

Mild SARS-CoV-2 Infections and Neutralizing Antibody Titers

Francesco Bonfante, DVM,^{a,*} Paola Costenaro, MD, DTM&H,^{b,*} Anna Cantarutti, PhD,^c Costanza Di Chiara, MD^b Alessio Bortolami, DVM, PhD,^a Maria Raffaella Petrara, PhD,^d Francesco Carmona, BSc,^e Matteo Pagliari, PhD,^a Chiara Cosma, MD,^f Sandra Cozzani, MD,^b Eva Mazzetto, DVM,^a Giovanni Di Salvo, MD,^g Liviana Da Dalt, MD,^g Paolo Palma, MD,^{h,i} Luisa Barzon, MD,^j Giovanni Corrao,^{k,c} Calogero Terregino, MD,^a Andrea Padoan, MD,^l Mario Plebani, MD,^{l,f} Anita De Rossi, PhD,^{d,e} Daniele Donà, MD, PhD,^b Carlo Giaquinto, MD^b

abstract

BACKGROUND: Recent evidence suggests that neutralizing antibodies (nAbs) to severe acute respiratory syndrome coronavirus 2 may persist over time; however, knowledge regarding pediatric subjects is limited.

METHODS: A single-center, prospective observational study was conducted on 57 family clusters of coronavirus disease 2019, including children of neonatal and pediatric age attending the University Hospital of Padua (Italy). For each patient, blood samples were collected for both the quantification of nAbs through a plaque reduction neutralizing test and the detection of antinucleocapsid-spike protein immunoglobulin G and/or immunoglobulin M.

RESULTS: We analyzed 283 blood samples collected from 152 confirmed coronavirus disease 2019 cases (82 parents and 70 children or older siblings of median age of 8 years, interquartile range: 4–13), presenting asymptomatic or with mildly symptomatic disease. Despite the decrease of immunoglobulin G over time, nAbs were found to persist up to 7 to 8 months in children, whereas adults recorded a modest declining trend. Interestingly, children aged <6 years, and, in particular, those aged <3 years, developed higher long-lasting levels of nAbs compared with older siblings and/or adults.

CONCLUSIONS: Mild and asymptomatic severe acute respiratory syndrome coronavirus 2 infections in family clusters elicited higher nAbs among children.



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^aDivision of Comparative Biomedical Sciences, Istituto Zooprofilattico Sperimentale delle Venezie, Padua, Italy; ^bDivision of Pediatric Infectious Diseases, Department for Women's and Children's Health, University of Padua, Padua, Italy; ^cLaboratory of Healthcare Research and Pharmacoepidemiology, Division of Biostatistics, Epidemiology and Public Health, Department of Statistics and Quantitative Methods, University of Milano-Bicocca, Milan, Italy; ^dDepartment of Surgery, Oncology and Gastroenterology, Section of Oncology and Immunology, University of Padua, Padua, Italy; ^eIstituto Oncologico Veneto, IRCCS, Padua, Italy; ^fDepartment of Laboratory Medicine, University Hospital of Padua, Padua, Italy; ^gDepartment for Women's and Children's Health, University of Padua, Italy; ^hAcademic Department of Pediatrics (DPUO), Research Unit of Clinical Immunology and Vaccinology, Bambino Gesù Children's Hospital, Rome, Rome, Italy; ⁱDepartment of Systems Medicine, Tor Vergata University of Rome, Rome, Italy; ^jDepartment of Molecular Medicine, University of Padua, Italy; ^kNational Centre for Healthcare Research and Pharmacoepidemiology, University of Milano-Bicocca, Milan, Italy; ^lDepartment of Medicine-DIMED, University of Padua, Italy, Padua, Italy

*Contributed equally as co-first authors

Drs Giaquinto and Donà designed the study and supervised the project; Dr De Rossi designed the study and contributed to the writing; Dr Costenaro designed the study and wrote the manuscript; Dr Bonfante designed the study, performed the investigations, and wrote the manuscript; Dr Di Chiara designed the study and provided reagents and samples; Dr Cozzani designed the study; Drs Bortolami, Pagliari, Mazzetto, and Barzon performed the investigations and provided reagents and samples; Dr Petrara performed the investigations, provided reagents and samples, and contributed to the writing; Dr Cantarutti performed the statistical analysis and wrote the manuscript; Prof Corrao performed the statistical analysis; Dr Padoan contributed to the writing; Dr Plebani

WHAT'S KNOWN ON THIS SUBJECT: Children and adolescents usually present with asymptomatic or mild coronavirus disease 2019 cases; however, they are key in transmitting severe acute respiratory syndrome coronavirus 2 infection. Recent findings revealed that neutralizing antibodies (nAbs) persist up to 6 months in convalescent adults; however, little is known about nAbs kinetics in children.

WHAT THIS STUDY ADDS: Younger children develop higher levels of nAbs during the first 7 to 8 months after asymptomatic or mild symptomatic coronavirus disease 2019, compared with older siblings and adults. The long-lasting levels of nAbs may lead to durable protection and higher viral clearance, reducing shedding and transmission.

