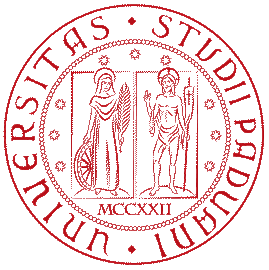

UNIVERSITÀ DEGLI STUDI DI PADOVA

Corso di Laurea Magistrale in Medicina e Chirurgia

Dipartimento Di Salute Della Donna e del Bambino

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TESI DI LAUREA

Evaluation of anti-SARS-CoV-2 S-RBD antibodies production and their persistence over time among a cohort of family clusters, including children and parents, after SARS-CoV-2 infection

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ANNO ACCADEMICO 2021-2022

SUMMARY

ABSTRACT

Background. While it is known that SARS-CoV-2 antibodies may persist in adults 12 months after the infection, there are few data on the pediatric population. We previously proved that children recovered from mild or asymptomatic COVID-19 present an intense early neutralizing antibodies (NAbs) production: they were found to persist up to 7-8 months in children while adults recorded a modest declining trend.

To date, the longer-term kinetics of Abs in children remains to be investigated.

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

Materials and methods. From April 2020 to August 2021, a single-center, prospective observational cohort study was conducted on 252 family clusters of COVID-19 evaluated 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.

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.

Results. Among 902 study participants, 697 were confirmed COVID-19 cases, including 351 children/older siblings aged of 8.6±5.1years, and 346 parents with 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, $p<0.0001$). The 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 significant decline over time.

Conclusions. In this study, we confirmed the different kinetics of the SARS-CoV-2 S-RBD IgG across several age classes of asymptomatic/mild COVID-19 cases. We proved that antibodies persisted until 12 months after infection in all age groups, with a significant peak Abs titer inversely related to age. Indeed, we found that the magnitude of SARS-CoV-2 S-RBD IgG Abs is higher among younger children than older siblings and adults at all follow-up time points.

ABSTRACT (ITALIAN VERSION)

Background. Mentre è ormai noto che negli adulti gli anticorpi anti-SARS-CoV-2 possono persistere per più di 12 mesi dall'infezione, ci sono pochi dati riguardanti la persistenza di anticorpi a lungo termine nella popolazione pediatrica. In uno studio precedente abbiamo dimostrato che i bambini guariti dall'infezione lieve o asintomatica presentano un'intensa precoce produzione di anticorpi neutralizzanti (NAbs), che si è visto persistere fino a 7-8 mesi, mentre negli adulti si è registrato un modesto declino.

Ad oggi, rimane da investigare la cinetica degli anticorpi nei bambini nel lungo periodo.

Scopo dello studio. Descrivere la risposta umorale e la cinetica a lungo termine degli anticorpi anti-SARS-CoV-2 S-RBD IgG nei bambini e i loro genitori a seguito dell'infezione.

Materiali e metodi. È stato condotto uno studio di coorte osservazionale, prospettico, monocentrico, su 252 famiglie valutate da aprile 2020 ad agosto 2021 presso l'ambulatorio di follow-up post-COVID-19 istituito presso il Dipartimento di Salute della Donna e del Bambino di Padova.

Tutti i pazienti con infezione confermata all'arruolamento sono stati sottoposti a un follow-up sierologico a 1-4, 5-10, e oltre 10 mesi dell'infezione con la quantificazione delle IgG anti-SARS-CoV-2 S-RBD tramite metodica a chemiluminescenza.

Risultati. Tra i 902 partecipanti allo studio, sono stati confermati 697 casi di COVID-19, di cui 351 bambini e adolescenti (età di 8,6±5,1 anni) e 346 genitori (età 42,5±7,1 anni). Il 96.5% di essi ha avuto un'infezione asintomatica o lieve.

I bambini hanno mostrato titoli di IgG anti-S-RBD significativamente più elevati dei genitori in tutti i time-point follow-up, e in particolare i bambini al di sotto dei 3 anni hanno sviluppato un titolo di IgG anti-S-RBD ben 5 volte superiore rispetto agli adulti (304.8 [139-516.6] kBAU/L vs 55.6 [24.2-136.0] kBAU/L, $p<0.0001$).

L'analisi longitudinale di 56 partecipanti allo studio campionati almeno due volte durante il follow-up ha dimostrato la persistenza degli anticorpi fino a oltre 10 mesi dall'infezione in tutte le classi di età, nonostante un progressivo declino significativo nel tempo.

