iii. **ABSTRACT (244/250)**

BACKGROUND: Despite 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 seeks 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 children (SY), stratified according to WHO clinical classification. We measured SARS-CoV-2 viral load using ddPCR and qPCR in longitudinally collected nasopharyngeal swabs samples. To define anti-SARS-CoV-2 antibodies we measured neutralization activity and total IgG load (Diasorin). We also evaluated antigen-specific B and CD8+T-cells, using a labelled S1+S2 protein and ICAM expression, respectively. Plasma protein profiling was performed with Olink.

RESULTS: Virological profiling showed that AS had lower viral load at diagnosis (p=0.004) and faster virus clearance (p=0.0002) compared to SY. Anti-SARS CoV-2 humoral and cellular response did not appear to be associated with the presence of symptoms. AS and SY 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 associated to symptomatology.

25 **CONCLUSION:** We demonstrated the development of anti-SARS-CoV-2 humoral and cellular

26 response with any regards to symptomatology, suggesting the ability of both SY and AS to

27 contribute towards herd immunity. The virological profiling of AS suggested that they have lower

28 virus load associated with faster virus clearance.

29

30 **KEY WORDS:** SARS-CoV-2, Asymptomatic, Symptomatic, Ag-specific cellular response,

31 neutralization humoral activity

32

33   iv.   **MAIN TEXT**

34 **INTRODUCTION**

35 While SARS-CoV-2 immunization programs have started in most countries, the achievement of

36 herd immunity still seems far ahead. Indeed, due to different restrictive measures amongst countries

37 and the lack of vaccination programs for children, the risk for second epidemic waves and health

38 systems overburden remains high. Children infected by SARS-CoV-2 usually present with a milder

39 course of disease compared to COVID-19 adults, with a consistent proportion being fully

40 asymptomatic (AS). Pathogenic reasons underlying such differences have been poorly defined[1].

41 Children play a crucial impact on SARS-CoV-2 transmission and on epidemic waves, especially in

42 school settings[2-4]. Test and trace interventions, implemented by government policies for epidemic

43 control may fail in the pediatric population, where AS range between 5 and 16%[5-8] and where a

44 consistent proportion remain undiagnosed[9]. In this scenario, some scientific questions arise about

45 AS: i) do they present the same virological features of symptomatic (SY)? ii) do they develop an

46 adaptive and protective immune memory response against SARS-CoV-2? iii) are there

47 inflammatory cytokines profiles associated with clinical manifestations?

48

49 Several hypotheses have been advanced in the attempt to explain the AS status of SARS-CoV-2

50 infected patients, but no specific investigations on pediatric population are available. The humoral

51 SARS-CoV-2 responses showed a lower level of anti-S IgG in children compared to adults[10],

52 considering both pediatric patients developing multi-inflammatory syndrome associated to COVID-

53 19 (MIS-C) and in those with a milder clinical presentations. Our results in MIS-C [11] showed the

54 absence of pre-existing humoral responses upon other "common cold coronaviruses" in comparison

55 to mild COVID19 children. However, the true influence of pre-existing humoral and T-cell

56 responses towards coronaviruses [12] on mitigating symptoms in children still need to be defined. In

57 addition, the magnitude of the inflammatory phase associated with viral infection in severe cases [13]

58 may represent a distinctive feature of AS children compared to SY.

59

60 In the present work, we attempt to define virological and immunological characteristics of 15 AS

61 patients, compared to 51 SY in order to define their ability to produce anti-SARS-CoV-2 immunity.

62 AS and SY patients were further compared to 11 SARS-CoV-2 negative (CoV-2-) patients that

63 were enrolled for suspicion of COVID-19, but that tested negative to both nasopharyngeal swab and

64 serology.

