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ABSTRACT

During the COVID-19 pandemic, Portugal has experienced three distinct SARS-CoV-2 infection waves. We previously documented the prevalence of SARS-CoV-2 immunity, measured by specific antibodies, in September 2020, six months after the initial moderate wave. Here we show the seroprevalence changes six months later, up to the second week of March 2021, shortly following the third wave, which was one of the most severe in the world, and two months following the start of the vaccination campaign. A longitudinal epidemiological study was conducted, with a stratified quota sample of the Portuguese population. Serological testing was performed, including ELISA determination of antibody class and titres. The proportion of seropositives, which was 2.2% in September 2020, rose sharply to 17.3% (95% CI: 15.8% – 18.8%) in March 2021. Importantly, circulating IgG and IgA antibody levels were very stable six months after the initial determination and up to a year after initial infection, indicating long-lasting natural immunity against SARS-CoV-2. Moreover, vaccinated people had higher IgG levels from 3 weeks post-vaccination when compared with previously infected people at the same times post-infection.

INTRODUCTION

On January 30th, 2020 the WHO declared that the outbreak SARS-CoV-2 constituted a Public Health Emergency of International Concern (PHEIC), followed by its characterization as a pandemic on March 11th. Since then, the infection has spread to almost every country in the world, with variable attack rates. Accurate estimates of anti-SARS-CoV-2 antibody seroprevalence in the population remain critical to inform policy to contain and bring to an end the ongoing pandemic. Seroprevalence studies can uniquely determine population exposure and correlate with the quality of immunity, and are more inclusive than polymerase chain reaction (PCR)-based virus-detection strategies. For example, they will include the prevalence of asymptomatic and pauci-symptomatic cases, individuals often missed in symptom-based infection screenings. Importantly, longitudinal seroprevalence studies provide a quantification of the evolution of exposure over time and associated demographics. Moreover, longitudinal designs can inform on the duration of antibody seropositivity. From the start, uncertainties regarding immune response and the duration of immunity against a novel mucosal corona virus were raised. We now have reports of good levels of antibodies, present at least 6 months post-infection, and

T cell immunity (*1-10*). But the majority of these studies use specific samples, such as health care workers, and do not provide a complete cross-sectional picture of the population.

In many countries, the epidemic has proceeded in waves. Portugal is a good example of this pattern. The first case of SARS-CoV-2 infection was officially reported on March 2nd, 2020, at the beginning of the first wave, which led to multiple early containment measures (Figure 1). There was a second much larger rise in incidence of infection in the months of October-November, followed by a severe third wave during January of 2021, when Portugal was for a few weeks one of the countries in the world with the most cases per million people (Figure 1).

However, the true level of incidence of the infection is difficult to ascertain from official case reports, as has been shown by serological prevalence studies from multiple countries (*11-14*). For example, we conducted a national level study based on a stratified quota sample of the prevalence of people positive for antibodies against SARS-CoV-2, in September 2020, before the second wave of infection, as an indicator of past infection, and concluded that 3-4 times as many people had been infected than the official case number reports (*15*). Moreover, this factor of extra infections was different among age groups, being ~9-fold in people younger than 18 years. This difference between number of registered cases and actual infections can be due to the number of asymptomatic or mild infections that go undetected, and overall testing policies.

Here, we report the results of a follow-up seroprevalence study performed from March 1st to March 17th, 2021 after the large increase of cases seen in January. At this time, in early March, there were ~810 thousand confirmed cases by the Portuguese health authorities, 58.3% of these cases occurring in December and January (Figure 1) (*16*). The current study is based on a sample randomly selected from the participants with known serological status in the first study (*15*). The participants constitute a national-level sample, stratified by age group and population density. Our primary objective was to assess the proportion of people with SARS-CoV-2 specific antibodies in Portugal, and how this varied by age group and population density. An important issue to determine an accurate estimate of the proportion of previous infections is how long antibodies can be detected after natural infection (*1, 7, 17, 18*). Thus, in this follow-up study, we also analyzed the antibody levels of people, who had tested positive in the first study, many of whom were infected during the first wave, up to ~1 year before. Finally, because the vaccine rollout was initiated in Portugal at the end of December, we also kept track of the fraction of people vaccinated. Altogether, we found a 17.3% seroprevalence level in Portugal and

that the vast majority of people maintain detectable antibodies, with some of these people almost 1 year after initial infection. This result provides insights in SARS-CoV-2-specific antibody waning.

METHODS

Study design, population and sample size

We conducted an observational, follow-up study to our previous non-institutionalized population seroprevalence study from September 2020. From the participants in that study, we invited for the current study a sample from among those who were seronegative – we call this the previously negative cohort (NC) – and all the participants (n=296) who tested positive for total antibodies against SARS-CoV-2 – we call this the previously positive cohort (PC). The global sample size was determined to allow no more than a 1.5% margin of error, at a 95% confidence level, for an expected prevalence of antibody positivity of 10%. With these assumptions, we estimated a sample of 2260 people, 2000 in the NC and 260 in the PC. To reach the desired sample size in the NC, we invited 3000 people from among those participants in our September study who were seronegative, which included over 13000 people (*15*). These 3000 people were selected randomly in strata by age group (<18, 18 to 54, ≥55 years) and by population density of the place of residence (≤500, >500 people/km^2). In addition, using appropriate probabilities of inclusion in the sample, we ensured that the distributions by sex, household size and level of formal education were consistent with those in the overall population Portugal.

We used the PC cohort to analyze the evolution of the antibody levels in people who tested positive in the previous study.

Informed consent was obtained from all participants aged 16 years or older. In addition, parental or legal guardian consent was required for all participants below 18 years old. Participants were excluded if they had any contraindication for phlebotomy. Prior diagnosis of SARS-CoV-2 infection was not an exclusion criterion.

The study was conducted in compliance with data protection regulations in Portugal and was approved by the Ethics Committee of the Centro Académico de Medicina de Lisboa (CAML – the Lisbon Academic Medical Center), under reference # 484/20 of 23rd of February 2021.

