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Improving knowledge on SARS-CoV-2 infection among family clusters including children: from the implementation of effective infection prevention and control measures to an integrated evaluation of epidemiological, clinical, and immune-virological characteristics of COVID-19

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A voi, meraviglie mie
Jacopo, Sofia e Alice
affinché non perdiate mai la curiosità
e l'entusiasmo, nell'imparare cose nuove

A Luca,
che mi ha sempre sostenuto in ogni follia

Ai miei genitori,
a cui devo tutto

SUMMARY

Since December 8th 2019, the date of symptoms' onset of the first known case of Coronavirus Disease 2019 (COVID-19) described in Wuhan City, Hubei Province, China, the world has been affected and unavoidably changed by the highly transmissible and pathogenic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. Soon after the identification of the novel Coronavirus by Chinese scientists, Italy was the first European country to recognize and describe COVID-19 cases among the autochthon population [2]. So far, COVID-19 has affected over 562 Million people worldwide, leading to more than 6 Million deaths (COVID-19 Dashboard, Center for Systems Science and Engineering, Johns Hopkins University – updated on July 18th, 2022 at 09:20 AM).

The global spread of COVID-19 infection soon became a new and unavoidable opportunity for research, significantly in line with my initial PhD proposal of "evaluation, development and implementation of multimodal strategies aimed at improving infection prevention/control strategies among paediatric and adult patients". For this reason, since the early beginning of the pandemic, a **project aimed at improving knowledge on SARS-CoV-2 infection among children and their families** was developed at the Department for Women's and Children's Health of the University Hospital of Padua, and it was integrated within my PhD plan, becoming my main research topic soon.

Aim of this Ph.D thesis is to improve knowledge on SARS-CoV-2 infection, ranging from the study and the implementation of effective infection prevention and control measures to contain the in-hospital spread of SARS-CoV-2 (work package 1), to the clinical characterization of first cases of COVID-19 observed among the children and other vulnerable populations (work package 2), up to the assessment of the medium and long-term immunological, clinical and psychological findings of family members recovered by a household cluster of COVID-19 (work package 3). Most of these aspects have been addressed by the establishment of comprehensive care and follow-up program for families affected by COVID-19, such as the *COVID-19 Family Cluster Follow-up Clinic (CovFC),* at the Department for Women's and Children's Health of the University Hospital of Padua. Since March 2020, the follow-up program has been providing an integrated and multimodal evaluation and care for children, older siblings, and parents enrolled in the prospective COVID-19 cohort named "*CASE cohort*", after receiving their consent. The core of the current research project regards the immunological and clinical research conducted on the "*CASE cohort*", as significant findings were provided, contributing to increasing knowledge on this complex and still largely unknown field.

TABLE OF CONTENTS

Chapter 1

INTRODUCTION AND AIMS

Since December 2019, the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has been spreading worldwide, responsible for the coronavirus disease-2019 (COVID-19) pandemic, thus representing a major concern for healthcare providers [1]. Italy was the first European country to recognize and manage COVID-19 cases among the autochthon population: on 21 February 2020, a resident of the municipality of Vo', near Padua (Italy), died of pneumonia, and he was the first COVID-19 related death detected in Italy [2]. So far, COVID-19 has affected over 562 Million people worldwide, leading to more than 6 Million deaths (COVID-19 Dashboard, Center for Systems Science and Engineering, Johns Hopkins University – updated on July 18th, 2022 at 09:20 AM).

On the 10th of January 2020, the first genome sequence of the novel coronavirus of zoonotic origin was published [3]. Genetic sequencing of the virus firstly isolated from bronchoalveolar lavage fluid from Chinese patients with severe pneumonia of "unknown origin" showed that SARS-CoV-2 is a novel virus belonging to the Betacoronavirus genus, Coronaviridae family [4] as for the two other highly pathogenic coronaviruses severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), that caused fatal respiratory illness in 2002 and 2012, respectively [5]. Four structural proteins of primary importance were further identified: the spike (S) that binds the cellular receptor for viral entry, the nucleocapsid (N) protein necessary for viral replication, and the envelope (E) and membrane (M) proteins. The S protein is composed of S1 and S2 subunits, and through the receptor-binding domain (RBD) S1 subunit is responsible for the binding between the virus and the host cell receptor angiotensin-converting enzyme 2 (ACE2) of epithelial cells in the respiratory tract, inducing the entry of the virus [6]. Soon after entering the cells, SARS- CoV-2 starts replicating and migrating down to the respiratory tract, up to the alveolar epithelial cells, causing pneumonia.

Epidemiological studies conducted on family clusters and nosocomial spread of COVID-19 provided the first evidence of human-to-human transmission of SARS-CoV-2 [7,8]. From December 2019, this virus rapidly spread among several countries worldwide, and on March 11th 2020, the World Health Organization officially declared the global COVID-19 outbreak as a pandemic [9]. First observational data from the adult population showed that most cases (81%) were affected by mild or moderate symptoms; however, severe pneumonia occurred in almost 15% of patients, and 5% were critical, complicated by respiratory failure, septic shock, and/or multiple organ dysfunction or failure [1,10]. Further studies highlighted that COVID-19 disease presentation and severity differ across age classes, and major risk factors for developing acute respiratory distress syndrome and death were identified, such as older age (>60 years) and underlying comorbidities (particularly obesity, cardiovascular disease, diabetes, chronic renal diseases, cancer, and immune deficiency) [11,12].

Since the beginning of the pandemic, it has been observed that children were less severely affected by SARS-CoV-2 infection than adults [13,14], often resulting in underdiagnosis given the mild or asymptomatic clinical course [15]. Several hypotheses have been proposed to explain this apparently reduced susceptibility to severe

SARS-CoV-2 infection. It has been shown that children have a reduced expression of ACE2 receptors and TMPRSS2 proteases required for SARS-CoV-2 viral entry [16], leading probably to a decreased viral replication or lesser susceptibility to pulmonary infection. In addition, the frequent exposure to common-cold human coronaviruses (HCoVs) leading to the development of non-neutralizing antibodies may provide some level of protection in recognizing SARS-CoV-2 early in infection [17]. Moreover, the efficient early control of inflammation due to a robust anti-viral innate immune response that appears earlier in children than adults may be the key to better early controlling of infection and limiting the disease course as reported in children [18–20].
Although children develop less severe COVID-19, it has been proved that they play an important role in spreading the virus [21,22]. In addition, since the beginning of the first European wave of COVID-19, observational data have highlighted that a severe and life-threatening immunological complication may occur in children 4-6 weeks after COVID-19, named multi-system inflammatory syndrome (MIS-C) [23]. Furthermore, it was observed that MIS-C mainly presented with respiratory signs, cardiac impairment (with or without respiratory signs), mucocutaneous signs and sometimes gastrointestinal and neurologic involvement, requiring intensive care unit support [24].

The advanced characterization of the molecular biology and physiopathology of COVID-19 led to the development of new drugs and treatment strategies that differ between the phase I of the disease so-called "viremic phase", occurring early in the course of COVID-19 and characterized by high viral replication, and the later phases, observed in some patients, that are firstly characterized by a pulmonary disease (phase II) with possible further progression to a hyperinflammatory state (phase III) that cause severe systemic complications with high mortality [25] . Evidence showed that patients with severe pneumonia and acute respiratory distress syndrome (ARDS), occurring weeks after a SARS-CoV-2 infection, are characterized by a marked increase in inflammatory markers including D-dimer, C-reactive, ferritin and cytokines (IL-6, TNF-alpha) and chemokines, similarly to the cytokine storm syndrome secondary to CAR-T [26,27]. A high increase in inflammatory markers was also observed in children affected by MIS-C [28].
Large, randomized control studies have been conducted worldwide, providing evidence for the further development and update of international guidelines that are currently available for clinicians (https://www.covid19treatmentguidelines.nih.gov). Among those, the RECOVERY trial provided the first evidence supporting the use of dexamethasone to treat severe pneumonia[29]. In addition, several antivirals were further approved by the Food and Drug Administration (FDA) and are currently used to prevent viral replication and reduce the risk of hospitalization and death for severe COVID-19. Oral antivirals mostly active in the early phase of infection have shown to be effective in preventing hospitalization and disease progression through various mechanisms, such as blocking SARS-CoV-2 entry through the nucleoside analogue monlupiravir [30] or by inhibiting the activity of SARS-CoV-2 3-chymotrypsin-like protease (3CLpro) and RNA-dependent RNA polymerase (RdRp) through the SARS-CoV-2 main protease nirmatrelvir booster with ritonavir[31]. In addition, the intravenous antiviral remdesivir has been extensively used in hospitalized patients, acting as a SARS-CoV-2 nucleotide analogue RNA polymerase inhibitor and leading to lethal viral

mutagenesis, even though its efficacy is still under debate [32,33]. Several anti-SARS-CoV-2 monoclonal antibodies (mAbs) have also been developed, targeting the spike protein. However, the effectiveness of the different anti-SARS-CoV-2 mAb therapies dramatically depends on the circulating variant, and their role in the treatment of COVID-19 remains variable [34]. As the Omicron variant of concern (VOC) has become the dominant variant, Bebtelovimab is currently the only mAb indicated within seven days of symptoms onset, in subjects older than 12 years (https://www.covid19treatmentguidelines.nih.gov/therapies).

In the early beginning of the COVID-19 pandemic, I soon realized that COVID-19 infection had to become a new and unavoidable opportunity for research, being in line with my initial proposal of "evaluation, development and implementation of multimodal strategies aimed at improving infection prevention/control strategies among paediatric and adult patients". The project **"Improving knowledge on SARS-CoV-2 infection among family clusters including children: from the implementation of effective infection prevention and control measures to an integrated evaluation of epidemiological, clinical and immune-virological characteristics of COVID-19**" was developed at the Department for Women's and Children's Health of University Hospital of Padua and it became my main research topic.

At that time, the following research questions were developed:

1. *What are the main infection prevention and control strategies to contain the in-hospital spread of SARS-CoV-2 infection?*
   - 1.1 What are the main actions to be set up to contain the spread of SARS-CoV-2 infection within an Emergency Department?
2. *How SARS-CoV-2 infection is transmitted, and how does it clinically present among different populations?*
   - 2.1 How is SARS-CoV-2 transmitted among the paediatric population?
   - 2.2 How is the clinical presentation of SARS-CoV-2 among infected children?
   - 2.3 What is the impact of SARS-CoV-2 on the most vulnerable population, including people living with HIV?
3. *What is the immunological and clinical impact of SARS-CoV-2 infection among COVID-19 family clusters, including children?*
   - 3.1 What are the magnitude and the dynamic of humoral response elicited by a SARS-CoV-2 infection?
   - 3.2 How does a SARS-CoV-2 infection elicit the cellular-mediated response?
   - 3.3 How long do anti-SARS-CoV-2 antibodies persist after COVID-19 infection?
   - 3.4 What is the clinical performance of current available SARS-CoV-2 serological assays?
   - 3.5 Is there any cardiac impairment in children with COVID-19?
   - 3.6 What is the psychological impact of COVID-19 and home isolation among children and parents experiencing a COVID-19 family cluster?

Based on the research questions mentioned above, three main study objectives were developed, and, focusing on them, three different work packages (WPs) were developed:

1. **Objective 1 - Work package 1 (WP1)**. "*Implementing infection prevention and control strategies to contain the in-hospital spread of SARS-CoV-2 infection*". Results of WP1 are presented in Chapter 2 (Sub-Chapters 2.1 and 2.2).
2. **Objective 2 - Work package 2 (WP2)**. "*Improving knowledge on viral transmission and clinical characteristics of SARS-CoV-2 infection among several populations*". Results of WP2 are presented in Chapter 3 (Sub-Chapters 3.1, 3.2 and 3.3).
3. **Objective 3 - Work package 3 (WP3)**. "*Improving knowledge on the medium and long term immunological and clinical impact of SARS-CoV-2 infection among children and adults recovered by a COVID-19 family cluster*". Results of WP3 are presented in Chapter 4 (Sub-Chapters 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6).

Description of Work Packages (WPs).

WP1 - ***Infection prevention and control strategies to contain the in-hospital spread of SARS-CoV-2 infection*****.**

The first phase of my work constitutes WP1 and is described in Chapter 2 (Sub-Chapters 2.1 and 2.2). WP1 focuses on the implementation of infection prevention and control strategies to contain the in-hospital spread of SARS-CoV-2 infection. We described the efforts made to reorganize the Paediatric Department of Women's and Children's Health, Padua University Hospital, since the beginning of the SARS-CoV-2 epidemic. Focusing on these aspects, we published two papers.

Chapter 2.1 describes our efforts to merge scientific evidence available at the beginning of the COVID-19 pandemic with the clinical and organizational issues faced within the first month of coexistence with COVID-19. More in detail, this chapter describes the operational measures set up at the Pediatric Emergency Department to contain the spread of SARS-CoV-2. According to the epidemiologic and clinical risk factors, four different pathways were developed to address children/adolescents with suspected COVID-19. The strict application of the measures led to quick identification, isolation, and management of all positive children, preventing SARS-CoV-2 intrahospital spread in the first wave of the COVID-19 pandemic.

Chapter 2.2 describes in detail the multilevel interventions set up at the Department of Women's and Children's Health of Padua University Hospital to prevent the SARS-CoV-2 in-hospital spread. More in detail, measures set up were (a) to revise the distribution of the clinical areas in order to create both designated COVID-19 and COVID-19-free areas with their access, (b) to reinforce infection prevention control (IPC) measures for all healthcare workers and administrative staff and (c) to reinforce IPC measures for patients adopting the new

"double-gate approach": a phone call pre-triage and nasopharyngeal swab for SARS-CoV-2 detection before the admission of all patients and caregivers.

WP2 - ***Viral transmission and clinical characteristics of SARS-CoV-2 infection among several populations*****.**

The second part of my PhD thesis constitutes WP2 and is described in Chapter 3 (Sub-Chapters 3.1, 3.2 and 3.3). This part was essentially developed through the collection and review of published studies available at the beginning of the pandemic, conducted in parallel with the retrospective analysis of patient-based data collection of the first COVID-19 cases observed among children attending the Department of Women's and Children's Health of Padua University Hospital. Focusing on these aspects, I have contributed to the publication of 3 articles.

Chapter 3.1 describes two infants evaluated in March 2020, both tested positive for SARS-CoV-2 at rectal swab (in addition to nasopharyngeal swab). It reviews the literature on viral transmission in children, suggesting that fecal shedding with environmental contamination may play an important role in the viral spread.

Chapter 3.2 is a multicenter retrospective analysis of clinical records of 127 SARS-CoV-2-infected children evaluated in 23 sites in Italy, including the COVID-19 Pediatric Ward of the Department of Women's and Children's Health, University of Padua. The study evaluates the mode of presentation, risk factors, the severity of disease presentation and early outcome of the first pediatric COVID-19 cases observed in Italy.

Chapter 3.3 systematically reviews the knowledge available on SARS-CoV-2 infection in people living with HIV from the beginning of the pandemic until June 2020. The study provides the first systematic characterization of cases of COVID-19, with or without laboratory confirmation, among people living with HIV/AIDS (PLWHA). MEDLINE, EMBASE and Google Scholar databases were systematically searched, using free-text terms for "SARS-CoV-2 AND HIV AND children AND adults". Moreover, reference lists from eligible articles were reviewed to identify other potentially relevant papers. The last search was conducted on May 28th, 2020.

WP3 - ***Medium and long-term immunological and clinical impact of SARS-CoV-2 infection among children and adults recovered by a COVID-19 family cluster*****.**

Lastly, the most important part of my work constitutes WP3, and it is described in Chapter 4 (Sub-Chapters 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6). This work package includes the research conducted to explore the immunological and clinical findings developed in the medium and long term period of time, after a SARS-CoV-2 infection. Several findings have been provided through a prospective observation of Italian families, including children and adults, recovered by a COVID-19 family cluster.

Since the beginning of the pandemic, evidence showed that SARS-CoV-2 spreads among families with different clinical presentations and outcomes according to age classes, with children often presenting with asymptomatic infection[2]. At that time, we perceived that families who recovered from COVID-19 needed to be clinically and psychologically supported and followed up over time. In addition, we had the insight that the prospective observation of the whole family cluster of COVID-19 would have provide essential findings on children and adults experiencing SARS-CoV-2 infection, and at the same time sharing the same environmental context. For this reason, since the end of March 2020, we have implemented an integrated evaluation for children and their families experiencing COVID-19 infection among their households through the *COVID-19 Family Cluster Follow-up Clinic (CovFC),* at the Department of Women's and Children's Health. Through a strong collaboration with the Family Pediatricians of Veneto Region, families recovered from COVID-19, including children, older siblings, and their parents, have been referred to our clinic and prospectively enrolled in the "*CASE cohort*", four or more weeks after SARS-CoV-2 infection. Inclusion criteria for enrollment were: a) having children of pediatric age (<15 years) and b) one or more family member/s with a history of confirmed COVID-19. At enrolment, a comprehensive assessment of children and adults occurred, provided by a Paediatrician and/or an Infectious Diseases specialist. During each visit, data on demographic parameters, past medical history, clinical evaluation, past SARS-CoV-2 virological assays at nasal-pharyngeal swab (date of tests and result) and vaccinal status, including the SARS-CoV-2 vaccine (from when it was available) were collected. Specifically, the following evaluations were included:

a) for all subjects (irrespectively of history of confirmed COVID-19):
   - clinical evaluation
   - a blood sample collection for the detection of SARS-CoV-2 antibodies (for all parents and children)

b) in addition, only for children with confirmed SARS-CoV-2 infection:
   - blood tests (full blood count, C-reactive protein, liver and renal function, NT-proBNT, troponin and any other test according to the clinical evaluation)
   - standard transthoracic echocardiogram (TTE) and cardiological assessment
   - any other instrumental assay, if clinically required (e.g. Chest X-Ray, pulmonary function tests…)

Moreover, a psychological support was proposed to a sub-cohort of families evaluated during the first and second waves of the SARS-CoV-2 pandemic, including the distribution of a web-based survey and, when needed, the opportunity of having a free-of-charge psychological interview.

A multidisciplinary network was set up, including Medical Doctors (Paediatricians and Infectious Diseases Consultants) and Nurses, a Virologist, Immunologists, Medical Biologists, Psychologists, and a Statistician. The connection of different specialists allowed a continuous sharing of knowledge, contributing to defining and operationally setting up the various phases of the research, over time. The following Departments were directly involved in the research studies:

- Family Pediatricians of Veneto Region;
- Pediatric and Congenital Cardiology Unit, Department for Women's and Children's Health, University Hospital of Padua;
- Division of Comparative Biomedical Sciences, Istituto Zooprofilattico Sperimentale delle Venezie, Padua;
- Department of Surgery, Oncology and Gastroenterology, Section of Oncology and Immunology, University of Padua, Padua;
- Department of Laboratory Medicine and Department of Medicine-DIMED, University-Hospital of Padua;
- Research Unit of Congenital and Perinatal Infections, Bambino Gesù Children's Hospital, Rome;
- Department of Molecular Medicine, University of Padua;
- Department of Statistics and Quantitative Methods, Division of Biostatistics, Epidemiology and Public Health, Laboratory of Healthcare Research and Pharmacoepidemiology, University of Milano-Bicocca, Milan.

At each follow-up visit, all family members were sampled for blood collection after being informed and after providing written consent for them (parents/siblings older than 18 years) and their children. All patients with persistence of clinical signs and/or with detection of positive SARS-CoV-2 serology at enrolment were followed up for longitudinal clinical and serological evaluation. Follow-up was interrupted in cases of serology negativization, in asymptomatic cases.

Blood samples were collected in EDTA-coated tubes to further separate cells and plasma by Ficoll procedure for immunological assays. Plasma and cellular samples were appropriately stored at -80°C and liquid nitrogen, respectively, until use. In the first pandemic wave, SARS-CoV-2 IgG and IgM detection was conducted using the CLIA MAGLUMI 2019-nCoV IgM and IgG on the analytical system MAGLUMI 2000 Plus (New Industries Biomedical Engineering Co, Ltd, Shenzhen, China) [35] . However, from September 2020, in line with emerging data comparing the diagnostic accuracy of novel serological tests, the new chemiluminescent immunoassay (CLIA) for the detection of the anti-receptor binding domain (RBD) antibodies (Abs) against SARS-CoV-2's spike (S) protein was used (MAGLUMI™2000 Plus, Snibe Diagnostics, New Industries Biomedical Engineering Co., Ltd [Snibe], Shenzhen, China); results were expressed in kiloastronomical unit (kBAU/L) [36]. Samples recording titers > 4.33 kBAU/L were considered positive. The serological assays were done at the Department of Laboratory Medicine and Department of Medicine-DIMED, University-Hospital of Padua.

From March 2020 to March 2021, most families were also tested for the quantification of SARS-CoV-2 neutralizing antibodies with a high throughput method for Plaque Reduction Neutralization Test (PRNT) [35]. Biosafety level 3 laboratory setting located at the Division of Comparative Biomedical Sciences, Istituto Zooprofilattico Sperimentale delle Venezie (Padua), was used for PRNT tests. The neutralization titer was defined as the reciprocal of the highest dilution resulting in a reduction of the control plaque count >50% ($PRNT_{50}$). Samples recording titers 1:10 were considered positive according to a previous validation conducted

on a panel of archive samples collected in 2018 in Italy. The sudden increase in the enrollment rate of further pandemic waves made it not sustainable to apply both serological assays, given the high economic and operational costs posed by the PRNT. Therefore, considering that previous validation exercises proved the high correlation between the two assays [36], from March 26th, 2021, all family members were tested for Snibe anti-SARS-CoV-2 S-RBD IgG levels.

A web-based case report form (CRF) was created using the REDCap platform (Vanderbilt University, Tennessee) hosted on the server of the University of Padova. Patient-based data collected at enrolment and clinical follow-up visits were anonymized and entered the web-based database. According to the national regulation, a study protocol was elaborated and communicated to the Ethical Committee (Prot. N° 0070714 of November 24th,2020; amendment N°71779 of November 26th, 2020). For research purposes, data on clinical and laboratory findings of all patients were extracted from the hospital's electronic medical records and analysed anonymously. Parents or legally authorized representatives were informed of the research proposal and provided their written consent for both the collection and use of biological specimens and the routine patient-based data.

Five articles were published, and one paper was recently submitted to a peer-reviewed journal. Four articles were done on the medium- and long-term immunological response after COVID-19 infection among children, older siblings, and adults:

Chapter 4.1 evaluates the production and persistence of naturally acquired SARS-CoV-2 neutralizing antibodies (nAbs) among different age classes of children and adults belonging to the *CASE cohort*. We analysed 283 blood samples collected from 152 confirmed COVID-19 cases (82 parents and 70 children/older siblings of median age of 8 years, IQR 4-13) presenting with asymptomatic or mildly symptomatic disease. Despite the decrease of IgG over time, SARS-CoV-2 neutralizing antibodies (nAbs) were found to persist up to 7-8 months in children, while adults recorded a modest declining trend. Interestingly, children under six years of age, mainly under three, developed higher long-lasting levels of nAbs compared to older siblings and/or adults.

Chapter 4.2 analyses the cellular immune profile of SARS-CoV-2-infected adults and children of the *CASE cohort* compared to uninfected age-class matched relatives: we explored the immune profiles of activation, senescence, exhaustion, and regulatory cells among 152 patients with confirmed COVID-19 (by PCR and/or serology) and we evaluated the relationship with neutralizing antibodies and viral load in asymptomatic and mild symptomatic COVID-19 children and adults.

Chapter 4.3 investigates the analytical and clinical performance of a SARS-CoV-2 RBD IgG assay (Snibe diagnostics), automated on a high throughput platform, and it evaluates the correlation of IgG levels with SARS-CoV-2 neutralizing antibodies detected through plaque reduction neutralization (PRNT50) test. A series of 546 samples were evaluated, including 171 negative and 168 positive SARS-CoV-2 subjects and a further group of 207 subjects of the COVID-19 family clusters follow-up cohort.

Chapter 4.4 is a single-centre, prospective observational cohort study that assesses the long-term anti-SARS-CoV-2 S-RBD IgG kinetics in children following SARS CoV-2 infection, up to 18 months after infection. Compared to the previous study, it includes a larger cohort of 252 COVID-19 family clusters belonging to the *CASE cohort.* Subjects underwent a serological follow-up at 1-4, 5-10, and >10 months after infection with quantification of anti-S-RBD IgG by chemiluminescent immunoassay. Among those, 351 were children/older siblings aged 8.6±5.1 years, and 346 were parents aged 42.5±7.1 years; 96.5% of cases had asymptomatic/mild COVID-19. Findings are reported in the Result session, Chapter 4.

Moreover, the prospective follow-up of subjects enrolled in the *CASE cohort* has characterized the clinical and psychological impact of asymptomatic/mildly symptomatic COVID-19. Two studies were done on this topic (one was published, and a second one was recently submitted):

Chapter 4.5 describes the cardiac involvement of SARS-CoV-2 infection in pediatric patients recovered by asymptomatic or mildly symptomatic SARS-CoV-2 infection. The research is a case-control study conducted on 53 paediatric patients with a mean age of 7.5 years belonging to the *CASE cohort*. Children underwent a standard transthoracic echocardiogram and speckle tracking echocardiographic study at least three months after diagnosis and were compared with 32 healthy controls.

Chapter 4.6 explores the resilience and the psychological impact of COVID-19 among family clusters of the *CASE cohort*. The study reports results from a single-center, cross-sectional web-based survey conducted on families attending the COVID-19 Family Cluster Follow-up Clinic. The survey included two main sessions: a first part ("*Questionnaire A – Family*") that was elaborated ad hoc by a team including two Psychologists, a Social Assistant, Pediatricians and an Infectious Diseases Specialist, aimed at exploring the quality of relationships within the family actors, retrospectively referring to the time before, during and after COVID-19. A second part was further elaborated ("*Questionnaire B - Children*") and specifically dedicated to the children's behavior observed during COVID-19 and to the children's ability to adapt to COVID-19 disease and home isolation. According to the age of children, the questionnaire included two mutually exclusive parts: "*Questionnaire B/1 – pre-school children*" and "*Questionnaire B/2 -Pediatric Symptoms Checklist*". Among the 176 surveys distributed from March to October 2020, 75 were collected from 66 families (97 parents and 129 children). Results of the web-based survey are presented in Chapter 4.

Details on study design, methods and statistical analysis are described in the Methods section for each individual study, in the Results. Results are presented in Chapters 2, 3 and 4 and they are articulated according to the three different work packages.

Chapter 2

RESULTS

Infection prevention and control strategies to contain the in-hospital spread of SARS-CoV-2 infection

2.1. Children's Hospital Management in the COVID-19 Era: The Reorganization of a Tertiary Care Pediatric Emergency Department in Northern Italy

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Abstract.

In the Veneto Region, an exponential spread of patients affected by 2019 novel Coronavirus disease (COVID-19) has been observed after February 21st. Since then, we have been evaluating children suspected or confirmed for SARS-CoV-2 infection. A protocol for pediatric hospital reorganization and children management has been developed since the beginning of the epidemic. A pre-triage area has been created at the immediate entrance of the pediatric emergency room, for all uncritical pediatric patients. According to the epidemiologic and clinical risk factors, all children/adolescents have been addressing to one of the four different pathways created. The strict application of this protocol has been leading to quickly identification, isolation, and management of all positive children, preventing SARS-CoV-2 intrahospital spread.

Keywords: COVID-19; children; clinical pathway; hospital; pediatric department.

Introduction.

The global health crisis of SARS-CoV-2 pandemic is changing the world (1, 2). In the Veneto Region, an exponential spread of patients affected by 2019 novel Coronavirus disease (COVID-19) has been observed since February 21st, the day of the first COVID-19 positive adult admitted to the University Hospital of Padua. Since then, several actions have been immediately taken to ensure a prompt recognition of children suspected or confirmed for SARS-CoV-2 infection and to guarantee both urgent care to COVID-19 infected children and the safety of all healthcare workers and other non-infected children of the Pediatric Department of the University Hospital of Padua (3).

Case definition for pediatric suspected COVID-19 was adapted from the definition provided by the Italian Ministry of Health, therefore a suspected case was defined as a child/adolescent with fever (TC> 37.5 C axillary) and/or respiratory symptoms (rhinitis, cough, and dyspnea) and/or gastrointestinal symptoms (vomiting, diarrhea) with or without close contact with a probable or confirmed case of COVID-19, within the previous 14 days (4, 5). Traveling in high-risk areas was no longer considered as epidemiologic risk factor, because Italy was declared "red zone" at the beginning of March. COVID-19 confirmed cases were those with a positive nasopharyngeal swab test for SARS-CoV-2, detected by qualitative polymerase-chain reaction (PCR) (4).

This protocol was the result of all our daily efforts to merge scientific evidence that were available with clinical and organizational issues faced within the first month of coexistence with COVID-19 (6–9). The aim of this paper is to share our experience in order to support other pediatricians in different settings in dealing with the structural reorganization of Pediatric Departments facing with COVID-19 pandemic.

Setting.

In March 2020, this hospital reorganization was, firstly, set up in the PED of the Department of Woman's and Child's Health at Padua University Hospital and it was subsequently promoted to all the Pediatric departments of Veneto Region.

Our Children's Hospital provides primary and secondary care for a metropolitan area of 350,000 people (45,000 younger than 15 years) and tertiary care for a regional and extra-regional population, with ~26,000 PED visits per year and an overall hospital admission rate from PED of around 7 out of 100 visits.

Policy Options and Implications.

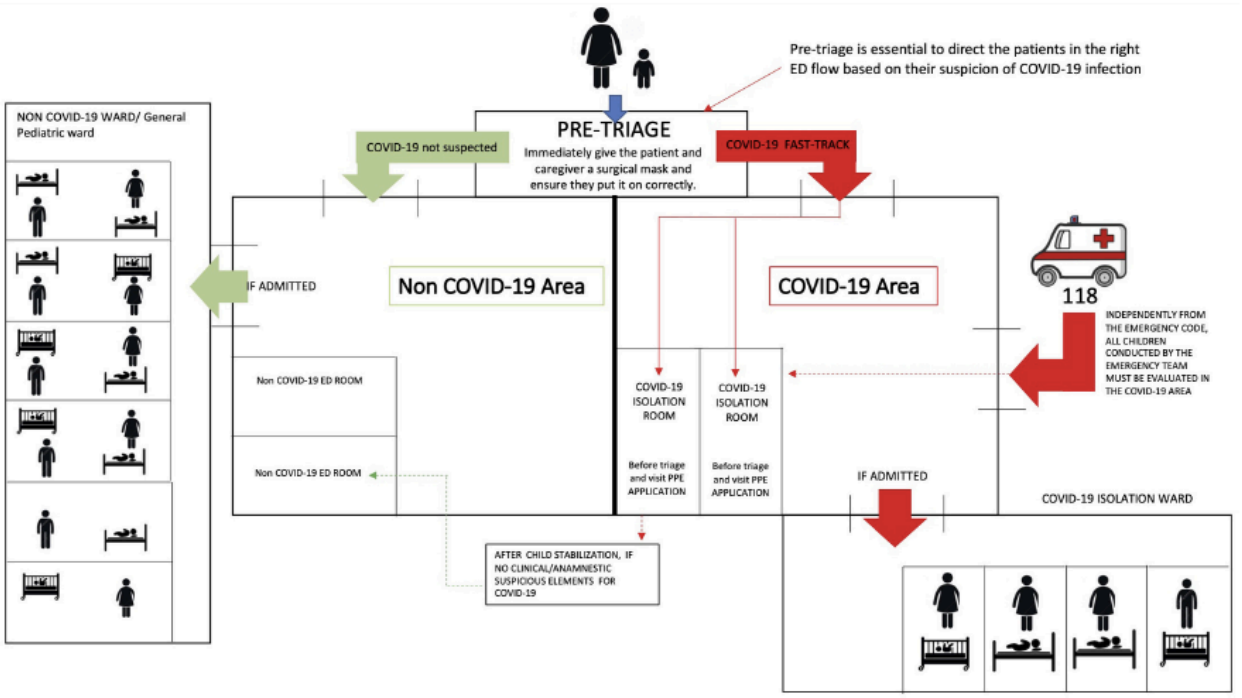
Several operational steps have been set up in order to reorganize the PED and the Pediatric Units.

Reorganization of the Emergency Room (ER) (Figure 1)

ER Presentation of Uncritical Patient.

A pre-triage area had to be created at the immediate entrance of every pediatric or general emergency room (ER), to evaluate all uncritical pediatric patients that autonomously come from home. The pre-triage evaluation is crucial to promptly identify patients at risk of COVID-19, before hospital admission. The evaluation must be performed by trained healthcare workers with adequate personal protective equipment (PPE) and a surgical mask must be given to the child and his/her caregiver.

Figure 1. Pediatric Emergency Department reorganization in the COVID-19 era.



Following the pre-triage, two different areas have to be created in order to separately direct patients and their caregivers to specifically dedicated areas, such as:

- Suspected COVD-19 child/adolescent area (COVID-19 Area), ideally provided with at least one negative pressure isolation room.
- Not suspected COVID-19 child/adolescent area (Not COVID-19 Area).

The two Areas must be physically separated, with dedicated healthcare workers in order to avoid grouping and contamination among suspected/confirmed cases and those who are not.

In the COVID-19 Area, a dedicated waiting room have to be set up for the child/adolescent and his/her caregiver, with recommendation of wearing surgical masks and keeping at least 1 m of distance from other patients. Whenever these conditions are not feasible, the child/adolescent and his/her caregiver have to be isolated in the evaluation room.

ER Presentation of Critically Ill Patient.

All patients urgently conducted to the emergency room by an emergency team have to be directly sent to the isolation room of COVID-19 Area, being assisted by healthcare workers equipped with adequate PPE, since a pre-triage assessment is not performed.
As soon as the child/adolescent is defined as stable, he/she have to be transferred to the Not COVID-19 Area if absence of any epidemiologic risks and/or signs and symptoms suspicious of COVID-19.

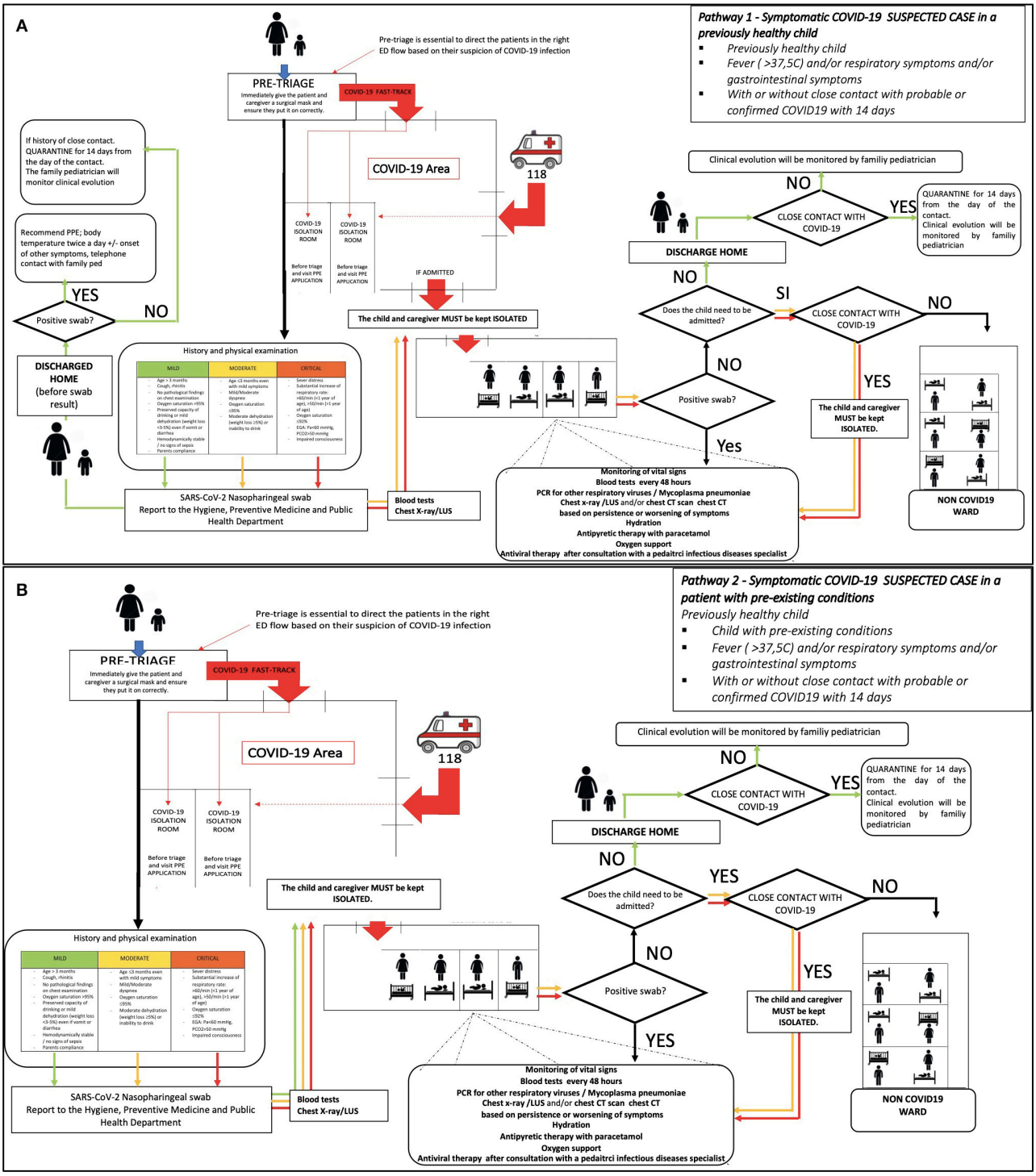
Reorganization of Pediatric Units.
Every hospital with an ER must arrange rooms for suspected or confirmed COVID-19 pediatric patients, separated from the non-COVID-19 patients' ward.

Whenever available, the child/adolescent should be placed in a negative pressured room or, if it is not possible, in a single-bed room with its own bathroom setting up all precautions needed for respiratory diseases, such as standard precautions and adjunctive protection devices for agents transmittable by droplets and/or through aerosol and/or contact. All patients with confirmed COVID-19 should be assisted by dedicated staff. The patient's caregiver should always wear a surgical mask and should not exit the room. Indeed, visitors have not to be allowed. Meals have to be served with disposable cutlery to both the patient and his/her caregiver.
In case both the child/adolescent and his/her caregiver are affected by COVID-19 infection, it would be reasonable to hospitalize them together in a dedicated room of the pediatric ward, to guarantee the family unity, if the caregiver is asymptomatic or has mild symptoms (which normally do not require hospitalization).
If parents/caregivers require hospitalization due to clinical conditions, the child and caregivers have to be referred to an Infectious Diseases ward and a dedicated pediatric consultant should be activated.
In case of a critically ill child/adolescent with confirmed COVID-19, the patient should be immediately transferred to the referral hospitals with COVID-19 dedicated pediatric intensive care units.

Pathways for Child/Adolescent With Suspected or Confirmed Infection by SARS-CoV-2.
After being evaluated at pre-triage, patients should be stratified according to clinical characteristics, past history including concomitant co-morbidities, and epidemiological risk of COVID-19. The following four different pathways have been defined:

Pathway 1—Symptomatic COVID-19 SUSPECTED CASE in a previously healthy child (Figure 2A)
• Previously healthy child.
• Fever (>37.5°C) and/or respiratory symptoms and/or gastrointestinal symptoms.
• With or without close contact with probable or confirmed COVID19 cases in the previous 14 days.

Figure 2. (A) Pathway 1—management of a previously healthy child/adolescent, suspected, or probable case of COVID-19. **(B)** Pathway 2—management of a child/adolescent with chronic disease /immunosuppression suspected or probable case of COVID-19.



Pathway 2—Symptomatic COVID-19 SUSPECTED CASE in a patient with pre-existing conditions (Figure 2B)

• Child with pre-existing conditions.

• Fever (>37.5°C) and/or respiratory symptoms and/or gastrointestinal symptoms.

• With or without close contact with probable or confirmed COVID19 cases in the previous 14 days.

Pathway 3—CLOSE CONTACT with confirmed case of COVID-19, with no symptoms of infection (Figure 3A)

• Child/adolescent reporting close contact with a confirmed case of COVID-19 within 14 days.

• No fever and/or respiratory and/or gastrointestinal symptoms.

• ± COVID-19 unrelated clinical symptoms.

Pathway 4—NOT SUSPICIOUS CASE (Figure 3B)

• Child/adolescent not reporting COVID-19 symptoms in particular fever (CT > 37.5°C) and/or respiratory and/or gastrointestinal symptoms.

• Without close contact with COVID-19 cases in the previous 14 days.

Figure 3. (A) Pathway 3—management of an asymptomatic child/adolescent with confirmed "close contact."

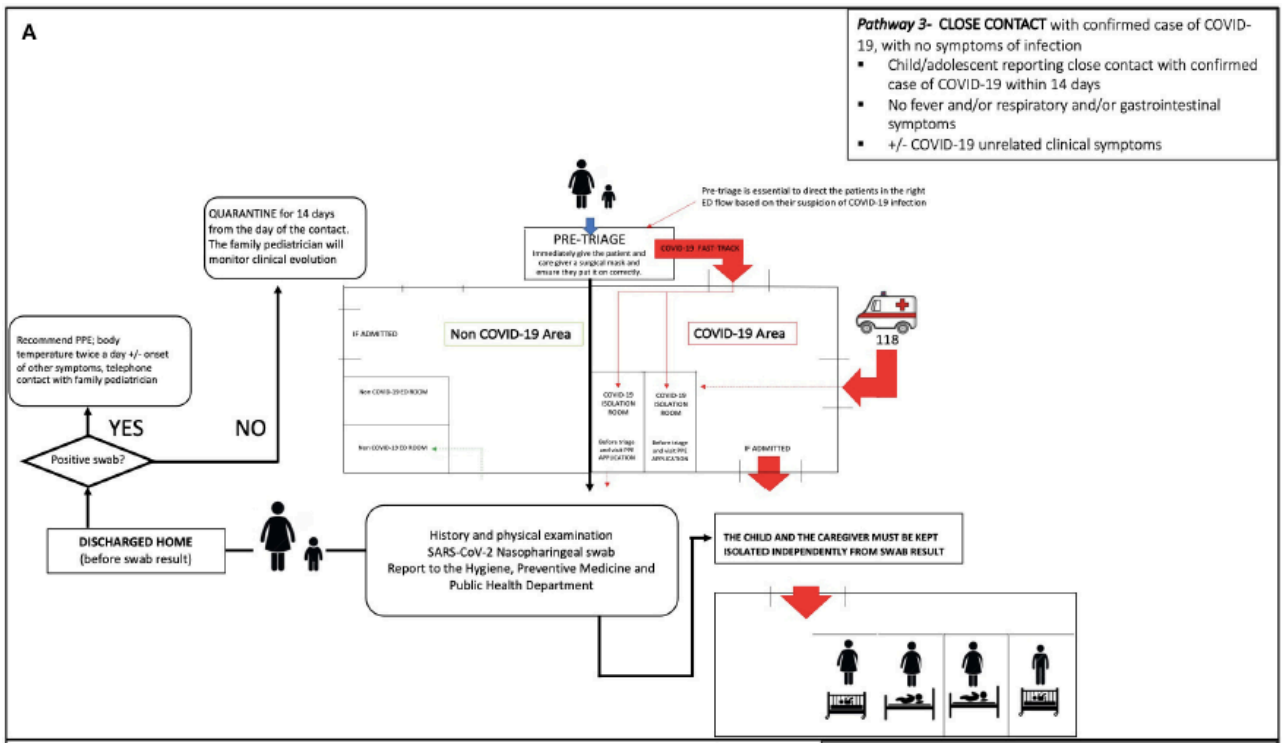
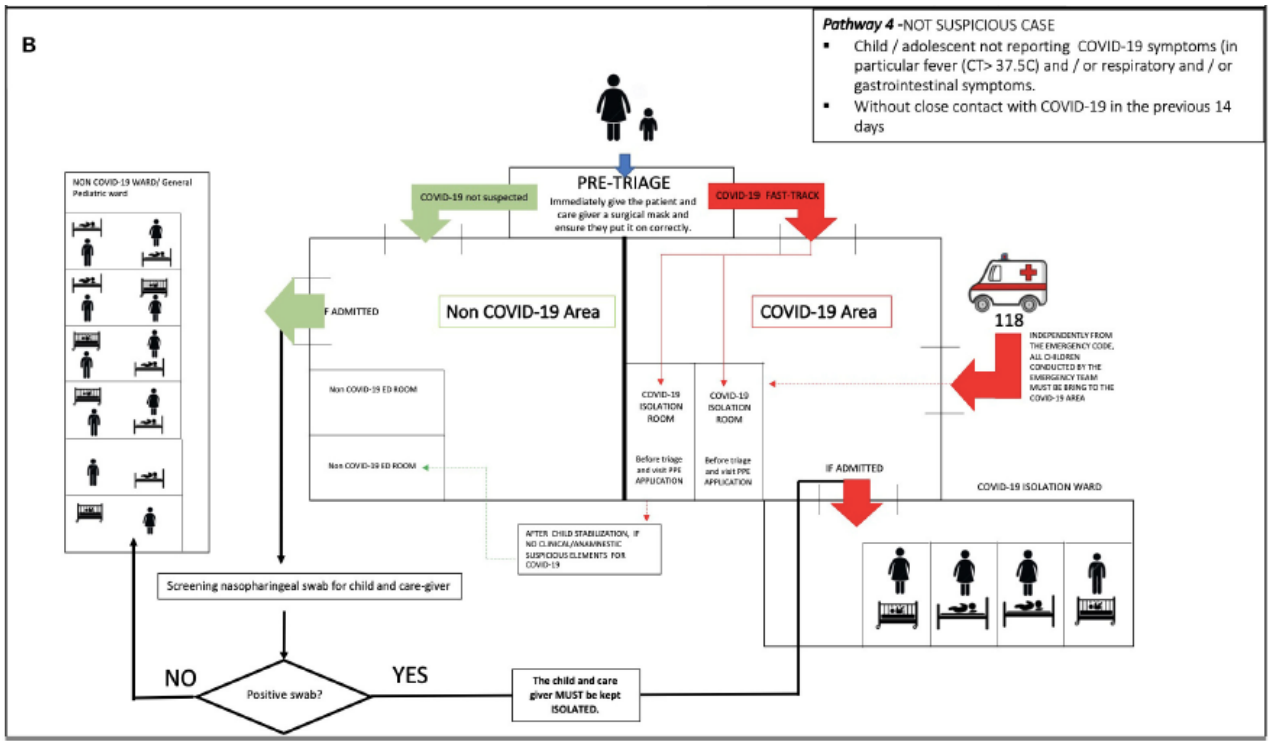


Figure 3. (B) Pathway 4—management of a child/adolescent NOT SUSPECTED for COVID-19.



Pathway 1 - "Symptomatic COVID-19 Suspected Case in a Previously Healthy Child" (Figure 2A).

Patients identified at pre-triage as "COVID-19 SUSPECTED CASE" and not affected by any chronic condition should be sent to the evaluation room of COVID-19 Area, after wearing a surgical mask.

After being evaluated for family history and past medical history, including immunizations and allergies, the child and his/her caregiver should be clinically evaluated and classified as "mild—moderate or critical" according to the following proposed classification (Table 1). This score was formulated on the basis of available evidence, at the time of protocol set up (6–9). To define moderate/critical clinical syndrome just one of the Table 1 criteria is need.

After clinical evaluation, all children/adolescents have to be tested with:

• Nasopharyngeal swab for SARS-CoV-2.

• Rectal swab for SARS-CoV-2 (whenever possible).

We strongly suggest testing also the caregiver with a nasopharyngeal swab for SARS-CoV-2 since familiar clusters are frequent.

Table 1. Clinical classification for SARS-CoV-2 infected children.

Mild	Moderate	Critical
- Age > 3 months - Cough, rhinitis - No pathological findings on chest examination - Oxygen saturation >95% - Preserved capacity of drinking or mild dehydration (weight loss <3–5%) even if vomit or diarrhea Hemodynamically stable/no signs of sepsis - Parents compliance	- Age ≤3 months even with mild symptoms - Mild/Moderate dyspnea - Oxygen saturation ≤95% - Moderate dehydration (weight loss ≥5%) or inability to drink	- Sever distress - Substantial increase of respiratory rate: >60/min (<1 year of age), >50/min (>1 year of age) - Oxygen saturation ≤92% - EGA: Pa<60 mmHg, PCO2>50 mmHg - Impaired consciousness

The following further tests have to be performed in case of moderate/critical clinical presentation:
i. Chest X-Ray and/or lung ultra-sound (LUS) and/or chest CT scan, using portable diagnostic tools (to avoid any transfer of infected patients to other areas).
ii. Blood tests: full blood count, renal and liver function, glucose, CRP, PCT, hemogasanalysis, urinalysis, if septic myocardial enzymes, PT, PTT, fibrinogen, FDP, LDH, ferritin, lactate.

The pediatrician will decide whether to discharge or admit the child/adolescent according to clinical criteria (while waiting for tests results).
If the patient has mild symptoms can be discharged before swabs result, with strict indication for home isolation while waiting for the results. The results have to be reported to the caregiver or to the pediatrician within 24–48 h. Children and their families have to be referred to the family pediatrician (FP) and/or general practitioner (GP), for further follow-up.

Based on swab's results, the child home-based management has to be organized as follows:
- In case of a positive result for COVID-19, the child discharged from the ER should undergo home isolation and caregivers must apply contact or droplets precaution (if negatives) to prevent viral transmission. Body temperature should be checked twice a day as the onset of respiratory and gastrointestinal symptoms. In case of worsening of symptoms, they should be further referred to the ER. Home isolation have to be kept for at least 14 days, at the end of that period two consecutive nasopharyngeal swab for SARS-CoV-2 have to be performed: isolation should end only if both swabs are negative. Active surveillance for 14 days from last contact with COVID-19 have to be applied to all “close contacts.”
- In case of a negative swab, a 14-day home isolation should be recommended (from the last day of contact) to al close contacts with a confirmed COVID-19 case. The clinical evolution will be monitored by the GP.

All suspected/confirmed COVID-19 cases classified as moderate/severe have to be hospitalized in a dedicated area, regardless of COVID-19 swab pending results. Critically ill children/adolescents with suspected/confirmed COVID-19 should be stabilized and referred to hospitals with COVID-19 dedicated pediatric intensive care units, particularly if the patient is younger than 1 year of age, due to the higher risk of worsening conditions.

The further management of admitted children/adolescents will change according to the results of nasopharyngeal swab test:
- In case of positive result, the patient should be kept hospitalized in the confirmed/suspected COVID-19 dedicated ward (regular or ICU). Please refer to section 4-Management of COVID-19 positive hospitalized patient.
- In case of negative result, the child or adolescent should be transferred to another non-COVID-19 ward, only in the absence of epidemiological risk factors. In case of history of close contact, the patient should be kept isolated in the COVID-19 ward.

Pathway 2 - "Symptomatic Suspected/Probable" COVID-19 Case in an Immunocompromised Patient/Patient With Chronic Disease (Figure 2B).

Patients identified at pre-triage as "COVID-19 SUSPECTED CASE" should be sent to the evaluation room of COVID-19 Area, after wearing a surgical mask. Please see section Pathway 1—"Symptomatic COVID-19 Suspected Case in a Previously Healthy Child" (Figure 2A) for the clinical evaluation.
All symptomatic children/adolescents with suspected COVID-19 and with concomitant immunosuppression/chronic disease have to be admitted to the hospital for observation.

The further management of admitted children/adolescents changes according to the results of nasopharyngeal swab test:
- in case of a positive result, the patient should be hospitalized in a confirmed/suspected COVID-19 dedicated ward (pediatric ward or PICU). Please refer to section Management of COVID-19 positive hospitalized patient. Home isolation with active surveillance should be applied to all "close contacts," for 14 days from patient's last contact.
- in case of negative result, the child or adolescent should be transferred to another non-COVID-19 ward, only in the absence of epidemiological risk factors. In case of history of close contact, the patient should be kept isolated in the COVID-19 ward. If clinical conditions do not require hospitalization, the patient should be discharged recommending home isolation and active surveillance only in case of a referred COVID-19 close contact. Clinical evolution should be monitored by the GP.

Pathway 3—Close Contact With a Confirmed COVID-19 Case, Asymptomatic for COVID-19 Symptoms (e.g., ER Admission for Different Clinical Issues) (Figure 3A).

Patients identified at pre-triage as "COVID-19 SUSPECTED CASE" and not affected by any chronic condition should be sent to the evaluation room of COVID-19 Area, after wearing a surgical mask. After COVID-19 test has been performed, the pediatrician should evaluate whether to discharge or hospitalize the child/adolescent according to disease severity.

If the child/adolescent is clinically stable, he/she should be discharged before the COVID-19 test result and home isolation have to be recommended until the availability of test result, that should be reported to the caregiver or to GP within 24–48 h. In case of children/adolescents with clinical course requiring hospitalization, they should be referred to a COVID-19 dedicated ward.

Pathway 4—Non-suspected Case (Figure 3B).

The healthcare worker of pre-triage has to address the patient and his caregiver to the triage in the NOT COVID-19 Area. They will be evaluated according to the disease that drove them to the ER.

The nasopharyngeal swab screening has to be performed to the child and caregiver only if hospitalization is needed, to prevent in-hospital virus spread from asymptomatic children.

Management of COVID-19 Positive Hospitalized Patient.

Monitoring

- Vital parameters: Temperature, heart rate, respiratory rate, blood pressure, and oxygen saturation.
- Blood tests.
- Viral PCR for other respiratory viruses and Mycoplasma pneumoniae.
- Chest x-ray or CT scan if respiratory symptoms persist or worsen.

Supportive Care

- Hydration with appropriate caloric and electrolytic intakes.
- Antipyretic drugs: paracetamol as first line and ibuprofen as second line (there is no clinical evidence that defines a correlation between ibuprofen and the worsening of clinical conditions due to evolution of COVID-19. As the national guidelines and EMA suggest, patients may continue the use of NSAIDs (10).

Steroid Therapy

The use of corticosteroids is not contraindicated for concomitant treatment of other underlying diseases (e.g., asthma) if benefits are greater than the risks. In patients with chronic use of corticosteroids any modification has to be arranged with the referent specialist/consultant.

Respiratory Support

- Moderate cases: oxygen mask with a target of oxygen saturation >95%.

- Oxygen with HFNC if the target is not achieved with the mask. In this case, we do suggest contacting the nearest center with a dedicated PICU.
- Severe cases: CPAP, NIV with early intubation, and mechanical ventilation.

Target Therapy

Because of poor evidence, any antiviral and/or immunomodulatory therapy should be considered case by case and defined after Pediatric Infectious Diseases consultation. For each specific case, a multidisciplinary team that includes a Pediatric Infectious Diseases consultant must review the last evidences on antiviral and/or immunomodulant treatments for Covid-19, in order to consider if the patient can be included in any trial and/or if he/she can apply for the compassionate use of any further treatment, including the use of Remdesivir, a nucleotide analog prodrug that inhibits viral RNA polymerases of SARS-CoV-2, the use of hyperimmune plasma from patients recovered from Covid-19 and/or the use of any immunomodulant treatment including targeted anti-inflammatory products such as interleukin inhibitors, interferons, kinase inhibitors, and others (11–13).

Confirmed COVID-19 hospitalized patients must be kept isolated till clinical recovery. This is defined by 48-72 of apyrexia AND respiratory/gastrointestinal symptoms resolution. When discharged the patents should continue isolation till two negative swabs results 24 h apart.

Actionable Recommendations.

During this first month of COVID-19 emergency, our Department faced mostly organizational issues in children management. Although during the lockdown period (6th March−4th May) the ER utilization had a significant reduction (−75%) a series of critical issues arose as the need of different areas and isolation rooms for infected patients and the need of clear pathways for the management of all patients according to different epidemiologic and/or clinical characteristics. Between the 6th March and 4th May, 1,291 patients were evaluated and 416 (32.2%) were tested for SARS-CoV-2. Two-hundred and fifty-nine children (20.1%) sought medical evaluation for fever and/or respiratory or gastrointestinal symptoms (pathways 1 and 2). All patients received the SARS-CoV-2 test and for 6/416 this turned positive. All close contact with a confirmed COVID-19 case admitted to our ER (24/416) were tested and 4/24 (16.7%) were found positive (pathway 3). Most of the children (83.1%) were referred to our ER for non-COVID-19 related problems. As per pathway 4, SARS-CoV-2 tests were performed only in case of hospital admission: 92/416 children were tested, all with negative results (Table 2).

