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Mild SARS-CoV-2 Infections and Neutralizing Antibody Titers

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Abbreviations:
CLIA, chemiluminescence immunoassay
COVID-19, Coronavirus disease 2019
CovFC, COVID-19 Family Cluster Follow-up Clinic
ddPCR, digital droplet Polymerase Chain Reaction
FP, family pediatrician
GMT, geometric mean titer
hCoV, human coronaviruses
nAbs, neutralizing antibodies
NPS, nasal-pharyngeal swab
PRNT, Plaque Reduction Neutralizing Test
RT-PCR, real-time Polymerase Chain Reaction

Article summary: High levels of SARS-CoV-2 neutralizing antibodies (nAbs) are found up to 7-8 months after asymptomatic/mild COVID-19, particularly in children aged less than 6 years.

What's Known on This Subject:

Children/adolescents usually present with asymptomatic/mild COVID-19 diseases; however, they are key in transmitting SARS-CoV-2 infection. Recent findings showed 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 7-8 months after asymptomatic/mild symptomatic COVID-19, compared to older siblings/adults. The long-lasting

levels of nAbs may lead to durable protection and higher viral clearance, reducing shedding and transmission.

Abstract

Background. Recent evidence suggests that neutralizing antibodies to SARS-CoV-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 COVID-19, 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 neutralizing antibodies (nAbs) through a Plaque Reduction Neutralizing Test (PRNT) and the detection of anti-nucleocapsid-spike protein IgG/IgM.

Results. We analyzed 283 blood samples collected from 152 confirmed COVID-19 cases (82 parents and 70 children/older siblings of median age of 8 years, IQR 4-13), presenting asymptomatic or with mildly symptomatic disease. Despite the decrease of IgG over time, nAbs were found to persist up to 7-8 months in children while adults recorded a modest declining trend. Interestingly, children under 6 years of age, and in particular under 3 years developed higher long-lasting levels of nAbs compared to older siblings and/or adults.

Conclusion. Mild and asymptomatic SARS-CoV-2 infections in family clusters elicited higher neutralizing antibodies among children.

Introduction

European countries have been facing a "third wave" of the novel Coronavirus disease 2019 (COVID-19) pandemic and the spread of several 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 after seasonal and SARS-like coronavirus infection, where nAbs can persist, respectively up to one 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 modelling 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/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 (CovFC), at the Department of Women's and Children's Health of the University Hospital of Padua (Veneto Region, Italy). From March 1st to September 4th 2020, 57 families were enrolled meeting the following inclusion criteria: a) having children of pediatric age (<15 years); b) any family member (e.g. mother and/or father and/or any son/daughter) with a history of COVID-19. Families were enrolled in the

program 4-8 weeks after the end of either isolation or hospitalization, and after referral from the family pediatrician (FP). 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 older than 18 years of age, including older siblings and parents, and legally authorized representatives of subjects under 18 years of age, 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 (Prot. N° 0070714 of November 24th, 2020; amendment N°71779 of November 26th, 2020).

Data collection and definitions

Information collected during the clinic were entered into a web-based database using the REDCap® 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 *confirmed COVID-19 cases* if they had a record of virological positivity for SARS-CoV-2 by real-time RT-PCR according to routine diagnostic molecular protocols[16] and/or resulted positive by either of the two 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; 2) for asymptomatic cases: the date of the first positive molecular assay or, in those with only serologically confirmed COVID-19 and with negative/undetermined nasal-pharyngeal swab (NPS), by the family outbreak temporal sequence, coinciding with the date of

symptoms onset in a virologically confirmed SARS-CoV-2 family outbreak (Supplementary Figure S2). Subjects that were asymptomatic and had no analytical evidence of SARS-CoV-2 infection were considered non-COVID-19 cases. The severity of COVID-19 was scored as mild, moderate, severe, critical, following the WHO classification [17]. For stratification purposes individuals were divided, based on both social and biological development, in toddlers (<3 years), pre-school children (3-<6 years), school-age children (6-<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 IgM/IgG [18]. Further details on the two assays are reported in the Supplementary Materials.

SARS-CoV-2 viral load measurement

A selection of NP swabs 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 home-made multiplex quantitative assay based on One-Step digital droplet PCR (ddPCR)[19]. Results were expressed as SARS-CoV-2 copies/5 µl. Further details are reported in the Supplementary Materials.

Statistical analyses

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

The humoral response was assessed comparing the geometric mean titer (GMT), and the 95% confidence interval (95% CI), of IgM, IgG, and $PRNT_{50}$ values, in the overall dataset including both independent and subject-paired samples, stratified by age classes and by time between serological sampling and baseline, categorizing subjects into three intervals, namely 1-2, 3-6 and 7-8 months. The one-way ANOVA and the independent samples t-test were performed, where 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, 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 confidence intervals considerably in the unadjusted analyses, so correlation structures were omitted from all analyses. Among a sub-cohort of subjects that agreed to be sampled again after enrolment, a dependent t-test for subject-paired samples was used to compare the GMT and 95% CI.

To test the robustness of our datasets against selection bias, we conducted a chi-square test and verified the homogeneity within each age class and time window of a) the temporal distribution of serological samplings (p=0.4363) and b) the proportion of cases identified by virological/serological methods (p=0.6568). Moreover, we conducted a chi-square test to verify

among subjects who contributed with either 1, 2 or three samples the homogeneity of gender (p=0.6082), age (p=0.0973), family position (p=0.3971) and severity of symptoms (p=0.6947).

The diagnostic sensitivity of the CLIA and PRNT assays were assessed on subjects with a positive NPS. 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 using the Statistical Analysis System software (version 9.4; SAS Institute, Cary, North Carolina). Statistical significance was set at the .05 level. All P values were 2-sided. Graphs were made using GraphPad Prism version 9 (GraphPad Software, La Jolla, CA).

RESULTS

From March 1st to December 3rd 2020, we prospectively evaluated 57 family clusters of COVID-19 (Supplementary Figure S1). 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 (111/209) were considered confirmed COVID-19 cases, together with individuals that had no record of virological positivity but showed evidence of seropositivity by either of the two serological tests adopted in this study (44/209). Descriptive analysis and additional information on *baseline* identification are provided as Supplementary Material (Table S1; Supplementary Figure S2). Three out of 73 children were excluded from the analyses (see supplement legend to Figure S1). In total 152 confirmed COVID-19 cases were studied: 70 children/older siblings and 82 parents with median ages of 8 (interquartile range (IQR), 4-13) and 42 years (IQR, 34-46), respectively. Out of 152 cases, 38, 97 and 17 were sampled once, twice and three times, respectively.

