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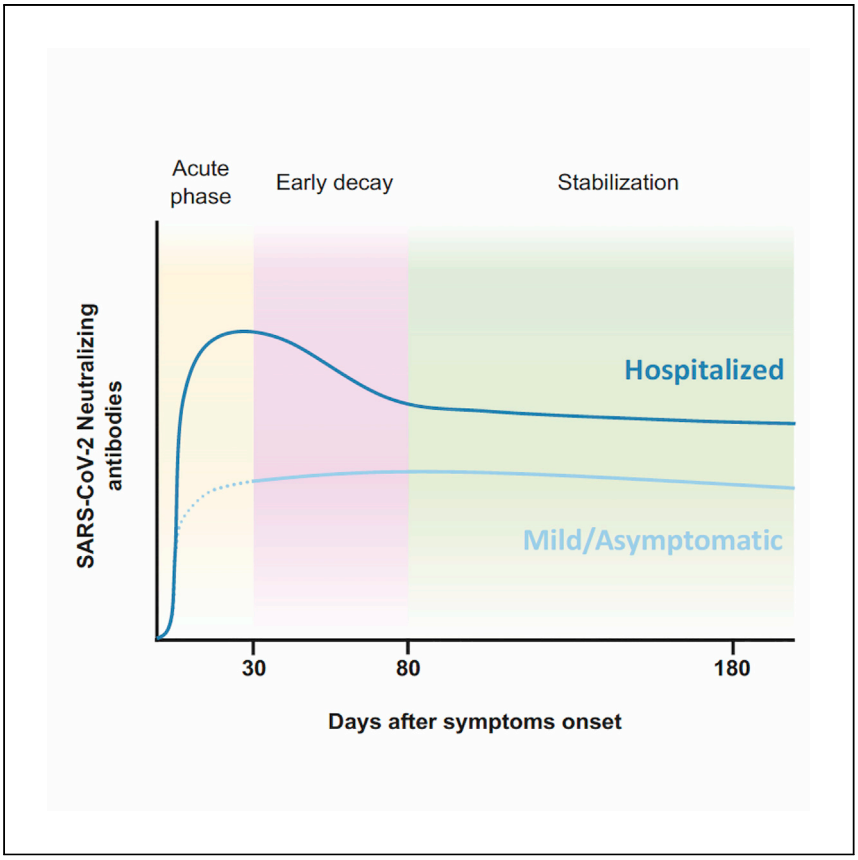
Clinical and Translational Report

Stable neutralizing antibody levels 6 months after mild and severe COVID-19 episodes



Pradenas et al. describe the kinetics of neutralizing antibodies against SARS-CoV-2 and demonstrate their association with clinical severity and their stability for at least 6 months, despite constant decay of IgG titers. These findings help us to understand the mid-term immune response and the impact on herd immunity.

Edwards Pradenas, Benjamin Trinité, Víctor Urrea, ..., Jorge Carrillo, Bonaventura Clotet, Julià Blanco

jblanco@irsicaixa.es

HIGHLIGHTS

Neutralizing activity against SARS-CoV-2 is maintained for at least 6 months

Anti-RBD and anti-S2 IgG titers show a constant decay

Maintenance of neutralizing activity suggests a potential evolution of the immunity

Hospitalized patients maintain higher neutralizing capacity than non-hospitalized

Translation to Patients

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Clinical and Translational Report

Stable neutralizing antibody levels 6 months after mild and severe COVID-19 episodes

Edwards Pradenas,[1,10] Benjamin Trinité,[1,10] Víctor Urrea,[1] Silvia Marfil,[1] Carlos Ávila-Nieto,[1] María Luisa Rodríguez de la Concepción,[1] Ferran Tarrés-Freixas,[1] Silvia Pérez-Yanes,[1,2] Carla Rovirosa,[1] Erola Ainsua-Enrich,[1] Jordi Rodon,[3] Júlia Vergara-Alert,[3] Joaquim Segalés,[4,5] Victor Guallar,[6,7] Alfonso Valencia,[6,7] Nuria Izquierdo-Useros,[1] Roger Paredes,[1,8] Lourdes Mateu,[8] Anna Chamorro,[8] Marta Massanella,[1] Jorge Carrillo,[1] Bonaventura Clotet,[1,8,9] and Julià Blanco[1,9,11,*]

SUMMARY

Background: Understanding mid-term kinetics of immunity to SARS-CoV-2 is the cornerstone for public health control of the pandemic and vaccine development. However, current evidence is rather based on limited measurements, losing sight of the temporal pattern of these changes.

Methods: We conducted a longitudinal analysis on a prospective cohort of COVID-19 patients followed up for >6 months. Neutralizing activity was evaluated using HIV reporter pseudoviruses expressing SARS-CoV-2 S protein. IgG antibody titer was evaluated by ELISA against the S2 subunit, the receptor binding domain (RBD), and the nucleoprotein (NP). Statistical analyses were carried out using mixed-effects models.

