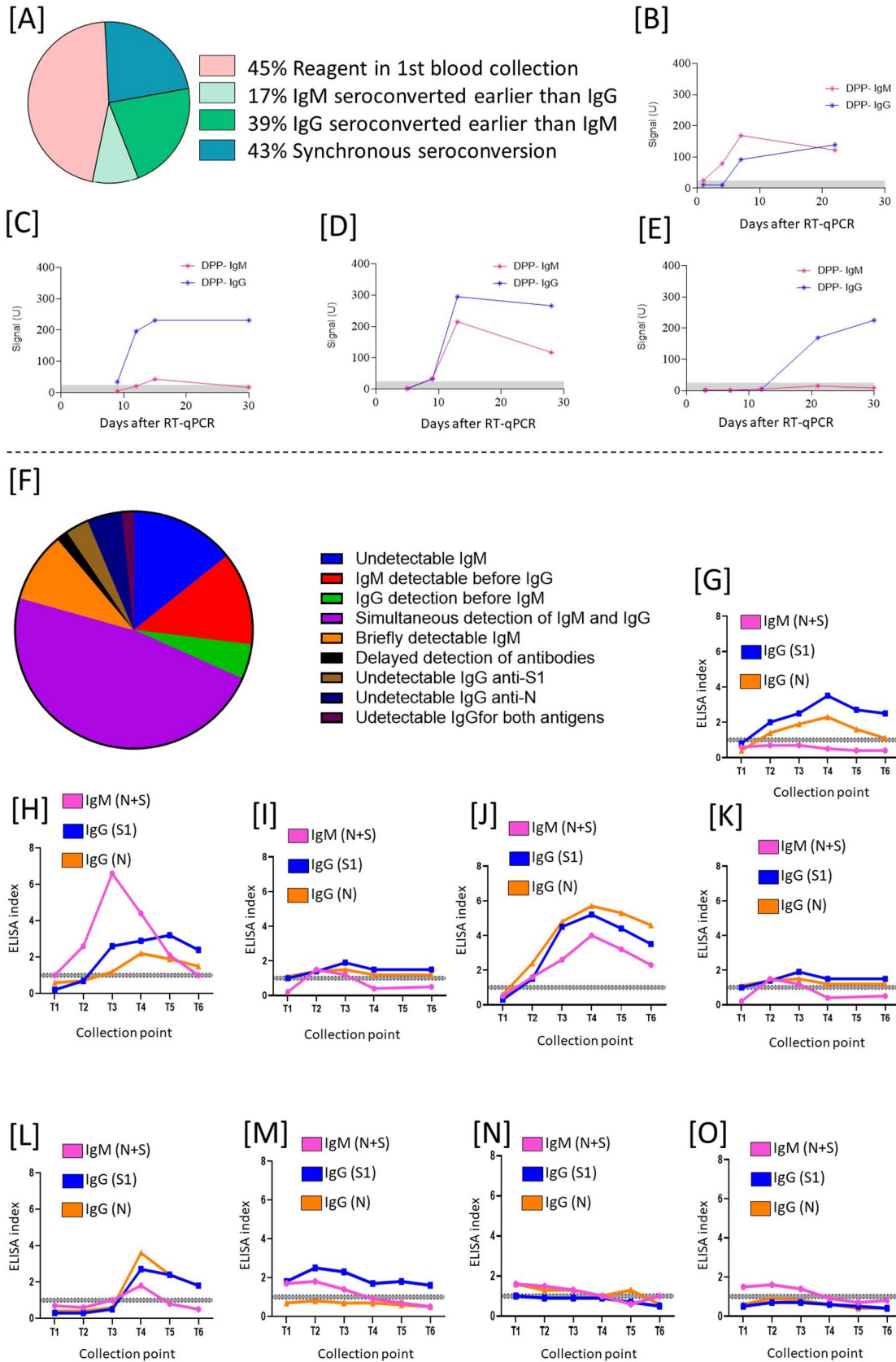
### IgM and IgG levels

Initially, 77% (50/65) of the tested subjects had at least one of the specific antibodies detected by DPP or ELISA; meanwhile, 23% of the enrolled participants (15/65) were excluded from the study as they failed to produce antibodies, as measured by either method. The combination of DPP/ELISA testing showed nine different seroconversion patterns, four observed in DPP and ELISA, and five observed by ELISA only. Both methods indicated subjects with undetectable IgM (6% DPP/18% ELISA), IgM detectable before IgG (17% DPP/16% ELISA), IgG detectable earlier than IgM (39% DPP/6% ELISA), and simultaneous detection of IgM and