Analyzing all 283 blood samples collected from confirmed COVID-19 cases, we observed that nAbs persisted in the population, (Figure 1A) recording a modest non-significant decline ($p=0.1062$) over a median period of 132 days (IQR, 79-187) from baseline. When samples were stratified by age, children under 6 years where the only class with a slightly increasing trend over time, as opposed to children of 6-15 years and adults, although only for subjects ≥15 years of age we recorded a statistical support for the regression line ($p=0.0166$). A further correlation analysis confirmed that nAbs inversely correlated with age (Pearson ρ=-0.4144, $p<0.0001$), irrespective of time. To better characterize this picture, we conducted a regression model of age against $PRNT_{50}$ titers overall and within age classes. Overall, regression was significant (estimated slope – 0.0423, $p<0.0001$), while the only significant regression within different age groups was observed for children under 6 years (estimated slope -0.2561, $p=0.0084$) (Figure 1B).

To better evaluate how age affected antibody titers over time, we stratified data by both age and baseline interval (Supplementary Table S2; Figure 2). Adults (cases >15 years) showed the lowest geometric mean titer (GMT) of nAbs, at all intervals. At 1-2 months after infection, children under 3 years had a GMT of 1:276, while adults had a GMT of 1:62. The 4.5-fold difference increased to 7.9-fold, in the 3-6 months window, as children under 3 reached a GMT of 1:340, while adults recorded a GMT of 1:43. At intermediate and late time points, children < 3 and those aged 3-6 years recorded significantly higher GMTs than children aged 6-15 years.

In a longitudinal serological assessment, we analyzed subject-paired plasmas from 76 subjects who were sampled a first and a second time around day 72 (SD, ± 22) and 169 (SD, ± 26) from baseline (time window 1), respectively (Supplementary Table S3). Moreover, we analyzed plasma from 50 subjects (of which 12 had contributed to time window 1), who were sampled a first and a second/third time around day 99 (SD, ± 35) and 234 (SD, ± 10) from baseline (Figures 3A-C;

Tables 1 and S3) (time window 2). In time window 1, we observed an increase of nAbs titers for children under 6 years (slope 0.0076), while children of 6-<15 years and subjects over 15 years recorded a slight decreasing trend with estimated slopes of -0.0046 and -0.0047, respectively (Figures 3A-B). In time window 2, children <6 and those aged 6-<15 years recorded a modest increase (slope 0.0019) and a minimal decrease (slope -0.0004) of nAbs titers, respectively, while in adults we observed a declining trend (slope of -0.0057) with a significant 40% reduction of nAbs titers (p=0.0021) over time (Figure 3C). Interestingly, serological data by CLIA indicated a steady and significant decrease of IgG over time (Table 1), and a negativization in 54% (29/53) and 79% (27/34) of the seropositive subjects in the first and second time windows, respectively, as opposed to the 3% (2/75) and 2% (1/50) of the $PRNT_{50}$ positive subjects. Almost all samples tested negative by CLIA IgM, at both timepoints in both groups, irrespective of age.

Since 14 cases had been assigned hypothetical baselines coinciding with the onset of symptoms of a family member (Supplementary Figure S2), 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 under-represented groups of children aged 6-<15 years and 3-<6 years at intermediate and late time-points (Table S4).

We compared the performance of PRNT and CLIA on a set of 194 samples collected from 111/152 confirmed COVID-19 cases who had a real-time RT-PCR positive NPS, recording sensitivities of 0.95, (184/194) and 0.48 (93/194), respectively (Figure 3D). Moreover, evaluating 264/283 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 (NPV) of the CLIA in predicting seropositivity months after infection (Supplementary Table S5).

We further explored whether nAbs correlated with either clinical presentation or viral load. Differences in the distribution of clinical presentations between age classes were non-significant (Figure 4A) and nAbs titers did not significantly differ between subjects showing mild or no symptoms (Figure 4B).

For 63/111 COVID-19 confirmed cases that had recorded virological positivity, the original swab was available for viral load quantification by ddPCR. In order to select a biologically relevant period of infection and standardize comparisons, we focused on a subgroup of 32/63 subjects for whom swabs had been collected within 4 days from symptom onset and serological samplings had been taken within 1-2 months. We observed that adults recorded a mean viral load of $10^{7.88}$ copies, while children under 6 and those aged 6-<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=0.2409) whereas $PRNT_{50}$ titers directly correlated with viral load among children (Supplementary Table S6).

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 potently neutralizing humoral immunity against SARS-CoV-2 is critical to increase survival and may protect against re-infection with other circulating strains of SARS-CoV-2 in adults [20]. In children it was recently shown that the onset of high titers of nAbs is associated with shorter viral shedding at nasal-pharyngeal level [19] but not with clinical presentation, in the short term follow-up (Cotugno N et al, manuscript in preparation).

The current study describes a longitudinal comparison of the magnitude/persistence of nAbs against SARS-CoV-2, among asymptomatic and mildly symptomatic toddlers, pre-school children, school-age subjects and parents, in family clusters of COVID-19. In our cohort, antibodies neutralizing SARS-CoV-2 virus persisted over a period of 2-8 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 under 6 years, and in particular toddlers under 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 1-10 years, proving these features to be age-dependent, in a cohort of subjects aged 1-24 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 one study [24] stratification by age was done below/above 24 years and children and adults were sampled around 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 towards *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 under 6 years of age have a narrow strain-specific hemagglutinating inhibition activity, while 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 (hCoV) might impair the response to SARS-CoV-2 in adults, while the less experienced immune repertoire of children could favor a prompt selective response. Recent work published by Selva et al. [28] supports this hypothesis proving that infection in elderly patients associates with antibodies targeting the cross-reactive S2 and NP proteins, while 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 al. [29] proved that in adult patients, an expansion of B-cell clones against seasonal hCoVs 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 demonstrate for the first time that mildly affected children under 6 years displayed increasing nAbs levels, over a period of 236 days from infection. Interestingly, children aged 6-<15 plateaued around the same period, while adults showed a significant decline in nAbs, recording a 40% decrease between 3 and 7 months from infection. Similarly, Lau et al. [10] estimated for adults that 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 al. [9] identified five profiles of antibody responses and observed that the persistence of high nAbs up to 6-7 months correlated with high levels of pro-inflammatory cytokines and the severity of COVID-19 in adults, predicting declines between 96-580 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 1-2 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 timelines were not coincidental, since 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 whom 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 re-infection. 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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Table 1. Subject-paired serological data of 76 subjects who were sampled twice around periods of 72 days (SD, ± 22) and 169 days (SD, ± 26) from baseline and data from 50 subjects, for whom paired samples were available around 99 days (SD, ± 35) and 234 days (SD, ± 10) from baseline.