65

66 **METHODS**

67 **Study participants**

68 Sixty-six SARS-CoV-2 infected children (CoV-2+) and 11 SARS-CoV-2 negative controls (CoV-2)

69 were enrolled from March to April 2020 at Bambino Gesu' Children's Hospital in Rome for the

70 CACTUS (Immunological studies in **C**hildren **A**ffe**CT**ed by COVID and ac**U**te re**S**piratory

71 diseases). The study was approved by local ethical committee and written informed consent was

72 obtained from all participants or legal guardians. Age, gender, clinical and routine laboratory

73 characters are described in Table 1. Inclusion criteria for positive cases was detection of SARS-

74 CoV-2 in nasopharyngeal (NP) swab using SARS-CoV-2 real-time reverse transcriptase–

75 polymerase chain reaction (RT-PCR) tests (GeneXpert, Cepheid, Sunnyvale, CA; 250 copies/mL

76 sensitivity, 100% specificity). Serology was performed as additional confirmatory test using

77 LIAISON® SARS-CoV2 S1/S2 IgG test (DiaSorin, Stillwater, MN, USA). CoV-2+ were stratified

78 according to WHO clinical classification (https://www.who.int/publications/i/item/WHO-2019-

79 nCoV-clinical-2021-1) as follow: i) asymptomatic CoV-2+ (AS), patients without any symptoms

80 despite confirmed SARS-CoV-2 infection, that were summoned to the hospital since they belonged

81 to the same nuclear family of symptomatic patients; ii) symptomatic CoV-2+ (SY), patients. We

82 also included 11 children (SARS-CoV-2 negative) with suspected SARS-CoV-2 infection, but

83 tested negative for 2 consecutive NP swabs (performed 24 hours apart) and for SARS-CoV-2 S1/S2

84 IgG.

85 **Sample collection and storage**

86 Prior to therapy initiation, venous blood was collected in EDTA tubes and processed within 2 hrs.

87 Plasma was isolated from blood and stored at -80°C. Peripheral blood mononuclear cells (PBMCs)

88 were isolated by Ficoll and cryopreserved in liquid nitrogen. NP swab preserving media was stored

89 at -80°C until use. Virological analysis was performed on NP collected at diagnosis and every 48

90 hours up to virus clearance. Serologic analysis were performed on vials collected at diagnosis and

91 after 10-14 days (herein referred to as "late phase").

92 **SARS-CoV-2 viral load measurement in swabs by ddPCR**

93 NP swabs were collected by using flocked swabs in liquid-based collection and transport systems.

94 Total nucleic acids were purified from 200µl NP swab preserving media and eluted in a final

95 volume of 100µl. Copies of SARS-CoV-2 were quantified by a home-made multiplex quantitative

96 assay based on One-Step RT-ddPCR, as previously described[14,15]. Each sample was run at least in

97 duplicate. Results were expressed as SARS-CoV-2 copies/5µl.

98

99

Allplex™ 2019-nCoV Assay

NP swabs were longitudinally collected from CoV-2+ and analyzed using the multiplex Allplex™ 2019-nCoV Assay (Seegene, Seul, South Corea) following manufacturer's instructions. The analyzed genes were RdRp and N gene of COVID-19 and E gene of *Sarbecovirus*.

Virus titration by focus forming assay (FFA)

FFA was performed as previously described *(Cotugno N. *, Ruggiero A.*, Bonfante F.* [...] Palma P., in press Cell reports)*. Focus forming units per ml (FFU/ml) were counted after acquisition of pictures at a high resolution of 4800 x 9400dpi, on a flatbed scanner.

Ab-mediated neutralizing activity measured with Plaque reduction neutralization test (PRNT)

A high- throughput PRNT method was developed *in-house*, as described before *(Cotugno N. *, Ruggiero A.*, Bonfante F.* [...] Palma P., in press Cell reports)*. 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 titre of 1:10 as the seropositive threshold.

Ag-specific B-cells by Flow Cytometry

Ag-specific B-cells were analyzed used a S1+S2 Spike SARS-CoV-2 PE-labelled protein as described before[16-18]*(Cotugno N. *, Ruggiero A.*, Bonfante F.* [...] Palma P., in press Cell reports)*. Gating strategy is shown in Fig. 2B. Data analyses were performed using Kaluza software (Beckman Coulter).

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

120 Following a previously validated method[19] ICAM-1-Fc were labeled in house with polyclonal anti-

121 human Fc-PE F(ab′)2 fragments. Full labeling and staining protocol details can be found in the

122 Supplementary information file.

123 **Olink assay**

124 Proteins in plasma were analyzed through a multiplexed proximity ligation assay previously

125 described[20].