Serological tests and procedures

All blood collections and serological tests were done by Centro de Medicina Laboratorial Germano de Sousa (CMLGS), an ISO 9001:2015 certified private laboratory, which performs serological tests for SARS-CoV-2 according to the clinical guidelines issued by the Directorate-General of Health (DGS), within the Portuguese Ministry of Health. CMLGS coordinated blood collection through their national network of collection sites, which allowed the participants to visit the center that was more convenient for them. Each participant donated 7-9 ml of blood collected into tubes with separation gel and without any anti-coagulant, for a 4-5ml serum sample, obtained by centrifugation. All samples were transported to the central laboratory, according to usual procedures, where they were assayed.

Total antibodies against SARS-CoV-2 were assessed using a chemiluminescent immunoassay test, Siemens® SARS-CoV-2 Total (COV2T) (Advia Centaur Siemens, Siemens Healthcare, Portugal). The overall sensitivity and specificity of this test are 98.1% and 99.9%, respectively (*19*), which we used to correct the seroprevalence estimates with the Rogan-Gladen estimator (*20*).

A majority of the samples that tested positive for total antibodies were sent to Biobanco-iMM, Lisbon Academic Medical Center, and stored at -80 C. Then they were further tested to quantify the level of antibodies. To this end we used our in-house developed protocol described in detail in (*1, 21*). Briefly, flat-bottom 96-well plates (Microlon plates medium binding; Greiner) were coated with recombinant protein RBD or Spike prepared in PBS at a concentration of 2 µg/ml (50 µl/well). Plates were blocked with 200 µl/well of 3% nonfat milk powder in PBS-1%T for 1 hour at room temperature and then washed with PBS-T 3x, 6x or 10x, as described previously (*1*). Serum samples were diluted in PBS-0.1%T + 1% nonfat milk powder, added (100 µl/well) and incubated for 1–2 h at room temperature, washed with PBS-T 3×, 6× or 10×. Hereafter several antibody isotypes, namely IgG, IgM, and IgA antiSARS-CoV2 were detected using HRP-labeled goat anti-human IgG Fc (Abcam, ab97225), IgM mu chain (Abcam, ab97205), IgA alpha chain (Abcam, ab97215), respectively. OD at 450nm was measured via SPARK (TECAN) plate reader. Each plate contained Quality control (QC) samples, composed of a pool of positive samples, tested in a high and low dilution.

To compare antibody titers after vaccination with those after natural infection, we included additional samples previously processed with identical method and reported in Figueiredo et al. (*1*). These samples, from our previous study, were obtained shortly after infection and provide a better comparison with the vaccinated people, who we sampled in the current study shortly after vaccination.

Data collection and outcomes

All participants completed a questionnaire with socio-demographic, general health and clinical/epidemiological questions regarding SARS-CoV-2 exposure, including symptoms of interest, as well as vaccination status. The full questionnaire was presented before (*15*).

The primary outcome was the proportion of serological positive cases in each of the twelve strata (six for each cohort), defined as the fraction of participants who tested positive for SARS-CoV-2 specific antibodies in the COV2T assay. With these fractions, we inferred the seroprevalence in the Portuguese population, adjusting for the weights of the strata and correcting for sensitivity and specificity of the tests. The secondary outcome included the proportion of previously positive people that remained positive, and the quantification of any decline in antibody levels.

Statistical analyses

We used sample weights to adjust the seroprevalence extrapolating from our strata (age groups and population density of the place of residence) to the whole population. In addition, we combined the results of seroprevalence in the two cohorts NC and PC, with appropriate weights for each, based on the results of the previous study (*15*), to obtain the overall estimation of seroprevalence in Portugal. We performed these calculations in two ways, excluding or including the people, who indicated that they had been vaccinated before the study.

The prevalence was calculated as weighted proportion and to calculate confidence intervals, we used the methodology described in (*22*), *i.e.*, we used the exact limiting terms for the binomial parameter adapted for weighted proportions and combined stratum specific confidence intervals with the use of the adequate rescaling factor as proposed in (*22*).

We compared continuous variables (such as antibody titres) using non-parametric tests (Wilcoxon matched pairs for paired design, Mann-Whitney for unpaired designs and Kruskal-Wallis to compare more than two groups, with Tukey correction for multiple comparisons). To analyze antibody levels with time since infection (or vaccination), we used the dates of known PCR positive test or COVID-19 symptoms, when known. We then calculated the decay of (log) antibody titers over time using linear-mixed effects models, where participant was the random factor. The half-life of antibodies is then given

by log(1/2)/slope of decay. We used general linear models to control for time since infection, when studying differences of antibody levels by gender, age, body mass index (BMI) and smoking status. We did not input any missing values. All statistical analyses were two-sided, the significance level was α=0.05, and reported confidence intervals are at the 95% level. Statistical analyses were done using R (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism 6.01 (GraphPad Software, San Diego, California USA).

RESULTS

SARS-CoV-2 antibody seroprevalence in the Portuguese population

We conducted a follow-up to our seroprevalence study of September 2020, with blood collections for serological assays between March 1st and March 17th, 2021. From the participants in that study, we recruited 2172 people, who had previously tested negative (negative cohort - NC), and 263 people, who had previously tested positive (PC). In the NC, 43.6% were men (n=948), and 10.3% (n=224) were younger than 18, 46.3% (n=1006) were between 18 and 54-years old, and 43.4% (n=942) were 55 years or older. We asked about the participant status regarding vaccination against SARS-CoV-2 and 173 (7.1%) participants indicated that they had at least one dose of a vaccine (156 in the NC, and 17 in the PC). In Table 1, we show the characteristics of the two cohorts.

We tested all participants for specific antibodies against SARS-CoV-2 (see Methods for details). Not including vaccinated people, there were 2016 people in the NC and 246 people in the PC, of these 214 tested positive in the NC and 239 in the PC. With these results, and adjusting for the strata (age groups and population density of place of residence), as well as sensitivity and specificity of the test, the global seroprevalence found in this study was 13.1% (95% CI: 11.8% -14.6%) due to natural infection only (Table 2). In addition, considering the vaccinated people, who had already developed antibodies (more below), the fraction of seropositive in the population increases to 17.3% (95% CI: 15.8% -18.8%).