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European countries have been facing a third wave of the novel coronavirus disease 2019 (COVID-19) pandemic and the spread of several severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants. With the advent of vaccines,¹ longitudinal studies of both convalescent and vaccinated patients are of fundamental importance to understand the kinetics of humoral response and infer correlates of protection for both infection and disease. In this respect, the titration of neutralizing antibodies (nAbs) is key to determine the concentration of antibodies preventing cells to be infected by SARS-CoV-2.²

Studies including convalescent adults reported that humoral immunity against SARS-CoV-2 may be short-lived, particularly in persons with mild illness.³⁻⁵ However recent findings provided evidences of nAbs persisting up to 6 months,⁶⁻¹⁰ as with seasonal and SARS-like coronavirus infection, after which nAbs can persist, respectively, up to 1 or several years.^{11,12}

SARS-CoV-2 infection in children is less severe than in adults,¹³ resulting in underdiagnosis given the mild or asymptomatic clinical course.¹⁴ However, children and adolescents are key in the transmission of infection.¹⁵ Little is known about the kinetics of SARS-CoV-2 nAbs in pediatric populations. Understanding the differences in the antibody response between adults and children has important scientific and public health implications, including design of risk-based surveillance programs, cost-effective vaccination campaigns, and mathematical modeling of clinical outcome.

In this study, we evaluated the role of age as a determinant of the production and persistence of

naturally acquired nAbs among a cohort of family clusters of COVID-19, including adults and children who recovered from asymptomatic or mild symptomatic infections.

METHODS

Study Design and Population

A single-center, prospective study was conducted on Italian family clusters of COVID-19 attending the COVID-19 Family Cluster Follow-up Clinic, at the Department of Women's and Children's Health of the University Hospital of Padua (Veneto Region, Italy). From March 1, 2020 to September 4, 2020, 57 families were enrolled meeting the following inclusion criteria: (1) having children of pediatric age (aged <15 years); (2) any family member (eg, mother and/or father and/or any son or daughter) with a history of COVID-19. Families were enrolled in the program 4 to 8 weeks after the end of either isolation or hospitalization and after referral from the family pediatrician. Evaluation of children and relatives included data collection on demographic parameters and past medical history, clinical evaluation and the collection of a blood sample for a characterization of the immune response to SARS-CoV-2. All subjects aged >18 years, including older siblings and parents, and legally authorized representatives of subjects aged <18 years, were informed of the research proposal and provided written consent for the collection and use of biological specimens and routine patient-based data for research purposes. Families were invited to return to the clinic for longitudinal blood collection. The protocol was communicated to the ethical committee according to the national regulation (Protocol N° 0070714 of November 24, 2020; amendment number 71779 of November 26, 2020).

Data Collection and Definitions

Information collected during the clinic was entered into a Web-based database by using the Research Electronic Data Capture platform (Vanderbilt University, Tennessee) hosted in the server of the University of Padova. For this study, data were collected retrospectively from the existing clinical files and analyzed anonymously. Subjects were considered patients with confirmed COVID-19 if they had a record of virological positivity for SARS-CoV-2 by real-time polymerase chain reaction (RT-PCR) according to routine diagnostic molecular protocols¹⁶ and/or resulted positive by either of the 2 serological tests adopted in this study. For each confirmed COVID-19 case, a baseline date was defined as follows: (1) for symptomatic cases, the first date between the onset of symptoms or the date of first positive SARS-CoV-2 molecular assay result; (2) for asymptomatic cases: the date of the first positive molecular assay result or, in those with only serologically confirmed COVID-19 and with negative or undetermined nasal-pharyngeal swab (NPS) results, by the family outbreak temporal sequence, coinciding with the date of symptoms onset in a virologically confirmed SARS-CoV-2 family outbreak (Supplemental Fig 6). Subjects who were asymptomatic and had no analytical evidence of SARS-CoV-2 infection were considered to not have COVID-19. The severity of COVID-19 was scored as mild, moderate, severe, or critical, following the World Health Organization classification.¹⁷ For stratification purposes, individuals were divided on the basis of both social and biological development, into toddlers (<3 years), preschool-aged children (3 to <6 years), school-aged children (6 to <15 years) and sexually mature subjects

(>15 years). These age classes were deemed instrumental for a translation of results into the context of school-targeted vaccination and sero-surveillance campaigns.

Serological Assays

Plasma was stored at -80°C before testing for the quantification of nAbs through a high-throughput method for plaque reduction neutralizing test (PRNT).¹⁸ Another aliquot was analyzed with the chemiluminescence immunoassay (CLIA) MAGLUMI 2019-nCoV Immunoglobulin M (IgM) and Immunoglobulin G (IgG).¹⁸ Further details on the 2 assays are reported in the Supplemental Information.

SARS-CoV-2 Viral Load Measurement

A selection of NPSs of enrolled subjects that had been originally screened at the Padova University Hospital were made available for quantification of the viral load. Copies of SARS-CoV-2 were quantified by a homemade multiplex quantitative assay on the basis of a 1-step digital droplet polymerase chain reaction (ddPCR).¹⁹ Results were expressed as SARS-CoV-2 copies per 5 μl . Further details are reported in the Supplemental Information.