Table 2. Padua PED 5-month experience stratified by four different pathways.

	Phase 1 (6 March–4 May 2020)			Phase 2 (5 May–31 July 2020)		
	PED evaluations n = 1,291			PED evaluations n = 3,593		
	SARS-CoV-2 nasopharyngeal swab performed		SARS-CoV-2 nasopharyngeal swab not performed	SARS-CoV-2 nasopharyngeal swab tests performed		SARS-CoV-2 nasopharyngeal swab not performed
	416		875	768		2,825
	Positive	Negative		Positive	Negative	
Pathway #1—SUSPECTED/PROBABLE COVID-19 case in a previously healthy patient	6	253	0	0	587	0
Pathway #2—SUSPECTED/PROBABLE COVID-19 case in an immunocompromised patient/patient with chronic disease	1	40	0	0	45	0
Pathway #3—CLOSE CONTACT with confirmed COVID-19 case. Asymptomatic for COVID-19 symptoms	4	20	0	5	14	0
Pathway #4—NON-COVID-19 SUSPECTED case	0	92	875	0	117	2,825
Total	11	405	875	5	763	2,825

After the lockdown period, with the gradual return to the usual PED workload, this protocol became even more crucial to guarantee the safety of all healthcare workers and other non-infected children admitted to the hospital. Three-thousand and ninety-three patients were evaluated and 768 (21.4%) were tested for SARS-CoV-2. Despite the increase in ER evaluations for fever and/or respiratory or gastrointestinal symptoms (587 vs 259 visits), none of these resulted positive for SARs-CoV-2. On the other hand, 5/19 (26.3%) children close contact with a confirmed COVID-19 case were found positive. One-hundred and seventeen were referred to our ER for non-COVID-19 related problems (Table 2).

Moreover, starting from March 4th, all health care workers have been screened every 10 days for SARS-CoV-2 through a nasopharyngeal swab. No cases of intra-department infection were documented among the healthcare workers and other admitted patients since all the preventive procedures described above were implemented.

Based on this 5-month experience, the following widely generalizable recommendations have been identified to face with COVID-19 pandemic:

- A pre-triage assessment has to be organized at the immediate entrance of the ER, in order to promptly guarantee patients identification and to address them to the most appropriate pathway.
- Clear and differentiated pathways have to be created for the management of all pediatric patients, according to different epidemiologic and/or clinical characteristics.
- Different areas and isolation rooms have to be arranged in order to separate SARS-CoV-2 infected and/or suspected patients from patients unlikely to be affected by COVID-19.

- Education of health care workers on infection prevention and control measures and on COVID-19 related operational procedures and standards has to be considered as a priority, in order to minimize the risk of in-hospital spread of SARS-CoV-2 infection.

Conclusions.

In-hospital spread of SARS-CoV-2 infection can be avoided through the implementation of clear and practical procedures aimed at promptly recognize and address pediatric patients and their caregivers, soon after being admitted to the pediatric emergency room. These measures, including a pre-triage area and specific and well-differentiated pathways based on patient's epidemiologic and clinical risk factors, are simple to implement and extremely important to quickly identify, isolate and manage all positive children, therefore must be planned and realized. We believe that our experience may be transferred to other similar settings, as a support for the implementation of hospital-based protocols aimed at containing the spread of SARS-CoV-2 infection, both at local and global levels.

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2.2. COVID-19 Pandemic: Perspective of an Italian Tertiary Care Pediatric Center

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Abstract.

Since February 2020, Italy has been faced with the dramatic spread of novel Coronavirus SARS-CoV-2. This impetuous pandemic infection forced many hospitals to reorganize their healthcare systems. Predicting a rapid spread of the SARS-CoV-2 virus within our region, the Department for Women's and Children's Health promptly decided (i) to revise the distribution of the clinical areas in order to create both designated COVID-19 and COVID-19-free areas with their own access, (ii) to reinforce infection prevention control (IPC) measures for all healthcare workers and administrative staff and (iii) to adopt the new "double-gate approach": a phone call pre-triage and nasopharyngeal swab for SARS-CoV-2 detection before the admission of all patients and caregivers. Between 21 February 2020 till 04 May 2020, only seven physicians, two nurses and two of the administrative staff resulted positive, all during the first week of March. No other cases of intra-department infection were documented among the healthcare workers since all the preventive procedures described above were implemented. It is predicted that similar situations can happen again in the future, and thus, it is necessary to be more prepared to deal with them than we were at the beginning of this COVID-19 pandemic.

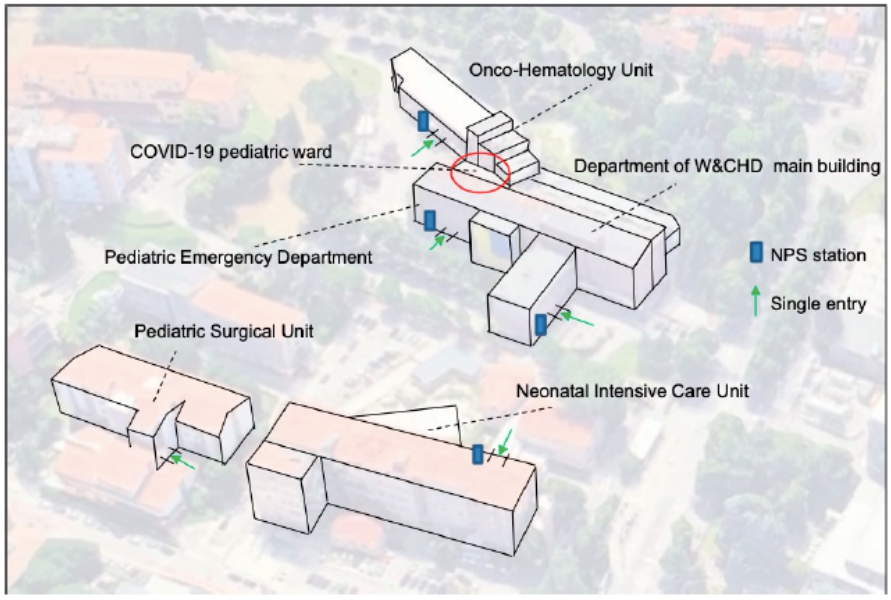
Keywords: COVID-19; children; hospital; infection prevention and control; pediatric department.

Introduction.

On 21 February 2020, the first COVID-19 positive case in a 76-year old man in Vo, a small town in the province of Padua, Italy, was reported. The same evening, his two grandchildren were evaluated in the Pediatric Emergency Room of Padua University Hospital due to household exposure and that represented the first contact with the SARS-CoV-2 virus in our hospital.

The Department for Women's and Children's Health (W and CHD) of Padua is one of the eight departments according to which the hospital is articulated; functionally it is a "Children's Hospital within a Hospital", functioning as a tertiary pediatric care center. It is composed of 16 Divisions serving the Veneto Region, an area of 4,700,000 inhabitants of which the 13.5% represent the cohort of children aged 0–14 years. It counts 211 beds of which 104 (49%) are dedicated to the different pediatric units (of which 8 are for Pediatric Intensive Care), 75 (36%) to the neonatal areas (of which 31 are for Neonatal Intensive Care) and 32 (15%) to the various pediatric surgical divisions (Figure 1). The staff of the Department is composed of 222 physicians (including 100 residents), 330 nurses and 75 other personnel. In 2019 the hospital admissions had been 10,428, the outpatient visits 180,000, births 2820 and accesses to the Pediatric Emergency Department (PED) slightly over 24,000. Considering that every child entering the W and CHD is accompanied by at least 1 caregiver, the number of accesses should actually be doubled. The clinical wards for the inpatient activity and ambulatory services are hosted in a five story high main building except for the Onco-Hematology and Stem Cell Transplant Unit, the Surgical Divisions and the Neonatal Units which are operating in satellite buildings attached to the main one (Figure 1).

Figure 1. Distribution of clinical wards with their own single access and nasopharyngeal swab (NPS) station: a representative map.



Predicting a rapid spread of the SARS-CoV-2 virus within our region, in the afternoon of February 24th the Chief Executive Officer (CEO) of Padua University Hospital called for an emergency meeting with all the department chairmen and the mandates received were:

- to ensure the protection of the healthcare workers, as the top priority;
- to rigorously implement all the conventional rules emanated by the WHO for preventing the infection
- to minimize the risk of admitting into hospital asymptomatic COVID-19 positive patients;
- to adapt/transform some hospital areas in order to be able to admit and treat suspected/confirmed; COVID-19 pediatric patients
- To ensure continuity of treatment when appropriate and needed.

This impetuous pandemic infection put a tremendous amount of stress on many hospitals which were forced to reorganize their healthcare systems [1]. In just 10 days, the Chinese government built two hospitals in Wuhan for treating confirmed COVID-19 cases. As time passes, China and other countries completely reorganized their healthcare systems by rapidly building new temporary hospitals [2,3]. In our setting, the most rapid and efficient strategy was to completely reorganize pre-existing hospital areas in order to face with the emergency.

Therefore, to fulfill all the given tasks, the W and CHD promptly decided:

- To revise the distribution of the clinical areas in order to create both designated COVID-19 and COVID-19-free areas with their own access.
- To reinforce infection prevention control (IPC) measures for all healthcare workers and administrative staff
- To reinforce IPC measures for patients with a new “double-gate approach”: a phone call pre-triage and nasopharyngeal swab (NPS) for SARS-CoV-2 detection before the admission of all patients and caregivers.

One of the key elements, among the many, which composed the comprehensive overall policy the University Hospital of Padua adopted to contain the COVID-19 epidemic, was to free the request of diagnostic NPSs on a 24/7 basis. Importantly, the routes to request NPS were highly facilitated and a very fast track (within three hours) was also made available to handle critical clinical situations.
Despite the fact that it was predicted that children could have been less frequently and less severely affected than adults [4], it also highly affected tertiary care pediatric institutions such as the W and CHD.

At the end of two months of the so called ”Phase I” of the COVID-19 pandemic that in Italy lasted between the last week of February till 4 May 2020, the W and CHD decided to run a preliminary critical assessment of the actions undertaken in this phase as per mandate of the CEO of the Hospital.

Multilevel Interventions to Prevent SARS-CoV-2 In-Hospital Spread.

1. Redistribution of Clinical Areas in Order to Create COVID-19 and COVID-19 Free Areas with Their Own Access

To monitor all the people entering the W and CHD, the access to the building was modified in order to have only one single entering point to the Department for each building (without considering the PED entrance, which always remained available) (Figure 1). Moreover, right outside of each entry point an NPS station was set up to perform NPSs to all healthcare workers and patients with caregivers who were attended for urgent or non-deferrable hospitalizations.

The PED and the Pediatric Emergency ward were designated for the evaluation and treatment of suspected/confirmed COVID-19 cases. A pre-triage area was created at the immediate entrance of the pediatric emergency room, for all non-critical pediatric patients. The pre-triage area served to screen patients with epidemiological risk or clinical signs and/or symptoms of possible COVID-19 infection. This served to point them towards an ad-hoc separate entry pathway leading to a so-called COVID-19 area, totally separated from the rest of the emergency room with a good ventilation, where they could be visited. Furthermore, another 24/7 emergency area with two short-stay isolation rooms, was set up in the Onco-Hematology building for in-treatment oncologic and transplanted patients.

Our Pediatric Emergency Unit was transformed in an exclusive COVID-19 ward. The existing six two- or three-bed rooms, were all transformed into single-bed rooms. All patients in the COVID-19 ward were assisted by dedicated staff. The patient's caregiver had to wear a surgical mask and was not allowed to exit the room. No visits were allowed.

Medical shifts were reorganized to allow a lower patient/staff ratio. Education of health care workers on preventive measures set up was guarantee, also leading to minimize occupational stress [5].

Residents' rotations were suspended for two months and they continue to work in the same ward they were before the pandemic started. A dedicated COVID-19 team was created.

All conventional large face-to-face meetings were moved to telematic platforms and all administrative staff was directed to work remotely.

The presence of volunteers and play-therapists was interrupted. All school activities run in-house were also suspended. Gatherings of people close to all vending machines were banned.

2. Reinforcing of IPC Measures for All Healthcare Workers and Administrative Staff

All healthcare workers entering the Department hospital were required to have their body temperature checked on daily basis; the use of surgical masks was made mandatory, except for those working in the COVID-19 area

who had to wear FFP2 masks and dedicated personal protective equipment at all times. Moreover, anesthesiologists had to wear FFP3 masks. A strict hand hygiene policy has been applied.
Starting from 4 March, a periodical screening with NPSs for all hospital personnel and administrative staff was implemented. Generally, NPS screening was performed on a 20-day basis, but for those healthcare workers dealing with COVID-19 patients, all anesthesiologists and those working with fragile patients (immunosuppressive children, children with chronic diseases or premature babies and/or the ones admitted to the neonatal intensive care unit) NPSs were performed every 10 days.
Initially, for those who had been in close contact with COVID-19 case, a 14-day home isolation was recommended. After the first two weeks of March, however, the rules changed and close contacts, if asymptomatic, were allowed to resume work upon obtaining an NPS every 48 h and then, from the beginning of April, every 5 days.

2. Reinforcing of IPC Measures for Patients with the New "Double-Gate Approach": Phone Call Pre-Triage and NPSs for All Admitted Patients and Caregivers

On a daily basis, the head nurses were required to call in the families who were scheduled to be admitted to the hospital, within the 1–2 days prior to the admission to run a telephone pre-triage, based on a standardized questionnaire aiming to pick-up epidemiologic risk factors or signs and symptoms of COVID-19 infection in the patient or in his/her accompanying family member. In case of positive results, the admission was rescheduled.Since SARS-CoV-2 infected patients could remain asymptomatic, after the telephone pre-triage, children and caregivers were requested to come to the hospital for receiving an NPS and then sent back home in order to be admitted as soon as the results were available (usually at the most within 48 hours).
On the day of admission, all children and their caregivers were asked to self-complete the same questionnaire used for the telephone triage; they also had their body temperature checked. Inpatient access was allowed only upon obtainment of a negative test result for SARS-CoV-2 for both the patient and caregiver. Finally, all the people were encouraged to respect the social distances and a strict hand hygiene policy. It was requested to have only one caregiver for each patient and always the same for the entire duration of the hospitalization.

Differently, the child and the caregiver entering the hospital for an outpatient visit were screened only by the telephone pre-triage a couple of day before the visit. Upon entering the hospital, the same inpatients' screening was applied. In order to guarantee the social distance in the waiting rooms, the seats were organized to respect at least one-meter of distance from each other and the appointments were distributed over a longer-than-usual time schedule with patient and caregiver's place assignment.
In order to reduce overcrowding and ensure proper continuation of treatment, telemedicine and home-based treatments were increased to offer blood tests, antibiotic and chemotherapy, provided by doctors and nurses of the Onco-Hematology unit.

Results of Intervention.

Since the end of February, the city of Padua, with a population of about 300,000 inhabitants, had 3844 confirmed infections with 281 SARS-Cov-2 attributable deaths [6].

In this ten-week period a total of 3382 NPSs were performed on healthcare workers. In total, 3371 (99.7%) of these came back negative. Only seven physicians (four consultants and three residents), two nurses and two of the administrative staff resulted positive, all during the first week of March (Table 1).

Table 1. Summary of NPS HCWs surveillance.

W and CHD Areas	Number of HCWs	Number of NPS	Number of Negative NPS	Number of Positive NPS
Main building	255	700	697	3
Onco-Hematology Unit	107	900	900	0
NICU	91	728	725	3
Pediatric Emergency Department	60	554	554	0
Pediatric Surgery Unit	80	500	495	5
Total	593	3382	3371	11

Upon reconstructing their recent histories of potential exposures, it turned out that ten of them (10/11, 91%) were unintentionally exposed to a SARS-CoV-2 infected person outside the hospital.

No other cases of intra-department infection were documented among the healthcare workers since all the preventive procedures described above were implemented. No doctor, nurse or other personnel working in the Department became infected; despite the fact that infected healthcare workers continue to attend the hospital for at least two days, before the positive results of the NPS became available, no other colleagues with whom they were working were infected.

Around six thousand pre-admission telephone pre-triages have been carried out, over 132 remote medical consultations and 275 accesses at the patients' domicile have been performed.

Through the "double-gate" approach a total of 1885 pre-admission NPSs have been performed to children and their caregiver with non-deferable admissions (Table 2).

Table 2. NPS Surveillance in Children and Their Caregivers for Non-Deferrable or Urgent Hospital Admissions.

W and CHD Areas	Number of Hospital Admissions (Including DH Activity)	Number of NPS (Patient + Caregiver)	Number of Negative NPS	Number of Positive NPS
Main building	377	720	719	1
Onco-Hematology Unit	95	188	188	0
NICU	93	426	423	2
Pediatric Emergency Department	92	185	185	0
Pediatric Surgery Unit	183	366	366	0
Total	840	1885	1882	3

Among these, only three asymptomatic COVID-19 caregivers were identified. One was the mother of a child coming weekly to the day-hospital for an enzyme replacing therapy and the other two were the paucisintomatic parents of a newborn. These three people were also wearing a mask during their staying in the hospital. None of the personnel they got in contact with were infected, including their respective children. These three asymptomatic COVID-19 patients were then sent home to complete a quarantine and prevented to come into the hospital. Finally, none of the children who had an NPS done pre-admission turned out to be an asymptomatic positive carrier of SARS-CoV-2 virus.

In addition, it should be noted that thanks to the decision of postpone all elective admissions, to telephone triage and most likely also an element of fear of entering the hospital, the number of regular hospital admissions registered in the months of March and April 2019 in comparison to the ones registered in the same time year of 2020 dropped from 1306 to 937 and the day-hospital admission from 387 to 188.

Although the activity of the PED significantly reduced (−75%), from 21 February 1291 patients have been evaluated and 416 (32.2%) have been tested for SARS-CoV-2 virus. Three hundred (300/1291, 23.2%) were evaluated because of fever and/or respiratory or gastrointestinal symptoms. All patients received the COVID-19 test and for seven (7/300, 2.3%) this turned positive. All close contact with a confirmed COVID-19 case (24/1291) admitted to our PED were tested and four (4/24 16.7%) were found positive. Most children (66.8%) were referred for non-COVID-19 related problems and they were tested only in case of hospital admission. In total, 92/967 (9.5%) were tested, all with negative results.

Conclusions.

The final result of this analysis clearly indicates that during the two months when the spread of the SARS-CoV-2 in Padua and in the Veneto region reached its peak and the numbers of people infected, at the time this analysis was ended, was still very high, the W and CHD remained a COVID-19-free environment. Indeed, in that time period no member of staff, resident, patient and her/his own caregiver during their in- or outpatient stay in the Department got infected by the SARS-CoV-2 infection.

It is difficult to pinpoint exactly what contributed to this success; we can only state that the comprehensive measures which were implemented resulted very effective at a time during which the entire city of Padua was locked and the “Entropy” of the Department decreased significantly. Furthermore, the expected small number of children infected by SARS-CoV-2 and thus the actual very small number of infected children who were admitted should also be considered in reading these positive results [7,8].

The main question which remains unanswered is indeed if the policy of freeing the use of NPSs and thus of using this test to screen all the people working and entering the Department played a major role in making the W and CHD a SARS-CoV-2-free Hospital. Relevant human and financial resources had to be invested. Two shifts of nurses, each one composed of three to four nurses, had to be fully dedicated to the NPS station in order to run it efficiently and smoothly. Other nurses, respectively working in the PED, in the Neonatology

and in the Onco-hematology divisions were involved in obtaining NPSs to their own medical personnel considering that those units elected, for functional reasons, to deal with their doctors and nurses independently. On top of that, one should consider the financial cost of obtaining the NPSs and of the subsequent analyses. Obviously, a direct cost-to cost confrontation between the one necessary for obtaining an NPS and the one necessary to deal with even a single doctor and/or nurse infected is unconceivable. However, the rationale for that policy, considered of course in the context of all the general and local procedures which were implemented, should be strongly re-read based on our findings.

It should be further acknowledged that, as already reported in the literature, these aggressive organizational and structural measures were generally well accepted [5]. The extensive use of PPE and the reorganization of separated areas with good ventilation were very effective in relieving the concern the staff of the Department and the patients and their caregivers experienced in those days.

This positive personal feeling was quite important in continuing to provide an effective care to the patients and in preventing the patients to not seek for medical care just for the fear of the SARS-CoV-2 infection. It is predicted that similar situations can happen again in the future, and thus, it is necessary to be more prepared to deal with them than we were at the beginning of this COVID-19 pandemic.

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Chapter 3

RESULTS

Viral transmission and clinical characteristics of SARS-CoV-2 infection among several populations

3.1. Fecal-Oral Transmission of SARS-CoV-2 In Children: is it Time to Change Our Approach?

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Abstract

Starting from 2 pediatric cases of COVID-19, with confirmation at nasopharyngeal and rectal swabs, we considered the lesson learnt from previous Coronavirus epidemics and reviewed evidence on the current outbreak. Surveillance with rectal swabs might be extended to infants and children, for the implications for household contacts and isolation timing.

Keywords: COVID-19, children, gastrointestinal, rectal swab

Introduction.

In Italy, we have been currently experiencing the effects of severe acute respiratory syndrome (SARS)-CoV-2 global spread, started at the end of 2019 in Wuhan. Findings from the impact of COVID-19 pandemic on pediatric patients are few. However, children seem to present more often gastrointestinal symptoms than adults, with reported vomiting, abdominal pain and diarrhea.[1] Additionally viral shedding has also been reported in children without gastrointestinal symptoms and has been linked to a possible long-term fecal-oral transmission.[2]

In March 2020, 2 infants with SARS-CoV-2 infection were admitted to our Pediatrics Department. The first one, a 5-month-old boy, presented respiratory and gastrointestinal symptoms with diarrhea. Nasopharyngeal and rectal swabs were taken, with a positive result. He was discharged in mandatory home isolation, afebrile and asymptomatic. The second one, 2-months-old, presented only mild respiratory symptoms. After COVID-19 infection was confirmed with nasopharyngeal swab, despite the absence of gastrointestinal symptoms and based on the findings of the previous case, he also underwent a rectal swab, that tested positive for SARS-CoV-2 on day 3 from onset.

According to the recommended dispositions provided by Italian Ministry of Health, the follow-up of these patients, to avoid contagion and uncontrolled spread of the disease, implies mandatory strict home isolation until the finding of 2 subsequent negative results at nasopharyngeal swab. However, there are no current official disposals concerning rectal swabs, for further investigations, with no implications on isolation timing.

State of the art and future directions for SARS-CoV-2 starting from lessons learnt from previous epidemics.

During the SARS outbreak in 2002 to 2003, there were reports of viral RNA being found in fecal samples, occasionally even after 30 days after symptoms onset, determining a risk for the stools to become a source of contamination of airdrops and several environmental surfaces.[3] In children, SARS-CoV infection was associated to gastrointestinal symptoms, but there is no evidence of rectal swabs being performed for diagnosis and further surveillance. In Middle East Respiratory Syndrome-CoV epidemic in 2012, there was proof of viral RNA detection in fecal specimens in adults, but there were no data about surveillance in children, despite reported occurrence of gastrointestinal symptoms.[1] As for COVID-19 outbreak, the most relevant international evidence is reported in Table Table1.1. Zhang et al[4] reported that SARS-CoV-2 RNA was found in stool specimens and rectal swabs, often with a higher number of positivities than oral samples in a later phase of disease. These findings might suggest that, if feasible, “non-infectivity” should not rely only on negativity of oral swabs, as the virus might still be shed in the body fluids.[4]

Table 1. Evidence of SARS-CoV-2 Detection in Stools and Rectal Swabs in Adults and Children.

TABLE 1. Evidence of SARS-CoV-2 Detection in Stools and Rectal Swabs in Adults and Children

	Country	Patients	Symptoms	Stool Sample (RT-PCR)	Live Virus Detection (Electron Microscopy)	Rectal Swab (RT-PCR)
Xu et al[2]	China	10 children (2 months to 15 years)	Non-specific: Fever Respiratory Gastrointestinal (3 pts), asymptomatic (1 pt).	—	—	8 positive (max.positivity 13 days after discharge)
Zhang et al[4]	China	16 adults	—	—	—	10 positive
Wang et al[5]	China	1070 specimens in 205 adults	Respiratory and/or gastrointestinal	44/153 samples positive	2 findings (no gastrointestinal symptoms)	—
Zhang et al[6]	China	1 adult	Asymptomatic after discharge	Positive (10 days after discharge)	—	—
Holshue et al[7]	US	1 adult	Fever Respiratory Gastrointestinal	Positive (until 7 days from onset)	—	—
He et al[9]	China	1 newborn	Gastrointestinal	—	—	Positive after discharge
Park et al[10]	Korea	1 child (10 years)	Fever	Positive (until 17 days from onset)	—	—
Tang et al[11]	China	1 child	Asymptomatic	Positive (until 17 days from onset)	—	—

Wang et al[5] reported detection of viral RNA in respiratory tract swabs and stools, with 44/153 positive fecal samples. Four positive fecal samples showed high copy numbers with a mean cycle threshold (Ct) value of 31 4 ($<2.6 \times 10^4$ copies/mL). Live virus was found in feces of patients without diarrhea, suggesting a systemic infection. The spread of the virus by also non-respiratory routes might justify the rapid diffusion of infection and testing of samples from multiple sites may be useful to enhance sensitivity and decrease false-negative results. Zhang et al[6] reported an adult case that presented after initial negativization positive sputum and fecal samples, with the latter still testing weakly positive 10 days after discharge. The hospital revised criteria for discharge, requiring 2 consecutive negative samples from the respiratory tract and stool alike. Holshue et al[7] published the first adult case in the US, showing high viral loads in nasopharyngeal specimens at onset, with a tendency to decrease following disease course, with increasing Ct values. In addition, viral detection in stool samples occurred at 7 days from onset.

An initial concern was the sensitivity and specificity of SARS-CoV-2 PCR tested in stools. Recently, the same level of accuracy has been demonstrated for stool samples and pharyngeal swabs, regardless of symptoms and with no correlation to disease severity.[8] Concerning the pediatric population, evidence on the use of rectal swabs or viral detection in stools is reported in Table 1. Xu et al[2] reported ten pediatric PCR-confirmed cases of SARS-CoV-2 infection, all with non-specific symptoms. The children's pattern of viral shedding was monitored with subsequent nasopharyngeal and rectal swab samples: 8 patients showed persistent positivity on rectal swabs, with

2 of them remaining positive for up to 13 days after discharge, also after nasopharyngeal swabs had turned negative. Viral loads showed that shedding from the gastrointestinal tract may be higher and more long-lasting compared with the respiratory tract. As reviewed by He et al,[9] a newborn presenting vomiting and diarrhea as first symptoms was tested positive for SARS-CoV-2, showing negativization of pharyngeal swab after treatment but a persistently positive rectal swab. Park et al[10] reported a pediatric case with positive nasopharynx, throat and feces samples on admission. By day 16 from symptoms onset, throat swab samples had turned negative, while on day 17, viral RNA was still found on feces and with weak positivity on nasopharyngeal samples. Last, Tang et al[11] showed how an asymptomatic child presented a positive fecal sample for up to 17 days since the last exposure, with reported negative samples from the respiratory tract.

Droplets are the main human-to-human mechanism of transmission of SARS-CoV-2, but fecal shedding with environmental contamination may play an important role in viral spread. As pointed out by Li et al,[12] there is a great number of infections not being documented, especially in paucisymptomatic or asymptomatic individuals, which may have helped the fast diffusion of the virus.[12] The clinical pattern of disease presentation among children may have facilitated viral dissemination. Moreover, there is evidence supporting viral viability in environmental settings that may predispose fecal-oral transmission, with recent evidence supporting that SARS-CoV-2 can remain viable in aerosols up to 3 hours, and for 72 hours on solid surfaces, similarly to SARS-CoV.[13]

Not only the importance of correct hand hygiene should be encouraged by every mean, but severe measures must also be observed handling the feces of infected patients, and sewage from hospitals requires proper disinfection. Current evidence brings concerns on excluding SARS-CoV-2 infection by single time point nasopharyngeal swabs, with sensitivity being dependent on the test's characteristics and technique of collection of the samples, with increasing data hinting at fecal transmission as an important alternative route.

Conclusions.

Since gastrointestinal symptoms seem to be more frequently reported in children than adults, and in view of current evidence of fecal shedding, there are implications for every child being admitted or home-isolated, and for household contacts. Indeed, rectal swabs should be considered especially in children for diagnosis as well as to better define the duration of isolation, along with findings from nasopharyngeal swabs.
Further evidence on gastrointestinal involvement and excretion of SARS-CoV-2 in stools is necessary to confirm fecal viral loads regardless of enteric symptoms, and to better explore viral RNA detection in the early incubation or late convalescence stages. A negativity in both nasopharyngeal and stool samples might be considered as a standard requirement for cessation of mandatory isolation, especially in those settings where there is a risk of infecting vulnerable populations (eg, retirement homes).

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3.2. Gastrointestinal Symptoms in Severe COVID-19 Children

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Abstract.

There are growing evidence of clinical manifestations other than acute respiratory syndrome in severe acute respiratory syndrome associated with coronavirus 2-infected children. In our multicenter retrospective analysis, we observed among 127 severe acute respiratory syndrome associated with coronavirus 2 positive children that the presence of gastrointestinal symptoms was more frequently associated with severe and critical phenotype (P = 0.029). Moreover, having gastrointestinal symptoms was more frequently reported in patients who developed cardiac impairment.

Introduction.

As of May 7, the Italian National Institute of Health reported 3752 cases of severe acute respiratory syndrome associated with coronavirus 2 (SARS-CoV-2) in Italian children <18 years of age, 140 of them requiring hospital admission. Since the first outbreak, a global effort has been made to collect clinical and laboratory findings on patients with SARS-CoV-2 infection. The lower airway is the primary target of the infection; however, the disease spectrum in adults goes from asymptomatic subjects to sever illness including 5.0% subjects requiring intensive care unit (ICU) admission, 2.3% who underwent invasive mechanical ventilation, and 1.4% who died.1 Data suggest that children are less likely to develop severe symptoms compared with adults.2 Also, there are growing evidence of clinical manifestations other than acute respiratory syndrome in pediatrics suggesting that coronavirus diseases 2019 (COVID-19) spectrum and pathogenesis in children are yet to be unravel. In this report, we describe the results of our preliminary analysis of a cohort of hospitalized pediatrics COVID-19 patients focusing on mode of presentation, presence of comorbidities, severity of disease, and early outcome.

Materials and methods.

We conducted a multicenter retrospective analysis of clinical record of SARS-CoV-2-infected children in 23 different sites in Italy. From February 21, 2020, to May 1, 2020, subjects less than 18 years of age with a positive result on high throughput sequencing or real-time reverse transcriptase-polymerase chain reaction assay of nasal/pharyngeal swab specimens were included. The study was approved by the ethical committees of the coordinating center in Milan (protocol number 2020/ST/061).

Data regarding recent exposure history, clinical symptoms or signs, and laboratory findings on admission were extracted using a common clinical record form. Radiologic assessments and laboratory testing were performed according to the clinical care needs of the patient. The Student's t test, the $\chi 2$ method, and Fisher's exact test were used as appropriate for statistical analysis to compare continuous and categorical variables. A P value <0.05 was chosen as cutoff for significance. Data were analyzed with StataMed (version 12.0).

Results.

Overall, 127 children were included; 44 were female (34.9%) and the median age was 4.8 years (interquartile range, 0.3–8.5); 57 (45%) <12 months of age. Eight of 127 (6.7%) were admitted in ICU, 14 of 127 (12%) required oxygen therapy, 5 (4%) were noninvasive ventilation, and 1 patient required mechanical ventilation during the hospitalization. The severity of the COVID-19 in our children was defined using previously published criteria3; 7.9%, 48.8%, and 27.7% of their clinical features were defined respectively as asymptomatic, mild, or moderate accounting for 84.4% of our cohort; 8.7% was severe and 7.1% was critical.

Age class, sex, and ethnic group did not show a different distribution among the severity categories (P = 0.57, 0.62, and 0.375 Fisher exact test; Table 1).

Table 1. - Association of Clinical Characteristics With Severity Score and ICU.

	Asymptomatic, mild, or moderate		Severe or critical			Not ICU		ICU		
	N = 107		N = 20			N = 111		N = 8		
Characteristics	N	%	n	%	*P*[a]	N	%	n	%	*P*[a]
Age median (IQR, y)	1.6 (0.3, 7.9)		4.3 (0.3, 10.1)		0.393[b]	1.6 (0.3, 7.9)		5.5 (0.4, 10.1)		0.497[b]
Age group					0.845					0.854
Newborn	5	4.7	1	5.0		6	5.4	0	0.0	
Infant	44	41.1	7	35.0		44	39.6	3	37.5	
Children	42	39.2	8	40.0		46	41.4	3	37.5	
Adolescent	16	15.0	4	20.0		15	13.5	2	25.0	
Male	68	64.2	14	70.0	0.799	71	64.5	5	62.5	1.000
Presentation										
Fever	85	79.4	20	100.0	0.023	92	82.9	8	100.0	0.352
Respiratory symptoms	68	63.6	14	70.0	0.799	74	67.3	4	50.0	0.441
Respiratory symptoms only	46	43.0	7	35.0	0.624	44	39.6	2	25.0	0.468
Cough	52	48.6	9	45.0	0.812	57	51.4	2	25.0	0.812
Rhinorrhea	43	40.2	6	30.0	0.460	46	41.4	0	0.0	0.022
Wheezing	4	3.7	0	0.0	1.000	3	2.7	0	0.0	1.000
Dyspnea	5	4.7	5	25.0	0.009	7	6.4	2	25.0	0.114
GI symtomps	26	24.3	10	50.0	0.029	31	27.9	4	50.0	0.232
GI symptoms only	13	12.1	5	25.0	0.160	14	12.6	3	37.5	0.087
Vomit	6	5.6	6	30.0	0.004	6	5.4	6	75.0	0.004
Diarrhea	20	18.7	8	40.0	0.044	20	18.0	8	100	0.044
Abdominal pain	6	5.6	2	10.0	0.611	8	7.2	0	0.0	1.000
Comorbidities	14	13.1	6	30.0	0.088	16	14.4	3	37.5	0.115
Chronic cardiac conditions	3	2.8	2	10.0	0.176	4	3.6	1	12.5	0.298
GI disorder	2	1.9	2	10.0	0.117	2	1.8	1	12.5	0.190
Obese	1	0.9	2	10.0	0.064	3	2.7	0	0.0	1.000
Chronic kidney disease	2	1.9	0	0.0	1.000	2	1.8	0	0.0	1.000
Chronic neurologic disease	0	0.0	2	10.0	0.024	1	0.9	0	0.0	1.000
Immunologic condition	2	1.9	0	0.0	1.000	1	0.9	0	0.0	1.000
CXR positive	25	43.9	13	65.0	0.125	35	51.5	3	37.5	0.711
Complication	23	21.5	19	95.0	<0.001	35	31.5	7	87.5	0.003
Viral pneumonia	16	15.0	9	45.0	0.004	24	21.6	1	12.5	0.468
Bronchiolitis	8	7.5	1	5.0	0.570	9	8.1	0	0.0	0.522
Bacterial pneumonia	0	0.0	2	10.0	0.024	1	0.9	1	12.5	0.130
ARDS	0	0.0	2	10.0	0.024	1	0.9	1	12.5	0.130
Pleural effusion	0	0.0	1	5.0	0.157	0	0.0	1	12.5	0.067
Myocardial involvement	0	0.0	6	30.0	<0.001	2	1.8	4	50.0	<0.001
Bacteremia	0	0.0	1	5.0	0.157	0	0.0	1	12.5	0.067
Coagulation disorder	0	0.0	1	5.0	0.157	0	0.0	1	12.5	0.067
AKI	0	0.0	1	5.0	0.157	0	0.0	1	12.5	0.067
Liver dysfunction	0	0.0	1	5.0	0.157	0	0.0	1	12.5	0.067
Myositis	1	0.93	0	0.0	0.843	1	0.9	0	0.0	0.933

AKI indicates acute kidney injury; ARDS, acute respiratory distress syndrome; CXR, chest radiograph; GI, gastrointestinal; ICU, intensive care units; IQR, interquartile range.

Twenty of 127 patients (15.7%) had at least 1 comorbidity. Five (3.9%) had chronic cardiac condition, 4 (3.1%) had gastrointestinal (GI) disorder, 3 (2.4%) were obese, 2 (1.6%) had chronic kidney disease, chronic neurologic disorder, and immunologic condition, respectively. Only 1 medically complex patient (defined as children who required long-term dependence on life support) was included. Comorbidities distribution was not different among severity classes ($P = 0.08$ Fisher exact test). Moreover, the ICU admission rate was similar in patients with comorbidities and those without ($P = 0.115$ Fisher exact test).

The most common symptoms reported on admission were fever (82.7%), cough (48%), and rhinorrhea (38%). Seventy-seven of 127 (60.6%) presented with respiratory symptoms (cough, rhinorrhea, wheezing, and dyspnea).

Thirty-six out 127 (28.3%) had GI symptoms (vomit, diarrhea, abdominal pain), of them twenty-eight (22%) had diarrhea, 12 (9,4%) vomit, and 8 (6.3%) abdominal pain.

The presence of GI symptoms at the admission was differently distributed throughout severity classes ($P = 0.006$). Having GI symptoms was more frequently associated with severe and critical phenotype ($P = 0.029$). Interestingly, a history of GI symptoms was positively associated with cardiac involvement as clinical complications, in presence of other symptoms ($P = 0.007$) or alone ($P = 0.004$).

Roughly, a third of the children presented lower respiratory tract complications as viral pneumonia and bronchiolitis. Viral pneumonia was more frequently reported in severe phenotype ($P = 0.004$), while admission rate to ICU was equally distributed among these patients. Chest radiogram was performed in 77 patients (65%) on admission, and infiltrates were found in 38 of 77 (50%). Respectively, 20 and 15 patients had bilateral and monolateral infiltrates, for 3 of them it was not specified. In 4 of 77 (5.2%), atelectasis and pleural effusion were found. The presence of infiltrates at the chest radiogram did not correlate with severity clinical score or ICU admission rate ($P = 0.125$ and 0.71 Fisher exact test, respectively).

Discussion.

In the present study, we reported that most SARS-CoV-2-infected children had fever and respiratory symptoms. Similarly, Shekerdemian et al[4] reported that most of the patients included in the North American Pediatric Intensive Care Unit cohort presented respiratory symptoms, but they also state that only 1 child of their cohort presented GI symptoms, speculating that these may be associated with milder clinical presentation.

In children, common circulating human coronaviruses can cause GI symptoms in up to 57% of cases, and this presentation is more common in children than adults.[5] Increasing evidence showed that the GI tract may represent a target for SARS-CoV-2 due to the expression of the angiotensin-converting enzyme 2, a major virus receptor. We reported, differently to published data, that a history of gastrointestinal (GI) was positively correlated with a worst severity score (severe and critical) and a higher ICU admission rate. The same result was found, in an pooled analyses of adult cohorts, where GI were correlated to increased odds of critical disease and higher prevalence of complications.[6]

Interestingly, in our cohort having GI was more frequently reported in patients who developed cardiac impairment as complications of SARS-CoV-2 infection. The development of hyperinflammatory syndromes and Kawasaki-like disease in children exposed to SARS-CoV-2 infection has been recently brought to attention. Riphagen et al[7] reported 8 cases of hyperinflammatory syndrome with cardiac involvement, all of them presenting with fever and significant GI symptoms (diarrhea, vomit, abdominal pain), according to our current results and to what we have previously reported.[8]

In recent studies,[4,9] comorbidities have been frequently reported in patients requiring admission to ICU. In the North American Pediatric Intensive Care Unit cohort, authors reported that up to 80% of patients included had

comorbidities. The most common comorbidity reported was medically complex defined as long-term dependence on technologic support.[4] In agreement with this cohort, Parri et al, in a SARS-CoV-2 positive cohort of pediatric patients admitted at Italian Emergency Departments, reported that 9 patients of 100 need mechanical ventilation and, among them, 6 (66%) had comorbidities.[9] In the present study, only 20 (16%) children with previous medical condition were included, 3 of them required ICU. The presence of preexisting medical conditions was not different in severe and critical patients when compared with mild, moderate, and asymptomatic ones. Moreover, the ICU admission rate was similar in patients with and without comorbidities.

There are several limitations to our study. First, the limited sample size. Second, children have been classified using a severity score previously applied to other pediatric cohorts, which is mainly designed on respiratory symptoms and lung involvement. The score criteria could explain the higher frequency of viral pneumonia among severe phenotype but not among patients requiring ICU admission. However, critical cases are defined not only by the progression to respiratory failure (acute respiratory distress syndrome) but also to life-threating organ dysfunction (shock, myocardial injury, acute kidney injury). Therefore, in the present study, the subset of critical patients includes not only patients with respiratory failure but also with other life-threating conditions. Finally, there are evidence that COVID-19-related multisystemic inflammatory syndrome could be a complication in the disease spectrum. Although a better understanding of timing between GI and its onset would be of great interest, we could not provide such information in the current study.

Conclusions.

The intention of this short report is to bring to attention that COVID-19 disease spectrum in children is far from been described in a universally shared way. Other manifestations from respiratory are often the cause of severe illness, as we reported. Having preexisted medical conditions is not associated with worse outcome and consequently, severe clinical presentation must be considering also in previously healthy children.

GI symptoms seem to be a clinical warning for children evaluated in any clinical settings when SARS-CoV-2 infection is suspected, independently of comorbidities. Pathogenetic mechanisms causing severe phenotypes in SARS-CoV-2-infected children need to be deepened by multidisciplinary approach as well we need more data to define a suitable clinical severity score for COVID-19 in children.

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3.3. SARS-CoV-2 infection in people living with HIV: a systematic review

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Summary.

Background and setting. Little is known about SARS-CoV-2 impact on some vulnerable subgroups, such as people living with HIV/AIDS (PLWHA). In our study we reviewed the current knowledge on SARS-CoV-2 cases in PLWHA.

Methods. A systematic review was conducted by searching the MEDLINE, EMBASE and Google Scholar databases. Studies reporting data on PLWHA affected by SARS-CoV-2 were considered for inclusion. The aim of this study was the systematic characterization of cases of SARS-CoV-2 infection among PLWHA, particularly focusing on age, clinical findings at diagnosis, radiological features, therapeutic management and clinical outcomes.

Results. Twenty three relevant articles were identified, which reported 164 adults with both HIV and SARS-CoV-2 infection. Of those, the large majority were males (120/142, 84.5%), often with one or more comorbidities. Fifteen cases needed intensive care treatment and 16 died. For each group, respectively three patients had underlying comorbidities. There were no studies on children. The included studies were mostly retrospective or case series/reports (19 studies). The overall risk of bias was moderate, due to the study types and characteristics.

Conclusion. It is still unclear if HIV infection may influence SARS-CoV-2 infection and disease course, however some PLWHA and particularly males affected by ARV-related complications may be at greater risk of severe Covid-19 course.

Introduction.

SARS-CoV-2 pandemic is now a global threat, and little is known about its impact on some vulnerable subgroups, such as immunosuppressed patients. Recent evidence highlighted that immunosuppression may not increase the risk of pulmonary disease severity, as SARS-CoV-2-related damage to lung tissue has shown to be related to a dysregulated host innate immune response.[1-3] People living with HIV/AIDS (PLWHA) represent a special population, particularly those with severe immunosuppression and a high viral load, as being at higher risk of infections, including common viral infections. In addition, PLWHA treated with long-term antiretroviral therapy are known to be at higher risk of developing several chronic comorbidities, particularly cardiovascular diseases, diabetes, dyslipidemia, renal diseases and other metabolic complications.[4]

For this reason, it may be supposed that PLWHA in long-term treatment with antiretrovirals (ARVs) may be at higher risk of severe disease and/or complications, if infected by SARS-CoV-2. In contrast, it has been suggested that some ARVs such as lopinavir/ritonavir (LPV/r) may have an antiviral effect against SARS-CoV-2[5] and based on these considerations it may also be supposed that PLWHA treated with protease-inhibitors (PI) and particularly with LPV/r may be at lower risk of SARS-CoV-2 infection. However, according to recent data from studies on LPV/r, including the worldwide RECOVERY trial, no clear benefit in terms of time to clinical improvement has been demonstrated for the use of this drug compared to standard of care in Covid-19.[6, 7]

Therefore, there is still a strong interest in exploring the impact of SARS-CoV-2 infection among PLWHA, worldwide. The aim of this study is to systematically review the current knowledge on SARS-CoV-2 infection among children and adults living with HIV.

Methods.

Criteria for considering studies for this review.

Randomized controlled trials (RCTs), prospective and retrospective studies, systematic reviews, case series and case reports, also pre-prints, were considered for inclusion in the review, if reporting data on pediatric or adult patients with Covid-19, including those without laboratory confirmation, and HIV.

Objectives.

The primary objective of this study was the systematic characterization of currently reported cases of Covid-19, with or without laboratory confirmation, among PLWHA. In particular, the primary analysis focused on age, clinical findings at diagnosis, radiological features and therapeutic management of PLWHA affected by Covid-19, and their clinical outcome defined as recovery, need of intensive care for mechanical ventilation and mortality.

Data source and search strategy.

A systematic review was carried out according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.[8] MEDLINE (Ovid MEDLINE[R] ALL 1946 to May 28, 2020) EMBASE (1974–2020 Week 22) and Google Scholar databases were systematically searched, using free text terms for SARS-CoV-2 AND HIV AND children AND adults. Moreover, reference lists from eligible articles were reviewed to identify other potentially relevant papers. The authors of articles reporting data on HIV patients were individually contacted by e-mail with enquiries about potentially missing or unreported data, but we could not retrieve any further information by this means. The last search conducted was on May 28th, 2020. The full search strategy and the flow chart for study selection are available in the Supplementary material. The systematic review was registered in PROSPERO, with ID CRD42020183355. Two reviewers (CM and PC) independently screened the titles, abstracts and full texts of retrieved articles to assess the eligibility of studies for inclusion. Duplicate references were removed, and disagreements were resolved by a consensus to generate the final list of papers.

Study selection and risk of bias assessment.

Clinical trials were assessed using the criteria and standard methods of the Cochrane risk of bias (RoB) tool for randomized trials.[9] RoB was assessed using six criteria: risk of selection bias (random sequence generation, allocation concealment), performance bias (blinding of participants and personnel), detection bias, attribution bias (incomplete outcome data), reporting bias (selective reporting) and other bias. The risk of bias for non-randomized studies of interventions (retrospective studies, case series and case reports) was assessed according to the Quality Assessment Tool for Observational Cohort and Cross-Sectional Studies.[10] For each criterion, the included studies were classified by quality rating as good, fair, or poor by two reviewers (CM and PC) and disagreements were resolved by discussion.

Studies reporting data on 1) clinical features, diagnostic/therapeutic management and/or pre-defined outcomes (need for intensive care support, mortality, recovery, composite outcomes) and related to 2) adults and children living with HIV affected by 3) SARS-CoV-2 4) published in English were included. Studies reporting data on SARS-CoV and MERS-CoV outbreaks or on patients, children, and adults, without mention of SARS-CoV-2 or HIV, book chapters and extracts from internet blogs or presentations were excluded.

Data extraction.

Data on study characteristics (study design), patients, and outcome measures were extracted using a specific form designed by one reviewer (CM) and checked by the other reviewer (PC). Disagreements between reviewers regarding extracted data were resolved through discussion and consensus of a third reviewer (DD). The following information was extracted: first author name; year of publication; country; age and gender of patients; ARV; darunavir therapeutic drug monitoring (DRV TDM, to exclude poor patient compliance to the therapy; NB: therapeutic range > 500 ng/mL); CD4 (last value before Covid-19), cells/mcl; CD4 (last value before Covid-19), cells/mcl; viral load (VL), other comorbidities, use of ACE-inhibitors, nasopharyngeal swab for SARS-CoV-2;

SARS-CoV-2 IgG-IgM; Covid-19 symptoms; Chest X-Ray/CT scan; lab tests; duration of symptoms (before hospitalization), days; hospitalization; Covid-19 treatment; non-invasive ventilation (NIV), mechanical ventilation, composite outcome, length of follow-up, days (from first visit). There was no funding source for this study.

Results.

Among the 291 papers from EMBASE and Medline search, the 898 articles from Google Scholar, with 12 further papers identified through manual search, 23 articles were included, with a total of 164 adult HIV patients being diagnosed with Covid-19 (Table 1.). There were no published studies on children.

Risk of bias assessment.

The included studies were mostly retrospective or case series/reports (19 studies), with only one RCT, one systematic review and one prospective survey (Table **1**). The overall risk of bias was moderate, due to the study types and characteristics.

Risk of bias assessment for Clinical Trials.

The risk of bias assessment for the only retrieved RCT is summarized in Supplementary Materials (Figure Supplementary **3**), showing a low risk of bias.

Table1. Results of systematic review.

