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Virological and immunological features of SARS-COV-2 infected children with distinct symptomatology

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Abstract

Background: Although SARS-CoV-2 immunizations have started in most countries, children are not currently included in the vaccination programs; thus, it remains crucial to define their anti-SARS-CoV-2 immune response in order to minimize the risk for other epidemic waves. This study sought to provide a description of the virology ad anti-SARS-CoV-2 immunity in children with distinct symptomatology.

Methods: Between March and July 2020, we recruited 15 SARS-CoV-2 asymptomatic (AS) and 51 symptomatic (SY) children, stratified according to WHO clinical classification. We measured SARS-CoV-2 viral load using ddPCR and qPCR in longitudinally collected nasopharyngeal swab samples. To define anti-SARS-CoV-2 antibodies, we measured neutralization activity and total IgG load (DiaSorin). We also evaluated

Nicola Cotugno and Alessandra Ruggiero are first sharing authorships.

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antigen-specific B and CD8+T cells, using a labeled S1+S2 protein and ICAM expression, respectively. Plasma protein profiling was performed with Olink.

Results: Virological profiling showed that AS patients had lower viral load at diagnosis ($p = .004$) and faster virus clearance ($p = .0002$) compared with SY patients. Anti-SARS-CoV-2 humoral and cellular response did not appear to be associated with the presence of symptoms. AS and SY patients showed similar titers of SARS-CoV-2 IgG, levels of neutralizing activity, and frequency of Ag-specific B and CD8+ T cells, whereas pro-inflammatory plasma protein profile was found to be associated with symptomatology.

Conclusion: We demonstrated the development of anti-SARS-CoV-2 humoral and cellular response with any regard to symptomatology, suggesting the ability of both SY and AS patients to contribute toward herd immunity. The virological profiling of AS patients suggested that they have lower virus load associated with faster virus clearance.

KEYWORDS

Ag-specific cellular response, asymptomatic patients, neutralization humoral activity, SARS-CoV-2, symptomatic patients

1 | INTRODUCTION

While SARS-CoV-2 immunization programs have started in most countries, the achievement of herd immunity still seems far ahead. Indeed, due to different restrictive measures among countries and the lack of vaccination programs for children, the risk for second epidemic waves and health system overburden remains high. Children infected by SARS-CoV-2 usually present with a milder course of disease compared with COVID-19 adults, with a consistent proportion of being fully asymptomatic (AS).[1] Pathogenic reasons underlying such differences have been poorly defined.[2] Children play a crucial role in SARS-CoV-2 transmission and in epidemic waves, especially in school settings.[3–5] Test and trace interventions, implemented by government policies for epidemic control, may fail in the pediatric population, where AS patients range between 5% and 16%[6–9] and where a consistent proportion remain undiagnosed.[10] In this scenario, some scientific questions arise about AS patients: (i) Do they present the same virological features of symptomatic (SY) patients?; (ii) do they develop an adaptive and protective immune memory response against SARS-CoV-2?; and (iii) are there inflammatory cytokines profiles associated with clinical manifestations?

Several hypotheses have been advanced in the attempt to explain the AS status of SARS-CoV-2–infected patients, but no specific investigations on pediatric population are available. The humoral SARS-CoV-2 responses showed a lower level of anti-S IgG in children than in adults,[11] considering both pediatric patients developing multi-inflammatory syndrome associated with COVID-19 (MIS-C) and those with a milder clinical presentations. Our results in MIS-C[12] showed the absence of preexisting humoral responses upon other "common cold coronaviruses" in comparison with mild COVID-19 children. However, the true influence of preexisting humoral and T-cell responses toward coronaviruses[13] on mitigating symptoms in children still needs to be

Key Message

SARS-CoV-2–infected children were presented with a milder course of disease compared with COVID-19 adults, with a consistent proportion of being fully asymptomatic (AS). Such difference in clinical presentations is still poorly defined, even though this population plays a crucial role in SARS-CoV-2 transmission. In this work, we attempted to fill this gap in knowledge by providing a virological and immunological characterization of SARS-CoV-2–infected children with distinct symptomatology. Comparison of SARS-CoV-2 viral load between AS and symptomatic (SY) patients showed that AS patients had lower viral load at admission and faster virus clearance. Immunological analysis revealed that AS patients are able to develop SARS-CoV-2–specific adaptive immunity at similar level compared with SY patients in terms of total SARS-CoV-2 Ab, Ab-mediated neutralization, and Ag-specific B and CD8 T cells. Conversely, the analysis of plasma profiling showed differences between AS and SY patients, supporting that pro-inflammatory mechanisms may drive worse clinical outcome in SY patients. Overall, our results show that AS patients have lower viral load in upper respiratory tract at diagnosis suggesting a lower infectivity potential compared with SY patients. Furthermore, development of both humoral and cell-mediated immunity is not associated with symptomatology, demonstrating that AS patients importantly contribute to achieve herd immunity at similar levels compared with SY patients. These data may inform alternative diagnostic algorithm to establish mitigated restrictive measures for AS patients.

defined. In addition, the magnitude of the inflammatory phase associated with viral infection in severe cases[14] may represent a distinctive feature of AS children compared with SY children.