Statistical Analyses

Descriptive statistics were used for comparing the distribution of sex, age, disease-related symptoms, and pediatric comorbidities between patients infected with COVID-19 and uninfected patients.

The humoral response was assessed by comparing the geometric mean titer (GMT) and the 95% confidence interval (CI) of IgM, IgG, and PRNT₅₀ values in the overall data set, including both independent and subject-paired

samples, stratified by age classes and by time between serological sampling and baseline, categorizing subjects into 3 intervals, namely 1 to 2, 3 to 6 and 7 to 8 months. The 1-way analysis of variance and the independent samples *t* test were performed, when appropriate. Associations between antibody titers, baseline intervals and age, were assessed with linear regression models. Strength of associations between variables was assessed by Pearson correlation coefficient by using the logarithm (base 10) of the antibody titers, given data skew.

Use of the robust variance estimator to account for correlations within patients with multiple blood samplings did not change the CIs considerably in the unadjusted analyses, so correlation structures were omitted from all analyses. Among a subcohort of subjects that agreed to be sampled again after enrollment, a dependent *t* test for subject-paired samples was used to compare the GMT and 95% CI.

To test the robustness of our data sets against selection bias, we conducted a χ^2 test and verified the homogeneity within each age class and time window of (1) the temporal distribution of serological samplings ($P = .4363$) and (2) the proportion of cases identified by virological or serological methods ($P = .6568$). Moreover, we conducted a χ^2 test to verify among subjects who contributed with either 1, 2, or 3 samples the homogeneity of sex ($P = .6082$), age ($P = .0973$), family position ($P = .3971$) and severity of symptoms ($P = .6947$).

The diagnostic sensitivity of the CLIA and PRNT assays were assessed on subjects with a positive NPS result. Considering the PRNT assay as reference method for the

validation of immunoassays for SARS-CoV-2, we calculated measures of diagnostic accuracy of the CLIA assay.

Analyses were performed by using the Statistical Analysis System software (version 9.4; SAS Institute, Inc, Cary, NC). Statistical significance was set at the .05 level. All *P* values were 2-sided. Graphs were made by using GraphPad Prism version 9 (GraphPad Software, Inc, La Jolla, CA).

RESULTS

From March 1, 2020, to December 3, 2020, we prospectively evaluated 57 family clusters of COVID-19 (Supplemental Fig 5). A serological assessment was performed at least once on 209 recruited subjects. Subjects who had previously tested positive for SARS-CoV-2 by real-time RT-PCR (111 of 209) were considered to have confirmed COVID-19, together with individuals who had no record of virological positivity but showed evidence of seropositivity by either of the 2 serological tests adopted in this study (44 of 209). Descriptive analysis and additional information on baseline identification are provided as Supplemental Information (Supplemental Table 2, Supplemental Fig 6). Three out of 73 children were excluded from the analyses (see Supplemental Fig 5). In total, 152 confirmed COVID-19 cases were studied: 70 children or older siblings and 82 parents with median ages of 8 (interquartile range [IQR], 4–13) and 42 years (IQR, 34–46), respectively. Of 152 cases, 38, 97, and 17 were sampled once, twice and 3 times, respectively.

Analyzing all 283 blood samples collected from confirmed COVID-19 cases, we observed that nAbs persisted in the population, (Fig 1A) recording a modest

