



Dynamics of Reverse Transcription-Polymerase Chain Reaction and Serologic Test Results in Children with SARS-CoV-2 Infection

Alfredo Tagarro, PhD, MD^{1,2,3}, Francisco José Sanz-Santaefemia, MD⁴, Carlos Grasa, PhD, MD⁵, Elena Cobos, PhD, MD³, Julia Yebra, MD¹, Jose Antonio Alonso-Cadenas, MD⁶, Fernando Baquero-Artigao, MD⁵, Juan Miguel Mesa-Guzmán, MD¹, Beatriz Pérez-Seoane, MD¹, Cristina Calvo, PhD, MD⁵, María Luisa Herreros, PhD, MD¹, Cristina Epalza, PhD, MD^{3,7}, Susana Melendo, MD⁸, Sara Dominguez-Rodriguez, MD³, Paula Vidal, MD⁹, Mónica Pacheco, MD¹, Alvaro Ballesteros, BSc³, María Bernardino, MD², Sara Villanueva-Medina, MD⁷, Paula Rodríguez-Molino, MD⁵, Sandra Miragaya Castro, MD⁶, Jacques Rivière, PhD, MD⁸, Rosa Garcés, MD⁹, Begonia Santiago, PhD, MD¹⁰, Victoria Fumadó, MD¹¹, María Urretavizcaya-Martínez, MD¹², María Luz García-García, PhD, MD¹³, María Penín, PhD, MD¹⁴, Fernando Cava, PhD, MD¹⁵, Elena Sáez, BSc¹⁵, María Isabel Iglesias-Bouzas, MD¹⁶, Blanca Herrero, MD¹⁷, Teresa de Jesús Reinoso, MD¹, and Cinta Moraleda, PhD, MD^{3,7}, on behalf of EPICO-AEP Working Group*

Objectives To determine the time to reverse transcription-polymerase chain reaction (RT-PCR) negativity after the first positive RT-PCR test, factors associated with longer time to RT-PCR negativity, proportion of children seroconverting after proven severe acute respiratory syndrome coronavirus 2 infection, and factors associated with the lack of seroconversion.

Study design The Epidemiological Study of Coronavirus in Children of the Spanish Society of Pediatrics is a multicenter study conducted in Spanish children to assess the characteristics of coronavirus disease 2019. In a subset of patients, 3 serial RT-PCR tests on nasopharyngeal swab specimens were performed after the first RT-PCR test, and immunoglobulin G serology for severe acute respiratory syndrome coronavirus 2 antibodies was performed in the acute and follow-up (<14 and ≥14 days after diagnosis) phase.

Results In total, 324 patients were included in the study. The median time to RT-PCR negativity was 17 days (IQR, 8-29 days), and 35% of patients remained positive more than 4 weeks after the first RT-PCR test. The probability of RT-PCR negativity did not differ across groups defined by sex, disease severity, immunosuppressive drugs, or clinical phenotype. Globally, 24% of children failed to seroconvert after infection. Seroconversion was associated with hospitalization, persistence of RT-PCR positivity, and days of fever.

Conclusions Time to RT-PCR negativity was long, regardless of the severity of symptoms or other patient features. This finding should be considered when interpreting RT-PCR results in a child with symptoms, especially those with mild symptoms. Seroprevalence and post-immunization studies should consider that 11 in 4 infected children fail to seroconvert. (*J Pediatr* 2022;241:126-32).

Studies of serial respiratory viral load and serum antibody responses from children infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are limited.¹ SARS-CoV-2 detection by reverse transcription-polymerase chain reaction (RT-PCR) testing of respiratory samples is used widely to diagnose and monitor infection. A better understanding

From the ¹Pediatrics Department, Hospital Universitario Infanta Sofía; ²Pediatrics Research Group, Europea University of Madrid; ³Fundación de Investigación Biomédica Hospital 12 de Octubre. Instituto de Investigación 12 de Octubre (imas12); ⁴Pediatrics Department, Hospital Infantil Universitario Niño Jesús; ⁵Pediatrics, Infectious and Tropical Diseases Department, Hospital Universitario La Paz, Instituto Investigación Hospital La Paz (IDIPaz); ⁶Emergency Department, Hospital Infantil Universitario Niño Jesús; ⁷Pediatrics Department, Hospital Universitario 12 de Octubre; ⁸Infectious Diseases and Pediatric Immunology Unit, Department of Pediatrics, Hospital Universitario Vall d'Hebron, Madrid; ⁹Department of Pediatrics Department, Hospital Clínico Universitario Lozano Blesa Zaragoza, Aragón; ¹⁰Pediatric Infectious Diseases Unit, Department of Pediatrics, Hospital Universitario Gregorio Marañón, Madrid; ¹¹Pediatric Infectious Diseases Unit, Department of Pediatrics, Hospital Universitario Sant Joan de Deu Barcelona, Barcelona; ¹²Pediatrics Department, Complejo Hospitalario de Navarra, Pamplona; ¹³Pediatrics Department, Hospital Universitario Severo Ochoa Madrid; ¹⁴Pediatrics Department, Hospital Universitario Príncipe de Asturias; ¹⁵Microbiology Department, UR Salud, Hospital Universitario Infanta Sofía; ¹⁶Pediatric Intensive Care Unit, Hospital Infantil Universitario Niño Jesús; and the ¹⁷Oncology Department, Hospital Infantil Universitario Niño Jesús, Madrid, Spain

*List of additional members of the EPICO-AEP Working Group is available at www.jpeds.com (Appendix).