	Age < 6 years (n= 16)			Age < 6 years (n= 11)		
	First sample	**Second sample (5-6 months)**	**p-value§**	**First sample**	**Latest sample (7-9 months)**	**p-value§**
Mean days from baseline (STD)	64.2 (13.1)	156.6 (20.8)		92.2 (43.8)	236.7 (9.3)	
	GMT (95% CI)	**GMT (95% CI)**		**GMT (95% CI)**	**GMT (95% CI)**	
IgM (kAU/L) ¥	0.7 (0.6 - 1)	0.7 (0.5 - 1.1)	0,5856	0.8 (0.4 - 1.3)	0.7 (0.4 - 1.3)	0,234
IgG (kAU/L) ¥	4.7 (2.9 - 7.5)	1.1 (0.7 - 1.8)	< 0.0001	3.2 (1.3 - 7.8)	0.2 (0.1 - 0.4)	< 0.0001
PRNT (endpoint titer)	146.7 (83 - 259.5)	246.8 (146.7 - 415.1)	0,1246	193.3 (106.9 - 349.5)	233.5 (138.1 - 394.9)	0,5175

	Age 6-<15 years (n=16)			Age 6-<15 years (n=10)		
	First sample	**Second sample (5-6 months)**	**p-value§**	**First sample**	**Latest sample (7-9 months)**	**p-value§**
Mean days from baseline (STD)	72.6 (27.1)	178.9 (25.5)		105.9 (33.9)	234.1 (11.4)	
	GMT (95% CI)	**GMT (95% CI)**		**GMT (95% CI)**	**GMT (95% CI)**	
IgM (kAU/L) ¥	0.6 (0.4 - 0.8)	0.5 (0.3 - 0.7)	0,0857	0.4 (0.3 - 0.6)	0.3 (0.2 - 0.4)	0,0038
IgG (kAU/L) ¥	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 (endpoint titer)	118.1 (58.6 - 238)	83.9 (43.9 - 160.4)	0.2087	139.3 (62.4 - 310.9)	134.5 (68.5 - 264.3)	0.2275

	Age ≥ 15 years (n=44)			Age ≥ 15 years (n=29)		
	First sample	**Second sample (5-6 months)**	**p-value§**	**First sample**	**Latest sample (7-9 months)**	**p-value§**
Mean days from baseline (STD)	74.9 (22.8)	173.7 (23.6)		102.6 (35.2)	234.3 (10.2)	
	GMT (95% CI)	**GMT (95% CI)**		**GMT (95% CI)**	**GMT (95% CI)**	
IgM (kAU/L) ¥	0.7 (0.6 - 0.9)	0.4 (0.3 - 0.6)	< 0.0001	0.5 (0.4 - 0.7)	0.3 (0.3 - 0.5)	0.0003
IgG (kAU/L) ¥	2.3 (1.5 - 3.6)	0.5 (0.3 - 0.8)	< 0.0001	2.4 (1.3 - 4.3)	0.4 (0.2 - 0.6)	< 0.0001
PRNT (endpoint titer)	64.3 (48 - 86.1)	47 (32.5 - 67.8)	0.0654	63 (46.6 - 85.1)	38.1 (24.2 - 60)	0.0021

¥ Missing data are handled in the analysis

ł One-way ANOVA

The following acronyms refer to: GMT, Geometric Mean Titer; 95% CI, 95% confidence interval; PRNT, Plaque Reduction Neutralization Test.

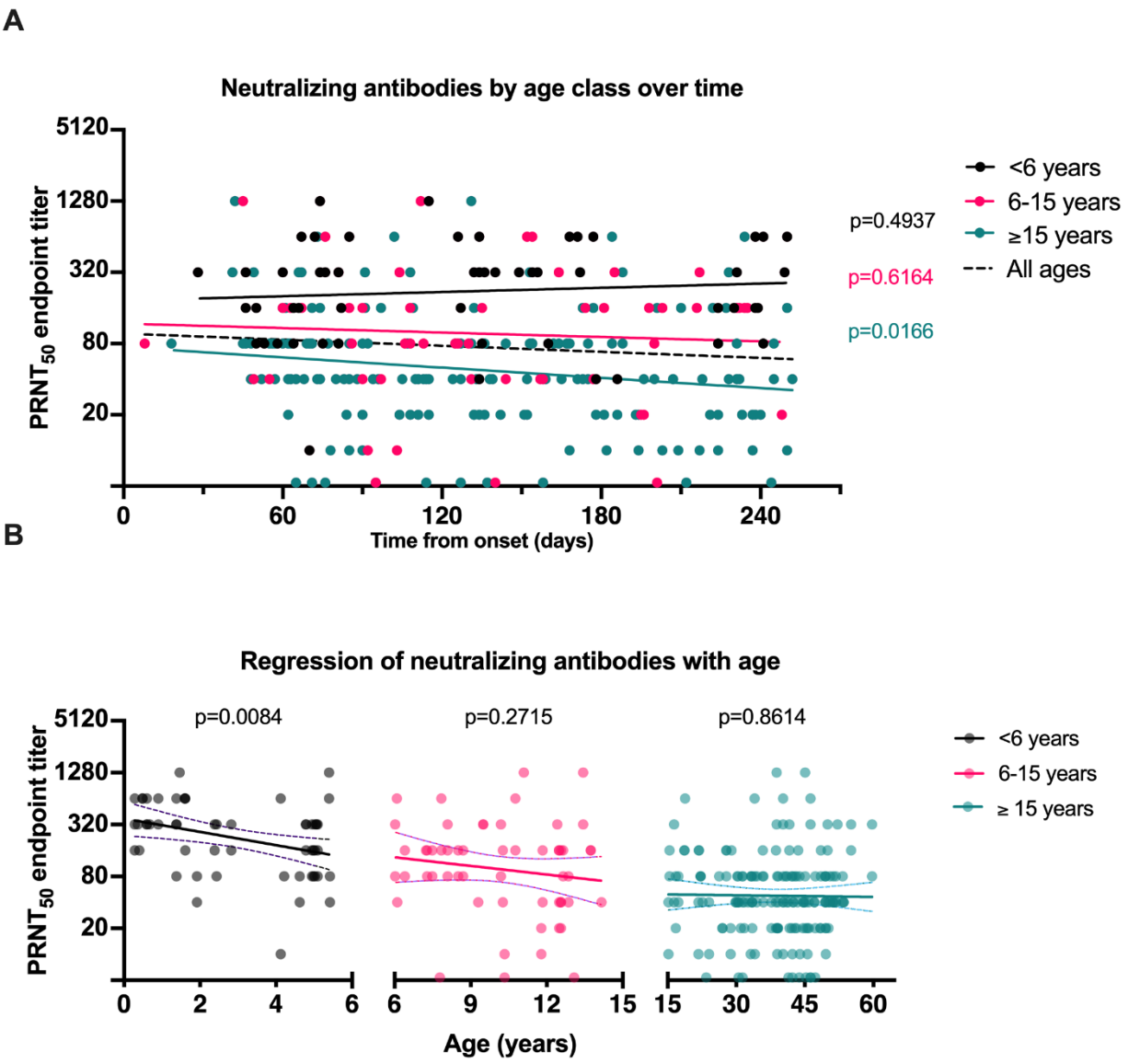

Figure 1. Stability of SARS-CoV-2 neutralizing antibodies titers over time. (A) $PRNT_{50}$ titers from 283 serum samples collected at a median time of 132 days (IQR, 79-187) from infection onset, overall and stratified by three age classes including children <6 years (n=55; R^2 0.0089, p= 0.4937), children ≥6 age <15 (n=58; R^2 0.0047, p= 0.6164) and older siblings/adults ≥15 years of age (n=170; R^2 0.0341, p= 0.0166). (B) Reduced $PRNT_{50}$ titers observed at increasing age, at linear regression analysis conducted among children <6 years (n=55; R^2 0.1239, p= 0.0084), children ≥6 age <15 (n=58; R^2 0.0224, p= 0.2715), and older siblings/adults of ≥15 years of age (n=170; R^2 0.0002, p= 0.8614).