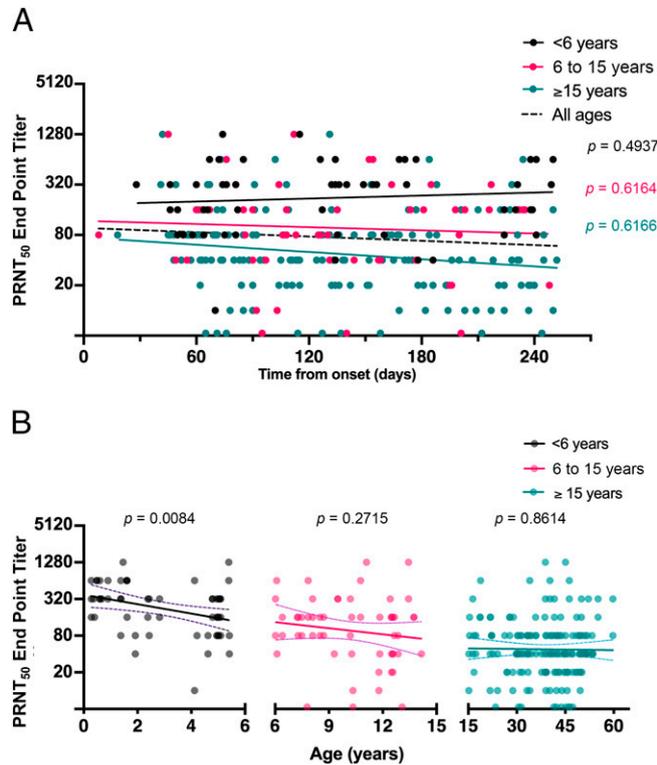


FIGURE 1

Stability of SARS-CoV-2 nAb titers over time. A, PRNT₅₀ titers from 283 serum samples collected at a median time of 132 days (IQR, 79–187) from infection onset, overall and stratified by 3 age classes, including children aged <6 years ($n = 55$; R^2 0.0089, $P = .4937$), children aged ≥ 6 and <15 years ($n = 58$; R^2 0.0047, $P = .6164$) and older siblings and adults aged ≥ 15 years of age ($n = 170$; R^2 0.0341, $P = .0166$). B, Reduced PRNT₅₀ titers observed at increasing age, at linear regression analysis conducted among children <6 years ($n = 55$; R^2 0.1239, $P = .0084$), children aged ≥ 6 and <15 years ($n = 58$; R^2 0.0224, $P = .2715$), and older siblings and adults of ≥ 15 years of age ($n = 170$; R^2 0.0002, $P = .8614$).

nonsignificant decline ($P = .1062$) over a median period of 132 days (IQR, 79–187) from baseline. When

samples were stratified by age, children aged <6 years were the only class with a slightly increasing

trend over time, as opposed to children aged 6 to 15 years and adults, although only for subjects

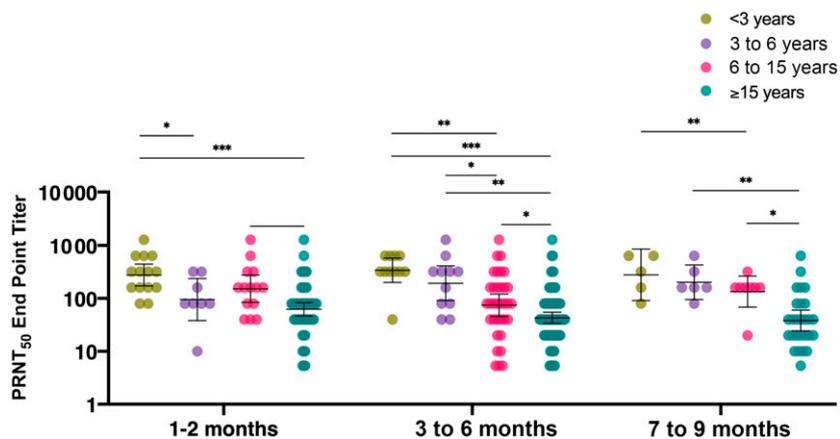


FIGURE 2

Differences in nAbs (PRNT₅₀) titers observed among 4 classes of age. PRNT₅₀ titers from 194 serum samples were stratified by age (aged <3 years, aged ≥ 3 and <6 years, aged ≥ 6 and <15 years, and aged ≥ 15 years), at 1 to 2 months, 3 to 6 months, and after disease onset (baseline); * $P < .05$; ** $P < .001$; *** $P < .0001$; Student's t test.