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COVID-19	Coronavirus disease 2019
C _t	Cycle threshold
EPICO-AEP	Epidemiological Study of Coronavirus in Children of the Spanish Society of Pediatrics
IgG	immunoglobulin G
MIS-C	Multisystem inflammatory syndrome in children
RT-PCR	Reverse transcription-polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2

of the dynamics of RT-PCR and serological testing in children with SARS-CoV-2 infection (coronavirus disease 2019 [COVID-19]) may help to inform healthcare decisions, including quarantine strategies and duration.

The severity and progression of SARS-CoV-2 infection has multifactorial associations, including host immunity, and an adequate antibody response might confer protection to future SARS-CoV-2 infection. The dynamics of the antibody response has been well-described in adults.² Studies of multi-system inflammatory syndrome in children (MIS-C) suggest that not all patients develop detectable antibodies despite a strong dysregulated immune response.³ Knowing the proportion of children who do not develop a detectable antibody response and the factors related to the absence of seroconversion might be useful for the diagnosis of post-COVID-19 conditions and reinfections.

The aims of the present study were to determine the time to RT-PCR negativity after the first positive RT-PCR test result, to investigate factors associated with a delayed RT-PCR negative result, to assess the rate of seroconversion after proven SARS-CoV-2 infection, and to examine the factors associated with the absence of seroconversion.

Methods

The Epidemiological Study of Coronavirus in Children of the Spanish Society of Pediatrics (EPICO-AEP) is a multicenter cohort study to assess the characteristics of COVID-19 in Spanish children. The registry includes patients aged 0-18 years evaluated in any 1 of the 76 hospitals of the network, with positive SARS-CoV-2 RT-PCR test or a diagnosis of MIS-C following the World Health Organization criteria.³ Children were tested for SARS-CoV-2 by RT-PCR if they pre-

sented with symptoms consistent with SARS-CoV-2 infection. The study was approved by the Ethics Committee of Hospital 12 de Octubre, Madrid (code 20/101), and the other participating hospitals. Patients were enrolled after signed or verbal consent from parents or guardians and patients older than 12 years.

For the present substudy, patients with SARS-CoV-2 infection confirmed by RT-PCR were offered serial follow-up visits with 3 associated RT-PCR testing episodes if the hospital and the patient's situation permitted. Additionally, the interval recommended between serial RT-PCR testing episodes was 1 week, but the timing depended on logistics, the situation at the center, and the participant. Serology was performed in the acute phase (<14 days after first positive RT-PCR test result) and in the follow-up phase (≥ 14 days after first positive RT-PCR test result).

Eligible participants for this study were those patients with a positive RT-PCR at diagnosis from March 1, 2020, to March 18, 2021, who had at least 2 PCR tests in follow-up. Patients with a diagnosis of MIS-C were excluded because the interval between the SARS-CoV-2 infection and the diagnosis of MIS-C usually is 4-6 weeks and most children with MIS-C are not diagnosed with acute infection.

Laboratory Methods

Respiratory samples were obtained from nasopharyngeal swabs. RT-PCR was performed according to standardized techniques at each center. RT-PCR was considered positive according to the limits provided by the manufacturers. For analysis purposes (Figure 1), negative was considered as 45 cycle thresholds (C_t). In all centers, immunoglobulin G (IgG) antibodies against the SARS-CoV-2 surface S1 domain of the spike protein or against the internal

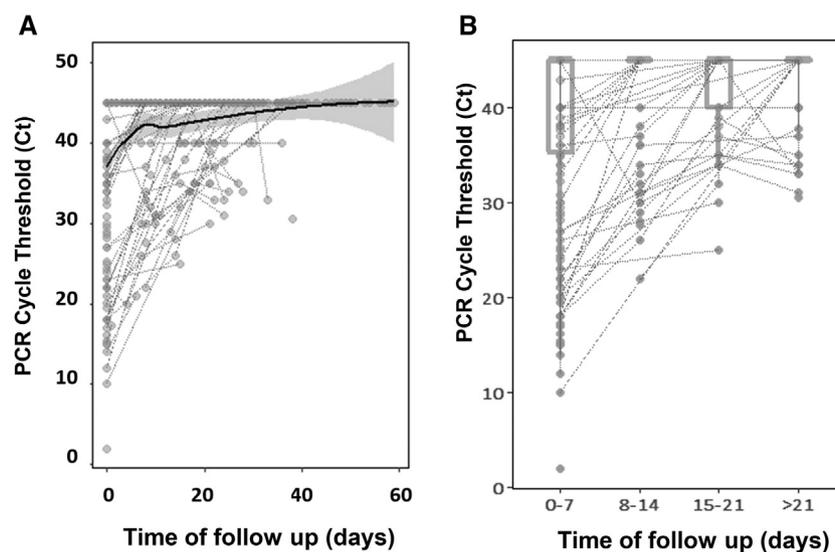


Figure 1. A, C_t over time. The line represents an inference of the trajectory of C_t s until negativity. Negative values are presented as a C_t of 45. Negative values beyond 60 days are not presented. B, Box-and-whiskers distributions of C_t across weeks. Most of the samples had a high C_t value. At diagnosis, the median C_t was 38.5 (IQR, 35.5-40.0).

nucleocapsid protein were measured in ethylenediaminetetraacetic acid plasma samples using standardized commercial techniques.

Data Management and Statistical Analyses

An electronic case report form was built, and the responsible physician of each center completed the form with clinical data and laboratory test results. Data included age, sex, comorbidities, immunosuppressant medication, date of diagnosis, syndromic diagnosis, and serial RT-PCR and serology test results.

Baseline characteristics were described using frequencies for categorical variables and medians (IQR) for continuous variables, both in the total population and after stratification based on RT-PCR test result. The χ^2 or Fisher exact test (<5 frequencies) was applied to assess differences across groups for categorical variables, and the Mann-Whitney test was used for continuous variables.