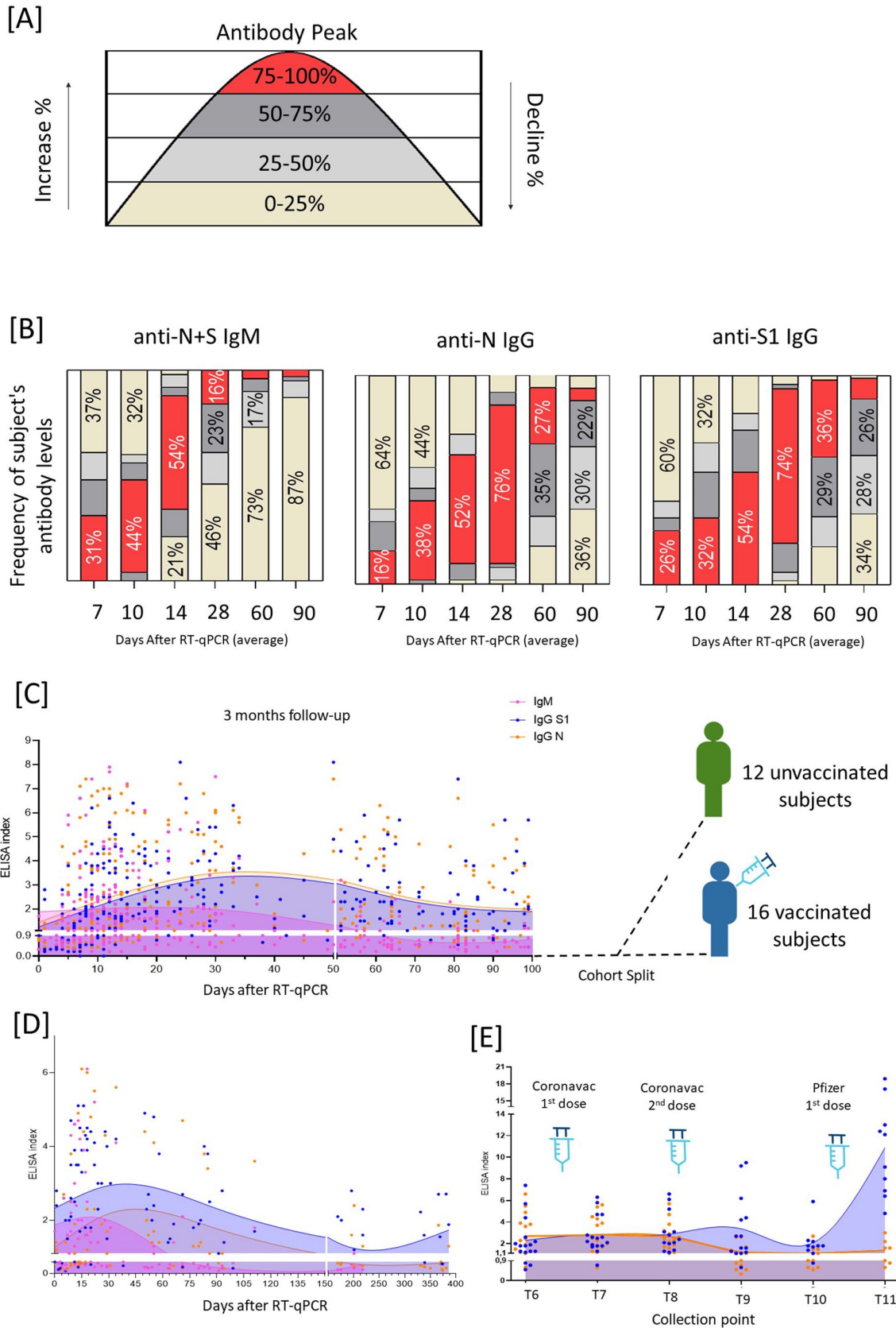
IgG (43% DPP/60% ELISA) (Figure 2(A) to (J)). The patterns detected only in ELISA were IgM briefly detectable (12%), delayed detection of antibodies (2%), undetectable IgG anti-S1 (4%), undetectable IgG anti-N (6%), and undetectable IgG for both antigens (2%) (Figure 2(K) to (O)). To determine antibody kinetics during infection and convalescence periods, we first monitored anti-S1 and anti-N, IgG, and IgM for approximately 100 days (three months) in six serial collection time points (Figure 1). Most subjects reached maximum IgM detection in 14 days ACI (after confirmed infection) and dropped to undetectable levels near two months ACI. Anti-S1 and anti-N IgGs peaked between 30 and 40 days ACI and started to decrease, losing almost 50% of detection in the first three months of the follow-up. IgG seroconversion was similar for both antigens, although anti-S1 presented higher levels than anti-N in the follow-up period (Figure 3(B)). After three months, the cohort was divided into two groups: those who remained unvaccinated (*n* = 12) and those who were vaccinated (*n* = 16) with two doses of Coronavac or one dose of Pfizer (Figure 3(C)). For the unvaccinated group, after 100 days, the decrease rate of IgG became different for anti-N and anti-S1: anti-N IgG levels dropped below the ELISA cutoff between 140 and 150 days ACI, whereas IgG anti-S1 decrease slower, and remained detectable for up to 350–400 days ACI (Figure 3(D)). The vaccinated group, on the other hand, presented a different dynamic, as expected. The Coronavac first and second doses kept the ELISA indexes for IgG (anti-N and S1) similar to before vaccination antibody levels, although five months after the Coronavac second dose, an increase of IgG anti-S1 levels was observed whereas anti-N IgG levels dropped below the ELISA cutoff. Approximately eight months after Coronavac's second dose, anti-S1 IgG dropped to the lowest levels, but antibody levels were quickly boosted after the Pfizer vaccine booster dose; anti-NS1 antibody levels raised from an average index of 2.1–10.9, representing a 519% increase. As expected, anti-N IgG did not show any increase after the vaccine booster dose (Figure 3(E)).