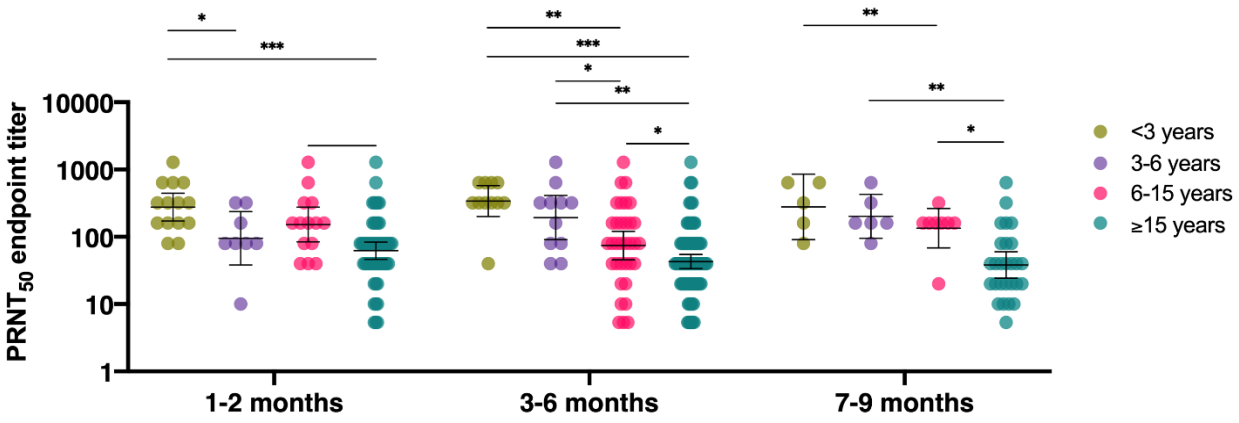

Figure 2. Differences in neutralizing antibodies ($PRNT_{50}$) titers observed among four classes of age. $PRNT_{50}$ titers from 194 serum samples were stratified by age (< 3, ≥3 age <6, ≥6 age <15 and ≥15 years of age), both at 1-2 months, 3-6 months and after disease onset (baseline); * p-value <0.05, ** p-value <0.001, *** p-value <0.0001, Student's T test.

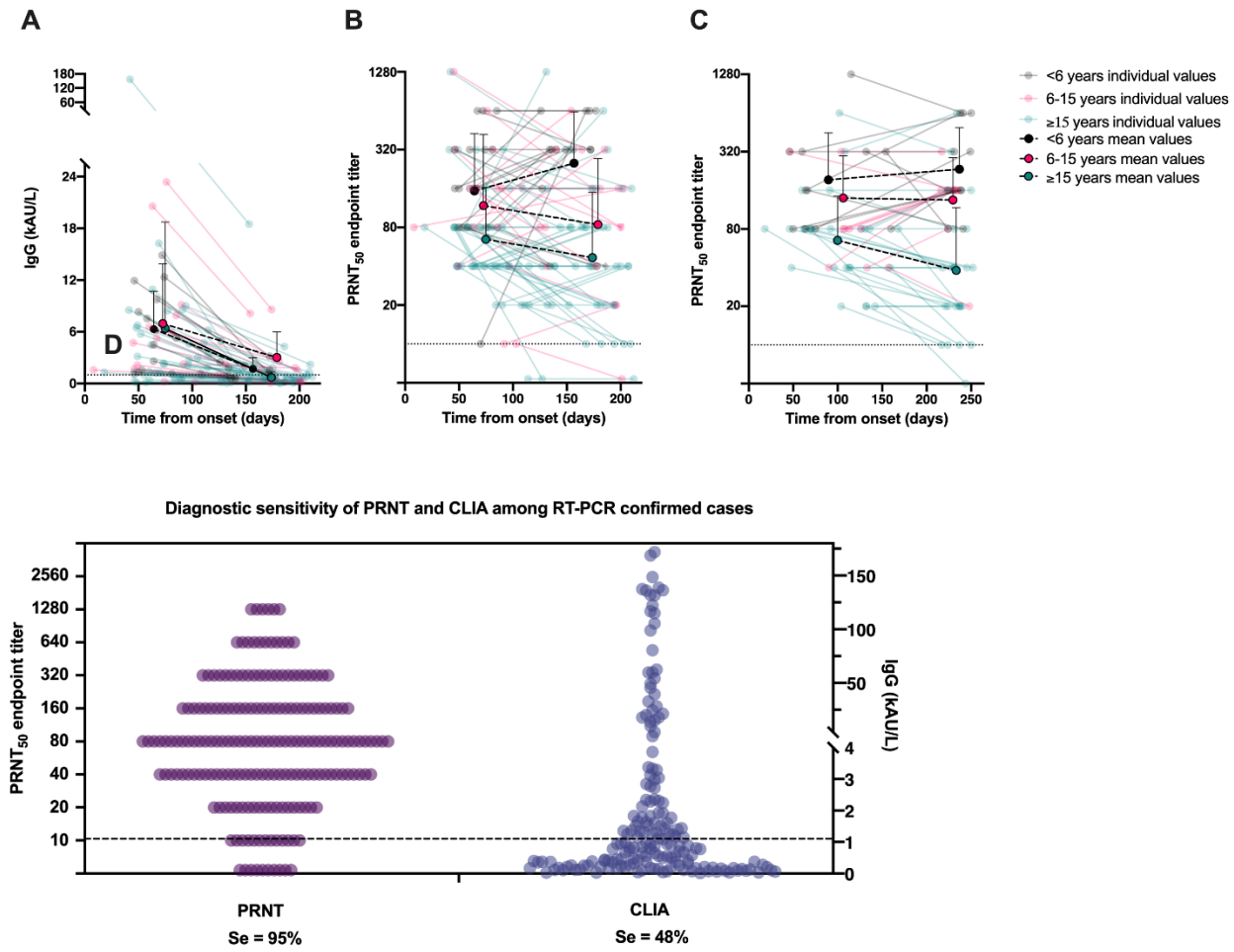

Figure 3. Performance of SARS-CoV-2 CLIA IgG and PRNT titers over time. (A) Decreasing levels of SARS-CoV-2 CLIA IgG levels observed for all classes of age (<6, ≥6 age <15 and ≥15 years; Paired T-Test p-values < 0.0001 across all groups), at longitudinal subject-paired serological assessment of 76 subjects sampled firstly at 72 days (SD, ± 22) and a second time around 169 days (SD, ± 26) after baseline. (B) Kinetics of $PRNT_{50}$ over time, for the same samples shown in (A). (C) Kinetics of $PRNT_{50}$ over time, in a subject-paired evaluation of 50 subjects, for whom paired samples were available around 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 $PRNT_{50}$ 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 cut-off value for $PRNT_{50}$ and CLIA assays, respectively.