Source	Study design	Patientsage gender	ARV	CD4 cells/mcl and VL	Other comorbidities	NF swab for SARS-CoV-2	COVID-19 symptoms	Chest X-Ray/ CT scan	Hospitali-zation	COVID-19 treatment	ICU/ invasive ventilation	Mortality	Clinical outcome
Zhu et al, *2020 (China)*[11]	Case report	1 61, F	NR	NR	Smoker Type II diabetes	+	Fever Respiratory symptoms	CT scan: bilateral ground glass opacities	1/1	LPV/r Gamma-globulins MPDN, Antibiotic therapy	0/1	0/1	Clinical improvement (recovered and discharged)
Chen et al, *2020 (China)*[12]	Case report	1 24, M	TDF, 3TC, EFV	NR	NR	+	Fever, respiratory symptoms	CT scan: multiple patchy subpleural-shadows right lung	1/1	LPV/r IFN	0/1	0/1	Recovered and discharged
Zhao et al, *2020 (China)*[13]	Case report	1 38, M	TDF, 3TC, EFV	250, <500	Previous HCV, treated	- (Positive serologies)	Fever Muscle aches	CT scan: right lower pneumonia	1/1	Oseltamivir IFN alpha	0/1	0/1	Recovered
Riva et al, *2020 (Italy)*[25]	Case series	3							3/3		1/3	0/3	Recovered (3/3), discharged (1/3)
		62, M	DRV/cobi, 3TC	441, <20	Hypertension, ischemic heart disease	+	Fever Respiratory symptoms	X-Ray: bilateral interstitial thickening	Yes	LPV/r HC tocilizumab Remdesivir (stop DRV/cobi)	Yes	No	Clinical improvement
		63, M	TDF/FTC DRV/cobi	743, <20	Hypertension	+	Fever	X-Ray: bilateral interstitial thickening	Yes	LPV/r HC (stop DRV/cobi)	No	No	Clinical improvement
		57, F	DRV/cobi RAL	NR	hypercholesterolemia	+	Fever Respiratory symptoms	X-Ray: interstitial thickening at the right lung	Yes	DRV/cobi HC	No	No	Clinical improvement
Drain et al, *2020 (USA)*[21]	Systematic review of case reports	2				2/2					1/2	1/2	
		66, M USA	NR	NR, undetectable	NR	+	NR	pneumonia	Yes	HC	Yes	Yes	
		50, M China	NR	NR	NR	+	NR	NR	NR	Human IGs MPDN IFN alpha -2b	NR	No	Clinical improvement
Guo et al, *2020 (China)*[14]	Observational study (survey)	9/1178				7/9						1/9	NR
		Median 57.0 (47.5-51.5), M	NRTI + NNRTI	101-350, <20	NR	+	NR	CT scan: typical findings	NR	NR	NR	No	Clinical improvement
				101–350, <20		+	NR		NR	NR	NR	No	Clinical improvement
				>350, NR		+	NR		NR	NR	NR	No	Clinical improvement
				>350, NR		+	NR		NR	NR	NR	No	

Source	Study design	Patientsage gender	ARV	CD4 cells/mcl and VL	Other comorbidities	NF swab for SARS-CoV-2	COVID-19 symptoms	Chest X-Ray/ CT scan	Hospitali- zation	COVID-19 treatment	ICU/ invasive ventilation	Mortality	Clinical outcome
													Clinical
				>350, NR		+	NR		NR	NR	NR	No	Clinical improvement
				>350, NR		+	NR		NR	NR	NR	Yes	
				>350, NR		–	NR		NR	NR	NR	No	Clinical improvement
		Median 57.0 (47.5–51.5), F		>350, NR		–	NR		NR	NR	NR	No	Clinical improvement
		NR	NR	NR		+	asymptomatic		No	No	No	No	
Huang et al, 2020 *(China)*[15]	Retrospective study	2/34 (adults)	NR	NR	NR	+	NR	NR	1/1	Yes, not specified	NR	0/2	
Wang et al, 2020 *(China)*[16]	Retrospective case series	2/138 (adults)	NR	NR	NR	+	Respiratory symptoms	CT scan: bilateral involvement	1/1	NR	0/2	NR	Clinical improvement
Wang et al, 2020 *(China)*[17]	Retrospective case series	1/125 Mean 38.76 (children included) Sex NR	NR	NR	NR	+	NR	CT scan (findings not specified)	1/1	Antiviral therapy (not specified)	0/1	0/1	Recovered
Borba et al, 2020 *(Brazil)*[28]	RCT	1/81 60, F	NR	NR	Hypertension, diabetes, CKD	+	Respiratory symptoms	NR	1/1	High dosage CQ	NR	1/1	
Schweitzer et al, 2020 *(Switzerland)*[29]	Case report	1 50, M	NR	NR	NR	+	Fever Respiratory symptoms Headache	Post-mortem CT scan: extensive bilateral pneumonia with ARDS	0/1	No	0/1	1/1	Found dead at home, in home-isolation, not admitted for self-reported symptoms
Yan et al, 2020 *(USA)*[22]	Retrospective study	15/128 Median 53.5 (40–65) Sex NR	NR	NR	NR	+	NR	NR	2/15	NR			
		2							Yes		NR	NR	Inpatient/ discharged
		13							No		No	NR	Ambulatory
Molina et al, 2020 *(France)*[30]	Case series	1/11 mean 58.7 (20–77) Sex NR	NR	NR	NR	+	NA (Fever 10/11)	NR	1/1	HC AZ	NR	NR	
Magagnoli et al, 2020 *(USA)*[23]	Retrospective study	9/368 median > 65 M	NR	NR	NR	+	NR	NR	9/9	HC 1/9	NR	NR	
										HC + AZ 4/9			
										No			

Source	Study design	Patients age gender	ARV	CD4 cells/mcl and VL	Other comorbidities	NF swab for SARS-CoV-2	COVID-19 symptoms	Chest X-Ray/ CT scan	Hospitalization	COVID-19 treatment	ICU/ invasive ventilation	Mortality	Clinical outcome
										HC 4/9			
Blanco et al, 2020 *(Spain)*[31]	Case series	5							5/5		2/5	0/5	
		40, transgender	TDF/FTC, DRV/cobi	616, <50	None	+	Fever, Respiratory symptoms, Malaise, Headache	X-Ray: normal	Yes	TDF/FTC, DRV/cobi (ART maintained)	No	No	Recovered
		49, M	Abacavir, 3TC, Dolutegravir	445, <50	Hypothyroidism	+	Fever, Respiratory symptoms (pneumonia)	X-Ray: bilateral ground-glass opacities	Yes	TDF/FTC LPV/r IFN beta-1b HC Antibiotic therapy Tocilizumab	Yes (ICU + invasive ventilation)	No	Inpatient, ECMO ongoing
		29, M	TDF/FTC, DRV/cobi	604, <50	None	+	Fever, Upper respiratory symptoms, Malaise, Headache, Dispnoea	X-Ray: normal	Yes	TDF/FTC LPV/r HC Antibiotic therapy	No	No	Recovered
		40, M	Abacavir, 3TC, Dolutegravir	1140, <50	Asthma	+	Fever, Lower respiratory symptoms, Malaise, Headache, Dispnoea (pneumonia)	X-Ray: right basal interstitial infiltrate	Yes	TDF/FTC LPV/r HC Antibiotic therapy Inhaled steroids	No	No	Recovered
		31, transgender	No ART	13, 45 500	None	+	Fever, Lower respiratory symptoms, Dispnoea (pneumonia)	X-Ray: right basal pneumonia with pleural effusion	Yes	TDF/FTC DRV/cobi IFN beta-1b HC Antibiotic therapy Steroids	Yes (ICU + NIV)	No	Recovered
Boldrini et al, 2020 *(Italy)*[26]	Case report	60 M	NR	NR	Haepatocarcinoma (metastatic)	NR	Fever, Respiratory symptoms	CT scan: suspicious findings	0/1	NR	0/1	0/1	Home care
Härter et al, 2020 *(Germany)*[32]	Case series	33	33/33	Median 670 <50 (30/33)	20/33	29/33	Cough (25/32); Fever (22/32); arthralgia/ myalgia (7/32); Headache (7/32) Sore throat (7/32) Sinusitis (6/32) Anosmia (6/32)	NRs	14/33	NR	6/14 (2 had detectable viremia)	3/32	10/14 discharged 1/14 inpatient, out of ICU
		Mean 48 (26-82) 30/33 M	NRTIs (31/33) INSTI (20/33) Non-NRTIs (9/33) PI (4/33)		Hypertension (10/20); COPD (6/20); HBV coinfection (5/20); Resolved HBV (4/20)	(2/33 BAL/ sputum)							

Source	Study design	Patientsage gender	ARV	CD4 cells/mcl and VL	Other comorbidities	NF swab for SARS-CoV-2	COVID-19 symptoms	Chest X-Ray/ CT scan	Hospitali-zation	COVID-19 treatment	ICU/ invasive ventilation	Mortality	Clinical outcome
					Diabetes (4/20); Cardiovascular disease (3/20); Renal insufficiency (2/20); Chronic HBV (1/20)								
Wu et al, *2020 (China)*[18]	Case series	2				2/2			2/2		0/2	0/2	
		60 M	TDF, 3TC, EFV	NR	Lymphoma, Pulmonary Tuberculosis, Diabetes	+	Fever, Myalgia	CT scan: bilateral multiple ground-glass opacities	Yes	ART continued, Oseltamivir, Antibiotic therapy	No	No	Recovered and discharged
		47 M	No (new diagnosis)	NR	Pneumocysitis pneumonia	+	Fever, Myalgia, Respiratory symptoms, diarrhoea	CT scan: multiple ground glass opacities	Yes	Ribavirin and Umifenovir	No	No	Recovered and discharged
Wang et al, *2020 (China)*[19]	Case report	37 M	NR	34 /	Syphilis	+	Fever, Respiratory symptoms	CT scan: multiple bilateral infiltrations	1/1	Arbidol, MPDN, Tocilizumab, antibiotic therapy	0/1	0/1	NR
Aydin et al, *2020 (Turkey)*[33]	Case series	4				4/4			4/4		1/4	1/4	
		34 M	No compliance	2.8 434 782	HBV, Bipolar disorder	+	Fever, Respiratory symptoms	CT scan: multiple bilateral ground glass opacities	Yes	TDF/FTC LPV/r, Oseltamivir, Antibiotic therapy	No	No	Recovered and discharged
		44 M	TDF/ FTCdolutegravir	1385 negative	Obesity, Diabetes, COPD, Hypertension	+	Fever, Respiratory symptoms	X-Ray, CT scan: ground glass opacities	Yes	HC, azithromycin and oseltamivir	Yes	Yes	Sudden cardiac arrest
		35 M	TAF/FTC EVG/c	/ Negative	NR	+	Weakness, Respiratory symptoms, diarrhea	CT scan: bilateral ground glass infiltrations	Yes	HC, Oseltamivir	No	No	Recovered and discharged
		36 M	TAF/FTC/EVG/c	NR	NR	+	Fever, Respiratory symptoms	CT scan: bilateral extended ground glass opacities	Yes	HC, Oseltamivir, Antibiotic therapy	No	No	Recovered and discharged
Su et al, *2020 (China)*[20]	Case report	32 M	Zidovudine 3TC, EFV	430 NR	NR	+ on sputum	Fever, Respiratory symptoms	CT scan: multifocal bilateral multifocal ground-glass opacities	1/1	Antibiotic therapy, Oseltamivir, LPV/r, IN-alpha, Stop EFV	0/1	0/1	Recovered and discharged
Gervasoni et al, *2020 (Italy)*[27]	Retrospective study	47	TAF/FTC/ bictegravir (10); ABC/3TC/ dolutegravir (10); TAF/FTC + INI (6);			26/47 (2/47 positive-serologies; 19/47	Fever (41) Respiratory symptoms (33) Diarrhea (7) Myalgia (4)	12/47 CT scan with confirmation of pneumonia	13/47	HC (8), LPV/r (5), Tocilizumab (2), Remdesivir (1) Antibiotic therapy (13)	2/47	2/47	45/47 fully recovered

Source	Study design	Patientsage gender	ARV	CD4 cells/mcl and VL	Other comorbidities	NF swab for SARS-CoV-2	COVID-19 symptoms	Chest X-Ray/ CT scan	Hospitali-zation	COVID-19 treatment	ICU/ invasive ventilation	Mortality	Clinical outcome
			Dolutegravir/3TC (5); Dolutegravir + boosted PI (5)			clinical diagnosis)	Headache (3) Asymptomatic (1)						
		Mean 50 ± 11 36/47 M	TAF/FTC/ bictegravir (8); ABC/3TC/ dolutegravir (9); TAF/FTC + INI (5); Dolutegravir/3TC (5); Dolutegravir + boosted PI (3)	Mean 630 ± 296 33/36 < 20	Dyslipidemia (8); Hypertension (9); Hepatitis C or B coinfection (4); Renal disease (4); Diabetes mellitus (2); Epilepsy (2); Cardiovascular disease (1); Neoplasms (3); Gastritis (1); Organ trasnplantation (1); COPD (1)	20/36	Fever (32) Respiratory symptoms (23) Diarrhea (5) Myalgia (2) Headache (1)	10/36	11/36	HC (5), LPV/r (4), Tocilizumab (2), Remdesivir (1) Antibiotic therapy (9)	2/36	2/36	34/36 fully recovered
		Mean 53 ± 12 11/47 F	TAF/FTC/ bictegravir (2); ABC/3TC/ dolutegravir (1); TAF/FTC + INI (); Dolutegravir/3TC (0); Dolutegravir + boosted PI (2)	Mean 658 ± 279 11/11 < 20	Dyslipidemia (7); Hypertension (5); Hepatitis C or B coinfection (1); Diabetes (1); Cardiovascular disease (1)	6/11	Fever (9) Respiratory symptoms (10) Diarrhea (2) Myalgia (2) Headache (2)	2/11	2/11	HC (3), LPV/r (1), Tocilizumab (2), Remdesivir (0) Antibiotic therapy (6)	0/11	0/11	11/11 fully recovered
Karmen-Tuohy et al, 2020 (USA)[24]	Retrospective study	21 Mean 60.04 ± 11.77 19/21 M	21/21 on HAART	19/21 had CD4 count. Median 298 (6/19: <200) 17/21 had VL: 15/17 < 50	Hypertension (7); Hyperlipidemia (4); Coronary disease (1); Peripheral vascular disease (1); Asthma or COPD (4); Diabetes (4); Malignancy (3); CKD (4); BMI 28.1 ± 5.37	21/21	NA	Abnormal X-Ray: 19/21	21/21	Antibiotic therapy for superimposed bacterial pneumonia (3/21)	6/21 ICU; 5/21 invasive ventilation	6/21 (3 had bacterial pneumonia)	NA

Abbreviations: ARV: antiretroviral therapy; VL: viral load, NF: nasopharyngeal, ICU: Intensive Care Unit; NR: not reported; LPV/r: Lopinavir/Ritonavir; MPDN: Methylprednisolone; DRV/cobi: Darunavir/Cobicistat; 3TC: Lamivudine; HC: hydroxychloroquine; TDF/FTC: Tenofovir/emtricitabine; RAL: Raltegravir; IGs: Immunoglobulins; IFN: Interferon; NRTI: Nucleoside reverse transcriptase inhibitor; NNRTI: Non-nucleoside reverse-transcriptase inhibitors; EFV: Efavirenz; CQ: chloroquine, NIV: non-invasive ventilation; INSTI: integrase strand transfer inhibitors; EVG/c: elvitegravir/cobicistat; COPD: chronic obstructive pulmonary disease; CKD: chronick kidney disease; HAART: highly active antiretroviral therapy; NA: not applicable.

Risk of bias assessment for observational studies.

The risk of bias assessment tool for non-RCTs is reported in Supplementary Materials (Figure Supplementary **4**). Most studies were retrospective and rated "fair", with case series and case reports being rated "poor", due to the study type and subsequent limitations.

Ten of the included studies were conducted in China,[11-20] 4 in the USA,[21-24] 3 in Italy[25-27] and one respectively in Brazil, Switzerland, France, Spain, Germany and Turkey.[28-33] Overall, we found a total of 164 adults with both HIV and SARS-CoV-2 infection. Of those, the diagnosis of Covid-19 was confirmed by a positive nasopharyngeal swab for SARS-CoV-2 in 135 cases, by bronchoalveolar lavage (BAL) positivity in two cases and by positive SARS-CoV-2 serologies (eg, IgM and total Igs) in five cases, while 24 patients were defined as "clinically confirmed Covid-19" according to their clinical findings combined with a typical radiological pattern, despite the lack of a virological confirmation.[26, 27, 32] No pediatric HIV patients were described.

According to the study setting, 47 patients were retrieved from studies conducted in the United States, 21 from China, 91 from Europe (51 from Italy, 33 from Germany, five from Spain, one from France and one from Switzerland), four from the Middle East (Turkey) and one from South America (Brazil). Eighteen studies had available data on gender,[11-14, 18-21, 23-29, 31-33] accounting for 142 patients: the large majority were males (120/142, 84.5%) with only 20 females (14%) and two transgenders. Patient age was reported for 57 cases[11-13, 18-21, 25, 26, 28, 29, 31-33] with a median age of 47 years (IQR 36-59). The eight patients reported by Guo et al[14] had a median age of 57 years (IQR 47.5-61.5), while the patients in the study conducted by Magagnoli et al[23] were slightly older, as one patient was included in the sub-cohort with a median age of 70 (IQR 60-75), four were from a sub-cohort with a median age of 68 (IQR 59-74) and four with a median age of 69 (IQR 59-75). The 13 HIV-positive patients reported by Yan et al were younger, as they were included in a sub-cohort of 102 patients with a median age of 43 years (IQR 34-54) while two cases were among those with a median age of 53.5 years (IQR 40-65).[22] The single patient affected by HIV reported by Molina et al[30] was included among patients with a median age of 58.7 years (20–77) and the one reported by Wang et al[17] was among those with a median age of 38.76 years. Last, Gervasoni et al included 47 patients with a mean age of 51 ± 11 years,[27] while Karmen-Tuohy et al reported 21 patients with a mean age of 60.04 ± 11.77.[24]

At the time of SARS-CoV-2 infection, ongoing ARV therapy was reported for 104 patients (63.4%). Of those, 19 were receiving a non-nucleoside reverse transcriptase inhibitors (NNRTI)-based regimen (18.4%), 18 (17.3%) were on a PI-based treatment and 55 (52.9%) were on an integrase strand transfer inhibitor (INSTI)-based treatment. Only one patient was ARV-naïve at the time of SARS-CoV-2 infection and the remaining ten patients were on treatment with other regimens, while data on ARV treatment were not reported for 60 patients. The immunological status at the time of SARS-CoV-2 infection was reported for 118 (71.9%) patients: the large majority (89/118, 75.4%) had CD4 > 350 cells/μl and of those 74 had high CD4 (≥500 cells/μl) while for six cases

the exact value was not reported. Of the remaining 29 patients, 25 had a CD4 count between 101 and 350 cells/μl[11, 32] and only four were severely immunosuppressed.[19, 31-33] Viral load (VL) detection for HIV was reported for 111 patients: all but four had an undetectable VL (< 500 cp/ml) and among those, 106 had a VL <50 cp/ml [14, 21, 24, 25, 27, 31-33].

Patients' comorbidities were reported in 101 cases. Of those, the most common were hypertension (35 patients), dyslipidemia (20 patients) and diabetes (15 patients), often combined with other conditions such as ischemic heart disease, chronic obstructive pulmonary disease (COPD) and chronic kidney disease. Other reported conditions were hypothyroidism, hepatocarcinoma, lymphoma, pulmonary tuberculosis, obesity, gastritis, solid organ transplantation, HBV co-infection, bipolar disorder, syphilis, asthma, and previous hepatitis C, treated. Thirty-three patients had no comorbidities and for the remaining 63 patients data were not available.
Sixteen articles reported clinical findings at first evaluation for SARS-CoV-2 infection [11-14, 16, 18-20, 25-29, 31-33], accounting for 104 patients. Among those, only two patients were asymptomatic[14, 27]; of those, the patient described by Gervasoni et al was tested because she was provided with healthcare insurance, while the other asymptomatic patient reported by Guo et al was tested because he reported close contact with confirmed or suspected COVID-19 patients. For all the other patients, the main symptoms were respiratory, such as dry cough, dyspnoea and/or fever. Eight patients also had diarrhea, seven myalgia and six headaches.

Data on radiological findings (CT scan and/or chest X-ray) were reported in 16 studies, accounting for 101 patients [11, 12, 14, 16-21, 24-27, 29, 31, 33]. Sixty-one patients had findings of pneumonia, of which 15 had bilateral involvement or multiple foci. In one case an extensive bilateral pneumonia with acute respiratory distress syndrome (ARDS) was observed at post-mortem CT scan.[29] In five patients, pneumonia was localized in the right lung and for 40 cases radiological information was reported as “typical findings”, “suspicious findings”, “abnormal X-ray” or “pneumonia” without specifying the radiological pattern. Last, two patients had no pathologic findings at chest X-ray.[31] Unfortunately, patient-related data on radiological findings were not provided for the remaining 63 patients.

Twenty-two studies reported data on patient management in terms of need of hospitalization or home care management [11-13, 15-33]. Sixty-nine out of 155 patients (44.5%) did not require hospitalization, while 86 (55.5%) were hospitalized. Among the hospitalized patients, data on further management (eg, need of non-invasive ventilation and/or mechanical ventilation) were available for 38 patients, of which 15 were transferred to ICU and required mechanical ventilation.
Nineteen studies reported the Covid-19 related treatment regarding 66 HIV-infected patients. Of those, 25 patients received hydroxychloroquine (HC) alone (3/25) or in combination with other drugs, such azithromycin (6/25), and/or oseltamivir (3/25) and/or interferon beta 1b (2/25) and/or remdesivir (2/25) and/or tocilizumab (4/25) and/or

steroids (2/25). Of the 35 remaining patients not treated with HC, 21 received antibiotics and/or remdesivir (1) and/or tocilizumab (3) and/or umifenovir (2) and/or oseltamivir (4) and/or steroids (3) and/or inhaled interferon alpha (4) and/or human immunoglobulins (2). As for the ARV therapy, at the time of SARS-CoV-2 infection, 14 patients were treated with LPV/r, of which three were already receiving LPV/r. Four patients were switched from darunavir/cobicistat (two cases) and from efavirenz (two cases) to LPV/r. Nine patients receiving long-term therapy with darunavir/cobicistat continued the same drug during Covid-19. In two cases, ARV treatment at the time of Covid-19 was not reported.

Clinical outcome of HIV-infected patients with SARS-CoV-2 infection was reported in 22 studies (Table **2**) [11-22, 24-29, 31-33]. Fifteen patients out of 139 with available data (10.8%) were transferred to ICU for mechanical ventilation (of those, five died). Among 152 patients with available data, 16 deaths were reported (10.5%, 15 males and one female); 11 of them had Covid-19 confirmation by positive SARS-CoV-2 nasopharyngeal swab, while for the remaining five patients data were not provided. It must be mentioned that among the 136 survivors, four were still hospitalized at the time of the study report.[19, 25, 31, 32] Of those, one patient was alive but still in ICU and treated with extracorporeal membrane oxygenation (ECMO).[31] Among the 16 deaths, seven cases had a relatively high CD4 count (six with >350 cell/µl and one with 298 cell/µl), while only one was severely immunosuppressed and for the eight remaining cases the immunological status was not reported. Three of the patients who died presented chronic conditions such as hypertension and diabetes. Of those, one had also a chronic kidney disease while two had concomitant COPD (of those, one had obesity); two patients had no comorbidities and for the remaining 11 deaths data were not specified.

Table 2. Results–Overall clinical outcomes

	Total patients n = 164
Alive and no invasive ventilation *(n,%)*	123 (75)
Alive and invasive ventilation *(n,%)*	4 (2.4)
Alive, not reported invasive ventilation *(n,%)*	11 (6.7)
Dead *(n,%)*	16 (9.8)
Unreported outcome *(n,%)*	10 (6.1)

Results.

To our knowledge, this is the first systematic review of Covid-19 in PLWHA to evaluate clinical features, diagnostic and therapeutic management and outcomes. Despite the impressive spread of SARS-CoV-2 all around the world, studies on Covid-19 in HIV patients are still few. Evidence of SARS-CoV-2 infection among PLWHA

has been emerging since early February 2020, while data on Chinese adults affected by Covid-19 have been published since January 2020.

According to the WHO/Global Health Observatory, there were 37.9 million (32.7–44.0 million) PLWHA at the end of 2018, with an estimated prevalence ranging from 0.6 to 0.9% of adults aged 15–49 years worldwide living with HIV, even though the burden differs significantly among countries and regions.[34] Undoubtedly, Africa is still the most strongly affected area, with nearly one case living with HIV in every 25 adults (3.9%) and accounting for more than two-thirds of people living with HIV worldwide[34] but there were no retrieved studies on Covid-19 and HIV from African Countries. The limited period of time occurred between the beginning of the Covid-19 pandemic in Africa and our last search (May 28th, 2020) can be an explanation. However, several other reasons may also explain the current lack of studies from Africa. First, the real number of African patients with Covid-19, including HIV co-infected cases, may be underestimated, considering that African healthcare systems are often weak and inadequately economically supported, leading to a reduced laboratory capacity of diagnosing SARS-CoV-2 infection and to the presence of weak surveillance systems, affecting the quality of data collection and report.[35] In addition, a reduction in seeking care may be observed for African PLWHA, due to several reasons, including poverty, long distance to reach healthcare facilities and stigma related to both HIV and Covid-19.
Even though Covid-19 is now affecting many countries, including those with moderate/high HIV prevalence, due to the limited period of time occurring between the beginning of Covid-19 pandemic and our last search, this systematic review identified and included only 164 HIV-positive adults with SARS-CoV-2 infection, mostly described as case reports and case series. The large majority were males (84.5%, 120/142 with available data) aged between 40-65 years, and mostly with a good immune-virological profile, as 75.4% (89/118) had a CD4 cell count >350 μL and 96.4% (107/111) had an undetectable VL. In 101/118 patients one or more comorbidities were reported, mostly hypertension (35 patients), dyslipidemia (20 patients) and diabetes (15 patients). Considering that nearly 50% of European PLWHA are older than 50 years and often report cardiovascular and chronic lung disease,[34] these findings strongly suggest that PLWHA chronically exposed to long-term ARV-related side effects must be considered a vulnerable population during SARS-CoV-2 pandemic. These results are in line with what has been reported for HIV-negative SARS-CoV-2 infected patients, regardless of disease severity, as reported in some large cohorts.[36, 37]

More than half (55.5%, 86/155) of patients with available data was hospitalized and of those, 15 patients were transferred to intensive care units for mechanical ventilation. Of 152 patients with available data on outcomes, 10.5% (16 cases) had an unfavorable outcome (death). From our findings, Covid-19 outcome seems to be unrelated to concomitant uncontrolled HIV infection, characterized by severe immune-suppression and high viral replication. In fact, 12 out of 16 deaths had an undetectable VL and seven had a relatively high immunity, while only one patient was severely immunosuppressed. However, the overall lack of data, particularly on the immune-

virological status, did not allow to make any conclusion on their impact in terms of disease severity and mortality, among PLWHA affected by Covid-19. We can therefore conclude that no clear evidence is available so far in favor of a different disease course or even a more serious illness in PLWHA than in HIV-negative people, while current evidence on Covid-19 suggests that the risk of disease severity increases with age, male gender and with chronic medical conditions, like cardiovascular disease, chronic lung disease, obesity and diabetes.[38]

One third of patients included in our review was managed as outpatients: Guo et al and Gervasoni et al respectively reported one asymptomatic outpatient[14, 27] while Yan et al reported 13 patients with ambulatory management with a favorable outcome.[22] Also, the 50-year-old patient reported by Schweitzer and colleagues was in home-isolation at the time of Covid-19 diagnosis: he was not admitted because of self-reported mild symptoms, with subsequent rapid deterioration and eventually death, without receiving treatment.[29] According to current evidence, the host response is determinant for the development of a more severe disease, with a disproportionate response or a dysregulated innate immunity being potentially responsible for tissue and organ damage during SARS-CoV-2 infection.[1] The status of mild immunosuppression, as noted by other authors, may be related to a subsequent milder disease presentation and course.[2, 39] Despite the lack of evidence, it may be hypothesized that HIV patients contracting SARS-CoV-2 have a chance to be asymptomatic or paucisymptomatic, and therefore not to be tested for SARS-CoV-2 and/or not to be admitted to hospitals, in case of infection. Further studies are needed to confirm this hypothesis.

The limited number and quality of included studies on Covid-19 and HIV/AIDS, and particularly the lack of studies from high HIV prevalence countries, did not allow to make any conclusion on the risk of SARS-CoV-2 infection among PLWHA. It is worth noting that Richardson and colleagues published a large cohort of 5700 hospitalized patients with Covid-19 in the New York City (NYC) area, among which only 43 (0.8%) had reported HIV infection as a comorbidity.[37] We could not consider those patients for inclusion in our review, because, although the study focused on the clinical outcomes during hospitalization, especially mechanical ventilation, renal replacement therapy and death, such clinical data or outcomes were not available for the HIV subgroup. Several factors may explain the potential low prevalence of HIV among Covid-19 cases in this setting, including behavioral factors, such as the potential inclination to show a fear or shame of seeking care or the difficulty in the access to healthcare services because of healthcare insurance costs.

A debate is still open regarding the potential protective effect of some ARVs in SARS-CoV-2 infection, as some drugs and particularly LPV/r have been shown to have some activity against coronaviruses. According to recent data, however, the use of LPV/r is not supported for the treatment of Covid-19. Cao et colleagues conducted the first randomized, controlled, open-label trial of LPV/r in patients with severe Covid-19.[40] A total of 199 hospitalized patients were considered, of whom 99 were assigned to the treatment group, and 100 received the standard of care. The authors found no benefit of LPV/r in terms of clinical improvement beyond the standard of

care - no reduction was found in viral RNA loads or in duration of viral RNA detectability as compared with standard supportive care alone. Osborne and colleagues conducted a systematic benefit-risk assessment for the use of LPV/r in severe Covid-19 compared to standard of care, screening several studies and finding no clear benefit as for time to clinical improvement.[7] Moreover, results for the LPV/r arm of the RECOVERY trial, established in March 2020 as a RCT to test potential treatments for Covid-19, were unblinded at the end of June 2020, showing no clinical benefit from the use of this drug in hospitalized patients.[6]

Based on literature included in our systematic review, among the 104 patients with available data, at the time of SARS-CoV-2 infection most cases were on an INSTI-based regimen (52.9%) while only 17.3% of patients were on a PI-based treatment before SARS-CoV-2 infection (nine with darunavir/cobicistat, three with lopinavir/ritonavir and six with an unspecified PI). Findings from Guo et al, on a cohort of 1174 HIV positive patients, are noteworthy on this matter.[12] According to this survey, only 8/1174 HIV positive patients had a diagnosis of Covid-19 and among those, all had a high CD4 cell count and an undetectable VL, none were taking PIs (LPV/r or DRV). In addition, none of the 178 PLWHA treated with LPV/r (119 cases) or DTG (59 cases) developed signs/symptoms of Covid-19. At adjusted logistic regression, older age was the only variable associated with an increased risk of Covid-19.[12]

Currently available literature published on Covid-19, though, is not supporting the use of any antiretroviral drugs including PIs, either for prophylaxis or treatmentTo date, there is no evidence to support switching patients with HIV from their usual antiretroviral therapy, or to justify HIV-negative people taking antiretrovirals, if not for PrEP to prevent HIV acquisition.

Limitations and Strengths.

This review has several limitations. First, the research occurred over a brief two-month period. Second, most of the eligible articles came from Chinese reports, with few reports from other countries and notably, no published studies from African countries. Third, due to the paucity of patients of our small sample, we were unable to conduct statistical analyses. Fourth, almost all the included studies had observational designs, and many were case series or case reports. This is not surprising, considering the timing of this systematic review in terms of the pandemic start, but certainly represents a considerable limitation, because of the nature of the included studies, often presenting a weaker methodological quality of reporting evidence, compared to RCTs.

To our knowledge, this is the first systematic review to summarize the current evidence on new SARS-CoV-2 infection in PLWHA, pointing out the clinical and therapeutic lack of knowledge. Another strength of this systematic review is the presence of a very low selection bias, as 137 patients were positive for SARS-CoV-2 at nucleic acid test and of the remaining 27 cases, five had confirmed serology and the remaining had clinical and radiological diagnosis.

Conclusion.

It is still unclear how HIV status may influence the risk of acquiring SARS-CoV-2 infection and/or the risk of developing a more severe disease course, but these preliminary data show no clear evidence for a higher Covid-19 infection rate or different disease course in PLWHA, compared to HIV-negative people. However, it must be considered that some PLWHA and particularly males exposed to long-term ARV may have recognized ARV-related risk factors for possibly severe Covid-19 complications, such as diabetes, hypertension and/or cardiovascular diseases, therefore being at greater risk of severe Covid-19 course.[41]

More evidence is needed to state the real burden of Covid-19 among PLWHA. Thus, until then, all PLWHA should employ precautions, with additional safety measures for people with severe immune-suppression and/or ARV-related comorbidities.

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Chapter 4

RESULTS

Medium and long term immunological and clinical impact of SARS-CoV-2 infection among children and adults recovered by a COVID-19 family cluster

4.1. Mild SARS-CoV-2 Infections and Neutralizing Antibody Titers

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Abstract.

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

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

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

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

Introduction.

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

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

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

In this study, we evaluated the role of age as a determinant of the production and persistence of naturally acquired nAbs among a cohort of family clusters of COVID-19, including adults and children who recovered from asymptomatic or mild symptomatic infections.

Methods.

Study Design and Population.

A single-center, prospective study was conducted on Italian family clusters of COVID-19 attending the COVID-19 Family Cluster Follow-up Clinic, at the Department of Women's and Children's Health of the University Hospital of Padua (Veneto Region, Italy). From March 1, 2020 to September 4, 2020, 57 families were enrolled meeting the following inclusion criteria: (1) having children of pediatric age (aged <15 years); (2) any family member (eg, mother and/or father and/or any son or daughter) with a history of COVID-19. Families were enrolled in the program 4 to 8 weeks after the end of either isolation or hospitalization and after referral from the family pediatrician. Evaluation of children and relatives included data collection on demographic parameters and past medical history, clinical evaluation and the collection of a blood sample for a characterization of the immune response to SARS-CoV-2. All subjects aged >18 years, including older siblings and parents, and legally authorized

representatives of subjects aged <18 years, were informed of the research proposal and provided written consent for the collection and use of biological specimens and routine patient-based data for research purposes. Families were invited to return to the clinic for longitudinal blood collection. The protocol was communicated to the ethical committee according to the national regulation (Protocol N° 0070714 of November 24, 2020; amendment number 71779 of November 26, 2020).

Data Collection and Definitions.

Information collected during the clinic was entered into a Web-based database by using the Research Electronic Data Capture platform (Vanderbilt University, Tennessee) hosted in the server of the University of Padova. For this study, data were collected retrospectively from the existing clinical files and analyzed anonymously. Subjects were considered patients with confirmed COVID-19 if they had a record of virological positivity for SARS-CoV-2 by real-time polymerase chain reaction (RT-PCR) according to routine diagnostic molecular protocols[16] and/or resulted positive by either of the 2 serological tests adopted in this study. For each confirmed COVID-19 case, a baseline date was defined as follows: (1) for symptomatic cases, the first date between the onset of symptoms or the date of first positive SARS-CoV-2 molecular assay result; (2) for asymptomatic cases: the date of the first positive molecular assay result or, in those with only serologically confirmed COVID-19 and with negative or undetermined nasal-pharyngeal swab (NPS) results, by the family outbreak temporal sequence, coinciding with the date of symptoms onset in a virologically confirmed SARS-CoV-2 family outbreak (Supplemental Fig 6). Subjects who were asymptomatic and had no analytical evidence of SARS-CoV-2 infection were considered to not have COVID-19. The severity of COVID-19 was scored as mild, moderate, severe, or critical, following the World Health Organization classification [17]. For stratification purposes, individuals were divided on the basis of both social and biological development, into toddlers (<3 years), preschool-aged children (3 to <6 years), school-aged children (6 to <15 years) and sexually mature subjects (>15 years). These age classes were deemed instrumental for a translation of results into the context of school-targeted vaccination and sero-surveillance campaigns.

Serological Assays.

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

SARS-CoV-2 Viral Load Measurement.

A selection of NPSs of enrolled subjects that had been originally screened at the Padova University Hospital were made available for quantification of the viral load. Copies of SARS-CoV-2 were quantified by a homemade

multiplex quantitative assay on the basis of a 1-step digital droplet polymerase chain reaction (ddPCR)[19]. Results were expressed as SARS-CoV-2 copies per 5 µl. Further details are reported in the Supplemental Information.

Statistical Analyses.

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

The humoral response was assessed by comparing the geometric mean titer (GMT) and the 95% confidence interval (CI) of IgM, IgG, and PRNT50 values in the overall data set, including both independent and subject-paired samples, stratified by age classes and by time between serological sampling and baseline, categorizing subjects into 3 intervals, namely 1 to 2, 3 to 6 and 7 to 8 months. The 1-way analysis of variance and the independent samples t test were performed, when appropriate. Associations between antibody titers, baseline intervals and age, were assessed with linear regression models. Strength of associations between variables was assessed by Pearson correlation coefficient by using the logarithm (base 10) of the antibody titers, given data skew. Use of the robust variance estimator to account for correlations within patients with multiple blood samplings did not change the CIs considerably in the unadjusted analyses, so correlation structures were omitted from all analyses. Among a subcohort of subjects that agreed to be sampled again after enrollment, a dependent t test for subject-paired samples was used to compare the GMT and 95% CI.

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

The diagnostic sensitivity of the CLIA and PRNT assays were assessed on subjects with a positive NPS result. Considering the PRNT assay as reference method for the validation of immunoassays for SARS-CoV-2, we calculated measures of diagnostic accuracy of the CLIA assay.

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

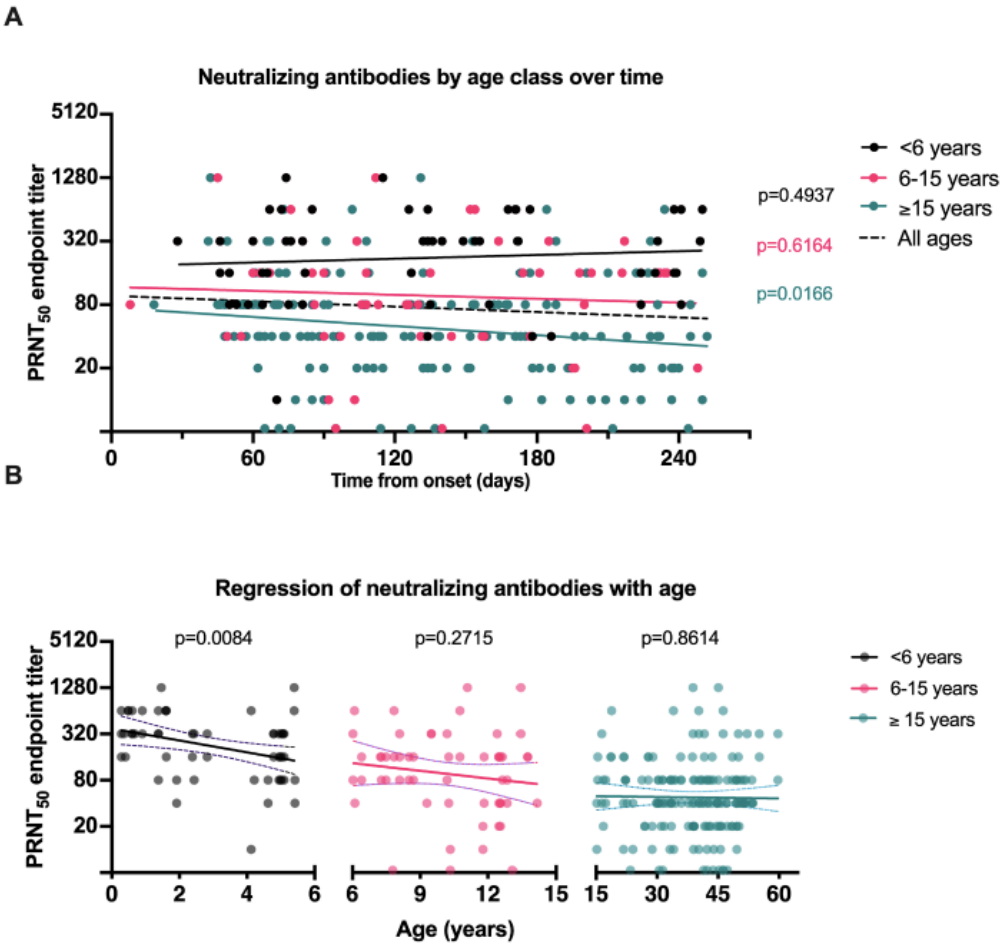
Results.

From March 1, 2020, to December 3, 2020, we prospectively evaluated 57 family clusters of COVID-19 (Supplemental Fig 5). A serological assessment was performed at least once on 209 recruited subjects. Subjects who had previously tested positive for SARS-CoV-2 by real-time RT-PCR (111 of 209) were considered to have

confirmed COVID-19, together with individuals who had no record of virological positivity but showed evidence of seropositivity by either of the 2 serological tests adopted in this study (44 of 209). Descriptive analysis and additional information on baseline identification are provided as Supplemental Information (Supplemental Table 2, Supplemental Fig 6). Three out of 73 children were excluded from the analyses (see Supplemental Fig 5). In total, 152 confirmed COVID-19 cases were studied: 70 children or older siblings and 82 parents with median ages of 8 (interquartile range [IQR], 4–13) and 42 years (IQR, 34–46), respectively. Of 152 cases, 38, 97, and 17 were sampled once, twice and 3 times, respectively.

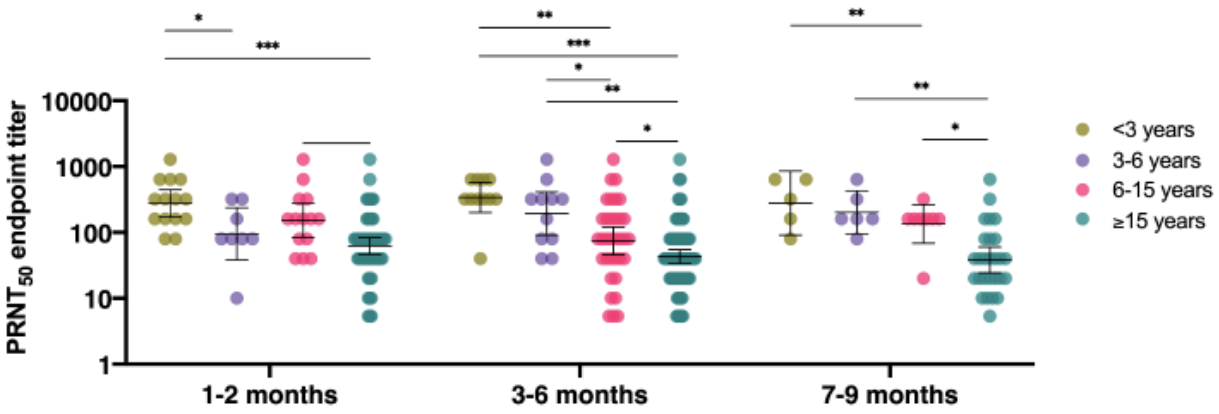
Analyzing all 283 blood samples collected from confirmed COVID-19 cases, we observed that nAbs persisted in the population, (Fig 1A) recording a modest nonsignificant decline (P = .1062) over a median period of 132 days (IQR, 79–187) from baseline. When samples were stratified by age, children aged <6 years were the only class with a slightly increasing trend over time, as opposed to children aged 6 to 15 years and adults, although only for subjects ≥15 years of age we recorded a statistical support for the regression line (P = .0166). A further correlation analysis confirmed that nAbs inversely correlated with age (Pearson $\rho = -0.4144$, $P < .0001$), irrespective of time. To better characterize this picture, we conducted a regression model of age against PRNT50 titers overall and within age classes. Overall, regression was significant (estimated slope: −0.0423, P < .0001), whereas the only significant regression within different age groups was observed for children aged <6 years (estimated slope −0.2561, P = .0084) (Fig 1B).

Figure 1. Stability of SARS-CoV-2 nAb titers over time. A, PRNT50 titers from 283 serum samples collected at a median time of 132 days (IQR, 79–187) from infection onset, overall and stratified by 3 age classes, including children aged <6 years (n = 55; R2 0.0089, P = .4937), children aged ≥6 and <15 years (n = 58; R2 0.0047, P = .6164) and older siblings and adults aged ≥15 years of age (n = 170; R2 0.0341, P = .0166). B, Reduced PRNT50 titers observed at increasing age, at linear regression analysis conducted among children <6 years (n = 55; R2 0.1239, P = .0084), children aged ≥6 and <15 years (n = 58; R2 0.0224, P = .2715), and older siblings and adults of ≥15 years of age (n = 170; R2 0.0002, P = .8614).



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

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



In a longitudinal serological assessment, we analyzed subject-paired plasmas from 76 subjects who were sampled a first and a second time on approximately day 72 (SD ±22) and 169 (SD ±26) from baseline (time window 1), respectively (Supplemental Table 4). Moreover, we analyzed plasma from 50 subjects (of which, 12 had contributed to time window 1), who were sampled a first and a second or third time on approximately day 99 (SD ±35) and 234 (SD ±10) from baseline (Fig 3 A–C, Table 1, and Supplemental Table 4) (time window 2). In time window 1, we observed an increase of nAbs titers for children aged <6 years (slope 0.0076), whereas children aged 6 to <15 years and subjects aged >15 years recorded a slight decreasing trend with estimated slopes of −0.0046 and −0.0047, respectively (Fig 3 A–B). In time window 2, children aged <6 years and those aged 6 to <15 years recorded a modest increase (slope 0.0019) and a minimal decrease (slope −0.0004) of nAbs titers, respectively, whereas, in adults, we observed a declining trend (slope of −0.0057) with a significant 40% reduction of nAbs titers (P = .0021) over time (Fig 3C). Interestingly, serological data by CLIA indicated a steady and significant decrease of IgG over time (Table 1), and a negativization in 54% (29 of 53) and 79% (27 of 34) of the seropositive subjects in the first and second time windows, respectively, as opposed to the 3% (2 of 75) and 2% (1 of 50) of the subjects who tested positive for PRNT50. Almost all samples tested negative by CLIA IgM at both time points in both groups, irrespective of age.

Figure 3. Performance of SARS-CoV-2 CLIA IgG and PRNT titers over time. A, Decreasing levels of SARS-CoV-2 CLIA IgG levels observed for all classes of age (aged <6 years, aged ≥6 and <15 years, and aged ≥15 years; paired t test P < .0001 across all groups), at longitudinal subject-paired serological assessment of 76 subjects sampled firstly at 72 days (SD ± 22) and a second time at ~169 days (SD ± 26) after baseline. B, Kinetics of PRNT50 over time, for the same samples revealed in A. C, Kinetics of PRNT50 over time in a subject-paired evaluation of 50 subjects, for whom paired samples were available at ~99 days (SD ± 35) and 234 days (SD ± 10) from baseline. The dotted line represents the limit of detection. D, Diagnostic sensitivity of CLIA IgG and PRNT50 assays evaluated through testing of 194 samples from 111 virologically confirmed SARS-CoV-2 subjects. The dashed line represents the limit of detection and the manufacturer-recommended cutoff value for PRNT50 and CLIA assays, respectively.

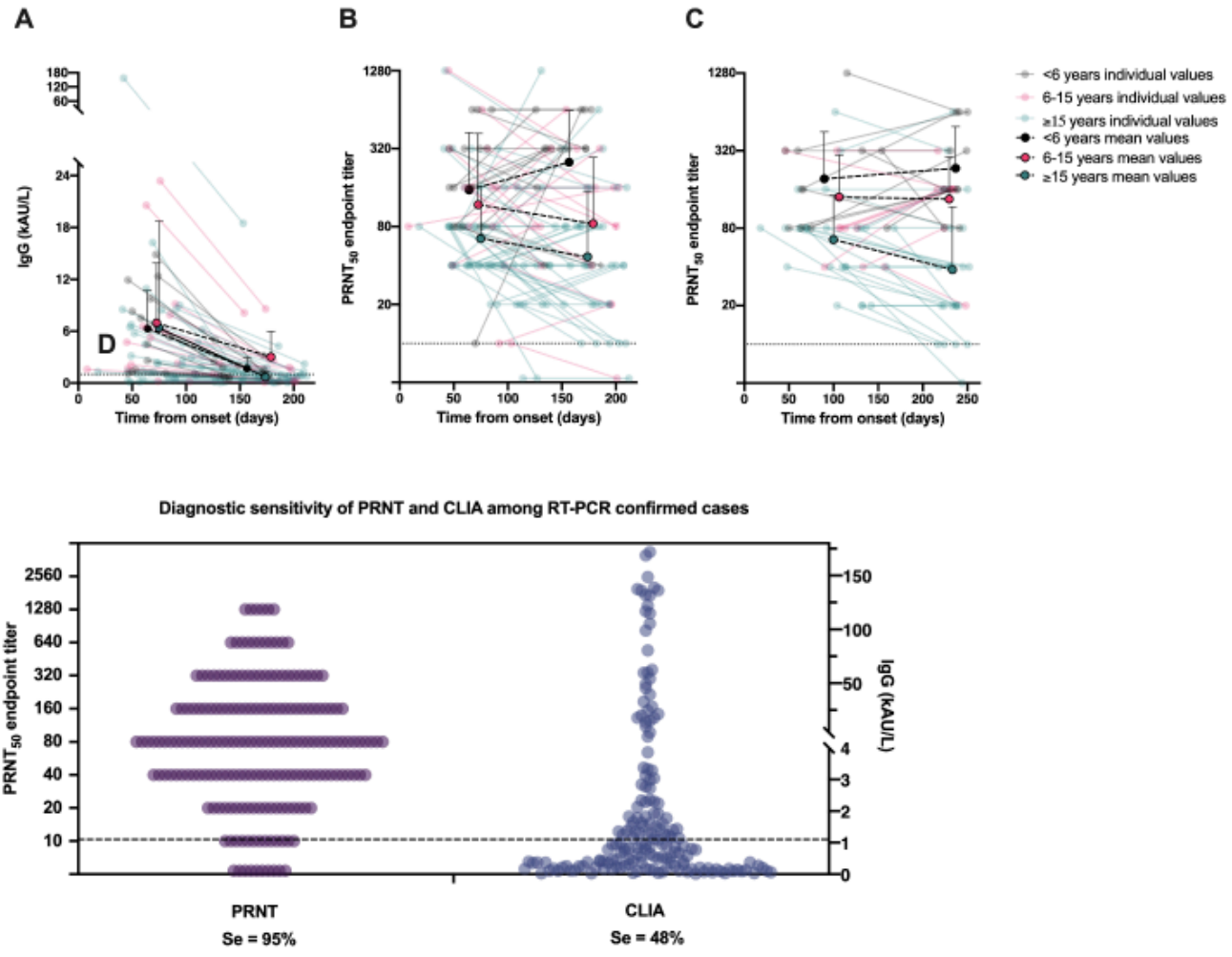


Table 1. Subject-paired serological data of 76 subjects who were sampled twice around periods of 72 days (SD, ± 22) and 169 days (SD, ± 26) from baseline and data from 50 subjects, for whom paired samples were available around 99 days (SD, ± 35) and 234 days (SD, ± 10) from baseline.

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

	Age 6-<15 years (n=16)			Age 6-<15 years (n=10)		
	First sample	Second sample (5-6 months)	p-value§	First sample	Latest sample (7-9 months)	p-value§
Mean days from baseline (STD)	72.6 (27.1)	178.9 (25.5)		105.9 (33.9)	234.1 (11.4)	
	GMT (95% CI)	GMT (95% CI)		GMT (95% CI)	GMT (95% CI)	
IgM (kAU/L) ¥	0.6 (0.4 - 0.8)	0.5 (0.3 - 0.7)	0,0857	0.4 (0.3 - 0.6)	0.3 (0.2 - 0.4)	0,0038
IgG (kAU/L) ¥	3.7 (1.9 - 7)	1.1 (0.6 - 2.3)	< 0.0001	2.4 (0.8 - 7)	0.4 (0.2 - 1.2)	< 0.0001
PRNT (endpoint titer)	118.1 (58.6 - 238)	83.9 (43.9 - 160.4)	0.2087	139.3 (62.4 - 310.9)	134.5 (68.5 - 264.3)	0.2275

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

¥ Missing data are handled in the analysis

ǂ One-way ANOVA

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

Because 14 cases had been assigned hypothetical baselines coinciding with the onset of symptoms of a family member (Supplemental Fig 6), we assumed that the considerable uncertainty of these values required a sensitivity analysis. The analysis verified that results and conclusions were robust against inclusion or exclusion of these 14 cases (data not shown). Nonetheless, we decided to include them, given that their exclusion would decrease underrepresented groups of children aged 6 to <15 years and 3 to <6 years at intermediate and late time points (Supplemental Table 5).

We compared the performance of PRNT and CLIA on a set of 194 samples collected from 111 of 152 confirmed patients with COVID-19 who had a positive real-time RT-PCR NPS result, recording sensitivities of 0.95, (184 of 194) and 0.48 (93 of 194), respectively (Fig 3D). Moreover, evaluating 264 of 283 samples for which both PRNT and IgG values were available, irrespective of the virological status of the donors, we found a moderate concordance but a poor negative predictive value of the CLIA in predicting seropositivity months after infection (Supplemental Table 6). We further explored whether nAbs correlated with either clinical presentation or viral load. Differences in the distribution of clinical presentations between age classes were nonsignificant (Fig 4A), and nAbs titers did not significantly differ between subjects showing mild or no symptoms (Fig 4B).

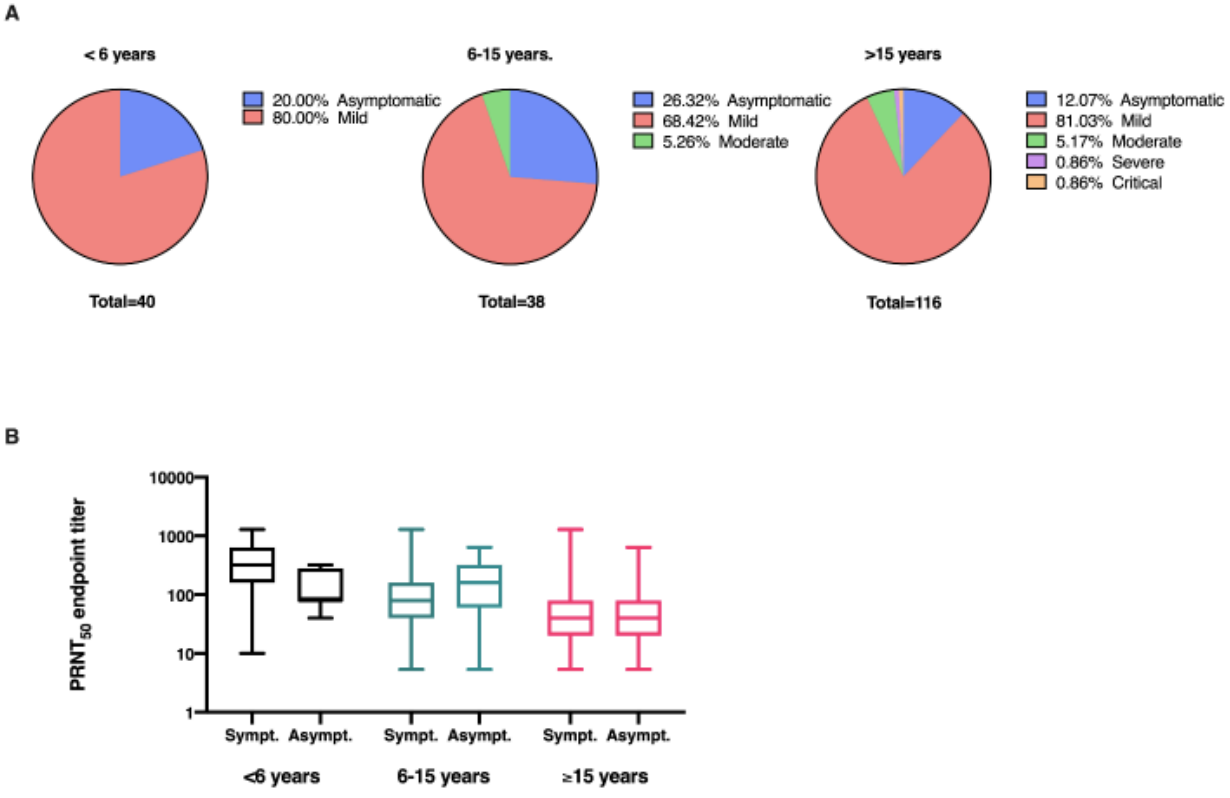


Figure 4. nAb titers according to COVID-19 disease severity. A, Clinical presentation of COVID-19 in children aged <6 years, aged ≥6 and <15 years, and aged ≥15 years, according to the World Health Organization COVID-

19 clinical classification. B, PRNT50 titer distribution among either asymptomatic or symptomatic subjects, stratified by age class and represented by box plots revealing minimum, maximum, median, first, and third quartiles (aged <6 years, aged ≥6 and <15 years, and aged ≥15 years; Wilcoxon test, P = .0548, P = .8409, and P = .6230, respectively).

For 63 of 111 COVID-19 confirmed cases that had recorded virological positivity, the original swab was available for viral load quantification by ddPCR. To select a biologically relevant period of infection and standardize comparisons, we focused on a subgroup of 32 of 63 subjects for whom swabs had been collected within 4 days from symptom onset and serological samplings had been taken within 1 to 2 months. We observed that adults recorded a mean viral load of 107.88 copies, whereas children aged <6 years and those aged 6 to <15 years had mean values of 107.65 and 106.79 copies, respectively. Differences in viral load between age classes were not significant (P = .2409), whereas PRNT50 titers directly correlated with viral load among children (Supplemental Table 7).

Discussion.

The role of antibodies on the clearance of established SARS-CoV-2 infection and clinical outcomes is still unclear. Recent data suggest that the development of potently neutralizing humoral immunity against SARS-CoV-2 is critical to increase survival and may protect against reinfection with other circulating strains of SARS-CoV-2 in adults [20]. In children it was recently revealed that the onset of high titers of nAbs is associated with shorter viral shedding at nasal-pharyngeal level[19] but not with clinical presentation in the short-term follow-up.

In the current study, we describe a longitudinal comparison of the magnitude and persistence of nAbs against SARS-CoV-2, among asymptomatic and mildly symptomatic toddlers, preschool-aged children, school-aged subjects, and parents, in family clusters of COVID-19. In our cohort, antibodies neutralizing SARS-CoV-2 virus persisted over a period of 2 to 8 months from infection, recording only a modest decline. This result is in line with previous studies using PRNT and surrogate-neutralization–based-assays[7–10,21,22] describing a minimal decline of nAbs in adult populations. Surprisingly, nAbs inversely correlated with age, and children aged <6 years, and, in particular, toddlers aged <3 years, had the highest titers throughout early, intermediate, and late times from infection onset. Our data strengthens and expands recent work published by Yang et al,[23] who described higher surrogate neutralizing ability and avidity of antibodies in children aged 1 to 10 years, proving these features to be age-dependent, in a cohort of subjects aged 1 to 24 years, early after recovery. In contrast with our findings, other studies indicated that nAbs in children were lower than in adults [24,25]. However, in 1 study,[24] stratification by age was done by age <24 years or >24 years, and children and adults were sampled on ∼5 and 12 days from hospital

admission, respectively; in the other study,[25] authors compared children with mildly affected adults previously selected as plasma donors at the hospital. We believe these selection and sampling biases might account for discrepancies with data reported in our study. Interestingly, in the latter study,[25] anti-S IgG and nAbs inversely correlated with age among children.

Strains encountered in childhood imprint adaptive immunity. Subsequent exposure to antigenically related viruses directs the antibody response largely toward known conserved epitopes and less against novel immunodominant proteins, blunting the neutralizing potential [26]. Recently, this mechanism has been explored for influenza, proving that children aged <6 years have a narrow strain-specific hemagglutinating inhibition activity, whereas adults have a back-boost response to past infections [27]. In light of this, we hypothesize that an original antigenic sin driven by repeat exposure to endemic human coronaviruses might impair the response to SARS-CoV-2 in adults, whereas the less experienced immune repertoire of children could favor a prompt selective response. Recent work published by Selva et al [28] supports this hypothesis, proving that infection in elderly patients associates with antibodies targeting the cross-reactive S2 and NP proteins, whereas, in children, the response is dominated by antibodies with high Fc-effector function targeting the immunodominant S1 protein of SARS-CoV-2. In addition, Westerhuis et al [29] proved that, in adult patients, an expansion of B-cell clones against seasonal human coronaviruses dominates the response, generating antibodies poorly reactive with SARS-CoV-2.

Another relevant result of our study is the persistence of nAbs in children. We reveal for the first time that mildly affected children aged <6 years displayed increasing nAbs levels, over a period of 236 days from infection. Interestingly, children aged 6 to <15 years plateaued at approximately the same period, whereas adults showed a significant decline in nAbs, recording a 40% decrease between 3 and 7 months from infection. Similarly, Lau et al [10] estimated that, for adults, the decline of PRNT titers would reach undetectable levels between 133 and 416 days from infection depending on clinical severity and reported a 50% decrease between 3 and 6 months from infection for mild cases. In addition, Chia et al [9] identified 5 profiles of antibody responses and observed that the persistence of high nAbs up to 6 to 7 months correlated with high levels of proinflammatory cytokines and the severity of COVID-19 in adults, predicting declines between 96 and 580 days.
In light of this, it is important to observe that, in our cohort, severity of infection and mean viral loads did not differ significantly among age classes; besides, the presence of mild symptoms was not a predictor of higher nAbs. Nonetheless, in children, viral load estimated at baseline correlated with magnitude of nAbs evaluated after 1 to 2 months, suggesting that a higher exposure to the antigen results in stronger humoral responses.

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

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

In the absence of correlates of protection for nAbs acquired after infection, it is not advisable to translate our data into predictions of a superior immunity of children to reinfection. According to clinical studies and experimental animal work, superior nAbs for SARS-CoV-2 might translate into protection from COVID-19 disease and higher viral clearance in the upper respiratory tract, leading to a reduction in shedding and transmission [19,32]. It is of the utmost importance to identify age- and time-matched correlates of protection to finally translate serological data into useful elements for the design of vaccines and immunization campaigns for SARS-CoV-2.

Supplementary materials.

Serological assays.