For the time-to-event analysis, the time to RT-PCR negativity was analyzed using Kaplan-Meier estimates according to socio-demographic and clinical variables including sex, age, days of fever, diagnostic syndrome and severity, and the log-rank test was used for comparison of curves. The time to RT-PCR negativity and association with potential risk factors was assessed using a generalized multivariable linear model. Variable selection for the model was performed through the stepwise Akaike Information Criterion. Two-tailed *P* values were considered significant when the *P* values was less than .05.

Locally estimated scatterplot smoothing splines were fitted to the C_t curves to plot the dynamics of extinct viral load along time to follow-up. For this plot, a C_t of 45 was considered as negative or undetectable. R software was used for all analyses (R Development Core Team, Vienna, Austria, 2009).

Results

RT-PCR Test Results

A total of 324 children from 50 centers were included in the present study and followed for a median of 20 days (IQR, 13-32; range, 0-120 days). The median age was 4.5 years (IQR, 0.6-11.6 years), 188 (58%) children were male, and the median days of fever at diagnosis was 2 (IQR, 0-4). All patients had 2 (100%), 3 (162/324 [50%]), or 4 (86/324 [26.5%]) RT-PCR tests performed during follow-up. The median time between serial RT-PCR tests was 11 days (IQR, 7.0-17.0 days).

A total of 248 of the 324 patients (76.5%) had a negative RT-PCR test result in 1 or more of the serial samples after the first positive RT-PCR; 27 of the 324 (8.3%) did not have a negative result in any of 3 serial samples after the first positive RT-PCR; 49 of the 324 (15.1%) did not complete the protocol of 3 serial samples and were still positive when they were lost to follow-up. Among 27 children who completed 3 sampling episodes but did not achieve RT-PCR negativity, the median time of follow-up was 27 days (IQR, 22-34 days). Among children who did not have 3 tests performed and did not achieve negativity and were lost to follow-up, the median time to follow-up was 14 days (IQR, 9-16 days).

Overall, the median time to RT-PCR negativity was 17 days (IQR, 8.0-29.2). A total of 89 of 248 participants (35.8%) had persistent positive RT-PCR 4 weeks after the first RT-PCR. Notably, 6 children remained positive 90 days after diagnosis. The C_t values increased with time since first positive RT-PCR test.

According to the Kaplan-Meier function estimate, the median time from first positive to first negative test result was 22 days (95% CI, 19-25). The probability of remaining positive at 14 days was 0.66 (95% CI, 0.61-0.71), at 21 days was 0.5 (95% CI, 0.45-0.56), and at 28 days 0.37 (95% CI, 0.31-0.43) (Figure 2; available at www.jpeds.com). The probability of a RT-PCR negative result did not differ between groups with different severity (hospitalized vs not hospitalized), receiving or not receiving immunosuppressive medication, or across different clinical phenotypes at presentation (Figure 3).

A Cox multivariable analysis was performed to investigate whether confounding might have attenuated the suggested association with severity of disease. The covariates included in the raw model were sex, phenotype, age, days of fever, and severity. However, according to the backward stepwise selection method, only sex was selected to be included in the final best fitting model (coefficient, 0.85; 95% CI, 0.66-1.1; *P* = .229). We did not find any factor influencing the probability of achieving a negative RT-PCR test result. There were no differences between patients who achieved or did not achieve negativity during the follow-up period by sex, age, days of fever at diagnosis, clinical phenotype at presentation, immunosuppressive medication, hospitalization, or need for pediatric intensive care unit admission or high-flow oxygen therapy.

The C_t values at diagnosis were available for 84 of 275 patients (30%). Most samples had a high C_t value. At diagnosis, the median C_t was 38.5 (IQR, 35.5-40.0). The C_t information of all the serial RT-PCRs was available for 46 of 84 patients (54%). The trajectory line of C_t s until negativity was reached quickly (around day 5) (value of 45) (Figure 1). After day 8, the median C_t value of the remaining positive patients was above the threshold of positive C_t . We observed a weak, but significant, positive correlation between initial C_t value and time to negativity (*R* = -0.35; *P* = .03)—the higher the C_t , the longer the time to negativity.

Serology

Among 324 children, a total of 136 participated in serology protocol: a first IgG serology test performed within 14 days from the first positive RT-PCR, and a follow-up IgG serology test 14 or more days after the first positive RT-PCR test result. Only 59 of 136 patients (43.3%) completed the protocol with both tests. The time between the first and second IgG test was 28.0 days (IQR, 25.5-34.5; range, 14-45 days).

The first SARS-CoV-2 IgG test was positive within the first 14 days in 43 of 136 children (31.6%) and was negative in 93 of the 136 children (68.4%). Of those with the first negative IgG test result, the follow-up IgG was available for 49 of 93 children (52.7%). Follow-up IgG was positive in 27 of 49 children (55.1%); therefore, 22 of these 49 (44.9%) did not seroconvert