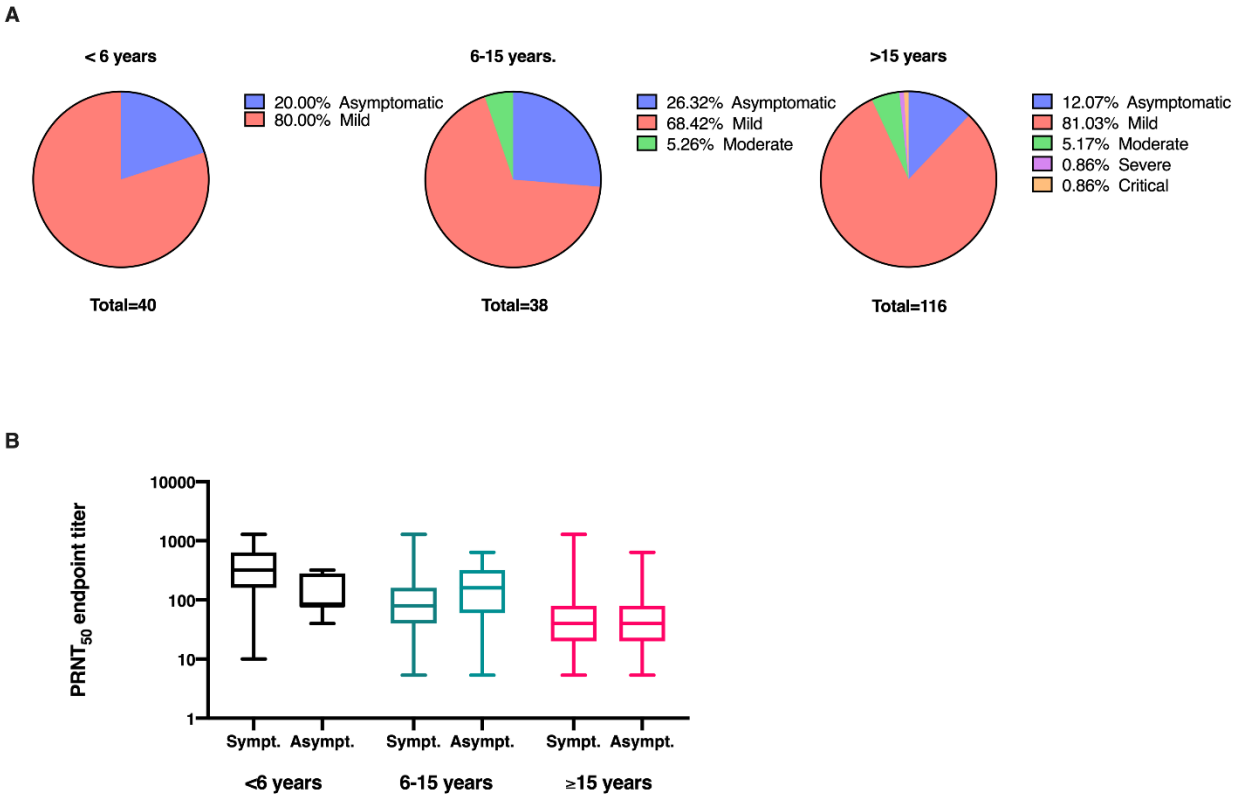

Figure 4. Neutralizing antibodies titers according to COVID-19 disease severity. (A) Clinical presentation of COVID-19 in children aged <6 years, ≥6 age <15 and ≥15 years, according to the WHO COVID-19 Clinical Classification. (B) $PRNT_{50}$ titer distribution among either asymptomatic or symptomatic subjects, stratified by age class and represented by box plots showing minimum, maximum, median, first and third quartiles (<6, ≥6 age <15 and ≥15 years; Wilcoxon test, p=0.0548, p=0.8409 and p=0.6230, respectively).

Supplementary Materials

Serological assays

Blood samples were collected in EDTA-coated tubes to further separate cells and plasma by Ficoll procedure. Plasma and cellular samples were appropriately store at -80°C and liquid nitrogen, respectively, until use. A high-throughput method for Plaque Reduction Neutralizing Test (PRNT) was used for the quantification of neutralizing antibodies in plasma samples [16]. Samples were heat-inactivated by incubation at 56°C for 30 min and 2-fold dilutions were prepared in Dulbecco modified Eagle medium (DMEM). The dilutions, mixed to a 1:1 ratio with a virus solution containing approximately 25 focus-forming units (FFUs) of SARS-CoV-2, were incubated for 1 h at 37 °C. Fifty microliters of the virus-serum mixtures were added to confluent monolayers of Vero E6 cells, in 96-wells plates and incubated for 1 h at 37 °C, in a 5% CO2 incubator. The inoculum was removed and 100 ml of overlay solution of Minimum essential medium (MEM), 2% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 U/ml) and 0.8% carboxy methyl cellulose was added to each well. After a 26-h incubation, cells were fixed with a 4% paraformaldehyde (PFA) solution. Visualization of plaques was obtained with an immunocytochemical staining method using an anti-dsRNA monoclonal antibody (J2, 1:10,000; Sci- cons) for 1 hour, followed by 1 h incubation with peroxidase-labeled goat anti-mouse antibodies (1:1000; DAKO) and a 7 min incubation with the True Blue (KPL) peroxidase substrate. FFUs were counted after acquisition of pictures on a flatbed scanner. Biosafety Level 3 laboratory setting was used for PRNT tests. The neutralization titer was defined as the reciprocal of the highest dilution resulting in a reduction of the control plaque count >50% (PRNT50). Samples recording titers equal to or above 1:10 were considered as positive according to a previous validation conducted on a panel of archive samples collected in 2018 in Italy[1].