Blood samples were collected in EDTA-coated tubes to further separate cells and plasma by Ficoll procedure. Plasma and cellular samples were appropriately store at -80°C and liquid nitrogen, respectively, until use. A high-throughput method for Plaque Reduction Neutralizing Test (PRNT) was used for the quantification of neutralizing antibodies in plasma samples [18]. Samples were heat-inactivated by incubation at 56°C for 30 min and 2-fold dilutions were prepared in Dulbecco modified Eagle medium (DMEM). The dilutions, mixed to a 1:1 ratio with a virus solution containing approximately 25 focus-forming units (FFUs) of SARS-CoV-2, were incubated for 1 h at 37 °C. Fifty microliters of the virus-serum mixtures were added to confluent monolayers of Vero E6 cells, in 96-wells plates and incubated for 1 h at 37 °C, in a 5% CO2 incubator. The inoculum was removed and 100 ml of overlay solution of Minimum essential medium (MEM), 2% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 U/ml) and 0.8% carboxy methyl cellulose was added to each well. After a 26-h incubation, cells were fixed with a 4% paraformaldehyde (PFA) solution. Visualization of plaques was obtained with an immunocytochemical staining method using an anti-dsRNA monoclonal antibody (J2, 1:10,000; Sci- cons) for 1 hour, followed by 1 h incubation with peroxidase-labeled goat anti-mouse antibodies (1:1000; DAKO) and a 7 min incubation with the True Blue (KPL) peroxidase substrate. FFUs were counted after acquisition of pictures on a flatbed scanner. Biosafety Level 3 laboratory setting was used for PRNT tests. The neutralization titer was defined as the reciprocal of the highest dilution resulting in a reduction of the control plaque count >50% (PRNT50). Samples recording titers equal to or above 1:10 were considered as positive according to a previous validation conducted on a panel of archive samples collected in 2018 in Italy1.

Sera from the same donors were analyzed with the chemiluminescence immunoassay (CLIA) MAGLUMI™ 2019-nCoV IgM/IgG on the analytical system MAGLUMI™ 2000 Plus (New Industries Biomedical Engineering Co., Ltd [Snibe], Shenzhen, China). IgG/IgM immunocomplexes are formed upon addition of a recombinant antigen expressing the full-length spike and nucleocapsid proteins of SARS-CoV-2. According to the manufacturer's inserts (271 2019-nCoV IgM, V2.0, 2020-03 and 272 2019-nCoV IgG, V1.2, 2020-02), the 2019-nCoV IgM cut-off is 1.0 AU/mL, while the 2019-nCoV IgG cut-off is 1.1 AU/mL. The assay is intended for qualitative detection and differentiation of IgM and IgG antibodies. The combined sensitivity and specificity of IgG/IgM is declared to be 95.6% and 96.0%, respectively.

SARS-CoV-2 viral load measurement.

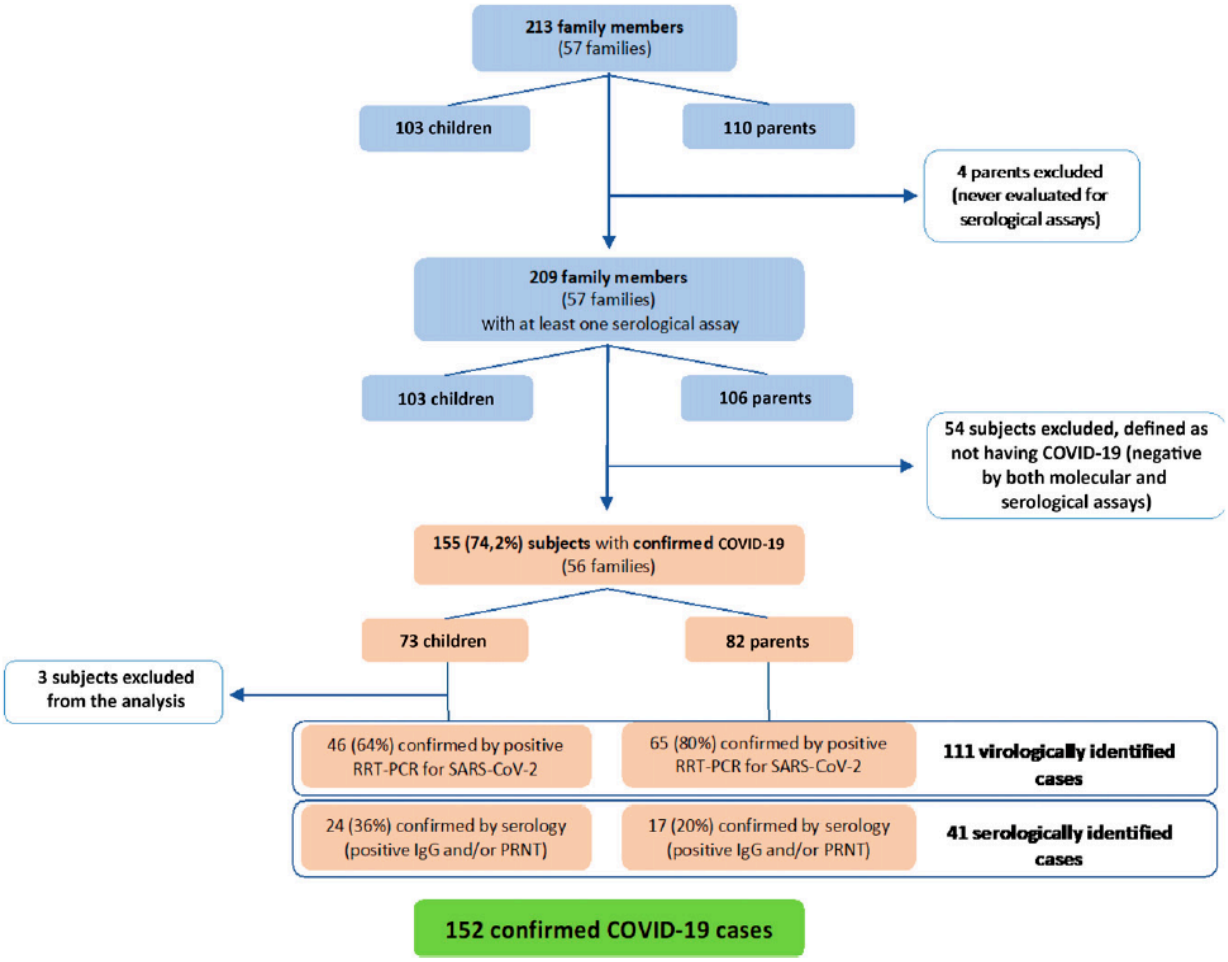
A selection of nasopharyngeal (NP) swabs of enrolled subjects that had been originally screened at the Padova University Hospital were made available for quantification of the viral load. NP swabs tested were collected by using flocked swabs in liquid-based collection and transport systems. Total nucleic acids were purified from 200µl media and eluted in a final volume of 100µl. Copies of SARS-CoV-2 were quantified by a home-made multiplex

quantitative assay based on One-Step digital droplet PCR (ddPCR). The reaction mixture consisted of 5µl of supermix (Bio-Rad, CA, USA), 2µl of reverse transcriptase, 2µl of DTT final concentration 300mM, forward and reverse primers of SARS-CoV-2 E gene to a final concentration of 400nM each and probe to a final concentration of 200nM and 5µl of nucleic acids were eluted from nasopharyngeal swab samples into a final volume of 20 µl. Housekeeping GAPDH was employed to verify the good quality of RNA extracted and amplified under the same conditions using the GAPDH Kit (PE Applied Biosystems, Waltham, MA, USA) [19]. Each well of the prepared mix was loaded into an 8-channel cartridge and 70µl of the Droplet Generation Oil for Probes (Bio-Rad) were added. Droplets were formed in the QX200TM Droplet Generator (Bio-Rad). Droplets in the oil suspension were transferred into a 96 well plate and placed into a Mastercycler (Eppendorf, Hamburg, Germany) with the following cycling parameters: 42-50°C for 60 min; 95°C for 10min; 95°C for 30sec and 60°C for 1 min; the last two passages were repeated for 40 cycles followed by 98°C for 10 min. The droplets were then read by the QX200TM Droplet Reader (Bio-Rad) and the results were analyzed with the QuantaSoftTM Analysis Software 1.7.4.0917 (Bio-Rad) 2. Wells with less than 10000 droplets were discarded from the analysis. Each sample was run at least in duplicate. Results were expressed as SARS-CoV-2 copies/5µl.

Supplementary Figure 1.

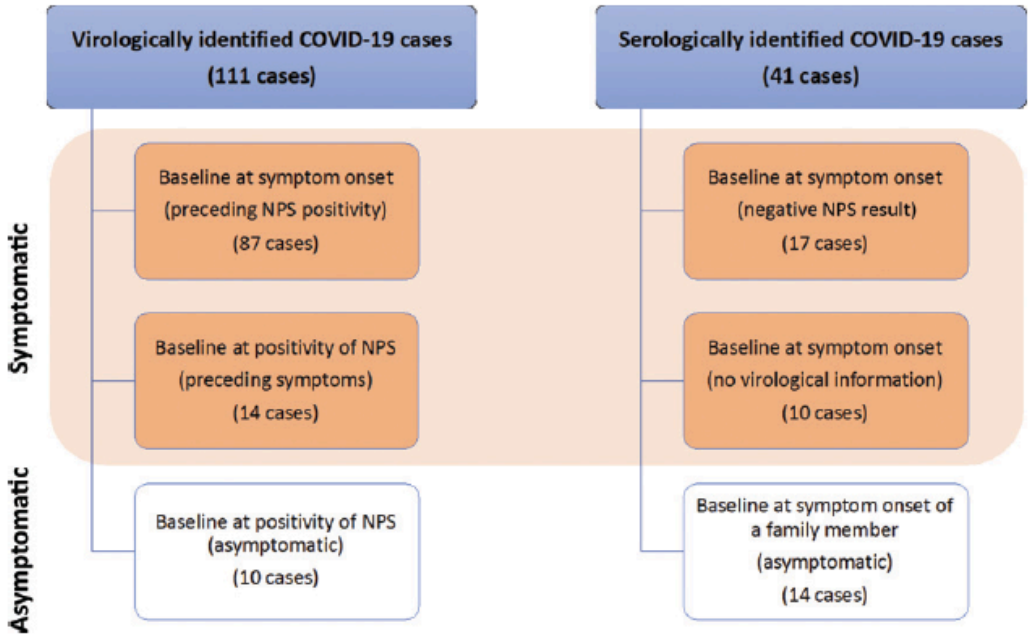
Flow chart of family clusters of COVID-19 observed from March 1st to the September 4th 2020, at the COVID-19 follow-up clinic of the Pediatric Department, Department of Women's and Children's Health, University of Padua. [a] Three children who tested positive for SARS-CoV-2 nAbs (PRNT) were further excluded from the analysis because they constituted peculiar cases if compared with the general cohort: in fact, 2 children presented with multisystem inflammatory syndrome in children 4 to 6 weeks after COVID-19 onset, and 1 newborn of a mother who tested positive for COVID-19 presented positive for SARS-CoV-2 nAbs (PRNT) detected 51 days after birth that could be related to maternal immunity and not seroconversion (SARS-CoV-2 molecular assay was never performed

at birth). RRT-PCR, real time reverse-transcriptase polymerase chain reaction.



Supplemental Figure 2.

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



Supplemental Table 2. Descriptive analysis of the 57 families observed at the Department of Women's and Children's Health of the University Hospital of Padua (Italy), overall (n= 209) and stratified by familiar status as children or older siblings (n =103) and parents (n= 106).

	Overall			Children and Older Siblings			Parents		
	Positive for COVID-19 (n = 155)	Negative for COVID-19 (n = 54)	P^a	Positive for COVID-19 (n = 73)	Negative for COVID-19 (n = 30)	P^a	Positive for COVID-19 (n = 82)	Negative for COVID-19 (n = 24)	P^a
Female sex, n (%)	81 (52.3)	23 (42.6)	.22	36 (49.3)	12(40)	.39	45 (54.9)	11 (45.8)	.44
Mean age (SD)	25.8 (17.7)	23.4 (19.5)	.37	8.75 (6.3)	7.12(5.7)	.26	40.9(8.3)	43.7 (7.4)	0.13
Age class, n (%)									
<6 y	28 (18.1)	15 (27.8)	.28	28 (38.4)	15 (50)	.63	0 (0)	0 (0)	—
≥6 and <15 y	34 (21.9)	12 (22.2)	—	34 (46.6)	12 (40)	—	0 (0)	0 (0)	—
≥15 y	93 (60)	27 (50)	—	11 (15.1)	3 (10)	—	82 (100)	24 (100)	—
Symptomatic, n (%)	128 (82.6)	15 (27.8)	<.001	56 (76.7)	8 (26.7)	<.001	72 (87.8)	7 (29.2)	<.001
WHO classification, n (%)									
Asymptomatic	27 (17.4)	39 (72.2)	<.001	17 (23.3)	22 (73.3)	<.001	10 (12.2%)	17 (70.8)	<.001
Mild	118 (76.1)	15 (27.8)	—	53 (68.8)	7 (26.9)	—	65 (79.3)	7 (29.2)	—
Moderate	6 (3.9)	—	—	1 (1.3)	—	—	5 (6.1)	—	—
Severe	1 (0.6)	—	—	0 (0)	—	—	1 (1.2)	—	—
Critical	1 (0.6)	—	—	0 (0)	—	—	1 (1.2)	—	—
MIS-C	2 (1.3)	—	—	2 (2.6)	—	—	0 (0)	—	—
Pediatric comorbidities									
No	—	—	—	57 (78.1)	27 (90)	.26	—	—	—
Yes[b]	—	—	—	16 (21.9)	3 (10)	—	—	—	

MIS-C, multisystem inflammatory syndrome in children; WHO, World Health Organization; —, not applicable.

[a] Student's t test, χ^2 test, Fisher's exact test when appropriate.

[b] The following comorbidities were found among 16 children who tested positive for COVID-19: premature birth (n = 1), asthma (n = 5), allergy (n = 1), congenital heart disease (n = 1), rheumatic disease (n = 1), chronic neuropathy (n = 1), immunodeficiency (n = 2), cleft lip and palate (n = 1), kidney or ureteral disease (n = 1).

Supplemental Table 3. Serological data of 283 plasma samples obtained from 152 confirmed COVID-19 cases (38 independent samples, 245 dependent samples obtained from 114 cases) among age Ccasses, overall and stratified by time from baseline.

	<3 Years	≥3–<6 Years	≥6–<15 Years	≥15 Years	P[a]
All data, irrespective of onset					
n	30	25	58	170	—
GMT (95% CI)					
IgM (kAU/L)[b]	0.7 (0.6–0.9)	0.8 (0.6–1.1)	0.4 (0.4–0.5)	0.5 (0.4–0.5)	.0024
IgG (kAU/L)[b]	1.4 (0.7–2.5)	1.5 (0.8–2.8)	1.5 (1–2.3)	0.9 (0.7–1.2)	.1055
PRNT (end point titer)	298.6 (221.4–402.6)	155.6 (100.9–239.9)	96.7 (68.8–135.8)	47.8 (40.2–56.7)	<.0001
At 1–2 mo from onset					
n	14	8	14	57	—
GMT (95% CI)					
IgM (kAU/L)[b]	0.7 (0.6–0.9)	0.8 (0.5–1.2)	0.6 (0.5–0.8)	0.6 (0.5–0.7)	.4902
IgG (kAU/L)[b]	3.8 (2–7.3)	4.9 (2.4–9.8)	3.9 (1.8–8.1)	1.6 (1–2.7)	.0915
PRNT (end point titer)	275.8 (171.4–443.8)	95.1 (38.1–237.8)	152.3 (83.8–276.6)	62.2 (46.4–83.5)	<.0001
At 3–6 mo, from onset					
n	11	11	34	84	—
GMT (95% CI)					
IgM (kAU/L)[b]	0.7 (0.4–1.2)	0.8 (0.4–1.4)	0.4 (0.3–0.6)	0.5 (0.4–0.6)	.1481
IgG (kAU/L[b]	0.9 (0.5–1.7)	1.6 (0.7–3.7)	1.5 (1–2.4)	0.8 (0.6–1.2)	.1863
PRNT (end point titer)	340.8 (200.8–578.5)	193.3 (91–410.6)	74.2 (45.6–120.6)	42.9 (33.7–54.7)	<.0001
At 7–9 mo, from onset					
n	5	6	10	29	—
GMT (95% CI)					
IgM (kAU/L)[b]	0.7 (0.4–1.3)	0.7 (0.3–2)	0.3 (0.2–0.4)	0.3 (0.3–0.5)	.0203
IgG (kAU/L)[b]	0.1 (0.1–0.2)	0.3 (0.1–0.7)	0.4 (0.2–1.2)	0.4 (0.2–0.6)	.4997
PRNT (end point titer)	278.6 (90.7–855.6)	201.6 (95.1–427.3)	134.5 (68.5–264.3)	38.1 (24.2–60)	<.0001

—, not applicable.
[a] One-way analysis of variance.
[b] Missing data are handled in the analysis.

Supplemental Table 4. Temporal distribution of sample collection among subjects who contributed to the study with either 1, 2, or 3 plasma samples.

	First Sample		Second Sample			Third Sample	
Time From Baseline	1–2 mo	3–6 mo	1–2 mo	3–6 mo	7–9 mo	3–6 mo	7–9 mo
Subjects with only 1 sample (*n* = 38)	21	17	0	0	0	0	0
Subjects with only 2 samples (*n* = 97)	52	45	0	62[a]	35[b]	0	0
Subjects with 3 samples (*n* = 17)	17	0	3	14[a]	0	2	15[b]
Total No. samples per period	90	62	3	76	35	0	17
Total No. samples (*n* = 283)	152[c]	—	114[c]	—	—	17[c]	—

—, not applicable.
[a] Second samples included in subject-paired analyses of time window 1 (total of 76).
[b] Second and third samples included in subject-paired analyses of time window 2 (total of 50).
[c] Total No. samples represents the combined total across times from baseline for each sample group.

Supplemental Table 5. Distribution of plasma samples across age classes and baseline intervals.

	Age Classes, *n* (%)				
Baseline Intervals	<3 y (*n* = 30)	≥3–<6 y (*n* = 25)	≥6–<15 y (*n* = 58)	≥15 y (*n* = 170)	Total (*n* = 283)
1–2 mo	14 (15.1)	8 (8.6)	14 (15.1)	57 (61.3)	93 (100.0)
3–6 mo	11 (7.9)	11 (7.9)	34 (24.3)	84 (60.0)	140 (100.0)
7–9 mo	5 (10.0)	6 (12)	10 (20)	29 (58.0)	50 (100.0)

Supplemental Table 6. Estimators of diagnostic accuracy and test agreement of the MAGLUMI 2019-nCoV IgG with the PRNT assay as gold standard method.

	Estimate	95% CI
Sensitivity	0.52	0.46–0.58
Specificity	0.85	0.65–1.0
Positive predictive value	0.99	0.96–1.0
Negative predictive value	0.08	0.04–0.13
Cohen's κ	0.08	0.02–0.13
Overall percent agreement	0.54	0.92–0.97
Positive percent agreement	0.52	0.46–0.58
Negative percent agreement	0.85	0.58–0.96

Estimates are calculated by using the contingency table, plotting 237 of 255 serological samples tested in the study.

Supplemental Table 7. Correlation between SARS-CoV-2 viral load (genome copies) detected by means of ddPCR in NPSs collected within 4 days from symptom onset and PRNT titers assessed 1–2 months later, overall and stratified for classes of age.

	NPSs Collected Within 4 Days From Symptom Onset		
	n	Pearson Coefficient	*P*
All ages	32	−0.00796	.9655
<15 y	13	0.67250	.0118
≥15 y	19	−0.29453	.2209

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4.2. Asymptomatic and Mild SARS-CoV-2 Infections Elicit Lower Immune Activation and Higher Specific Neutralizing Antibodies in Children Than in Adults

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Abstract.

Background: The immune response plays a pivotal role in dictating the clinical outcome in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-infected adults, but it is still poorly investigated in the pediatric population.

Methods: Of 209 enrolled subjects, 155 patients were confirmed by PCR and/or serology as having coronavirus disease 2019 (COVID-19). Blood samples were obtained at a median of 2.8 (interquartile, 2.1–3.7) and 6.1 (5.3–7.2) months after baseline (symptom onset and/or first positive virus detection). The immune profiles of activation, senescence, exhaustion, and regulatory cells were analyzed by flow cytometry. Neutralizing antibodies (nAbs) were detected by a plaque reduction neutralization test. In available nasopharyngeal swabs at baseline, SARS-CoV-2 levels were quantified by digital droplet PCR (ddPCR).

Results: Overall, COVID-19 patients had higher levels of immune activation, exhaustion, and regulatory cells compared to non-COVID-19 subjects. Within the COVID-19 group, activated and senescent cells were higher in adults than in children and inversely correlated with the nAbs levels. Conversely, Tregs and Bregs regulatory cells were higher in COVID-19 children compared to adults and positively correlated with nAbs. Higher immune activation still persisted in adults after 6 months of infection, while children maintained higher levels of regulatory cells. SARS-CoV-2 levels did not differ among age classes.

Conclusions: Adults displayed higher immune activation and lower production of anti-SARS-CoV-2 nAbs than children. The different immune response was not related to different viral load. The higher expression of regulatory cells in children may contribute to reduce the immune activation, thus leading to a greater specific response against the virus.

Introduction.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease (COVID)-19, emerged in China in late December 2019 and subsequently spread globally. SARS-CoV-2 has affected children less severely than adults (1) because a large majority of children usually present with asymptomatic or paucisymptomatic outcomes (2), and only a minority develop severe/critical COVID-19 and/or a COVID-19-related multisystem inflammatory syndrome (MIS-C) (3). It was first hypothesized that children had a milder disease because of the lower expression levels of the angiotensin-converting enzyme 2 (ACE-2) receptor, and thus lower viral load than adults (1), but so far, there is no evidence of a lower degree of tissue expression or function of ACE-2 in children (4), and emerging data suggest that viral loads do not differ significantly between young and old age groups (5–7).

The immune response plays a pivotal role in dictating the clinical outcome in SARS-CoV-2-infected patients. Nonetheless, while a large number of studies have been conducted in adults, the disease has been poorly investigated in the pediatric population. In adults, SARS-CoV-2 infection induces spike-specific neutralizing antibodies (8) and a specific response via T cells (9). However, high levels of immune activation and an overproduction of proinflammatory cytokines have been consistently described in SARS-CoV-2-infected adults, and this pattern has been associated with the severe clinical outcome of COVID-19 (10–12).

To date, very little is known about the immunopathogenesis of pediatric COVID-19. In asymptomatic/mildly symptomatic children, peripheral blood lymphocytes remain mostly in the normal range, suggesting less immune dysfunction (13, 14), but few data are available concerning their specific immune response against the virus (15) and their status of immune activation and cytokines storm (16). Children have yet to be included in clinical trials of the COVID-19 vaccine, thus understanding the immunopathogenesis of COVID-19 may provide important clues for effective treatments of this disease and the best strategy to fight infection in the pediatric population.

In this study, we studied the immune profiles of activation, senescence, exhaustion, and regulatory cells, and we analyzed their relationship with neutralizing antibodies and viral load in asymptomatic and mild symptomatic COVID-19 children and adults belonging to the same family cluster.

Materials and Methods.

Study Population and Sampling.

A single-center, prospective study was conducted on Italian family clusters of COVID-19 attending the COVID-19 Family Cluster Follow-up Clinic (CovFC), at the Department of Women's and Children's Health of the University Hospital of Padova (Veneto Region, Italy). From March 1 to September 4, 2020, 57 families were enrolled who met the following inclusion criteria: (a) having children of pediatric age (<15 years) and/or (b) any family member with an history of confirmed COVID-19. Families were enrolled 4–8 weeks after the end of either

isolation or hospitalization and after referral from family pediatricians. Evaluation of children and relatives included data collection on demographic parameters and medical history. Parents or legally authorized representatives were informed of the research proposal and provided written consent for the collection and use of biological specimens and routine patient-based data for research purposes. The protocol was communicated to the Ethical Committee according to the national regulation (Prot. No. 0070714 of November 24, 2020; amendment no. 71779 of November 26, 2020).

A total of 209 family members were enrolled (Supplementary Figure S1). One hundred fifty-five subjects were considered confirmed COVID-19 cases if they had a record of virological positivity for SARS-CoV-2 by real-time PCR and/or resulted positive by either of the two serological tests adopted in the study (CLIA) MAGLUMI™ 2019-nCoV IgM/IgG and/or by plaque reduction neutralizing test (PRNT) (17). For each confirmed COVID-19 case, the baseline date was defined as follows: (1) for symptomatic case, the date of the onset of symptoms or the date of first positive SARS-CoV-2 molecular assay; (2) for asymptomatic cases, the date of the first positive molecular assay or, in those with only serologically confirmed COVID-19 and with negative/undetermined nasopharyngeal swab (NPS), by the family outbreak temporal sequence, coinciding with the date of symptoms onset in the family cluster. Fifty-four subjects that were asymptomatic and had no analytical evidence of SARS-CoV-2 infection were considered non-COVID-19 cases.

For all enrolled family members, a blood sample was collected in ethylenediaminetetraacetic acid (EDTA)-containing tube at median of 2.8 [interquartile (IQR), 2.1–3.7] months after baseline and for 116 members a follow-up sample at 6.1 (5.3–7.2) months after baseline. Plasma and cells were separate by Ficoll–Paque gradient (Pharmacia, Uppsala, Sweden). Plasma was collected, centrifuged, and appropriately stored at −80°C until use. Cells were appropriately stored at liquid nitrogen.

SARS-CoV-2 Viral Load Quantification.

A selection of 41 NPS of enrolled subjects, collected at a median of 3 (1–5) days after symptoms and originally screened at University Hospital of Padova, was made available in order to quantify the viral load. Levels of SARS-CoV-2 were quantified using a home-made multiplex quantitative assay based on One-Step RT digital droplet PCR (ddPCR) (15, 17). Results were expressed as SARS-CoV-2 copies/5 µl.

Flow Cytometry.

Cells were thawed, washed, and stained for 20 min in the dark with the Live/Dead Fixable Near-IR Dead Cell Stain Kit (Life Technologies, Carlsbad, CA, USA) and the following labeled monoclonal antibodies: anti-CD3 [fluorescein isothiocyanate (FITC)], anti-CD4 [peridinin chlorophyll protein (PerCP)], anti-CD38 [phycoerythrin (PE)], anti-HLA-DR [allophycocyanin (APC)], anti-CD279 (programmed cell death 1, PD-1) (PE-Cy7), anti-CD57 (PE), anti-CD21 (BV421), anti-CD27 (PE-Cy7), and anti-IgD (PE) (Becton-Dickinson, San Diego, CA, USA); anti-CD8 (VioGreen), anti-CD28 (APC), anti-CD19 (VioBright515), and anti-CD10 (APC) (Miltenyi

Biotec, Auburn, California USA). Cells were then washed and resuspended in phosphate-buffered saline (PBS) supplemented with 1% paraformaldehyde. Tregs were determined using anti-CD4 (BB515), anti-CD25 (BV421), anti-CD127 (PE-CF594) (Becton-Dickinson, San Diego, CA, USA), and combined membrane and intracytoplasmic staining for anti-FoxP3 (AlexaFluor 647) using a transcription factor buffer set according to the manufacturer's protocol (Becton-Dickinson, San Diego, CA, USA). All samples were analyzed using an LSRII flow cytometer (Becton-Dickinson, San Diego, CA, USA). A total of 50,000 events were collected in the lymphocyte gate using morphological parameters (forward and side-scatter). Data were processed with FACSDiva Software (Becton-Dickinson) and analyzed using Kaluza Analyzing Software v.1.2 (Beckman Coulter) (Supplementary Figure S2).

Circulating Levels of PAMPS, DAMPS, and Cytokines.

DNA was extracted from 200 µl of plasma using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) and eluted in 50 µl of AE buffer. To quantify circulating levels of 16S ribosomal (r)DNA and mitochondrial (mt)DNA, two quantitative methods based on real-time PCR assay were performed with primer pair and probe as previously described (18). Results were expressed as 16S rDNA copies/µl plasma and as mtDNA copies/µl plasma.

Plasma samples were thawed at room temperature and circulating levels of interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)-α were quantified with Fluorokine MAP Human IL-6 kit, Fluorokine MAP Human IL-10 kit (R&D Systems), and Fluorokine MAP Human TNF-α/TNFSF2 kit designed for using the Luminex 200TM according to the manufacturer's instructions (18).

Statistical Analysis.

The immune response was assessed by comparing the median (IQR) of viral load, levels of PRNT values, and the proportion of immune activation, senescence, exhaustion, and regulatory cells, in the overall dataset, and stratified by age classes (age <6 years, 6≤ age <15 years, and age ≥15 years) between COVID-19 infected and non-infected patients. Comparisons were made by the Wilcoxon rank sum test and Kruskal–Wallis test, as appropriate.

Strength of associations between the immunology response and antibody titers, the time between first serological sampling and baseline, age, and the viral load (where possible) among infected patients was assessed by Spearman's correlation coefficients overall and stratified by age classes.

A linear-log regression model was used to assess the association between the immunology response, and the infection, using the logarithm transformation given data skew, adjusting by age. While a log–log model was used to assess the association between the immunology response and the infection, adjusting by age and time between first serological sampling and baseline.

Finally, among 116 subjects who recorded the second peripheral blood sample, a dependent non-parametric Wilcoxon signed-rank test for subject-paired samples was used to compare the median and IQR among age classes.

Analyses were performed using the Statistical Analysis System software (version 9.4; SAS Institute, Cary, NC, USA). Statistical significance was set at the 0.05 level. All p values were two-sided.

Results.

Patients' Characteristics.

Descriptive characteristics of patients are shown in Table 1. In total, 152 confirmed COVID-19 cases were studied: 70 children/older siblings and 82 parents with a median age of 8.0 (4.3–12.6) and 41.7 (33.5–46.5) years, respectively. In addition, 54 non-COVID-19 cases were studied as controls: 30 children/siblings and 24 parents, with a median age of 5.4 (3.2–8.8) and 42.1 (38.7–45.3) years, respectively. Most of COVID-19 children (75.7%) and adults (79.3%) were mild symptomatic, according to WHO guidelines (19). To better evaluate the immunological profile, the cohort was further stratified based on both social and biological development into children of pediatric age [<6 years (preschool children) and 6≤ age <15 years school age, but still pediatric subjects] and sexually mature subjects (≥15–60 years, defined as adults) (17).

Table 1. Descriptive analysis of the 57 families studied at the Department of Women's and Children's Health of the University Hospital of Padova (Italy).

	Overall			Children/older siblings			Parents		
	Non-COVID-19 (n = 54)	COVID-19 (n = 152)	*p-value* §	Non-COVID-19 (n = 30)	COVID-19 (n = 70)	*p-value*§	Non-COVID-19 (n = 24)	COVID-19 (n = 82)	*p-value*§
Female n (%)	23 (42.6%)	78 (51.3%)	0.27	12 (40%)	33 (47.1%)	0.51	11 (45.8%)	45 (54.9%)	0.44
Age Median (IQR)	15.6 (4.5–41.6)	28.6 (8.2–42.1)	0.33	5.4 (3.2–8.8)	8 (4.3–12.6)	0.21	42.1 (38.7–45.3)	41.7 (33.5–46.5)	0.13
Age classes n (%)									
<6 years	15 (27.8%)	27 (17.8%)	0.24	15 (50%)	27 (38.6%)	0.52	–	–	
6≤ age <15	12 (22.2%)	32 (21.1%)		12 (40%)	32 (45.7%)		–	–	
≥15 years	27 (50%)	93 (61.2%)		3 (10%)	11 (15.7%)		24 (100%)	82 (100%)	
WHO classification* n (%)									
Asymptomatic	–	25 (16.5%)		–	16 (22.9%)		–	10 (12.2%)	
Mild	–	119 (78.3%)		–	53 (75.7%)		–	65 (79.3%)	
Moderate	–	6 (4%)		–	1 (1.4%)		–	5 (6.1%)	
Severe	–	1 (0.7%)		–	0 (0%)		–	1 (1.2%)	
Critical	–	1 (0.7%)		–	0 (0%)		–	1 (1.2%)	
Pediatric comorbidities n (%)									
No	–	–		27 (90%)	54 (77.1%)	0.13	–	–	
Yes**	–	–		3 (10%)	16 (22.9%)		–	–	

§*Student's t-test, χ^2 test, Fisher exact test where appropriate.*

**WHO, World Health Organization.*

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

Immunological Profile: Comparison Between COVID-19 and Non-COVID-19 Subjects and Between Age Classes at First Sampling.

For all enrolled subjects, a first peripheral blood sample was available at a median of 2.8 (2.1–3.7) months after baseline. Differences in the immunological parameters among COVID-19 and non-COVID-19 cases were explored by a univariate linear-log regression model and a multivariate model adjusted by age (Supplementary Table S1): the two groups differed in their percentages of both T (CD4 and CD8) and B-activated cells and Tregs and Bregs (Supplementary Table S1). Furthermore, COVID-19 adults showed higher percentages of senescent CD4 and CD8 cells and CD8 exhausted cells compared to non-COVID-19 adults, while no significant differences occurred between COVID-19 and non-COVID-19 children (Supplementary Table S2).

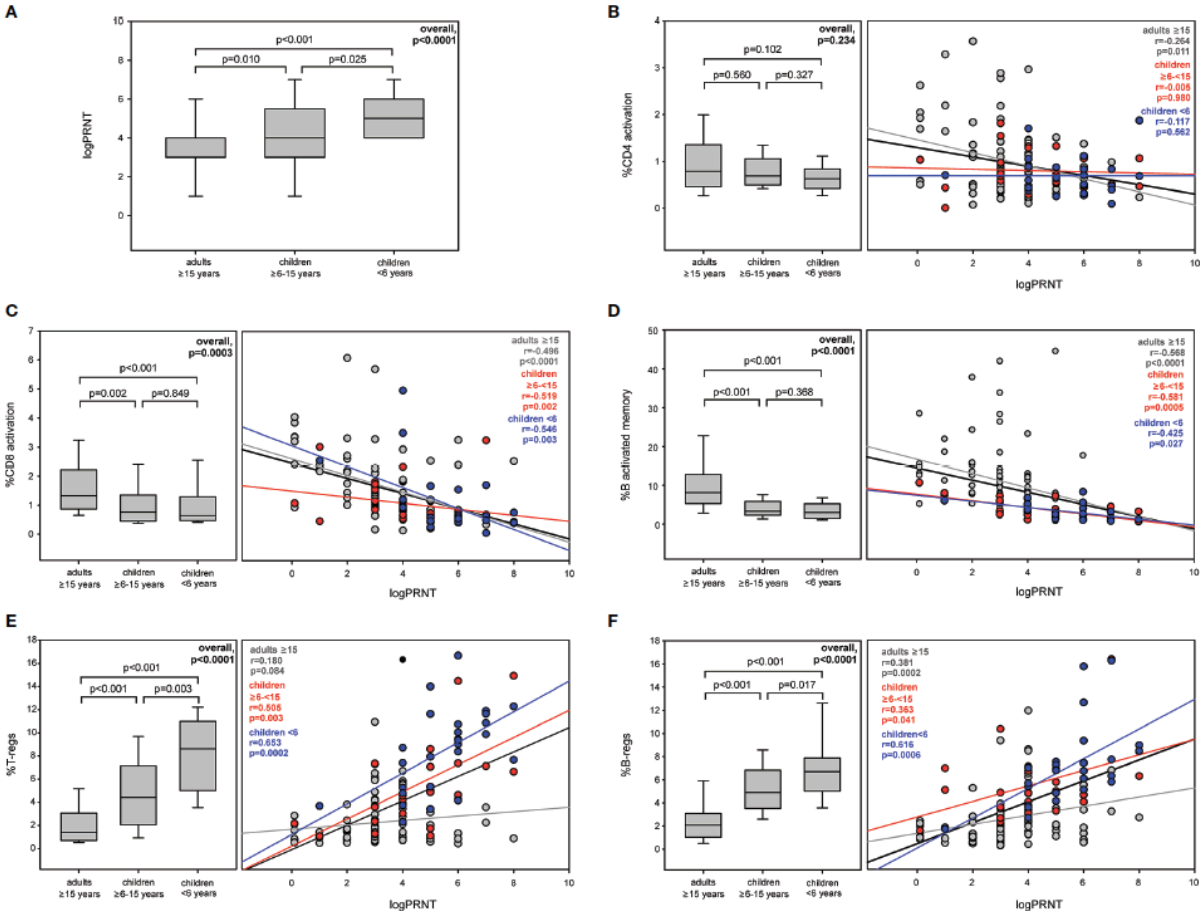
Within the COVID-19 group, the percentage of activated cells was higher in adults than in children aged 6–15 and <6 years (Table 2 and Figures 1B–D). Moreover, COVID-19 adults had higher levels of immune senescent T and B cells compared to children, and a higher expression of CD4 and CD8 exhausted cells compared to children (Table 2).

Table 2. Immunological parameters in COVID-19 age classes at first sampling.

Immunological parameters	Children <6 years (N = 27) Median (IQR)	Children 6≤ age <15 years (N = 32) Median (IQR)	Adult ≥ 15 years (N = 93) Median (IQR)	*Overall p-value**
%CD4 activation (CD4+HLA-DR+CD38+)	0.63 (0.39–0.87)	0.58 (0.45–1.06)	0.78 (0.46–1.33)	0.234
%CD8 activation (CD8+HLA-DR+CD38+)	0.68 (0.47–1.44)	0.87 (0.51–1.32)	1.26 (0.86–2.2)	**0.0003**
%B activated memory (CD19+CD10−CD21−CD27+)	3.04 (1.51–5.17)	3.86 (2.48–5.83)	8.06 (4.89–12.63)	**<0.0001**
%CD4 senescence (CD4+CD28−CD57+)	0.56 (0.28–2.7)	0.64 (0.41–2.33)	2.14 (0.89–6.17)	**<0.0001**
%CD8 senescence (CD8+CD28−CD57+)	3.85 (1.77–7.75)	8.44 (4.06–13.54)	13.04 (10.44–20.38)	**<0.0001**
%B senescence (CD19+IgD−CD27−)	7.83 (5.04–15.42)	15.27 (8.73–16.89)	16.13 (11.8–21.6)	**<0.0001**
%CD4 exhaustion (CD4+PD-1+)	7.83 (3.43–16.17)	10.27 (5.61–15.17)	12.63 (6.7–20.57)	0.112
%CD8 exhaustion (CD8+PD-1+)	8.85 (5.82–13.28)	9.73 (6.63–16.56)	13.67 (8.63–20.98)	**0.014**
%T-regs (CD4+CD25+CD127−FoxP3+)	8.29 (4.66-10.79)	4.51 (2.66-7.22)	1.42 (0.71-3.3)	**<0.0001**
%B-regs (CD19+CD24hiCD38hi)	6.70 (4.79-8.43)	4.83 (3.52-6.76)	2.11 (1.06-3.14)	**<0.0001**
logPRNT	5 (4-6)	4 (3-5.5)	3 (3-4)	**<0.0001**

**Kruskal–Wallis test.*
Bold values refer to statistically significant p-values.

Figure 1. Levels and relationship between immune activation and Tregs and Bregs with PRNT at first sampling Levels of PRNT (A), activated CD4 (B), activated CD8 (C) and activated memory B cells (D), Tregs (E), and Bregs (F) in COVID-19 subjects among age classes and the relationship of these immunological markers with PRNT values at 2.8 (2.1–3.7) months after baseline.



The entire COVID-19 group had a greater expression of Tregs and Bregs cells than the entire non-COVID-19 group (Supplementary Table S1), and this was confirmed when analysis was performed in age subgroup (Supplementary Table S2). Notably, within the COVID-19 subjects, children <6 years had a higher expression of Tregs and Bregs when compared with COVID-19 children 6–15 years and adults (Table 2).

PRNT and Relationship With Immune Profile Among COVID-19 Subjects at First Sampling.

COVID-19 children <6 years had the highest titer of nAbs [5(4–6) vs. 4(3–6) vs. 3(3–4) logPRNT, overall, $p < 0.0001$] (Figure 1A) (17).

Overall, immune activation inversely correlated with PRNT titer (%CD4+HLA-DR+CD38+: $r = -0.201$, $p = 0.013$; %CD8+HLA-DR+CD38+: $r = -0.563$, $p < 0.0001$; %CD19+CD10−CD21−CD27+: $r = -0.636$, $p < 0.0001$)

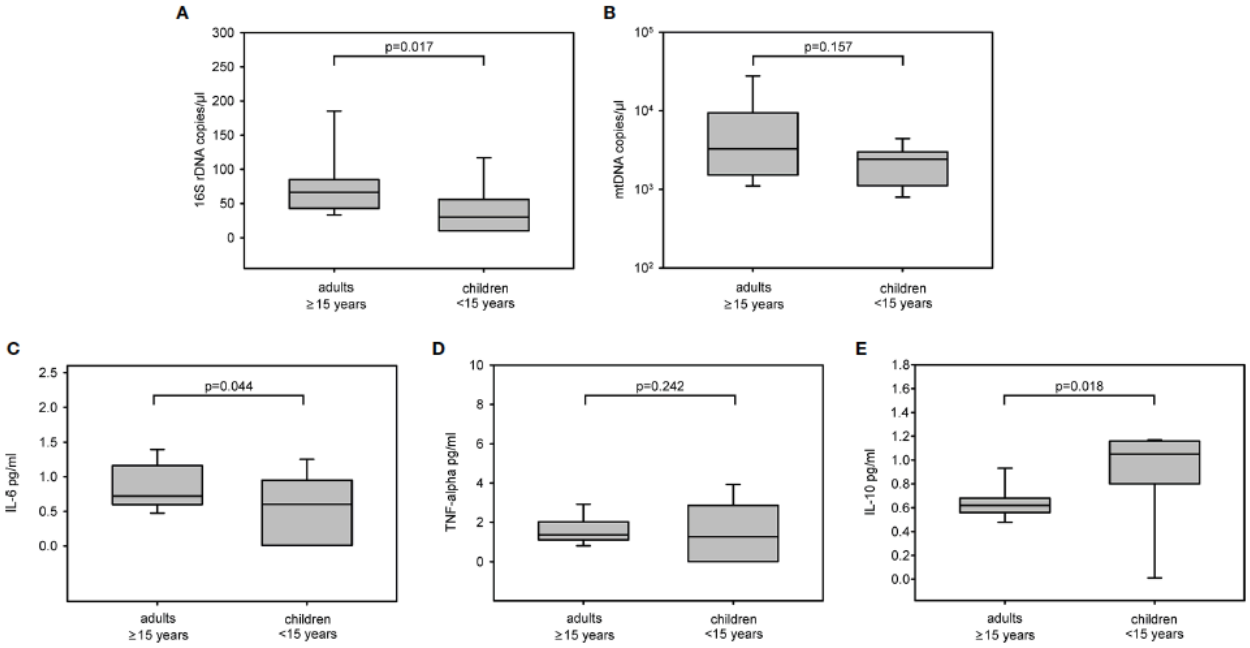
(Supplementary Table S3). This inverse relationship was confirmed in all age groups, and adults had the greatest negative association (Figures 1B–D). In addition, senescent CD4, CD8, and B cells were inversely associated with PRNT titer (%CD4+CD28−CD57+: r = −0.097, p = 0.237; %CD8+CD28−CD57+: r = −0.220, p = 0.006; %CD19+IgD−CD27− r = −0.309, p = 0.0001). Stratifying by age, in adults, PRNT inversely correlated with B-cell senescence (r = −0.222, p = 0.032), while in children 6–15 years, a mild negative correlation was found between CD4 and CD8 senescent cells and PRNT titer (%CD4+CD28−CD57+: r = −0.325, p = 0.069; %CD8+CD28−CD57+: r = -0.353, p = 0.047) (Supplementary Table S3). No correlation was found between CD4 and CD8 exhausted cells and PRNT (%CD4+PD−1+: r = 0.035, p = 0.669; %CD8+PD-1+: r = −0.025, p = 0.756). However, in adults, there was a positive relationship between PRNT and expression of PD-1 in CD4 and CD8 (%CD4+PD-1+: r = 0.256, p = 0.013; %CD8+PD-1+: r = 0.209, p = 0.045), no relationship was found in children (Supplementary Table S3).

Overall, Tregs and Bregs cells positively correlated with PRNT titer (%CD4+CD25+CD127−FoxP3+: r = 0.488, p < 0.0001, %CD19+CD24hiCD38hi: r = 0.548, p < 0.0001). Notably, this positive correlation was confirmed in children (Figures 1E, F), while in adults, there was no correlation between Tregs and log PRNT (Figures 1E, F).

Circulating Markers of Inflammation: PAMPs, DAMPs, and Cytokines at First Sampling.

In a subgroup of 49 available plasma samples from COVID-19 patients, collected at a median of 2.8 (2.1–3.7) months after baseline, circulating levels of PAMPs (16S rDNA), DAMPs (mtDNA), and cytokines (IL-6, TNF-α, and IL-10) were quantified. Given the low number of samples, the cohort was divided into adults (≥15 years, n = 29) and children (<15 years, n = 20). Adults had higher plasma levels of 16S rDNA [66 (43–86) vs. 32 (10–67) copies/µl, p = 0.017] and mtDNA [3,289 (1,451–10,497) vs. 2,553 (1,118–3,703) copies/µl, p = 0.157] than children (Figures 2A, B). IL-6 was significantly higher in adults than children [0.8 (0.7–1.2) vs. 0.7 (0.1–1.0) pg/ml, p = 0.044], and TNF-α tended to be higher in adults, but not significantly [1.4 (1.1–2.4) vs. 1.2 (0.01–2.4) pg/ml, p = 0.242]. Conversely, IL-10 was higher in children compared to adults [1.1 (0.8–1.2) vs. [0.6 (0.5–0.7) pg/ml, p = 0.018) (Figures 2C–E).

Figure 2. Circulating markers of PAMPs, DAMPs, and inflammatory cytokines in COVID-19 subjects at first sampling. 16S rDNA (A), mtDNA (B), IL-6 (C), TNF-a
(D) and IL-10 (E) levels in COVID-19 patients among age classes at 2.8 (2.1–3.7) months after baseline.



Viral Load and Relationship With Immune Profile.

SARS-CoV-2 viral load (VL) was measured by ddPCR on available NPS. No significant differences were found among the three age groups [185,067 (326–339,315) SARS-CoV-2 copies/5 µl in adults vs. 6,723 (3,427–114,587) children 6–15 years old vs. 21,106 (162–152,500) in children <6 years old, respectively, overall, p = 0.955). The relationship among VL and immunological parameters was evaluated by stratifying the cohort into two groups: children <15 years and adults ≥15 years. Overall, only a weak negative relationship was found between VL and activated CD4 (r = −0.272, p = 0.085) and B cells (r = −0.267, p = 0.092) (Table 3), and in children, VL tended to be inversely correlated with B activated memory cells (r= -0.463, p=0.053) and positively correlated with Bregs (r = 0.437, p = 0.070) (Table 3).

Table 3. Relationship between viral load and immunological parameters at first sampling.

Immunological parameters	All 41 NPS		Children aged <15 years		Adults aged ≥15 years	
	R spearman	p-value[§]	R spearman	p-value[§]	R spearman	p-value[§]
logPRNT	0.118	0.463	**0.523**	**0.026**	0.035	0.875
%CD4 activation (CD4+HLA-DR+CD38+)	**–0.272**	**0.085**	–0.375	0.126	–0.182	0.406
%CD8 activation (CD8+HLA-DR+CD38+)	–0.083	0.607	–0.307	0.216	0.059	0.788
%B activated memory (CD19+CD10–CD21–CD27+)	**–0.267**	**0.092**	**–0.463**	**0.053**	–0.202	0.356
%CD4 senescence (CD4+CD28–CD57+)	0.077	0.638	0.197	0.433	–0.023	0.919
%CD8 senescence (CD8+CD28–CD57+)	0.159	0.321	–0.141	0.576	0.334	0.119
%B senescence (CD19+IgD–CD27–)	–0.173	0.279	–0.358	0.145	–0.197	0.369
%CD4 exhaustion (CD4+PD-1+)	–0.016	0.919	–0.216	0.390	0.076	0.730
%CD8 exhaustion (CD8+PD-1+)	0.159	0.321	–0.222	0.376	0.289	0.180
%T-regs (CD4+CD25+CD127–FoxP3+)	0.087	0.589	0.271	0.276	0.026	0.906
%B-regs (CD19+CD24hiCD38hi)	–0.053	0.744	**0.437**	**0.070**	–0.154	0.483

[§]*Spearman correlation.*
Bold values refer to R spearman and p-values that are or tended to be significant.

Differences of Immunological Profile and Relationship With PRNT at Second Sampling.

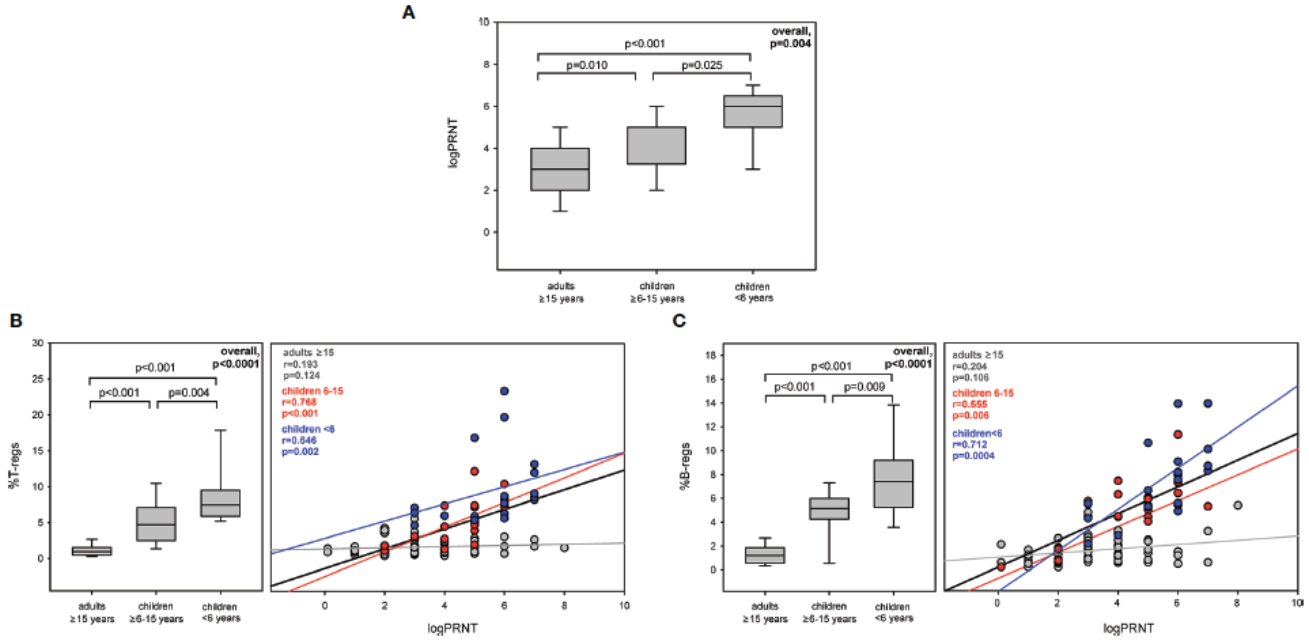
For a total of 116 subjects, a second peripheral blood sample was obtained after a median of 6.1 (5.3–7.2) months from baseline. No significant difference was seen in levels of nAbs from first and second samples in children, while they significantly decreased in adults [4 (3–4) vs. 3 (2–4) log PRNT, p = 0.004] (Table 4 and Figure 3A). Adults still maintained a higher level of immune activation (Table 4), which continued to be significantly higher than those observed in children [%CD4+HLA-DR+CD38+: 0.9 (0.6–1.1) vs. 0.6 (0.4–1) vs. 0.6 (0.5–0.8), overall p = 0.132; %CD8+HLA-DR+CD38+: 1.7 (0.9–2.7) vs. 0.8 (0.6–1.3) vs. 0.7 (0.5–1.1), overall p < 0.0001; %CD19+CD10−CD21−CD27+: 9.1 (4.7–11.8) vs. 3.3 (2.3–4.8) vs. 4.2 (2.5–5.7), overall p < 0.0001]. Percentage of CD4 senescent cells increased in all age classes (Table 4). Notably, Tregs and Bregs significantly decreased in adults (Table 4), while their percentages did not change in children, and children <6 years maintained the highest expression [%CD4+CD25+CD127−FoxP3+: 7.9 (6.1–10) vs. 4.7 (2.4–7.1) vs. 1.0 (0.5–1.5), overall p < 0.0001; %CD19+CD24hiCD38hi: 7.4 (5.3–8.9) vs. 5.2 (4.3–5.9) vs. 1.2 (0.6–1.9), overall p < 0.0001]. Moreover, 6 months after infection, the positive association persisted in children between PRNT titer and Tregs (r = 0.646, p = 0.002 in children aged <6 years; r = 0.768, p < 0.0001 in children aged 6–15 years) and between PRNT titer and Bregs (r = 0.712, p = 0.0004 in children aged <6 years; and r = 0.555, p = 0.006 in children aged 6–15 years), while no relationship was found in adults (Figures 3B, C).

Table 4. Differences of immunological parameters between first and second sampling.

	Children < 6 years) (n = 21)			Children 6–15 years (n = 26)			Adult > 15 years (n = 69)		
	First sample	Second sample	p-value[§]	First sample	Second sample	p-value[§]	First sample	Second sample	p-value[§]
Median (IQR) months	2.4 (1.9–2.8)	5.6 (4.5–5.9)		3.3 (2.6–3.8)	6.6 (5.9–7.5)		2.8 (2.1–3.6)	6.1 (5.4–7.3)	
logPRNT	5 (4–6)	6 (5–6.5)	0.521	5 (4–6)	5 (3–5)	0.672	4 (3–4)	3 (2–4)	**0.004**
%CD4 activation (CD4+HLA-DR+CD38+)	0.6 (0.5–0.8)	0.6 (0.5–0.8)	0.533	0.6 (0.5–1.1)	0.6 (0.4–1)	0.855	0.8 (0.4–1.3)	0.9 (0.6–1.1)	0.885
%CD8 activation (CD8+HLA-DR+CD38+)	0.7 (0.5–1.4)	0.7 (0.5–1.1)	0.750	0.8 (0.5–1.2)	0.8 (0.6–1.3)	0.618	1.2 (0.9–1.9)	1.7 (0.9–2.7)	0.058
%B activated memory (CD19+CD10–CD21–CD27+)	2.8 (1.5–4.8)	4.2 (2.5–5.7)	0.079	3.3 (2.3–4.5)	3.3 (2.3–4.8)	0.592	7.1 (4.1–11.7)	9.1 (4.7–11.8)	0.126
%CD4 senescence (CD4+CD28–CD57+)	0.6 (0.4–3.1)	3 (1.7–4.8)	**0.047**	0.8 (0.5–2.7)	6.5 (3.1–10)	**<0.0001**	2.5 (0.9–8.8)	6.3 (3.2–9.6)	**0.026**
%CD8 senescence (CD8+CD28–CD57+)	3.9 (2.0–7.8)	4.7 (2.7–7.9)	0.418	8.3 (4– 13)	8.4 (6.2–13.5)	0.956	13.8 (11–21.5)	14.7 (10.5–22.4)	0.484
%B senescence (CD19+IgD–CD27–)	10.9 (6.1–15.6)	7.8 (2.4–10.7)	0.051	14.9 (9–16.2)	13.4 (10.2–17.1)	0.303	16 (12.5–20.1)	16.5 (11.8–21.8)	0.868
%CD4 exhaustion (CD4+PD-1+)	7.7 (3.3–16.2)	7.3 (4.4–13.7)	0.927	9.2 (5.2–15.4)	10 (4.2–15.8)	0.825	13 (8.3–21.9)	11.5 (7.3–18.6)	0.430
%CD8 exhaustion (CD8+PD-1+)	9.5 (5.9–12.3)	7.8 (4.7–12.4)	0.870	9.6 (6.2–16.5)	9.9 (6.7–15)	0.719	14.2 (9.5–22)	12.2 (7.2–19.5)	0.213
%T-regs (CD4+CD25+CD127-FoxP3+)	8.3 (4.7–10)	7.9 (6.1–10)	0.498	4.5 (2.9–7)	4.7 (2.4–7.1)	0.658	1.4 (0.7–3.4)	1.0 (0.5–1.5)	**<0.0001**
%B-regs (CD19+CD24hiCD38hi)	6.1 (4.6-8.8)	7.4 (5.3–8.9)	0.468	5 (4–6.9)	5.2 (4.3–5.9)	0.310	2.1 (1.2–3)	1.2 (0.6–1.9)	**<0.0001**

[§]Wilcoxon signed-rank test.
Bold values refer to statistically significant p-values.