126 **Statistical analysis**

127 Statistical analyses were performed using R software (version 3.6.2) and GraphPad Prism 6

128 (GraphPad Software, Inc., San Diego, CA). Mann-Whitney test and Chi-square test were used to

129 continuous and discrete variables, respectively. D'Agostino-Pearson test was used to assess

130 normality distribution and use the appropriate test. The Area Under the curve (AUC) was calculated

131 whit the MESS R library. Plasma proteins were statistically processed as previously described[11].

132 Principal Component Analysis (PCA) was performed on proteomics data with the prcomp R

133 function, meanwhile, the PC contribution plot was done using the library factoextra. Statistical

134 significance was set at $p < 0.05$ and the test were two-tailed. Full details for statistical analysis can be

135 found in the supplementary information file.

136

137 **RESULTS**

138 **Study participants**

139 Overall, we analyzed 66 CoV-2+ and 11 CoV-2- children. Our results show that 15/66 (23%) were

140 asymptomatic (AS). No significant differences in terms of age, gender, platelets, white blood cell

141 count (WBC), neutrophils, lymphocytes and hemoglobin (Hb) emerged between symptomatic (SY)

142 and AS (Table 1). WBC and platelets resulted significantly lower in SY compared to CoV-2- while

143 C reactive protein (CRP) resulted significantly higher in CoV-2- compared to the CoV-2+ groups

144 (Table 1). Full spectrum of symptoms and therapy administered to symptomatic patients is shown in
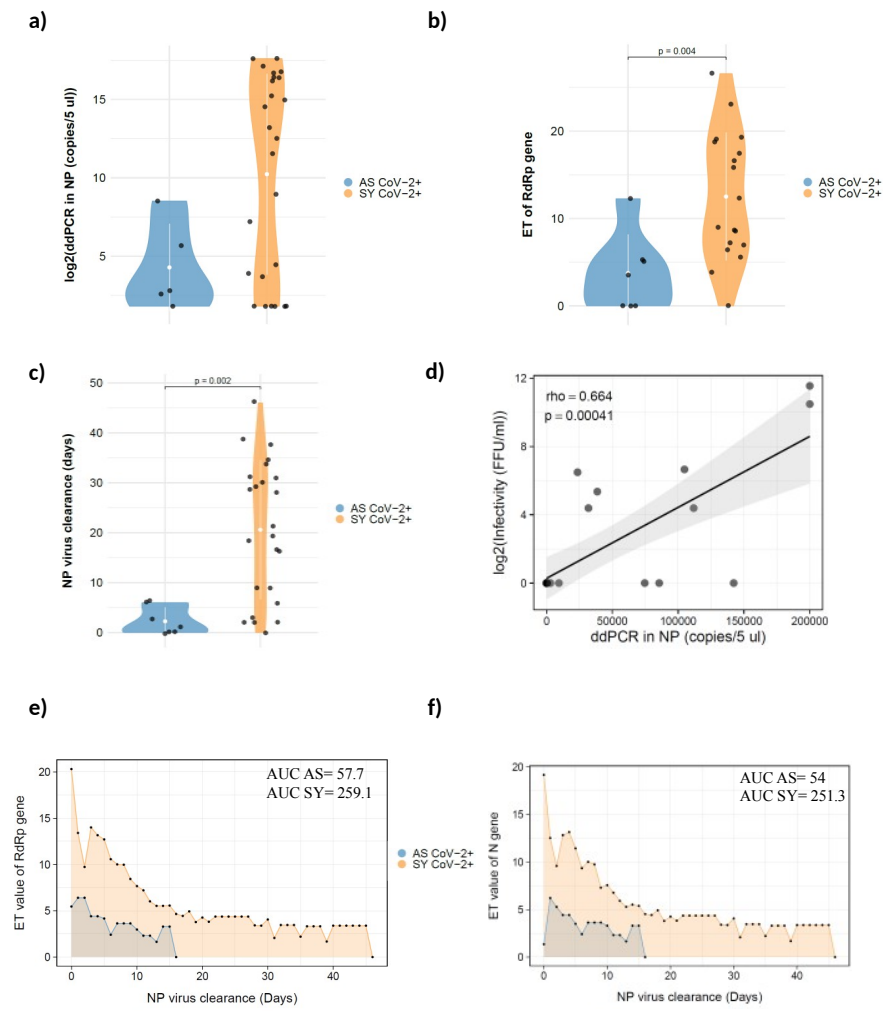
145 Supplementary Table 1.