Sera from the same donors were analyzed with the chemiluminescence immunoassay (CLIA) MAGLUMI™ 2019-nCoV IgM/IgG on the analytical system MAGLUMI™ 2000 Plus (New Industries

Biomedical Engineering Co., Ltd [Snibe], Shenzhen, China). IgG/IgM immunocomplexes are formed upon addition of a recombinant antigen expressing the full-length spike and nucleocapsid proteins of SARS-CoV-2. According to the manufacturer's inserts (271 2019-nCoV IgM, V2.0, 2020-03 and 272 2019-nCoV IgG, V1.2, 2020-02), the 2019-nCoV IgM cut-off is 1.0 AU/mL, while the 2019-nCoV IgG cut-off is 1.1 AU/mL. The assay is intended for qualitative detection and differentiation of IgM and IgG antibodies. The combined sensitivity and specificity of IgG/IgM is declared to be 95.6% and 96.0%, respectively.

SARS-CoV-2 viral load measurement

A selection of nasopharyngeal (NP) swabs of enrolled subjects that had been originally screened at the Padova University Hospital were made available for quantification of the viral load. NP swabs tested were collected by using flocked swabs in liquid-based collection and transport systems. Total nucleic acids were purified from 200µl media and eluted in a final volume of 100µl. Copies of SARS-CoV-2 were quantified by a home-made multiplex quantitative assay based on One-Step digital droplet PCR (ddPCR). The reaction mixture consisted of 5µl of supermix (Bio-Rad, CA, USA), 2µl of reverse transcriptase, 2µl of DTT final concentration 300mM, forward and reverse primers of SARS-CoV-2 E gene to a final concentration of 400nM each and probe to a final concentration of 200nM and 5µl of nucleic acids were eluted from nasopharyngeal swab samples into a final volume of 20 µl. Housekeeping GAPDH was employed to verify the good quality of RNA extracted and amplified under the same conditions using the GAPDH Kit (PE Applied Biosystems, Waltham, MA, USA) [2]. Each well of the prepared mix was loaded into an 8-channel cartridge and 70µl of the Droplet Generation Oil for Probes (Bio-Rad) were added. Droplets were formed in the QX200TM Droplet Generator (Bio-Rad). Droplets in the oil suspension were transferred into a 96 well plate and placed into a Mastercycler (Eppendorf, Hamburg, Germany) with the following cycling parameters: 42-50°C for 60 min; 95°C for 10min; 95°C for 30sec and 60°C for 1 min; the last two passages were repeated for 40 cycles followed by 98°C for 10 min. The droplets were then read by the QX200TM Droplet Reader (Bio-Rad) and the results were analyzed with the QuantaSoftTM Analysis Software

1.7.4.0917 (Bio-Rad) [2]. Wells with less than 10000 droplets were discarded from the analysis. Each sample was run at least in duplicate. Results were expressed as SARS-CoV-2 copies/5µl.

References

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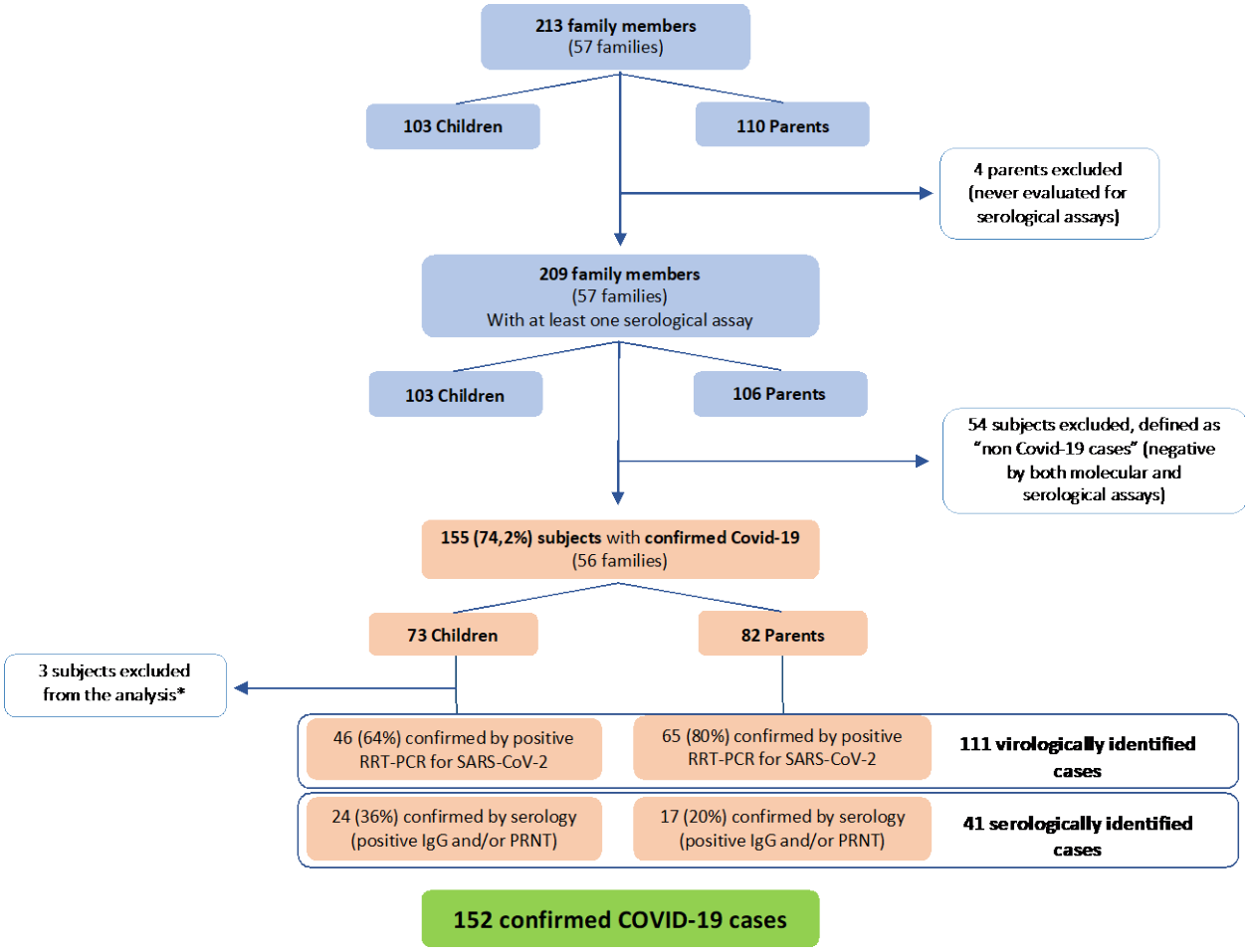
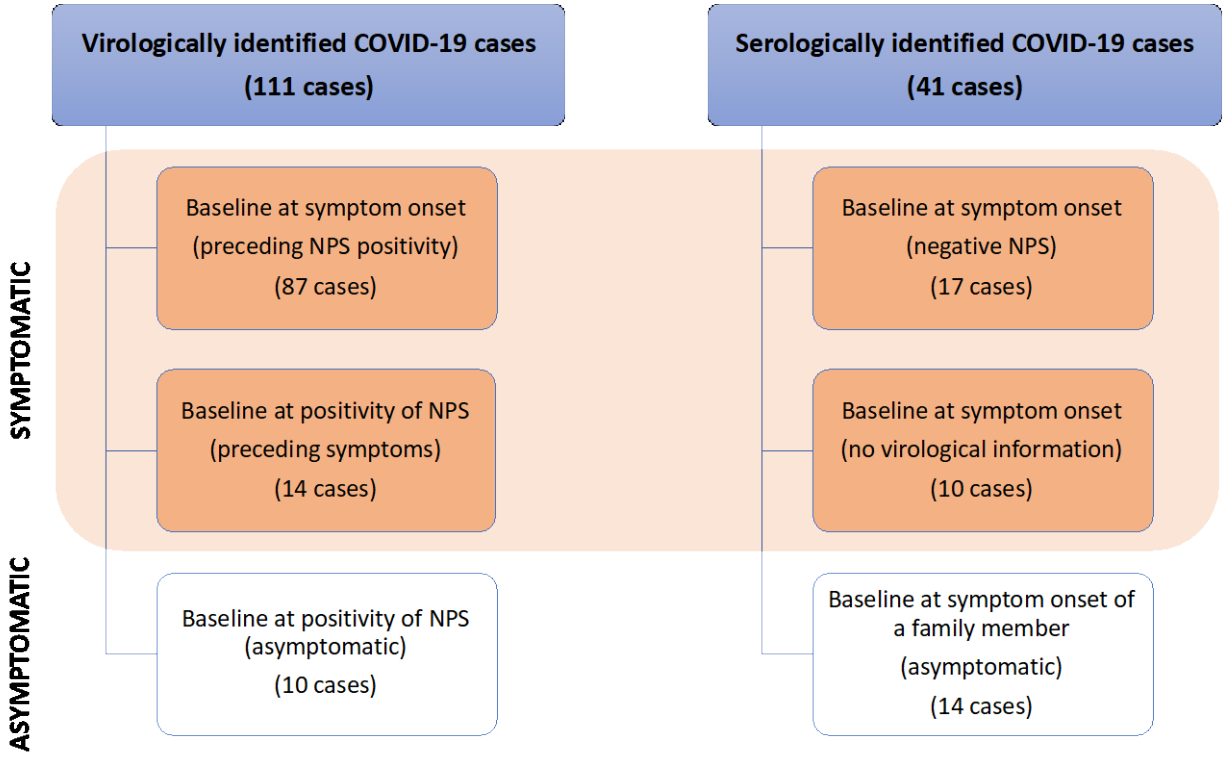
Supplementary Figure S1.