Findings: We found that individuals with mild or asymptomatic infection experienced an insignificant decay in neutralizing activity, which persisted 6 months after symptom onset or diagnosis. Hospitalized individuals showed higher neutralizing titers, which decreased following a 2-phase pattern, with an initial rapid decline that significantly slowed after day 80. Despite this initial decay, neutralizing activity at 6 months remained higher among hospitalized individuals compared to mild symptomatic. The slow decline in neutralizing activity at mid-term contrasted with the steep slope of anti-RBD, S2, or NP antibody titers, all of them showing a constant decline over the follow-up period.

Conclusions: Our results reinforce the hypothesis that the quality of the neutralizing immune response against SARS-CoV-2 evolves over the post-convalescent stage.

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Context and significance

Assessing the durability of neutralizing responses against SARS-CoV-2 is crucial to predict the level of protection in post-convalescent COVID-19 patients. We monitored for >6 months a cohort of 210 SARS-CoV-2-infected individuals with a wide range of symptoms (from asymptomatic infection to severe disease). Our results indicate that neutralizing antibodies are stable for at least 6 months after infection. However, individuals with mild or asymptomatic infection developed lower titers of neutralizing antibodies and could be at higher risk of reinfection. Despite the maintenance of neutralizing antibodies, total antibody titers slowly but gradually declined over time without apparent stabilization. This observation requires further analysis to evaluate the potential role of viral persistence or viral re-exposure in maintaining neutralization titers.

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INTRODUCTION

While the early humoral response after severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) infection has been thoroughly described,[1–5] current data on the decay of antibody levels beyond the convalescent stage depict a heterogeneous scenario with limited information on the neutralizing activity throughout the follow-up period.[6–8] Various authors have recently suggested more complex kinetics of neutralizing activity decay as compared to total antibody titers, with clonotype-, epitope-, or subject-specific patterns that evolve in terms of potency and resistance to epitope mutations.[9–11] In this study, we longitudinally evaluated the neutralizing humoral response, in mild/asymptomatic and hospitalized individuals infected by SARS-CoV-2, over a 6-month period. These mid-term kinetics showed stable behavior of the neutralizing response in both groups, despite a clear decrease in the total viral-specific humoral response.

RESULTS

Patient selection and early neutralizing responses

Our analysis included 210 patients with RT-PCR-confirmed SARS-CoV-2 infection, recruited during the first and second waves of the coronavirus disease 2019 (COVID-19) epidemic in Catalonia (northeast Spain). Of these, 106 (50.5%) had a mild or an asymptomatic infection, and 104 (49.5%) required hospitalization because of respiratory compromise (Table 1). As reported in our country,[12] the hospitalization group showed significantly older age and lower frequency of females (Table 1). We collected samples periodically throughout a maximum follow-up period of 242 days (mean follow-up time point of patients from the first COVID-19 wave was 201 days; Figure S1). Most of the study participants developed a neutralizing humoral response against SARS-CoV-2 HIV-based pseudoviruses that was confirmed using infectious viruses.[13] However, in line with trends reported elsewhere,[6,8] mildly affected or asymptomatic individuals developed a 10-fold lower maximal neutralization titer than those who required hospitalization when the full dataset was analyzed ($p < 0.0001$, Mann-Whitney test; Figure 1A). The higher number of determinations obtained from hospitalized individuals during the acute phase permitted the clear observation of a sharp initial response (Figures 1B and 1C), also reported in previous analyses of the early response.[1–5] This was visible for individuals recruited during both the first (March–June 2020) and the second (July–October 2020) waves of the COVID-19 pandemic in Catalonia. A longitudinal analysis fitted to a 4-parameter logistic model of increase defined a 30-day sharpening phase after symptom onset, irrespective of the wave in which hospital admission occurred. Half-maximal neutralization activity was achieved on day 10 (95% confidence interval [CI] 8–11); 80% maximal response, which corresponded to 3.97 logs (i.e., 9,333 reciprocal dilution), was achieved on day 14 (Figure 1D). Moreover, as reported previously using an infectious virus neutralization assay,[13] we could not find a gender impact on the elicitation of neutralizing antibodies in hospitalized individuals. Based on these findings, irrespective of gender and wave, we decided to set day 30 after symptom onset as a starting point for the longitudinal analysis of immune response at the mid-term.

Assessment of mid-term neutralizing responses

The longitudinal modeling of the neutralizing activity at mid-term in our cohort revealed a nearly flat slope (i.e., not significantly different from 0, with a half-life of

[1]IrsiCaixa AIDS Research Institute, Germans Trias i Pujol Research Institute (IGTP), Can Ruti Campus, UAB, 08916 Badalona, Catalonia, Spain

[2]Laboratorio de Inmunología Celular y Viral, Unidad de Farmacología, Sección de Medicina, Facultad de Ciencias de la Salud, Universidad de La Laguna (ULL), La Laguna 38071, Tenerife, Spain

[3]IRTA Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), Campus de la UAB, 08193 Bellaterra, Catalonia, Spain

[4]UAB, Centre de Recerca en Sanitat Animal (IRTA-UAB), Campus de la UAB, 08193 Bellaterra, Catalonia, Spain