146

147 **Virologic profiling of AS and SY SARS-CoV-2+**

148 In order to define virological differences according to the symptoms, we measured SARS-CoV-2

149 viral load by digital droplet PCR (ddPCR) and qRT-PCR on NP swabs collected at diagnosis and

150 longitudinally up to first negative test. The analysis revealed a lower viral load at diagnosis in AS vs

151 SY, found statistically significant for RdRp qRT-PCR (p=0.004) (Fig.1A and B). The virus

152 clearance expressed in days resulted significantly lower in AS vs SY (p=0.002) (Fig. 1C). To define

153 the virus potential of infectivity and its relation to viral load we used a focus forming assay (FFA)

154 and found a positive association with viral load suggesting that higher SARS-CoV-2 loads correlate

155 with higher infectivity potential (rho=0.66; p=0.0004) (Fig.1D). The longitudinal analysis

156 performed on NP collected every 48 hours after diagnosis revealed a lower viral area under the

157 curve for both RdRp gene (AUC AS= 57.7; AUC SY= 259.1; p = 0.08) and N gene (AUC AS= 54;

158 AUC SY= 251.3; p = 0.08) (Fig.1E and F).

159 **Both AS and SY were able to develop specific anti-SARS-CoV-2 humoral and cellular**

160 **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 at a "late 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, 5/15 (33%) AS and 17/51 (33%) SY had developed similar anti-SARS-CoV-2 antibody levels (Fig.2A upper panels). At "late phase", the majority of AS and SY had developed neutralizing antibodies (Fig.2A bottom panel, 83% for AS and 84% for SY).

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-), using and in-house fluorescently labeled probe expressing S1+S2 SARS-CoV-2 proteins (gating strategy in Fig.2B). Ag-specific B-cells were detectable in both AS and SY in

173   similar levels (Figure 2C). Also, the analysis of maturational subsets of CD19+ B-cells through the

174   surface expression of CD10, CD27, IgD and CD21, showed no differences between SY and AS

175   (data not shown).

176   We further assessed the frequency of Ag-specific CD8+T-cells at diagnosis in both groups as

177   previously described[19] (gating strategy in Figure 2D). The frequency of CD8+Ag-specific T-cells

178   was similar between AS and SY children both in terms of frequency (Fig.2E) and absolute counts

179   (not shown). The cytotoxic potential of ICAM+CD8+T-cells, was measured after SARS-CoV-2

180   peptide *in-vitro* stimulation by intracellular production of IL2, TNF-alpha and IFN-gamma.

181   Boolean analysis showed that TNF-alpha positive and TNF-alpha and IL-2 bi-functional

182   ICAM+CD8+T-cells were significantly higher in AS vs SY (p=0.003 and p=0.004 respectively)

183   (Fig.2F) suggesting how these cells in AS maintain an effective antiviral cytotoxic response. No

184   differences in terms of frequency of total CD8+ (Supplementary Figure 1) nor maturational subsets

185   were found among the groups (not shown).

186   **Plasma protein profile of SARS-CoV-2 children**

187   We deepened the characterization of AS and SY by investigating their immunological profiles at

188   admission, using two Olink panels focused on both immune response and inflammation. PCA

189   suggested that proteomic data could only partially define differences between AS and SY (Figure

190   3A), with the top 15 contributing factors including pro-inflammatory cytokines and chemokines

191   (CXCL10, LAMP3) (Fig.3B). We then further explored the levels of expression of each of the 121

192   plasma proteins analyzed, in AS vs SY. Several pro-inflammatory cytokines and chemokines such

193   as CXCL10 and CCL19 results higher in SY compared to AS, conversely others (MCP-4 and IL8),

194   were found lower in SY. Whilst this result confirms the role of inflammatory pathways in inducing

195   severe symptomatology manifestations, it also suggests that the proteomic profiling can only be

196   partially used for the identification of AS patients and that symptomatology severity may be driven

197   by mechanisms not related to inflammation.