### PRNT and neutralization potency index (NPI)

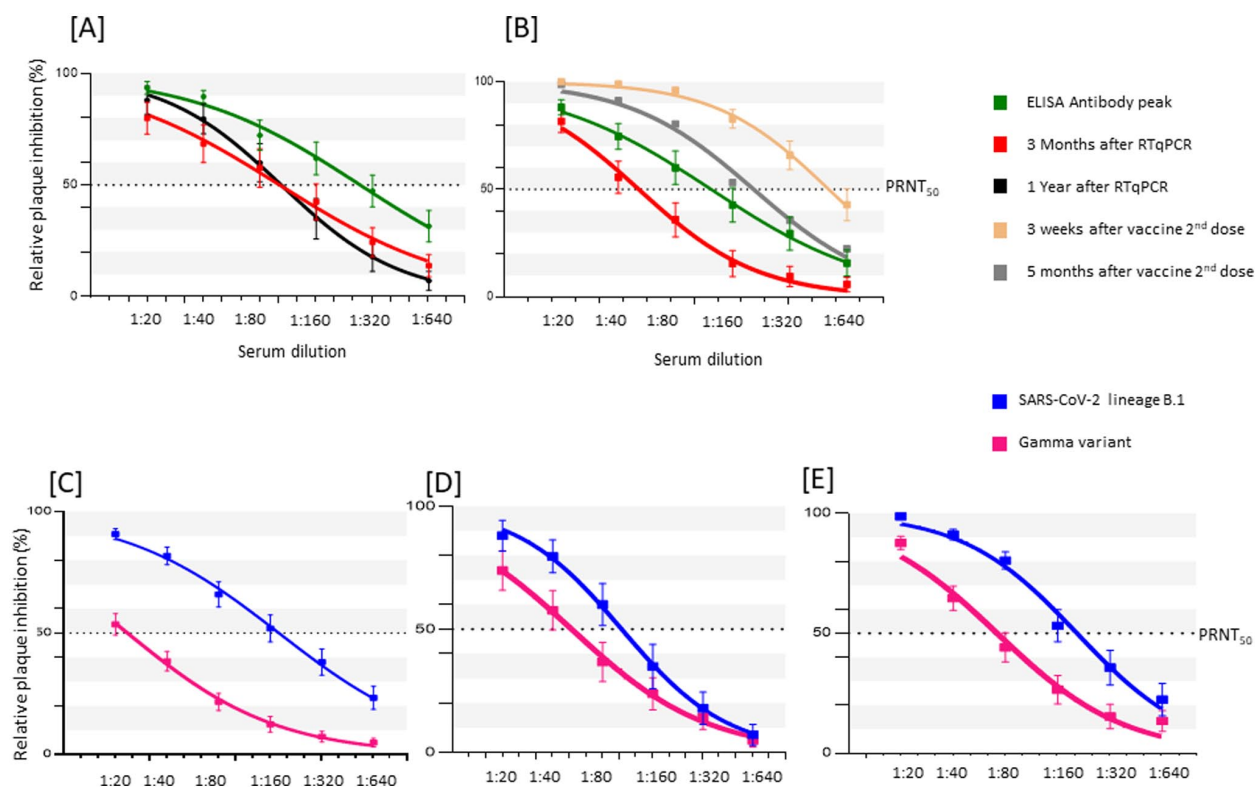
We evaluated the serum neutralizing activity at different time points by PRNT<sub>50</sub> for two SARS-CoV-2 lineages, B.1 and Gamma Variants. For unvaccinated individuals, we compared the ELISA antibody peak (approximately 28 days after ACI) to three months and one-year ACI, against the B.1 lineage. In those subjects, we observed that antibody peak had neutralizing titers of 1:275, whereas antibodies measured three months and one-year ACI presented approximately 2.6-fold less neutralizing power, with titers varying between 1:102 and 1:104 (*P* < 0.0001) (Figure 4(A)). For the vaccinated group, we compared the antibody peak ACI to three months ACI, and then to three weeks and five months after Coronavac's second dose. The higher neutralizing activity was detected three weeks and five months after the Coronavac vaccination, with titers of 1:513 and 1:206, respectively. Samples obtained at the antibody peak and three months ACI had titers of 1:123 and 1:57, respectively (Figure 4(B)). Finally, samples obtained after the Pfizer vaccine booster dose generated neutralizing activities above the threshold limit of 1:5120 (data not shown).



**Figure 2.** Patterns of seroconversion for IgM and IgG anti-SARS-CoV-2 in mildly symptomatic, non-hospitalized COVID-19 patients. (A) Frequency of DPP seroconversions. (B to E) Four different patterns of seroconversion were observed using the DPP system. (F) Frequency of ELISA seroconversions. (G to O) Nine different seroconversion patterns were observed in ELISA for IgG anti-N, IgG anti-RBD, and IgM anti-N + S. The gray line represents the test borderline; below the gray line, samples were considered negative and above the gray line were considered positive.