Flow chart of family clusters of COVID-19 observed from March 1st to the September 4th 2020, at the COVID-19 follow-up clinic of the Pediatric Department, Department of Women's and Children's Health, University of Padua.



* 3 children with positive SARS-CoV-2 neutralizing antibodies (PRNT) were further excluded from the analysis as they constituted "peculiar cases" if compared to the general cohort: in fact, 2 children presented MIS-C, 4-6 weeks after Covid-19 onset and 1 newborn of a Covid-19 positive mother presented positive SARS-CoV-2 neutralizing antibodies (PRNT) detected 51 days after birth that could be related to maternal immunity and not seroconversion (SARS-CoV-2 molecular assay was never performed at birth).

Supplementary Figure S2. Identification of cases and criteria for the definition of the *baseline time*, defined as the most likely onset of infection, for confirmed COVID-19 cases.



Supplementary Table S1. Descriptive analysis of the 57 families observed at the Department of Women's and Children's Health of the University Hospital of Padua (Italy), overall (n=209) and stratified by familiar status as children/older siblings (n=103) and parents (n=106).

	OVERALL			CHILDREN/OLDER SIBLINGS			PARENTS		
	COVID-19 positive (n=155)	**COVID-19 negative** (n=54)	*p-value* §	**COVID-19 positive** (n=73)	**COVID-19 negative** (n=30)	*p-value*§	**COVID-19 positive** (n=82)	**COVID-19 negative** (n=24)	*p-value*§
Female (percentage)	81 (52.3%)	23 (42.6%)	0.22	36 (49.3%)	12 (40%)	0.39	45 (54.9%)	11 (45.8%)	0.44
Mean age (SD)	25.8 (17.7)	23.4 (19.5)	0.37	8.75 (6.3)	7.12 (5.7)	0.26	40.9 (8.3)	43.7 (7.4)	0.13
Age classes (n, %):									
< 6 years	28 (18.1%)	15 (27.8%)	0.28	28 (38.4%)	15 (50%)	0.63	0 (0%)	0 (0%)	
6≤ age <15	34 (21.9%)	12 (22.2%)		34 (46.6%)	12 (40%)		0 (0%)	0 (0%)	
≥15 years	93 (60%)	27 (50%)		11 (15.1%)	3 (10%)		82 (100%)	24 (100%)	
Symptomatic (percentage):	128 (82.6%)	15 (27.8%)	<0.001	56 (76.7%)	8 (26.7%)	<0.001	72 (87.8%)	7 (29.2%)	<0.001
WHO classification* (n, %)**:**									
Asymptomatic	27 (17.4%)	39 (72.2%)	<0.001	17 (23.3%)	22 (73.3%)	<0.001	10 (12.2%)	17 (70.8%)	<0.001
Mild	118 (76.1%)	15 (27.8%)		53 (68.8%)	7 (26.9%)		65 (79.3%)	7 (29.2%)	
Moderate	6 (3.9%)	-		1 (1.3%)	-		5 (6.1%)	-	
Severe	1 (0.6%)	-		0 (0%)	-		1 (1.2%)	-	
Critical	1 (0.6%)	-		0 (0%)	-		1 (1.2%)	-	
MIS-C	2 (1.3%)	-		2 (2.6%)	-		0 (0%)	-	
Pediatric comorbidities:									
No	-	-	-	57 (78.1%)	27 (90%)	0.26	-	-	-
Yes**	-	-		16 (21.9%)	3 (10%)		-	-	

§ T student test, $\chi 2$ test, Fisher exact test where appropriate.

*WHO, World Health Organization; MIS-C, Multisystem Inflammatory Syndrome in Children.

**The following co-morbidities were found among 16 COVID-19 positive children: premature birth (n=1), asthma (n=5), allergy (n=1), congenital heart disease (n=1), rheumatic disease (n=1), chronic neuropathy (n=1), immune-deficiency (n=2), cleft lip and palate (n=1), kidney/ureteral disease (n=1).