In the present work, we attempt to define virological and immunological characteristics of 15 AS patients compared with 51 SY patients in order to define their ability to produce anti-SARS-CoV-2 immunity. AS and SY patients were further compared with 11 SARS-CoV-2–negative (CoV-2-) patients that were enrolled for suspicion of COVID-19, but that tested negative to both nasopharyngeal swab and serology.

2 | METHODS

2.1 | Study participants

Sixty-six SARS-CoV-2–infected children (CoV-2+) and 11 SARS-CoV-2–negative controls (CoV-2) were enrolled from March to April 2020 at Bambino Gesù Children's Hospital in Rome for the CACTUS (Immunological studies in **C**hildren **A**ffe**CT**ed by COVID and ac**U**te re**S**piratory diseases). The study was approved by local ethical committee, and written informed consent was obtained from all participants or legal guardians. Age, gender, and clinical and routine laboratory characteristics are described in Table 1. Inclusion criteria for positive cases were detection of SARS-CoV-2 in nasopharyngeal (NP) swab using SARS-CoV-2 real-time reverse transcriptase-polymerase chain reaction (RT-PCR) tests (GeneXpert, Cepheid, Sunnyvale, CA; 250 copies/mL sensitivity and 100% specificity). Serology was performed as additional confirmatory test using LIAISON® SARS-CoV-2 S1/S2 IgG test (DiaSorin, Stillwater, MN, USA). CoV-2+ patients were stratified according to WHO clinical classification (https://www.who.int/publications/i/item/WHO-2019-nCoV-clinical-2021-1) as follows: (i) asymptomatic CoV-2+ (AS) patients without any symptoms despite confirmed SARS-CoV-2 infection that were summoned to the hospital since they belonged to the same nuclear family of symptomatic patients; and (ii) symptomatic CoV-2+ (SY) patients. Full list and timing of symptoms are specified in Tables S1 and S2. All SARS-CoV-2–infected children were admitted to the hospital. We also included 11 SARS-CoV-2–negative children with suspected SARS-CoV-2 infection, which was excluded by: (i) two consecutive nasopharyngeal swabs, performed at enrollment and after 24 h; and (ii) SARS-CoV-2 serology, performed at discharge (approximately 7–10 days after admission).

2.2 | Sample collection and storage

Prior to therapy initiation, venous blood was collected in EDTA tubes and processed within 2 h. Plasma was isolated from blood and stored at −80°C. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll and cryopreserved in liquid nitrogen. PBMCs and plasma used for the "acute"-phase analysis were collected on the same day of first positive SARS-CoV-2 PCR. NP swab–preserving media were stored at −80°C until use. Virological analysis was performed on NP collected at diagnosis and every 48 h up to virus clearance. Serologic analysis was performed on vials collected at diagnosis and after 10–14 days (herein referred to as "post-acute phase").

2.3 | SARS-CoV-2 viral load measurement in swabs by ddPCR

Nasopharyngeal swabs were collected by using flocked swabs in liquid-based collection and transport systems. Total nucleic acids were purified from 200 μl NP swab–preserving media and eluted in a final volume of 100 μl. Copies of SARS-CoV-2 were quantified by a home-made multiplex quantitative assay based on One-Step

TABLE 1 Continuous data were presented as mean (SD) calculated on the total number of patients, unless otherwise stated.

	SARS-CoV-2+ (N = 66)			
	Asymptomatic (AS, *N* = 15)	**Symptomatic (SY, *N* = 51)**	***SARS-CoV-2 neg* (*N* = 11)**	***p* value**
Age mean years (SD)	4.6 (3.4)	7.4 (5.6)	6.2 (5.5)	n.s.
Male *N* (%)	6/15 (40%)	32/51 (63%)	5/11 (45%)	n.s.
Platelets, mean 10^3/μl (SD)	309.1 (97.3)	293.5 (134.4), *n* = 50	390.9 (201.5)	n.s.
WBC, mean 10^3/μl (SD)	7.4 (2.7)	6.4 (2.6)	9.4 (3.8)	SY vs SARS-CoV-2neg $p = .006$
Neutrophils, mean 10^3/μl (SD)	2.9 (1.4)	2.5 (1.8), *n* = 50	4.8 (3.1)	SY vs SARS-CoV-2neg $p = .003$
Lymphocytes, mean 10^3/μl (SD)	3.9 (1.9)	3.5 (2.2), *n* = 48	3.4 (1.3)	n.s.
Hb, mean g/dl (SD)	12.0 (2)	12.8 (1.7), *n* = 50	12.1 (1.4)	n.s.
CRP, mean mg/dl (SD)	0.46 (1.3), *n* = 10	1.6 (3.8), *n* = 41	6.5 (6.8), *n* = 11	AS vs SY $p = .01$ AS vs SARS-CoV-2neg $p = .0002$ SY vs SARS-CoV-2neg $p = .0001$