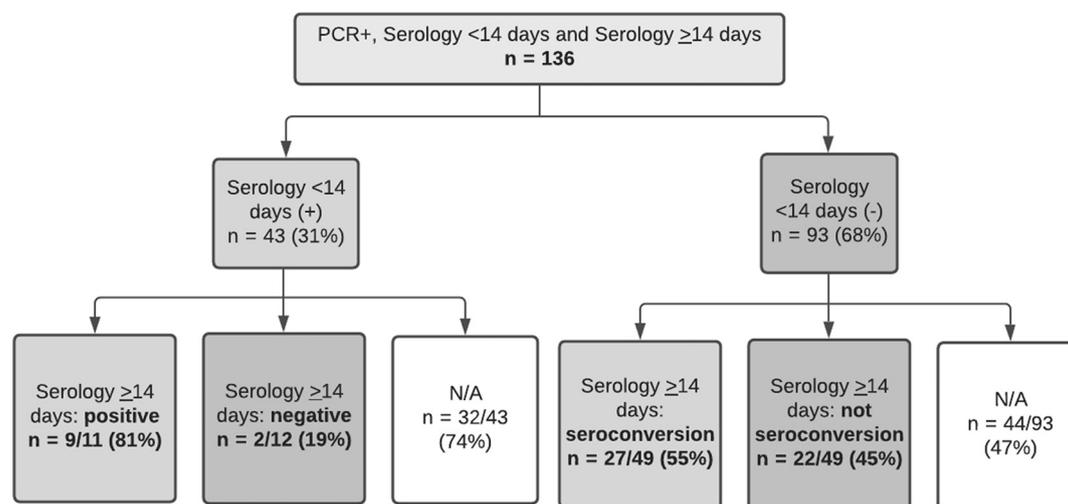


Figure 3. Flowchart of patients with serologic follow-up. Overall, at least 22/93 (23.6%) children with a recent RT-PCR positive test and available serum specimens did not develop detectable IgG SARS-CoV-2 antibodies. *N/A*, not applicable.

over the period of observation (Figure 4). For those who seroconverted, median time from first IgG negative to second IgG positive test was 18 days (IQR, 16.5-31.5). Overall, at least 22 of 93 children (23.6%) with a recent RT-PCR positive test result and available serologies did not develop detectable IgG antibodies against SARS-CoV-2.

We compared the 27 children who seroconverted with the 22 who did not (Table). We excluded from this analysis those patients with a positive IgG less than 14 days after the positive RT-PCR to exclude the possibility of a positive IgG from a prior infection 14 days previously. Absence of seroconversion was observed in 10 of 32 hospitalized patients (31.2%) and in 12 of 17 nonhospitalized patients (70.6%; $P = .02$). Similarly, among patients who never achieved a negative RT-PCR test result during the study follow-up, all patients seroconverted, whereas among patients who achieved a negative RT-PCR test result during the follow-up, 22 of the 40 (55%) did not seroconvert ($P = .02$). Patients who seroconverted had more days of fever (median, 3; IQR 1-4) than patients who did not seroconvert (median, 0; IQR, 0-3; $P = .017$).

Discussion

In our study, after diagnosis with clinical symptoms, the median time to RT-PCR test negativity in children with SARS-CoV-2 infection was 17 days, irrespective of the type or severity of presenting features, and 35% of children remained RT-PCR positive more for more than 1 month after the first positive RT-PCR. We also showed that at least 25% of patients did not develop SARS-CoV-2 IgG antibodies after confirmed infection, particularly those not hospitalized and those with early SARS-CoV-2 RT-PCR clearance.

Time to RT-PCR negativity did not differ by sex, disease severity, receipt of immunosuppressive drugs, or clinical phenotype. Kaplan-Meier analysis indicated a trend for

patients admitted to the pediatric intensive care unit remain positive for a slightly longer time than others, but the CIs overlapped, and the P value was not significant.

In adults with SARS-CoV-2 infection, RT-PCR positivity can persist for weeks after the resolution of symptoms; however, the median time to RT-PCR negativity is unclear. Some studies have reported a median time from 12 to 35 days, but there is large variability depending on age, the severity of the disease and the sample type.^{4,5} In a study of 68 children, patients in the 0- through 5-year age group had a median of 22 days to RT-PCR negativity, and patients aged 6 through 15 years had a median of 32 days, compared with 18 days for patients 16-22 years of age.⁶

In our study, children seemed to have more rapid clearance than adults, which might be related to the high median C_t observed (38; IQR, 35.5-40.0), indicating a low viral load. In adults, the median initial C_t value has been reported to be 28 (95% CI, 27-28).⁷ Our results are consistent with similar studies in children conducted with smaller sample sizes. A Korean study reporting 71 symptomatic children showed a median time to negativity of 17.6 days, a study from Singapore observed a median time of 16 days, and a study of 68 children from the US reported median duration of viral shedding (RT-PCR positivity) of 19.5 days (IQR, 12-39).^{1,6,8} However, it is important to consider that a proportion of patients in our study remained positive for more than 21 days after diagnosis. Consistent with our findings, it has been described that 19% of people continue to show nasopharyngeal RT-PCR positivity 2 or more weeks after the resolution of symptoms.⁹ Likewise, in an Italian study, 28 of 191 adults (14.7%) with a mean age of 51 years (IQR, 41-59), were persistently positive between days 41 and 60 after discharge.⁵ Prolonged or recurrent viral RNA detection from the nasopharynx does not necessarily indicate prolonged viral infectiousness. Isolation of infectious viruses from upper respiratory specimens more than 10 days after the onset of illness has