Supplementary Table S2. Serological data of 283 plasma samples obtained from 152 confirmed COVID-19 cases (38 independent samples, 245 dependent samples obtained from 114 cases) among age classes, overall and stratified by time from baseline.

All data, irrespective of onset

Age Classes (years)	< 3 (n=30)	3 - <6 (n=25)	6 - <15 (n=58)	≥ 15 (n=170)	p-valueł
	GMT (95% CI)	GMT (95% CI)	GMT (95% CI)	GMT (95% CI)	
IgM (kAU/L) ¥	0.7 (0.6 - 0.9)	0.8 (0.6 - 1.1)	0.4 (0.4 - 0.5)	0.5 (0.4 - 0.5)	0,0024
IgG (kAU/L) ¥	1.4 (0.7 - 2.5)	1.5 (0.8 - 2.8)	1.5 (1 - 2.3)	0.9 (0.7 - 1.2)	0.1055
PRNT (endpoint titer)	298.6 (221.4 - 402.6)	155.6 (100.9 - 239.9)	96.7 (68.8 - 135.8)	47.8 (40.2 - 56.7)	<0.0001

At 1 - 2 months, from onset

Age Classes (years)	< 3 (n=14)	3 - <6 (n=8)	6 - <15 (n=14)	≥ 15 (n=57)	p-valueł
	GMT (95% CI)	GMT (95% CI)	GMT (95% CI)	GMT (95% CI)	
IgM (kAU/L) ¥	0.7 (0.6 - 0.9)	0.8 (0.5 - 1.2)	0.6 (0.5 - 0.8)	0.6 (0.5 - 0.7)	0.4902
IgG (kAU/L) ¥	3.8 (2 - 7.3)	4.9 (2.4 - 9.8)	3.9 (1.8 - 8.1)	1.6 (1 - 2.7)	0.0915
PRNT (endpoint titer)	275.8 (171.4 - 443.8)	95.1 (38.1 - 237.8)	152.3 (83.8 - 276.6)	62.2 (46.4 - 83.5)	<0.0001

At 3 - 6 months, from onset

Age Classes (years)	< 3 (n=11)	3 - <6 (n=11)	6 - <15 (n=34)	≥ 15 (n=84)	p-valueł
	GMT (95% CI)	GMT (95% CI)	GMT (95% CI)	GMT (95% CI)	
IgM (kAU/L) ¥	0.7 (0.4 - 1.2)	0.8 (0.4 - 1.4)	0.4 (0.3 - 0.6)	0.5 (0.4 - 0.6)	0.1481
IgG (kAU/L) ¥	0.9 (0.5 - 1.7)	1.6 (0.7 - 3.7)	1.5 (1 - 2.4)	0.8 (0.6 - 1.2)	0.1863
PRNT (endpoint titer)	340.8 (200.8 - 578.5)	193.3 (91 - 410.6)	74.2 (45.6 - 120.6)	42.9 (33.7 - 54.7)	<0.0001

At 7 - 9 months, from onset

Age Classes (years)	< 3 (n=5)	3 - <6 (n=6)	6 - <15 (n=10)	≥ 15 (n=29)	p-valueł
	GMT (95% CI)	GMT (95% CI)	GMT (95% CI)	GMT (95% CI)	
IgM (kAU/L) ¥	0.7 (0.4 - 1.3)	0.7 (0.3 - 2)	0.3 (0.2 - 0.4)	0.3 (0.3 - 0.5)	0.0203
IgG (kAU/L) ¥	0.1 (0.1 - 0.2)	0.3 (0.1 - 0.7)	0.4 (0.2 - 1.2)	0.4 (0.2 - 0.6)	0.4997
PRNT (endpoint titer)	278.6 (90.7 - 855.6)	201.6 (95.1 - 427.3)	134.5 (68.5 - 264.3)	38.1 (24.2 - 60)	<0.0001

¥ Missing data are handled in the analysis ł One-way ANOVA

The following acronyms refer to: GMT, Geometric Mean Titer; 95% CI, 95% confidence interval; PRNT, Plaque Reduction Neutralization Test.

Supplementary Table S3.

Temporal distribution of sample collection among subjects who contributed to the study with either one, two or three plasma samples.

	First sample		Second sample			Third sample	
Time from baseline	**1-2 months**	**3-6 months**	**1-2 months**	**3-6 months**	**7-9 months**	**3-6 months**	**7-9 months**
Subjects with only one sample (n=38)	21	17	0	0	0	0	0
Subjects with only two samples (n=97)	52	45	0	62*	35§	0	0
Subjects with three samples (n=17)	17	0	3	14*	0	2	15§
Total number of samples per period	90	62	3	76	35	0	17
Total number of samples (n=283)	152		114			17	

* second samples included in subject-paired analyses of time window 1 (total of 76)

§ second/third samples included in subject-paired analyses of time window 2 (total of 50)

Supplementary Table S4. Distribution of plasma samples across age classes and baseline intervals.

	Age classes									
Baseline intervals	**< 3 years (n=30)**		**3 - <6 years (n=25)**		**6 - <15 years (n=58)**		**≥ 15 years (n=170)**		**Total (n=283)**	
	No.	**(%)**	**No.**	**(%)**	**No.**	**(%)**	**No.**	**(%)**	**No.**	**(%)**
1-2 months	14	(15.1)	8	(8.6)	14	(15.1)	57	(61.3)	93	(100.0)
3-6 months	11	(7.9)	11	(7.9)	34	(24.3)	84	(60.0)	140	(100.0)
7-9 months	5	(10.0)	6	(12)	10	(20)	29	(58.0)	50	(100.0)

Supplementary Table S5. Estimators of diagnostic accuracy and test agreement of the MAGLUMI™ 2019-nCoV IgG with the PRNT assay as gold standard method. Estimates are calculated using the contingency table of Table S5, plotting 237/255 serological samples tested in the study. Estimates are reported with 95% Confidence intervals.

	Estimate	**95% Confidence Intervals**	
Sensitivity	0.52	0.46	0.58
Specificity	0.85	0.65	1.0
Positive Predictive Value	0.99	0.96	1.0
Negative Predictive Value	0.08	0.04	0.13
Cohen's Kappa	0.08	0.02	0.13
Overall percent agreement	0.54	0.92	0.97
Positive percent agreement	0.52	0.46	0.58
Negative percent agreement	0.85	0.58	0.96

Supplementary Table S6. Correlation between SARS-CoV-2 viral load (genome copies) detected by means of ddPCR in NP swabs collected within 4 days from symptom onset and PRNT titers assessed 1-2 months later, overall and stratified for classes of age.

	NP swabs collected within 4 days from symptom onset		
	N	**Pearson coef.**	**p-value**
All ages	32	-0.00796	0.9655
<15 years	13	0.67250	0.0118
≥15 years	19	-0.29453	0.2209

Mild SARS-CoV-2 Infections and Neutralizing Antibody Titers

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