**Figure 3.** Antibody kinetics of anti-N IgG, anti-RBD IgG, and anti-N + S IgM. (A) The antibody curve was divided into areas (0–100% of antibodies) and each individual had normalized data in terms of its maximum peak. Then, it was possible to evaluate the frequency of subjects achieving different levels of antibody detection in terms of IgM (anti-N + S), IgG (anti-N), and IgG (anti-S). (B) Frequency of antibody levels detected 90 days cohort follow-up. (C) 100 Days follow-up, and cohort split into vaccinated and unvaccinated subjects. (D) Unvaccinated subject's follow-up. (E) Vaccinated subject's follow-up with vaccines given where point 1 represents the last sera sample before vaccination, point 2 represents two weeks after Coronavac first dose, point 3 represents three weeks after Coronavac second dose, point 4 represents five months after Coronavac second dose, point 5 represents sera day before Pfizer first dose, and point 6 represents one month after Pfizer booster dose. The curves were made by spline regression. The index cut-off for samples to be considered positive was  $\geq 1.1$ , and the borderline zone ranges from 0.8 to 1.1 (horizontal white bars).



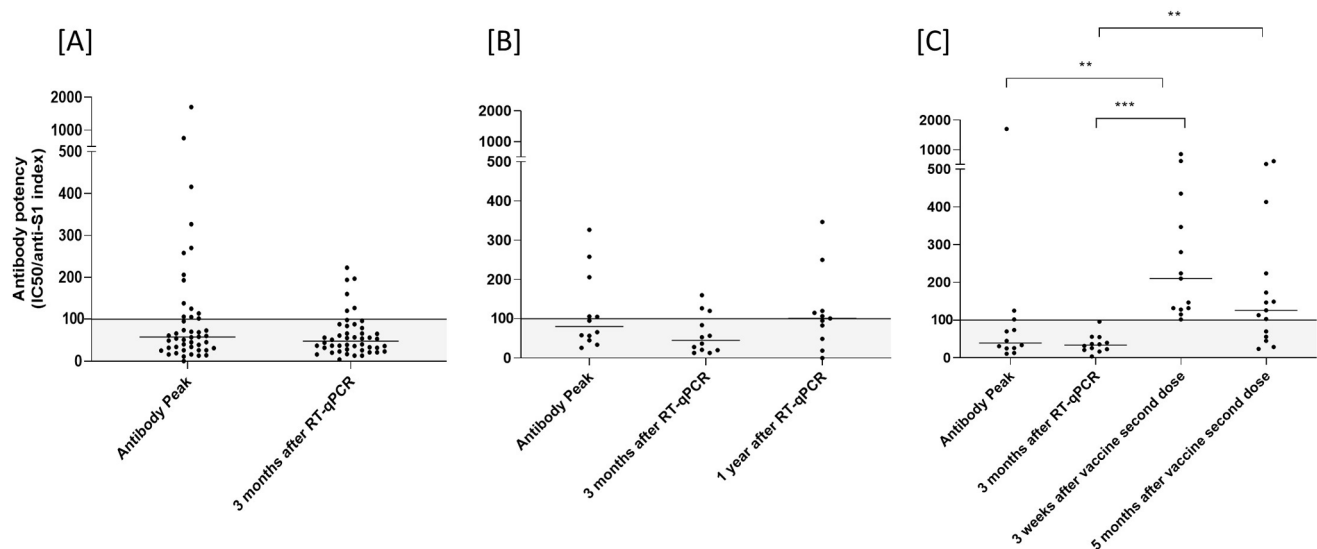
**Figure 4.** Serum neutralizing activity measured by PRNT<sub>50</sub>. In sera from COVID-19 patients in different time points. Plasma samples were incubated with Vero cells infected with SARS-CoV-2 lineages B.1 or Gamma to evaluate PRNT<sub>50</sub> (defined as reciprocal plasma sample dilution that showed 50% protection against cytopathic effects). (A) Unvaccinated individuals, one-year follow-up against the B.1 isolate ( $n=12$ ). (B) Vaccinated individual's follow-up against B.1 isolate ( $n=16$ ). (C) Sera from 28 days after confirmed infection (ACI) (antibody peak time point, measured by ELISA) against Gamma variant ( $n=28$ ). (D) Sera from unvaccinated subjects one-year AIC against Gamma variant ( $n=12$ ). (E) Sera from vaccinated individuals five months after the vaccine's second dose, against Gamma variants ( $n=16$ ). Each data point represents the mean for each group at the dilution level, and error bars represent SEM made in a duplicate assay for each plasma sample; two independent assays were done for all groups. The curves were calculated as nonlinear regression (curve fit).