Analyzing the results by the strata (Table 2), including all seropositive, whether by natural infection or vaccination, we found similar fraction of seropositivity by population density, 17.3% (95% CI: 15.0% - 19.6%) for high density (>500 people/km^2) and 17.0% (95% CI: 15.0%-19.0%) for low/medium density (≤500 people/km^2). Furthermore, in terms of age groups the seroprevalence was 15.0% (11.0%-19.6%) in people <18 years old, 20.2% (17.8%-22.4%) for the 18-54 years old, and 14.2% (95% CI: 12.1%-16.3%) for ≥55 years old, which reflects the priorities of vaccination early on in the campaign in Portugal. This

vaccination bias is clear if we compare these numbers with the seroprevalence estimated from natural infection only: 15.0% (11.0%-19.6%) in people <18 years old, 13.8% (11.7%-15.8%) for the 18-54 years old, and 10.9% (95% CI: 9.0%-12.9%) for ≥55 years old.

Evolution of SARS-CoV-2 antibody seroprevalence in the Portuguese population over the last 6 months

When we compare the results of seroprevalence obtained in this study, with its precursor 6 months before, we see that the overall prevalence increased from 2.2% to 13.1%, due to natural infection. The increase in seroprevalence was similar in the younger age group (<18 years) and intermediate age group (18-54 years) from 2.4% to 15.0% and from 2.3% to 13.8%, respectively; but smaller in the eldest group, from 1.9% to 10.9%. In terms of population density, in September we obtained a significantly higher prevalence in high population density regions, but now the relative gap narrowed, since we found, considering only natural infection, 13.5% prevalence in high-density regions and 12.5% in the other regions.

It is also interesting to compare the total number of cases estimated by these seroprevalence studies to the official number of cases reported by the Portuguese authorities. In September 2020, we found an overall prevalence of 2.2% o for antibodies against SARS-CoV-2 in the Portuguese population, corresponding to about 226 000 people, considering the 10.3 million people living in Portugal. In the current study, we found a prevalence of 13.1% antibody positive due to natural infected, corresponding to about 1 350 000 people. Assuming that it takes an average of two weeks from the time of infection for people to become seropositive (*23-25*), this seroprevalence reflects the extent of SARS-CoV-2 infection in Portugal two weeks before each study. Comparing with the cumulative confirmed cases in Portugal (58243 thousand on September 1st, 2020 and 797,525 on February 21st, 2021) (*16*), we can see that the multiplicative factor decreased from more than three to less than two, suggesting a higher testing rate.

In terms of vaccination, there were 0 people in September 2020, before any vaccine had been approved, and there were 7.1% of people that reported being vaccinated in the current study. This value compares well with the reported number of people with at least one dose of vaccination, at 6% on February 28th and 8% on March 14th (*26*)

Quantification of antibodies against SARS-CoV-2

In September 2020, in the first phase of this longitudinal study, we quantified, using our in-house ELISA assay (see Methods), the titers of antibodies in 204 people, who also participated in the March 2021 phase of the study. Now, i.e., 6 months following the first determinations, we performed the same quantification in 201 of those people (for the other 3 no sample was available). Of these, 13 had received at least one dose of a vaccine and were excluded from the analysis. Importantly all 188 (non-vaccinated) previously screened seropositive persons remained seropositive 6 months later. When comparing antibody levels, we found a reduction in IgM ($p=0.0006$) and IgG ($p=0.0095$) levels 6 months following the first assessment, but no significant difference in IgA ($p=0.15$) (Figure 2a). One individual had a substantial increase in IgG titre, suggesting a reinfection with SARS-CoV-2. We plotted the antibody titers since estimated date of infection (confirmed by PCR or suspected due to symptoms) (Figure 2b). Twenty-two subjects were removed due to an unknown date of infection. Overall, the data demonstrate the longevity of antibodies, up to 12 months after initial infection (Figure 2b). We calculated the half-life of antibody decay using a linear mixed-effect model, and found that $t_{½} \sim 29$ months for IgM, $t_{½} \sim 28$ months for IgG, and no decay for IgA. We then separated the participants into two groups, according to how long after infection their serostatus had been analyzed in September: i) less than 4 months or ii) more than 4 months. Participants in the former group show a reduction in IgM and IgG levels but stable IgA levels six months later (Figure 2c). Those participants for whom the first antibodies were determined 5-7 months after infection show more stable levels for IgM and IgG isotypes six months later (Figure 2d), although IgA showed a slight but significant decline. Collectively, this indicates that after the initial contraction phase, there is some waning of IgM and IgG antibodies levels, which subsequently stabilizes resulting in detectable levels anti-SARS-CoV-2 Spike antibodies for the first year after natural infection (Figure 2).

We also quantified the levels of antibodies for 194 newly infected and non-vaccinated people, of whom 178 knew an approximate date of infection. These more recently infected participants had levels of IgG and IgA antibodies higher than the titers detected in the participants who were seropositive in the first phase of the study in September 2020 (Figure 3a). The mean time since infection until assessment in the study in September was 151 days, whereas for the newly positive people in March, this median time was 76 days. This is consistent with the September 2020 phase of the study occurring several months after the first wave, in March-April 2020; and the March 2021 screening occurring shortly after the severe

third wave in January 2021. Categorizing the antibody levels according to month since infection indicated similar antibody levels raised after the first or second and third waves (Figure 3b).

We also analyzed the level of antibody in non-vaccinated participants by gender at birth, age, BMI, and smoking status controlling for time since infection when known, using general linear models. We found no differences by gender or BMI. However, when controlling for time since infection, older people tended to have higher levels of antibodies (IgM, IgG and IgA, $p=10^{-5}$, p=0.006 and p=0.022, respectively) than younger people, and non-smokers also had higher levels of antibodies (IgG and IgA, p=0.0078, p=0.012, respectively) than smokers, even when we controlled for age group and gender (p=0.021, p=0.013, for IgG and IgA, respectively).