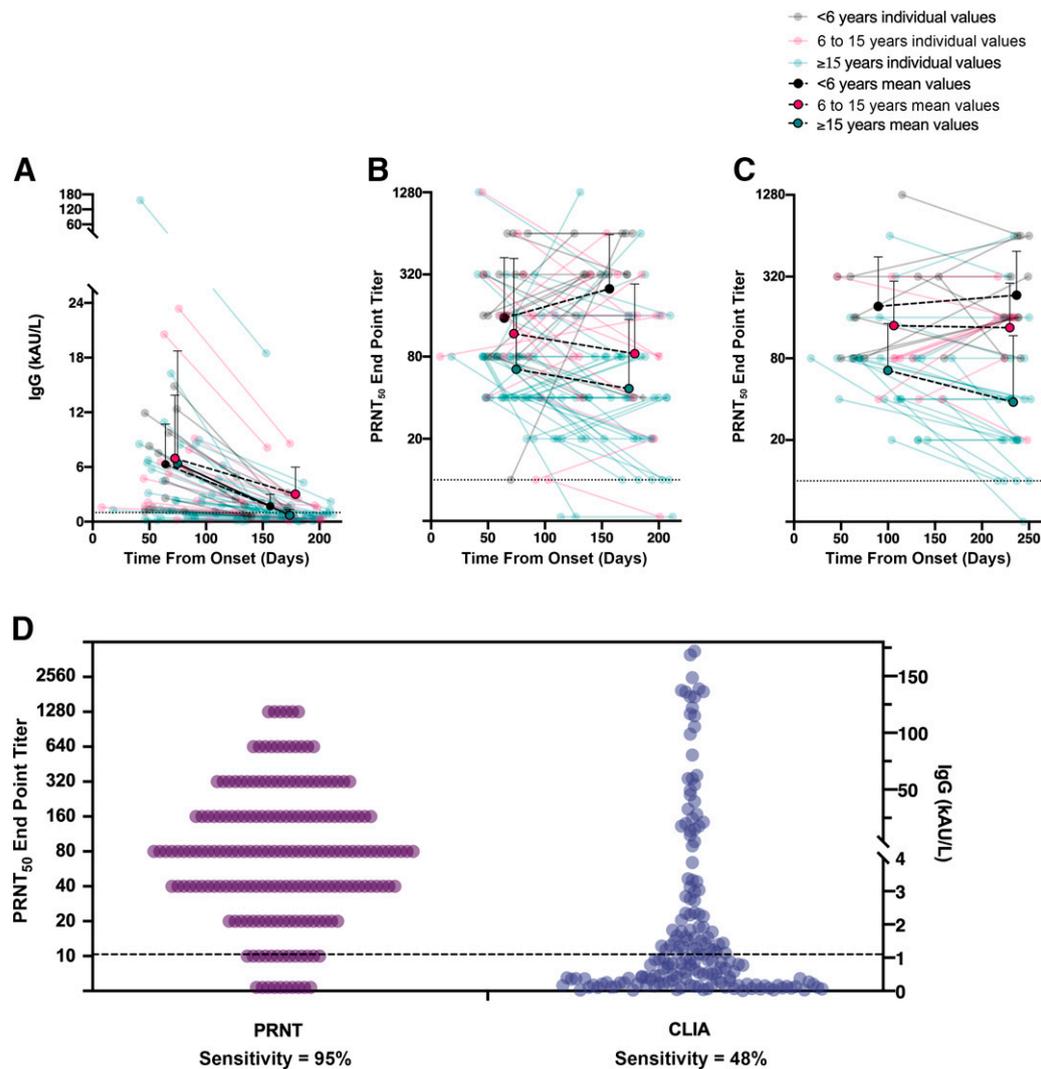


FIGURE 3

Performance of SARS-CoV-2 CLIA IgG and PRNT titers over time. A, Decreasing levels of SARS-CoV-2 CLIA IgG levels observed for all classes of age (aged <6 years, aged ≥6 and <15 years, and aged ≥15 years; paired *t* test $P < .0001$ across all groups), at longitudinal subject-paired serological assessment of 76 subjects sampled firstly at 72 days (SD ± 22) and a second time at ~169 days (SD ± 26) after baseline. B, Kinetics of PRNT₅₀ over time, for the same samples revealed in A. C, Kinetics of PRNT₅₀ over time in a subject-paired evaluation of 50 subjects, for whom paired samples were available at ~99 days (SD ± 35) and 234 days (SD ± 10) from baseline. The dotted line represents the limit of detection. D, Diagnostic sensitivity of CLIA IgG and PRNT₅₀ assays evaluated through testing of 194 samples from 111 virologically confirmed SARS-CoV-2 subjects. The dashed line represents the limit of detection and the manufacturer-recommended cutoff value for PRNT₅₀ and CLIA assays, respectively.

≥15 years of age we recorded a statistical support for the regression line ($P = .0166$). A further correlation analysis confirmed that nAbs inversely correlated with age (Pearson $\rho = -0.4144$, $P < .0001$), irrespective of time. To better characterize this picture, we conducted a regression model of age against PRNT₅₀ titers overall and within age classes. Overall, regression was significant

(estimated slope: -0.0423 , $P < .0001$), whereas the only significant regression within different age groups was observed for children aged <6 years (estimated slope -0.2561 , $P = .0084$) (Fig 1B).

To better evaluate how age affected antibody titers over time, we stratified data by both age and baseline interval (Supplemental

Table 3; Fig 2). Adults (patients aged >15 years) showed the lowest GMT of nAbs at all intervals. At 1 to 2 months after infection, children aged <3 years had a GMT of 1:276, whereas adults had a GMT of 1:62. The 4.5-fold difference increased to 7.9-fold in the 3 to 6 months window as children aged <3 years reached a GMT of 1:340, whereas adults recorded a GMT of 1:43. At intermediate and late time points,