Conclusioni. In questo studio abbiamo dimostrato la differente cinetica degli anticorpi anti-SARS-CoV-2 in diverse classi di età, in soggetti che sono stati asintomatici o lievemente sintomatici. Abbiamo dimostrato che gli anticorpi anti-SARS-CoV-2 S-RBD IgG persistono fino ad almeno 12 mesi dall'infezione in tutti i gruppi d'età, con un picco significativo del titolo anticorpale inversamente correlato all'età: infatti, è stato osservato che la risposta anticorpale è maggiore nei bambini più piccoli (soprattutto di età < 3 anni) rispetto ai fratelli maggiori e agli adulti in tutti i time-point follow-up.

1. INTRODUCTION

Coronaviruses are a large family of animal and human pathogens that usually cause mild to moderate upper-respiratory tract illnesses infections in humans. However, three coronaviruses caused more serious and fatal diseases: SARS coronavirus (SARS-CoV), which emerged in November 2002 and provoked Severe Acute Respiratory Syndrome (SARS); MERS coronavirus (MERS-CoV), which emerged in 2012 and caused Middle East Respiratory Syndrome (MERS); and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV.2), which emerged in 2019 and caused the ongoing coronavirus disease 2019 (COVID-19). (1)

Although, so far, no direct ancestor to the SARS-CoV-2 that can fully explain its emergence has been found (2), the current hypothesis is that it emerged in the Wuhan Seafood Market in Wuhan, in Hubei province, China, the city where it was firstly detected in December 2019. However, there are discrepancies both on the place and period of origin of the virus. Some authors stated that the first patient, so the one who may be the *first COVID-19 case*, the so-called "patient zero", had no direct link with the Wuhan Seafood Market. There is not even clarity on the earliest date of symptoms: some studies argued that there were subjects who went to the hospital for symptoms as early as November 2019, others in December 2019. However, Chinese doctors began to realize that they were dealing with a new and serious virus only in late December 2019, when similar symptoms continued to increase every day, and mostly originated from Wuhan. (3-6)

On January 11, 2020, the first related death was reported in China and two days later the first case of pneumonia caused by the novel coronavirus was recorded outside China. On January 30, WHO declared that the novel coronavirus outbreak constitutes a Public Health Emergency of International Concern (PHEIC) (7) and in early February 2020 announced that the disease caused by the novel coronavirus would be officially named Corona Virus Disease 2019 (COVID-19), and the virus responsible *SARS-CoV-2*. (8) The virus spread worldwide very fast, and on March 13, 2020 WHO declared that Europe had become the epicenter of the pandemic with more reported cases and deaths than the rest of the world combined, apart from the People's Republic of China. (9) (**Fig. 1**)

Globally, as of June 1st, 2022, there have been 527.603.107 confirmed cases of COVID-19, including 6.290.452 deaths reported to WHO. (10) Italy was one of the most countries affected by the pandemic, and from January 3, 2020 to June 1st, 2022, there have been 17.421.410 confirmed cases of COVID-19 with 166.697 deaths. (10) (**Fig. 1**)

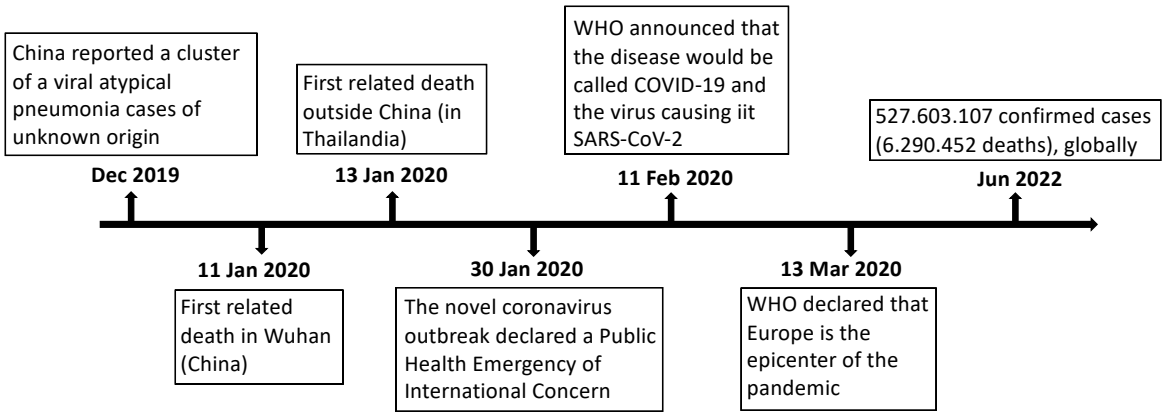

***Figure 1**: Timeline of COVID-19 pandemic.*

During these years of pandemic, the high numbers of infected people have brought health care systems worldwide to their knees, with overcrowding of hospitals, in addition to the serious economic and social problems. Various strategies have been adopted to halt the advancement of viral transmission, first of all lockdowns, which, however necessary, have contributed to develop social problems of clinical relevance, too, such as psychosocial disorders. To date, vaccines are the most effective tool to reach the desired herd immunity in a short period of time.