SARS-CoV-2 vaccination in Portugal started December 27th, 2020. We quantified antibody titers in 161 people, who reported vaccination between days 1 and 73 prior to providing a blood sample. Of these 13 were in seropositive participants from the first phase. Vaccines are intramuscular and in accordance, we observe little RBD-specific IgM, robust induction of IgG after the first 2 weeks and modest IgA (Figure 4a). Furthermore, within the group of confirmed seropositive after natural infection with SARS-CoV-2, as expected vaccination did not significantly increase RBD-specific IgM levels, but significantly boosted IgG and IgA levels (Figure 4b).

Comparing antibody levels generated by natural infection or the first dose of the vaccine (Figure 4c), in comparable time bins since infection or vaccination, showed high variability in the induced levels of antibodies, especially after natural infection; and that natural infection elicits stronger IgM, IgA and IgG responses early on, but, at least for IgG, vaccine induced-levels are higher from 3 weeks after dosing. These data underscore the potential of vaccines to maximize anti-RBD IgG responses.

DISCUSSION

We present the findings of a population-based longitudinal study, conducted with a stratified quota sample of the Portuguese population, covering two distinct periods during the SARS-CoV-2 pandemic. Our first study was in September 2020 after a modest first SARS-CoV-2 wave in March to May 2020 and showed a low seroprevalence of 2.2%, approximately 226,000 people (*15*). The current follow-up study followed the second and third wave, the latter of which was very severe, placing Portugal at the top of

number of cases and fatalities (per capita) in the world during January 2021. Furthermore, the Portuguese national vaccination program started on December 27th 2020, albeit at a small scale due to limited supply in the beginning of 2021 and with priority given to healthcare staff and elderly care home residents (this latter group is not included in our study). Reflecting the high SARS-CoV-2 incidence in this period and the initial vaccination effort, until March 2021 we found a seroprevalence of 13.1% due to natural infection, corresponding to about 1,350,000 people. Considering that the number of official PCR-confirmed cases reported at the beginning of September 2020 and in late February 2021 were ~58 thousand and ~797 thousand, respectively (*16*). The ratio between serological test and reported PCR positive cases reduced from approximately 3.9 to 1.7, reflecting the increased capacity for testing since the first wave hit Portugal (*27*). Furthermore, allowing for a typical delay of three weeks from infection to death, the 16684 deaths officially registered on March 14th 2021, suggest an overall infection fatality rate of 1.2%, at the top end of those reported in total populations using serology data (0.5%–1.2%) (*15, 28-30*), and increased from the 0.85% found in September 2020, reflecting the dire situation in Portugal during January 2021. When we also consider vaccinated people, the seroprevalence increased to 17.3% nationwide, approximately 1,782,000 people. This value is consistent with another (unpublished) study of seroprevalence in Portugal conducted between early February and late March, which found 15.5% seropositive people, including vaccinated (*31*).

We previously reported antibody kinetics during and shortly after acute SARS-CoV-2 infection (*1*). These kinetics shows a characteristic rapid increase peaking at 3-4 weeks post infection followed by a subsequent contraction phase, but with continued presence of SARS-CoV-2-specific antibodies at least until months 6-7 post infection, and with rapid increases of the three isotypes assessed, similar as reported for the SARS and MERS responses (*1, 32, 33*). An important question in SARS-CoV-2 immunology and also in the interpretation of serology studies, is how long seropositivity lasts. We made use of our established sensitive lab-based ELISA, to quantify the antibody levels in 188 people, who were seropositive in the first phase of our longitudinal study in September 2020, and who were re-assessed in March 2021. For many of these people, we know the date of diagnosis by PCR or the suspected date of infection by symptoms and epidemiological contact. We found an initial antibody waning for the isotypes IgM and IgG within the first four months after natural infection, with significantly reduced levels of SARS-CoV-2 RBD-specific IgM and IgG levels in longitudinal testing the same people. Of interest, IgA levels remained stable. After this initial decay, there is a plateau phase with those individuals initially sampled five-seven months after infection showing stable levels of anti-SARS-CoV-2 RBD IgM, IgG and IgA over time, with a trend indicating continued, but modest, antibody waning, maintaining detectable

antibody levels up to 13 months after the initial infection in all sera tested. Indeed, it is remarkable that in all 188 people for whom we quantified titers in September 2020, and who represent a cross-sectional sample of the Portuguese population, we could still detect antibodies (IgA, IgM, IgG) in March 2021. For these participants, we estimated very slow decays in antibody levels, with half-lives of >2 years, which is substantially longer than previously reported (*8*). This is likely explained by our finding that there seems to be two phases of decay, an initial contraction in antibody levels followed by a more stable plateau. The patients in the report by Dan et al. (*8*) were, in general, assayed earlier after infection than our participants. In fact, if we calculate the half-life of patients identified earlier than 4 months post-infection, it is much shorter at ~300 days for both IgM and IgG, closer to those reported previously.

Serological studies rely on maintenance of antibody levels to identify those who are immune and to quantify the extent of infection in populations. The presence of detectable anti-SARS-CoV-2 antibodies more than a year after the infection in a longitudinal study as we present here, suggests good quality immunity likely able to reduce illness severity upon reinfection and reduce future transmission in the population. In addition, it indicates the feasibility to retrospectively interrogate SARS-CoV-2 infection rates with help of serological studies at least a year into the pandemic.

Human studies have correlated the presence of antibodies prior to a human coronavirus (HCoV) challenge with protection from infection or symptoms (*34, 35*). SARS-CoV-2 immunity has been compared with HCoV-E229 infections after which antibodies may last for a period of up to two years, but are reported to be able to wane more rapidly within the first year (*36, 37*). Endemic HCoV, primarily HCoV-229E, HCoV-HKU1, HCoV-OC43, and HCoV-NL63, as opposed to SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) do not show high levels of pathogenicity. Tissue damage is an important criterion that determines the threat level to invoke the strength of immune response. Although early HCoV-229E antibody kinetics are similar, SARS anti-Spike antibodies have been detected for up to five years post infection, with some loss of circulating antibodies reported after 16 months (*38-40*). Anti-MERS antibodies are present at least up to one year after infection (*41*). Our results are in line with SARS and MERS serological studies, with SARS-CoV-2 invoking a similar immune response. Importantly, antibodies are only one characteristic of immune protection against disease. T cell responses against SARS have been reported 12 years post infection (*40*). SARS-CoV and MERS-CoV were contained, but with its global spread SARS-CoV-2 is likely to become an endemic virus able to re-infect when neutralising antibody levels are sufficiently low, but possibly without causing severe disease due to immunological memory (*42*).