Note: The Mann-Whitney test was used for comparison.

Abbreviations: n, Number of patients available for the analysis; N, Number of patients included in this study; n.s, not significant; SD, standard deviation.

RT-ddPCR, as previously described.[15–17] Each sample was run at least in duplicate. Results were expressed as SARS-CoV-2 copies/5 μl.

2.4 | Allplex™ 2019-nCoV assay

Nasopharyngeal swabs were longitudinally collected from CoV-2+ patients and analyzed in the clinic using the multiplex Allplex™ 2019-nCoV Assay (Seegene, Seoul, South Korea) following the manufacturer's instructions. This assay is routinely used in the clinic to determine SARS-CoV-2 infections, and it was certified and accepted by the Italian Minister of Health. The analyzed genes were RdRp and N gene of COVID-19 and E gene of *Sarbecovirus*.

2.5 | Virus titration by focus-forming assay (FFA)

Focus-forming assay was performed as previously described.[17] Focus-forming units per ml (FFU/ml) were counted after acquisition of pictures at a high resolution of 4,800 × 9,400 dpi, on a flatbed scanner.

2.6 | Ab-mediated–neutralizing activity measured with plaque reduction neutralization test (PRNT)

A high-throughput PRNT method was developed and validated *in-house*, as described before.[17] The serum neutralization titer was defined as the reciprocal of the highest dilution resulting in a reduction of the control plaque count >50% (PRNT50). We considered a titer of 1:10 as the seropositive threshold. This method was validated in both adults and children using pre-pandemic serum (2017–2018) isolated from age- and gender-matched individuals.[18,19]

2.7 | Ag-specific B cells by flow cytometry

Ag-specific B cells were analyzed used a S1+S2 Spike SARS-CoV-2 PE-labeled protein as described before.[17,20–22] Gating strategy is shown in Figure 2B. Data analyses were performed using Kaluza software (Beckman Coulter). Pre–COVID-19 era (2017–2018) PBMC samples from healthy age- and gender-matched controls were used to set the gate. The protocol is available in Cotugno N. et al (2021)[17] and included in the Appendix S1. PBMC timing of collection from first SARS-CoV-2–positive PCR is now reported in Table S2.

2.8 | Labeling of human recombinant ICAM-1-Fc multimers and CD8 Ag-specific T-cell analysis by FACS

Following a previously validated method,[23] ICAM-1-Fc **multimers** were labeled in-house with polyclonal anti-human Fc-PE F(ab′)2 fragments. Full labeling and staining protocol details can be found in the Appendix S1. Pre–COVID-19 era (2017–2018) PBMC samples from healthy age- and gender-matched controls were used to set the gate for the Ag CD8 T cells.

2.9 | Olink assay

Proteins in plasma were analyzed through a multiplexed proximity ligation as described in detail before.[24] Further details are given in the Appendix S1.

2.10 | Statistical analysis

Statistical analyses were performed using R software (version 3.6.2) and GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA). The Mann-Whitney test and the chi-square test were used for continuous and discrete variables, respectively. The D'Agostino-Pearson test was the appropriate test to assess normality distribution. The area under the curve (AUC) was calculated with the MESS R library. Plasma proteins were statistically processed as previously described.[12] The principal component analysis (PCA) was performed on proteomics data with the prcomp R function; meanwhile, the PC contribution plot was done using the library factoextra. Statistical significance was set at $p < .05$, and the test was two-tailed. Full details for statistical analysis can be found in the Appendix S1.

3 | RESULTS

3.1 | Study participants

Overall, we analyzed 66 CoV-2+ and 11 CoV-2- children. Our results show that 15 of 66 (23%) were asymptomatic (AS). No significant differences in terms of age, gender, platelets, white blood cell count (WBC), neutrophils, lymphocytes, and hemoglobin (Hb) emerged between symptomatic (SY) and AS (Table 1) patients. WBC and platelets were significantly lower in SY compared to CoV-2- patients, while C-reactive protein (CRP) resulted significantly higher in CoV-2- compared with the CoV-2+ groups (Table 1). Full information on timing of both symptoms and therapy administered to symptomatic patients is shown in Tables S1 and S2.