Figure 3. Levels and relationship between Tregs and Bregs with PRNT at second sampling. Levels of PRNT (A), Tregs (B), and Bregs (C) in COVID-19 subjects among age classes and the relationship of these immunological markers with PRNT values at 6.1 (5.3–7.2) months after baseline.



Discussion.

Most of the studies conducted in SARS-CoV-2-infected adults confirm the pivotal relevance of immune activation and cytokine storm in dictating the clinical outcome of the infection. Most of the SARS-CoV-2-infected children are asymptomatic or very mildly symptomatic, but the immunopathogenesis of COVID-19 is still poorly investigated in the pediatric population. In the present study, for the first time, we had the opportunity to study the immune profile of SARS-CoV-2-infected adults and children, clustered within the same families, and compared to uninfected age-class matched relatives.

Immunological response to SARS-CoV-2 has been widely studied in the adult population; several studies reported that COVID-19-infected patients expressed higher percentages of activated cells and exhausted cells compared to healthy controls (11, 20). Additionally, T-cell activation and exhaustion appears to correlate with disease severity in COVID-19 patients (9, 10, 21), and the immune activation persists despite viral clearance (12, 21). Our data confirmed a higher immune activation/exhaustion in asymptomatic/mildly symptomatic COVID-19 compared to non-COVID-19 adults, and the activation still persisted after 6 months from infection.

Higher levels of activated CD4 and CD8 T cells were described in COVID-19 pediatric patients with MIS-C (22–24). COVID-19 children without or with mild/moderate clinical manifestations showed similar frequencies of activated CD4 and CD8 cells compared to age-matched control (15, 22, 25). In agreement with these findings, the present study found no differences between COVID-19 and non-COVID-19 children, and for the first time, we demonstrated that COVID-19 adults, mostly asymptomatic/mildly symptomatic, have a higher expression of both activated T and B cells, not only compared to non-COVID-19 adults but also compared to COVID-19 children.

The immune activation is a major driver of immune senescence (26, 27); the continuous cell activation and expansion of immune cells leads to their senescent status with the loss of their function (28). Consistently with this concept, COVID-19 adults had significantly higher levels of senescent T and B cells than non-COVID-19 adults. It is well known that the release of exogenous pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) into circulation through binding Toll-like receptors (TLR-9 or TLR-4) is an important driver of cytokine storm, inflammation, and immune activation (29). The significantly higher levels of PAMPs, DAMPs, and IL-6 proinflammatory cytokine in COVID-19 adults compared to COVID-19 children may contribute to explaining the higher levels of immune activation and senescence in the former. The only exception was the IL-10. Notably, activated and senescent T and B cells inversely correlated with a production of anti-SARS-CoV-2 nAbs, thus suggesting that after infection in adults, immune activation exerts a strong influence on immune aging and drains resources from the immune system for the specific production of anti-SARS-CoV-2 antibodies.

In agreement with several studies indicating that children and adults do not differ for viral load (5–7), in this study, which was conducted in asymptomatic or mild symptomatic COVID-19 patients, levels of SARS-CoV-2 at baseline did not differ among age classes. Nonetheless, the impact of viral load on adults may differ from its effect on children. It is widely reported that viral load is higher in symptomatic than asymptomatic COVID-19 adults (30). The data available in the pediatric population are controversial. Some studies found higher levels in symptomatic children compared to asymptomatic children (25, 31), while other reports found no association of viral load and disease severity (5, 32). A recent study, conducted in COVID-19 children within their first week from baseline (symptom onset and/or first positive virus detection) demonstrated an inverse relationship between viral load and nAbs, and the estimation of virus under curve from NPS, collected every 48 h up to undetectable viral load, confirmed the impact of nAbs on virus clearance (15). Our data suggested that this relationship did not persist after viral clearance.

Regulatory T cells play a crucial role in suppressing excessive immune responses to pathogens, cancer cells, and transplanted organs and in preventing and controlling the development of autoimmune and allergic diseases (33). Regulatory B cells had a negative role in immune reaction and inflammation in humans (34). Data on Tregs and COVID-19 are conflictual: COVID-19 adult patients expressed higher percentages of Tregs compared to uninfected ones (11), but decreased numbers of circulating Tregs have been described in severe COVID-19 cases (35, 36). The reduced proportion of SARS-CoV-2-reactive regulatory T cells observed in hospitalized COVID-19 patients, compared to non-hospitalized ones, suggested that a defect in the generation of immunosuppressive SARS-CoV-2-reactive Tregs was associated with a severe clinical outcome (37). No data are available for Bregs to date. In our study, both Tregs and Bregs were significantly higher in asymptomatic/mildly symptomatic COVID-19 patients compared to non-COVID-19 subjects in all age classes. Interestingly, COVID-19 children, particularly those <6 years, had higher expression of Tregs and Bregs than COVID-19 adults, and notably, this is positively associated with production of nAbs. Tregs inhibit the activation of both innate and adaptive immune response via inhibitory surface molecules (like CTLA-4 and LAG-3) and by the secretion of immunosuppressive cytokines (i.e., IL-10, TGF-β, and IL-35) (38, 39). It has been recently reported that slow-progressors HIV-infected children secreted higher levels of IL-10 compared to those who progressed and had higher proliferation of Tregs (40). Similarly, it is possible that Tregs and Bregs in SARS-CoV-2-infected children constrains inflammation/immune activation, likely through the release of IL-10. Indeed, a significant positive association was found between IL-10 and Tregs in children ($r = 0.633$, $p = 0.011$). Interestingly, in children, and in particular in children <6 years of age, high levels of Tregs and Bregs cells persisted for over 6 months of follow-up, and the titer of nAbs, thus supporting the concept that these cells play a role in directing the host immune response.

A limitation of this study is that it includes only asymptomatic/mildly symptomatic COVID-19 children and adults. Nonetheless, our data demonstrated that even in the absence of severe disease, COVID-19 adults showed a higher

degree of hyperinflammation/immune activation than COVID-19 children, although levels of SARS-CoV-2 did not differ among classes. The immune activation, with higher release of PAMPs and DAMPs into circulation, leading to the overproduction of proinflammatory cytokine IL-6, might limit the production of anti-SARS-COV-2-neutralizing antibodies and impair the specific response in adults. Conversely, in COVID-19 children, the viral-induced inflammation may be mitigated by the higher expansion of regulatory T and B cells resulting in preserved resources for a higher specific production of anti-SARS-CoV-2-neutralizing antibodies. Further studies are needed to support the role of regulatory cells in this context.

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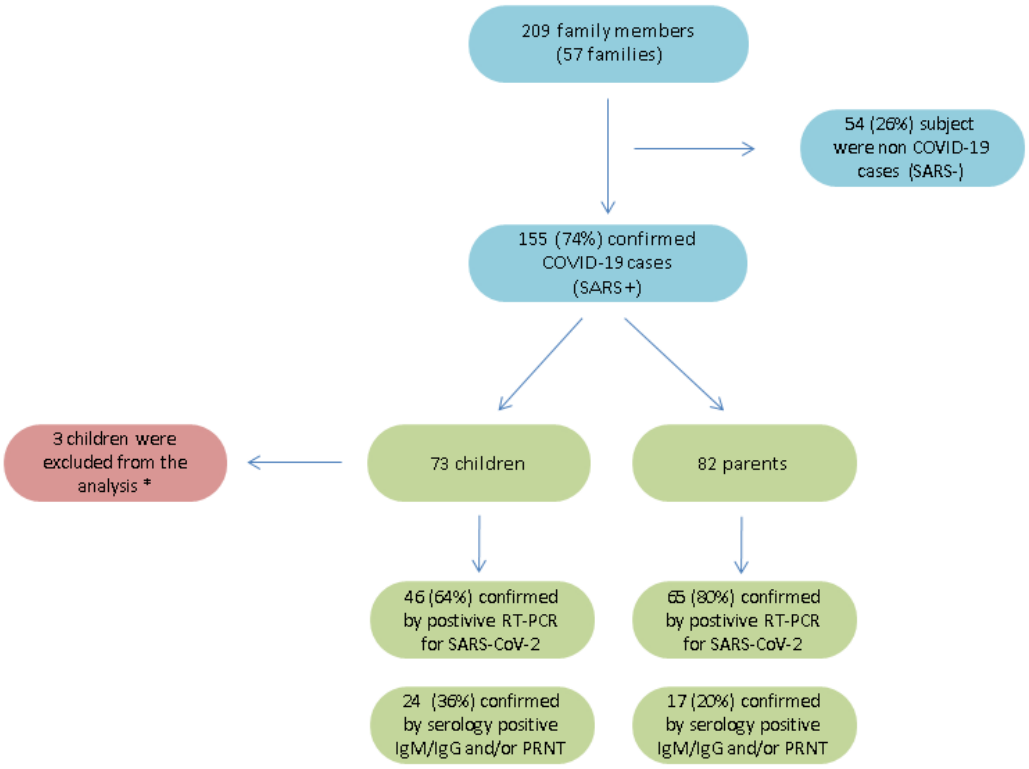
Supplementary materials.

Supplementary Figure S1. Flow chart of family clusters of COVID-19 enrolled from March 1st to the September 4th 2020, at the COVID-19 follow-up clinic of the Pediatric Department, Department of Women's and Children's Health, University of Padua.



*Three children were excluded from the analysis: 2 children presented MIS-C, Multisystem Inflammatory Syndrome in Children, 4-6 weeks after COVID-19 onset and 1 newborn of a COVID-19 positive mother presented positive SARS-CoV-2 neutralizing antibodies detected 51 days after birth that could be related to maternal immunity and not seroconversion (SARS-CoV-2 molecular assay was never performed at birth).

Supplementary Table S1. Immunological characteristics of the overall cohort of SARS-CoV-2-infected cases at first sampling.

Immunological markers	**non COVID-19** (N=54) Median [IQR]	**COVID-19** (N=152) Median	*p-value*[§]	Linear regression model*	
				β	*p-value*[**]
%CD4 activation (CD4+HLA-DR+CD38+)	0.41 [0.26-0.70]	0.70 [0.45-1.12]	**<0.0001**	0.45	**0.002**
%CD8 activation (CD8+HLA-DR+CD38+)	0.82 [0.56-1.08]	1.09 [0.68-1.82]	**0.001**	0.25	0.084
%B activated memory (CD19+CD10-CD21-CD27+)	2.16 [1.30-3.23]	5.61 [3.15-9.93]	**<0.0001**	1.03	**<0.0001**
%CD4 senescence (CD4+CD28-CD57+)	0.99 [0.51-2.96]	1.55 [0.51-4.16]	0.211	0.27	0.217
%CD8 senescence (CD8+CD28-CD57+)	9.64 [6.50-13.44]	11.16 [5.79-17.86]	0.356	0.00	0.904
%B senescence (CD19-IgD-CD27-)	9.00 [6.20-14.51]	15.24 [9.05-19.52]	**<.0001**	0.50	**0.001**
%CD4 exhaustion (CD4+PD-1+)	7.58 [4.80-11.65]	11.75 [6.13-18.02]	**0.024**	0.21	0.083
%CD8 exhaustion (CD8+PD-1+)	8.76 [5.10-13.46]	12.66 [6.96-19.21]	**0.013**	0.27	**0.018**
%T-regs (CD4+CD25+CD127-FoxP3+)	0.73 [0.39-1.99]	2.75 [1.01-5.65]	**<0.0001**	1.16	**<0.0001**
%B-regs (CD19+CD24hiCD38hi)	1.69 [0.90-2.70]	3.11 [1.69-5.75]	**<0.0001**	0.71	**<0.0001**

*adjusted by age
[§]Wilcoxon rank sum test
**t-test

Supplementary Table S2. Immunological parameters in COVID-19 and non-COVID-19 age classes at first sampling.

	children < 6 years			children 6-15 years			adult ≥ 15 years		
	non COVID-19 (N=15) Median [IQR]	**COVID-19** (N=27) Median [IQR]	**p-value**[§]	**non COVID-19** (N=12) Median [IQR]	**COVID-19** (N=28) Median [IQR]	**p-value**[§]	**non COVID-19** (N=27) Median [IQR]	**COVID-19** (N=93) Median [IQR]	**p-value**[§]
logPRNT	0.1 [0.1-0.1]	5 [4-6]	**<0.0001**	0.1 [0.1-0.1]	4 [3-5.5]	**<0.0001**	0.1 [0.1-0.1]	3 [3-4]	**<0.0001**
%CD4 activation (CD4+HLA-DR+CD38+)	0.36 [0.32-0.52]	0.63 [0.39-0.87]	0.083	0.53 [0.35-0.87]	0.58 [0.45-1.06]	0.437	0.41 [0.17-0.7]	0.78 [0.46-1.33]	**0.001**
%CD8 activation (CD8+HLA-DR+CD38+)	0.99 [0.72-1.17]	0.68 [0.47-1.44]	0.401	1.03 [0.72-1.78]	0.87 [0.51-1.32]	0.399	0.66 [0.38-0.85]	1.26 [0.86-2.2]	**<0.0001**
%B activated memory (CD19+CD10-CD21-CD27+)	1.74 [1.29-2.92]	3.04 [1.51-5.17]	0.052	2.34 [1.17-3.25]	3.86 [2.48-5.83]	**0.020**	2.3 [1.3-3.25]	8.06 [4.89-12.63]	**<0.0001**
%CD4 senescence (CD4+CD28-CD57+)	0.77 [0.17-2.48]	0.56 [0.28-2.7]	0.823	1.01 [0.7-2.27]	0.64 [0.41-2.33]	0.589	1.3 [0.68-4.38]	2.14 [0.89-6.17]	0.169
%CD8 senescence (CD8+CD28-CD57+)	6.65 [4.05-9.46]	3.85 [1.77-7.75]	0.068	13.39 [9.18-18.39]	8.44 [4.06-13.54]	0.123	10.83 [8.05-13.44]	13.04 [10.44-20.38]	**0.030**
%B senescence (CD19+IgD-CD27-)	7.08 [5.24-9.35]	7.83 [5.04-15.42]	0.294	10.49 [4.07-15.85]	15.27 [8.73-16.89]	0.159	11.76 [7.08-17.24]	16.13 [11.8-21.6]	**0.002**
%CD4 exhaustion (CD4+PD-1+)	6.03 [3.32-9.32]	7.83 [3.43-16.17]	0.096	10.55 [6.84-15.92]	10.27 [5.61-15.17]	0.702	7.97 [4.65-14.48]	12.63 [6.7-20.57]	0.093
%CD8 exhaustion (CD8+PD-1+)	4.65 [3.51-10.59]	8.85 [5.82-13.28]	0.088	11.09 [8.07-15.28]	9.73 [6.63-16.56]	0.802	9.23 [6.5-17.34]	13.67 [8.63-20.98]	**0.039**
%T-regs (CD4+CD25+CD127-FoxP3+)	1.21 [0.96-3.03]	8.29 [4.66-10.79]	**<0.0001**	0.7 [0.36-1.55]	4.51 [2.66-7.22]	**<0.0001**	0.63 [0.31-1.17]	1.42 [0.71-3.3]	**0.0003**
%B-regs (CD19+CD24hiCD38hi)	1.98 [0.81-2.86]	6.7 [4.79-8.43]	**<0.0001**	2.06 [1.18-3.07]	4.83 [3.52-6.76]	**<0.0001**	1.29 [0.89-2.16]	2.11 [1.06-3.14]	**0.045**

[§] Wilcoxon rank sum test

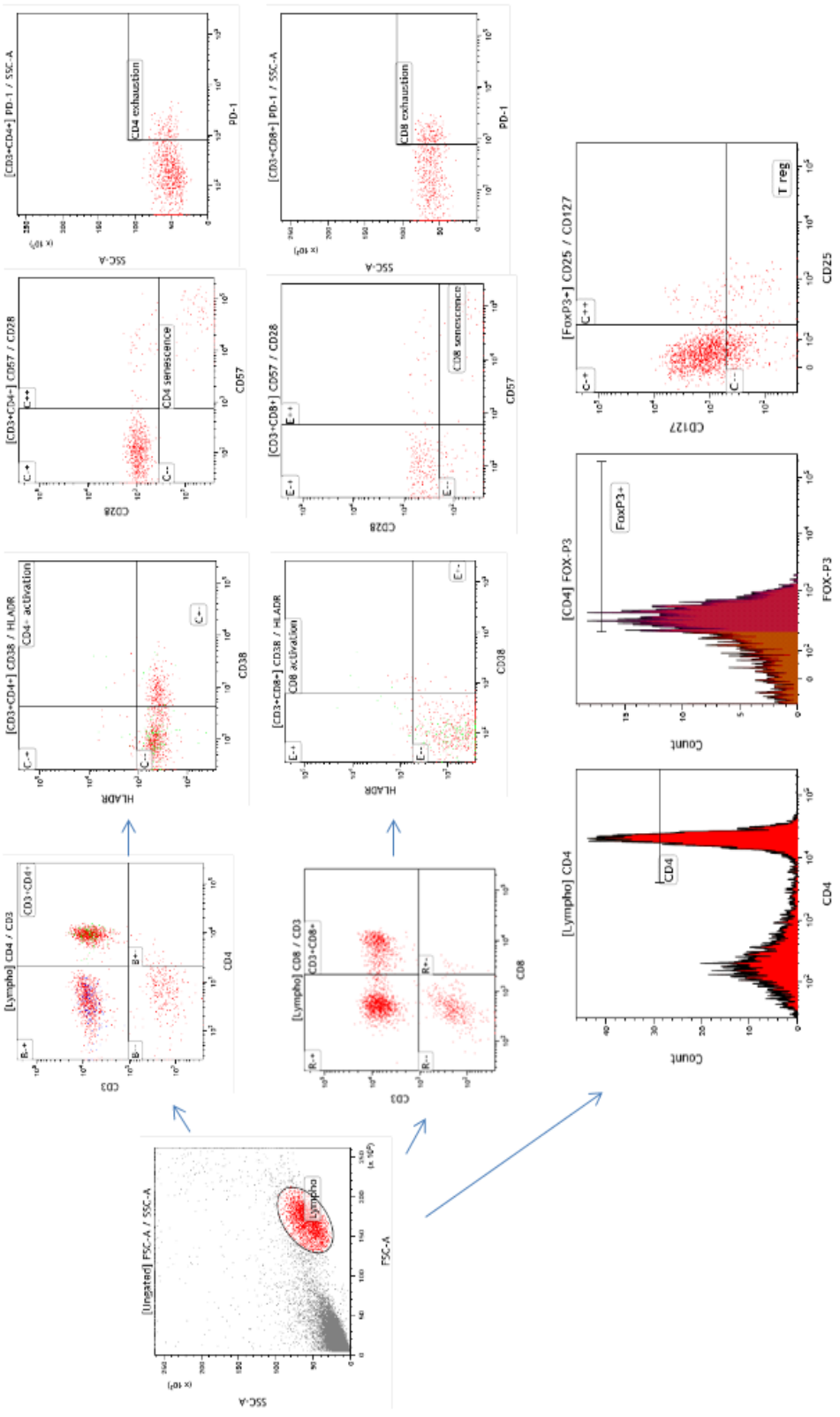
Supplementary Table S3. Relationship between PRNT values and immunological parameters at first sampling.

	logPRNT							
Immunological markers	**overall, n=152**		**children <6 years n=27**		**children 6-15 years n=28**		**adults >15 years n=93**	
	R spearman	p-value§	R spearman	p-value§	R spearman	p-value§	R spearman	p-value§
%CD4 activation (CD4+HLA-DR+CD38+)	-0.201	0.013	-0.117	0.562	-0.005	0.980	**-0.264**	**0.011**
%CD8 activation (CD8+HLA-DR+CD38+)	**-0.563**	**<.0001**	**-0.546**	**0.003**	**-0.519**	**0.002**	**-0.496**	**<.0001**
%B activated memory (CD19+CD10-CD21-CD27+)	**-0.636**	**<.0001**	**-0.425**	**0.027**	**-0.581**	**0.001**	**-0.568**	**<.0001**
%CD4 senescence (CD4+CD28-CD57+)	-0.097	0.237	0.099	0.625	-0.325	0.069	**0.209**	**0.046**
%CD8 senescence (CD8+CD28-CD57+)	**-0.220**	**0.006**	0.020	0.922	**-0.353**	**0.047**	0.161	0.122
%B senescence (CD19-IgD-CD27-)	**-0.309**	**0.0001**	-0.363	0.063	-0.116	0.526	**-0.222**	**0.032**
%CD4 exhaustion (CD4+PD-1+)	0.035	0.669	-0,0116	0.954	-0.125	0.494	**0.256**	**0.013**
%CD8 exhaustion (CD8+PD-1+)	-0.025	0.756	-0,12573	0.532	-0.042	0.827	**0.209**	**0.045**
%T-regs (CD4+CD25+CD127-FoxP3+)	**0.488**	**<0.0001**	**0.653**	**0.0002**	**0.505**	**0.003**	0.180	0.084
%B-regs (CD19+CD24hiCD38hi)	**0.548**	**<0.0001**	**0.616**	**0.001**	**0.363**	**0.041**	**0.381**	**0.0002**

§Spearman correlation

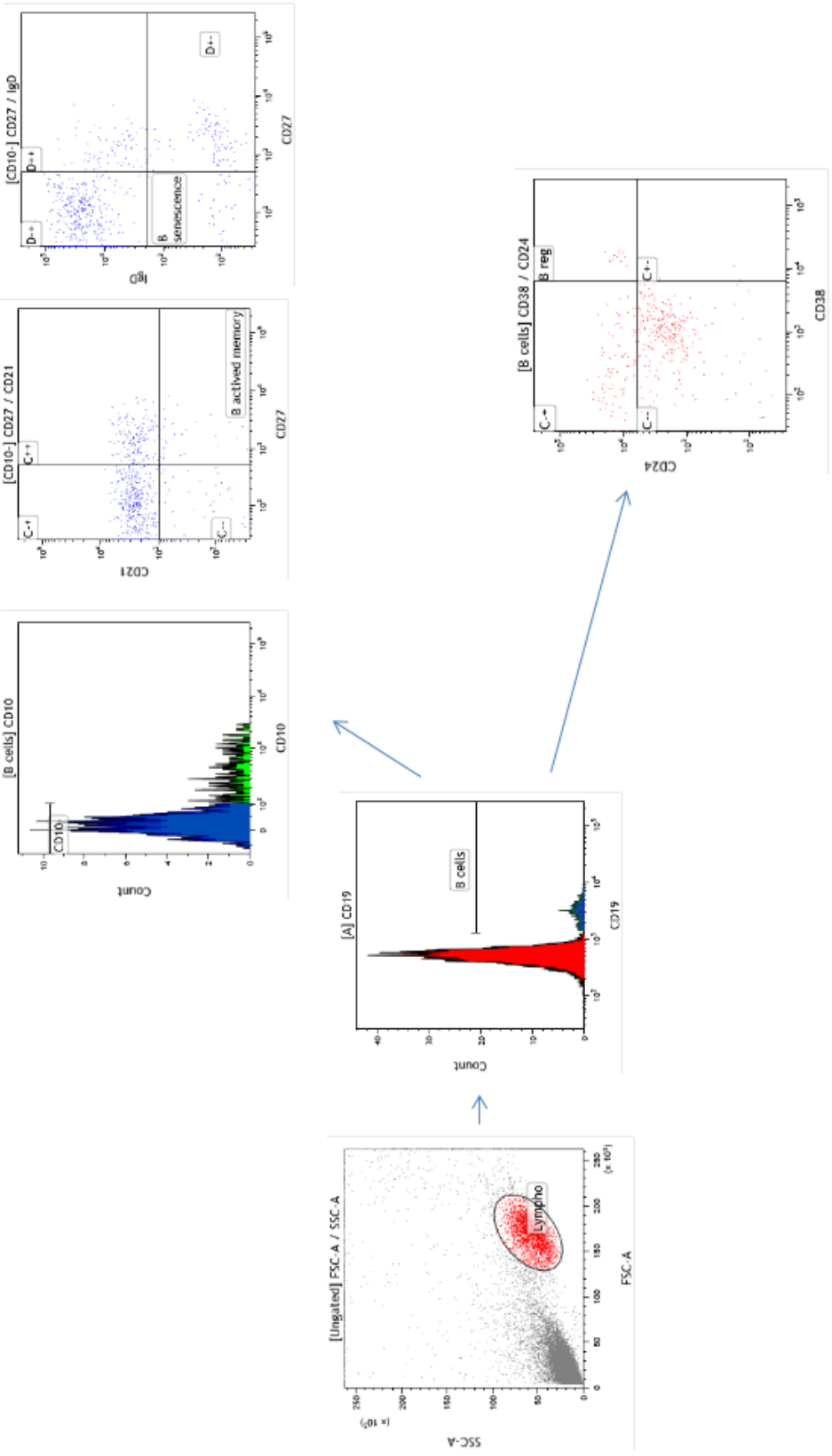
Supplementary Figure S2. Gating strategy. A) Flow cytometry gating strategy for CD4+ and CD8+ T cell activation (HLA-DR+CD38+), senescence (CD28-CD57+), exhaustion (PD-1+) and Tregs (CD4+CD25+CD127-FoxP3+).

A)



Supplementary Figure S2. Gating strategy. (B) Flow cytometry gating strategy for B cell activation (CD19+CD10-CD27+CD21-), senescence (CD19+IgD-CD27-) and Bregs(CD19+CD24hiCD38hi)**.**

B)



4.3. Analytical and clinical performances of a SARS-CoV-2 S-RBD IgG assay: comparison with neutralization titers

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Abstract.

Objectives. SARS-CoV-2 serology presents an important role in several aspects of COVID-19 pandemic. Immunoassays performances have to be accurately evaluated and correlated with neutralizing antibodies. We investigated the analytical and clinical performances of a SARS-CoV-2 RBD IgG assay, automated on a high throughput platform, and the correlation of the antibodies (Ab) levels with the plaque reduction neutralization (PRNT50) Ab titers.

Methods. A series of 546 samples were evaluated by SARS-CoV-2 RBD IgG assay (Snibe diagnostics), including 171 negative and 168 positive SARS-CoV-2 subjects and a further group of 207 subjects of the COVID-19 family clusters follow-up cohort.

Results. Assay imprecision ranged from 3.98 to 12.18% being satisfactory at low and medium levels; linearity was excellent in all the measurement range. Considering specimens collected after 14 days post symptoms onset, overall sensitivity and specificity were 99.0 and 92.5%, respectively. A total of 281 leftover samples results of the PRNT50 test were available. An elevated correlation was obtained between the SARS-CoV-2 RBD IgG assay and the PRNT50 titer at univariate (ρ=0.689) and multivariate (ρ=0.712) analyses.

Conclusions. SARS-CoV-2 S-RBD IgG assay shows satisfactory analytical and clinical performances, and a strong correlation with sera neutralizing activity.

Keywords. SARS-CoV-2; antibodies; clinical performances; immunoassays; neutralization; plaque reduction neutralization test; serology.

Introduction.

Current testing for SARS-CoV-2 largely depends on labor-intensive molecular techniques, particularly reverse transcription real-time polymerase chain reaction (rRT-PCR), but a body of evidence highlights that individuals with positive molecular tests represent only a small fraction of all infections [1], [2].
Serological assays for the accurate measurement of SARS-CoV-2 antibodies (Abs) are suboptimal tools for the early diagnosis of infection but provide important population-based data on pathogen exposure, on the prevalence of infection, also in asymptomatic subjects, and on the selection of convalescent plasma donors. Furthermore, SARS-CoV-2 serology represents a complementary tool of molecular virological assays to achieve a more accurate diagnosis in some "difficult" patients, for tracking transmission dynamics, gaining knowledge on population immunity levels and informing disease control policies [3]. In addition, serology plays a central role in clinical trials on vaccine development to provide evidence of potency and efficacy [4], [5] and in supporting decisions on population groups who should be prioritized in vaccine administration [6].
Many assays have been developed for SARS-CoV-2 Ab detection, including lateral flow tests, enzyme-linked immunosorbent assays (ELISA), chemiluminescent (CLIA) assays and other platforms (https://www.fda.gov/medical-devices/emergency-use-authorizations-medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices); some rely on whole inactivated virions, while others adopted viral subunits such as the nucleocapsid protein, or viral spike protein; however, key issues such as the correlation between circulating antibodies and their neutralizing ability and persistence over time have not been adequately addressed, yet. More recently, a body of evidence has been collected to demonstrate that the recombinant SARS-CoV-2 receptor binding domain (RBD) is a highly sensitive and specific antigen for the detection of antibodies induced by SARS-CoV-2 and that the levels of RBD-binding antibodies present a strong correlation with neutralizing antibodies in COVID-19 patients [7], [8].

Aim of this paper is the analytical and clinical evaluation of a SARS-CoV-2 RBD IgG assay, automated on a high throughput platform and the correlation of IgG levels with neutralizing antibodies.

Materials and methods.

Patients.
A total of 546 leftover serum samples from 168 COVID-19 patients (24 asymptomatic or with mild disease [Asympt/Mild], who recovered at home with supportive care and isolation, and 144 hospitalized subjects classified with mild or moderate/severe disease following WHO interim guidance [9]) and 171 SARS-CoV-2 negative subjects (97 pre-pandemic samples from healthy donors, 31 healthcare workers, 11 and 32 patients with rheumatic disease or with human immunodeficiency virus [HIV]) were included in the study. Furthermore, 207 subjects of the COVID-19 Family Cluster Follow-up Clinic (CovFC), set up at the Department of Women's and Children's Health of the University Hospital of Padua were studied. Families

were enrolled when complied with the following inclusion criteria: a) having children of pediatric age; b) having a history of medically confirmed COVID-19 or being a household member of a COVID-19 confirmed case.

All subjects underwent at least one nasopharyngeal swab test analyzed by rRT-PCR. Healthcare workers were considered negative (HCW) on the basis of at least three recent negative sequential molecular test results obtained between February 26th and May 29th, 2020. Information concerning family clusters past and recent history were collected retrospectively through both patients interviews and the revision of clinical files. Family subjects who had tested positive for SARS-CoV-2 by rRT-PCR and/or by either of the two serological tests adopted in this study were considered confirmed COVID-19 cases. For each confirmed COVID-19 case, a baseline was defined as the most likely onset of infection, based on different criteria. In detail, for patients reporting COVID-19 related symptoms, the baseline coincided with the onset of symptoms; in case of asymptomatic patients the baseline referred to the date the first positive NP swab was recorded. Among SARS-SARS-CoV-2 patients in family clusters, five were hospitalized for moderate disease, whereas the others were recovered at home.

The study protocol (number 23307) was approved by the Ethics Committee of the University-Hospital, Padova. All the patients were informed of the study and voluntarily agreed to participate, providing a written consent.

Analytical system under evaluation.

In this study, a commercially available immunoassay was evaluated, the anti-SARS-CoV-2 S-RBD IgG (Snibe Diagnostics, New Industries Biomedical Engineering Co., Ltd [Snibe], Shenzhen, China). SARS-CoV-2 S-RBD IgG is a chemiluminescent immunoassay (CLIA) that determines IgG Ab against the RBD of the Spike (S) protein of the virus, in human serum or plasma. All analyses were performed on MAGLUMI™ 2000 Plus (Snibe Diagnostics), with results expressed in kiloastronomical unit.

Repeatability and intermediate precision evaluation.

Precision estimation was performed using three human serum sample pools with different values, by means of triplicate measurements of same pool aliquots, performed for a total of five consecutive days. Nested analysis of variance (ANOVA) was used to estimate precision, following the CLSI EP15-A3 protocol [10]. The results for precision were compared to those claimed by the manufacturer, using the procedure recommended by EP15-A3.

Linearity assessment of Maglumi anti-SARS-CoV-2 S-RBD.

Linearity was assessed using two samples pools (high level pools), prepared with different levels of SARS-CoV-2 antibodies, serially diluted in low level pools, as specified in the CLSI EP06-A guideline (paragraph 4.3.1) [11]. The following high-level serum pools were prepared: 3.7 and 71 kAU/L. The pools were serially diluted with the corresponding low-level serum pools (0.181 and 0.59 kAU/L). All measurements were performed in triplicate. Polynomial regression was used to test deviation from linearity.

Plaque reduction neutralization test (PRNT).

A high-throughput PRNT method was used for the fast and accurate quantification of neutralizing antibodies in plasma samples collected from patients exposed to SARS-CoV-2, as described elsewhere [12]. Briefly, after heat-inactivation, samples were diluted in Dulbecco modified Eagle medium (DMEM) and then mixed with a virus solution containing 20–25 focus-forming units (FFUs) of SARS-CoV-2. After 1 h at 37 °C, 50 µL of the virus–serum mixtures were added to confluent monolayers of Vero E6 cells, in 96-wells plates and incubated for 1 h at 37 °C, in a 5% CO2 incubator. After 26 h of incubation and cells fixing, visualization of plaques was obtained with an immunocytochemical staining method using an anti-dsRNA monoclonal antibody (J2, 1:10,000; Scicons) for 1 h, followed by 1 h incubation with peroxidase-labeled goat anti-mouse antibodies (1:1,000; DAKO) and a 7 min incubation with the True Blue™ (KPL) peroxidase substrate. FFUs were counted after acquisition of pictures at a high resolution of 4,800 × 9,400 dpi, on a flatbed scanner. The serum neutralization titer was defined as the reciprocal of the highest dilution resulting in a reduction of the control plaque count >50% (PRNT50). From previous experiments, we defined a titer of 1:10 as the seropositive threshold [12].

Statistical analyses.

For evaluation of precision, an in-house developed R (R Foundation for Statistical Computing, Vienna, Austria) script for implementing the CLSI EP15-A3 protocol was used for ANOVA and for calculating the upper verification limit [10]. The GraphPad Prism version 9.1 for Windows (GraphPad Software, LLC) was employed to evaluate plaque reduction neutralization test results. Stata v16.1 (Statacorp, Lakeway Drive, TX, USA) was used to evaluate the assays' clinical performances. Bonferroni's adjusted p-value (B-adj) was calculated for multiple comparisons. For ROC analyses, the non-parametric empirical method was used to estimate the area under the ROC curve (AUC), while the 'diagt' module was used to estimate sensitivity, specificity, and positive and negative predictive values. Youden index (calculated as sensitivity + specificity-1) was used to estimate the best performances of the assay. Considering a type I error α=0.05, a power of 0.8 and with 249 positive and 249 negative subjects, a sensitivity (or specificity) of 0.95 can be considered significant with respect to values above or equal to 0.99 (null hypothesis). PASS 2020 Power Analysis and Sample Size Software (2020), NCSS, Kaysville, Utah, USA, was used for sample size and power analyses.

Results.

Patients 'characteristics.

Demographic characteristics of the subjects included in the study are reported in Table 1. The overall mean age was 42.5 years, with a standard deviation (±SD) of 22.6 (range 0.7–92.2 years). Excluding family clusters, the remaining subjects (n=337) presented a mean age (±SD) of 53.7 ± 16.9 years. A multivariate ANOVA analysis was performed considering Age as dependent variable and Gender and studied groups (F=56.55, p<0.001) as independent variables. The ages of family clusters differed from other groups (Bonferroni's

adjusted [B-adj] p<0.001 for all), except for Asympt/Mild positive patients (B-adj p=0.051). Age of negative healthcare workers (HCW), pre-pandemic subjects and Asympt/Mild patients were not statistically significant different (p=0.999), while these groups' age differ with respect to hospitalized COVID patients (B-adj p<0.0001). Age of Rheumatic disease/HIV patients differs from other groups (B-adj p<0.001 for all), with the exception of Asympt/Mild disease group (B-adj p=0.493). The percentage of females differed significantly from that of males (p<0.001), particularly in the Asympt/Mild disease group. For SARS-CoV-2 patients, the mean time interval from the onset of symptoms and serological determinations was 17.7 days (SD ± 16.3; range 1–103 days). In the family clusters, the mean time interval from the onset of symptoms and serological determinations was 148.2 days (SD ± 71.2; range 41–257 days). The differences in time from symptoms onset with respect to the studied groups of individuals were reported in Table 2.

Table 1. Demographic characteristics of the groups of subjects included in the study.

Types of individuals	n, %	Gender		Age, years, mean ± SD
		Females n, %	Males n, %	
Pre-pandemic	97 (28.6%)	62 (63.9)	35 (36.1%)	40.8 ± 11.9
Negative healthcare workers, HCW	31 (9.1%)	10 (32.3%)	21 (67.7%)	43.6 ± 12.0
Patients with rheumatic diseases and with human immunodeficiency virus, AI/HIV	43 (12.7%)	12 (27.9%)	31 (72.1%)	52.2 ± 12.7
Asymptomatic/mild SARS-CoV-2 positive patients, asympt/mild	24 (7.1%)	21 (87.5%)	3 (12.5%)	43.0 ± 13.9
Severe SARS-CoV-2 positive hospitalized patients, Sev	70 (20.6%)	24 (34.3%)	46 (65.7%)	61.3 ± 16.1
Critical SARS-CoV-2 positive hospitalized patients, critical	74 (21.9%)	14 (18.9%)	60 (81.1%)	68.1 ± 13.8
All samples excluding family clusters	339 (100%)	143 (42.2%)	196 (57.8%)	53.7 ± 16.9
Families with COVID-19 pediatric patients	207 (37.9%)	95 (45.9%)	112 (54.1%)	24.2 ± 18.4
Overall	546 (100%)	238 (43.6%)	308 (56.4%)	42.5 ± 22.6

Table 2. Disease severity, time from symptoms onset, percentage of positive samples to serological determination of anti-SARS-CoV-2 RBD IgG antibodies and PRNT50 titers, subdivided by the studied groups.

Types of individuals	Samples evaluated for SARS-CoV-2 antibodies, n, %	Days from symptoms onset and serology, mean ± SD	Percentage of samples with positive assays results, n, %	Samples tested for neutralization activity, n	Percentage of samples with neutralizing antibodies ($PRNT_{50}$≥1:10)
Pre-pandemic	97 (17.8%)	–	4 (4.1%)	20 (20.6%)	0
Negative healthcare workers, HCW	31 (5.7%)	–	8 (25.8%)	–	–
Patients with rheumatic diseases and with human immunodeficiency virus, AI/HIV	43 (7.9%)	–	2 (4.7%)	–	–
Asymptomatic/mild SARS-CoV-2 positive patients, asympt/mild	24 (4.4%)	73 ± 28.3[a]	22 (91.6%)	5 (20.8%)	5 (100%)
Severe SARS-CoV-2 positive hospitalized patients, Sev	70 (12.8%)	16.1 ± 17.5	60 (85.7%)	23 (32.9%)	23 (100%)
Critical SARS-CoV-2 positive hospitalized patients, critical	74 (13.5%)	19.3 ± 15.0	70 (94.6%)	26 (35.1%)	26 (100%)
Families with COVID-19 pediatric patients	207 (37.9%)	148.2 ± 71.2[b]	191 (92.2%)	207 (100%)	189 (91.3%)
Overall	546 (100%)	89.5 ± 84.2	194	281 (51.5%)	238 (84.7%)

[a]data available for only two patients; [b]statistically significant with respect to the time from symptom onset of severe and critical hospitalized patients (p<0.001) (one-way ANOVA, F=227.7, p<0.0001).

Repeatability and intermediate precision.

Results for precision of CLIA assay is reported in Table 3. Repeatability and within-laboratory precision were in accordance with the repeatability and intermediate precision conditions specified in the international vocabulary of metrology (VIM, JCGM 100:2012) for precision estimation within a five-day period. Obtained data show acceptable imprecision at low and medium levels, but significantly deviated from the values claimed by the manufacturer for the high-level control material.

Table 3. Precision results of Maglumi SARS-CoV-2 S-RBD IgG obtained using a 3 × 5 design (triplicate measurement for five consecutive days). Coefficient of variation (CV) are expressed in percentage (%) and were obtained by using pools of samples.

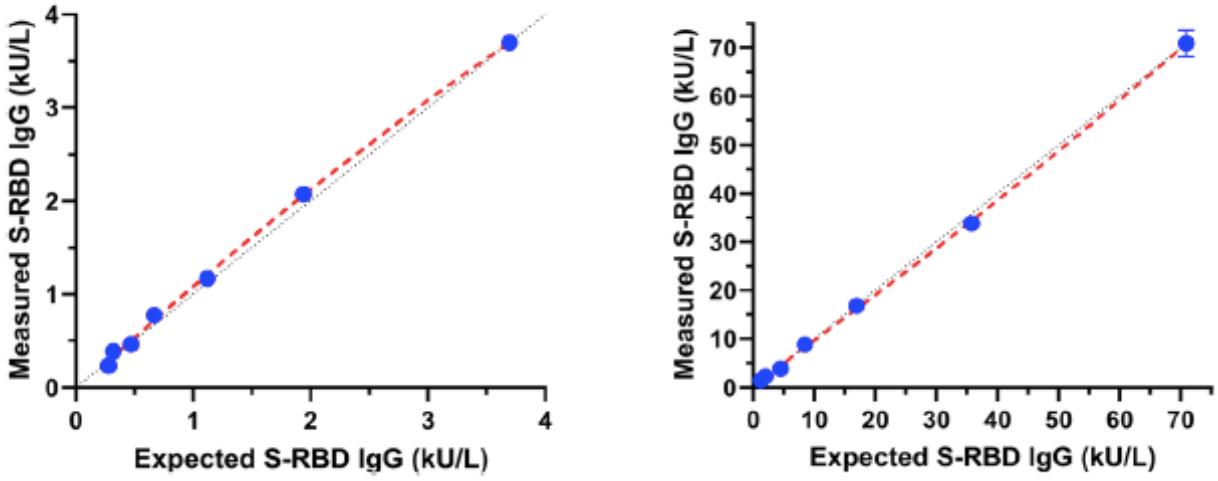
Measurand	Level	Design	Laboratory estimation of repeatability, CV%	Laboratory evaluation of intermediate precision – CV%
Anti-SARS-CoV-2 S-RBD IgG[a]	1.06 kAU/L[b]	3 × 5	5.32	12.18
	2.94 kAU/L		3.99	7.06
	6.14 kAU/L		3.98[c]	6.88[c]

[a]Performances were obtained from the Snibe Maglumi SARS-CoV-2 S-RBD IgG-en-EU, V1.2, 2020-08: declared precision specifications for repeatability and intermediate precision (repeatability and between days precision) were: 7.64% and 11.7%, respectively, at 0.55 kAU/L; 4.06% and 4.92%, respectively, at 2.42 kAU/L; 2.25% and 2.40%, respectively, at 5.11 kAU/L. [b]Manufacturer's precision at this level was estimated by applying linear interpolation estimation (6.63% for repeatability and 9.59% for intermediate precision). [c]Indicates that imprecision value was higher than that declared by manufacturers, also after the calculation of the upper verification limit (UVL) as suggested by EP15-A3 (UVL=3.32% for repeatability and UVL=3.39% for intermediate precision at level 5.11 kAU/L).

Linearity assessment.

Linearity results for Maglumi anti-SARS-CoV-2 S-RBD are summarized in Figure 1. Since the method is claimed to be quantitative, tested mixes were prepared for covering a wide range of values (the upper limit of the method without sample dilution is 100 kAU/L), including the manufacturers' cut-offs. Maglumi anti-SARS-CoV-2 S-RBD IgG does not deviate from linearity in the entire range of tested values, being the coefficients of the second-order polynomial non-statistically significant.

Figure 1. Linearity assessment of anti-SARS-CoV-2 S-RBD IgG assays, performed at two concentration levels.

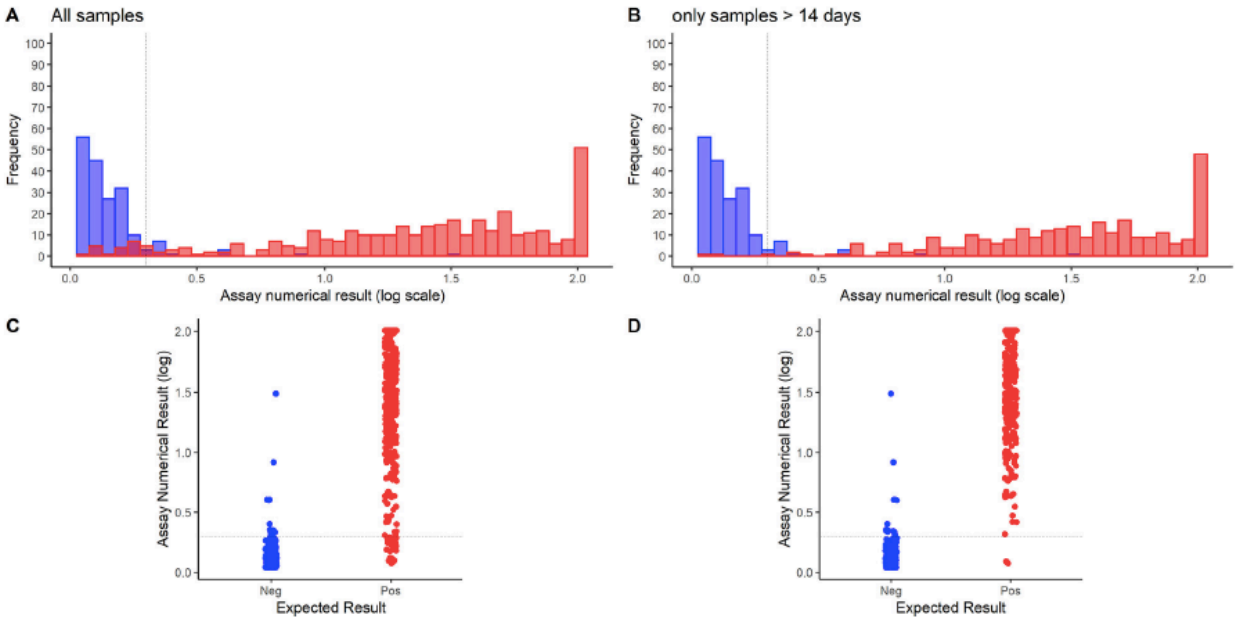


Evaluation of clinical performances.

For a total of 339 samples, including pre-pandemic (collected in 2015), negative HCW and AI/HIV subjects (collected between March 2020 and May 2020) and samples from patients hospitalized for COVID-19 (collected between April 2020 and November 2020), a total of 178 and 161 resulted negative and positive to SARS-CoV-2, respectively. In family clusters of COVID-19, out of 207 samples, 191 had a laboratory-confirmed past SARS-CoV-2 infection, and positivity were correctly identified by the assay in all cases under evaluation.

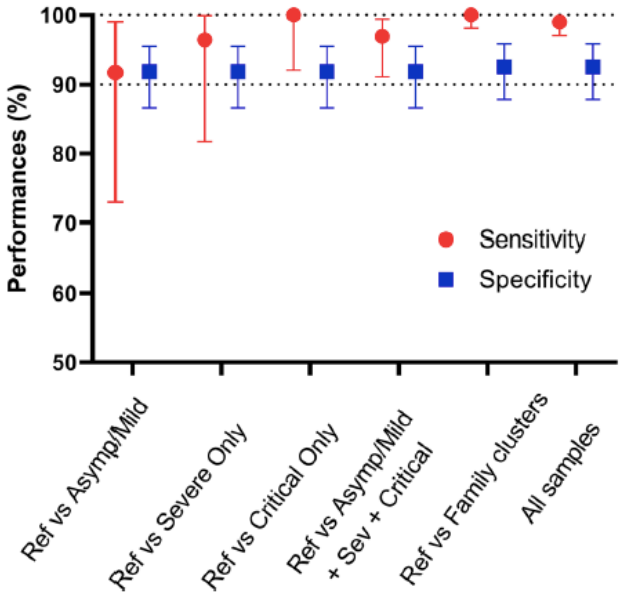
The distribution of log10 anti-SARS-CoV-2 S-RBD transformed results is reported in Figure 2, considering both overall individuals and only samples collected after 14 days post-symptoms onset. Considering only samples collected after 14 days post-symptoms onset, median and interquartile range (IQR) of anti-SARS-CoV-2 S-RBD Ab in SARS-CoV-2 patients were: 18.5 kAU/L (12.13–30.48 kAU/L) for Asympt/Mild, 52.1 kAU/L (34.1–78.0 kAU/L) for Severe and 79.1 (36.3–100 kAU/L) for Critical individuals; for family Clusters the median and IQR Ab level was 27.3 kAU/L (10.9–51.6 kAU/L). By using the Kruskal-Wallis test, significant differences were obtained comparing Asympt/Mild with Severe or Critical patients (Bonferroni's adjusted [Badj] p-value<0.001 for both), and between Severe or Critical patients with family clusters (Bonferroni's adjusted [Badj] p-value<0.001 for both); no statistical significance difference was observable between Severe and Critical SARS-CoV-2 patients (Badj p-value=0.117).

Figure 2. Frequency histograms and dot plots of log10 transformed anti-SARS-CoV-2 S-RBD IgG CLIA results (in kiloastronomical unit), considering all the studied individuals (A and C) and only samples collected after 14 days from symptom onset (B and D). Ref: all samples from negative individuals (pre-pandemic samples, healthcare workers, patients with rheumatic disease or with human immunodeficiency virus).



Sensitivities, specificities, and positive/negative likelihood ratios, estimated using the manufacturers' cut-offs and considering samples collected from 14 days post-symptoms onset, were reported in Figure 3 and Supplementary Table 1. Receiver operating characteristic (ROC) curves were further reported in the same table. A further analysis was performed, using the Youden index strategy, for identifying the most accurate cut-off; However, the cut-off calculated with Youden's index (0.96 kAU/L) does not significantly improve the clinical performances when compared to that recommended by the manufacturer (1.0 kAU/L).

Figure 3. Sensitivities and specificities of anti-SARS-CoV-2 S-RBD IgG, calculated considering only samples collected after 14 days from symptom onset. Different conditions were inspected and compared. Ref group includes SARS-CoV-2 negative samples from pre-pandemic specimens, healthcare workers and patients with rheumatic diseases and HIV.



Although sensitivity and specificity are helpful for clinical purposes, positive and negative predictive values (PPV and NPV) are more relevant in clinical decision making. Using two different scenarios of disease prevalence settings, (a) 4%, as found in a Veneto Region (Italy) survey [13] and (b) 10%, as described in a survey conducted in Geneva [14], PPV and NPV were then estimated. Considering performances derived from sera collected 14 days after the onset of symptoms on Asympt/Mild symptomatic subjects, mimicking a survey conducted in a population not reporting symptoms attributable to COVID-19, the PPV (95%CI) and NPV (95% CI) were 31.8% (21.8–43.9%) and 99.9% (98.6–100%) with a prevalence of 4%, and 55.4% (42.6–67.6%) and 99.0% (96.3–99.7%) with a prevalence of 10%.

CLIA results correlation with PRNT50 results.

Considering all individuals included in the study, a total of 281 leftover samples results of the PRNT50 test were available (Table 2 and Supplementary Figure 1). The relationship among the anti-SARS-CoV-2 S-RBD IgG and the corresponding PRNT50 titers is shown in Figure 4, panels A and B. Overall, positive associations were found between log10 PRNT50 titer and log10 Ab results. An elevated correlation was obtained (ρ=0.689, $p<0.001$) at univariate analyses. At multivariate analyses, performed including Age, Gender and the time from symptom onset and serological determination in the linear regression model, a similar correlation coefficient was found (R2 adj=0.508, ρ=0.712), being only Age (p=0.013) and time post symptom onset (p=0.041) statistically significant. In a further sub-analysis, including also disease severity, this latter variable results not significantly associated with log10 PRNT50 titer.

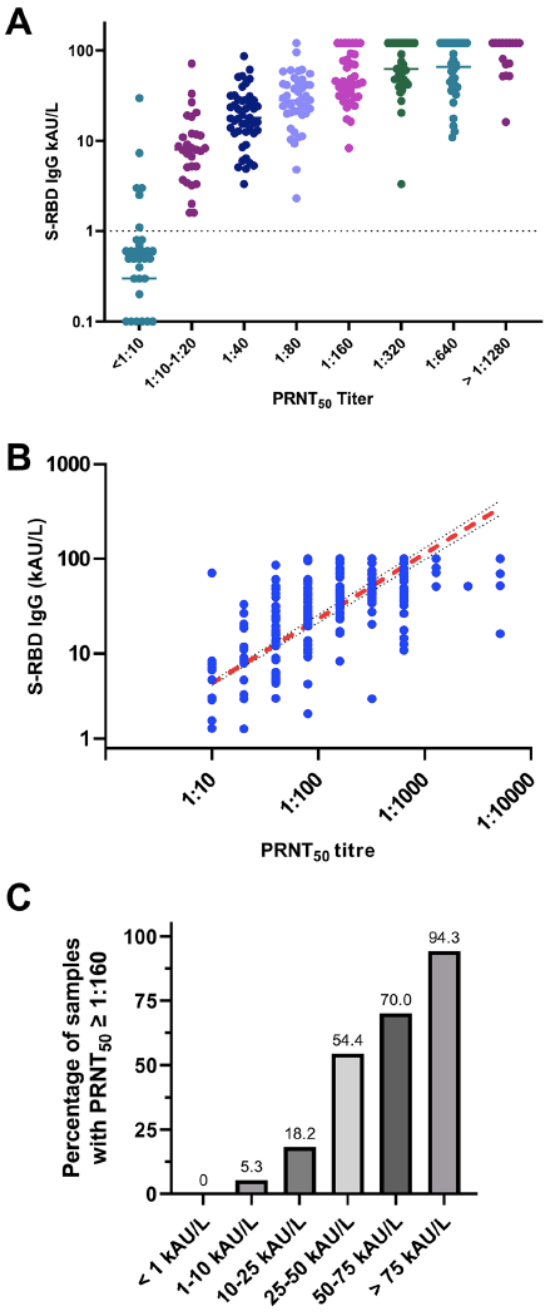


Figure 4. Correlation between the anti-SARS-CoV-2 S-RBD IgG CLIA results and PRNT50 titers. (A) dot plots presenting the CLIA results with respect to the different PRNT50 titers; (B) linear correlation of positive PRNT50 titers with respect to CLIA results (both in log10 scale); (C) percentage of samples with PRNT50 titers ≥1:160 and different ranges of CLIA results.

Since for COVID-19 convalescent plasma treatments a high neutralization titer is advisable, a further analysis was performed [15]. Figure 4 (panel C) shows the percentage of samples with a PRNT50 titer ≥1:160 with respect to the ranges of S-RBD IgG results. For CLIA result above 75 kAU/L, a neutralizing titer ≥1:160 was detected in the 94.3% of cases (5% of cases below 1:160 were three samples with PRTN50 equal to 1:80, 1:860 and 1:40).

Discussion.

This paper reports a head-to-head evaluation of the Snibe anti-SARS-CoV-2 S-RBD IgG CLIA analytical performances, since this assay is claimed to be quantitative and the evaluation of these characteristics is especially important for monitoring seroconversion and antibody persistence. Results showed that this assay presents excellent analytical performances, both for precision and linearity. The repeatability was less than 6% for all the studied levels, while intermediate precision was more elevated at the lowest level (1 kAU/L), which is close to the cut-off proposed by the manufacturer (Table 3). Precision performances statistically deviated from the manufacturer's claims only at the highest level (6.14 kAU/L), as the precision value reported inside the inserts at 5.11 kAU/L were 2.25 and 2.40%.
The adoption of serological testing for monitoring of Ab titers requires, in addition to assay robustness, a good method linearity, to effectively quantify differences between measured values. Our data demonstrate that anti-SARS-CoV-2 S-RBD IgG presents excellent linearity not only within the range of values including the cut-off (0.2–4 kA/L) but also for the highest values (from 5 to 70 kAU/L) (Figure 1); notably, these findings are relevant when considering that, in vaccinated subjects, Ab values above the limit of the method are often detected, requiring a further dilution step for delivering results (data not shown).