[5]Departament de Sanitat i Anatomia Animals, Facultat de Veterinària, UAB, 08193 Bellaterra, Catalonia, Spain

[6]Barcelona Supercomputing Center, 08034 Barcelona, Catalonia, Spain

[7]Catalan Institution for Research and Advanced Studies (ICREA), 08010 Barcelona, Catalonia, Spain

[8]Infectious Diseases Department, Fight against AIDS Foundation (FLS), Germans Trias i Pujol Hospital, 08916 Badalona, Catalonia, Spain

[9]University of Vic–Central University of Catalonia (UVic-UCC), 08500 Vic, Catalonia, Spain

[10]These authors contributed equally

[11]Lead contact

*Correspondence: jblanco@irsicaixa.es

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Table 1. Characteristics of individuals included in analysis

	Mild/asymptomatic (n = 106)	Hospitalized (n = 104)	p
Gender, female, n (%)	72 (68)	46 (44)	0.0006[a]
Age, y, median (IQR)	46.5 (38–54)	57.5 (46–66)	<0.0001[b]
Individuals with ≥2 samples, n (%)	52 (49)	59 (57)	0.278[a]
Wave of COVID-19 outbreak (first), n (%)	96 (91)	73 (70)	–
Severity, n (%)			
Asymptomatic	8 (8)	–	–
Mild	98 (92)	–	–
Hospitalized non-severe	–	59 (56.7)	–
Hospitalized severe	–	37 (35.6)	–
Hospitalized (intensive care unit)	–	8 (7.7)	–

IQR, interquartile range (25th and 75th percentiles).
[a]Chi-square test.
[b]Mann-Whitney test.

2,134 days) in individuals with asymptomatic infection or mild disease (Figure 2A). Conversely, the decrease in neutralizing activity in hospitalized individuals showed a 2-phase pattern, with a rapid decay (half-life 31 days) until day 80, which slowed down to a flat slope (half-life 753 days) from that time point on (Figure 2B). In agreement with previous data, suggesting a faster decay of neutralizing antibodies in male compared to female infected individuals,[9,14] we found significant gender differences in early decay; however, upon stabilization of neutralization titers after day 80, no gender impact was observed in our cohort (Figure S2).

The characterization of the neutralizing activity behavior at mid-term should ultimately project the proportion of post-convalescent individuals protected against new infections in the mid- and long-terms. The limited number of measures and lack of a clear threshold of neutralizing activity for preventing SARS-CoV-2 infection precluded assessing this outcome using survival analysis. Alternatively, we explored the neutralizing activity at the end of our 6-month follow-up period. Based on the mixed-effects model obtained from the longitudinal analysis, we estimated a stable mid-term neutralizing activity of 2.72 and 3.16 log for the mild/asymptomatic and hospitalized subgroups, respectively ($p < 0.0001$; likelihood ratio test; Figure 2C, dotted lines). This estimate was consistent with the observed values for the last measurement taken between days 135 and 242, a time frame centered on day 180 (Figure 2C, boxplots). Likewise, the value distribution at this time frame showed significant differences between mild/asymptomatic (median 2.5; interquartile range [IQR] 2.0–3.0) and hospitalized (3.0; 2.7–3.3) individuals ($p = 0.0012$, Mann-Whitney test). To date, no clear cutoff for a neutralizing activity that protects against new reinfection has been established. Nevertheless, data gathered from high attack rate events suggest that neutralizing activities between 1:161 and 1:3,082 are strong enough to prevent infection.[15] Hence, we assumed that reinfections would be unlikely among individuals above the 1:250 cutoff. Of the 23 hospitalized individuals with measurement beyond day 135, 21 (91%) had a mean neutralizing activity value above 1:250 and were thus considered long-term neutralizers. The corresponding proportion in the mild/asymptomatic group (42%; 19/45) was significantly lower ($p = 0.0052$, chi-square test; Figure 2D). Although this number must be considered cautiously due to the cutoff assumption, our finding suggests that hospitalized patients have a higher capacity for long-term neutralization, despite the faster initial decay in neutralization activity.