3.2 | Virological profiling of AS and SY SARS-CoV-2+ patients

In order to define virological differences according to the symptoms, we measured SARS-CoV-2 viral load by digital droplet PCR (ddPCR) and qRT-PCR on NP swabs collected at diagnosis and longitudinally up to first negative test. The analysis revealed a lower viral load at diagnosis in AS vs SY patients, found statistically significant for

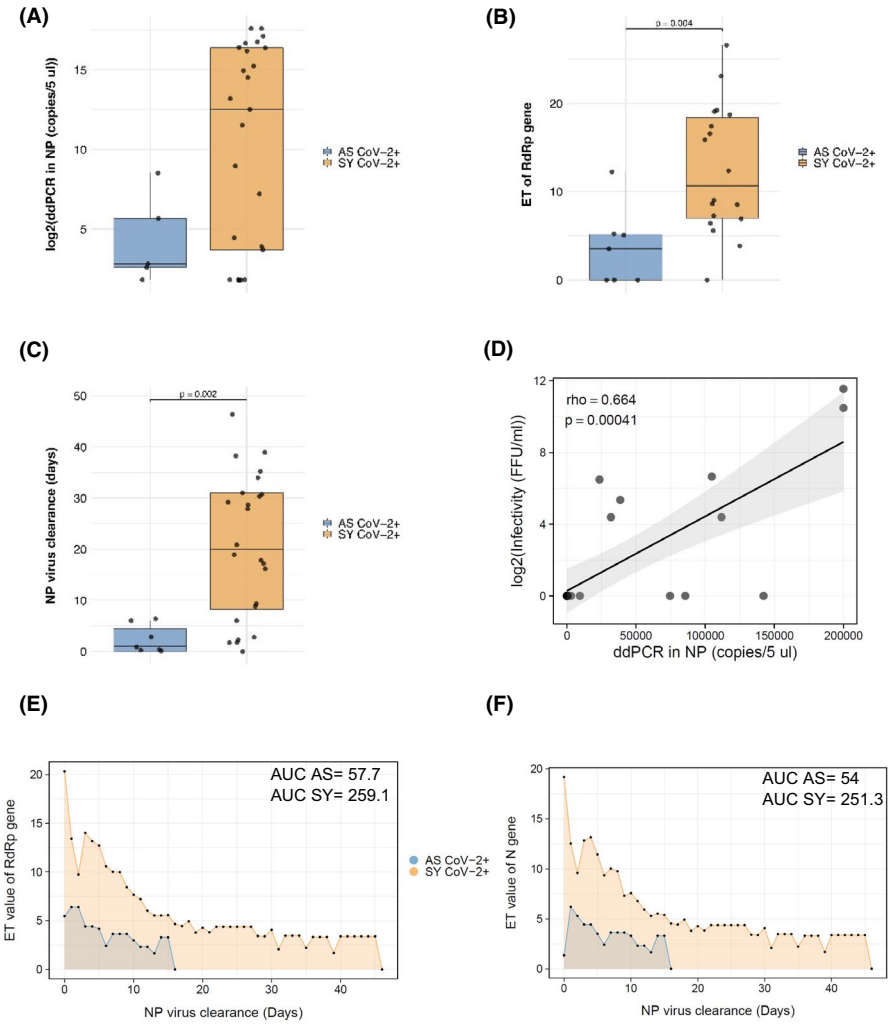

FIGURE 1 Virological analysis of SARS-CoV-2–infected children. (A) Log_2 ddPCR in NP swabs (copies/5 μl) in AS vs SY patients. (B) ET of RdRp gene and (C) NP virus clearance (days) in AS vs SY patients. (D) Association between infectivity (measured as FFU/ml) and ddPCR in NP swabs. (E) AUC for RdRp gene in AS vs SY patients. (F) AUC for N gene in AS vs SY patients. The Mann-Whitney test was used to define differences in (A–C); Spearman's test was used in (D), AUC was calculated as described in Methods. *p* values <.05 were considered significant

RdRp qRT-PCR (p = .004) (Figure 1A,B). The virus clearance expressed in days was significantly lower in AS vs SY (p = .002) patients (Figure 1C). To define the virus potential of infectivity and its relation to viral load, we used a focus-forming assay (FFA) and found a positive association with viral load, suggesting that higher SARS-CoV-2 loads correlate with higher infectivity potential (rho = 0.66, p = .0004) (Figure 1D). The longitudinal analysis performed on NP collected every 48 h after diagnosis revealed a lower viral area under the curve for both RdRp gene (AUC AS = 57.7; AUC SY = 259.1, p = .08) and N gene (AUC AS = 54; AUC SY = 251.3, p = .08) (Figure 1E,F).