On a large panel of blood samples, including pre-pandemic, negative HCW, and negative AI/HIV specimens and SARS-CoV-2 patients with different severity of disease (Asymp/Mild, Severe and Critical), using the pre-defined assay thresholds for calling test results positive or negative, overall sensitivity and specificity were around 97% and 92%, respectively (Figure 3 and Supplementary Table 1). The suboptimal specificity is related to the presence of some false-positive results obtained for 14 samples (including four pre-pandemic, two AI, and eight HCW specimens), and which may affect all currently available immunoassays. In agreement with the time-dependent nature of antibody response, different results are obtained assessing samples collected at least 14 days post symptoms onset [16]. Accordingly, two separate analyses were conducted. In the time frame from 14 days post symptom onset, using all negative subjects as references (Ref), better sensitivity results were achieved for critical rather than severe disease patients, despite the anti-SARS-CoV-2 S-RBD IgG did not differ between the groups of severe and critical patients. Comparing Ref and family clusters, performances of anti-SARS-CoV-2 S-RBD IgG were excellent, being sensitivity 100% and specificity above 92%; remarkably, all samples of this group were collected after 14 days post symptom onset. Considering samples from family clusters, a slight statistically significant time-dependent decrease of anti-SARS-CoV-2 S-RBD IgG was

observed, and linear regression allowed to estimate a change in Ab levels, with a confidence of 95%, from −0.17 to −0.04 kAU/L per day (Supplementary Figure 2) and in a further analysis, performed excluding individuals aged <30 years, findings confirmed the magnitude of the linear slope. These results are fully in accordance with our previously reported data [12], suggesting that, with the exception of some individuals, immunological memory remain persistently elevated for months up to 10 months [17], [18].

The relationship between SARS-CoV-2 antibodies and neutralizing activity remains an essential and open issue. In fact, SARS-CoV-2 neutralizing antibodies (NAb) titer is currently gaining importance for supporting vaccine development, and to aid convalescent plasma therapy. Therefore, due to the high demand for the neutralization test, a surrogate method to evaluate their levels in patients with varying severity of illness at a various time points is strongly advisable, also for circumventing the need to handle live virus in BSL-3 laboratories. Alternatively, recently developed surrogate virus neutralization assays are coming on the marker, and these methods should be assessed and validated more extensively in future before a widely utilization [19]. For this reason, we assessed the correlation between the plaque reduction neutralization, the gold standard methods for determining the titer of NAb, with anti-SARS-CoV-2 S-RBD IgG levels. Overall, the anti-SARS-CoV-2 S-RBD IgG levels showed a good dynamic range and the response of the method was highly correlated with PRNT50 titers (Pearson ρ=0.712 at multivariate analysis) (Figure 4). In addition, when the percentage of samples with a PRNT50 titers ≥1:160 was calculated with respect to the ranges of anti-SARS-CoV-2 S-RBD IgG, results above 75 kAU/L presented a neutralizing titer ≥1:160 in the 94.3% of samples. These results are in accordance with our previously reported findings, performed in different assays, which gave similar results of this anti-SARS-CoV-2 S-RBD IgG. Currently, a small number of studies have validated a range of commercially available SARS-CoV-2 serological assays against a live-virus neutralization test [12, 20–25], and in our knowledge this is the first study comparing Snibe anti-SARS-CoV-2 S-RBD IgG levels and PRTN50 titers. Walker et al. evaluated different commercially available assays for their correlation with the microneutralisation assay and reported values ranging from 69 to 100%, with assays measuring total antibodies being the most sensitive [18]. Differently, Legros et al. found that Diasorin SARS-CoV-2 S1/S2 kit anti-S IgG titers correlated highly with microneutralization nAb titers (Spearman's $\rho = 0.7075$) [21]. Patel et al., who evaluated five immunoassays with respect to NAb results, observed that the strongest correlation was ρ=0.81 (with the ELISA from Euroimmun) and the weakest correlation was ρ=0.40 (with Roche CLIA assay) [22].

This study presented several limitations. First, neutralizing antibodies were mainly tested in a well-defined cohort of family cluster, with sera collected at various time points and, therefore, should be confirmed in further studies; second, COVID-19 positive patients were selected retrospectively on the basis of available leftover samples, and third cross-reactivity with seasonal human coronaviruses was not assessed; therefore NPV and PPV could be overestimated. Another limitation of this study is that no longitudinal sera were analyzed and, therefore, we cannot exclude that some patients might have seroconverted at later time points.

In conclusion, the data reported in this study showed that anti-SARS-CoV-2 S-RBD IgG assay achieves excellent analytical and clinical performances. Since specificity results were not 100%, the assay might present a limited number of false-positive results and this characteristic could be further confirmed in a more representative number of samples. However, the correlation with sera neutralization activity was very elevated, demonstrating that the dynamic range of the assay is expanded enough to capture all clinically significant NAb results. Finally, an appropriate threshold could be derived for selecting samples for COVID-19 convalescent plasma therapy.

Further studies are needed to clarify whether the current generation of anti-SARS-CoV-2 neutralizing antibodies, and eventually circulating IgG antibodies, would retain clinical significance in samples of patients infected with virus variants, mainly B.1.351 and P.1, which may hence generate a class of antibodies non-reacting with the recombinant (RBD) antigen(s) of the evaluated assay [26].

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4.4. Long-term Immune Response to SARS-CoV-2 Infection Among Children and Adults After Mild Infection

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Abstract

Importance: Understanding the long-term immune response against SARS-CoV-2 infection in children is crucial to optimize vaccination strategies. While it is known that SARS-CoV-2 antibodies may persist in adults 12 months after infection, there is limited data in the pediatric population.

Objective: We describe the long-term anti-SARS-CoV-2 S-RBD IgG kinetics in children following SARS CoV-2 infection.

Design: Single-centre, prospective observational cohort study.

Setting: From April 2020 to August 2021, patients were enrolled consecutively at the COVID-19 Family Cluster Follow-up Clinic set up at the Department of Women's and Children's Health of the University Hospital of Padua.

Participants: A cohort of 252 COVID-19 family clusters underwent serological follow-up at 1-4, 5-10, and >10 months after infection with quantification of anti-S-RBD IgG by chemiluminescent immunoassay.

Exposure: SARS-CoV-2 infection.

Results: Among 902 study participants, 697 had confirmed SARS-CoV-2 infection, including 351 children/older siblings aged 8.6±5.1 years, and 346 parents aged 42.5±7.1 years; of those, 96.5% cases had asymptomatic/mild COVID-19. Children showed significantly higher S-RBD IgG titers than older patients across all follow-up time points, with an overall mean S-RBD IgG titer in patients <3 years of age five-fold higher than adults (304.8 [139-516.6] kBAU/L vs 55.6 [24.2-136.0] kBAU/L, $p<0.0001$). Longitudinal analysis of 56 study participants sampled at least twice during follow-up demonstrated the persistence of antibodies up to 10 months from infection in all age classes, despite a progressive decline over time.

Conclusions: In this cohort study of Italian children and adults following SARS-CoV-2 infection, we found different kinetics of SARS-CoV-2 antibodies across several age classes of asymptomatic/mild COVID-19 cases, which could help in optimizing COVID-19 vaccination strategies and prevention policies. Our work confers further evidence of sustained immune response in children up to one year after primary SARS-CoV-2 infection.

Introduction.

The ongoing SARS-CoV-2 pandemic has afflicted public health care systems worldwide. Vaccination is one of the most effective tools to achieve herd immunity in a short period. Consequently, a deeper understanding of the mechanisms related to long-term kinetics and durability of the immune response against SARS-CoV-2 is vital in optimizing vaccination strategies. In this respect, the anti- receptor-binding domain (RBD) antibodies against SARS-CoV-2's spike (S) protein, with their strong positive correlation with the neutralizing antibodies (NAbs), represent a reproducible, cost-effective, and precise tool to define the quality of the host's immune response against the virus.[1-6] Currently, scientific knowledge investigating the long-term persistence of anti-SARS-CoV-2 antibodies up to 12 months after the infection is mainly limited to adults,[7-15] whereas a gap concerning the pediatric population, which plays an essential role in silently spreading the virus,[16,17] still remains. To date, few studies[18-20] have reported on the production of NAbs and anti-S-RBD IgG up to 8 to 12 months after infection in children who had recovered from mild or asymptomatic COVID-19. To gain a greater understanding of the immune response in children after SARS-CoV-2 infection, we examine the long-term anti-S-RBD IgG kinetics in a prospective cohort of COVID-19 family clusters mostly affected by asymptomatic or mild disease.

Methods.

Study Design and Data Collection.

We conducted a single-center, prospective cohort study of families, including children, older siblings, and their parents, who attended the COVID-19 Family Cluster Follow-up Clinic at the Department of Women's and Children's Health, University Hospital of Padua. From April 1, 2020, to August 31, 2021, families were enrolled 4 or more weeks after infection if they had children younger than 15 years and at least one family member with a history of COVID-19 infection. Exclusion criteria were receipt of the SARS-CoV-2 vaccine and classification as non-COVID-19 cases. Parents or legally authorized representatives were informed of the research proposal and provided their written informed consent. The study protocol was approved by the ethical committee of the University of Hospital of Padua.

At enrollment, a pediatrician (C.D.C., P.C., or S.C.) collected data on demographic characteristics, medical history, and vaccination status and performed a clinical evaluation. A blood sample was collected from all patients for characterization of the immunologic response to SARS-CoV-2. All patients with positive SARS-CoV-2 serologic test results at enrollment were followed up for longitudinal clinical and serologic evaluation with a minimum of 1 and a maximum of 3 collected blood samples at 1 to 4 and/or 5 to 9 and/or 10 or more months up to 18 months after baseline. Data on new contacts with confirmed or probable COVID-19 and confirmed SARS-CoV-2 subsequent infections were collected at each visit. Follow-up was interrupted if patients received any SARS-CoV-2 vaccine or in case of negative serologic test

results. Data were anonymized and entered into a web-based database using the REDCap (Research Electronic Data Capture) platform (Vanderbilt University).

Serologic Assays.

Quantification of anti-SARS-CoV-2 S-RBD IgG antibodies was performed with commercially available chemiluminescent assays (Snibe Diagnostics, New Industries Biomedical Engineering Co Ltd [Snibe]). This method, previously validated elsewhere,[5] quantitatively determined the IgG antibodies to the RBD portion of the SARS-CoV-2 spike protein. All analyses were conducted on MAGLUMI 2000 Plus (Snibe Diagnostics), with results expressed in kilo-binding antibody units per liter (kBAU/L), in accordance with the World Health Organization International Standard for anti-SARS-CoV-2 immunoglobulin. Samples recording titers greater than 4.33 kBAU/L were considered positive.

A high-throughput method for the plaque reduction neutralization test (PRNT) was used to quantify NAbs in serum samples for a subgroup of patients infected by SARS-CoV-2 within the first and second waves.[19,21] The neutralization titer was defined as the reciprocal of the highest dilution resulting in a reduction of the control plaque count greater than 50% (PRNT50). Samples recording
titers of 1:10 or greater were considered positive (details are provided in the Supplementary Materials).

Case Identification and Definitions.

Study participants were considered to have confirmed COVID-19 cases if they had a record of virologic positivity for SARS-CoV-2 by reverse transcriptase-polymerase chain reaction and/or tested
positive on either of the 2 serologic tests adopted in this study. A confirmed SARS-CoV-2 subsequent infection was defined as the new detection of a positive SARS-CoV-2 virologic assay at nasopharyngeal swabbing, occurring 60 days or more after having recovered from a previous case of
COVID-19 confirmed by negative virologic assay results.[22,23] During the first wave of COVID-19 (from February 17 to September 18, 2020), all enrolled family members were systematically tested by both assays. However, during subsequent waves, a sudden increase in the enrollment rate brought us to reconsider the sustainability of applying both serologic assays, given the high economic and operational costs posed by the PRNT assay. Previous validation exercises had proven the high correlation between the 2 assays.5 For this reason, from March 26, 2021, all family members were tested for Snibe anti-SARS-CoV-2 S-RBD IgG levels. Patients enrolled in the study were included in the statistical analysis if a defined baseline date was present. For symptomatic COVID-19 cases, the baseline date was defined as the first date between the onset of symptoms or the date of first positive SARS-CoV-2 molecular assay result. For asymptomatic cases, the baseline date was defined as the date of the first positive molecular assay result or, in those with only serologically confirmed COVID-19 and with negative or undetermined nasopharyngeal swab results, by the family outbreak temporal sequence, coinciding with the date of symptom onset in the family cluster. Infants younger than 6 months were included in the analysis only in case of virologic confirmation of SARS-CoV-2 infection at nasopharyngeal swabbing. COVID-19 severity was scored as mild, moderate, severe, critical, or

multisystem inflammatory syndrome in children according to the World Health Organization classification.[24] Patients who were
asymptomatic and had no laboratory evidence of SARS-CoV-2 infection were considered non-COVID-19 cases. Three periods or COVID-19 waves were identified and defined as follows: a first
wave from February 17 to September 18, 2020; a second wave from September 19, 2020, to February
18, 2021; and a third wave from February 19 to September 20, 2021.

Statistical Analysis.

Descriptive statistics, the $\chi 2$ test, the Fisher exact test, and a 2-tailed, unpaired t test were used for categorical or continuous covariates. The antibody titer response was assessed by comparing the median and the IQR of anti-SARS-CoV-2 S-RBD IgG values in the overall data set, including independent and participant-paired samples, and stratified by age classes (<3 years, 3-5 years, 6-11 years, 12-17 years, and 18 years) and by the time between serologic sampling and baseline, categorizing patients into 3 intervals, namely, 1 to 4, 5 to 9, and 10 or more months. The Kruskal-Wallis test was performed accordingly.

Longitudinal analysis of time between serologic sampling and baseline was conducted on participant-paired plasma from a subcohort of COVID-19 cases tested at least twice for S-RBD IgG titers, stratified by age classes (<6, 6-17, and 18 years). The Wilcoxon rank-sum test was performed accordingly. Linear regression analysis was used to assess the association between anti-SARS-CoV-2 S-RBD IgG and NAbs, using the log2 of both variables given data skew. Despite the transformation of the variables into logarithm, the strength of the associations between variables was assessed by the Spearman correlation coefficient and its relative P value.

The use of the robust variance estimator to account for correlations within patients with multiple blood samplings did not change the CIs considerably in the unadjusted analyses, so correlation structures were omitted from all analyses. Analyses were performed using the SAS software, version 9.4 (SAS Institute Inc). Statistical significance was set at $P < .05$. All P values were 2-sided. Graphs were made using GraphPad Prism, version 9.2 (GraphPad Software). This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline.

Results.

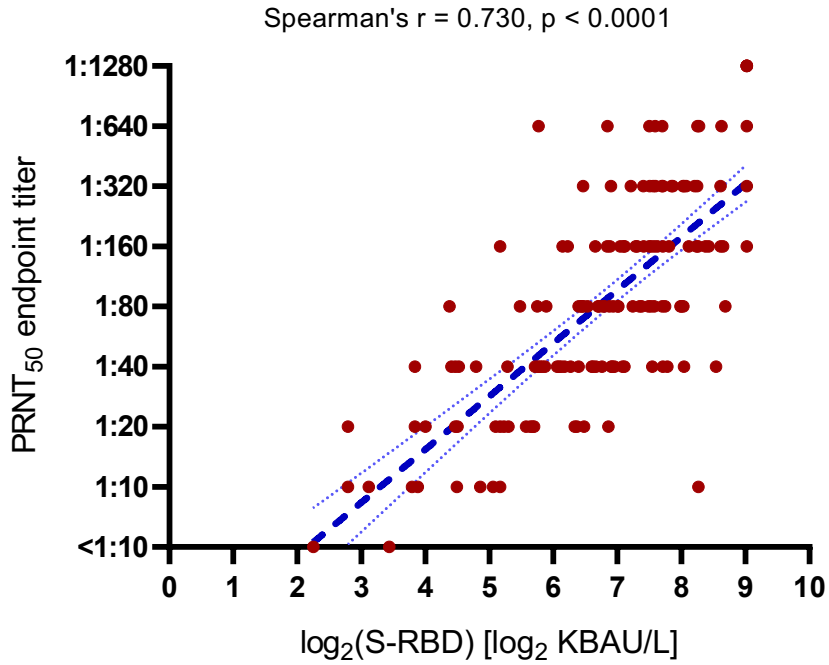
We prospectively evaluated 252 family clusters with COVID-19, for a total of 902 individuals. We excluded from the analyses 25 patients (2.8%) who had received at least 1 dose of SARS-CoV-2 vaccine before the first serologic follow-up and 180 patients (20.0%) defined as non-COVID-19 cases. A total of 575 patients (63.7%) who tested positive for SARS-CoV-2 by reverse transcriptase-
polymerase chain reaction, together with 122 individuals (17.5%) who had no record of virologic positivity but showed evidence of seropositivity, were considered COVID-19 cases (eFigure 1 in the

Supplement) and were included in the analysis. As a result, 697 patients with confirmed SARS-CoV-2 infection were analyzed, including 351 children or older siblings (mean [SD] age, 8.6 [5.1] years) and 346 parents (mean [SD] age, 42.5 [7.1] years). Among 697 cases, 674 (96.7%) were asymptomatic or mild. Descriptive characteristics of the patients and additional information on baseline time definition are reported in the Supplementary Table 1 and Supplementary Figure 2.

Correlation Between Anti-SARS-CoV-2 S-RBD IgG and NAbs.

From the 139 individuals who were tested in parallel with both serologic tests used in the study, a total of 172 samples were available for estimating the correlation between anti-SARS-CoV-2 S-RBD
IgG and NAbs, detected by chemiluminescent immunoassay and PRNT50, respectively. Overall, in the linear regression model, a positive correlation was found between PRNT50 log titers and log2 S-RBD IgG titers ($R2 = 0.47$, $\rho = 0.73$, $P < .001$) (Figure 1). A similar correlation between PRNT50 log titers and log2 S-RBD IgG was observed when samples were stratified according to follow-up time points and age classes.

Figure 1. Correlation between S-RBD IgG and NAbs titers in 139 patients analyzed simultaneously with both methods (172 sera). The dotted line represents the fitted line plot with confidence bands.



Long-term Kinetics of Anti-SARS-CoV-2 S-RBD IgG.

A total of 659 study participants had at least 1 anti-SARS-CoV-2 S-RBD IgG titer performed after infection. During follow-up, 657 (99.7%) still recorded positive titer results, whereas 2 of 659 patients (0.3%) with confirmed COVID-19 had negative antibody titer results after 64 and 556 days from baseline, respectively (Table 1; Supplementary Figures 3 and 4). During follow-up visits, none of the patients reported exposure to other patients with COVID-19 or a subsequent confirmed SARS-CoV-2 infection. However, we recorded an unexpected increase in S-RBD IgG titer for 17 patients. Considering the possibility of an unknown exposure to SARS-CoV-2, the last serum samples from these 17 patients were excluded from the analysis.

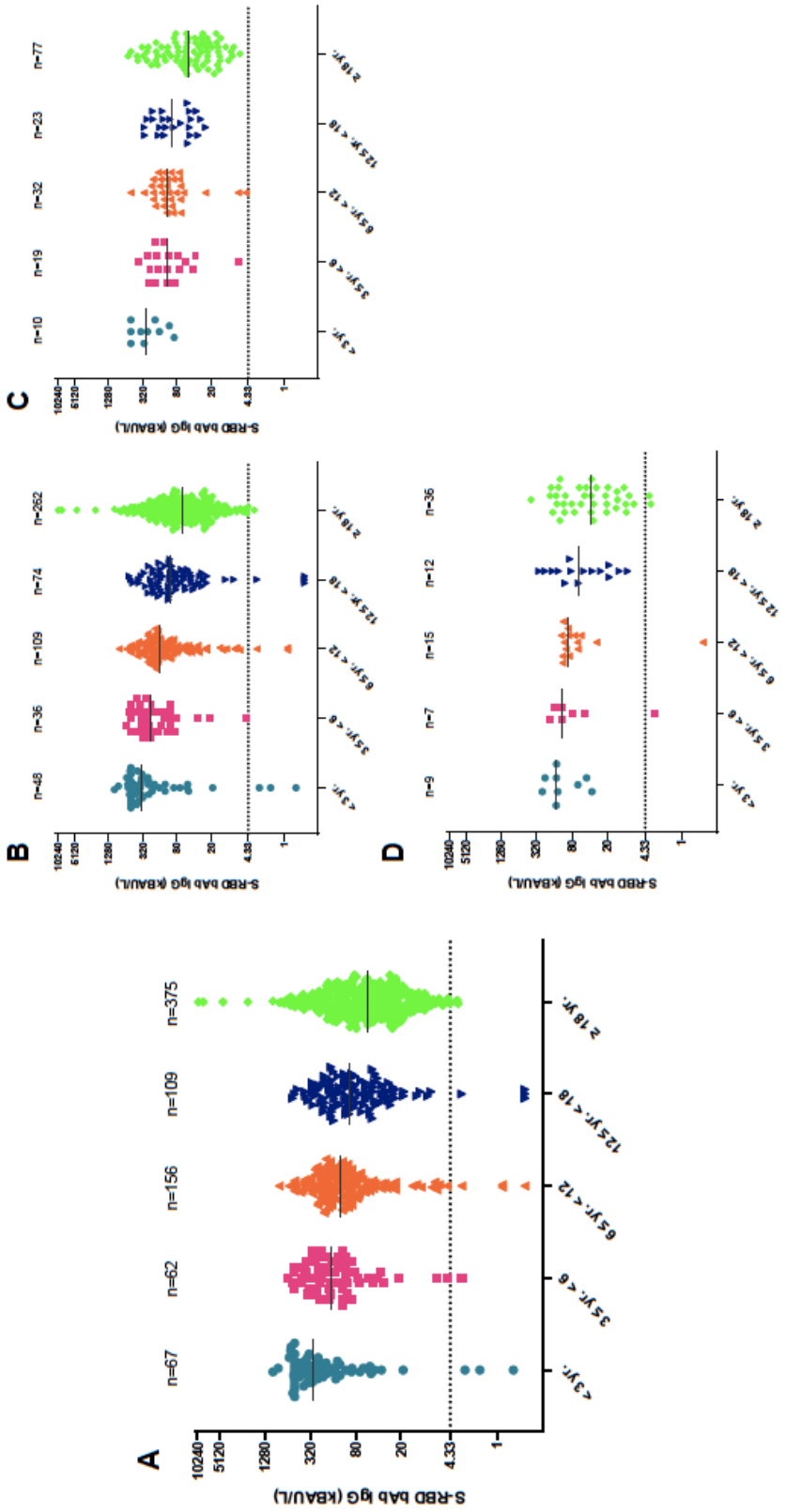
Table 1. Serologic Data of 769 Serum Samples Obtained From 659 Individuals With Confirmed COVID-19 Among Different Age Classes, Overall and Stratified by Time From Baseline.

All data, irrespective of onset						
Age Classes (yr.)	**< 3 yr. (n=67)**	**3 ≤ yr. < 6 (n=62)**	**6 ≤ yr. < 12 (n=156)**	**12 ≤ yr. < 18 (n=109)**	**≥ 18 yr. (n=375)**	**p-value‡**
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	
RBD	304.83 (139 - 519.6)	169.3 (103.1 - 277.1)	126.2 (74 - 207.8)	98.2 (44.7 - 169)	55.6 (24.2 - 136)	<0.0001
At 1 - 4 months, from onset						
Age Classes (yr.)	**< 3 yr. (n=48)**	**3 ≤ yr. < 6 (n=36)**	**6 ≤ yr. < 12 (n=109)**	**12 ≤ yr. < 18 (n=74)**	**≥ 18 yr. (n=262)**	**p-value‡**
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	
RBD	342.8 (179.5 - 519.6)	234.6 (113.5 - 347.9)	164.1 (79.1 - 236)	103.1 (46.3 - 170.2)	64.5 (26.2 - 140.9)	<0.0001
At 5 - 9 months, from onset						
Age Classes (yr.)	**< 3 yr. (n=10)**	**3 ≤ yr. < 6 (n=19)**	**6 ≤ yr. < 12 (n=32)**	**12 ≤ yr. < 18 (n=23)**	**≥ 18 yr. (n=77)**	**p-value‡**
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	
RBD	284.3 (162.5 - 519.6)	118.2 (70.6 - 192.5)	119.7 (77.4 - 165.2)	89.6 (45.9 - 170.2)	49.8 (22.5 - 114.7)	<0.0001
≥ 10 months, from onset						
Age Classes (yr.)	**< 3 yr. (n=9)**	**3 ≤ yr. < 6 (n=7)**	**6 ≤ yr. < 12 (n=15)**	**12 ≤ yr. < 18 (n=12)**	**≥ 18 yr. (n=36)**	**p-value‡**
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**	
RBD	146.2 (62.8 - 231.2)	115.6 (45.9 - 160.6)	90.6 (62.4 - 111.8)	48.6 (18.1 - 95.7)	36.7 (13.5 - 108.5)	0,0237

‡ Kruskal-Wallis Test

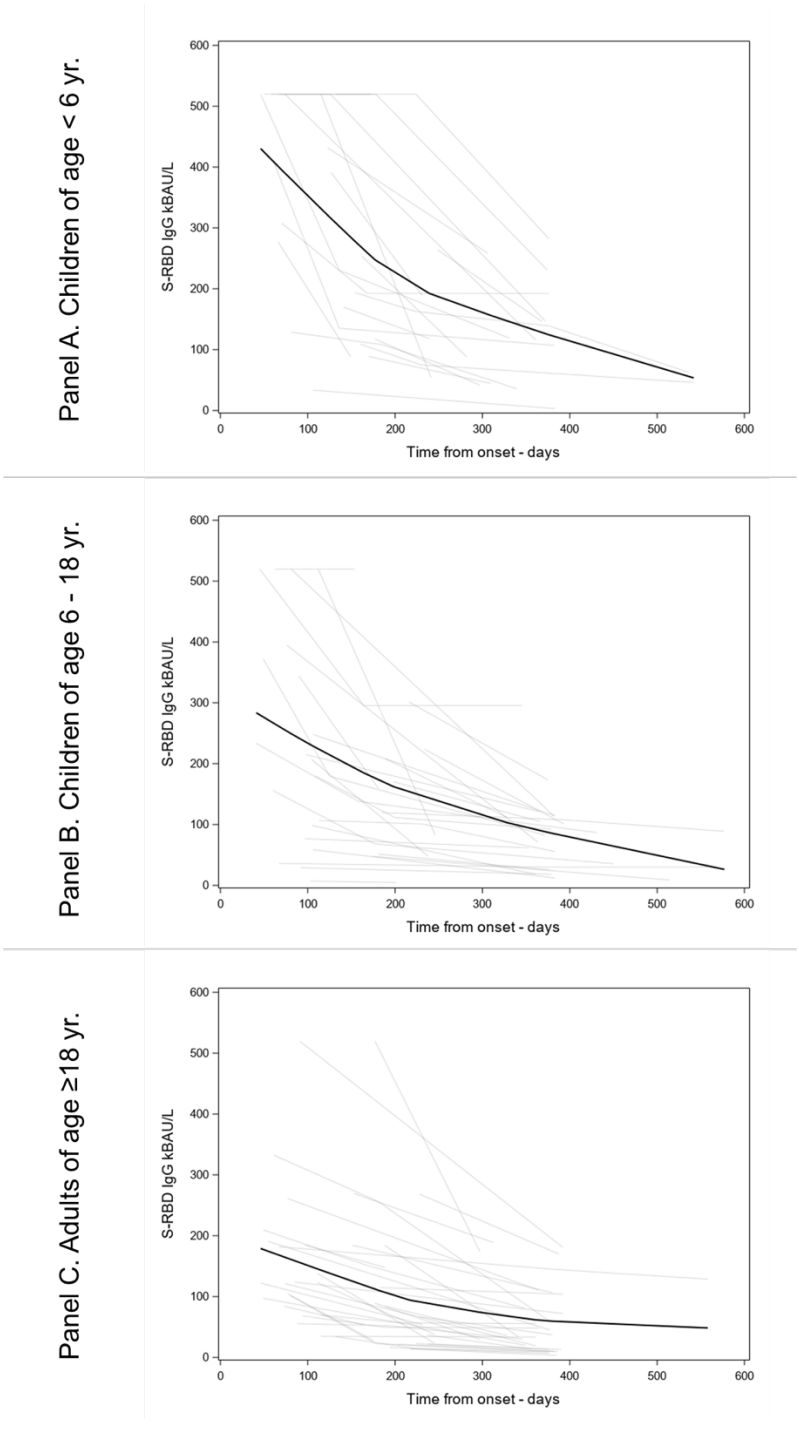
To better assess the association of age with the immunologic response, we analyzed 769 samples collected at 1 to 4 months (529 samples), 5 to 9 months (161 samples), and 10 or more months (79 samples) from baseline, stratifying among 5 classes of age (<3, 3-5, 6-11, 12-17, and 18 years of age) (Table 1 and Figure 2; Supplementary Figures 3 and 4). The S-RBD IgG titers differed among age classes (Table 1 and Figure 2; Supplementary Figures 3and 4). Overall, higher levels of antibodies were observed among younger children compared with older children, adolescents, and adults, with an overall median S-RBD IgG titer in patients younger than 3 years 5-fold higher than adults (304.8 [IQR, 139.0-516.6] kBAU/L vs 55.6 [24.2-136.0] kBAU/L, P < .001) (Table 1 and Figure 2; Supplementary Figures 3 and 4).

Figure 2. Distribution of S-RBD IgG titers according to age classes, overall (A, n=769) and stratified by time from baseline (B, C, D). Line and whiskers represent the median and interquartile range (25th and 75th percentile) of results. The dotted line at 4.33 kBAU/L correspond to the assay cut-off for discriminating positive from negative samples.



Differences in S-RBD IgG titers among all age classes, with younger children having significantly higher levels of antibodies, were also observed when samples were stratified by time of collection (at 1-4 months from infection, IgG anti-RBD levels ranged from 342.8 to 64.5 kBAU/L [P < .001]; at 5-9 months from infection, IgG anti-RBD levels ranged from 284.3 to 49.8 kBAU/L [P < .001]; and at10 months from infection, IgG anti-RBD levels ranged from 146.2 to 36.7 kBAU/L [P = .02]) (Table 1 and Figure 3).

Figure 3. Individual Kinetics of Spike Receptor-Binding Domain (S-RBD) IgG Titers in Patients With At Least 2 Time Points of Follow-up Regardless of the Time of the First Serum Collection According to Age Groups and Collection Time (n = 194 Serum Samples).



A longitudinal analysis was conducted on participant-paired plasma from a subcohort of 56 patients with COVID-19 tested at least twice for S-RBD IgG titers, with the first sample collected at 1 to 4 months from baseline. A first analysis was conducted on 31 patients who were sampled at a mean (SD) of 89.2 (38.6) and 199.2 (30.3) days from baseline, whereas a second analysis was conducted on 40 patients whose samples were collected at a mean (SD) of 81.9 (25.7) and 380 (47.7) days from baseline (referred to as medium and long intervals, respectively). Twenty-two patients were tested 3 times, contributing to both of these patient subgroups. Both analyses were stratified by 3 age subgroups: younger than 6 years, 6 to 18 years, and older than 18 years (Table 2).

Table 2. Subject-paired serological data of 56 patients who were sampled at least twice; overall, 31 patients were evaluated between a period of 1-4 months (89.2±38.6) and 5-9 months (199.2±30,3), and 40 patients between a period of 1-4 months (81,9±25,7) and ≥10 months (380±47,4) from baseline. Data are represented stratified by age classes.

	Age < 6 years (n= 8)				Age < 6 years (n= 10)		
	First sample	Intermediate sample	p-value§		First sample	Late sample	p-value§
	(1-4 months)	(5-9 months)			(1-4 months)	(≥ 10 monyhs)	
Mean days from baseline (STD)	98 (68.5 - 129.5)	205 (175 - 235)			72 (58 - 106)	373 (339 - 376)	
	Median (IQR)	Median (IQR)			Median (IQR)	Median (IQR)	
RBD	455.1 (238.9 - 519.6)	190.6 (113 - 519.6)	0,0625		475.6 (308 - 519.6)	132.7 (107 - 231.2)	0,002
	Age 6-18 years (n=10)				Age 6-18 years (n=15)		
	First sample	Intermediate sample	p-value§		First sample	Late sample	p-value§
	(1-4 months)	(5-9 months)			(1-4 months)	(≥ 10 monyhs)	
Mean days from baseline (STD)	96.5 (60 - 108)	190.5 (164 - 231)			92 (60 - 106)	379 (363 - 383)	
	Median (IQR)	Median (IQR)			Median (IQR)	Median (IQR)	
RBD	220.4 (155.9 - 519.6)	106.1 (68 - 158.9)	0,0039		180.3 (76.6 - 372.4)	71.4 (29.9 - 113.7)	< 0.0001
	Age ≥ 18 years (n=13)				Age ≥ 18 years (n=15)		
	First sample	Intermediate sample	p-value§		First sample	Late sample	p-value§
	(1-4 months)	(5-9 months)			(1-4 months)	(≥ 10 monyhs)	
Mean days from baseline (STD)	88 (73 - 111)	188 (178 - 224)			80 (61 - 94)	365 (361 - 386)	
	Median (IQR)	Median (IQR)			Median (IQR)	Median (IQR)	
RBD	104.8 (69.7 - 138.1)	52 (27.7 - 56.7)	0,0002		121.2 (68.4 - 209.6)	48.1 (19.9 - 80.5)	< 0.0001

§ Wilcoxon Signed-Rank Test

All 3 age groups exhibited persistence of S-RBD IgG titers at both intervals. Nonetheless, a progressive decrease in antibody levels was observed among all age classes and ranged from 2.0- to 2.3-fold reductions for the medium intervals and 2.5- to 3.6-fold reductions for the long intervals (Table 2).

To better investigate the decay in antibodies across age groups, the same analysis was conducted on a subcohort of 84 patients with COVID-19 tested at least twice for S-RBD IgG titers, regardless of the time of the first serum collection, for a total of 194 samples. Tracing a theoretical line obtained considering differences among individual antibody titers of all patients, we observed that all 3 age groups exhibited progressive decay in antibody titers; the rate of antibody waning was more rapid during the first 200 days and progressively slower thereafter. Compared with adults and children 6 years or older, children younger than 6 years showed an

apparently faster early waning of antibody titers (Figure 3). In addition, antibody titers remained detectable for 18 months (Figure 3).

Discussion.

In this cohort study, we evaluated the dynamic changes of the SARS-CoV-2 binding antibody titers in 252 family clusters mostly affected by asymptomatic or mild COVID-19 up to 12 months after initial infection. The findings suggest that anti-SARS-CoV-2 S-RBD IgG may persist more than a year from infection in all age groups, with antibody titers that inversely correlate with age.

This study strengthens and expands what we observed previously about the medium-term SARS-CoV-2 NAb response after COVID-19, analyzing preliminary data on the first 57 families affected by mild COVID-19 enrolled in our cohort.19 This study suggests that the magnitude of SARS-CoV-2 S-RBD IgG antibodies is higher among younger children compared with older siblings and adults at all follow-up points. Considering the 2 ends of the age spectrum of our cohort, we found that children younger than 3 years developed 5-fold higher levels of binding antibodies compared with individuals older than 18 years. These results align with prior studies[18,20,25,26] using PRNT50 and surrogate neutralization-based assays describing higher antibody titer and neutralizing ability in children than adults.

Our results demonstrate different antibody titers among mildly affected age groups, suggesting that factors such as specific cellular responses, genetics, environment, and stochastic variables may contribute to the high variation in immune response between individuals, irrespective of disease severity.[27,28] One study found that children had class-switched convergent cellular clones to SARS-
CoV-2 before the pandemic, with weak cross-reactivity to other coronaviruses, whereas adult blood
or tissues showed few clones.[29] Another study[30] reinforces our supposition, suggesting that infection in elderly patients is associated with antibodies targeting the cross-reactive S2 and NP proteins, whereas in children, the response is dominated by antibodies with high Fc-effector function targeting the immunodominant S1 protein of SARS-CoV-2. Conversely, Renk et al[20] recently observed that the repeated exposure to previous endemic human coronaviruses did not impair the humoral response to SARS-CoV-2. Finally, given that our family clusters were likely exposed to similar environmental factors, genetic attributes may also contribute to the different potency and durability of humoral responses.[31]

Three studies contrast with our findings, reporting no differences in the expression of specific antibodies between age classes or lower neutralizing activity in children compared with adults.[32-34]

However, Márquez-González et al[32] evaluated samples collected 3 weeks after infection, and 40% of pediatric patients were affected by malignant neoplasms at the time of COVID-19 diagnosis, implying a potential state of immunosuppression that may have altered the humoral response to infection. In the other studies,[33,34] children were compared with mildly affected adults selected as plasma donors, meaning that potentially only hyperimmune adults were selected.

Our work suggests that anti-S-RBD antibodies persist up to 18 months after infection, even in children. Stratifying individuals by age groups, we demonstrated that both children and adults experienced a decrease in anti-S-RBD IgG levels mostly during the first 200 to 300 days from infection. Of interest, children younger than 6 years showed a faster waning of antibody titers and then reached a plateau without a conversion to negative results. These results are in line with previous studies[7-14,20,35-39] conducted among both adults and children. In particular, Lau et al[39] observed that antibodies were detectable by spike RBD enzyme-linked immunosorbent assays in 92.6% of serum samples at 200 to 386 days from infection, despite showing an assay-dependent kinetics of antibody levels.

The persistence of a detectable S-RBD IgG titer more than 10 months after infection was observed in all age groups, regardless of whether the titers decreased over time. Remarkably, children younger than 6 years exhibited a median (IQR) S-RBD IgG titer of 132.7 (107-231.2) kBAU/L at 373 (339-376) days from baseline, and only 2 patients had results that converted to negative.

A previous study[40] estimated that the correlate of 50% protection from subsequent infection was 20% of the convalescent NAb titer. Relying on these findings, Lau et al[39] estimated that the threshold for 50% protection from subsequent infection for PRNT50 was 1:25.9 (95% CI, 1:24.7-1:27.6). As previously estimated by Padoan et al,[5] an S-RBD IgG titer greater than 70 kBAU/L is assumed to correspond to a PRNT50 titer greater than 1:20. In the current study, we demonstrated that the time-consuming PRNT50 correlated with the more available chemiluminescence assay on a large number of samples (n = 172), which could represent a promising open-access tool for estimation of serum's neutralizing power. In line with these findings, our data indicate that children younger than 6 years might be protected from subsequent infection up to 1 year.

However, as different virus variants emerge, the level of protective immunity may be compromised. Although it was observed that antibodies showed strong cross-reactivity to different variants, including Beta, Delta, Gamma, and Mu, for more than 1 year after infection,[35] future studies should also confirm the long-lasting response against Omicron. Moreover, to better understand the long-term persistence of immune protection against new emerging SARS-CoV-2 variants and to translate our data into estimations of immunity of children to subsequent infection, future research should include the evaluation of the longevity of B and T cells, which plays a key role in the human immune response. In fact, although we focused on the antibody responses to infection in this analysis, cellular immune responses are also likely to play an important role in protection against SARS-CoV-2 subsequent infection, as we and others have previously reported.[41] Children had a higher absolute number of circulating T cells and a high proportion of naive T cells than adults, thus enabling an efficient adaptive immune response to previously unrecognized microbial antigens, which persisted until 6 months after infection.[42]

Limitations.

Our study has several limitations. First, operational challenges related to the pandemic restrictions affected organization and access to the clinic; therefore, patients were evaluated with different follow-up times, and for a proportion of patients, intermediate follow-up was missing. Second, the baseline of infection for those

patients with COVID-19 without a positive nasopharyngeal swab result was identified through the only temporal reference to infection of the first symptomatic household and may be susceptible to temporal error. However, the initial temporal discrepancy, which may alter the evaluation of the acute phase of humoral response, was partially addressed by long-term follow-up.

Conclusions.

In this cohort study of Italian children and adults with SARS-CoV-2 infection, we found that anti-SARS-CoV-2 S-RBD IgG persisted until 12 months after infection in all age groups, with significant higher antibody peaks for younger individuals at every follow-up point. This study may provide an important basis to determine the schedule of COVID-19 vaccination in non-previously infected children and of booster immunization in pediatric patients who have already experienced COVID-19.

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Supplementary Materials.

Serological assays.

Blood samples were collected in EDTA-coated tubes to further separate cells and plasma by Ficoll procedure. Plasma and cellular samples were appropriately store at -80°C and liquid nitrogen, respectively, until use. In a subgroup of 172 samples (from 139 patients), a high-throughput method for Plaque Reduction Neutralizing Test (PRNT) was used for the quantification of neutralizing antibodies in plasma samples(1,2). Samples were heat-inactivated by incubation at 56°C for 30 min and 2-fold dilutions were prepared in Dulbecco modified Eagle medium (DMEM). The dilutions, mixed to a 1:1 ratio with a virus solution containing approximately 25 focus-forming units (FFUs) of SARS-CoV-2, were incubated for 1 h at 37 °C. Fifty microliters of the virus-serum mixtures were added to confluent monolayers of Vero E6 cells, in 96-wells plates and incubated for 1 h at 37 °C, in a 5% CO2 incubator. The inoculum was removed and 100 ml of overlay solution of Minimum essential medium (MEM), 2% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 U/ml) and 0.8% carboxy methyl cellulose was added to each well. After a 26-h incubation, cells were fixed with a 4% paraformaldehyde (PFA) solution. Visualization of plaques was obtained with an immunocytochemical staining method using an anti-dsRNA monoclonal antibody (J2, 1:10,000; Sci- cons) for 1 hour, followed by 1 h incubation with peroxidase-labeled goat anti-mouse antibodies (1:1000; DAKO) and a 7 min incubation with the True Blue (KPL) peroxidase substrate. FFUs were counted after acquisition of pictures on a flatbed scanner. Biosafety Level 3 laboratory setting was used for PRNT tests. The neutralization titer was defined as the reciprocal of the highest dilution resulting in a reduction of the control plaque count >50% (PRNT50). Samples recording titers equal to or above 1:10 were considered as positive according to a previous validation conducted on a panel of archived samples collected in 2018 in Italy(1).

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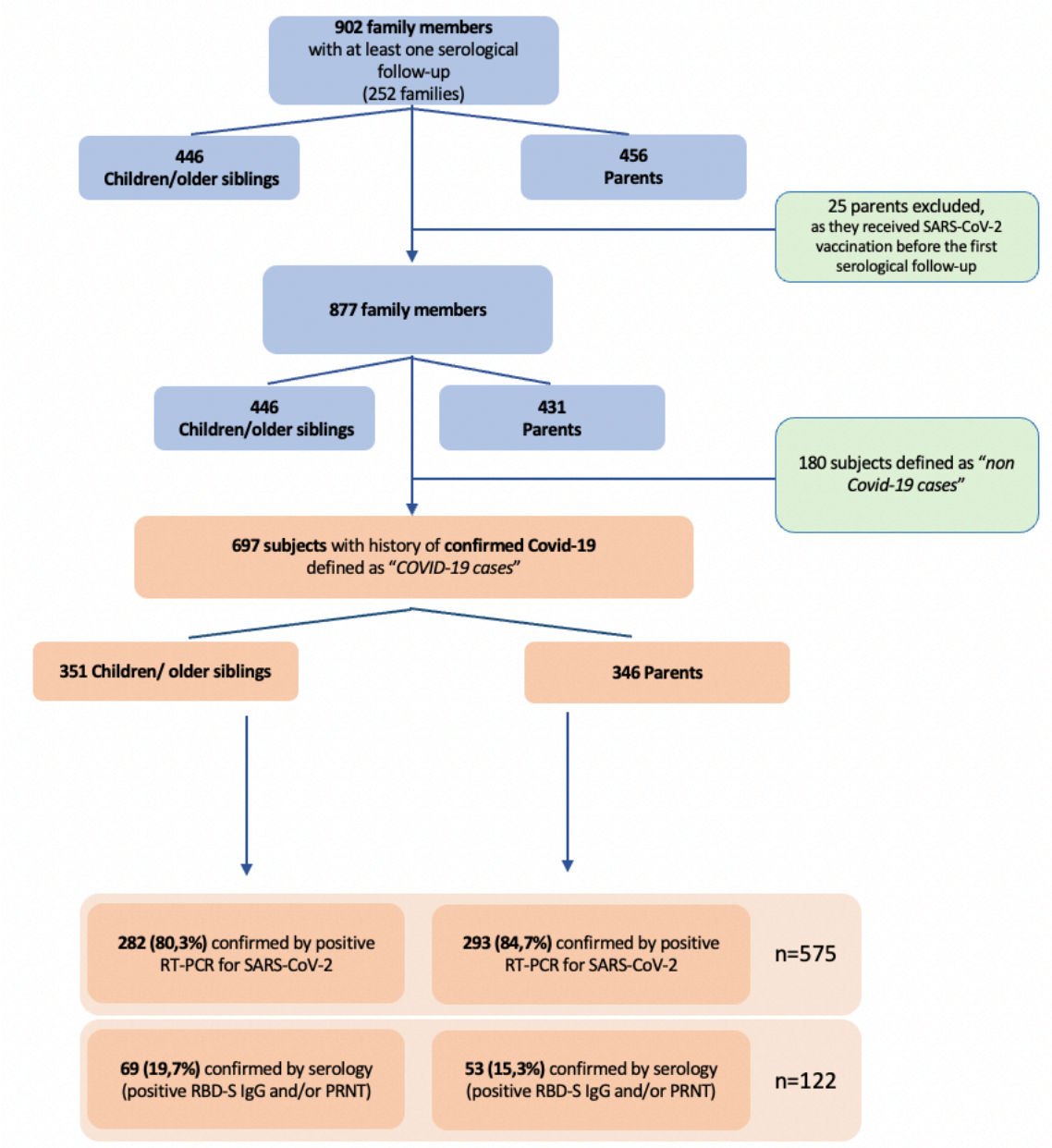
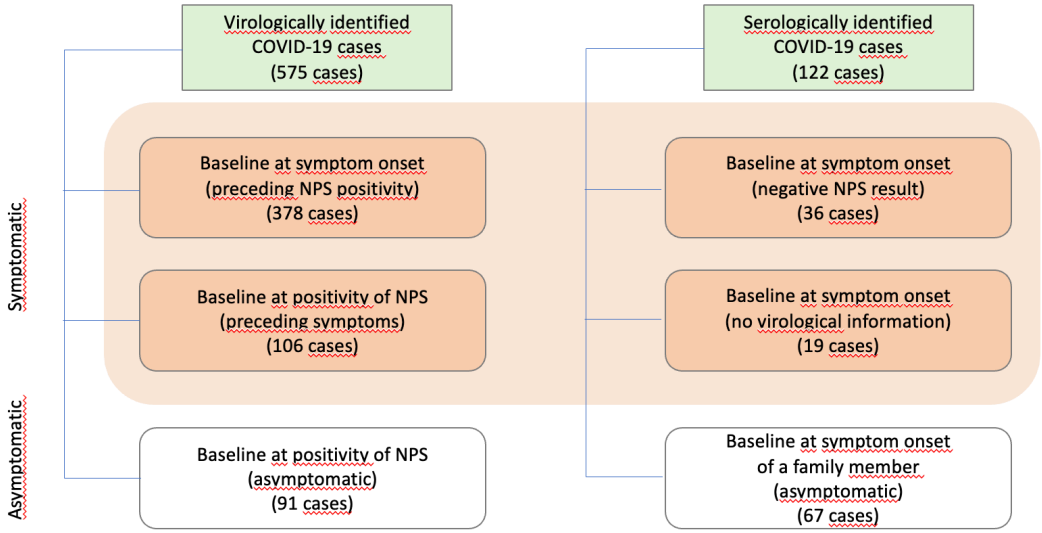
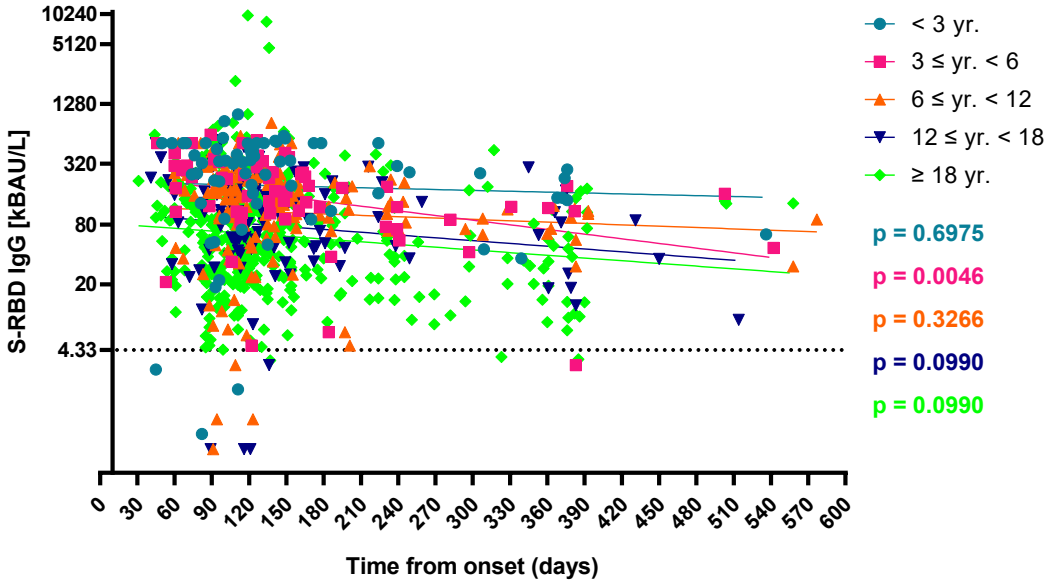
Supplementary Figure 1. Family Clusters of COVID-19 Observed From March 1st to August 6th 2021, at the COVID-19 Follow-up Clinic of Our Institution. Blue: whole cohort of enrolled subjects; green: individuals excluded from the analysis; orange: confirmed COVID-19 cases.



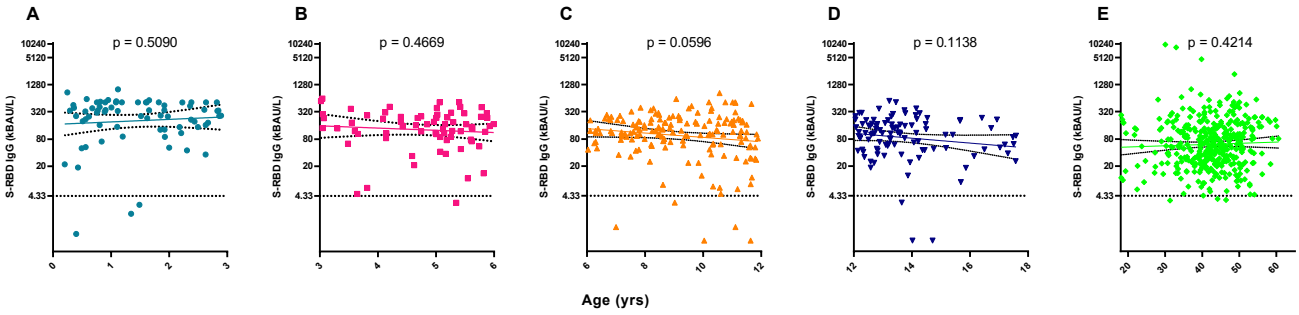
Supplementary Figure 2. Criteria for the Definition of the Baseline Time for COVID-19 Cases. For symptomatic cases the baseline of infection was defined as the onset of symptoms or the date of first positive SARS-CoV-2 molecular assay; while for asymptomatic cases as the date of the first positive molecular assay or by the family outbreak temporal sequence. Only for 67 (9,6%) asymptomatic cases with negative or not performed NPs, but with evidence of SARS-CoV-2 seropositivity, the baseline time was identified as the symptoms' onset of the first symptomatic family member.



Supplementary Figure 3. Distribution of S-RBD IgG Samples According to Time of Collection and Age Classes (n=769). Younger patients presented higher levels of Abs across all time points of samples collection. S-RBD IgG levels are reported in log2 scales. The dotted lines at 4.33 kBAU/L correspond to the assay cut-off for discriminating positive from negative samples.



Supplementary Figure 4. Distribution of S-RBD IgG Samples According to Age Classes Younger children presented a significantly higher levels of Abs than adults. S-RBD IgG levels are reported in log2 scales. The dotted lines at 4.33 kBAU/L correspond to the assay cut-off for discriminating positive from negative samples.



Supplementary Table 1. Demographic and Clinical Characteristics of the Analyzed Population, Overall (n=876) and Stratified by Familiar Status as Children or Older Siblings (n=446) and Parents (n=431).

	OVERALL					CHILDREN/OLDER SIBLINGS					PARENTS				
	COVID-19 negative		**COVID-19 positive**		*p-value* §	**COVID-19 negative**		**COVID-19 positive**		*p-value* §	**COVID-19 negative**		**COVID-19 positive**		*p-value* §
	(n=179)		(n=697)			(n=94)		(n=351)			(n=85)		(n=346)		
Female (n, %)	83	(46.4)	321	(46)	0.94	41	(43.6)	155	(44.2)	0.93	42	(49.4)	166	(48)	0.81
Age (mean, SD)	27.1	±18.6	25.4	±18.0	0.28	10.4	±5.9	8.6	±5.1	0.005	45.6	±6.1	42.5	±7.1	0.0003
Age classes (n, %):															
< 3 years	4	(2.2)	55	(8)		4	(4.3)	55	(15.7)		-	-	-	-	
3 ≤ years < 6	19	(10.6)	47	(6.7)		19	(20.2)	47	(13.4)		-	-	-	-	
6 ≤ years < 12	37	(20.7)	141	(20.2)	0.04	37	(39.4)	141	(40.2)	0.0002	-	-	-	-	-
12 ≤ years < 18	21	(11.7)	94	(13.5)		21	(22.3)	94	(26.8)		-	-	-	-	
≥ 18 years	98	(54.8)	360	(51.6)		13	(13.8)	14	(4)		85	(100)	346	(100)	
Symptomatic (n, %):	4	(2.2)	540	(77.5)	<0.0001	2	(2.1)	241	(68.7)	<0.0001	2	(2.3)	299	(86.4)	<0.0001
WHO classification* (n, %)**:**															
Asymptomatic	-	-	157	(22.5)		-	-	111	(31.9)		-	-	47	(13.6)	
Mild	-	-	516	(73.9)		-	-	231	(65.3)		-	-	285	(82.4)	
Moderate / severe	-	-	14	(2)	-	-	-	1	(0.3)	-	-	-	13	(3.8)	-
Critical	-	-	1	(0.1)		-	-	0	(0)		-	-	1	(0.3)	
MIS-C	-	-	9	(1.3)		-	-	9	(2.5)		-	-	0	(0)	
comorbidities:															
No						82	(87.2)	290	(82.6)	0.28	72	(84.7)	286	(82.4)	
Yes**						12	(12.8)	59	(17.4)		13 (15.3)		61	(17.6)	

§ T student test, $\chi 2$ test, Fisher exact test where appropriate. *WHO, World Health Organization; MIS-C, Multisystem Inflammatory Syndrome in Children. **The following co-morbidities were found among 59 COVID-19 positive children: premature birth (n=6), asthma (n=15), allergy (n=6), congenital heart disease (n=6), rheumatological disease (n=3), neuro-epileptic disease (n=5), metabolic disease (n=1), kidney/ureteral disease (n=4), endocrinological disease (n=2), gastrointestinal disease (n=4).

4.5. Left ventricular longitudinal strain alterations in asymptomatic or mildly symptomatic paediatric patients with SARS-CoV-2 infection

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Abstract

Aims.

Compared with adult patients, clinical manifestations of children's coronavirus disease-2019 (COVID-19) are generally perceived as less severe. The objective of this study was to evaluate cardiac involvement in previously healthy children with asymptomatic or mildly symptomatic severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection.

Methods and results.