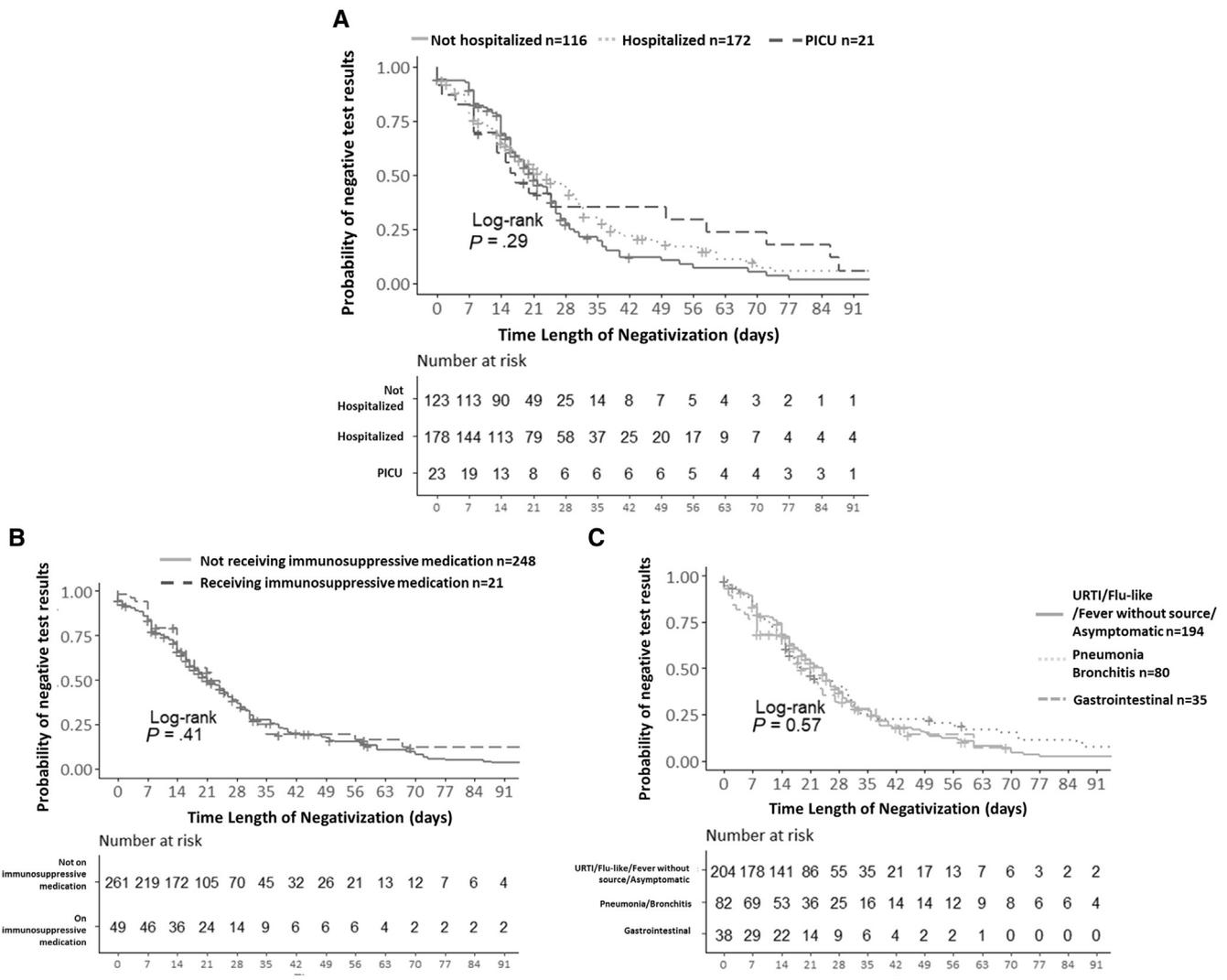


Figure 4. Probability of RT-PCR test negativity in all 324 participants.

only rarely been documented in patients who had a nonsevere infection and whose symptoms had resolved.¹⁰ Rather, our findings should be used to better understand the significance of a positive RT-PCR in children in different settings. A positive RT-PCR should not exclude other possible infections. Also, our findings can help to further our understanding of postinfectious complications such as MIS-C. In children with MIS-C, almost one-half of all patients have positive RT-PCR test results at presentation.³ Possibly, the persistence of the antigenic stimulus may stimulate immune activation, leading to the development of late complications.

Serologic tests play an important role in the clinical diagnosis of postinfectious patients or patients with late-stage SARS-CoV-2-related conditions. Our data suggest that negative SARS-CoV-2 serologic test results alone do not exclude previous SARS-CoV-2 in children. In a previous study, only one-half of the patients demonstrated adequate neutralizing antibodies during the time frame of specimen collection.⁶ This finding is important when considering postinfectious complications such as MIS-C. Interestingly, pa-

tients with mild disease and a shorter time to RT-PCR negativity in our study seroconverted less often than patients with more severe disease and who had more prolonged RT-PCR positivity, suggesting that the level and persistence of viral material may be key to developing antibodies. These findings also suggest that detectable antibodies by routine techniques are not a useful marker for clinical SARS-CoV-2 infection. In contrast, the absence of detectable antibodies may be related to early clearance, mild disease, and possibly a good innate response. We also should consider that some patients may not have initiated seroconversion at the time of the second test. The median time from RT-PCR positivity as studied by to seropositivity, by chemiluminescent testing, has been reported to be 18 days in children.⁶ Our data also should be considered for interpretation of seroprevalence studies, especially in children, who usually have mild disease seem to achieve PCR negativity earlier than adults and may remain seronegative.

Almost all adults seroconvert after confirmed COVID-19.⁹ However, the sensitivity of enzyme-linked immunosorbent

Table. Characteristics of initially IgG-negative patients who had follow-up IgG serology