We also analyzed the effects of vaccination on antibody levels. For the most part, we confirmed expected results, with increasing levels of IgG and IgA detected after the first two- or three-weeks post-vaccination, and a significant boost in antibody levels for those isotypes in people who had been infected before. It is important to note that even after just the first dose of vaccine (in Portugal at this time only the vaccines by AstraZeneca and Pfizer/Biontech were approved) the levels of IgG generated are substantial and after 3 weeks tend to be even higher than in natural infection, at a similar time. These results are important to inform models for most efficient vaccination strategies in the population (*43*).

Our study has some limitations. For logistic, cost and rapidity of the study, we opted in our first-phase study to use a stratified quota sample, which does not guarantee perfect proportionality to the population for demographic and epidemiological variables. However, we believe that true random samples, with more sophisticated sampling schemes, such as random household surveys, also suffer from these potential issues, due to large fraction of non-participation, typically 30%-50% (*11, 28, 44*) and the potential extra accuracy does not have impact in public health decisions. In the current phase, we sampled from the original participants, matching several characteristics to the Portuguese population for better representation. However, the 28% non-participation rate (2173 of 3000 invitations), in line with the numbers of other studies, may introduce some bias in the sample, which are only partly ameliorated by our stratification strategy. Another issue is that we measured antibody titres, but not neutralization levels, which is arguably a better surrogate of immune protection (*6, 10*). However, as a measure of past infection, how long antibodies last, and how to interpret serological studies, the levels of IgM, IgG and IgA as assayed here are the gold-standard. Moreover, antibody levels measured by ELISA and neutralization titers are typically well-correlated (*3*). It is also possible that some of the vaccinated people were infected before receiving the vaccine, and our assays does not distinguish between natural infection and vaccination. Thus, our estimate of natural infection could be slightly higher, however, this is mitigated by current guidelines in Portugal, which delay vaccination to previously infected people until a later phase of the campaign. Although, we did find cases of previously infected people, who were already vaccinated.

In spite of these limitations, our study is one of the few assessing seropositivity longitudinally in a large population-wide National sample, allowing calculation of antibody waning. Most other recent National-level studies with multiple serological assessment campaigns use a cross-sectional, rather than a longitudinal design (*31, 45-47*). One recent study with a longitudinal design was conducted in the Faroe

Islands (*48*), with results comparable to what we present here, especially in terms of two phases of decay and long duration of antibody positivity.

Overall, this study showed that even after the severe wave of infection during January of 2021, the overall seropositivity levels of the population in Portugal was only ~13%, reaching 17% when including the estimated vaccinated people. Two months onward from our study, in May 2021, the fraction of people with at least one dose of the vaccine was approaching 50%. We found that antibody levels are measurable in the vast majority of people with times since infection spanning 3 to 12 months. Indeed, all 188 people, who were positive in September 2020, still had detectable antibodies in March 2021. This indicates that antibody immunity against SARS-CoV-2 likely lasts more than one year.

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FIGURE LEGENDS

Figure 1. Comparison of the evolution of the COVID-19 pandemic in Portugal and other European countries. a) Total number of cases per million, b) total number of deaths per million, c) number of new cases per million and d) number of new deaths per million. Depicted are the evolution of the pandemic in neighboring countries (Spain, France, Italy) and other countries of similar size in number of inhabitants (Belgium, Czechia, Netherlands and Sweden). The large third wave when Portugal had very large number of cases and deaths in January is clearly seen.

Figure 2. Evolution of antibody levels between the September 2020 and the March 2021 phases of the longitudinal study. a) Comparison of IgM, IgG and IgA (from left to right in all rows) in the same people assayed in the two periods (n=188). b) Same as a) but considering the time since infection, which is the time of PCR positive test or symptoms, as reported by the participants (n=166). c) Same as b) but showing only participants who were infected less than 4 months before the September 2020 serology study (n=49), to compare with d) same as b) but showing only participants who were infected more than 4 months before the September 2020 serology study (n=117). P-values for two-sided Wilcoxon matched pairs test.

Figure 3. Comparison of antibody levels for people seropositive for the first time in September 2020 or March 2021 phases of the study. a) Comparison of the levels of antibodies (IgM, IgG and IgA) between participants who were positive in September 2020 (n=188) and those who were positive in March 2021 (n=194. b) Same as a), but binning the participants by time since infection, with participants positive in September 2020 in blue (n=166) and those positive in March 2021 in white (n=178). P-values for two-sided Mann-Whitney tests.

Figure 4. Antibody levels for participants with the first dose of SARS-CoV-2 vaccine. a) Levels of IgM, IgG and IgA (from left to right) for vaccinated participants (n=148) binned by time since the first vaccine dose. b) Boosting of antibody levels for the few participants who had been infected before receiving the vaccine (n=13). c) Comparison of antibody titres after natural infection (green, n=287, includes data from (*1*)) or first dose of vaccine (blue, n=148) by time in days since that event. P-values in a) are for Kruskal-Wallis test with Tukey correction for multiple comparisons; in b) are for two-sided Wilcoxon matched pairs; and in c) are for two-sided Mann-Whitney tests.