Concerning the measured antibody neutralizing capacity, all compared time points presented statistically significant differences. For instance, the antibody peak time point compared to five months after the Coronavac vaccine had a  $P$ -value of 0.0054; other comparisons had  $P$ -value  $< 0.0001$  among them. When evaluated against the SARS-CoV-2 Gamma variant, all tested sera lost efficiency to neutralize the virus. In the ELISA antibody peak time point, we observed a 7.7-fold decrease in neutralizing titers, falling from 1:179 against the B.1 virus lineage to 1:23 for the Gamma variant ( $P \leq 0.0001$ ) (Figure 4(C)). In samples obtained one-year ACI, we detected a two-fold decrease in neutralizing capacity, from 1:104 for B.1 lineage to 1:52 against the Gamma variant ( $P=0.0002$ ) (Figure 4(D)). Finally, serum obtained five months after Coronavac's second dose presented a 2.8-fold decrease, from 1:206 against B.1 to 1:71 against the Gamma variant ( $P \leq 0.0001$ ) (Figure 4(E)). In addition to determine the neutralization potency index (NPI) (PRNT<sub>50</sub>/ELISA index) of antibody samples, we first correlated the PRNT<sub>50</sub> data with the ELISA indexes for both S and N antigens, tested to establish the best fit, and obtained an average correlation of 0.38 for PRNT<sub>50</sub>/IgG anti-N and 0.73 for PRNT<sub>50</sub>/IgG anti-S1. We estimated a low neutralizing potency index below 100 and high potency above 100 as described.<sup>13</sup> Comparing the cohort ( $n=50$ ) NPI during antibody peak and three-month ACI, we observed different NPI variations among subjects with a median potency of 57.7 in the antibody peak

(approximately 28 days after ACI) against 47.5 measured three months ACI, with no significant differences (Figure 5(A)). In the unvaccinated group ( $n=12$ ), the timing follow-up showed median NPIs of 80.5, 45, and 101 at the antibody peak, three months, and one-year ACI, respectively, with no significant differences in potency among all time points (Figure 5(B)). Comparing the NPIs obtained for the vaccinated group ( $n=12-16$ ), we observed the lowest NPI during antibody peak and three months ACI (median NPI=39.5 and 34). Nonetheless, after the Coronavac full vaccination, the median NPI rose to 210 – three weeks, and 126 – five months after vaccination, representing an increase of antibody neutralizing potency of 531% and 318% compared to the antibody peak ACI time point, respectively. Statistically, significant differences were observed when we compared the three weeks after vaccine second dose time point to sera obtained three months ACI ( $P \leq 0.0001$ ); three months ACI compared to five months after Coronavac vaccine ( $P=0.0078$ ), and antibody peak time point to three weeks after Coronavac's second dose ( $P=0.0028$ ) (Figure 5(C)). We did not calculate the NPI after Pfizer's vaccine booster dose as the PRNT<sub>50</sub> was above the measurable threshold.

## Discussion

At the beginning of the Covid pandemic, there were many questions about how long the antibodies would last and



**Figure 5.** Neutralization Potency Indexes (NPI): (A) NPI comparison in three months ACI follow-up ( $n=50$ ). (B) NPI comparison in unvaccinated subject's follow-up ( $n=12$ ). (C) NPI comparison in vaccinated subjects' follow-up ( $n=12-16^*$ );  $P$  values are indicated as  $**P=0.0028$  for antibody peak versus two weeks after vaccine second dose and  $P=0.0078$  for three weeks after vaccine second dose versus five months after vaccine second dose;  $***P\leq 0.0001$ . NPI was calculated as PRNT50/ELISA anti-RBD index. A non-parametric Kruskal–Wallis was performed for group comparisons. The gray area represents a low NPI (below 100). Antibody peak is 28 days ACI.