At the beginning of the pandemic, there were not many infected children, so they were supposed to play no role in the progression of the pandemic. This was probably the reason why several studies examined COVID-19 infection and antibody response to it in adult population, while few studies included children.

Later, the epidemiological situation changed, with an increase in pediatric cases and it was understood that children contributed to fueling the pandemic, playing a key role in transmission of the virus. For this reason, it became necessary to study them, too, to investigate how SARS-CoV-2 affected children and how they

responded to it, as they had different clinical and serological characteristics compared to adults.

To stop the transmission sustained by this part of population, too, the best available strategy is vaccination. Furthermore, a targeted vaccination campaign in children is essential to prevent children from contracting severe COVID-19 infection and severe sequelae that can follow even a mild acute infection, namely post-COVID-19 syndromes (MIS-C and Long COVID).

While in adults the immune response induced by vaccination is well known, to date, there is still no studies on antibody response following vaccination in children because the vaccination programs in this population is very recent.

Studying how long the post-infection antibody titer lasts in children after the acute infection, we could try to predict the antibody response following vaccination, thus understanding the long-term vaccine efficacy and optimizing future COVID-19 vaccination strategies, the only way to end the pandemic.

1.1 VIROLOGY

1.1.1 CLASSIFICATION OF CORONAVIRUSES

Coronaviruses are enveloped, positive-stranded RNA viruses that constantly circulate among the population (as well as among animals) and usually cause mild respiratory disease (11). They affect humans and a wide variety of animal species. Many human coronaviruses come from bats, which are considered their natural hosts. However, how SARS-CoV-2 was transmitted from animals to humans is currently unknown. (12)

The family *Coronaviridae* contains the sub-family *Orthocoronaviridae*, also known as Coronavirus (CoV), divided into four *genera*: Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus. Among these, Alpha- and Betacoronavirus are human Coronavirus. Each genus is further subdivided into *sub-genera*, which are divided into *species*. Specifically, SARS-CoV, MERS-CoV and SARS-CoV-2 are three *species* included into the Betacoronavirus *genus* but

belonging to two different *sub-genera*: SARS-CoV-2 is a *Betacoronavirus* that belongs in the same *sub-genus* as SARS-CoV, namely Sarbecovirus, while MERS-CoV virus belongs to the *sub-genus* Merbecovirus, so it is less related to the first two. (**Fig.2**)