Characteristics	Total (n = 49)	Not seroconverted (n = 22)	Seroconverted (n = 27)	P value	OR [95% CI]
Sex				.827	
Male	27 (55.1%)	13 (59.1%)	14 (51.9%)		Ref. 1.00
Female	22 (44.9%)	9 (40.9%)	13 (48.1%)		1.33 [0.42-4.30]
Age (months)	NA = 1 (2.0%)	NA = 0 (0.0%)	NA = 1 (3.7%)	.041	
Median [IQR]	38.3 [1.83-130]	93.7 [13.9-163]	10.1 [1.23-78.4]		0.99 [0.98-1.00]
Fever days	NA = 9 (18.4%)	NA = 6 (27.3%)	NA = 3 (11.1%)	.017	
Median [IQR]	2.50 [0.00-4.00]	0.00 [0.00-3.00]	3.00 [1.00-4.00]		1.56 [1.04-2.35]
Immunosuppressive medication				1.000	
No	45 (91.8%)	20 (90.9%)	25 (92.6%)		Ref. 1.00
Yes	4 (8.16%)	2 (9.09%)	2 (7.41%)		0.80 [0.08-8.29]
Severity				.020	
Not hospitalized	17 (34.7%)	12 (54.5%)	5 (18.5%)		Ref. 1.00
Hospitalized (PICU and normal wards)	32 (65.3%)	10 (45.5%)	22 (81.5%)		5.01 [1.43-20.1]
Cycle threshold of RT-PCR at diagnosis	NA = 31 (63.3%)	NA = 10 (45.5%)	NA = 21 (77.8%)	.589	
Median [IQR]	38.5 [35.5-40.0]	38.0 [36.5-40.0]	40.0 [11.5-40.0]		0.96 [0.90-1.03]
PICU or high-flow oxygen necessity				1.000	
No	44 (91.7%)	20 (90.9%)	24 (92.3%)		Ref. 1.00
Yes	4 (8.33%)	2 (9.09%)	2 (7.69%)		0.84 [0.08-8.64]
Time between first (–) and second serology (+)				-	
Median [IQR]	-	-	28.0 [25.5-34.5]		-
Median [Range]			28.0 (14.0-45.0)		
RT-PCR negativity				.022	
No	6 (13.0%)	0 (0.00%)	6 (25.0%)		
Yes	40 (87.0%)	22 (100%)	18 (75.0%)		-
Clinical phenotype				.744	
Asymptomatic	5 (10.2%)	3 (13.6%)	2 (7.41%)		Ref. 1.00
URTI/flu-like/fever without source	30 (61.2%)	12 (54.5%)	18 (66.7%)		2.15 [0.29-20.8]
Pneumonia/bronchitis	7 (14.3%)	4 (18.2%)	3 (11.1%)		1.11 [0.09-14.8]
Gastrointestinal	7 (14.3%)	3 (13.6%)	4 (14.8%)		1.86 [0.17;25.4]

PICU, pediatric intensive care unit; NA, not available; PICU, pediatric intensive care unit; URTI, upper respiratory tract infection. Values in bold are statistically significant (P -value $<.05$).

assay (ELISA) tests targeting the nucleocapsid or spike protein, or both (combined) antigens is 0.79 (95% CI, 0.68-0.88), 0.80 (95% CI, 0.62-0.92), and 0.86 (95% CI, 0.68-0.91), respectively.⁴ From our data, we cannot state definitively whether the proportion of children who did not seroconvert did not develop a humoral response, or if the ELISA techniques were unable to detect the antibodies.

The strengths of our study include a large sample size, serial testing, and a multicenter approach. Our study has several limitations. As a multicenter study, the techniques used for virus detection and antibody assays were not uniform. Also, we did not analyze neutralizing antibodies or the cross-reactivity of serology tests for SARS-CoV-2, although the specificity of ELISA and chemiluminescent immuno assay ranges from 92% to 100%.⁴ Owing to the exceptional situation, mainly during the first months of the pandemic, several patients were lost to follow-up, and there was some variability in the timing between tests. C_t information was available for a proportion of participants only, so the analysis related to C_t should be interpreted with caution. Reporting C_t values as a tool for assessing with exactitude the viral load has been challenged.^{11,12} However, other reports have reported a good correlation between C_t value and viral loads in samples obtained from children with SARS-CoV-2 infection ($r = -1$), with lower values of viral loads in asymptomatic children and children aged less than 1 year.¹³ Some centers are reporting C_t values after normalization or in a semiquantitative format and, in practice, most centers use the C_t as a proxy for a high or low viral load. Regarding serologic results, because there was appreciable dropout from the 136

included to the 59 who completed the protocol, a selection bias should be considered. ■

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Reprint requests: Teresa de Jesús Reinoso, Pediatrics Department, Hospital Universitario Infanta Sofía, Madrid, Spain. E-mail: treinosol@gmail.com

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50 Years Ago in *THE JOURNAL OF PEDIATRICS*

50 Years into Understanding Hemolytic-Uremic Syndrome

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Hemolytic-uremic syndrome (HUS) was first described in 1955 by Gasser et al after identifying a clinical triad of anemia, acute kidney failure, and thrombocytopenia among pediatric patients who presented with gastroenteritis. However, its epidemiology, pathophysiology, and prognosis were uncertain at the time, given the small number of cases.

Five decades later, the overall incidence of HUS is said to be about 1-2 cases per 100 000 per year, with a peak incidence among ages 3-5 years old. HUS is predominantly associated with enterocolitis from the Shiga-toxin-producing *Escherichia coli* in US and Europe. In other countries, *Shigella dysenteriae* type 1 is the main bacteria isolated and, although uncommon, approximately 5% of cases are related with infection by *Streptococcus pneumoniae*.¹ HUS pathophysiology, although complex, is likely due to a renal insult from microangiopathy affecting the glomerulus and arterioles, leading to acute renal and multisystem organ failure, including the central nervous system.

Risk factors for developing HUS include certain autoimmune diseases, recent hemopoietic stem cell transplantation, malignant hypertension, malignancy, pre-existing nephropathy, use of nephrotoxic drugs, and hereditary cobalamin deficiency. A rare but severe form of the disease, known as an atypical HUS, has been described in patients with an overexpression of the alternative complement pathway: this is characterized by a genetic mutation of the complement pathway in patients with no known predisposing factors.²

Early identification and management allow correction of volume status. Pressor support has been shown to improve clinical outcomes. As intensive care units became more equipped to manage patients with HUS, with dialysis and eculizumab, the grim prognosis from HUS has improved with a 10-fold decrease in mortality rate from 30%-50% to 3%-5%. Nonetheless, the evident 10% risk of developing chronic kidney disease in these patients remains a current challenge faced by pediatricians and intensivists.³ Although the prognosis and treatment of HUS have progressed greatly the past 50 years, there is still a long way to go and many questions to be answered.