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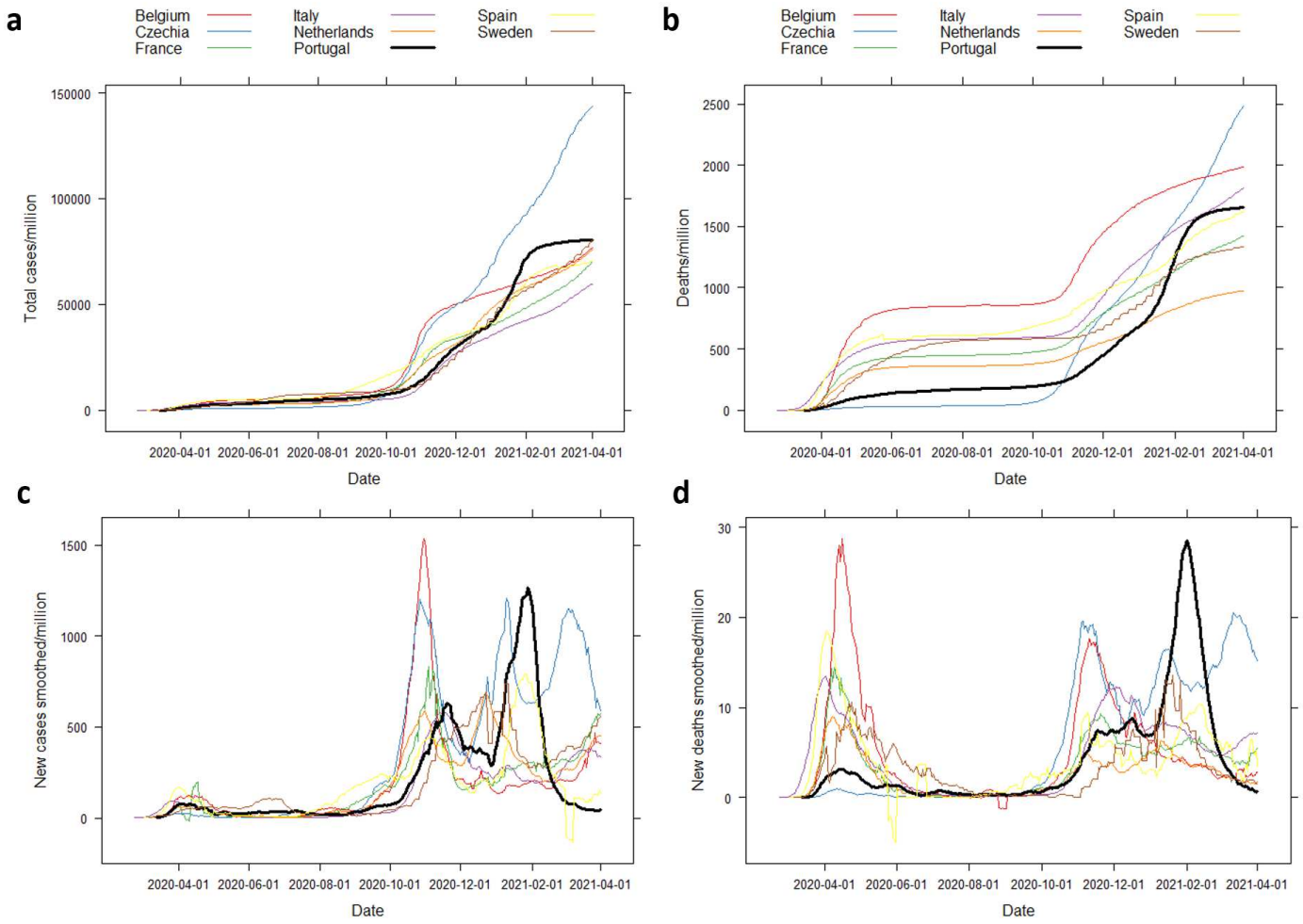
Table 1. Socio-demographic characteristics of the study sample.

	Cohort "NC"		Cohort "PC"	
	n	%	n	%
Sex				
Male	948	43.6%	126	47.9%
Female	1224	56.4%	137	52.1%
Age categories				
<18 years	224	10.3%	40	15.2%
18-54 years	1006	46.3%	132	50.2%
≥55 years	942	43.4%	91	34.6%
Population density				
Low or Medium	1162	53.5%	100	38.0%
High	1010	46.5%	163	62.0%
Household size				
1 person	390	18.0%	24	9.1%
2 to 4 people	1649	75.9%	216	82.1%
≥5 people	133	6.1%	23	8.7%
Education				
Less than high school	619	29.4%	73	27.8%
High school, post high school (no undergraduate degree)	749	35.5%	73	27.8%
Undergraduate or graduate degree	704	33.4%	108	41.1%
Other	37	1.8%	9	3.4%
Occupation				
Employed	1272	58.6%	156	59.3%
Unemployed	115	5.3%	13	4.9%
Student	263	12.1%	47	17.9%
Retired	438	20.2%	36	13.7%
Other, Disability, Homemaker	84	3.9%	11	4.1%
Professional sector				
Commerce, Industry and Building	229	18.0%	28	10.6%
Administration / services	338	26.6%	35	13.3%
Education	190	14.9%	21	8.0%
Health	121	9.5%	22	8.4%
Health (no clinic)	42	3.3%	7	2.7%
Transportation	43	3.4%	3	1.1%
Other	309	24.3%	40	15.2%
Current working arrangement (employed workers)				
Teleworking	345	13.2%	43	27.7%
Physically at work, only in contact with colleagues	386	14.8%	54	34.8%
Physically at work, no contact	72	2.8%	11	7.1%
Physically at work, contact with public	407	15.6%	43	27.7%
Mixed arrangements	62	2.3%	4	2.6%
Body Mass Index				
Normal or underweight	905	45.9%	95	42.0%
Overweight	713	36.2%	90	39.8%
Obese	353	17.9%	41	18.1%
Smoking status				
Non-smoker	1366	64.8%	198	75.3%
Ex-smoker	415	19.7%	44	16.7%
Smoker	328	15.6%	21	8.0%
Physical Exercise				
No	1141	52.5%	138	52.5%
Yes	1031	47.5%	125	47.5%
COVID-19 Vaccine				
No	2016	92.8%	246	93.5%
Yes	156	7.2%	17	6.5%

Table 2. SARS-CoV-2 antibody seroprevalence estimates in Portugal, March 2021. Estimates of seroprevalence for the population of Portugal, and by region of population density and age group. The n indicated in the table corresponds to the sample assessed, and is for information purposes.