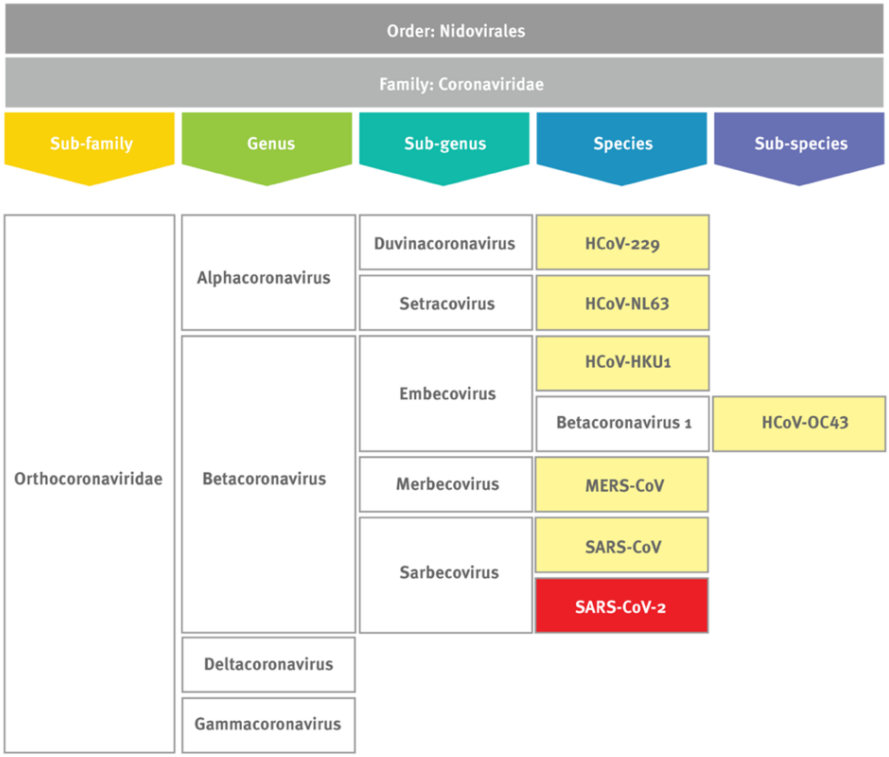

***Figure 2**: Human coronavirus taxonomy. The human coronaviruses are depicted in yellow and SARS-CoV-2 responsible for COVID-19 in red. Source: Adapted from the International Committee on Taxonomy of Viruses (ICTV).*

1.1.2 VIRAL STRUCTURE AND PATHOGENESIS

The coronaviral genome contains four major structural proteins: Spike (S), envelope (E), membrane (M), and nucleocapsid (N) protein. The S protein mediates attachment of the virus to the host cell surface receptors; the nucleocapsid (N) protein is necessary for viral replication, as well as the envelope (E) protein; the membrane (M) protein defines the shape of the viral envelope. (13) (**Fig. 3**)

To enter target cells, SARS-CoV-2 relies on its obligate cellular host receptor Angiotensin-Converting Enzyme 2 (ACE-2), to which it binds via the Spike protein (S). It also requires priming by cellular serine protease TMPRSS2 that cleavage protein S and allows fusion between viral and cellular membrane. (14)

The S protein is made up of the S1 and S2 subunits. In S1 there is a receptor-binding domain (RBD) that interacts with ACE-2 expressed by human host cells, allowing the virus enter into the cells. (15-19) Interestingly, it was seen that the binding affinity of these two structures in SARS-CoV-2 is much higher compared to SARS-CoV. (20) Furthermore, mutations in the SARS-CoV-2 RBD are associated with enhanced ACE-2 affinity and are considered to underpin key characteristics of variants, such as Delta and Omicron VOC (Omicron Variant of Concern), which show increased transmissibility and immune evasion. (21) Another noteworthy feature of ACE-2, especially in the pediatric population, is that it is present on multiple human epithelial surfaces, not only in the upper and lower respiratory tract (where it causes the most common flu-like symptoms of Covid-19, such as cough and cold), but also in the gastrointestinal tract, endovascular epithelia and heart, typical extrapulmonary localizations of the child, providing possible routes of entry for the virus and other types of symptoms. The ACE-2-RBD interaction and the mapping these receptors in different human body's district allowed us to better understand the pathogenesis of the main manifestations of COVID-19 disease both in children and adults. (22)

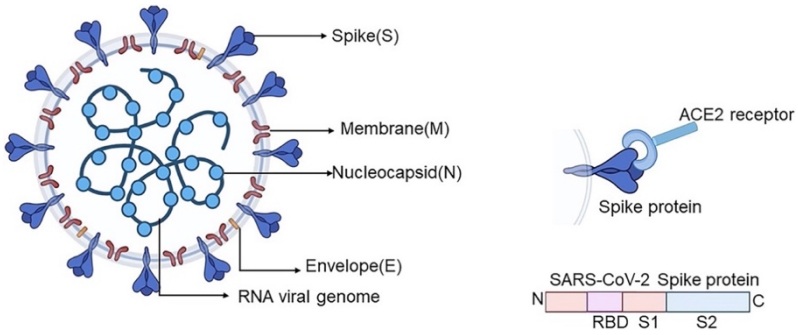

***Figure 3**: Figure 3: Schematic diagram of the SARS-CoV-2 coronavirus particle. Four structure proteins contain the envelope protein (E), nucleocapsid protein (N), spike protein (S), and membrane protein (M). SARS-CoV-2 virus enters the cell through the S protein on the surface of SARS-CoV-2 by means of binding with its receptor ACE2. The S protein is mainly divided into S1, which contains RBD, and S2 subunits. Source: Min L and Sun Q (2021) Antibodies and Vaccines Target RBD of SARS-CoV-2. Front: Mol. Biosci.*