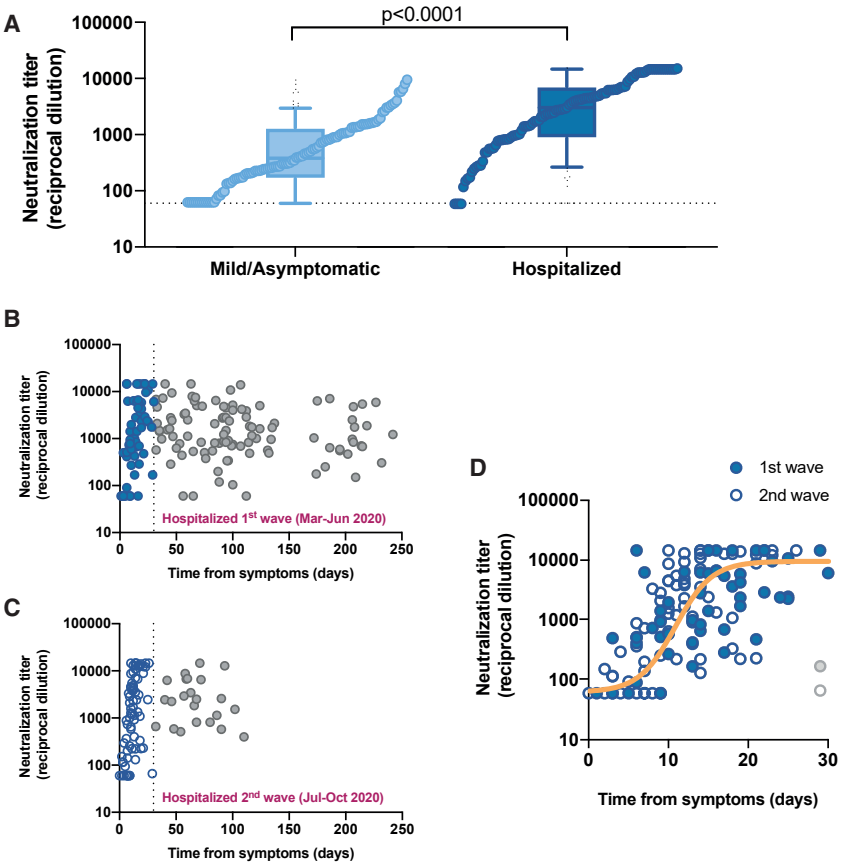

Figure 1. Neutralizing activity among study participants

(A) Maximal neutralization titer of 210 individuals recruited according to disease severity (light and dark blue for mild/asymptomatic and hospitalized individuals, respectively). Boxes show the median and the interquartile range and bars the 10th and 90th percentiles. Distributions were compared using the Mann-Whitney test. Individual values are ranked for comparative purposes.

(B and C) Longitudinal dot plot of neutralizing activity among hospitalized individuals admitted during the first (B) and second (C) waves of the COVID-19 epidemic in our area; filled (B) and empty (C) blue dots show the early (i.e., 30 days after diagnosis) increasing phase.

(D) Magnification of the early phase for individuals admitted during the first (filled symbols) and second (empty symbols) waves. No differences between waves were observed. The solid orange line shows the non-linear fit (mixed-model estimate) for the whole dataset (125 samples, 55 individuals analyzed). Two samples from late seroconverters (1 from each wave, gray dots) were excluded from the analysis.

Comparative analysis of neutralizing responses and immunoglobulin G (IgG) titers

It has recently been proposed that the kinetics of neutralizing activity may not mirror those of antibody titers.[11] Hence, we investigated the change in IgG titers in a subset of 28 individuals (14 in each severity group) with the most extended follow-up period. The analysis included antibodies against the S protein receptor-binding domain (RBD), the main target of SARS-CoV-2-specific neutralizing antibodies[16]; the S2 subunit of the S protein, which may also contribute to neutralizing activity and is more cross-reactive with other coronaviruses[17]; and the nucleoprotein (NP), which is very abundant, albeit unable to neutralize the SARS-CoV-2.16 The longitudinal analysis revealed a 1-phase significant ($p < 0.0001$) steady decay pattern of all tested antibodies, which was notably faster in anti-NP IgG (Figures 3A–3C). The half-lives of anti-RBD, anti-S2, and anti-NP antibodies for the period beyond day 30 were