Mariana Ramírez-Lara, MD

Karen González-Moctezuma, MD

Pediatrics Residency Program

Tecnológico de Monterrey

Escuela de Medicina y Ciencias de la Salud

Monterrey, Nuevo León, México

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Appendix

Additional members of the EPICO-AEP Working Group

Francisco José Sanz, María Isabel Iglesias-Bouzas, Jose Antonio Alonso Cadenas, Blanca Herrero (Hospital Infantil Universitario Niño Jesús, Spain), Teresa del Rosal, Ana Méndez-Echevarría, Talía Sainz, Clara Udaondo, Fernando Baquero, Cristina Calvo, Carlos Grasa, Paula R Molino, María José Mellado, María Ceano, Victor Galán, Paula Garcia Sanchez, Sonsoles San Roman (Hospital Universitario La Paz, Spain), Alicia Hernanz Lobo, Mar Santos, Marisa Navarro, Elena Rincón, David Aguilera, Begoña Santiago, Jorge Huerta, Eduardo Bardón, Jorge Lorente (Hospital Universitario Gregorio Marañón, Spain), Pablo Rojo, Daniel Blázquez, Luis Prieto, Elisa Fernández-Cooke, David Torres-Fernández, Ángela Manzanares, Jaime Carrasco, Cristina Epalza, Jesús Contreras, Sara Domínguez, Sara Villanueva, Arantxa Gonzalez (Hospital Universitario 12 de Octubre, Spain), Cinta Moraleda, Alfredo Tagarro, Elena Cobos, Álvaro Ballesteros, Sara Domínguez-Rodríguez (Instituto de Investigación 12 de Octubre, Spain), Gemma Pons, Silvia Simó, Miguel Lanaspá, Victoria Fumadó, Rosa María Pino, María Melé (Hospital Sant Joan de Déu, Spain), María Espiau, Jacques G. Rivière, Pere Soler-Palacín, Antonio Soriano Arandes, Natalia Mendoza (Hospital Universitari Vall d'Hebron, Spain), Mercedes Herranz, María Urretavizcaya Martínez (Complejo Hospitalario de Navarra, Spain), Fernando Cabañas, Fátima Ara, Marta Baragaño (Hospital Universitario Quirónsalud Madrid, Spain), Rut del Valle, Ana González-de-Zárate, Mónica Pacheco, María Luisa Herreros, Julia Yebra, Beatriz Pérez-Seoane, María Fernández, Teresa Raga, María de la Serna, Ane Plazaola, Juan Miguel Mesa, Rosa Batista, Ana Barrios, Ignacio Navarro, Jana Rizo, Teresa Reinoso, Alfonso Cañete (Hospital Universitario Infanta Sofía, Spain), María Dolores Martín, Elena Sáez, Olga Nerea Coya, Fernando Cava (BR Salud, Spain), Enrique Otheo, Juan Carlos Galán, José Luis Vázquez, Carmen Vázquez, Victor Quintero (Hospital Universitario Ramón y Cajal, Spain), Lola Falcón, Olaf Neth, Peter Olbrich, Walter Goicoechea, Cati Márquez, Marisol Camacho, Inés Marín Cruz (Hospital Universitario Virgen del Rocío, Spain), Laura Martín (Hospital Universitario Regional de Málaga, Spain), Lucía Figueroa (Hospital de Villalba, Spain), María Llorente (Hospital Universitario del Sureste, Spain), María Penin, Claudia García, María García, Teresa Alveredo (Hospital Universitario Príncipe de Asturias, Spain), M^a Inmaculada Olmedo, Agustín López, María Jose Pérez (Hospital Universitario Puerta de Hierro, Spain), Elvira Cobo (Hospital Fundación Alcorcón, Spain), Mariann Tovizi (Hospital del Tajo, Spain), Pilar Galán (Hospital Universitario de Fuenlabrada, Spain), Beatriz Soto, Sara Guillén (Hospital de Getafe, Spain), Adriana Navas (Hospital Universitario Infanta Leonor, Spain) M. Luz García (Hospital de Leganés, Spain), Sara Pérez (Hospital de Torrejón, Spain), Amanda Bermejo,