3.3 | Both AS and SY patients were able to develop specific anti-SARS-CoV-2 humoral and cellular responses

To assess whether asymptomatic children presented similar ability to induce protective and neutralizing humoral response, we quantified serum levels of SARS-CoV-2–specific Ab and Ab-neutralizing activity at diagnosis and in the "post-acute phase" (10–14 days after diagnosis). No differences emerged in terms of both SARS-CoV-2 IgG and Ab-mediated neutralization activity (PRNT) (Figure 2A) between the groups at both time points. At diagnosis, 5 of 15 (33%) AS and 17 of 51 (33%) SY patients had developed anti-SARS-CoV-2 similar antibody levels (Figure 2A, upper panels; SARS-CoV-2 IgG); furthermore, approximately half of both AS (8/15, 53%) and SY (29/51, 57%) patients developed strong neutralizing activity. In the "post-acute phase," the majority of AS and SY patients had developed neutralizing antibodies (Figure 2A, bottom panel; 83% for AS and 84% for SY). In order to define the ability of these patients to maintain an Ab response over time, a 3-month follow-up visit was performed. Although the majority of patients were lost at follow up, we could analyze 17 of 66 patients (1 AS and 16 SY). Overall, the majority of patients including the asymptomatic patients showed PRNT activity, with 3 of 17 (18.7%) resulting to be negative (data not shown).

We also investigated SARS-CoV-2–specific cellular immunity in peripheral blood prior to any therapy initiation. We studied Ag-specific B cells gated on switched memory B cells (CD10-CD19+CD27+IgD-) (gating strategy for B-cell populations in Figure S1), using and in-house fluorescently labeled probe expressing S1+S2 SARS-CoV-2 proteins (gating strategy in Figure 2B). Ag-specific B cells were detectable in both AS and SY patients in similar levels (Figure 2C). Also, the analysis of maturational subsets