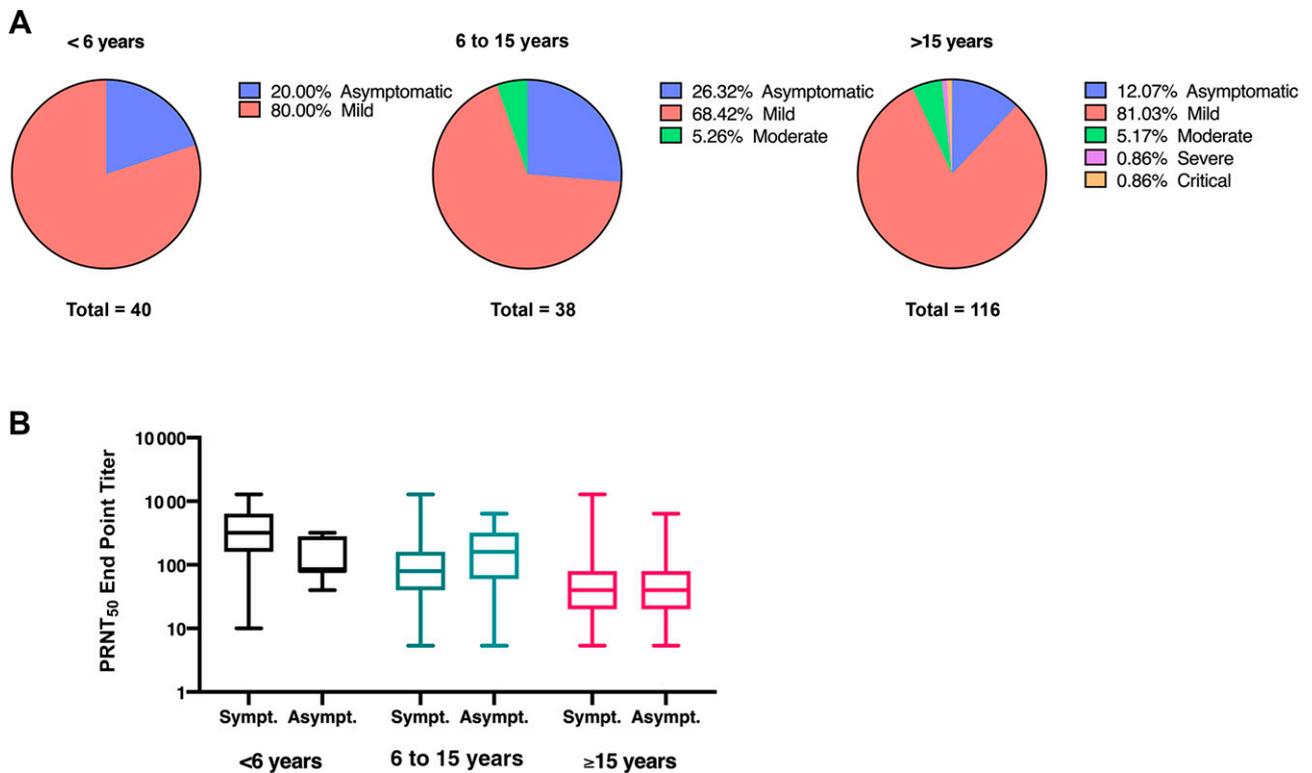


FIGURE 4 nAb titers according to COVID-19 disease severity. A, Clinical presentation of COVID-19 in children aged <6 years, aged ≥6 and <15 years, and aged ≥15 years, according to the World Health Organization COVID-19 clinical classification. B, PRNT₅₀ titer distribution among either asymptomatic or symptomatic subjects, stratified by age class and represented by box plots revealing minimum, maximum, median, first, and third quartiles (aged <6 years, aged ≥6 and <15 years, and aged ≥15 years; Wilcoxon test, $P = .0548$, $P = .8409$, and $P = .6230$, respectively).

children aged <3 years and those aged 3 to 6 years recorded significantly higher GMTs than children aged 6 to 15 years.

In a longitudinal serological assessment, we analyzed subject-paired plasmas from 76 subjects who were sampled a first and a second time on approximately day 72 (SD ±22) and 169 (SD ±26) from baseline (time window 1), respectively (Supplemental Table 4). Moreover, we analyzed plasma from 50 subjects (of which, 12 had contributed to time window 1), who were sampled a first and a second or third time on approximately day 99 (SD ±35) and 234 (SD ±10) from baseline (Fig 3 A–C, Table 1, and Supplemental Table 4) (time window 2). In time window 1, we observed an increase of nAbs titers for children aged <6

years (slope 0.0076), whereas children aged 6 to <15 years and subjects aged >15 years recorded a slight decreasing trend with estimated slopes of –0.0046 and –0.0047, respectively (Fig 3 A–B). In time window 2, children aged <6 years and those aged 6 to <15 years recorded a modest increase (slope 0.0019) and a minimal decrease (slope –0.0004) of nAbs titers, respectively, whereas, in adults, we observed a declining trend (slope of –0.0057) with a significant 40% reduction of nAbs titers ($P = .0021$) over time (Fig 3C). Interestingly, serological data by CLIA indicated a steady and significant decrease of IgG over time (Table 1), and a negativization in 54% (29 of 53) and 79% (27 of 34) of the seropositive subjects in the first and second time windows,

respectively, as opposed to the 3% (2 of 75) and 2% (1 of 50) of the subjects who tested positive for PRNT₅₀. Almost all samples tested negative by CLIA IgM at both time points in both groups, irrespective of age.

Because 14 cases had been assigned hypothetical baselines coinciding with the onset of symptoms of a family member (Supplemental Fig 6), we assumed that the considerable uncertainty of these values required a sensitivity analysis. The analysis verified that results and conclusions were robust against inclusion or exclusion of these 14 cases (data not shown). Nonetheless, we decided to include them, given that their exclusion would decrease underrepresented groups of children aged 6 to <15 years and 3 to <6 years at

TABLE 1 Subject-Paired Serological Data of 76 Subjects Who Were Sampled Twice at Periods of ~72 Days (SD ±22) and 169 Days (SD ±26) From Baseline and Data From 50 Subjects, for Whom Paired Samples Were Available at ~99 Days (SD ±35) and 234 Days (SD ±10) From Baseline