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-cells SARS-CoV-2+. Quantification of SARS-CoV-2 using ddPCR in NP swabs revealed a lower virus load associated with reduced infectivity in AS patients compared to SY. Besides, these data suggested that virus clearance in the upper respiratory system (NP) was faster in AS patients than SY.

As previously reported, pediatric population experience milder clinical manifestation resulting in a higher rate of asymptomatic and undiagnosed patients compared to adults[1,9]. The mechanisms behind such differences are still poorly defined, which renders very difficult the timely identification of AS to prevent virus spread that could fuel further epidemic waves as consequence of school re-openings. Our data suggested that overall AS had lower viral load and associated *in vitro* infectivity, alongside capacity to clear the virus faster compared to SY. This difference could suffer of an inescapable bias given by the fact the time of infection cannot be determined, as discussed in other studies[21,22]. On the other hand, several studies in adults have shown that SARS-CoV-2 load is typically lower after seroconversion[23,24] underpinning the close relationship between development of humoral response and viral load reduction. In our population, seroconversion rate in AS vs SY at diagnosis was comparable. Whilst this finding partially compensate for the bias discussed above and strengthen the data about AS being less infectious, we acknowledge that further study in bigger population are needed to define the virological characteristics of AS.

We then investigated the immunological profiling in relation to symptomatology, showing that both AS and SY were capable to produce 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

223   the site of infection[25]. Our results did not identify any difference in terms of maturational subset

224   distribution and frequency between AS and SY SARS-CoV-2 infected children, as previously

225   shown in adults[26]. On the other hand, our data showed that AS had higher levels of polyfunctional

226   Ag-specific CD8+T-cells compared to SY, demonstrated by the higher frequency of CD8+T-cell

227   producing both TNF-alpha and IL-2 upon *in vitro* stimulation with SARS-CoV-2 peptides.

228   Although this result cannot discriminate whether this is the cause or the effect of symptomatology,

229   it clearly suggests the presence of an effective adaptive immunity in AS. We further evaluated

230   seroconversion rate at a later time point and found no difference between AS and SY. Further, our

231   data confirmed what found in adults [24] showing that approximately 15% were still seronegative at

232   10-14 days after diagnosis, regardless the symptomatology. Our data interestingly suggest that AS

233   have an intact ability to seroconvert, hence contributing to Ab-mediated herd immunity at similar

234   levels compared to SY. In a recent study, similar levels of anti-S IgG were found in

235   paucisymptomatic SARS-COV-2 infected children and COVID19 adults not requiring

236   hospitalization[27]. However, few uncertainties remain on how the presence of neutralizing antibodies

237   translates in a protected status, since higher antibody concentrations have been observed in adult

238   patients with severe disease than in those with mild disease[28,29]. In line with this, it must be noted as

239   a suboptimal antibody response could fuel the production of inflammatory cytokines through

240   antibody-dependent enhancement[30].

241

242   To define the cytokines profiles of these patients and its association with clinical course of SARS-

243   CoV-2 infection, we analyzed plasma proteome. In a recent work[11], we demonstrated that severe

244   COVID-19 manifestations such as the multisystem inflammatory syndrome (MIS-C) were

245   characterized by a specific cytokine storm with unique features as compared to mild COVID-19 and

246   Kawasaki disease. In this patients' cohort, we find that proteomics could only partially discriminate

247   between AS and SY, which may be a reflection of the characteristics of our cohort which lacked of

248   severe cases such as MIS-C. On the other hand, in line with another study in adults[31], SY appeared

249 to have higher levels of pro-inflammatory cytokines, confirming the role of the cytokines storms in

250 driving severe clinical outcome.

251

252 This study presents some limitations. First, we could only include a small group of the AS, albeit

253 this is a reflection of the clinical reality, which could not be resolved considering that the

254 recruitment included only hospital admissions and not home-assisting surveillance. As previously

255 stated, the infection onset cannot be clearly defined especially in AS children and this could affect

256 the viral load at diagnosis. Further longitudinal studies on larger cohorts with quantitative correlates

257 of viral dynamics and an adult COVID19 cohort for comparison, would be crucial to confirm our

258 observations.