		Total			Non-vaccinated only		
		n	Seroprevalence	95% CI	n	Seroprevalence	95% CI
Overall		2435	17.3%	15.8 -18.8	2262	13.1%	11.8-14.6
Population density	High (>500/km^2)	1173	17.3%	15.0-19.6	1104	13.5%	11.5-15.7
	Low/Medium (<500/km^2)	1262	17.0%	15.0-19.0	1158	12.5%	10.7-14.4
Age group	< 18 years	264	15.0%	11.0-19.6	264	15.0%	11.0-19.6
	18-54 years	1138	20.2%	17.8-22.4	1047	13.8%	11.7-15.8
	≥55 years	1033	14.2%	12.1-16.3	951	10.9%	9.0-12.9

Figure 1. Comparison of the evolution of the COVID-19 pandemic in Portugal and other European countries. a) Total number of cases per million, b) total number of deaths per million, c) number of new cases per million and d) number of new deaths per million. Depicted are the evolution of the pandemic in neighboring countries (Spain, France, Italy) and other countries of similar size in number of inhabitants (Belgium, Czechia, Netherlands and Sweden). The large third wave when Portugal had very large number of cases and deaths in January is clearly seen.



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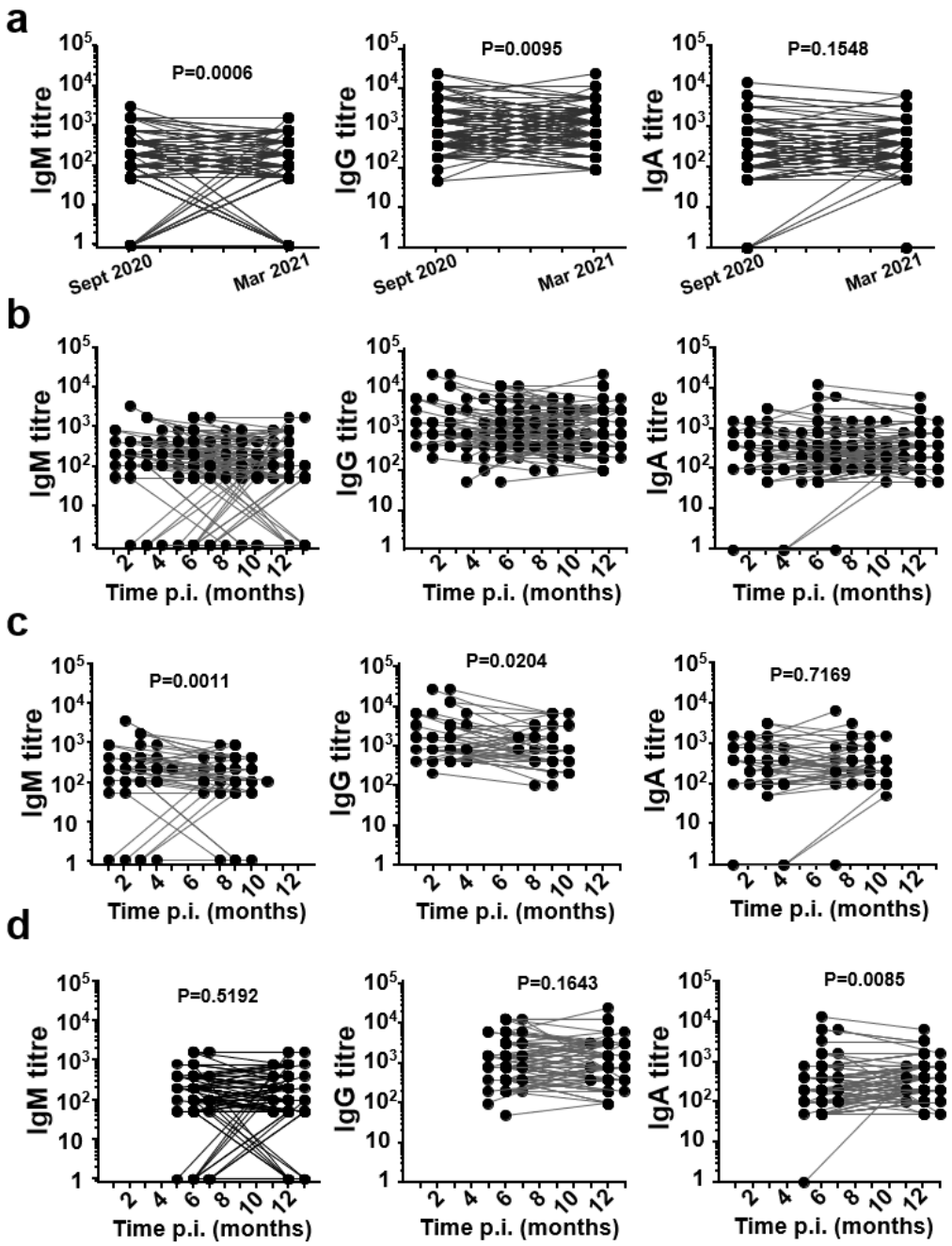