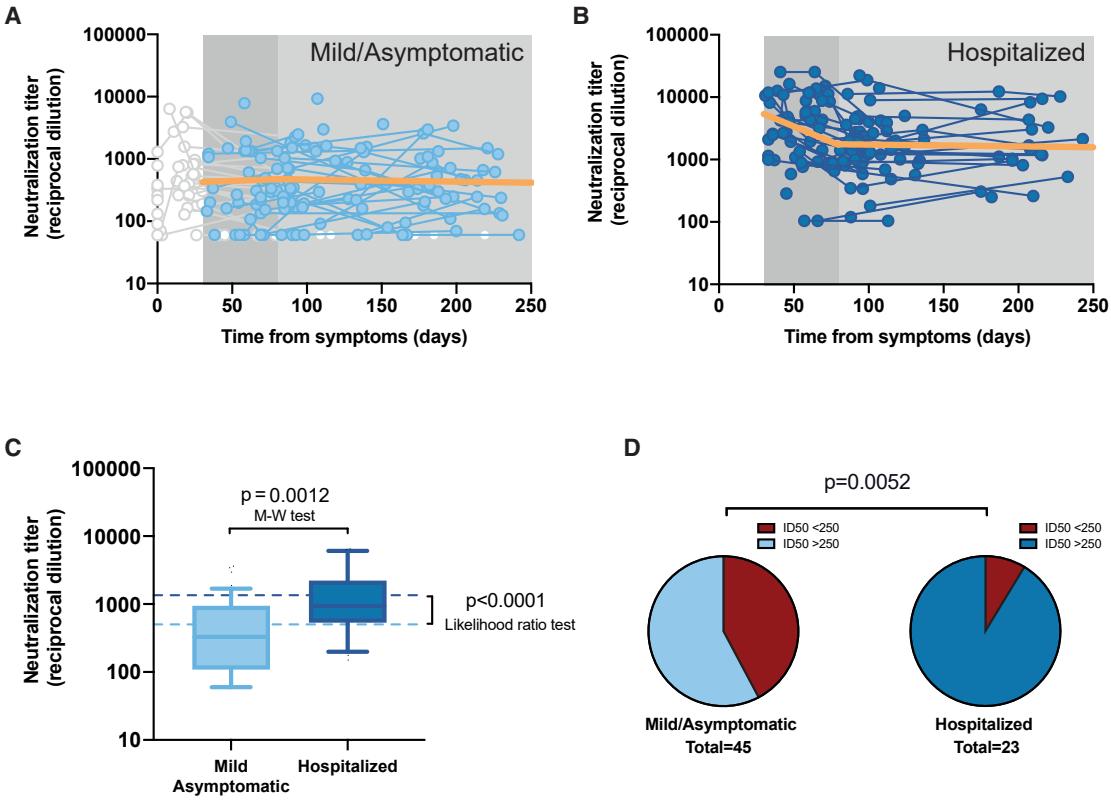

Figure 2. Longitudinal analysis of neutralizing activity

(A) Individual measurements (dots) and linear mixed model (solid orange line) of the longitudinal analysis for mild or asymptomatic individuals beyond day 30 (single-phase slope −0.00014; p = 0.75, likelihood ratio test; estimated half-life 2,134 days). Time points preceding day 30 as well as participants only showing undetectable titers were excluded from the analysis; values are shown but grayed out.

(B) The corresponding analysis for hospitalized individuals (the slopes of the linear fit for the first and second phase were −0.0096 [p = 0.0002] [half-life 31 days] and −00004 [half-life 753 days] [p = 0.78], respectively).

(C) Distribution of neutralizing activity 6 months after infection in both disease severity groups. Experimental values of mean neutralizing activities in the period 135–242 days as summarized in boxplots (as in Figure 1A; Mann-Whitney test for comparative analysis) and modeled data as dotted lines (likelihood ratio test for comparative analysis).

(D) Frequency of long-term neutralizers (i.e., individuals with mean neutralizing activity >250 in the 135–242 days period) in each severity subgroup (chi-square test p value is shown).

86, 108, and 59 days, respectively. These values were consistent with those reported by Wheatley et al.,[11] estimated on a 160-day time frame. Although the limited sample size of this sub-analysis precluded independent modeling of the decay in mild/asymptomatic and hospitalized patients, the latter showed significantly higher titers of anti-S2 at the end of the follow-up period (Figure S3), whereas no significant differences were found in other antibodies regarding disease status. Interestingly, in this subset of individuals, the decay in antibody titers contrasted with the behavior of neutralizing activity, which fitted to a 2-phase model—as in the whole dataset—with a rapid decay until day 80 (slope 0.014, half-life 22 days) and a flat slope (i.e., not significantly different from 0) afterward (Figure 3D).

DISCUSSION

Complementary data on the binding affinity and B cell clone abundance at the same time points would provide a more comprehensive picture to explain this divergent trend. However, our findings support the hypothesis of Gaebler et al.,[10] who suggested that the accumulation of IgG somatic mutations—and subsequent production of antibodies with increased neutralizing potency—allow the maintenance of