259

260 In conclusion, this study demonstrated that AS has lower viral load and associated *in vitro*

261 infectivity in upper respiratory tract, compared to SY children. Development of both humoral and

262 cell-mediated immunity is not associated with symptomatology, suggesting that importantly AS

263 contribute to achieve herd immunity at similar levels compared to SY. During later time points, the

264 rate of failure in achieving seroconversion is similar in AS and SY: this data may inform alternative

265 diagnostic algorithm to establish mitigated restrictive measures. Additional studies investigating the

266 long-term maintenance of humoral and cell-mediate immunity in these populations are warranted.

267

272

IMPACT STATEMENT

273

274 SARS-CoV-2-infected children present with a milder course of disease compared to COVID-19

275 adults, with a consistent proportion of fully asymptomatic (AS). Such difference in clinical

276 presentations are still poorly defined, even though this population plays a crucial role on SARS-

277 CoV-2 transmission. In this work, we attempted to fill this gap in knowledge by providing a

278 virological and immunological characterization of SARS-CoV-2 infected children with distinct

279 symptomatology. Comparison of SARS-CoV-2 viral load between AS and symptomatic (SY)

280 showed that AS patients had lower viral load at admission and faster virus clearance.

281 Immunological analysis revealed that AS are able to develop SARS-CoV-2 specific adaptive

282 immunity at similar level compared to SY in terms of total SARS-CoV-2 Ab, Ab-mediated

283 neutralization, and Ag-specific B and CD8 T-cells. Conversely, the analysis of plasma profiling

284 showed differences between AS and SY, supporting that pro-inflammatory mechanisms may drive

285 worse clinical outcome in SY. Overall our results show that AS have lower viral load in upper

286 respiratory tract at diagnosis suggesting a lower infectivity potential compared to SY. Furthermore,

287 development of both humoral and cell-mediated immunity is not associated with symptomatology,

288 demonstrating that AS importantly contribute to achieve herd immunity at similar levels compared

289 to SY. This data may inform alternative diagnostic algorithm to establish mitigated restrictive

290 measures for AS.

	SARS-CoV-2 + (N= 66)		SARS-CoV-2 neg	p value
	Asymptomatic (AS, N= 15)	Symptomatic (SY, N= 51)	(N= 11)	
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 10³/uL (SD)	309.1 (97.3)	293.5 (134.4), n=50	390.9 (201.5)	n.s.
WBC count mean 10³/uL (SD)	7.4 (2.7)	6.4 (2.6)	9.4 (3.8)	SY vs SARS-CoV-2neg p=0.006
Neutrophils (mean 10³/uL (SD)	2.9 (1.4)	2.5 (1.8), n=50	4.8 (3.1)	SY vs SARS-CoV-2neg p=0.003
Lymphocytes mean 10³/uL (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=0.01 AS vs SARS-CoV-2neg p=0.0002 SY vs SARS-CoV-2neg p=0.0001

291

292 **Table 1.** Continuous data were presented as mean (SD) calculated on the total number of patients, unless otherwise stated. Mann Whitney test was used for
293 comparison. SD=standard deviation. n.s = not significant. N=number of patients included in this study. n=number of patients available for the analysis.

REFERENCES

1   Brodin, P. Why is COVID-19 so mild in children? *Acta paediatrica* **109**, 1082-1083, doi:10.1111/apa.15271 (2020).

2   Panovska-Griffiths, J. *et al.* Determining the optimal strategy for reopening schools, the impact of test and trace interventions, and the risk of occurrence of a second COVID-19 epidemic wave in the UK: a modelling study. *The Lancet. Child & adolescent health* **4**, 817-827, doi:10.1016/S2352-4642(20)30250-9 (2020).

3   Head, J. R. *et al.* The effect of school closures and reopening strategies on COVID-19 infection dynamics in the San Francisco Bay Area: a cross-sectional survey and modeling analysis. *medRxiv : the preprint server for health sciences*, doi:10.1101/2020.08.06.20169797 (2020).

4   Esposito, S., Cotugno, N. & Principi, N. Comprehensive and safe school strategy during COVID-19 pandemic. *Italian journal of pediatrics* **47**, 6, doi:10.1186/s13052-021-00960-6 (2021).

5   Parri, N., Lenge, M., Buonsenso, D. & Coronavirus Infection in Pediatric Emergency Departments Research, G. Children with Covid-19 in Pediatric Emergency Departments in Italy. *The New England journal of medicine* **383**, 187-190, doi:10.1056/NEJMc2007617 (2020).