\*Not all 16 vaccinated participants were available for blood collection in all time points.

what their neutralizing power would be. The first longitudinal studies were quite worrisome, as they indicated that detectable humoral responses could last as little as three months.<sup>14,15</sup> Later cross-sectional cohort studies pointed to the detection of antibodies for a longer period.<sup>16</sup>

The long follow-up over time in our study has given us a clearer view of the big picture concerning the anti-SARS-CoV-2 humoral responses after infection, both in terms of the most representative portion of the infected populations and the longevity and potency of the antibody responses. Indeed, we were able to detect the presence of antibodies with neutralizing capacity up to one year after COVID-19, even in non-vaccinated individuals. This is an important result because this is something increasingly difficult to assess today due to the growing vaccination of the world's population.

Our initial screenings showed that not all subjects with SARS-CoV-2 infections confirmed by molecular or antigen tests present positive results in serological tests. Our analyses identified that 23% of the patients had no detectable levels of either anti-S1 or anti-N SARS-CoV-2-specific antibodies, which was similar to the rate found in a previous study.<sup>8</sup> Among the other participants of the study, we identified different patterns of antibody seroconversion, which is also consistent with other published works.<sup>4,17–19</sup>

Our data provide evidence that although the detection of antibodies declines over time, the remaining antibodies are efficient in neutralizing the virus, one could speculate that even with more neutralizing capacity. This happens because there are fewer detectable antibodies over time, but at the same time, there is no significant difference in antibody neutralization potency index in the different time points evaluated. Although it is not possible to state that these subjects did not have contact with SARS-CoV-2 again in the period between collections – which could have boosted the humoral responses – it is known that the immune system evolves

and adapts over time,<sup>20</sup> improving its efficiency in fighting pathogens. Of course, the appearance of variants with the ability to evade previous immune responses jeopardizes the protective effect of these antibodies.

In addition, it was possible to observe that vaccination changes the profile of antibody-mediated immunity. We observed that two doses of the Coronavac vaccine did not significantly increase the antibody index in ELISA, but it significantly increased the potency of these antibodies, providing a higher neutralization capacity than that acquired by the natural infection. Previous studies<sup>21</sup> have suggested that the use of heterologous vaccination protocols greatly improves the immune response. In this study, we show that after the Pfizer booster dose, the antibody index in ELISA increased significantly when compared to post-Coronavac vaccine levels, and even at higher levels than those obtained after natural infection, exceeding neutralizing levels of 1:5120. Unfortunately, it was not possible to calculate the neutralization power of these post-dose booster antibodies due to the method's detection limit threshold (1:5120). We also examined the trends of antibody neutralization against the Gamma Variant as a way to infer how the appearance of SARS-CoV-2 mutants can impact the protective power of specific antibodies, and indeed antibodies were less protective, as already described and published in numerous studies.<sup>11</sup> It is important to mention that our cohort was recruited between June and November 2020 when this variant had not yet been detected.<sup>22</sup>

These findings corroborate the fact that even though the vast majority of COVID-19 survivors can develop specific antibodies, and these antibodies can maintain their neutralizing potency index for one year or more, being vaccinated even after SARS-CoV-2 infection is still the best strategy to develop and maintain a robust neutralizing response against SARS-CoV-2.

## AUTHORS' CONTRIBUTIONS

Conceptualization: FGdF, LAFA, and FFB; methodology: LAFA, FFB, SARS, PLP, DAT-T, and FG; validation: LAFA and FFB; formal analysis: LAFA and FFB; investigation: LAFA and FFB; data curation: LAFA and FFB; writing – original draft preparation: LAFA and FFB; writing – review and editing: LAFA and FFB; visualization: FGdF; supervision: FGdF, APSMF, SMRT, and JLP-M; project administration: FGdF; funding acquisition: FGdF, APSMF, and SMRT. All authors have read and agreed to the published version of the manuscript.

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## DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.


## ETHICAL APPROVAL

This study was approved by the Ethics Committee of the Federal University of Minas Gerais (UFMG) (CAAE: 1686320.0.0000.5149).

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## SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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