Pablo Mendoza (Hospital de Móstoles, Spain), Gema Sabrido (Hospital Rey Juan Carlos, Spain), María José Hernández (Hospital Central de la Defensa, Spain), Ana Belén Jiménez (Fundación Jiménez Díaz, Spain), Arantxa Berzosa, José Tomás Ramos, Marta Illán (Hospital Clínico San Carlos, Spain), Ana López, Nerea Gallego (Hospital Universitari Son Espases, Spain), Beatriz Ruiz (Hospital Universitario Reina Sofía, Spain), Santiago Alfayate, Ana Menasalvas, Eloísa Cervantes (Hospital Clínico Universitario Virgen de la Arrixaca, Spain), María Méndez (Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Spain), Ángela Hurtado (Instituto Hispalense de Pediatría, Spain), Cristina García, Inés Amich, Yolanda Ruiz (Hospital San Pedro, Spain), Manuel Oltra, Álvaro Villarroya-Villalba, Adela Cañete, Bienvenida Argiles (Hospital Universitari i Politècnic La Fe, Spain), Angustias Ocaña (Hospital La Moraleja, Spain), Isabel Romero, María Fernanda Guzmán (Hospitales Madrid, Spain), M.J. Pascual (Hospital Nisa, Spain), María Sánchez-Códez (Hospital Universitario Puerta del Mar, Spain), Elena Montesinos (Consorci Hospital General Universitari de València, Spain), Julia Jensen, María Rodríguez (Hospital Universitario Infanta Cristina, Spain), Gloria Caro (Hospital Universitario Infanta Elena, Spain), Neus Rius, Alba Gómez (Hospital Universitari Sant Joan de Reus, Spain), Rafael Bretón (Hospital Clínico Universitario de Valencia, Spain), Margarita Rodríguez, Julio Romero, Juan Francisco Pascual Gazquez (Hospital Universitario Virgen de las Nieves, Spain), Ana Campos (Hospital Universitario Sanitas La Zarzuela, Spain), Mercedes García (Hospital de Mérida, Spain), Rosa María Velasco (Complejo Hospitalario de Toledo, Spain), Zulema Lobato (Althaia, Xarxa Assistencial Universitària de Manresa, Spain), Fernando Centeno, Elena Pérez, Alfredo Cano (Hospital Universitario Río Hortega, Spain), Paula Vidal (Hospital Clínico Universitario Lozano Blesa, Spain), Corsino Rey, Ana Vivanco, Maruchi Alonso (Hospital Universitario Central de Asturias, Spain), Pedro Alcalá, Javier González de Dios, Laura Ureña Horno (Hospital General Universitario de Alicante, Spain), Eduard Solé, Laura Minguell (Hospital Universitari Arnau de Vilanova, Spain), Itziar Astigarraga, Olatz Villate, Susana García Obregón (Hospital Universitario de Cruces, Spain), M^a Ángeles Vázquez, Miguel Sánchez, Leticia Martínez Campos (Hospital Universitario Torrecárdenas, Spain), Elena Díaz (Hospital Virgen de la Luz, Spain), Eduardo Consuegra, Susana Riesco, Almudena González, Maika Mendoza (Hospital Universitario de Salamanca, Spain), María Cabanillas (Complejo Asistencial Universitario de Palencia, Spain), Yeray Novoa-Medina, Elena Colino-Gil, Ana Reyes-Domínguez, Luis Peña-Quintana (Hospital Universitario Materno Infantil de las Palmas, Spain), Elisa Garrote, Maite Goicoechea (Hospital Universitario de Bazarro, Spain), Irene Centelles (Hospital General Universitari de Castelló, Spain), Santiago Lapeña, Sara Gutiérrez, Soraya Gutiérrez (Complejo Asistencial Universitario de León, Spain), Amparo Cavalle (PIUS Hospital de Valls, Spain), José María Olmos (Hospital Mare de Déu dels Lliris, Spain), Alejandro Cobo, Sara Díaz, Cristina Martínez Faci, Macarena

Gonzalez Cruz, Beatriz Castro (Hospital Universitario de Canarias, Spain), Beatriz Jiménez, Cristina Alvarez Alvarez (Hospital Universitario Marqués de Valdecilla, Spain), Raúl González (Hospital Sant Joan d'Alacant, Spain), Miguel Lafuente, Matilde Bustillo (Hospital Infantil de Zaragoza, Spain), Natividad Pons, Julia Morata (Hospital Lluís Alcanyis, Spain), Elsa Segura (Hospital Universitario Son Llatzer de Palma de Mallorca, Spain), María Bernardino (Universidad Europea de Madrid, Spain), Marta Pareja León (Complejo Hospitalario Universitario de Albacete, Spain), Ana Domingo Ruiz (Hospital de Manacor, Spain),

Eider Oñate, Nagore Garcia de Andoin Baran (Hospital Universitario Donostia, Spain), Nerea Domínguez-Pinilla (Hospital Virgen de la Salud, Spain), María Teresa Coll Sibina (Hospital General de Granollers, Spain), María Jesús García García (Hospital Universitario de Cáceres, Spain), Marta Osuna (Hospital HM Montepríncipe, Spain), Raquel Portugal (Hospital Universitario de Burgos, Spain), Leonor García Maset (Hospital de Sagunto, Spain), Belén Sevilla (Hospital Universitario San Cecilio Granada, Spain), Noelia Berciano Jiménez (Hospital Virgen Macarena Sevilla, Spain).

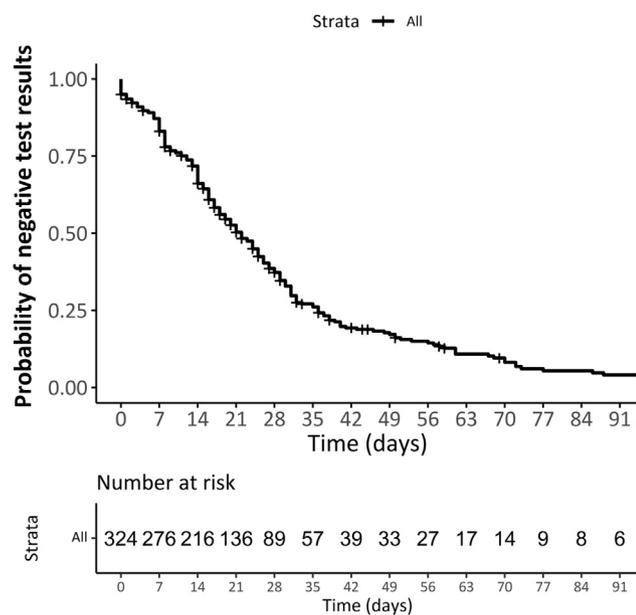


Figure 2. Probability of RT-PCR negativity by **A**, severity, **B**, immunosuppressive medication or **C**, phenotype. *PICU*, pediatric intensive care unit; *URTI*, upper respiratory tract infection.