6   Lu, X. *et al.* SARS-CoV-2 Infection in Children. *The New England journal of medicine* **382**, 1663-1665, doi:10.1056/NEJMc2005073 (2020).

7   Lu, X., Xiang, Y., Du, H. & Wing-Kin Wong, G. SARS-CoV-2 infection in children - Understanding the immune responses and controlling the pandemic. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology* **31**, 449-453, doi:10.1111/pai.13267 (2020).

8   Dong, Y. *et al.* Epidemiology of COVID-19 Among Children in China. *Pediatrics* **145**, doi:10.1542/peds.2020-0702 (2020).

9   Li, R. *et al.* Substantial undocumented infection facilitates the rapid dissemination of novel coronavirus (SARS-CoV-2). *Science* **368**, 489-493, doi:10.1126/science.abb3221 (2020).

10   Weisberg, S. P. *et al.* Distinct antibody responses to SARS-CoV-2 in children and adults across the COVID-19 clinical spectrum. *Nature immunology* **22**, 25-31, doi:10.1038/s41590-020-00826-9 (2021).

11   Consiglio, C. R. *et al.* The Immunology of Multisystem Inflammatory Syndrome in Children with COVID-19. *Cell* **183**, 968-981 e967, doi:10.1016/j.cell.2020.09.016 (2020).

12   Grifoni, A. *et al.* Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans with COVID-19 Disease and Unexposed Individuals. *Cell* **181**, 1489-1501 e1415, doi:10.1016/j.cell.2020.05.015 (2020).

13   Peng, Y. *et al.* Broad and strong memory CD4(+) and CD8(+) T cells induced by SARS-CoV-2 in UK convalescent individuals following COVID-19. *Nature immunology* **21**, 1336-1345, doi:10.1038/s41590-020-0782-6 (2020).

14   Corman, V. M. *et al.* Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eurosurveillance* **25**, doi:10.2807/1560-7917.ES.2020.25.3.2000045 (2020).

15   Rampazzo, E. *et al.* Telomere length and telomerase levels delineate subgroups of B-cell chronic lymphocytic leukemia with different biological characteristics and clinical outcomes. *Haematologica* **97**, 56-63, doi:10.3324/haematol.2011.049874 (2012).

16   Cotugno, N. *et al.* Early antiretroviral therapy-treated perinatally HIV-infected seronegative children demonstrate distinct long-term persistence of HIV-specific T-cell and B-cell memory. *Aids* **34**, 669-680, doi:10.1097/QAD.0000000000002485 (2020).

17   Cotugno, N. *et al.* Artificial Intelligence Applied to in vitro Gene Expression Testing (IVIGET) to Predict Trivalent Inactivated Influenza Vaccine Immunogenicity in HIV Infected Children. *Frontiers in immunology* **11**, 559590, doi:10.3389/fimmu.2020.559590 (2020).

18   Cotugno, N. *et al.* Higher PIK3C2B gene expression of H1N1+ specific B-cells is associated with lower H1N1 immunogenicity after trivalent influenza vaccination in HIV infected children. *Clinical immunology* **215**, 108440, doi:10.1016/j.clim.2020.108440 (2020).