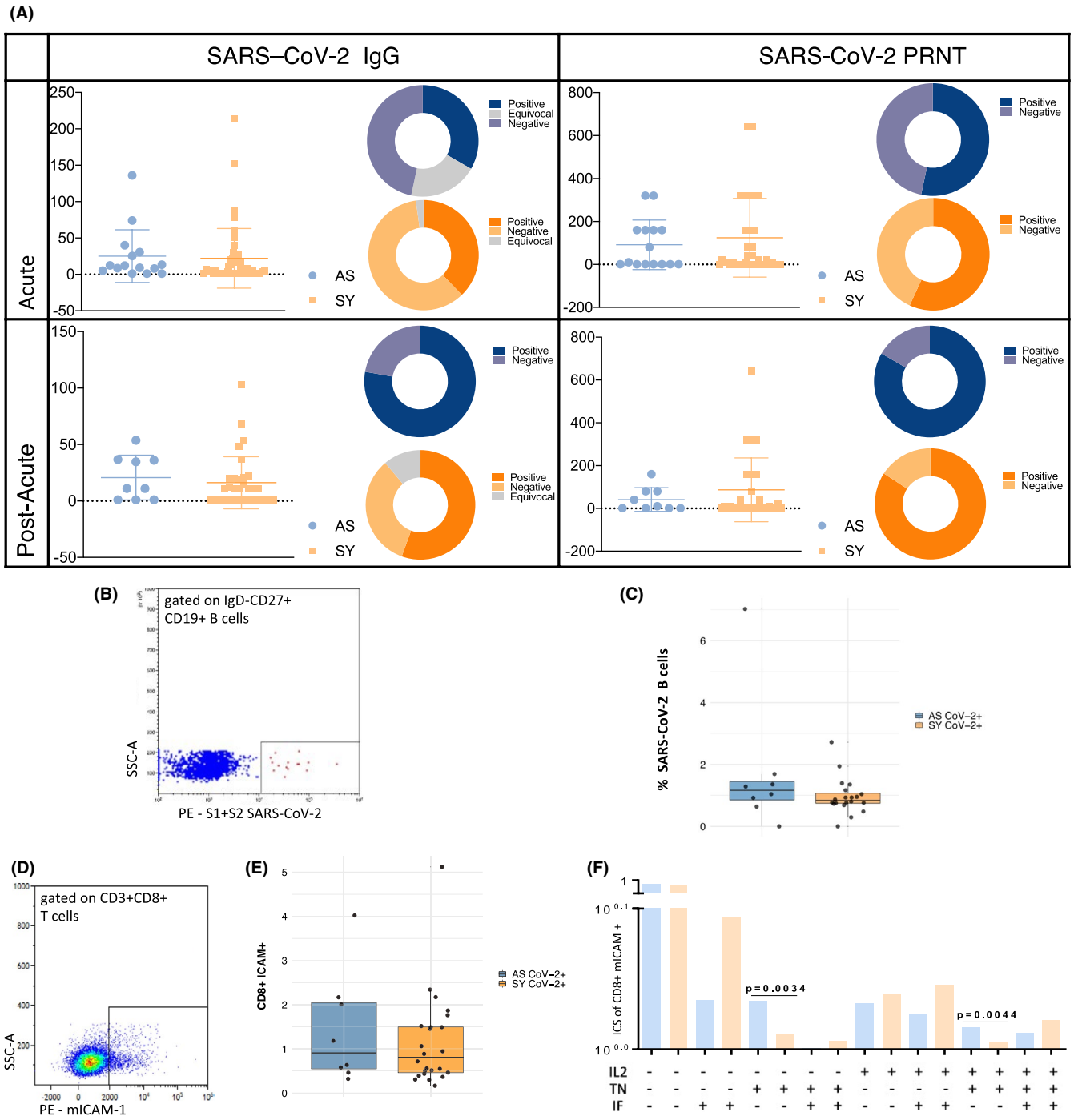

FIGURE 2 SARS-CoV-2 seroconversion and Ag-specific B and CD8 T cells in children with distinct symptomatology. (A) Left-hand side represents SARS-CoV-2 Ab titers at admission (upper dot plots) and at "post-acute phase" (lower dot plots). Right-hand side represents SARS-CoV-2 PRNT at admission (upper dot plots) and at "post-acute phase" (lower dot plots). Neutralization values were identified by dilution factor. Contingency plots show frequency of seronegative, seropositive, and equivocal results in both phases, following the same pattern described for the dot plots. Gating strategy (B) and frequencies of Ag-specific CD19+IgD-CD27+ switched B cells (C) are shown in AS and SY patients. Gating strategy (D) and frequencies of Ag-specific ICAM+CD8+ T cells (E) are shown in AS and SY patients. Box plot in (F) shows results from Boolean gating of intracellular staining analysis from ICAM+CD8 T cells. The Mann-Whitney test was used to define differences, *p* values <.05 were considered significant

of CD19+ B cells through the surface expression of CD10, CD27, IgD, and CD21 showed no differences between SY and AS patients (Figure S1F). We further assessed the frequency of Ag-specific CD8+T cells at diagnosis in both groups as previously described[23] (gating strategy in Figure 2D). The frequency of CD8+Ag-specific T cells was similar between AS and SY children in terms of both frequency (Figure 2E) and absolute counts (not shown). As expected, Ag-specific CD8+T cells showed an enrichment within the memory

subsets with 33% effector memory (CD45RA-CCR7–) and 31% central memory (CD45RA-CCR7+) (Figure S2A). No differences were found between the groups in terms of distribution of Ag-specific T cell in the maturational subsets (Figure S2B). The cytotoxic potential of ICAM+CD8+T cells was measured after SARS-CoV-2 peptide *in vitro* stimulation by intracellular production of IL-2, TNF-alpha, and IFN-gamma (gating strategy in Figure S2). Boolean analysis showed that TNF-alpha–positive and TNF-alpha and IL-2 bifunctional ICAM+CD8+T cells were significantly higher in AS vs SY ($p = 0.003$ and $p = 0.004$, respectively) (Figure 2F) patients, suggesting how these cells in AS patients maintain an effective antiviral cytotoxic response. No differences in terms of frequency of total CD8+ nor maturational subsets were found among the groups (Figure S1).

We further evaluated natural killer (NK) frequency and distribution to define whether innate immune determinants could distinguish between patients presenting with distinct symptomatology. In line with a recent report on adult patients,[25] our analysis did not show any significant difference in the frequency of total NK cells between SY compared with asymptomatic AS CoV-2+ patients (Figure S3). Our analysis further showed no difference in terms of CD56bright and CD56dim frequency.

3.4 | Plasma protein profile of SARS-CoV-2 children

We deepened the characterization of AS and SY patients by investigating their immunological profiles at admission, using two Olink panels focused on both immune response and inflammation. PCA suggested that proteomic data could only partially define differences between AS and SY (Figure 3A) patients, with the top 15 contributing factors including pro-inflammatory cytokines and chemokines (CXCL10, LAMP3) (Figure 3B). We then further explored the levels of expression of each of the 121 plasma proteins analyzed, in AS vs SY patients. Albeit limited in power by the small sample size, this analysis shows that only few pro-inflammatory cytokines and chemokines including CXCL10 and CCL19 were higher in SY than in AS patients.