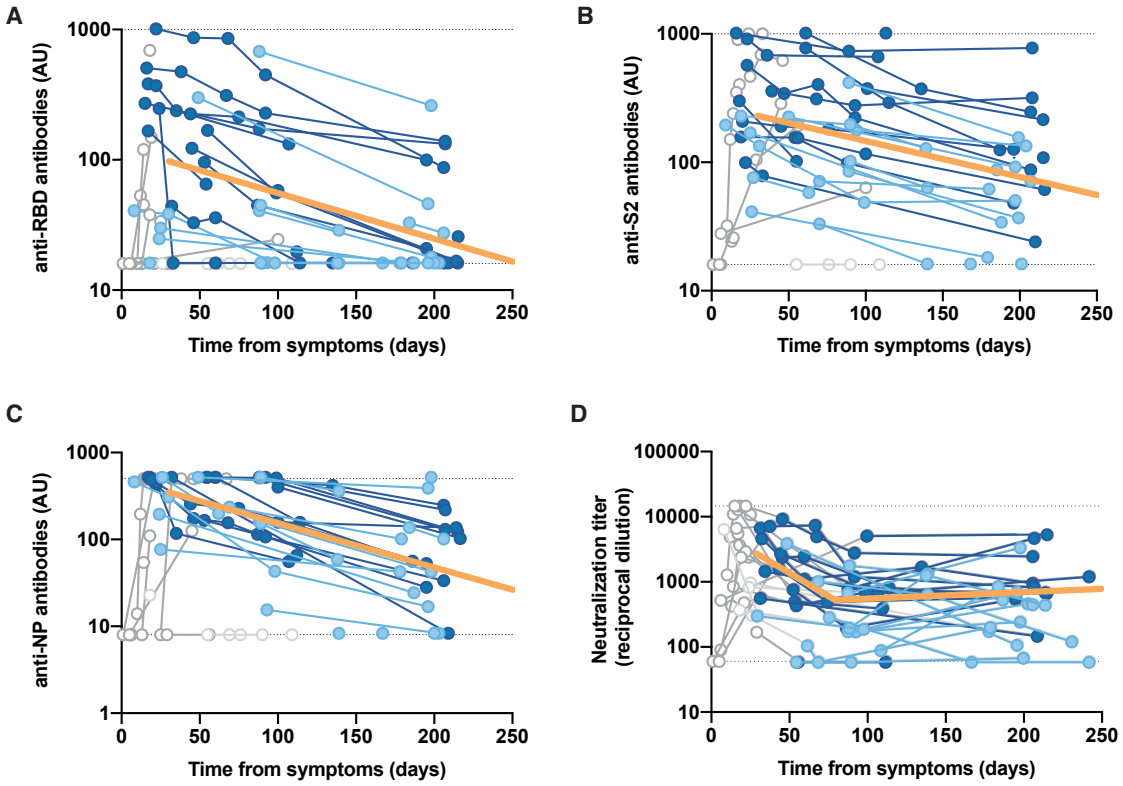

Figure 3. Longitudinal analysis of IgG titers

(A) Anti-receptor binding domain (RBD).

(B) Anti-S2.

(C) Anti-nucleoprotein.

(D) Overall neutralizing activity in the same set of samples. All of the analyses were performed on a subset of individuals with the largest follow-up (n = 14 for mild/asymptomatic in light blue and n = 14 for hospitalized in dark blue; total no. samples 94). Solid orange lines show the linear mixed model estimate for the period beyond day 30.

Kinetics of antibody decay (A–C) were calculated excluding time points preceding the maximal values for each patient. Kinetics of neutralizing antibodies excluded samples preceding day 30 (as in Figures 2A and 2B). All of the excluded values are shown but grayed out.

neutralizing activity levels, despite the decline in specific antibody titers. Of note, our follow-up period encompassed 2 waves of the COVID-19 outbreak in our country. Individuals infected during the first wave were likely to be exposed to high viral pressure in their environment, potentially favoring further virus exposure that may also contribute to maintaining humoral responses, adding to the mechanism proposed by Gaebler et al.[10]

Our longitudinal analysis supplements current evidence regarding mid-term immunity against SARS-CoV-2[8,10,11] and confirms the slow decay and mid-term maintenance of neutralizing activity observed in other cohorts, with a 5%-to-11% prevalence of hospitalized patients.[8,10] In this regard, the 2-phase behavioral pattern of neutralizing activity observed in hospitalized individuals suggests that the rapid decay reported in previous characterizations[7] may be due to the abundance of individuals in this early phase. Furthermore, apparent inconsistencies found between the declines of neutralizing activity and IgG titers reinforce the idea proposed by other authors that the behavior of antibody titers may not mirror the neutralizing activity. Interestingly, differences in decline were observed not only between neutralizing activity and anti-N antibodies, which do not contribute to neutralization, but also for anti-S2 and anti-RBD antibodies, which are major determinants of

neutralization.[16,17] The current evidence on immunity to SARS-CoV-2 infection suggests stability of neutralizing activity, pointing toward an optimistic scenario for the establishment of infection- or vaccine-mediated herd immunity. Still, long-term data available on other human coronaviruses show waning of antibodies 1–2 years after infection,[18,19] with uncertainty regarding the immune response behavior in the context of vaccine-mediated immunity.[20] The continuity of our prospective cohort of individuals recovered from SARS-CoV-2 infection will provide novel insights into the long-term kinetics of the immune response.

Limitations of Study

Our analysis is limited by the reduced sample size, particularly in the acute phase for mild/asymptomatic subgroup, for which we failed to define the kinetics of neutralizing response development and to identify a 2-phase pattern decay. Despite the limited sample size, the availability of multiple measures along the follow-up period allowed us to provide a longitudinal perspective on neutralizing activity and antibody titer behavior.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.medj.2021.01.005.

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AUTHOR CONTRIBUTIONS

J.B. and B.C. designed and coordinated the study. E.P., B.T., S.M., C.A.-N., M.L.R., F.T.-F., S.P.-Y., C.R., E.A.-E., J.R., J.V.-A., J.S., and N.I.-U. performed and analyzed the neutralization and ELISA assays. V.U. performed the statistical analysis. R.P., L.M., A.C., M.M., V.G., A.V., and J.C. selected the patients and coordinated the data. J.B. and Gerard Carot-Sans drafted the manuscript, and all of the authors made substantial contributions to the revision of the subsequent versions. All of the authors approved the submitted version of the manuscript and agreed both to be personally accountable for their own contributions and to ensure answer to questions related to the accuracy or integrity of any part of the work.