	A			B		
	First Sample	Second Sample (5–6 mo)	<i>P</i> ^a	First Sample	Latest Sample (7–9 mo)	<i>P</i> ^a
Aged <6 y						
(<i>n</i> = 16 for A and <i>n</i> = 11 for B)						
Mean days from baseline (SD)	64.2 (13.1)	156.6 (20.8)	—	92.2 (43.8)	236.7 (9.3)	—
GMT (95% CI)						
IgM (kAU/L) ^b	0.7 (0.6–1)	0.7 (0.5–1.1)	.5856	0.8 (0.4–1.3)	0.7 (0.4–1.3)	.0234
IgG (kAU/L) ^b	4.7 (2.9–7.5)	1.1 (0.7–1.8)	<.0001	3.2 (1.3–7.8)	0.2 (0.1–0.4)	<.0001
PRNT (end point titer)	146.7 (83–259.5)	246.8 (146.7–415.1)	.1246	193.3 (106.9–349.5)	233.5 (138.1–394.9)	.5175
Aged 6–<15 y						
(<i>n</i> = 16 for A and <i>n</i> = 10 for B)						
Mean days from baseline (SD)	72.6 (27.1)	178.9 (25.5)	—	105.9 (33.9)	234.1 (11.4)	—
GMT (95% CI)						
IgM (kAU/L) ^b	0.6 (0.4–0.8)	0.5 (0.3–0.7)	.0857	0.4 (0.3–0.6)	0.3 (0.2–0.4)	.0038
IgG (kAU/L) ^b	3.7 (1.9–7)	1.1 (0.6–2.3)	<0.0001	2.4 (0.8–7)	0.4 (0.2–1.2)	<0.0001
PRNT (end point titer)	118.1 (58.6–238)	83.9 (43.9–160.4)	.2087	139.3 (62.4–310.9)	134.5 (68.5–264.3)	.2275
Aged ≥15 y						
(<i>n</i> = 44 for A and <i>n</i> = 29 for B)						
Mean days from baseline (SD)	74.9 (22.8)	173.7 (23.6)	—	102.6 (35.2)	234.3 (10.2)	—
GMT (95% CI)						
IgM (kAU/L) ^b	0.7 (0.6–0.9)	0.4 (0.3–0.6)	<.0001	0.5 (0.4–0.7)	0.3 (0.3–0.5)	.0003
IgG (kAU/L) ^b	2.3 (1.5–3.6)	0.5 (0.3–0.8)	<.0001	2.4 (1.3–4.3)	0.4 (0.2–0.6)	<.0001
PRNT (end point titer)	64.3 (48–86.1)	47 (32.5–67.8)	.0654	63 (46.6–85.1)	38.1 (24.2–60)	.0021

—, not applicable.

^a One-way analysis of variance.

^b Missing data are handled in the analysis.

intermediate and late time points (Supplemental Table 5).

We compared the performance of PRNT and CLIA on a set of 194 samples collected from 111 of 152 confirmed patients with COVID-19 who had a positive real-time RT-PCR NPS result, recording sensitivities of 0.95, (184 of 194) and 0.48 (93 of 194), respectively (Fig 3D). Moreover, evaluating 264 of 283 samples for which both PRNT and IgG values were available, irrespective of the virological status of the donors, we found a moderate concordance but a poor negative predictive value of the CLIA in predicting seropositivity months after infection (Supplemental Table 6).

We further explored whether nAbs correlated with either clinical presentation or viral load. Differences in the distribution of clinical presentations between age classes were nonsignificant (Fig 4A), and nAbs titers did not significantly

differ between subjects showing mild or no symptoms (Fig 4B).

For 63 of 111 COVID-19 confirmed cases that had recorded virological positivity, the original swab was available for viral load quantification by ddPCR. To select a biologically relevant period of infection and standardize comparisons, we focused on a subgroup of 32 of 63 subjects for whom swabs had been collected within 4 days from symptom onset and serological samplings had been taken within 1 to 2 months. We observed that adults recorded a mean viral load of $10^{7.88}$ copies, whereas children aged <6 years and those aged 6 to <15 years had mean values of $10^{7.65}$ and $10^{6.79}$ copies, respectively. Differences in viral load between age classes were not significant ($P = .2409$), whereas PRNT₅₀ titers directly correlated with viral load among children (Supplemental Table 7).

DISCUSSION

The role of antibodies on the clearance of established SARS-CoV-2 infection and clinical outcomes is still unclear. Recent data suggest that the development of potentially neutralizing humoral immunity against SARS-CoV-2 is critical to increase survival and may protect against reinfection with other circulating strains of SARS-CoV-2 in adults.²⁰ In children it was recently revealed that the onset of high titers of nAbs is associated with shorter viral shedding at nasal-pharyngeal level¹⁹ but not with clinical presentation in the short-term follow-up.