We analysed a cohort of 53 paediatric patients (29 males, 55%), mean age 7.5 ± 4.7 years, who had a confirmed diagnosis of SARS-CoV-2 infection and were asymptomatic or only mildly symptomatic for COVID-19. Patients underwent standard transthoracic echocardiogram and speckle tracking echocardiographic study at least 3 months after diagnosis. Thirty-two age, sex, and body surface area comparable healthy subjects were used as control group. Left ventricular ejection fraction was within normal limits but significantly lower in the cases group compared to controls (62.4 ± 4.1% vs. 65.2 ± 5.5%; P = 0.012). Tricuspid annular plane systolic excursion (20.1 ± 3 mm vs. 19.8 ± 3.4 mm; P = 0.822) and left ventricular (LV) global longitudinal strain (−21.9 ± 2.4% vs. −22.6 ± 2.5%; P = 0.208) were comparable between the two groups. Regional LV strain analysis showed a significant reduction of the LV mid-wall segments strain among cases compared to controls. Furthermore, in the cases group, there were 14 subjects (26%) with a regional peak systolic strain below −16% (−2.5 Z score in our healthy cohort) in at least two segments. These subjects did not show any difference regarding symptoms or serological findings.

Conclusion.

SARS-CoV-2 infection may affect left ventricular deformation in 26% of children despite an asymptomatic or only mildly symptomatic acute illness. A follow-up is needed to verify the reversibility of these alterations and their impact on long-term outcomes.

Introduction.

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), responsible for the coronavirus disease-2019 (COVID-19) pandemic, has rapidly spread worldwide and represents a major concern for healthcare providers.1 SARS-CoV-2 infects host cells through ACE2 receptors, which are largely expressed in the lungs and heart. Furthermore, cardiac involvement seems to be the result of either direct viral damage or indirect effect, secondary to virus infection's immunological response. COVID-19 has been able to induce myocardial injury, myocardial infarction, myocarditis, and Takotsubo syndrome in a relevant number of adult patients.2–4 While most children with COVID-19 present with mild symptoms and generally have a good prognosis,5,6 data about the role of cardiovascular involvement in children with COVID-19 is still scarce. Growing evidence shows that some children, following COVID-19 recovery, may develop a severe multisystem inflammatory syndrome (MIS-C) with cardiac involvement in up to 80% of cases,7 such as reduced left ventricular (LV) systolic function, heart failure, and coronary artery abnormalities.8–10 However, little is known regarding cardiac involvement in paediatric patients with asymptomatic or mildly symptomatic SARS-CoV-2 infection.

Thus, this study aims to perform a detailed cardiac assessment, including standard echocardiography and speckle tracking echocardiography (STE), in previously healthy children who had an asymptomatic or mildly symptomatic SARS-CoV-2 infection.

Methods.

Study design and population.

A single-centre, retro-prospective observational study has been conducted on Italian family clusters of SARS-CoV-2 infection evaluated between 1 March and 10 September 2020. The study was carried out by the COVID-19 Family Cluster Follow-up Outpatient Clinic (CovFC), set up at the Department for Women's and Children's Health (W&CHD) of Padua University Hospital, in Veneto region, Italy. The CovFC programme included a clinical assessment provided by either Paediatrician trained on infectious diseases and/or by an Infectious Diseases specialist, an immunological assay for SARS-CoV-2 in both children and parents, and an echocardiographic evaluation only for children younger than 18 years old who had got infected. The study protocol was approved by the Institutional Review Board.

Families were enrolled 1–3 months after COVID-19 infection, through different institutional channels: (i) after hospitalization or after isolation upon diagnosis in the COVID-19 emergency room of the W&CHD; (ii) after receiving a home-based evaluation provided by family paediatricians in the Veneto Region. Inclusion criteria were as follows: (i) having children of paediatric age (0–18 years old); (ii) having a history of at least one confirmed intra-family COVID-19 case; and (iii) providing written informed consent.

The first evaluation included: (i) patient-based data collection; (ii) clinical evaluation of all children; (iii) collection of a blood sample for serological assessment for SARS-CoV-2 for all children/older siblings and their parents; and (iv) standard transthoracic echocardiogram (TTE) for only children with COVID-19

infection confirmed by either a positive SARS-CoV-2 molecular assay at nasal-pharyngeal swab (NPS) or a positive serology.

Data collection and definitions.

All information concerning the past and recent history were collected retrospectively at first evaluation through both patients' interviews and clinical file revision. Data were prospectively collected from enrolment according to a case report form and entered into a web-based database using the REDCap platform (Vanderbilt University, Tennessee). Patient-based data collection included demographic information (sex, date of birth), comorbidities and vaccination history, data on COVID-19-related diagnosis (such as clinical features, management infection details, time and result of SARS-CoV-2 molecular assays at NPS), and follow-up (serological assays, cardiac evaluations including echocardiogram). Authorized staff involved in data entry were provided with passwords for secure access to data. All data were collected, maintaining confidentiality, and were anonymized for statistical analysis.

Subjects who had previously tested positive for SARS-CoV-2 by RT-PCR were considered 'confirmed COVID-19' cases, together with patients with no record of virological positivity SARS-CoV-2 but showed evidence of seroconversion by either of the two serological tests adopted in this study, explained below. Subjects who had no record of infection or seroconversion were considered 'non-COVID-19 cases'; therefore, they were excluded from the analysis. For all COVID-19 cases, a 'baseline time' was defined as the most likely onset of infection, based on either symptoms outset or time of first virological positivity at molecular assay. Furthermore, for subjects with an asymptomatic infection and negative/not done NPS but with a serologically confirmed COVID-19, a baseline time was derived by the family outbreak temporal sequence.

The severity of COVID-19 was scored as mild, moderate, severe, and critical following the WHO classification based on clinical features, laboratory testing, and chest radiograph imaging (when available).11

Control group.

Thirty-two healthy controls, comparable for age, sex, and weight, were consecutively recruited from the Pediatric Cardiology Outpatient Clinic of the W&CHD. Controls were enrolled among subjects referred for atypical chest pain or innocent murmur, otherwise healthy, not on any medication, and with normal cardiac evaluation, EKG, and echocardiogram. Specifically, we retrospectively selected only the subject with adequate quality and suitable views of 2D TTE in order to perform longitudinal strain (LS) analysis.

Serological assays.

Subjects were sampled to collect sera and detect IgG and IgM targeting a recombinant nucleocapsid (N)-spike (S) protein of SARS-CoV-2, with the chemiluminescence immunoassay MAGLUMI™ 2019-nCoV IgM/IgG on the analytical system MAGLUMI™ 2000 Plus (New Industries Biomedical Engineering Co., Ltd [Snibe], Shenzhen, China). According to the manufacturer's inserts (271 2019-nCoV IgM, V2.0, 2020-03 and 272 2019-nCoV IgG, V1.2, 2020-02), the 2019-nCoV IgM cut-off is 1.0 AU/mL, while the 2019-nCoV IgG cut-

off is 1.1 AU/mL. From the same subjects, plasma samples were taken to perform a 50% plaque reduction neutralization test (PRNT50). The neutralization titer was defined as the reciprocal of the highest dilution resulting in a reduction of the control plaque count >50% (PRNT50). Samples recording titers equal to or above 1:10 (or 1 on a log10 scale) were considered positive according to a previous validation conducted on a panel of archive samples collected in 2020 in Italy.12

Cardiac evaluation.

Children recognized as COVID-19 cases underwent standard cardiac evaluation within 6 months from baseline time, including electrocardiogram (ECG) and TTE. Standard TTE study was performed using the GE Vivid E9 Ultrasound System (GE Healthcare, USA) following the recommendations for cardiovascular imaging during COVID-19 pandemic.13,14 Left ventricular ejection fraction (LVEF) was calculated by TTE using the modified Simpson method (biplane method of disks), while LS analysis of the left ventricle, through 2D STE analysis, was performed offline using GE EchoPac Software (GE Healthcare, USA). Our methodology for STE study has been previously described.15,16 Briefly, the best apical four-, two- and three-chamber views to visualize the LV segments were selected. Afterward, three points (two annular and one apical) were positioned, enabling the software to track the myocardium semi-automatically throughout the heart cycle. The region of interest was adjusted with careful inspection of the endocardial border, and manual correction was performed if needed. The automated algorithm allowed global longitudinal strain (GLS) to be calculated. Left ventricular LS by speckle tracking was defined as the average peak negative value on the strain curve during the systole (end of T-wave on the ECG) of all the studied segments.17 The peak negative systolic strain value for each regional LV segment was also analysed. Analysis of the standard TTE and STE was performed by an experienced echocardiographer blind to the clinical data. Therefore, we compared echocardiographic results with the control group. Coronary arteries diameter was measured on 2D TTE and the respective z-scores were estimated based on previous reported data.18

Reproducibility.

The data of reproducibility of our Echo Lab for standard TTE parameters as well as for STE has been already published.19

Statistical analysis.

Categorical variables were presented as percentage (%), and continuous variables as mean ± standard deviation. Shapiro–Wilk test and histogram were used to test normality for each variable. Student's t-test was performed for normally distributed continuous variables and Mann–Whitney U test for non-parametric continuous variables. Chi-square test was performed for categorical variables to examine if there were significant differences between the groups. In multiple hypothesis testing, the Bonferroni correction test was used to control the occurrence of false positives. Statistical analysis was performed using STATA 14.0 MP (StataCorp LP, TX, USA).

Results.

From 1 March to 10 September 2020, we enrolled 67 children among the COVID-19 cases who had a full cardiac evaluation. Among these, 64 had asymptomatic or only mildly symptomatic COVID-19 infection (WHO stages 0 or 1), while the remaining three patients had an infection with more than mild symptoms (WHO ≥ 2). Therefore, they were excluded from our analysis. All 64 included children were previously healthy, without evidence of previous cardiac disorders. A cardiac evaluation, including ECG and echocardiography, was performed after a mean time of 3.7 ± 1.6 months since COVID-19 disease's onset (baseline time). ECG showed sinus rhythm in all cases. Only five patients (9%) presented an abnormal ECG (four cases presented anomalies in the repolarization phase and one patient sinus bradycardia). Interestingly, there was no significant difference in the prevalence of ECG abnormalities among the two groups of patients with different degree of LV LS impairment. In the offline echocardiogram review, 11 patients were excluded because of inadequate quality of 2D scans (≥2 segments not visualized) to perform STE analysis (Figure 1). The remaining 53 patients formed our cases group.

Figure 1. Patient inclusion flowchart.

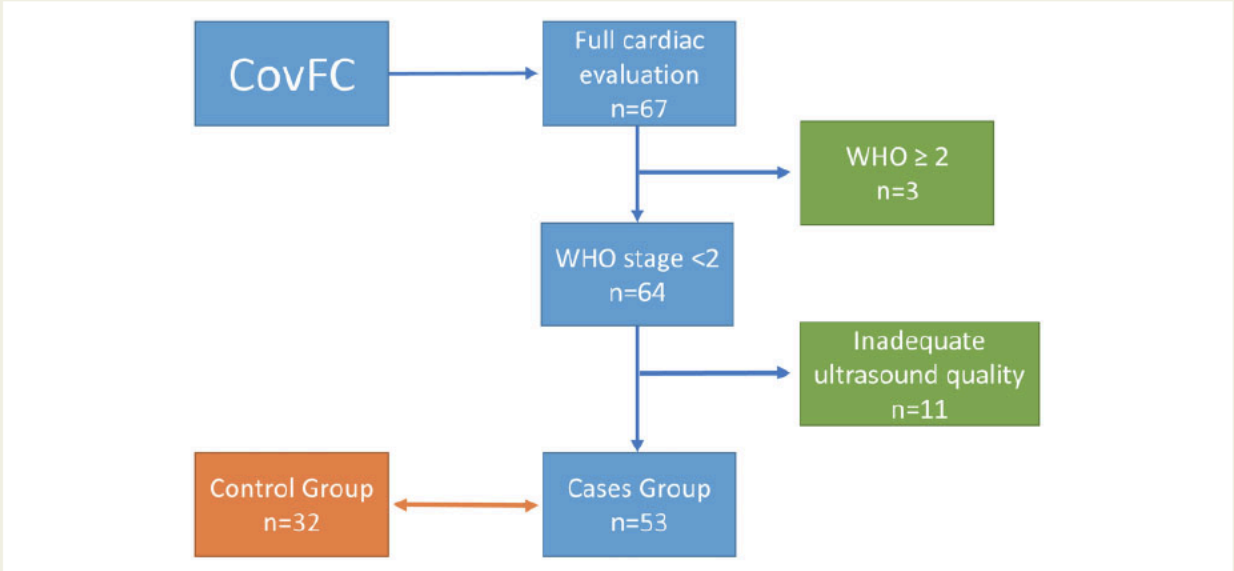

Figure 1 Patient inclusion flowchart. The flowchart shows patients enrolment from the COVID-19 Family Cluster Follow-up Outpatient Clinic (CovFC), with mild or no symptoms and full echocardiogram to perform speckle tracking analysis.

Cases (n = 53) and controls (n = 32) were comparable for age (7.5 ± 4.7 years vs. 8 ± 4.9 years; P = 0.673), gender (29 males—55% vs. 18 males—56%; X2 = 0.019; P = 0.89), and body surface area (0.98 ± 0.3 m2 vs. 0.8 ± 0.4 m2, P = 0.17) (Table 1). Among the cases, 12 patients (23%) had asymptomatic COVID-19 infection (WHO = 0), while the remaining 41 (77%) showed only mild symptoms (WHO = 1) (Table 2). According to SARS-CoV-2 serological assays performed after a mean time of 96 ± 41 days from baseline, 2019-nCoV IgM mean value was 0.7 ± 0.54 AU/mL, 2019-nCoV IgG mean value was 5.3 ± 6.45 AU/mL, and PRNT Log10 mean value was 4.7 ± 1.71.

Table 1. Cases and controls demographical characteristics.

Table 1 Cases and Controls demographical characteristics

	Cases ($n = 53$)	Controls ($n = 32$)	*P*-value
Gender (males)	29 (55%)	18 (56%)	$X^2 = 0.019$; $P = 0.89$
Age (years)	7.5 ± 4.7	8 ± 4.9	0.673
BSA (m^2)	0.98 ± 0.3	0.8 ± 0.4	0.17

BSA, body surface area.

Table 2. Clinical features of children with COVID-19.

Table 3. Cases and controls standard echo characteristics.

Table 2 Clinical features of children with COVID-19

	N (%)
Asymptomatic patients (WHO = 0)	12 (23)
Mildly symptomatic patients (WHO = 1)	41 (77)
Fever	28 (53)
Congestion or runny nose	8 (15)
Cough	9 (17)
Myalgia	2 (4)
Arthralgia	1 (2)
Sore throat	4 (8)
Smell and taste changes	2 (4)
Abdominal pain	2 (4)
Fatigue	4 (8)
Headache	4 (8)
Nausea or vomiting	3 (6)
Diarrhoea	5 (10)
Loss of appetite	1 (2)
Cutaneous rash	3 (6)
Two or more symptoms	22 (42)

Table 3 Cases and Controls standard echo characteristics

	Cases ($n = 53$)	Controls ($n = 32$)	*P*-value
LVEDd (mm)	35.9 ± 7.6	35.8 ± 7.7	0.96
LVEDd (z-score)	-0.87 ± 1.45	-0.7 ± 1.4	0.63
LVESd (mm)	22.9 ± 4.0	23.8 ± 6.1	0.68
LVESd (z-score)	-0.20 ± 1.68	-0.22 ± 1.2	0.95
E/A ratio	1.82 ± 0.31	1.7 ± 0.3	0.62
Dec time	140 ± 38.8	139.9 ± 27.5	0.99
E/E′ ratio	5.8 ± 1.6	5.8 ± 1.4	0.93
TAPSE (mm)	20.1 ± 3	19.8 ± 3.4	0.82
LVEF (%)	62.4 ± 4.1	65.2 ± 5.5	**0.01***
GLS (%)	-21.9 ± 2.4	-22.6 ± 2.5	0.21

GLS, global longitudinal strain; LVEDd, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; LVESd, left ventricular end-systolic diameter; TAPSE, tricuspid annular plane systolic excursion.
**P*-value < 0.05.

Standard echocardiographic study.

Left ventricular end-diastolic diameter was similar among cases and controls (35.9 ± 7.6 mm vs. 35.8 ± 7.7 mm; P = 0.964) (Table 3). LVEF was significantly lower in the cases group than controls (62.4 ± 4.1% vs. 65.2 ± 5.5%; P = 0.012), although both in the normal range. All cases showed an LVEF ≥ 55%. Among cases, we could not appreciate a correlation between LVEF value and time from infection diagnosis (Ro −0.19, P = 0.17). Furthermore, the cases group showed no LV diastolic dysfunction with a mean E/A ratio of 1.9 ± 0.55 and mean E/E′ ratio of 6.4 ± 1.3. We did not appreciate any significant coronary artery dilation (z scores all <+2) or pericardial effusion among cases. Finally, right ventricular longitudinal function, measured

using tricuspid annular plane systolic excursion parameter, was comparable among the two groups (20.1 ± 3 mm vs. 19.8 ± 3.4 mm; P = 0.822).

Speckle tracking echocardiographic analysis.

LV global longitudinal strain (LV-GLS) did not show any difference between the two groups (−21.9 ± 2.4% vs. −22.6 ± 2.5%; P = 0.208). Similar to LVEF, we did not highlight a correlation between LV-GLS and time from infection diagnosis (Ro 0.21, P = 0.13). However, strain segmental analysis of the LV showed significant strain reduction of the LV mid-wall segments among cases, compared to controls. On the other hand, two apical segments displayed higher deformation in cases compared to controls (Table 4). Thus, there was a higher base to apex gradient in our patients' cohort than in controls (Figure 2).

Figure 2. Left ventricular longitudinal strain Bull's Eye plot.

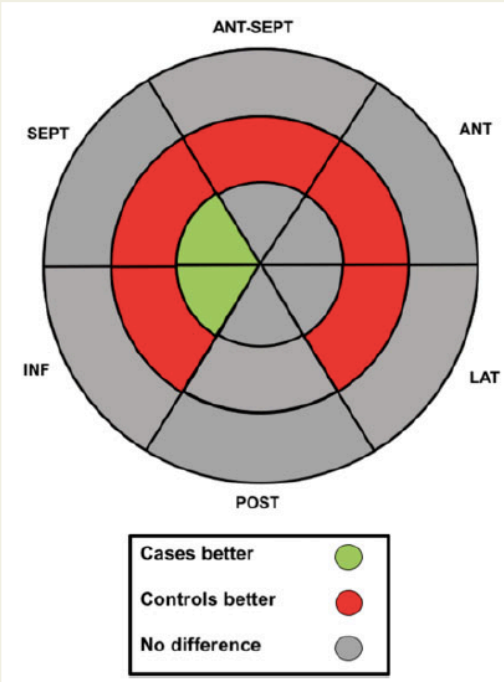

Figure 2 Left ventricular longitudinal strain Bull's Eye plot. Bull's eye representation of segmental LV longitudinal strain differences between cases and controls. In red, the segments with reduced deformation in cases compared to controls. In green, the segments with increased strain values among cases compared to controls. In grey, the segments with no statistical difference between the two groups.

Furthermore, in the cases group, there were 14 subjects (26.4%) with a strain lower than −16% (corresponding to the mean strain value minus 2.5 SD in our studied healthy cohort) in ≥2 segments. COVID-19 subjects with more compromised LV regional LS (i.e. ≥2 segments below −16%) did not show any difference compared to the remaining cases regarding the presence of symptoms, serological findings (IgM, IgG, and PRNT log10), or age. For the latter, a not statistically significant trend was documented towards older age in the most affected sub-group (9.4 ± 4.9 vs. 6.9 ± 4.5, P = 0.09) (Table 5).

Table 4. Regional LV longitudinal strain deformation analysis.

Table 4 Regional LV longitudinal strain deformation analysis

LV segmental LS	Cases ($n = 53$)	Controls ($n = 32$)	*P*-value
LV GLS (%)	-21.9 ± 2.4	-22.6 ± 2.5	0.208
LV basal septum	-18.7 ± 2.6	-21.1 ± 6.3	0.021
LV mid septum	-21.6 ± 3.8	-25.5 ± 6.1	**<0.001***
LV apical septum	-26.1 ± 4.8	-20.7 ± 5.1	**<0.001***
LV apical lateral	-24.5 ± 5.3	-21.8 ± 2.9	0.048*
LV mid antero-lateral	-20.7 ± 3.7	-25.4 ± 8.4	**<0.001***
LV basal antero-lateral	-19.6 ± 6.1	-20.3 ± 6.2	<0.617
LV A4C LS (%)	-21.9 ± 2.9	-22.2 ± 2.8	0.582
LV basal inferior	-21.4 ± 3.3	-21.5 ± 4.8	0.846
LV mid inferior	-22.9 ± 2.9	-25.5 ± 5.0	0.005
LV apical inferior	-26.5 ± 4.1	-20.6 ± 11.3	**0.002***
LV apical anterior	-23.4 ± 5.2	-19.4 ± 5.0	0.011
LV mid anterior	-21.3 ± 4.3	-27.4 ± 8.8	**<0.001***
LV basal anterior	-21.2 ± 3.7	-25.2 ± 8.3	0.006
LV A2C LS (%)	-22.8 ± 2.9	-22.9 ± 4.8	0.923
LV basal posterior	-19.1 ± 5.9	-22.2 ± 4.9	0.027
LV mid posterior	-20.5 ± 3.4	-25.0 ± 5.6	**<0.001***
LV apical posterior	-23.3 ± 4.5	-21.5 ± 2.9	0.086
LV apical septal	-24.2 ± 5.8	-20.7 ± 5.5	0.020
LV mid antero-septal	-21.5 ± 4.0	-27.3 ± 8.6	**<0.001***
LV basal antero-septal	-19.1 ± 3.4	-20.3 ± 5.5	0.225
LV A3C LS (%)	-21.2 ± 3.0	-22.8 ± 2.4	0.014

A2C, apical 2 chamber; A3C, apical 3 chamber; A4C, apical 4 chamber; GLS, global longitudinal strain.
*P-value < 0.002 (Bonferroni correction).

Table 5. Clinical and serological characteristics of COVID-19 cases.

Table 5 Clinical and serological characteristics of COVID-19 cases

	≥2 LV segments abnormal ($n = 14$)	<2 LV segments abnormal ($n = 39$)	*P*-value
Age (years)	9.4 ± 4.9	6.9 ± 4.5	0.09
Symptoms (WHO = 1) (%)	11 (79%)	30 (77%)	$X^2 = 0.016$; $P = 0.89$
Time from diagnosis (days)	118 ± 39	104 ± 41	0.27
LVEF (%)	61 ± 3.4	63 ± 4.3	0.14
GLS (%)	-19.6 ± 1.6	-22.7 ± 2.1	<0.001*
Positive NPS (%)	11 (79%)	30 (77%)	$X^2 = 0.016$; $P = 0.89$
IgM (AU/mL)	0.617 ± 0.58	0.76 ± 0.52	0.44
IgG (AU/mL)	4.78 ± 8.56	5.46 ± 5.72	0.75
PRNT $\log_{10}$	4.1 ± 1.8	4.9 ± 1.7	0.17

COVID-19 patients divided in two subgroups according to the number of LV segments with reduced LV longitudinal strain (below -16%).
GLS, global longitudinal strain; LVEF, left ventricular ejection fraction; NPS, nasal-pharyngeal swab; PRNT, plaque reduction neutralization test.
*P-value < 0.05.

Discussion.

Our study provides new insights on the cardiac impact of COVID-19 among paediatric patients, showing for the first time that 26% of children recovered from an asymptomatic or mildly symptomatic COVID-19 present a mild subclinical cardiac involvement at 3 months after the infection. This finding is of great interest as the cardiac involvement does not correlate to the severity of COVID-19 clinical manifestations.
In contrast to adults, SARS-CoV-2 usually leads to a mild illness in children.20,21 However, it has been described that MIS-C associated with SARS-CoV-2 infection may occur, presenting with significant cardiac involvement in up to 80% of cases7 and often leading to myocardial abnormalities with a significant reduced LVEF, and abnormal LV regional LS, valve regurgitation and coronary arteries dilation.10,22–25 On the other side, lack of knowledge still regards the cardiovascular involvement in children with asymptomatic and/or mildly symptomatic COVID-19.

Recent evidence suggests myocardial and pericardial involvement in young athletes after COVID-19 recovery, with a significant proportion of them showing pericardial enhancement on CMR.26,27 In our population, we did not find signs of pericardial effusion on TTE, however, the shorter timing of imaging in respect to active viral disease and different sensitivity of used imaging modalities might explain this only apparent difference. In our paediatric COVID-19 cohort, standard echocardiography showed preserved LVEF, although significantly lower than controls, and normal LV diastolic function. Despite mean LV GLS in COVID-19 children did not differ significantly from that of the control group, we found differences regarding regional strain analysis of the left ventricle, with the most affected segments in the COVID-19 group being the mid-wall ones and the basal anterior, posterior and septal inferior ones compared with the control group. Conversely, the apical segments showed higher deformation in the COVID-19 group. This finding is in agreement with the distribution of affected areas of the left ventricle in MIS-C patients, which does not follow coronary distribution.28 Our data are of particular interest since we demonstrated subclinical cardiac involvement even in children who had an asymptomatic or mildly symptomatic COVID-19, persisting at least 3 months after the infection. In accordance with Piccinelli et al.29 we found that apical segments were spared or even showed increased deformation, increasing the base to apex gradient. This pattern has already been described in another cardiac diseases like systemic hypertension.30

In COVID-19 adult patients, Croft et al.31 reported a lower mean LV GLS than in healthy populations, even in the presence of preserved LVEF. Moreover, a recent study observed significantly increased mortality alongside with decrease in LV GLS in adult patients with COVID-19.32 Interestingly, we found a good proportion of our cohort (26.4%) having a regional strain value <−16% in at least two LV segments. Due to the significant inter-vendor variation in normal values, this cut-off value was calculated based on our control group mean LV GLS value minus 2.5 SD. This value was significantly below the normality range proposed in a large meta-analysis in children by Levy et al.,33 and significantly lower than the 5th percentile for LS

proposed for healthy children by Cantinotti et al.34 Although abnormal systemic inflammatory response following infection is a described mechanism of indirect myocardial injury, in our subset of patients with reduced LS we could not demonstrate any difference regarding symptoms or levels of SARS-CoV-2 antibodies. These data suggest that COVID-19 clinical features and the degree of the immune system response following the infection may not predict the subclinical myocardial injury's extension. Thereafter, the virus, per se, could directly affect the heart in a significant proportion of patients. Our study may suggest the need to implement a cardiac evaluation for virtually all children affected by COVID-19, irrespectively from their initial clinical presentation. Besides, the clinical value and reversibility of the abnormal longitudinal myocardial deformation properties should be further investigated in order to evaluate the necessity of an extended cardiac follow-up of this cohort of patients.

The single-centre nature of our study may constitute a major limitation. However, we believe that this should be considered a pilot study enhancing further research, including larger cohorts, on this topic. COVID-19 cases were enrolled at least three months after SARS-CoV2 infection. This delay may have influenced the proportion with an abnormal regional strain of the left ventricle. Nevertheless, one-quarter of our cohort presented LV deformation abnormalities late after infection. Unfortunately, we were not able to enroll a higher number of control subject due to the nature of our Institution (tertiary care hospital), the time constrains of COVID-19 and the very limited access to elective cases during the COVID pandemic. Finally, the control group was not matched with cases but resulted in comparable demographic characteristics.

Conclusion.

SARS-CoV-2 infection may affect LV cardiac mechanics in a quarter of asymptomatic or mildly symptomatic children, with persistence at least three months after the infection. The cardiac involvement does not seem to be related to the SARS-CoV-2 humoral response nor the clinical presentation of COVID-19.

The clinical significance of these findings is unclear and should be further investigated. Moreover, the development of dedicated paediatric follow-up programmes would be able to verify the reversibility of these alterations.

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4.6. Psychological impact and resilience of children and parents experiencing a Covid-19 family cluster

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Abstract

Behavior and mental health may be impaired during SARS-CoV-2 infection; therefore, we explored the psychological impact in COVID-19 family clusters. A cross-sectional web-based survey was conducted on families attending the COVID-19 Follow-up Clinic, at the Department for Women's and Children's Health, Padua (Italy). From March to October 2020, 75 survey were collected from 66 families (97 parents and 129 children); almost 70% of subjects had COVID-19, mostly asymptomatic/mildly symptomatic, and median time from infection to survey compilation was 164,7 days (SD 56). Most (>87%) parents reported positive relationships within family members either before, during, or after COVID-19. More than one third of children and adolescents showed a scarce ability to adapt to isolation. Among 31 pre-school children of median age of 3 years (SD 1,7), a change of one or more functions was reported for 74,2% of cases irrespectively of COVID-19 status, particularly a change in circadian rhythm (25%), in relationship with parents (42,8%) and poor emotional control (36%). Among 74 children of 10,9 years median age (SD 2,7) 8,1% had a score indicating a disease, however a significant impairment in attention was reported for 16,7%, while anxiety/depression and problems with conduct for 5,6% and 6,5% of cases, respectively.

Keywords

COVID-19, children, adolescents, family cluster, SARS-CoV-2, resilience, psychological

Introduction

Coronavirus Disease 2019 (COVID-19), caused by the highly transmissible and pathogenic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has so far affected about 554 million people worldwide, leading to more than 6.3 million deaths (WHO Coronavirus (COVID-19) Dashboard, July 13th 2022, available at https://covid19.who.int/). The global spread of the COVID-19 pandemic has been severely affecting public health systems and economies worldwide, as well as it has changed our approach to infectious diseases.
Before the development of SARS-CoV-2 vaccines, the adoption of stringent social restrictions combined with other infection prevention measures were the only strategies to contain the viral spread thus reducing SARS-CoV-2-related morbidity and mortality (1). If such approaches allowed to reduce the burden of COVID-19 and the risk of overwhelming healthcare resources, on the other hand, the social distancing mandate, as occurred during the lockdown, has been associated with negative impacts on the physical and mental health of individuals (2,3). This sudden situation forced people to engage all the resources on the emotional-relational level to cope with the event, especially for families with children for whom the relationships and socio-educational support foundations for development have been lacking. The search for a new adaptation at intra-family and socio-relational levels seems to represent the effort to counteract the impotence and the anguish of death evoked by the pandemic.

Several studies reported high rates of stress, psychological distress, anxiety and depression during the first year of the pandemic in both pediatric and adult populations, reflecting the impact of the COVID-19 pandemic on the mental health and wellbeing of children, adolescents and adults (4–8). Although preliminary evidence showed a correlation with the immune dysfunction, including nonspecific neuroinflammation and antineural autoimmune dysregulation (9), recently studies observed no differences between infected and uninfected subjects, highlighting the importance of quarantine and social distance as a pathophysiological mechanism of neurocognitive, behavioral, and mental health consequences in the general population (8). Firstly, it was shown that physical environment changes, including reduction of spaces, lack of natural views, and reduction of sunlight exposure that people experienced during quarantine, especially in the first pandemic wave, had a relevant impact on people's mental health (10,11). In addition, the current COVID-19 pandemic has profoundly altered our life in a very short period of time; the smart-working and home-schooling introduction, home-shopping implementation, and virtual meeting adoption lead to profound changes in lifestyle habits among individuals. Moreover, the role of fear of unknown contagious diseases, anxiety for family members or friends infected with the novel SARS-CoV-2, limited access to health care services, and stigma of infectious diseases, leading to isolation from others and withdrawal, have a considerable impact on children and adults' emotional wellbeing and mental health (12).

The restrictive measures and social isolation have especially implied an unprecedented strong impact on families' daily routines (13,14), resulting in changes in domestic dynamics (15). The homeschooling

conditions and the implementation of screen time and virtual meeting strategies among kids and adolescents have impacted caring skills, enhancing parents' feelings of inability to care for their children (16). In addition, parental worry related to losing their own work, financial instability, and anxiety to return to a sense of normality, could affect the partner's relationship and consequently negatively impacted children's mental health (17). Moreover, since the children and adolescents' psychological wellbeing depends also on the educational and playful activities they usually carry out, the lockdown from COVID-19, with the interruption of daily experiences, reflected in behavioral consequences and on social problems with peers and negative feelings (18,19).

Given the clinical and social relevance of families' mental health, it is important to implement the knowledge on the psychosocial impact of the current pandemic in adults and children who experienced COVID-19 in their households. Furthermore, assessing the resilience of children to a stressful event would allow understanding the personal resources of children and adolescents to cope with difficulties. Herein, conducting an online survey on COVID-19 family clusters attending the Infectious Diseases Unit of the Department of Women's and Children's health of the University of Padua, we aimed to explore the impact of the COVID-19 pandemic in a multidimensional and multi-professional model of work. From this perspective, we evaluated the psycho-social characteristics of a cohort of Italian families observed during the first year of the pandemic, including children and their parents.

Methods

Study design and participants

We provide findings from a single-center, cross-sectional study aimed at exploring the psychological impact of experiencing SARS-CoV-2 infection among one or more family member/s. The study was conducted on Italian families attending the COVID-19 Family Cluster Follow-up Clinic (CovFC), at the Department of Women's and Children's Health of the University Hospital of Padua (Veneto Region, Italy). From March 2020, families were referred to the Clinic by their family pediatrician (FP) 4-12 weeks after the end of isolation, if meeting the following inclusion criteria: a) having children of pediatric age, and b) at least one family member with a history of COVID-19. At enrolment, a pediatrician collected data on demographic parameters, past medical history, vaccinal status, and performed a clinical evaluation. At each follow-up visit, blood samples were collected from all cases for serological assessment of SARS-CoV-2 infection, through either the detection of the anti-receptor binding domain (RBD) antibodies against SARS-CoV-2 spike protein (MAGLUMI™2000 Plus, Snibe Diagnostics, New Industries Biomedical Engineering Co.) and/or the quantification of SARS-CoV-2 neutralizing antibodies with a high throughput method for Plaque Reduction Neutralization Test (PRNT) (20).

From March to October 31st, 2020 at each follow-up visit families were informed of the opportunity to be supported by a psychosocial team. Health Care Workers (HCWs) collected the consent of the parents/tutors to be contacted for participating in the psychosocial project. After receiving their consent, a psychologist contacted each family by phone, to explain the goal of the study; an ad-hoc questionnaire was further sent by email. The questionnaires were collected until December 2020. Participation in the study was not related to an economical compensation. The study was approved by the Ethical Committee of the Department (Prot. N° 0070714 of November 24th,2020; amendment N°71779 of November 26th, 2020). Parents or legally authorized representatives were informed of the research proposal and provided written consent for use of the routine patient-based data for research purposes.

Study Instruments.

A web-based survey was developed and on-line distributed to a cohort of 88 Covid-19 family clusters enrolled at CovFC. The online survey was developed using the REDCap® platform (Vanderbilt University, Tennessee) hosted on the server of the University of Padua and it was shared by email to all parents that explicitly agreed to take part in the study, for the period of time from March 2020 to April 2021.

A questionnaire was elaborated for the retrospective collection of data concerning the psycho-social impact of families experiencing Covid-19 and the children's ability to adapt to SARS-CoV-2 related disease and home isolation. The survey included two main sessions: the first part, defined as "*Questionnaire A – Family"*, was elaborated ad hoc by a team including two Psychologists, a Social Assistant, Paediatricians, and an Infectious Diseases Specialist. The questionnaire specifically explored the quality of relationships within the family actors retrospectively referring to the time either before, during, and after Covid-19.

A second part was further elaborated, defined as "*Questionnaire B - Children*" and specifically dedicated to the children's behavior observed during Covid-19 and to the children's ability to adapt to Covid-19 disease and to home isolation. According to the age of children, "*Questionnaire B - children*" included two mutually exclusive parts: "*Questionnaire B/1 – pre-school children*" and "*Questionnaire B/2 -Pediatric Symptoms Checklist* ". The "*Questionnaire B/1 – pre-school children*" was elaborated at hoc by the team for children of less than 6 years of age, while for "*Questionnaire B/2 - Pediatric Symptoms Checklist* " the Pediatric Symptom Checklist (PSC) was used as a validated tool to evaluate children aged from 6 to 17 years, to improve the recognition of psychosocial problems (21). All the questions were completed by the parent. For "*Questionnaire A – Family*" both parents were allowed to provide their answer, however for "*Questionnaire B - Children*" specifically dedicated to children, only one parent was asked to provide answers. In the case of multiple children, the parent was asked to compilate one survey for each child. The PSC questionnaire provides a total scores and three specific subscales scores: the "Attention Problems" subscale which deepens the attention and concentration impairment; the "Internalizing Problems" subscale which investigates the anxiety and depressive symptoms; the "Externalizing Problems" subscale that investigates the behavioral and the conduct impairments.

Clinical data collection and definitions

Clinical data collected during follow-up visits were entered into a web-based database using the REDCap® platform. Data were also collected retrospectively from the existing clinical files and analyzed anonymously. Subjects were considered *confirmed COVID-19 cases* if they had a record of virological positivity for SARS-CoV-2 by real-time RT-PCR and/or resulted positive by either of the two serological tests adopted in this study. For each confirmed COVID-19 case, a *baseline date* was defined as follows: 1) for symptomatic cases: the first date between the onset of symptoms or the date of first positive SARS-CoV-2 molecular assay; 2) for asymptomatic cases: the date of the first positive molecular assay or, in those with only serologically confirmed COVID-19 and with negative/undetermined nasal-pharyngeal (NP) swab, by the family outbreak temporal sequence, coinciding with the date of symptoms onset in the family cluster. Subjects that were asymptomatic and had no analytical evidence of SARS-CoV-2 infection were considered non-COVID-19 cases. The severity of COVID-19 was scored as mild, moderate, severe, critical, following the WHO classification (22).

Two periods of time or "Covid-19 waves" were identified and defined as follows: a first wave occurring from February 17th to September 18th, 2020, and a second wave from September 19th, 2020 to February 18th, 2021.

Statistical analysis.

A descriptive analysis of children and adults belonging to families that provided answers from at least one survey of the two proposed *"Questionnaire A - Family"* and *"Questionnaire B- Children"* was conducted. Counts and percentiles were provided for children and parents, overall and stratified by survey editors. Demographic data, comorbidities, Covid-19 diagnosis and clinical presentation, and time from disease to survey compilation were evaluated.

Answers provided on "*Questionnaire A -Family*" were evaluated, overall and stratified for Covid-19 pandemic waves (1st wave versus 2nd wave) according to the time of Covid-19 diagnosis and the non-parametric Fisher exact test and the Mann-Whitney test were used to assess differences among either categorical or continuous covariates, respectively.

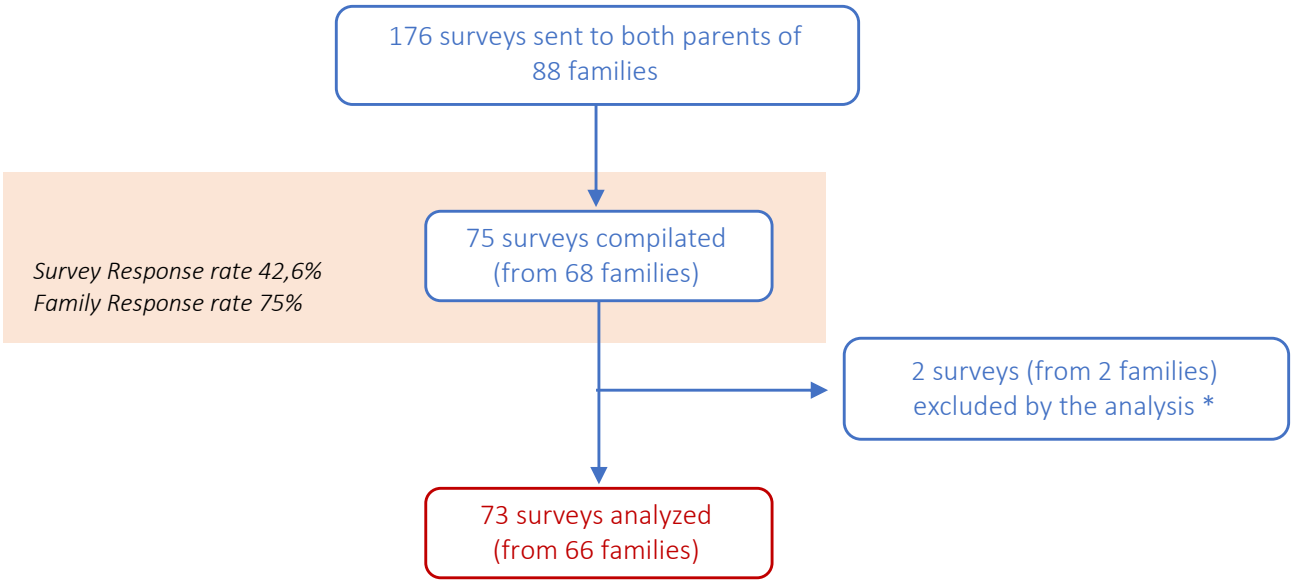
Data collected by the "*Questionnaire B/1 – pre-school children"* and by the *"Questionnaire B/2 - Pediatric Symptoms Checklist"* were evaluated, overall and stratified between confirmed Covid-19 versus Covid-19 negative cases and the Fisher exact test and T test were used to assess differences among either categorical or continuous covariates. Statistical analysis was conducted using Stata-IC v. 15.1; a P value <0.05 was considered to be statistically significant.

Results

From March 2020 to April 2021, 176 web-based surveys were distributed by email to mothers and fathers of a cohort of 88 families attending the CovFC. Seventy-five surveys from 68 families were filled out, accounting for a 75% (68/88) of family response rate: for 7 families answers came from both parents (accounting for 14

surveys) while for the remaining 59 families only one parent provided answers (accounting for 59 surveys). Two surveys from 2 families were excluded from the analysis (figure 1). After the compilation of the questionnaire, 42 (47.7%) families requested an online based clinical psychological interview. In addition, in 5 pediatric cases we highlighted the need for a psychological and/or psychiatric deepening, therefore they were sent to a dedicated neural-psychiatric service.

Figure 1. Flow-chart of 73 surveys analyzed out of 176 *"Questionnaire A - Family"* distributed to 97 parents, enrolled at CovFC from March 2020 to December 2020.



*Two surveys from 2 families were excluded by the analysis because of a) one family did not agree to be enrolled at CovFC and b) one family attended the CovFC only once for probable Covid-19, however both virological and serological assays resulted negative for SARS-CoV-2 among all subjects.

Overall, 66 families were included in the analysis, accounting for 97 parents of median age of 43 years (SD 7.8) and 129 children with median age of 10 years (SD 5,6). Among those, 74.2% of parents and 71.3% of children/adolescents had confirmed Covid-19, the large majority presenting with mild symptomatic or asymptomatic infection. Seventy-three parents (66 families) answered the *"Questionnaire A - Family"*, of those 58 (79.5%) were mothers e 15 were fathers of median age of 43.3 years (SD 7.7); 90.4 % of them were Italian. Descriptive analyses were reported in table 1.

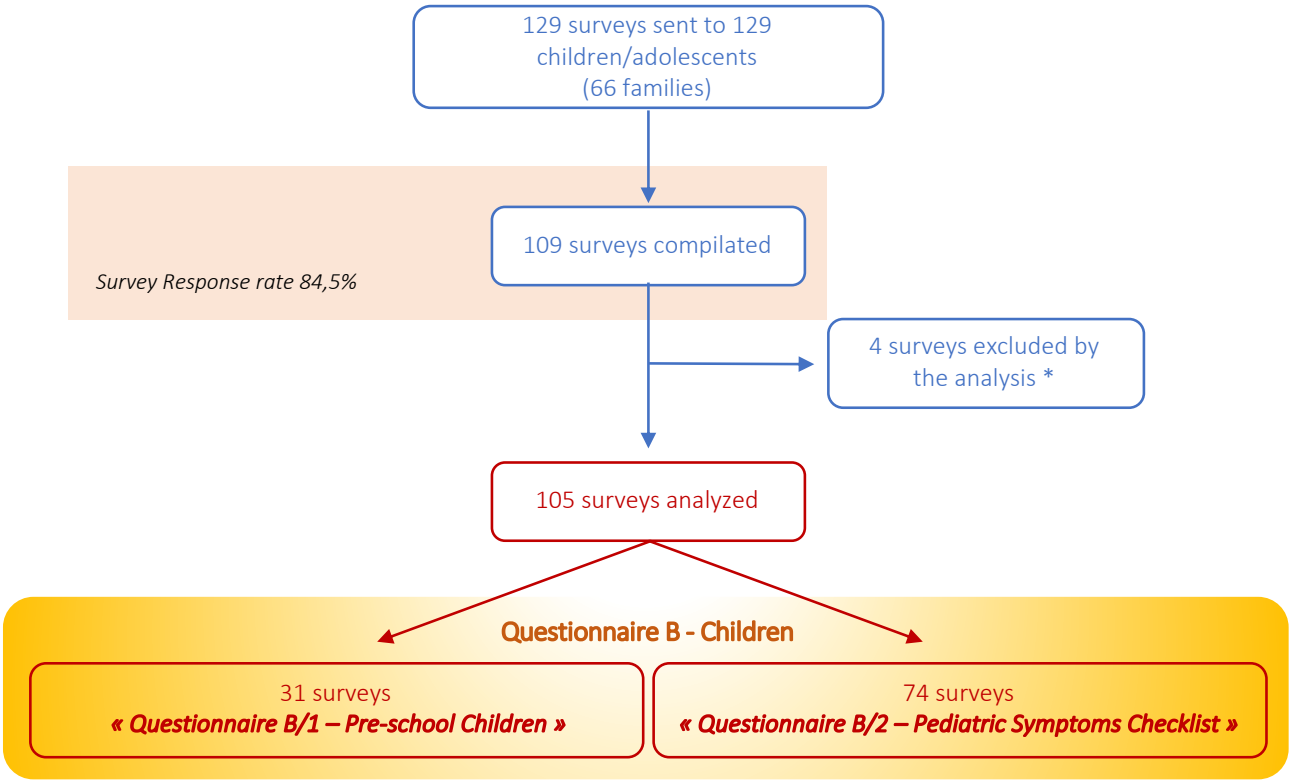
Table 1. Descriptive analysis of 66 families (n=226) attending the survey *"Questionnaire A - Family"* and with *"Questionnaire B- Children"*.

	Children (n=129)	***Parents***	
		Overall (n=97)	**Survey's editors (n=73)**
Time (days) from baseline to survey compilation *(MEAN, DS)**	-	-	164,71 (56,03)
Age at survey compilation *(mean/DS)*	10 (5,6)	43 (7,8)	43,3 (7,2)
Gender *(female), n (%)*	58 (44,9%)	63 (64,9%)	58 (79,5%)
Nationality			
Italian	120 (93%)	89 (91,7%)	66 (90,4%)
Other	9 (6,9%)	8 (8,2%)	7 (9,6%)
Comorbidities§	22 (17%)	10 (10,3%)	10 (13,7%)
Positive SARS-CoV-2 Nasal-Pharyngeal Swab *(n, %)*	77 (59,7%)	64 (65,9%)	52 (71,2%)
Confirmed COVID-19 cases *(n, %)*	92 (71,3%)	72 (74,2%)	55 (75,3%)
COVID-19 clinical presentation (among confirmed cases)*:*			
asymptomatic	20 (21,7%)	5 (6,9%)	4 (7,3%)
Mild COVID-19	71 (77,2%)	61 (84,7%)	49 (89,1%)
Moderate/severe COVID-19	0	4 (4,1%)	1 (1,8%)
Critical COVID-19	1 (MIS-C)	1 (1%)	1 (1,8%)
Pandemic wave			
1st wave	72 (55,8%)	67 (69%)	44 (60,3%)
2nd wave	57 (44,2%)	30 (30,9%)	29 (39,7%)

*For parents with confirmed Covid-19, we evaluated the period of time occurred from "date of baseline" to "date of survey compilation"; in case of editors that were negative for Covid-19, we evaluated the period of time from the more recent "date of baseline" reported for a positive household to the date of survey compilation,
§ Children's comorbidities were growth disorders (n=2), prematurity (n=2), asthma (n=2), chronic lung disease (n=1), congenital heart disease (n=3), reumathic disease (n=1), or other (10); none had congenital or acquires immune disease nor tumor.

After the completion of the *"Questionnaire A – Family",* a further survey specifically dedicated to children and adolescents was proposed. According to the age of each child, parents were allowed to fill out the *"Questionnaire B/1 – pre-school children"* and/or the *"Questionnaire B/2 - Pediatric Symptoms Checklist",* aimed at investigating the psychological impact of Covid-19 among children and adolescents. Only one parent was allowed to fill out the survey dedicated to each son/daughter. Among the 129 surveys proposed, 109 surveys were collected; 4 surveys were excluded from the analysis as they were referred to adolescents of >= 18 years of age (figure 2).

Figure 2. Flow-chart of 105 surveys analyzed out of 129 *"Questionnaire B – Children"* distributed to parents, enrolled at CovFC from March 2020 to December 2020.



*Four surveys were excluded by the analysis because they referred to adolescents of >= 18 years of age.

Results from the "*Questionnaire A – Family*"

Findings from *"Questionnaire A - Family"* are reported in Table 2. Overall, the median time from COVID-19 disease (defined as "*baseline date*") to survey compilation was 164.7 days (SD 56 days). Most families (95.8%) shared the diagnosis of Covid-19 with people other than close contacts, however, 53.4% of families were worried thinking about a people's behavioural change, after the communication of Covid-19. In 30.5% of cases, a people's behavioural change towards the family was observed, with a significant negative impact for 72.7% of families that experienced Covid-19 during the 1st wave, compared to those of the 2nd wave.

Parents were asked to describe the quality of the human relationship experienced within the family before, during, and after Covid-19, through a scale from 0 to 5 points (0=worse, 1=negative, 2=more negative than positive, 3=more positive than negative, 4=positive, 5=excellent). Overall, families reported positive quality of relationships with partners and children, and within siblings with no significant differences comparing the first and second pandemic waves. As expected, the most "critical" period was the time of Covid-19 infection and particularly during the 2nd wave, where 17.9% of parents experienced a negative relationship with their partner, the 10.4% with children/adolescents, and the 16% observed a negative relationship within siblings.

Table 2. Results from the "*Questionnaire A – Family"* provided by 73 parents (66 families).

	Total (n=73)	***1st WAVE (n=44)***	***2nd WAVE (n=29)***	***p-value****
Time from baseline to survey compilation*, days (mean, DS)*	164,7 (56)	165,3 (64,8)	163,7 (40,2)	0,1724
Diagnosis of Covid-19 was shared with people other than close contacts *(yes/total answer)*	69/72 (95,8%)	42/43 (97,7%)	27/29 (93,1%)	0,561
Fear of a people's behavioural change, after the communication of Covid-19 *(yes/total answer)*	39/73 (53,4%)	22/44 (50%)	17/29 (58,6%)	0,485
Objective finding of people's behavioural change, after the communication of Covid-19 *(yes/total answer)*	22/72 (30,5%)	11/43 (25,6%)	11/29 (37,9%)	0,304
Type of observed behavioral change *(negative)*	8/22 (36,4%)	8/11 (72,7%)	0/11 (0%)	*0,001*
BEFORE COVID -19				
Quality of the relationship with the partner (>=3)	65/67 (97%)	38/39 (97,4%)	27/28 (96,4%)	1,000
Quality of the relationship with son/daughters (>=3)	73/73 (100%)	44/44 (100%)	29/29 (100%)	-
Quality of the relationship within siblings (>=3)	42/42 (100%)	17/17 (100%)	25/25 (100%)	-
DURING COVID-19				
Quality of the relationship with the partner (>=3)	59/66 (89,4%)	36/38 (94,7%)	23/28 (82,1%)	0,125
Quality of the relationship with son/daughters (>=3)	68/71 (95,8%)	42/42 (100%)	26/29 (89,6%)	0,064
Quality of the relationship within siblings (>=3)	34/39 (87,2%)	13/14 (92,9%)	21/25 (84%)	0,636
AT SURVEY COMPILATION				
Quality of the relationship with the partner (>=3)	65/68 (95,6%)	38/40 (95%)	27/28 (96,4%)	1,000
Quality of the relationship with son/daughters (>=3)	72/73 (98,6%)	43/44 (97,7%)	29/29 (100%)	1,000
Quality of the relationship within siblings (>=3)	40/41 (97,6%)	15/16 (93,7%)	25/25 (100%)	0,390
Responder was working before Covid-19 (yes)	56/72 (77,8%)	36/44 (81,8%)	20/28 (71,4%)	0,386
Impact of COVID-19 on work (yes)	20/66 (30,3%)	13/39 (33,3%)	7/27 (25,9%)	0,593
Negative impact	8/9 §			

* p-value for the non-parametric Fisher exact test (for categorical variables) or Mann-Whitney test (for quantitative variables)
** all percent values have been calculated according to total answer (handling missing values)
§ we specify that of those, only 1 person resigned from work.

Results from the *"Questionnaire B – Children"*

Findings from the *"Questionnaire B/1 – pre-school children"* are reported in Table 3. Overall, 31 surveys were filled out by parents, referring to 31 children of median age of 3 years (SD 1,7), 64.5% of those with confirmed COVID-19. Children's ability to adapt to home isolation was investigated through a scale elaborated ad hoc, ranging from 0 to 5 (0=very easy, 1=quite well, 2=easier than difficult, 3= more difficult than easy, 4=quite difficult, 5= very difficult). Overall, a scarce ability of the child to adapt to isolation (>=3) was noticed for 35.5% of children, as indicated by their parents. During isolation at home, a change of one or more functions was reported for 74.2% of children, with no differences between COVID-19 positive and negative children. The most frequent alterations referred to were: a change in circadian rhythm (25%), a change in the emotional expression and with poor control (36%), and a change in the relationship with both parents (42.8%) and siblings (28%). No differences were observed among COVID-19 positive and negative children.

Table 3. Findings from the *"Questionnaire B/1 – pre-school children"* referring to 31 children less than 6 years of age.

	TOT (n=31)	COVID-19 CASE (n=20)	Covid-19 negative (n=11)	p-value*
Age at survey compilation, mean (DS)	3,01 (1,71)	2,85 (1,86)	3,33 (1,43)	0,232
Gender (female)	14/31 (45,2%)	9/20 (45%)	5/11 (45,5%)	1,000
Comorbidities (yes)	5/31 (16,1%)	4/20 (20%)	1/11 (9%)	0,631
Scarce ability to adapt to isolation (>=3)	11/31 (35,5%)	7/20 (35%)	4/11 (36,4%)	1,000
Functional change occurred during isolation (yes)	23/31 (74,2%)	13/20 (65%)	10/11 (90,9%)	0,203
Change in circadian rhythm (yes)	7/28 (25%)	4/18 (22,2%)	3/10 (30%)	0,674
Change in nutrition (yes)	3/25 (12%)	3/15 (20%)	0/10 (0%)	0,250
Change in sphincteric control (yes)	2/22 (9,1%)	2/12 (16,7%)	0/10 (0%)	0,481
Speech alteration (yes)	3/26 (11,5%)	3/16 (18,7%)	0/10 (0%)	0,262
Play alteration (yes)	5/27 (18,5%)	4/17 (23,5%)	1/10 (10%)	0,621
Change in body care (yes)	4/23 (17,4%)	0/12 (0%)	4/11 (36,4%)	0,037
Change in emotional expression and control (yes)	9/25 (36%)	4/14 (28,6%)	5/11 (45,5%)	0,434
Change in relationship with parents (yes)	12/28 (42,8%)	6/17 (21,4%)	6/11 (21,4%)	0,441
Change in relationship with siblings (yes)	7/25 (28%)	4/14 (28,6%)	3/11 (27,3%)	1,000

*Fisher exact test for categorical variables, T-test for quantitative variables

Findings from the *"Questionnaire B/2 - Pediatric Symptoms Checklist"* are reported in Table 4. Overall, among 82 children/adolescents aged >=6 to <18 years, 74 surveys were filled out with at least one answer, by their parents (90.2% response rate). Mean age of 74 children was 10.9 years (SD 2,7), of those 39.2% were female and 22.9% had one or more comorbidities. Overall, a scarce ability to adapt to isolation (>=3) was noticed for the 36.9% of children/adolescents, as indicated by their parents. For all cases, one or more functional changes were observed during isolation.