1.1.3 TRANSMISSION AND INFECTIOUSNESS

Direct person-to-person transmission is the primary form of transmission of SARS-CoV-2, chiefly through close personal contact (within 1-2 meters) via respiratory particles (*droplets*) containing the virus emitted when an infected person coughs, sneezes, or talks. The risk of transmission seems highest in indoor poorly ventilated places, where there is no constant air exchange, such as restaurants, buses, etc. Indeed, it has been seen that infections take place mostly in settings where individuals are residing or working at a close distance and among household contacts, while infectiveness in outdoor spaces seems less probable. To infect another individual, the virus must be inhaled or needs to come in direct contact with the mucosa, for example bringing contaminated hands to the eyes, nose, and mouth. (23)

Even if the primary route of COVID-19 infection is through the respiratory system, viral RNA was also detected in surfaces, and transmission through contact with contaminated fomites and by touching contaminated surfaces is considered possible, although their role in the viral spread remains unclear. (24-26)

The risk of becoming infected with SARS-CoV-2 is a combination of susceptibility (host biological factors), environmental factors associated with exposure type (work, shopping, schools, etc.), and exposure intensity (level of community transmission and preventive measures like masks). It is difficult to separate the influence of these factors on the risk of children and adults becoming infected. Moreover, transmission of SARS-CoV-2 from infected but asymptomatic individuals has been well reported and RT-PCR cycle threshold values, as well as the persistence of the viral RNA in symptomatic and asymptomatic individuals appears to be similar (27), suggesting that also asymptomatic patients must be isolated to control the viral spread.

The precise time-lapse in which an infected individual can transmit the virus is still debated. It seems that infectiousness begins some days before any manifestation of symptoms and the affected patients are more likely to spread the virus during the first days of the infection. The incubation period of SARS-CoV-2 appears to be about the same for children and adults, up to 14 days with an average

of 5-6 days from exposure to symptom onset. The problem is that with the emergence of variants, information on transmissibility and incubation/infectious periods continues to evolve. (28) In a work by Tsang et al. (29), viral load in oropharyngeal samples was highest during the first week after the symptoms' onset and then dropped with time, as confirmed by other studies as well, which documented that the infectiousness peaked between two days before and one day after symptoms onset and declined seven days after, making transmission happens most likely in household clusters in a pre-symptomatic stage. (30) There have been cases in which viral RNA has been detected after several months (31), but prolonged viral RNA presence does not seem to indicate prolonged infectiousness.

1.1.4 EPIDEMIOLOGY AND TRANSMISSION OF SARS-COV-2 IN CHILDREN

Over time, in the various pandemic waves, the incidence of COVID-19 in pediatric population changed and, if at the beginning of pandemic children seemed spared by the virus, in the following waves reported COVID-19 cases among children spiked dramatically. This trend is motivated by several factors: first of all, every virus tends to affect the elderly first, as they are fragile, and then reach the child as well; secondly, children were the most protected part of the population, as they remained in their houses, because of schools, gym, parks closure, coming into contact only with their parents, who for a long time represented their only possible source of contagion, indeed most children infected with COVID-19 became infected via a family member (households contacts); finally, in children the vaccination campaign began later than in the rest of the population, thus exposing them to a greater susceptibility.

Furthermore, at the beginning of pandemic children did not even seem to be a vector of SARS-CoV-2 transmission. In fact, during the first pandemic waves, most pediatric cases have been described inside family clusters with an adult as the *index patient*, and without documentation of child-to-child or child-to-adult transmission. But these findings must be interpreted with caution because these cases were identified after implementation of strict physical distancing measures (closure of the meeting places), limiting the exposure of children to close contacts outside their family members. (23, 32-34)

Subsequent studies showed that children and adolescents can not only get COVID-19, but also spread the virus to other children and adults. To date, although children are more likely to develop a less severe COVID-19 than adults, they play an essential role in spreading the virus, contributing to the disastrous worldwide evolution of the pandemic. Infected children seem to drop SARS-CoV-2 with nasopharyngeal viral loads comparable or higher (in the first 2 days of symptoms) than hospitalized adults (35), even if a study (36) presenting a multicenter investigation on over five thousand SARS-CoV-2 cases confirmed by RT-PCR assay found no discernable difference in the amount of viral nucleic acid among young children and adults. Besides, transmission by asymptomatic children has also been reported (37), suggesting that also non-symptomatic children can play a decisive role in viral transmission and could be unknowingly silent spreaders of infection in populations.