In the current study, we describe a longitudinal comparison of the magnitude and persistence of nAbs against SARS-CoV-2, among asymptomatic and mildly symptomatic toddlers, preschool-aged children, school-aged subjects, and parents, in family

clusters of COVID-19. In our cohort, antibodies neutralizing SARS-CoV-2 virus persisted over a period of 2 to 8 months from infection, recording only a modest decline. This result is in line with previous studies using PRNT and surrogate-neutralization-based-assays^{7-10,21,22} describing a minimal decline of nAbs in adult populations. Surprisingly, nAbs inversely correlated with age, and children aged <6 years, and, in particular, toddlers aged <3 years, had the highest titers throughout early, intermediate, and late times from infection onset. Our data strengthens and expands recent work published by Yang et al,²³ who described higher surrogate neutralizing ability and avidity of antibodies in children aged 1 to 10 years, proving these features to be age-dependent, in a cohort of subjects aged 1 to 24 years, early after recovery. In contrast with our findings, other studies indicated that nAbs in children were lower than in adults.^{24,25} However, in 1 study,²⁴ stratification by age was done by age <24 years or >24 years, and children and adults were sampled on ~5 and 12 days from hospital admission, respectively; in the other study,²⁵ authors compared children with mildly affected adults previously selected as plasma donors at the hospital. We believe these selection and sampling biases might account for discrepancies with data reported in our study. Interestingly, in the latter study,²⁵ anti-S IgG and nAbs inversely correlated with age among children.

Strains encountered in childhood imprint adaptive immunity. Subsequent exposure to antigenically related viruses directs the antibody response largely toward known conserved epitopes and less against novel

immunodominant proteins, blunting the neutralizing potential.²⁶ Recently, this mechanism has been explored for influenza, proving that children aged <6 years have a narrow strain-specific hemagglutinating inhibition activity, whereas adults have a back-boost response to past infections.²⁷ In light of this, we hypothesize that an original antigenic sin driven by repeat exposure to endemic human coronaviruses might impair the response to SARS-CoV-2 in adults, whereas the less experienced immune repertoire of children could favor a prompt selective response. Recent work published by Selva et al²⁸ supports this hypothesis, proving that infection in elderly patients associates with antibodies targeting the cross-reactive S2 and NP proteins, whereas, in children, the response is dominated by antibodies with high Fc-effector function targeting the immunodominant S1 protein of SARS-CoV-2. In addition, Westerhuis et al²⁹ proved that, in adult patients, an expansion of B-cell clones against seasonal human coronaviruses dominates the response, generating antibodies poorly reactive with SARS-CoV-2.

Another relevant result of our study is the persistence of nAbs in children. We reveal for the first time that mildly affected children aged <6 years displayed increasing nAbs levels, over a period of 236 days from infection. Interestingly, children aged 6 to <15 years plateaued at approximately the same period, whereas adults showed a significant decline in nAbs, recording a 40% decrease between 3 and 7 months from infection. Similarly, Lau et al¹⁰ estimated that, for adults, the decline of PRNT titers would reach undetectable levels between 133 and 416 days from infection depending on clinical severity and reported a 50%

decrease between 3 and 6 months from infection for mild cases. In addition, Chia et al⁹ identified 5 profiles of antibody responses and observed that the persistence of high nAbs up to 6 to 7 months correlated with high levels of proinflammatory cytokines and the severity of COVID-19 in adults, predicting declines between 96 and 580 days.

In light of this, it is important to observe that, in our cohort, severity of infection and mean viral loads did not differ significantly among age classes; besides, the presence of mild symptoms was not a predictor of higher nAbs. Nonetheless, in children, viral load estimated at baseline correlated with magnitude of nAbs evaluated after 1 to 2 months, suggesting that a higher exposure to the antigen results in stronger humoral responses.

In line with other reports,^{30,31} we observed a dramatic drop in the sensitivity of a CLIA assay targeting a spike-nucleoprotein-fused antigen, confirming the importance of selecting immunoassays that are specifically validated for assessing antibodies over long periods of time.

Our study has several limitations. The processes of enrollment, case definition, and identification of time lines were not coincidental because we relied on retrospective heterogeneous diagnostic evaluations related to the structure of the clinic. This potentially led to biases in the identification of baseline intervals, especially for pediatric cases with no virological record of positivity, for which mild symptoms reported by parents were the only temporal reference to infection. Nonetheless, information from other family members and the long duration of the study potentially reduced the weight of these indeterminate values; moreover, sensitivity

analyses confirmed our conclusions against the exclusion of few cases.

In the absence of correlates of protection for nAbs acquired after infection, it is not advisable to translate our data into predictions of a superior immunity of children to reinfection. According to clinical studies and experimental animal work, superior nAbs for SARS-CoV-2 might translate into protection from COVID-19 disease and higher viral clearance in the upper respiratory tract, leading to a reduction in

shedding and transmission.^{19,32} It is of the utmost importance to identify age- and time-matched correlates of protection to finally translate serological data into useful elements for the design of vaccines and immunization campaigns for SARS-CoV-2.

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ABBREVIATIONS

CI: confidence interval
CLIA: chemiluminescence immunoassay
COVID-19: coronavirus disease 2019
ddPCR: digital droplet polymerase chain reaction
GMT: geometric mean titer
IgG: immunoglobulin G
IgM: immunoglobulin M
IQR: interquartile range
nAb: neutralizing antibody
NPS: nasal-pharyngeal swab
PRNT: plaque reduction neutralizing test
RT-PCR: real-time polymerase chain reaction
SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

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Address correspondence to Francesco Bonfante, DVM, Istituto Zooprofilattico Sperimentale delle Venezie, viale dell'Università 10, 35020 Legnaro, Padua, Italy. E-mail: FBonfante@izsvenezie.it

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