The Pediatric Symptom Checklist (PSC) was adopted and 35 items parent-reported were proposed with a single choice answer of a score ranging from 0 to 1 (0=never, 1=sometimes, 2= often). Based on previous studies (21,23), reaching a cutoff of >=28 indicates a possible problem. In our cohort, the 8.1% (6/74) of children were reported as having a score of >=28, with no differences between confirmed Covid-19 cases and negative subjects. The most common psychosocial alterations reported were "Tires easily, has little energy" (51.4%), "Less interested in school" (59.7%), "Distracted easily" (66.7%), "Is afraid of new situations" (51.4%), "Is irritable, angry" (54.2%), "Has trouble concentrating" (52.8%), and "Wants to be with you more than before" (54.3%). No differences among covid-19 positive and negative subjects were observed, for all items.

Among all cases, 5 out of 69 (7.3%) children used to get hurt frequently and all of them were Covid-19 cases. In addition, the inability to express emotions ("he/she does not show feelings") was reported for 16 out of 69 (23,2%) children. Parents reported the "blamed others for his or her troubles" for 31.3% of children.

Table 4. Findings from the Pediatric Symptoms Checklist named *"Questionnaire B/2 - Pediatric Symptoms Checklist"* referring to 74 children/adolescents aged >=6 to <18 years.

	TOT (n=74)	COVID-19 CASES (n=62)	Covid-19 negative (n=12)	p-value*
Age at survey compilation, mean (DS)	10,9 (2,7)	10,3 (2,7)	10,9 (2,7)	0,7749
Gender (female)	29 (39,2%)	23 (37%)	6 (50%)	0,521
Comorbidities (yes)	17 (22,9%)	14 (22,6%)	3 (25%)	1,000
Scarce ability to adapt to isolation (>=3)	27/73 (36,9%)	23/61 (37,7%)	4/12 (33,3%)	1,000
Functional change occurred during isolation, any (yes)	74 (100%)	62 (100%)	12 (100%)	-
Pediatric Symptom Checklist (PSC) scores of >=28	6/74 (8,1%)	5/62 (8%)	1/12 (8,3%)	1,000
Attention Problems subscale (>=7)	12/72 (16,7%)	11/60 (18,3%)	1/12 (8,3%)	0,676
Internalizing Problems subscale (>=5)	4/74 (5,4%)	4/62 (6,5%)	0/12	1,000
Externalizing Problems subscale (>=7)	4/72 (5,6%)	4/60 (6,7%)	0/12	1,000
Complains of aches/pains (>=1)	23/70 (32,9%)	21/59 (35,6%)	2/11 (18,2%)	0,318
Spends more time alone (>=1)	28/71 (39,4%)	24/60 (40%)	4/11 (36,4%)	1,000
Tires easily, has little energy (>=1)	37/72 (51,4%)	33/60 (55%)	4/12 (33,3%)	0,214
Fidgety, unable to sit still (>=1)	34/72 (47,2%)	30/60 (50%)	4/12 (33,3%)	0,354
Has trouble with a teacher (>=1)	17/72 (23,6%)	15/60 (25%)	2/12 (16,7%)	0,719
Less interested in school (>=1)	43/72 (59,7%)	36/60 (60%)	7/12 (58,3%)	1,000
Acts as if driven by a motor (>=1)	24/72 (33.3%)	21/60 (35%)	3/12 (25%)	*0,739*
Daydreams too much (>=1)	31/72 (43,1%)	25/60 (41,7%)	6/12 (50%)	0,751
Distracted easily (>=1)	48/72 (66,7%)	40/60 (66,7%)	8/12 (66,7%)	1,000
Is afraid of new situations (>=1)	37/72 (51,4%)	31/60 (51,7%)	6/12 (50%)	1,000
Feels sad, unhappy (>=1)	26/72 (36,1%)	20/60 (33,3%)	6/12 (50%)	0,330
Is irritable, angry (>=1)	39/72 (54,2%)	33/60 (55%)	6/12 (50%)	0,762
Feels hopeless (>=1)	7/72 (9,7%)	6/60 (10%)	1/12 (8,3%)	1,000
Has trouble concentrating (>=1)	38/72 (52,8%)	32/60 (53,3%)	6/12 (50%)	1,000
Less interest in friends (>=1)	18/72 (25%)	15/60 (25%)	3/12 (25%)	1,000
Fights with others (>=1)	31/72 (43%)	29/60 (48,3%)	2/12 (16,7%)	*0,057*
Absent from online school (>=1)	16/72 (22,2%)	14/60 (23,3%)	2/12 (16,7%)	1,000
School grades dropping (>=1)	25/72 (34,7%)	22/60 (36,7%)	3/12 (25%)	0,524
Is down on him or herself (>=1)	16/72 (22,2%)	13/60 (21,7%)	3/12 (25%)	0,722
Visits doctor with finding nothing wrong (>=1)	5/72 (6,9%)	3/60 (4,2%)	2/12 (2,8%)	0,191
Has trouble sleeping (>=1)	21/71 (29,6%)	17/59 (28,8%)	4/12 (33,3%)	0,740
Worries a lot (>=1)	36/74 (48,6%)	30/62 (48,4%)	6/12 (50%)	1,000
Wants to be with you more than before (>=1)	38/70 (54,3%)	32/59 (54,3%)	6/11 (54,5%)	1,000
Feels he or she is bad (>=1)	8/70 (11,4)	7/59 (11,9%)	1/11 (9,1%)	1,000
Takes unnecessary risks (>=1)	5/69 (7,3%)	5/58 (8,6%)	0/11	0,585
Gets hurt frequently (>=1)	5/69 (7,3%)	5/58 (8,6%)	0/11	0,585
Seems to be having less fun (>=1)	23/70 (32,9%)	18/59 (30,5%)	5/11 (45,5%)	0,485
Acts younger than children his or her age (>=1)	16/68 (23,5%)	15/57 (26,3%)	1/11(9,1%)	0,437
Does not listen to rules (>=1)	28/68 (41,2%)	24/57 (42,1%)	4/11 (36,4%)	1,000
Does not show feelings (>=1)	16/69 (23,2%)	14/58 (24,1%)	2/11 (18,2%)	1,000
Does not understand other people's feeling (>=1)	12/69 (17,4%)	10/58 (17,4%)	2/11 (18,2%)	1,000
Teases others (>=1)	27/67 (40,3%)	24/57 (42,1%)	3/10 (30%)	0,728
Blames others for his or her troubles (>=1)	21/67 (31,3%)	17/57 (29,8%)	4/10 (40%)	0,713
Takes things that do not belong to him/her (>=1)	9/67 (13,4%)	7/57 (12,3%)	2/10 (20%)	0,614
Refuses to share (>=1)	16/66 (24,4%)	13/56 (23,2%)	3/10 (30%)	0,695

The scores relating to the "*Attention Problems*", "*Internalizing Problems*" and "*Externalizing problems*" subscales were then analyzed. On the "*Attention Problems*" subscale (derived from the sum of the following items: "Fidgety, unable to sit still", "Daydreams too much", "Distracted easily", "Has trouble concentrating", "Acts if is driven by a motor") a cut-off > 7 indicates an impairment condition. In our cohort, the Attention impairment was referred for the 16.7% of the total. A trend (p=0.06) was identified in the comparison between COVID 19 confirmed cases (18.3%) and negative (8.3%). On the Internalizing problems subscale (derived from the sum score of the following items: "Feels sad or unhappy", "Feels hopeless", "Is down on him or herself", "Worries a lot") a cut-off > 5 indicates an impairment condition. In our cohort, the anxiety and

depressive impairment were referred for 4/72 (5.6%). On the Externalizing problems subscale (derived from the sum of the following items: “fight with others”, “does not listen to rules”, “does not understand other people feelings”, “teases others”, “blamed others for his / her troubles”, “takes things that do not belong to him/her”, “refuses to share”) a cut-off > 7 indicates an impairment condition. In our cohort, behavioral and conduct impairment was referred by parents for 4/62 of the total (6.5%). No difference between covid-19 confirmed cases and negative was found but all the internalizing and externalizing impairment conditions were referred for confirmed positive covid-19 children and adolescents.

Discussion

Our study explored the psychological effects of the COVID-19 pandemic on children and their parents. Using an electronically distributed survey, we evaluated the family’s relational and behavioral changes and the children’s ability to adapt to COVID-19 disease and home isolation among a cohort of 66 Italian family clusters of COVID-19 including 129 children/adolescents, observed at the Department of Women’s and Children’s Health of the University Hospital of Padua during the first year of the SARS-CoV-2 pandemic.

We retrospectively investigated changes in children’s and caregivers’ behavioral wellbeing and daily routine before, during, and at least 3 months after their isolation, according to parents’ perceptions. Despite more than half of parents being afraid of a people’s behavioral changes after the communication of infection, almost all of them shared the COVID-19 diagnosis with others outside their family, both during the 1st and 2nd wave of Covid-19 pandemic.

This is the first socially relevant result we observed in our cohort. Most of the families shared the diagnosis with other people, and more than half expressed concern about the possible change of attitude following this communication. In 30% of cases, there was a change of attitude with a major negative impact in reference to the first wave. Compared to other infectious disease outbreaks, that led communities to marginalize subjects who were infected (24,25), our results showed that people would appear to assess better with disease-related stigma during the SARS-CoV-2 pandemic. This is in contrast with other findings reporting a high level of social stigma among adults who recovered from COVID-19. Yuan et al (26), in a cross-sectional study comprising 154 COVID-19 survivors and 194 healthy controls, observed that COVID-19-related stigma is still commonly experienced among COVID-19 survivors even though the outbreak has been well-contained. In this regard, it must be considered that we were dealing with a selected sample of families who accepted the proposal to socialize and shared their experience of the disease.

In our family cohort, we examined how the current pandemic afflicted the relationship among households. Overall, most parents reported a positive quality of the relationship with the partner, with son/daughters, and within siblings either referring to the period before, during, and after COVID-19, although some of them report changes in their children's behavior towards them. Our results showed that satisfactory intrafamilial relationships persist even though the family experiences stressful events, such as fear of illness, worry of

lacking own economic stability, isolation from others and peers, and changes in business affairs and in daily life.

In light of this, previous studies reported that parental distress due to COVID-19 can have a relevant impact on children's psychological health (27,28). These results are in line with our study, as we observed at first that 30% of parents had an impact of COVID-19 on work with an unavoidable related stress (Table 2) and on the other side, parents reported a scarce ability to adapt to isolation in less than 38% of their children, both kids under 6 years of age and adolescents (Tables 3 and 4), reflecting a possible linkage between parental and children distress, as reported by other studies (29) .

In addition, our findings showed that all caregivers noticed at least one functional change in their child's behavior during the lockdown. For children younger than 6 years, change in emotional expression and control followed by change in circadian rhythm were the most prominent changes reported by approximately one thirds and one fourth of the caregivers respectively. This data suggests that even preschoolers have been affected by the changes caused by isolation. In this developmental stage, the understanding of events passes yet through direct experience. For preschooler children could be difficult to construct a mental representation of the threat linked to the pandemic, as the infection is an invisible threat. Furthermore, for the preschooler children the language might not represent the best tool for the emotional expression. For these reasons, the childhood alterations may appear as non-specific and occur in the form of alterations in bodily functions or in the quality of the emotional experience.

According to the Pediatric Symptom Checklist (PSC) scores used in this study, an increase in lack of school interest, distractibility, tiredness and easy fatigue, anxiety, worry about the new situation, angry and irritability, and difficult in concentrating were also noted in children older than 6 years and adolescents by approximately one third to one out of two of the participant caregivers. For most of the school age children and adolescents, parents referred a decreased interest in the school activities suggesting the primary importance of the school setting and of the role of interpersonal and peer relationships in motivation to learn at any level of education. We noticed no differences in resiliency and functional changes both in children and adolescence when they were stratified according to COVID-19 diagnosis. This may reflect the significant role of the environmental restrictions adopted during the pandemic on children and adolescents' behavioral changes, rather than a disease-related impact.

The overall prevalence rate of psychosocial dysfunction as measured by the PSC in school-aged children was 8.1%, slightly lower than rates reported by previous studies 12% (30) conducted in the pediatric general population with the PSC parents' version. Higher psychological distress scores were found with the youth self-report form of the PSC-17 with preadolescents and adolescents (31). In our cohort, we can consider an underestimation effect linked to the younger age of our cohort (10 years of median age), the retrospective data collection and the indirect collection through parents. No difference was observed among either COVID-19 positive or negative children, suggesting that COVID-19 may not increase the risk of developing a psychosocial disfunction. However, we must underline that almost all the subjects reported with anxiety-depressive problems and with behavioral or conduct problems are all subjects with confirmed diagnosis of

COVID 19 like most subjects with attention problems. The lack of difference noticed between COVID-19 infected and uninfected cases may be due to the small number of the negative COVID-19 group. We can also hypothesize that the COVID 19 diagnosis may have contributed to increasing the time of isolation both towards the outside world and within one's own family and thus making the emotional and relational experience of subjects with confirmed covid diagnosis more complex than negative subjects.

Despite being descriptive, our findings suggested that the severe quarantine measures adopted during the current pandemic may lead to a psychological impact on children and adolescents. These findings agree other studies on the psychological wellbeing consequences of the COVID-19 lockdown in Italian, Spanish and Chinese children (32,33).

Our study has several limitations. Firstly, at the time of the survey's distribution, all family members were healthy and well recovered from COVID-19, consequently the parents were retrospectively referring to the time of COVID-19, having time to rework the experience. However, we believe that this can also constitute a strength, as parents probably provided a more objective and less emotional answer as the time of survey compilation occurred 164 days (IQR 56) after their COVID-19 related isolation. In addition, while the PSC is a validated score using to evaluate mental health in children and adolescences, the Questionnaire B1, evaluating the behavioral response of younger children to the same stressful situations, where constructed ad hoc by our specialists therefore it needs to be validated to further studies. Third, not having enrolled families of children with significant disabilities and/or living with social-economic disadvantage, our results may underestimate the intra-family difficulties and the behavioral changes encountered during social isolation. In fact, almost all families observed belonged to the middle class and were very sensitive and motivated in attending the clinical follow-up. By recruiting subjects who voluntarily attend the CovFC following detailed clinical and serological post-covid evaluations, we may have selected families who enjoy a privileged social and intrafamilial situation.

In conclusion, in our Italian cohort of COVID-19 family clusters we observed the maintenance of satisfactory intra-family balance and relationships among households during a psychologically stressful event such as the lockdown. However, almost one third of children and adolescents showed a reduced ability to adapt to isolation, and alteration of psychological functions were observed, particularly related to attention impairment.

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Chapter 5

DISCUSSION AND CONCLUSION

COVID-19 pandemic suddenly upset the beginning of my second year of PhD. At that time, I rapidly changed perspectives as a medical doctor to care for people infected and as a researcher to improve the scientific knowledge of COVID-19. However, very few data were available at that time, particularly on paediatric SARS-COV-2 infection. Therefore, my research project was conducted primarily focusing on children. Several aspects were evaluated, from assessing the clinical characteristics of first cases of COVID-19 across different age classes and populations to the characterization of the human and cellular immune response elicited by the virus over time. In addition, from a public health point of view, it was essential to work in a team to implement infection prevention and control measures to contain the in-hospital spread of SARS-CoV-2 and to share our experience with the rest of the world.

Within my PhD project, I have contributed to the publication of several papers, exploring different but complemental aspects of the impact of the novel virus SARS-CoV-2 among children and their parents, according to the following three main study objectives, as defined in Chapter 1:

4. To explore and implement infection prevention and control strategies to contain the in-hospital spread of SARS-CoV-2.
5. To improve knowledge on viral transmission and clinical characteristics of SARS-CoV-2 infection among children and other vulnerable populations.
6. To improve knowledge on the medium and long-term immunological and clinical impact of SARS-CoV-2 infection among children and adults recovered by COVID-19 family clusters.

The main findings of my PhD thesis are presented in the following paragraphs.

MAIN FINDINGS

Infection prevention and control strategies to contain the in-hospital spread of SARS-CoV-2.

At the beginning of the SARS-CoV-2 pandemic, there was an urgent need for evidence-based protocols and guidelines on the most effective infection prevention and control (IPC) measures to be implemented to contain the spread of SARS-CoV-2 at the Hospital level. At that time, all Departments and Hospitals, including our Department for Women's and Children's Health at the University Hospital of Padua, had to be rapidly reorganized. Two studies were published during the first months of the COVID-19 pandemic in Italy regarding this field.

The study "*Children's Hospital management in the COVID-19 era: the reorganization of a tertiary care Paediatric Emergency Department in Northern Italy*" (Chapter 2.1) [37], describes the operational measures set up at the Paediatric Emergency Department to contain the spread of SARS-CoV-2, at the beginning of SARS-

CoV-2 pandemic. According to the epidemiologic and clinical risk factors, four different pathways were developed to address children/adolescents with suspected COVID-19. The strict application of the measures led to quick identification, isolation, and management of all positive children, preventing SARS-CoV-2 intrahospital spread in the first wave of the COVID-19 pandemic.

The communication "*COVID-19 Pandemic: Perspective of an Italian Tertiary Care Pediatric Center*" (Chapter 2.2) [38] describes in detail the multilevel interventions set up at the Department for Women's and Children's Health of Padua University Hospital to prevent the in-hospital spread of SARS-CoV-2. Measures set up at the Department for Women's and Children's Health were (a) to revise the distribution of the clinical areas in order to create both designated COVID-19, and COVID-19-free areas with their own access, (b) to reinforce infection prevention control (IPC) measures for all healthcare workers and administrative staff and (c) to reinforce IPC measures for patients adopting the new "double-gate approach": a phone call pre-triage and nasopharyngeal swab for SARS-CoV-2 detection before the admission of all patients and caregivers. After setting up IPC measures, in a ten-week observation period, a total of 3382 nasal-pharyngeal swabs (NPSs) were performed on healthcare workers, and 99.7% (3371) resulted negative. Only seven physicians, two nurses and two of the administrative staff tested positive. However, upon reconstructing their recent histories of potential exposures, it turned out that ten of them (91%) were unintentionally exposed to a SARS-CoV-2 infected person outside the hospital. No other cases of intra-department infection were documented among the healthcare workers since all the preventive procedures described above were implemented. The study constituted one of the first operational guidelines on pediatric hospital reorganization, developed at the early beginning of the COVID-19 pandemic.

Viral transmission and clinical characteristics of SARS-CoV-2 infection among several populations.

A few weeks after the detection of the first COVID-19 related death in Italy[2], the first pediatric cases of COVID-19 were observed and managed at the Department for Women's and Children's Health of the University Hospital of Padua. At that time, clinical data on the first cases of COVID-19 were retrospectively collected, and available evidence was examined, including case series and first observational cohort data on COVID-19 transmission and infection, particularly among the most vulnerable populations such as children and immune-depressed patients. Three papers were published.

The brief report "*Fecal-Oral Transmission of SARS-CoV-2 In Children: is it Time to Change Our Approach?"* (Chapter 3.1) was published early in pandemic [39]. It describes two infants evaluated in March 2020 at the Department of Women's and Children's Health; both tested positive for SARS-CoV-2 at rectal swab. The first one, a 5-month-old boy, presented respiratory and gastrointestinal symptoms with diarrhea and SARS-CoV-2 was detected at both nasopharyngeal and rectal swabs. The second one, 2-months-old, presented only mild

respiratory symptoms, and despite the absence of gastrointestinal symptoms, a rectal swab tested positive for SARS-CoV-2 on day three from the onset. We reviewed the literature on viral transmission in children available at that time, suggesting that fecal shedding with environmental contamination may play an important role in the viral spread. Further studies confirmed our preliminary observation, showing that a consistent proportion of patients eliminate SARS-CoV-2 RNA through fecal shedding, with persistence up to several weeks after COVID-19 and impacting person-to-person transmission [40–42].

After that, I contributed to providing real-life findings on the mode of presentation, risk factors, the severity of disease presentation and early outcome of the first pediatric cases of COVID-19 through the participation in a multicenter retrospective analysis of clinical records of 127 SARS-CoV-2-infected children evaluated in 23 different sites in Italy, including our Pediatric Department. The brief report "*Gastrointestinal symptoms in severe Covid-19 children"* (Chapter 3.2) was published in 2020 [43]. Among the 127 children observed (34,9% female) with a median age of 4.8 years (IQR 0.3–8.5), 45% were <12 months of age. Most children (84,4%) were asymptomatic or with mild/moderate disease, while 8,7% had severe and 7,1% had critical COVID-19. Fourteen (12%) children required oxygen, and 8 (6.7%) were admitted to ICU and among those, 1 case required mechanical ventilation. At admission, the most common symptoms reported were fever (82.7%), cough (48%), and rhinorrhea (38%). Seventy-seven out of 127 (60.6%) presented with respiratory symptoms (cough, rhinorrhea, wheezing, and dyspnea) and 36 (28.3%) had gastrointestinal (GI) symptoms (vomit, diarrhea, abdominal pain). The 15.7% of patients had at least one comorbidity: 3.9% had a chronic cardiac condition, 3.1% had GI disorders, 2.4% were obese, and 1.6% had chronic kidney disease, chronic neurologic disorder, and immunologic condition. Comorbidities distribution was not different among severity classes (P = 0.08, Fisher exact test) and among children admitted to ICU (P = 0.115 Fisher exact test). GI symptoms at admission were distributed differently throughout severity classes: GI symptoms were more frequently associated with the severe and critical phenotype (P = 0.029).

Interestingly, a history of GI symptoms was positively associated with cardiac involvement as clinical complications, either in the presence of other symptoms (P = 0.007) or alone (P = 0.004). Roughly a third of the children presented lower respiratory tract complications such as viral pneumonia and bronchiolitis. On admission, a chest radiogram was performed in 77 patients (65%) and infiltrates were found in 38 of 77 (50%). The presence of infiltrates at the chest radiogram did not correlate with severity clinical score or ICU admission rate (P = 0.125 and 0.71 Fisher exact test, respectively). It is known that common circulating human coronaviruses can cause GI symptoms in up to 57% of children [44]. The evidence available at the time of paper writing showed that the GI tract represents a target for SARS-CoV-2 due to the expression of the angiotensin-converting enzyme 2, a major virus receptor. Our data showed that a history of gastrointestinal (GI) symptoms would be related to the worst severity score (severe and critical) and a higher ICU admission rate. The same result was found in another study, conducted in a pooled analysis of adult cohorts, where GI symptoms were correlated to increased odds of critical disease and higher prevalence of complications [45]. We also observed that having GI was more frequently reported in patients who developed cardiac impairment as complications

of SARS-CoV-2 infection, anticipating what would be described by further studies on SARS-CoV-2 hyperinflammatory syndrome defined as MIS-C [46]. Our study has several limitations, at first, the limited sample size and the retrospective design.

Lastly, the impact of SARS-CoV-2 infection on people living with HIV was explored by conducting the systematic review "*SARS-CoV-2 infection in people living with HIV: a systematic review"* (Chapter 3.3*)* [47]. The study provided the first systematic characterization of cases of COVID-19, with or without laboratory confirmation, among people living with HIV/AIDS (PLWHA). Among the 291 papers from EMBASE and Medline search, the 898 articles from Google Scholar, with 12 other papers identified through manual search, 23 articles were included, with a total of 164 adult HIV patients being diagnosed with Covid-19. There were no studies on children, and the included studies were mainly retrospective or case series/reports; none came from an African country. The overall risk of bias was moderate due to the study types and characteristics. Most patients included were males (84.5%, 120/142 with available data) aged between 40-65 years, and mainly with a good immune-virological profile, as 75.4% had a CD4 cell count >350/μL and 96.4% had an undetectable VL. In 101/118 patients, one or more comorbidities were reported, mostly hypertension, dyslipidemia, and diabetes, similarly to what reported for HIV-negative SARS-CoV-2 infected patients [48,49] . Considering that nearly 50% of European PLWHA are older than 50 years and often report cardiovascular and chronic lung disease, our findings suggested for the first time that PLWHA chronically exposed to long-term ARV-related side effects are vulnerable during the SARS-CoV-2 pandemic. More than half (55.5%, 86/155) of patients with available data were hospitalized, and of those, 15 patients were transferred to intensive care units for mechanical ventilation. Of 152 patients with available data on outcomes, 10.5% (16 cases) had an unfavorable outcome (death), the majority of those (12/16) had an undetectable HIV viral load (VL) and seven had a relatively high immunity. In contrast, only one patient was severely immune suppressed, suggesting that outcome is probably unrelated to concomitant uncontrolled HIV infection. At that time, the overall lack of data, particularly on the immune-virological status, did not allow to make any conclusion on their impact in terms of disease severity and mortality among PLWHA affected by COVID-19. However, our findings confirmed that comorbidities such as chronic kidney disease, diabetes mellitus, hypertension and chronic cardiac disease are major risk factors for SARS-CoV-2 disease severity, in PLWHA. Further findings from more recent systematic reviews suggest that COVID-19 disease course and mortality did not differ between HIV-positive and negative people [50,51]. On the other side, other studies highlighted that PLWHA seem to be at higher risk of COVID-19 disease severity and death compared to non-PLWHA [52] .

Medium and long-term immunological and clinical impact of SARS-CoV-2 infection among children and adults recovered by a COVID-19 family cluster.

The most important results achieved within my PhD course are provided by studies conducted on the "*CASE cohort*", the observational cohort followed up at the COVID-19 Follow-up Clinic (CovFC), set up at the Department of Women's and Children's Health. Five papers were published, strongly contributing to improving knowledge on clinical findings and medium and long-term humoral and cellular immune response to SARS-CoV-2 infection among children, their older siblings, and parents. In addition, a further study was recently submitted to a peer-reviewed journal.

Immunological findings from the "CASE cohort"

The production and persistence of naturally acquired SARS-CoV-2 neutralizing antibodies (nAbs) among the first 57 families observed at the CovFC was evaluated in the study "*Superior magnitude and persistence of SARS-CoV-2 neutralizing antibodies in children*" (Chapter 4.1) [35]. We analyzed 283 blood samples collected from 152 confirmed COVID-19 cases evaluated from March 1st to September 4th 2020, including 82 parents and 70 children/older siblings of median age of 8 years (IQR 4-13) presenting with asymptomatic or mildly symptomatic disease. For each patient, blood samples were collected for both the quantification of neutralizing antibodies (nAbs) through a plaque reduction neutralizing test (PRNT) and the detection of antinucleocapsid-spike protein immunoglobulin G detected through the Chemiluminescence Immunoassay (CLIA) MAGLUMI 2019-nCoV IgM and IgG on the analytical system MAGLUMI 2000 Plus (New Industries Biomedical Engineering Co, Ltd, Shenzhen, China). Despite an observed decrease of IgG over time, SARS-CoV-2 nAbs were found to persist up to 7-8 months in children, while adults showed a significant decline in nAbs, recording a 40% decrease between 3 and 7 months from infection. Surprisingly, nAbs inversely correlated with age and children under six years, and in particular toddlers under three years developed higher long-lasting levels of nAbs compared to older siblings and/or adults throughout early, intermediate, and late times from infection onset. In fact, at 1-2 months after infection, children under three years had a geometric mean titer (GMT) of 1:276 of PRNT, while adults had a GMT of 1:62. The 4.5-fold difference increased to 7.9-fold, in the 3-6 months window, as children under 3 reached a GMT of 1:340, while adults recorded a GMT of 1:43. Our results strengthened and expanded the work published by Yang et al. [53] who described higher surrogate neutralizing ability and avidity of antibodies in children aged 1-10 years, proving these features to be age-dependent. Strains encountered in childhood imprint adaptive immunity. Subsequent exposure to antigenically-related viruses directs the antibody response largely towards known conserved epitopes and less against novel immunodominant proteins, blunting the neutralizing potential [54]. Recently, this mechanism has been explored for influenza, proving that children under six years of age have a narrow strain-specific hemagglutinating inhibition activity, while adults have a back-boost response to past infections [55]. In light of this, we hypothesized that an original antigenic sin driven by repeat exposure to endemic human coronaviruses (hCoV) might impair the response to SARS-CoV-2 in adults, while the less experienced immune repertoire of

children could favor a prompt selective response. Recent work published by Selva et al.[56] supported this hypothesis proving that infection in elderly patients is associated with antibodies targeting the cross-reactive S2 and NP proteins, while in children, the response is dominated by antibodies with high Fc-effector function targeting the immunodominant S1 protein of SARS-CoV-2. In addition, recent evidence [57] proved that in adult patients, an expansion of B-cell clones against seasonal hCoVs dominates the response, generating antibodies poorly reactive with SARS-CoV-2.

Another relevant result of our study is the persistence of nAbs in children. We demonstrated for the first time that mildly affected children under six displayed increasing nAbs levels over 236 days from infection. Interestingly, children aged 6-<15 plateaued around the same period, while adults showed a significant decline in nAbs. Similarly, Lau et al.[58] estimated for adults that the decline of PRNT titers would reach undetectable levels between 133 and 416 days from infection depending on clinical severity and reported a 50% decrease between 3 and 6 months from infection for mild cases. In addition, Chia et al. [59] identified five profiles of antibody responses and observed that the persistence of high nAbs up to 6-7 months correlated with high levels of pro-inflammatory cytokines and the severity of COVID-19 in adults, predicting declines between 96-580 days.

The study has several limitations. The enrollment processes, case definition and identification of timelines were not coincidental since we relied on retrospective heterogeneous diagnostic evaluations related to the clinic's structure. This potentially led to biases in identifying baseline intervals, especially for pediatric cases with no virological record of positivity, for whom mild symptoms reported by parents were the only temporal reference to infection. Nonetheless, information from other family members and the long duration of the study potentially reduced the weight of these indeterminate values; moreover, sensitivity analyses confirmed our conclusions against the exclusion of a few cases.

Our paper has been endorsed by the commentary "*Duration of Effective Antibody Levels After COVID-19*" of Cruz A.T. [60], which concluded with the following sentences "Although many aspects of what comprises an effective immune response to SARS-CoV-2 require additional study, the work of Bonfante and Costenaro et al advances our understanding, demonstrating a more durable (and likely effective) response in younger children. Because children constitute an important contribution to the spread of COVID-19 through the population, knowledge of the potential susceptibility of children to reinfection is important in modeling COVID-19 epidemiology".

We further assessed the cellular-mediated response to SARS-CoV-2 in the study "*Asymptomatic and mild SARS-CoV-2 infections elicit lower immune activation and higher specific neutralizing antibodies in children than in adults*" (Chapter 4.2) [61]. The immune profile of 152 SARS-CoV-2-infected adults and children clustered within the same families was analyzed and compared to 54 uninfected age-class matched relatives. More in detail, we explored the immune profiles of activation, senescence, exhaustion, and regulatory cells among confirmed COVID-19 cases and uninfected controls, and we evaluated the relationship between neutralizing antibodies and viral load in asymptomatic and mildly symptomatic children and adults. Overall,

COVID-19 patients presented higher levels of immune activation, exhaustion, and regulatory cells compared to non COVID-19 subjects. Within the COVID-19 group, activated and senescent CD4+ and CD8+ cells were higher in adults than in children and inversely correlated with the nAbs levels detected through a plaque reduction neutralizing test (PRNT). Conversely, Tregs and Bregs regulatory cells were higher in COVID-19 children compared to adults and positively correlated with nAbs. Higher immune activation persisted in adults after six months of infection, while children maintained higher levels of regulatory cells; SARS-CoV-2 viral load did not differ among age classes.

As previously reported [62–65], our data confirmed a higher immune activation/exhaustion in asymptomatic/mildly symptomatic COVID-19 compared to non-COVID-19 adults, and the activation still persisted after six months from infection. Higher levels of activated CD4 and CD8 T cells were described in COVID-19 pediatric patients with MIS-C [28,66]. However, COVID-19 children without or with mild/moderate clinical manifestations showed similar frequencies of activated CD4 and CD8 cells compared to age-matched control [66–68]. In agreement with these findings, the present study found no differences between COVID-19 and non-COVID-19 children, and for the first time, we demonstrated that COVID-19 adults, mostly asymptomatic/mildly symptomatic, have a higher expression of both activated T and B cells, not only compared to non-COVID-19 adults but also compared to COVID-19 children. Notably, activated and senescent T and B cells inversely correlated with a production of anti-SARS-CoV-2 nAbs, thus suggesting that after infection in adults, immune activation exerts a strong influence on immune ageing and drains resources from the immune system for the specific production of anti-SARS-CoV-2 antibodies.

In our study, Tregs and Bregs were significantly higher in asymptomatic/mildly symptomatic COVID-19 patients than in non-COVID-19 subjects in all age classes. Interestingly, COVID-19 children, particularly those <6 years, had higher expression of Tregs and Bregs than COVID-19 adults, and notably, this was positively associated with the production of nAbs. Tregs inhibit the activation of both innate and adaptive immune responses via inhibitory surface molecules and the secretion of immunosuppressive cytokines (i.e., IL-10, TGF-β, and IL-35) [69]. It was previously reported that slow-progressors HIV-infected children secreted higher levels of IL-10 compared to those who progressed and had a higher proliferation of Tregs [70] . Similarly, it is possible that Tregs and Bregs in SARS-CoV-2-infected children constrain inflammation/ immune activation, likely through the release of IL-10. Indeed, a significant positive association was found between IL-10 and Tregs in children ($r = 0.633$, $p = 0.011$). Interestingly, in children, particularly under 6, high levels of Tregs and Bregs cells persisted for over six months of follow-up, and the titer of nAbs, thus supporting the concept that these cells play a role in directing the host immune response.

A limitation of this study is that it includes only asymptomatic/ mildly symptomatic COVID-19 children and adults. Nonetheless, our data demonstrated that even in the absence of severe disease, COVID-19 adults showed a higher degree of hyperinflammation/immune activation than COVID-19 children. The immune activation might limit the production of anti-SARS-COV-2-neutralizing antibodies and impair the specific response in adults. Conversely, in COVID-19 children, the viral-induced inflammation may be mitigated by the higher expansion of regulatory T and B cells resulting in preserved resources for higher specific production

of anti-SARSCoV-2-neutralizing antibodies. Further studies are needed to support the role of regulatory cells in this context.

During the first waves of COVID-19, all enrolled family members were systematically tested by the high throughput method for Plaque Reduction Neutralization Test (PRNT) for the detection of SARS-CoV-2 neutralizing antibodies. However, during subsequent waves, a sudden increase in the enrollment rate brought us to reconsider the sustainability of applying the test, given the high economic and operational costs posed by the PRNT assay. For this reason, the study "*Analytical and clinical performances of a SARS-CoV-2 S-RBD IgG assay: comparison with neutralization titers*" (Chapter 4.3) was conducted [71]. The study analyzes the analytical and clinical performance of a SARS-CoV-2 RBD IgG assay compared to SARS-CoV-2 neutralizing antibodies. The correlation between SARS-CoV-2 RBD IgG assay (Snibe diagnostics), automated on a high throughput platform, and SARS-CoV-2 neutralizing antibodies detected through plaque reduction neutralization test ($PRNT_{50}$) was assessed on 546 samples, including 171 negative and 168 positive SARS-CoV-2 subjects and on a further group of 207 subjects of the COVID-19 family clusters follow-up cohort. We demonstrated that anti- SARS-CoV-2 S-RBD IgG presents excellent linearity not only within the range of values including the cut-off (0.2–4 kA/L) but also for the highest values (from 5 to 70 kAU/L). Assay imprecision ranged from 3.98 to 12.18% being satisfactory at low and medium levels; linearity was excellent in all the measurement range. Considering specimens collected after 14 days post symptoms onset, overall sensitivity and specificity were 99.0 and 92.5%, respectively. From the analysis of a sub-group of 281 samples with available results of the $PRNT_{50}$, we found an elevated correlation between the SARS-CoV-2 RBD IgG assay and the $PRNT_{50}$ titer both at univariate (ρ=0.689) and multivariate (ρ=0.712) analyses. Based on this study, from March 26th 2021, all families enrolled at CovFC were tested only using Snibe anti-SARS-CoV-2 S-RBD IgG levels, as the sudden increase in the enrollment rate of further pandemic waves made not sustainable to apply both serological assays, given the high economic and operational costs posed by the PRNT.

Finally, we evaluated the long-term humoral response to SARS-CoV-2 infection in children and adults enrolled in the "*CASE cohort*" and the study "*Long-term Immune Response to SARS-CoV-2 Infection Among Children and Adults After Mild Infection*" (Chapter 4.4) [72] was recently published on JAMA Network Open. This prospective cohort study strengths and expands our previous findings on the magnitude and persistence of nAbs from the preliminary analysis conducted on 57 families enrolled at CovFC [35] . It evaluates 252 family clusters of COVID-19 attending the CovFC. All patients with confirmed infection at enrolment underwent serological follow-up at 1-4, 5-10, and >10 months after infection with quantification of anti-SARS-CoV-2 S-RBD IgG by chemiluminescent immunoassay. Among 902 subjects observed, 697 had confirmed SARS-CoV-2 infection, including 351 children/older siblings aged 8.6±5.1 years and 346 parents aged 42.5±7.1 years; of those, 96.5% cases had asymptomatic/mild COVID-19. A total of 659 study participants had at least one anti-SARS-CoV-2 S-RBD IgG titer performed after infection. During follow-up, 99.7% of them still recorded

positive titers, while 2/659 (0.3%) patients with confirmed COVID-19 negativized, after 64 and 556 days from baseline, respectively. None of these patients reported either exposure to other COVID-19 patients or a confirmed SARS-CoV-2 re-infection. However, we recorded an unexpected increase in S-RBD IgG titer for 17 patients. Considering the possibility of an unknown exposure to SARS-CoV-2, the last time-point sera of these 17 patients were excluded from the analysis. Children showed significantly higher S-RBD IgG titers than older siblings and parents across all follow-up time points, with an overall mean S-RBD IgG titer in patients <3 years of age five-fold higher than adults (282.3 [139-516.6] kBAU/L vs 56.7 [24.6-136.9] kBAU/L, p<0.001). These results align with prior studies using PRNT50 and surrogate-neutralization based-assays describing higher Abs titer and neutralizing ability in children than adults [35,73–76].

To explain this finding, we hypothesize that several factors such as specific cellular responses, genetic, environmental, and stochastic factors may be at the basis of a high variation in the immune response between individuals, irrespective of disease severity [68,77]. It has been shown that pre-pandemic children had class-switched convergent cellular clones to SARS-CoV2 with weak cross-reactivity to other coronaviruses, while adult blood or tissues showed few clones [78]. A recent paper [17] reinforces our supposition, suggesting that infection in elderly patients is associated with Abs targeting the cross-reactive S2 and NP proteins, while in children, the response is dominated by Abs with high Fc-effector function targeting the immunodominant S1 protein of SARS-CoV-2. Conversely, Renk et al. recently observed that repeated exposure to previous endemic human coronaviruses (HCoVs) did not impair the humoral response to SARS-CoV-2 [74]. Finally, given that our family clusters were likely exposed to similar environmental factors, genetic attributes may also contribute to the different potency and durability of humoral responses [79].

The longitudinal analysis of a sub-group of 56 subjects sampled at least twice during follow-up, with the first sample collected at 1-4 months from COVID-19 infection, demonstrated the long-term persistence of antibodies. We conducted the first analysis on 31 patients sampled at 89.2 (STD, ±38.6) and 199.2 (STD, ±30.3) days from baseline, while a second analysis was conducted on 40 patients evaluating samples collected at 81.9 (STD, ±25.7) and 380 (STD, ±47.7) days from baseline, to whom we will refer as medium and long intervals, respectively. Twenty-two patients were tested three times, contributing to both above-mentioned subgroups of patients. Both analyses were stratified by three age subgroups: <6 years, 6 to 18 years, and >18 years and all three age groups exhibited persistence of S-RBD IgG titers at both intervals. Remarkably, children aged <6 years exhibited a median S-RBD IgG titer of 132.7 (107-231.2) kBAU at 373 (339-376) days from baseline, and only two patients negativized. Nonetheless, a progressive decline of Abs levels was observed among all age classes and ranged between 2.0-2.3 fold and 2.5-3.6 fold reductions for the medium and long intervals, respectively.

To better investigate the decay in Abs across age groups, the same analysis was conducted on a sub-cohort of 84 COVID-19 cases tested at least twice for S-RBD IgG titers, regardless of the time of the first serum collection, for a total of 194 samples. Tracing a theoretical line obtained considering differences between individual Abs titers of all patients, disposed on the x-axis according to their collection time point, we observed that all of the three age groups exhibited progressive decay in Abs titer; the rate of Abs waning was more rapid

during the first 200 days and progressively slower thereafter. Compared to adults and children >6 years of age, children younger than six years showed an apparently faster early waning of Abs titers and then reached a plateau without Abs negativization, up to 18 months. These results are in line with recent studies conducted among both adults and children [74,80–83]. In particular, Lau et al. [83] observed that Abs were detectable by spike RBD ELISA assays in 92.6% of sera at 200-386 days from infection, despite showing an assay-dependent kinetics of Abs levels. In our study, the persistence of a detectable S-RBD IgG titer more than ten months after infection was observed in all age groups, regardless of whether they declined over time.

Finally, from the 139 individuals tested in parallel for both SARS-CoV-2 S-RBD IgG and the Plaque Reduction Neutralization Test ($PRNT_{50}$) for the detection of SARS-CoV-2 neutralizing antibodies, a total of 172 samples were available for estimating the correlation between the two assays. Overall, in the linear regression model, a positive correlation was found between $PRNT_{50}$ log titers and log2 S-RBD IgG titers (correlation coefficient R2 0.47; Spearman coefficient 0.73, $p<0.0001$). With this finding, we confirmed that $PRNT_{50}$ correlates significantly with the more available and easier-to-perform chemiluminescence assay, which could represent a promising "open-access" tool for the estimation of serum's neutralizing power.

Studies conducted before the advent of Omicron variants estimated that the correlate of 50% protection from re-infection was 20% of the convalescent NAbs titer [84]. Relying on these findings, Lau et al. [83] estimated that the threshold for 50% protection from re-infection for $PRNT_{50}$ was 1:25.9 (95% CI 1:24.7-1:27.6). As previously reported [36], an S-RBD IgG titer >70 kBAU/L is assumed to correspond to PRNT50 titer >1:20. In line with these findings, our data suggested that children <6 years might be protected from re-infection, up to 1 year. Although it was observed that mAbs can cross-neutralize different variants, including Beta, Delta, Gamma, and Mu for more than one year after infection [85], recent evidence has shown that with the rapid advance of the highly contagious Omicron variants, the level of protective immunity may be insufficient to protect by re-infections [85]. Recently, Dejnirattisai et al reported that the huge number of mutations observed in the Spike antigen of Omicron lead to escape from neutralization by either naturally acquired or vaccine-induced Abs, meaning that previously infected or vaccinated subjects can be re-infected by Omicron and probably by other emerging variants of concern (VoC) [86]. However, we can assume that protection from severe disease and mortality is likely to be preserved, as T cell response induced by a previous SARS-CoV-2 infection or vaccination plays a key role [61,87]. Future research should include the evaluation of B and T cells' longevity and role in preventing disease severity.

Our study has several limitations. First, operational challenges related to the pandemic restrictions affected both organization and access to the clinic; therefore, patients were evaluated with different time points of follow-up, and for a proportion of them, intermediate follow-up was missing. Second, the baseline of infection for those COVID-19 cases without positive NPs were identified through the only temporal reference to infection of the first symptomatic household and may be susceptible to temporal error. However, the initial temporal discrepancy, which may alter the evaluation of the acute phase of humoral response, was partially addressed by long-term follow-up.

Clinical findings from the "CASE cohort"

The cardiac involvement of SARS-CoV-2 infection in children recovered by asymptomatic or mildly symptomatic COVID-19 has been evaluated through the case-control study "*Left ventricle longitudinal strain alterations in asymptomatic or mildly symptomatic pediatric patients with SARS-CoV-2 infection*" (Chapter 4.5). Fifty-three pediatric patients of mean age of 7.5 years, underwent a standard transthoracic echocardiogram, and speckle tracking echocardiographic study at least three months after diagnosis and were compared with 32 comparable healthy controls. We found that the left ventricular ejection fraction was within normal limits but significantly lower in the cases group compared to controls (62.4 ± 4.1% vs 65.2 ± 5.5%; P = 0.012), while tricuspid annular plane systolic excursion and left ventricular (LV) global longitudinal strain (GLS) were comparable between the two groups. The regional LV strain deformation analysis showed a significant reduction of the LV mid-wall segments strain, and the most affected segments in the COVID-19 group were the mid-wall ones and the basal anterior, posterior and septal inferior ones, compared with the control group. With this finding, we demonstrated that a subclinical cardiac involvement might occur after asymptomatic or mildly symptomatic COVID-19, persisting at least three months after the infection.

Conversely, in our cohort, the apical segments showed higher deformation in the COVID-19 group. This finding agrees with the distribution of affected areas of the left ventricle in MIS-C patients, which does not follow coronaries distribution. In accordance with Piccinelli et al [88], we found that apical segments were spared or even showed increased deformation, increasing the base to apex gradient. This pattern has already been described in other cardiac diseases like systemic hypertension [89].

Furthermore, in the cases group, 14 subjects (26%) had a regional peak systolic strain below -16% (-2.5 Z score in our healthy cohort) in at least two segments, detected 118 days (SD 39) after COVID-19, suggesting for the first time that SARS-CoV-2 infection may affect left ventricular deformation despite an asymptomatic or only mildly symptomatic COVID-19. Due to the significant inter-vendor variation in normal values, this cut-off value was calculated based on our control group mean LV GLS value minus 2.5 SD. This value was significantly below the normality range proposed in a large meta-analysis in children by Levy et al.[90], and significantly lower than the 5th percentile for longitudinal strain proposed for healthy children by Cantinotti et al.[57.] Although abnormal systemic inflammatory response following infection is a described mechanism of indirect myocardial injury, in our subset of patients with reduced longitudinal strain we could not demonstrate any difference regarding symptoms or levels of SARS-CoV-2 antibodies, suggesting that the degree of the humoral response following the infection may not predict the subclinical myocardial injury's extension. More recently, Seidel F. et al. reported no evidence of myocardial inflammation, fibrosis, or functional cardiac impairment at cardiovascular magnetic resonance performed 42 days (37,8-54) after asymptomatic/mildly symptomatic COVID-19 in 18 children, compared with healthy controls [92].

The single-center nature of the study may constitute a major limitation. COVID-19 cases were enrolled at least three months after SARS-CoV2 infection. This delay may have influenced the proportion of children with an abnormal regional strain of the left ventricle. Nevertheless, one-quarter of our cohort presented LV deformation abnormalities late after infection. Unfortunately, we were not able to enroll a higher number of

control subjects due to the nature of our institution (tertiary care hospital), the time constraints of COVID-19 and the very limited access to elective cases during the COVID pandemic. Finally, the control group was not matched with cases but resulted in comparable demographic characteristics.

The single-center study "*Psychological impact and resilience of children and parents experiencing a family cluster of COVID-19*" (Chapter 4.6) provides evidence from a cross-sectional web-based survey distributed to 88 Covid-19 family clusters enrolled within the *CASE cohort* during the first two waves of the COVID-19 pandemic. The study aims at evaluating the psychological impact and the ability to adapt to the isolation of children and parents experiencing SARS-CoV-2 infection among one or more family member/s. A manuscript was submitted to the peer-reviewed journal Children MDPI, on July 18th 2022. Among the 176 surveys distributed (88 families) from March to October 2020, 75 were collected from 66 families, including 97 parents and 129 children; almost 70% of subjects had COVID-19, mostly asymptomatic/mildly symptomatic and median time from infection to survey compilation was 164,7 days (SD 56). Most (>87%) parents reported positive relationships with family members either before, during or after COVID-19. However, more than one-third of children and adolescents showed a scarce ability to adapt to isolation. Among 31 pre-school children of median age of 3 years (SD 1,7), a change of one or more functions was reported for 74,2% of cases irrespectively of COVID-19 status, particularly a change in circadian rhythm (25%), poor emotional control (36%) and changes in the relationship with parents (42,8%). Among 74 children with a median age of 10,9 years (SD 2,7), only 8,1% (6/74) had a score indicating a psychological disease; however, a significant impairment in attention was reported for 16,7% of cases – most of them were COVID-19 - and anxiety/depression and problems with conduct were significant for 5,6% and 6,5% of cases (all of them COVID-19), respectively.

Although we report descriptive results, our findings suggest that the severe quarantine measures adopted during the current pandemic may have a psychological impact on children and adolescents. However, the study has several limitations. Firstly, at the time of the survey's distribution, all family members were healthy and well recovered from COVID-19, consequently, the parents were retrospectively referring to the time of COVID-19, having time to rework the experience. However, we believe this can also constitute a strength, as parents probably provided a more objective and less emotional answer at the time of survey compilation, 164 days (IQR 56) after COVID-19. In addition, while the "*Questionnaire B/2 - Pediatric Symptoms Checklist"* is a validated score used to evaluate mental health in children and adolescents aged 6-17 years, the "*Questionnaire B/1 – pre-school children"* was constructed ad hoc by our specialists to evaluate the behavioral response of younger children to the same stressful situations. Therefore, it needs to be validated by further studies. Third, not having enrolled families of children with significant disabilities and/or living with social-economic disadvantages, our results may underestimate the intra-family difficulties and the behavioral changes encountered during social isolation.

CONCLUSION

In conclusion, this Ph.D thesis represents the first effort to implement the knowledge of SARS-CoV-2 infection among children and adults, trying to provide a comprehensive overview of different but complementary aspects, ranging from the implementation of infection prevention and control measures to contain the in-hospital spread of SARS-CoV-2, up to the characterization of the humoral and cellular immune response over time and stratified by age classes, and ending with the evaluation of clinical and psychological findings observed within COVID-19.

I believe that one of the major results of this research project remains the setting up and implementation of an effective outpatient care program specifically dedicated to children, older siblings and parents recovered from a COVID-19 family cluster. The *COVID-19 Family Cluster Follow-up Clinic (CovFC)* set up at the Department of Women's and Children's Health of the University Hospital of Padua has been providing care to families with a comprehensive clinical program through the collaboration of several professional figures working in a team as part of a multidisciplinary network. The connection of different figures has been allowing a continuous sharing of knowledge, actively contributing to carry on the integrated evaluation and study of epidemiological, clinical, and immune-virological characteristics of COVID-19 among children and adults.

Since March 2020, the "*CASE cohort*" has been expanding. At the current time, it includes more than 400 families, accounting for 724 children/older siblings and 738 parents that have been actively followed up over time. Several operational challenges have been faced since the pandemic's beginning, mostly related to restrictions affecting both organization and access to the clinic. Future efforts are needed to optimize and re-organize the clinical care program in line with the new emerging evidence, to deal with challenges mostly related to the rapid emergency of new and still unknown SARS-CoV-2 variants of concern (VoC). In addition, both efforts and extreme flexibility are required to continuously optimize the follow-up of patients, elaborating new research questions and objectives to carry on in providing new evidence, to fill existing gaps on SARS-CoV-2 infection and disease.

FUTURE PERSPECTIVES

Since the beginning of this project, it was clear that this unique cohort would be highly relevant to the long-term study of clinical and immunological of COVID-19 among children and adults. For this reason, the "*CASE cohort*" has been included in the three-year international research project "ORCHESTRA", aimed at tackling the coronavirus pandemic, led by the University of Verona and involving 26 partners (extending to a broader network of 37 partners) from 15 countries: Argentina, Belgium, Brazil, Congo, France, Gabon, Germany, India, Italy, Luxemburg, Netherlands, Romania, Slovakia, Spain, Venezuela. The project is financed by Horizon 2020 research and innovation programme under Grant Agreement Number 101016167 (https://orchestra-cohort.eu/). ORCHESTRA consists of 11 work packages with different tasks. Member vision

is to establish a large-scale international cohort to conduct retrospective and prospective studies to generate rigorous evidence to improve the prevention and treatment of COVID-19 and be better prepared for future pandemics. The study objectives are: 1) to develop evidence-based recommendations for effective prevention, protection, and optimized treatment of COVID-19 patients (including long-term consequences) with a particular focus on 'at risk' population, including healthcare workers and fragile individuals; 2) to assess the impact of environmental factors, socio-economic determinants, lifestyle, and confinement measures on the spread of COVID-19; 3) to provide knowledge on the efficacy of vaccines against SARS-CoV-2; 4) to provide a model for responsiveness for future pandemic outbreaks.

The *"CASE cohort*" has also been included in the international research project "SARS-COV-2 VARIANTS EVALUATION IN PREGNANCY AND PAEDIATRICS COHORTS" (acronym: VERDI), funded by the European Union (grant n°101045989). The VERDI consortium consists of 22 centers of excellence in Europe, the USA, South Africa, the Caribbean, the Middle East and Asia, coordinated by the University of Padua and Penta Foundation (Italy), with scientific coordination shared between the University of Padua and University College London.

Several aspects of SARS-CoV-2 infection are still unknown, and significant efforts must be made to fill the existing gaps, providing new evidence for clinicians and people affected by COVID-19. Considering the peculiar characteristics of the "*CASE cohort*" as much as the participation of the cohort within international collaborations, in the future, we expect that it will contribute to improving knowledge in several aspects, from the further characterization of cellular and humoral response to different SARS-CoV-2 VoC to the evaluation of the immunological response after pediatric vaccination against SARS-CoV-2, up to the assessment of the long-term clinical and psychological impact for the study of long COVID-19 disease in the pediatric population.

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ABBREVIATIONS

ACE2	Angiotensin-Converting Enzyme 2
AIDS	Acquired Immune Deficiency Syndrome
ARDS	Acute Respiratory Distress Syndrome
ARV	antiretrovirals
Bregs	regulatory B cells
CAR-T	Chimeric Antigen Receptor T-cell therapy
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CLIA	Chemiluminescence Immune Assay
COVID-19	Coronavirus Disease-2019
CovFC	COVID-19 Family Cluster Follow-up Clinic
CRF	Case Report Form
EDTA	Ethylenediamine tetraacetic acid
FDA	Food and Drug Administration
GLS	Global Longitudinal Strain
GMT	Geometric Mean Titer
HCoVs	Human Coronaviruses
HIV	Human Immunodeficiency Virus
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-35	Interleukin 35
IPC	Infection Prevention and Control
IQR	Inter Quartile Range
LV	Left Ventricular
mAbs	monoclonal Antibodies
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
MIS-C	Multi-System Inflammatory Syndrome - Children
NPs	Nasal Pharyngeal swab
PLWHA	People Living With HIV/AIDS
PCR	Polymerase Chain Reaction
PRNT	Plaque Reduction Neutralization Test
PSC	Pediatric Symptoms Checklist
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SD	Standard Deviation

RBD	Receptor Binding Domain
RdRp	RNA-dependent RNA polymerase
TGF-β	Transforming Growth Factor beta
TMPRSS2	Transmembrane Protease, Serine 2
TNF-alpha	Tumor Necrosis Factor alpha
TTE	Trans Thoracic Echocardiogram
Tregs	regulatory T cells
VL	Viral Load
VoC	Variants of Concern
WP	Work Package
3CLpro	3-Chymotrypsin-Like protease

LIST OF PUBBLICATIONS

Publications related to my Ph.D activities

Policy Brief Article. "*Children's Hospital management in the COVID-19 era: the reorganization of a tertiary care Paediatric Emergency Department in Northern Italy"*. Daniele Donà, Susanna Masiero, Paola Costenaro, Marco Todeschini Premuda, Sara Rossin, Giorgio Perilongo, Anna M. Saieva, Carlo Giaquinto, Liviana Da Dalt. Frontiers in Pediatrics. Manuscript ID: 594831. Received on: 14 Aug 2020. Revised on: 15 Sep 2020.Accepted for publication on 22 October 2020.

Communication. "*COVID-19 Pandemic: Perspective of an Italian Tertiary Care Pediatric Center*". Daniele Donà, Carlo Giaquinto, Eugenio Baraldi, Alessandra Biffi, Piergiorgio Gamba, Anna Maria Saieva, Luca Antoniello, Paola Costenaro, Susanna Masiero, Laura Sainati, Liviana Da Dalt and Giorgio Perilongo. Healthcare 2020, 8, 311; doi:10.3390/healthcare8030311.

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