344 19 Dimitrov, S. *et al.* Activated integrins identify functional antigen-specific CD8(+) T cells within
345   minutes after antigen stimulation. *Proceedings of the National Academy of Sciences of the United*
346   *States of America* **115**, E5536-E5545, doi:10.1073/pnas.1720714115 (2018).
347 20 Lundberg, M. *et al.* Multiplexed homogeneous proximity ligation assays for high-throughput protein
348   biomarker research in serological material. *Molecular and Cellular Proteomics* **10**,
349   doi:10.1074/mcp.M110.004978 (2011).
350 21 Meyerowitz, E. A., Richterman, A., Bogoch, II, Low, N. & Cevik, M. Towards an accurate and
351   systematic characterisation of persistently asymptomatic infection with SARS-CoV-2. *The Lancet.*
352   *Infectious diseases*, doi:10.1016/S1473-3099(20)30837-9 (2020).
353 22 Ooi, E. E. & Low, J. G. Asymptomatic SARS-CoV-2 infection. *The Lancet. Infectious diseases* **20**, 996-
354   998, doi:10.1016/S1473-3099(20)30460-6 (2020).
355 23 Sayampanathan, A. A. *et al.* Infectivity of asymptomatic versus symptomatic COVID-19. *Lancet* **397**,
356   93-94, doi:10.1016/S0140-6736(20)32651-9 (2021).
357 24 Peeling, R. W. *et al.* Serology testing in the COVID-19 pandemic response. *The Lancet. Infectious*
358   *diseases* **20**, e245-e249, doi:10.1016/S1473-3099(20)30517-X (2020).
359 25 Zheng, H. Y. *et al.* Vol. 17   541-543 (Springer Nature, 2020).
360 26 Qin, C. *et al.* Dysregulation of immune response in patients with coronavirus 2019 (COVID-19) in
361   Wuhan, China. *Clinical Infectious Diseases* **71**, 762-768, doi:10.1093/cid/ciaa248 (2020).
362 27 Weisberg, S. P. *et al.* Distinct antibody responses to SARS-CoV-2 in children and adults across the
363   COVID-19 clinical spectrum. *Nature immunology* **22**, doi:10.1038/s41590-020-00826-9 (2020).
364 28 Zhao, J. *et al.* Antibody Responses to SARS-CoV-2 in Patients with Novel Coronavirus Disease 2019.
365   *Clinical Infectious Diseases* **71**, 2027-2034, doi:10.1093/cid/ciaa344 (2020).
366 29 Peeling, R. W. *et al.* Vol. 20   e245-e249 (Lancet Publishing Group, 2020).
367 30 Iwasaki, A. & Yang, Y. The potential danger of suboptimal antibody responses in COVID-19. *Nature*
368   *reviews. Immunology* **20**, 339-341, doi:10.1038/s41577-020-0321-6 (2020).
369 31 Arunachalam, P. S. *et al.* Systems biological assessment of immunity to mild versus severe COVID-
370   19 infection in humans. *Science* **369**, 1210-1220, doi:10.1126/science.abc6261 (2020).

371

372

373

374

375

376

377

378

379

380

381

382

383

384

385

FIGURE LEGEND

387 **Figure 1. Virological analysis of SARS-CoV-2 infected children**. (A) Log$_2$ (ddPCR in NP swabs

388 (copies/5μl) in AS vs SY. (B) ET of RdRp gene (C) NP virus clearance (days) in AS vs SY (D)

389 Association between infectivity (measured as FFU/ml) and ddPCR in NP swabs (E) AUC for RdRp

390 gene in AS vs SY (F) AUC for N gene in AS vs SY. Mann Whitney test were used to define

391 differences in (A)-(B)-(C) were considered significant; Spearman's test was used in (D), AUC was

392 calculated as described in methods. p values lower than 0.05 were considered significant.

393 **Figure 2. SARS-CoV2 seroconversion and Ag-specific B and CD8 T-cells in in children with**

394 **distinct symptomatology. (A)** Left-hand side represents SARS-CoV-2 Ab titers at admission

395 (upper dot plots) and at "late phase" (lower dot plots). Right-hand side represents SARS-CoV-2

396 PRNT at admission (upper dot plots) and at "late phase" (lower dot plots). Neutralization values

397 were identified by dilution factor. Contingency plots show frequency of seronegative, seropositive

398 and equivocal results in both phases, following the same pattern described for the dot plots. Gating

399 strategy **(B)** and frequencies of Ag-specific CD19+IgD-CD27+switched B-cells **(C)** are shown in

400 AS and SY. Gating strategy **(D)** and frequencies of Ag-specific ICAM+ CD8+T-cells **(E)** are shown

401 in AS and SY. Box plot in (**F)** shows results from Boolean gating of intracellular staining analysis

402 from ICAM+ CD8 T cells. Mann Whitney test were used to define differences, p values lower than

403 0.05 were considered significant.

404 **Figure 3. Proteomic profile in AS vs SY.** PCA in (**A)** shows distribution according to protein

405 profile between AS and SY. (**B**) PC loading for PC4 is showed in the box plot. The red line

406 indicates the expected values. Violin plots in (**C**) shows differentially expressed proteins among the

407 groups.

408

409