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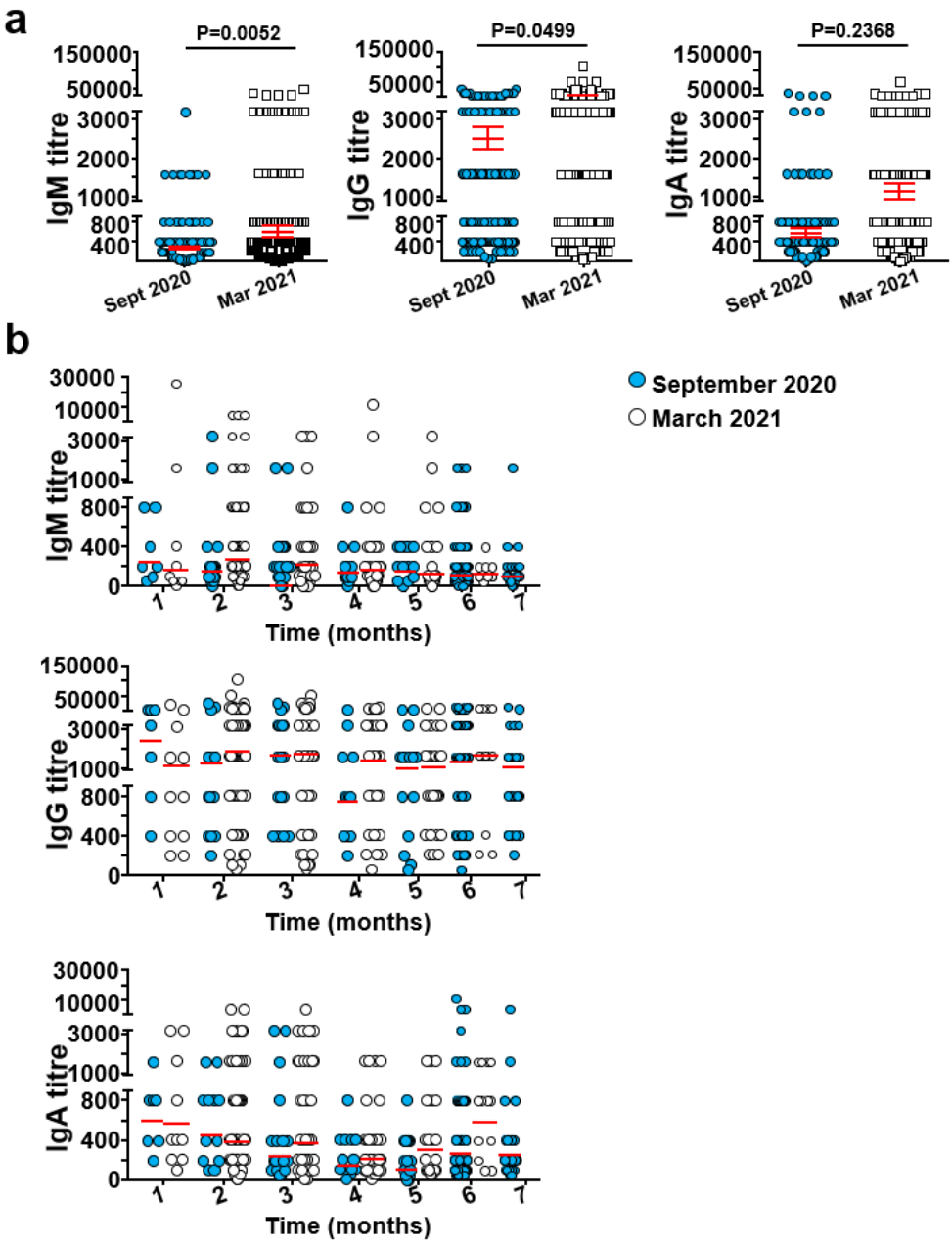

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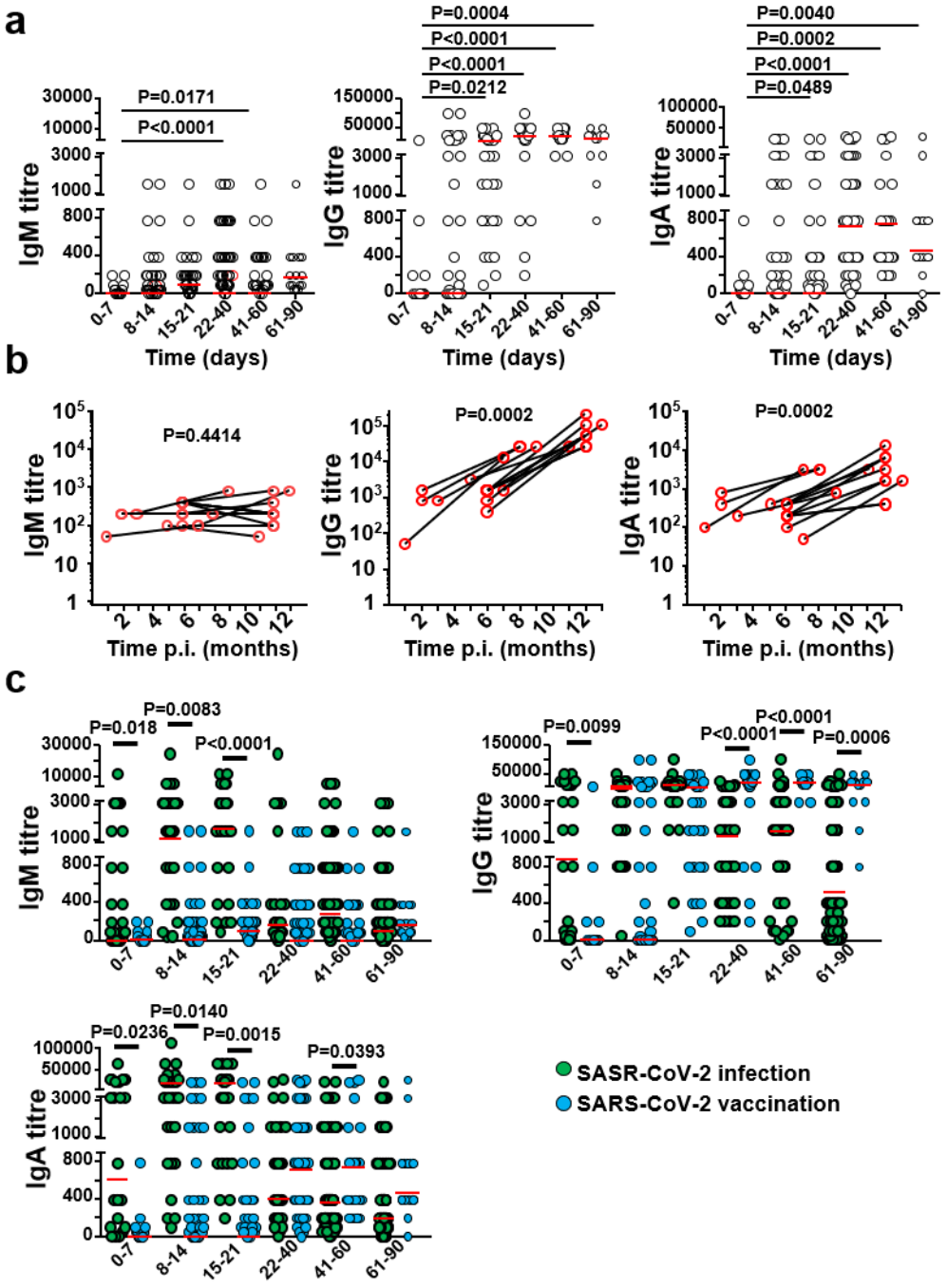

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