4 | DISCUSSION

This study provides a description of the virological and immunological profiles of 66 SARS-CoV-2–infected children with distinct symptomatology. In particular, this work attempted to contribute to the current need for a precise identification of asymptomatic pediatric patients in order to define public restrictive measures. We here investigated viral dynamics, SARS-CoV-2 humoral response, and Ag-specific B and CD8 T cell SARS-CoV-2+ patients. Quantification of SARS-CoV-2 using ddPCR in NP swabs revealed a lower virus load associated with reduced infectivity in AS patients compared with SY patients. Besides, these data suggested that virus clearance in the upper respiratory system (NP) was faster in AS patients than in SY patients.

As previously reported, pediatric population experience milder clinical manifestation resulting in a higher rate of asymptomatic and undiagnosed patients compared with adults.[1–10,26] The mechanisms behind such differences are still poorly defined, which renders very difficult the timely identification of AS patients to prevent virus spread that could fuel further epidemic waves as a consequence of school reopenings. Our data suggested that overall AS patients had lower viral load and associated *in vitro* infectivity, alongside capacity to clear the virus faster compared with SY patients. This difference could suffer from an inescapable bias given by the fact the time of infection cannot be determined, as discussed in other studies.[27,28] On the other hand, several studies in adults have shown that SARS-CoV-2 load is typically lower after seroconversion[29,30] underpinning the close relationship between development of humoral response and viral load reduction. In our population, seroconversion rate in AS vs SY patients at diagnosis was comparable. While this finding partially compensates for the bias discussed above and strengthens the data about AS patients being less infectious, we acknowledge that further study in bigger population is needed to define the virological characteristics of AS patients. We then investigated the immunological profiling in relation to symptomatology, showing that both AS and SY patients were capable of producing Ag-specific B and CD8 T cells. In a viral respiratory infection, virological control is also maintained thanks to cytotoxic activity of effector CD8+T lymphocytes at the site of infection.[31] Our results did not identify any difference in terms of maturational subset distribution and frequency between AS and SY SARS-CoV-2–infected children, as previously shown in adults.[32] On the other hand, our data showed that AS patients had higher levels of polyfunctional Ag-specific CD8+T cells compared with SY patients, demonstrated by the higher frequency of CD8+T cell producing both TNF-alpha and IL-2 upon *in vitro* stimulation with SARS-CoV-2 peptides. Although this result cannot discriminate whether this is the cause or the effect of symptomatology, it clearly suggests the presence of an effective adaptive immunity in AS patients. We further evaluated seroconversion rate at a later time point and found no difference between AS and SY patients. Further, our data confirmed what found in adults[30] showing that approximately 15% were still seronegative at 10–14 days after diagnosis, regardless of the symptomatology. Our data interestingly suggest that AS patients have an intact ability to seroconvert, hence contributing to Ab-mediated herd immunity at similar levels compared with SY patients. In a recent study, similar levels of anti-S IgG were found in paucisymptomatic SARS-COV-2–infected children and COVID-19 adults not requiring hospitalization.[11]

To define the cytokine profiles of these patients and its association with clinical course of SARS-CoV-2 infection, we analyzed plasma proteome. In a recent work,[12] we demonstrated that severe COVID-19 manifestations such as the multisystem inflammatory syndrome (MIS-C) were characterized by a specific cytokine storm with unique features as compared to mild COVID-19 and Kawasaki disease. Conversely, in the present patients' cohort, which lacked of