DECLARATION OF INTERESTS

J.B. and J.C. are founders of and shareholders in AlbaJuna Therapeutics; B.C. is a founder of and shareholder in AlbaJuna Therapeutics and AELIX Therapeutics (all unrelated to the present work). The other authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-6x-His clone HIS.H8	Thermo Fisher Scientific	Cat#MA1-21315; RRID: AB_557403
HRP-conjugated, F(ab')$_2$ goat anti-human IgG	Jackson ImmunoResearch	Cat#109-035-006; RRID: AB_2337578
Bacterial and virus strains		
pNL4-3.Luc.R-.E-	NIH ARP	Cat#3418
SARS-CoV-2.SctΔ19	This paper	N/A
pcDNA3.4-TOPO	GeneArt/Thermo Fisher Scientific	Cat#810330DE
pVSV-G	Clontech	[21]
Biological samples		
ELISA standard, positive plasma sample	This paper	N/A
Chemicals, peptides, and recombinant proteins		
S2 (Ser686-Pro1213)	Sino Biological	Cat#40590-V08B
RBD (Arg319-Phe541)	Sino Biological	Cat#40592-V08H
Nucleocapsid protein (NP)	Sino Biological	Cat#40588- V08B
MACS BSA solution	Miltenyi Biotec	Cat#130-091-376
Phosphate Buffered Saline	Thermo Fisher Scientific	Cat#10010015
o-Phenylenediamine dihydrochloride	Sigma-Aldrich	Cat#P8787-100TAB
H_2SO_4	Sigma-Aldrich	Cat#258105-1L-PC-M
Fetal Bovine Serum	Thermo Fisher Scientific	Cat#10270106
Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific	Cat#41966052
Expi293 Expression Medium	Thermo Fisher Scientific	Cat#A1435102
Opti-MEM I Reduced Serum Medium	Thermo Fisher Scientific	Cat#31985070
ExpiFectamine 293 Transfection Kit	Thermo Fisher Scientific	Cat#A14524
Versene	Thermo Fisher Scientific	Cat#15040033
Puromycin	Thermo Fisher Scientific	Cat#A1113803
DEAE-Dextran	Sigma-Aldrich	Cat#D9885-100G
BriteLite Plus Luciferase	PerkinElmer	Cat#6066769
Experimental models: cell lines		
Expi293F GnTI- cells	Thermo Fisher Scientific	Cat#A39240
HEK293T/hACE2 cells	Integral Molecular	Cat#C-HA101
Software and algorithms		
GraphPad Prism v8.4.3	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
R v4.0	R Foundation for Statistical Computing	https://www.r-project.org/
"nlme" R Package	R Foundation for Statistical Computing	https://cran.r-project.org/web/packages/nlme/index.html
Other		
GeneArt Gene Synthesis	Thermo Fisher Scientific	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Julià Blanco (jblanco@irsicaixa.es).

Materials availability

The plasmid pcDNA3.4 SARS-CoV-2.SctΔ19 is available upon request to the lead contact.

Data and code availability

This study did not generate any unique datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study overview and subjects

The study was approved by the Hospital Ethics Committee Board from Hospital Universitari Germans Trias i Pujol (PI-20-122 and PI-20-217) and all participants provided written informed consent before inclusion.

Plasma samples were obtained from individuals of the prospective KING cohort of the HUGTiP (Badalona, Spain). This is an observational cohort, no blinding or randomization was applied. The recruitment period lasted from March to October 2020, thus covering the first and second waves of COVID-19 outbreak in Catalonia (dadescovid.cat). The KING cohort included individuals with a documented positive RT-qPCR result from nasopharyngeal swab and/or a positive serological diagnostic test. In addition, we performed in all individuals a confirmatory ELISA test, analyzing IgG, IgM and IgA anti-RDB and anti-S2 responses, that has been developed in our center (https://www.irsicaixa.es/sites/default/files/detection_of_sars-cov-2_antibodies_by_elisa_-_protocol_by_irsicaixa_protected.pdf). Participants were recruited irrespective of age and disease severity—including asymptomatic status-in various settings, including primary care, hospital, and epidemiological surveillance based on contact tracing. Age under 18 was the sole exclusion criterion. Stratification of participants was performed according to the WHO progression scale:[22] asymptomatic or mild (levels 1-3), and hospitalized (levels 4-10). We collected plasma samples at the time of COVID-19 diagnosis and at 3 and 6 months. Additionally, hospitalized individuals were sampled twice a week during the acute phase.

Cell lines

HEK293T cells (presumably of female origin) overexpressing WT human ACE-2 (Integral Molecular, USA) were used as target for SARS-CoV-2 spike expressing pseudovirus infection. Cells were maintained in T75 flasks with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1μg/mL of Puromycin (Thermo Fisher Scientific, USA).

METHOD DETAILS

Humoral response determination

The humoral response against SARS-CoV-2 was evaluated with an in-house sandwich- ELISA using the following antigens (Sino Biological, Germany): S2 (Ser686-Pro1213), RBD (Arg319-Phe541), both potentially contributing to neutralizing activity; and whole nucleocapsid protein (NP), which is unrelated to neutralizing capacity. Nunc MaxiSorp plates were coated with 50 μL of anti-6x-His antibody clone HIS.H8 (2 μg/mL, Thermo Fisher Scientific) in PBS overnight at 4°C. After washing, plates were blocked with 1% BSA in PBS (Miltenyi Biotec, Germany) for two hours at room temperature. Antigens were added at 1 μg/mL concentration (50 μL/well) and incubated overnight at 4°C. Plasma samples were heat-inactivated before use (56°C for 30 minutes) and analyzed in duplicate in antigen-coated and antigen-free wells in the same plate. Serial dilutions of a positive plasma sample were used as standard. A pool of pre-pandemic plasmas from healthy controls was used as a negative control. Standards, negative control, and plasma samples were diluted in blocking buffer and were incubated (50 μL/well) for one hour at room temperature. The HRP-conjugated (Fab)2 goat anti-human IgG (Fc specific, Jackson

ImmunoResearch, UK) was then incubated for 30 minutes at room temperature. Plates were revealed with o-Phenylenediamine dihydrochloride (Sigma-Aldrich, USA) and reaction was stopped using 4N of H_2SO_4 (Sigma-Aldrich). Optical density (OD) at 492 nm with noise correction at 620 nm were used to calculate specific signal for each antigen after subtracting the antigen-free well signal for each sample. Standard curves were fitted to a 5-parameter logistic curve and data was expressed as arbitrary units (AU) according to the standard.

Pseudovirus generation and neutralization assay

HIV reporter pseudoviruses expressing SARS-CoV-2 S protein and Luciferase were generated. pNL4-3.Luc.R-.E- was obtained from the NIH AIDS Reagent Program.[23] SARS-CoV-2.SctΔ19 was generated (GeneArt) from the full protein sequence of SARS-CoV-2 spike with a deletion of the last 19 amino acids in C-terminal,[24] human-codon optimized and inserted into pcDNA3.4-TOPO. Expi293F cells were transfected using ExpiFectamine293 Reagent (Thermo Fisher Scientific) with pNL4-3.Luc.R-.E- and SARS-CoV-2.SctΔ19 at a 24:1 ratio, respectively. Control pseudoviruses were obtained by replacing the S protein expression plasmid with a VSV-G protein expression plasmid as reported.[21] Supernatants were harvested 48 hours after transfection, filtered at 0.45 μm, frozen, and titrated on HEK293T cells overexpressing WT human ACE-2 (Integral Molecular, USA). This neutralization assay has been previously validated in a large subset of samples.[13]

Neutralization assays were performed in duplicate. Briefly, in Nunc 96-well cell culture plates (Thermo Fisher Scientific), 200 $TCID_{50}$ of pseudovirus were preincubated with three-fold serial dilutions (1/60–1/14,580) of heat-inactivated plasma samples for 1 hour at 37°C. Then, $2x10^4$ HEK293T/hACE2 cells treated with DEAE-Dextran (Sigma-Aldrich) were added. Results were read after 48 hours using the EnSight Multimode Plate Reader and BriteLite Plus Luciferase reagent (PerkinElmer, USA). The values were normalized, and the ID_{50} (the reciprocal dilution inhibiting 50% of the infection) was calculated by plotting and fitting the log of plasma dilution versus response to a 4-parameters equation in Prism 8.4.3 (GraphPad Software, USA).

QUANTIFICATION AND STATISTICAL ANALYSIS

Continuous variables were described using medians and the interquartile range (IQR, defined by the 25th and 75th percentiles), whereas categorical factors were reported as percentages over available data. Quantitative variables were compared using the Mann-Whitney test, and percentages using the chi-square test. All experimental data were generated in duplicates. Kinetics of neutralizing activity and antibody titers (Log_{10} transformed to approximate to a normal distribution) were estimated from symptom onset—or serological diagnosis in asymptomatic individuals—and modeled using mixed-effects models in two steps. First, a 4-parameter logistic function was adjusted for the first 30 days after diagnosis using non-linear mixed models. Mid-term decay was analyzed using a piecewise regression with two decline slopes for data beyond 30 days, with a breakpoint at 80 days. For the latter analysis, linear mixed-effect models with random intercepts and slopes were used, and different breakpoints were tested; the best fit was chosen. For the longitudinal analysis of neutralizing activity, patients were grouped into two severity groups according to the WHO progression scale:[22] asymptomatic or mild (levels 1-3), and hospitalized (levels 4-10). Differences between the two severity groups were assessed using the likelihood ratio test. Association of neutralizing titers with gender was analyzed adjusting fitted models by gender and computing the corresponding likelihood ratio test. The longitudinal analysis of antibody titers was

performed on a subset of 28 individuals (14 in each severity group) with the highest number of measures during the follow-up; owing to the limited sample size, all individuals were analyzed as a single group. Analyses were performed with Prism 8.4.3 (GraphPad Software) and R version 4.0 (R Foundation for Statistical Computing). Mixed-effects models were fitted using "nlme" R package.