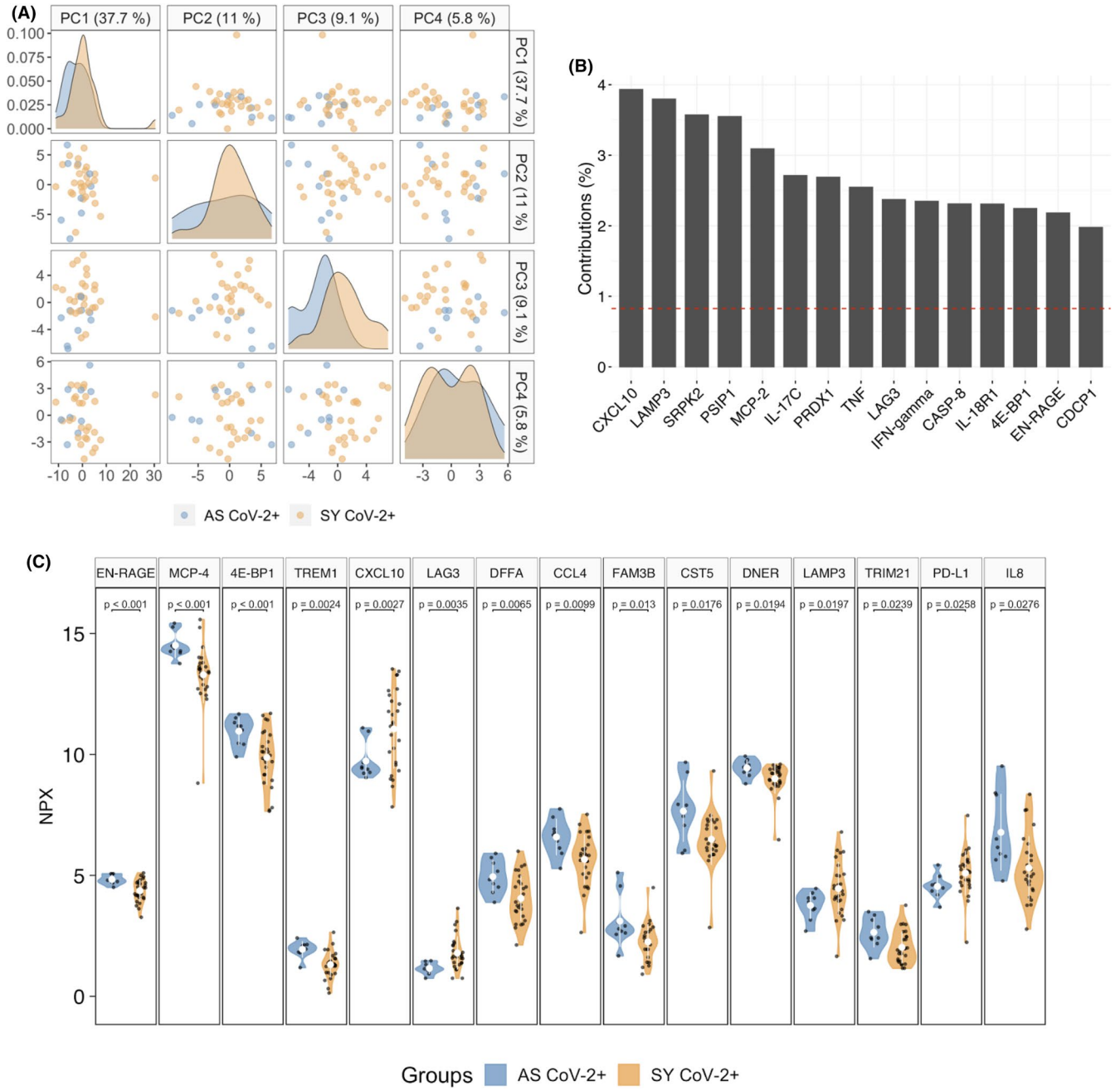

FIGURE 3 Proteomic profile in AS vs SY patients. PCA in (A) shows distribution according to protein profile between AS and SY patients. (B) PC loading for PC4 is showed in the box plot. The red line indicates the expected values. Violin plots in (C) show differentially expressed proteins among the groups

severe cases such as MIS-C, we find that proteomics could only marginally discriminate between AS and SY patients, as found in adults.[33] Further, in our cohort either NK cell frequency or CD56+CD16+ distribution was able to discriminate between AS and SY patients. In line with this, Ramaswamy et al[34] found that NK and CD8+ T-cell phenotype characteristics alone are not sufficient to define the highly symptomatic/severe cases of SARS-CoV-2 (eg, MIS-C), but a deepen study on cytotoxicity genes is required.

This study presents some limitations. First, we could only include a small group of the AS patients, albeit this is a reflection of the clinical reality, which could not be resolved considering that the recruitment included only hospital admissions and not home-assisting surveillance. As previously stated, the infection onset cannot be clearly defined especially in AS children, and this could affect the viral load at diagnosis. Further longitudinal studies on larger cohorts with quantitative correlates of viral dynamics and an adult COVID-19 cohort for comparison would be crucial to confirm our observations.

In conclusion, this study demonstrated that AS patients has lower viral load and associated *in vitro* infectivity in upper respiratory tract compared with SY children. Development of both humoral and cell-mediated immunity is not associated with symptomatology, suggesting that importantly AS patients contribute to achieve herd

immunity at similar levels compared with SY patients. During later time points, the rate of failure in achieving seroconversion is similar in AS and SY patients: These data may inform alternative diagnostic algorithm to establish mitigated restrictive measures. Additional studies investigating the long-term maintenance of humoral and cell-mediated immunity in these populations are warranted.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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APPENDIX 1

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