1.2 CLINICAL, LABORATORISTIC AND RADIOLOGICAL FEATURES OF COVID-19 IN CHILDREN

From the beginning of the COVID-19 pandemic, it became evident that the presentation and severity of the disease differ across age classes, and younger children, school children, and adolescents infected with SARS-CoV-2 remain mostly asymptomatic or mildly symptomatic, largely spared from severe respiratory illness compared to adults, where SARS-CoV-2 often caused a life-threatening COVID-19 pneumonia that required hospitalization.

The infection in children is mild in more than 95% of cases and this is reflected both in the clinic manifestations, with rare case of pneumonia and intensive care admission, and in laboratory and radiodiagnostic findings, which in most cases are less marked than adults.

Despite this, even children and adolescents can develop complications during infection, prolonged clinical symptoms, and severe sequelae up to life-threatening events. In these cases, clinical, laboratory and radiological manifestations differ from the more common ones. These patients are usually the

more fragile ones, due to their young age (< 1 years) or pre-existing comorbidities, that are to be considered as risk factors.

1.2.1 CLINICAL MANIFESTATIONS AND RISK FACTORS FOR SEVERE DISEASE

Children with symptomatic COVID-19 infection usually report fever with one or more associated respiratory symptoms, most frequently rhinitis and cough, practically indistinguishable from seasonal respiratory viral infections. (38) Other frequent symptoms are shortness of breath, myalgia, rhinorrhea, sore throat, headache, loss of smell and/or taste. In infants under 12 months old, respiratory symptoms may be minimal but, if present, they are similar to those seen in older children. Other clinical pediatric COVID-19 manifestations are feeding difficulty and fever from unknown origin (FUO). (39) Gastrointestinal manifestations, such as diarrhea, nausea, vomiting, and abdominal pain, could be present, also without respiratory symptoms.

The lack of specificity of signs or symptoms and the significant proportion of asymptomatic infections make symptom-based screening for identification of SARS-CoV-2 in children, particularly challenging. (40) On the other hand, this mild presentation contrasts with other typically pediatric respiratory infections, such as the respiratory syncytial virus (RSV) or influenza, which have a more significant burden in terms of disease severity and hospitalizations in young children than adolescents and adults. (41, 42)

The biological mechanisms for the age-related differences in severity and for the reduced susceptibility to severe SARS-CoV-2 infection in the pediatric population are still under investigation, but several hypotheses have been proposed. Firstly, the expression of ACE-2 receptors and TMPRSS2 required for SARS-CoV-2 viral entry increases with age (43), leading probably to a decreased viral replication or lesser susceptibility to pulmonary infection in the younger population. Secondly, the presence of pre-existing, non-neutralizing antibodies to the common-cold human coronaviruses (HCoVs), which could recognize SARS-CoV- 2 early in infection, through a viral interference, may sustain inflammation in adults by increasing viral entry and innate responses in macrophages. (44) Furthermore, the

efficient early control of inflammation due to a robust anti-viral innate immune response that appears early in children may be the key to better control the infection and mitigating the disease course. (45, 46) Still, children might react to the virus with a less intense immune response, indeed cytokines storms are at the basis of the pathogenesis of a severe COVID-19-disease. (47, 48)

Although COVID-19 in children is almost always asymptomatic or mild, some children can develop a severe form, even requiring hospitalization, critical care support, treatments in the intensive care unit, ventilator to breathe. Current evidence suggests that, although severe and protracted COVID-19 disease can also be found in healthy children, those with certain underlying medical conditions and infants (age < 1 year), due to the vulnerability given by the young age, are at increased risk for severe illness from SARS-CoV-2 infection. (49-51) Multicentre studies, as the one by Götzinger F. et Al. (52), show that most pediatric patients admitted to ICU have at least one underlying disease, and especially children younger than 1 month have higher risk of requiring ICU.

According to CDC surveillance network COVID-NET, as of May 22, 2021, 47.3% of all American children infected by SARS-CoV-2 showed no comorbidity at the moment of diagnosis, while the remaining 52.7% of them showed an underlying medical condition, including: (**Fig. 4**)

- Obesity – 34.2%;
- Asthma or other chronic lung diseases – 13.2%;
- Neurological conditions – 12.9 %;
- Metabolic conditions – 5.3%;
- Cardiovascular disease – 5.1%;
- Chronic lung disease – 5%;
- Immunosuppression – 4.7%;
- Hypertension – 1.8%;
- Gastrointestinal/liver disorders – 1.3%;
- Renal disease – 1.3%;
- Autoimmune disease – 4%;

- Other disease – 19.5% (such as diabetes, genetic conditions, congenital heart disease, malignant disorders, blood disorders such as Sickle Cell Disease).

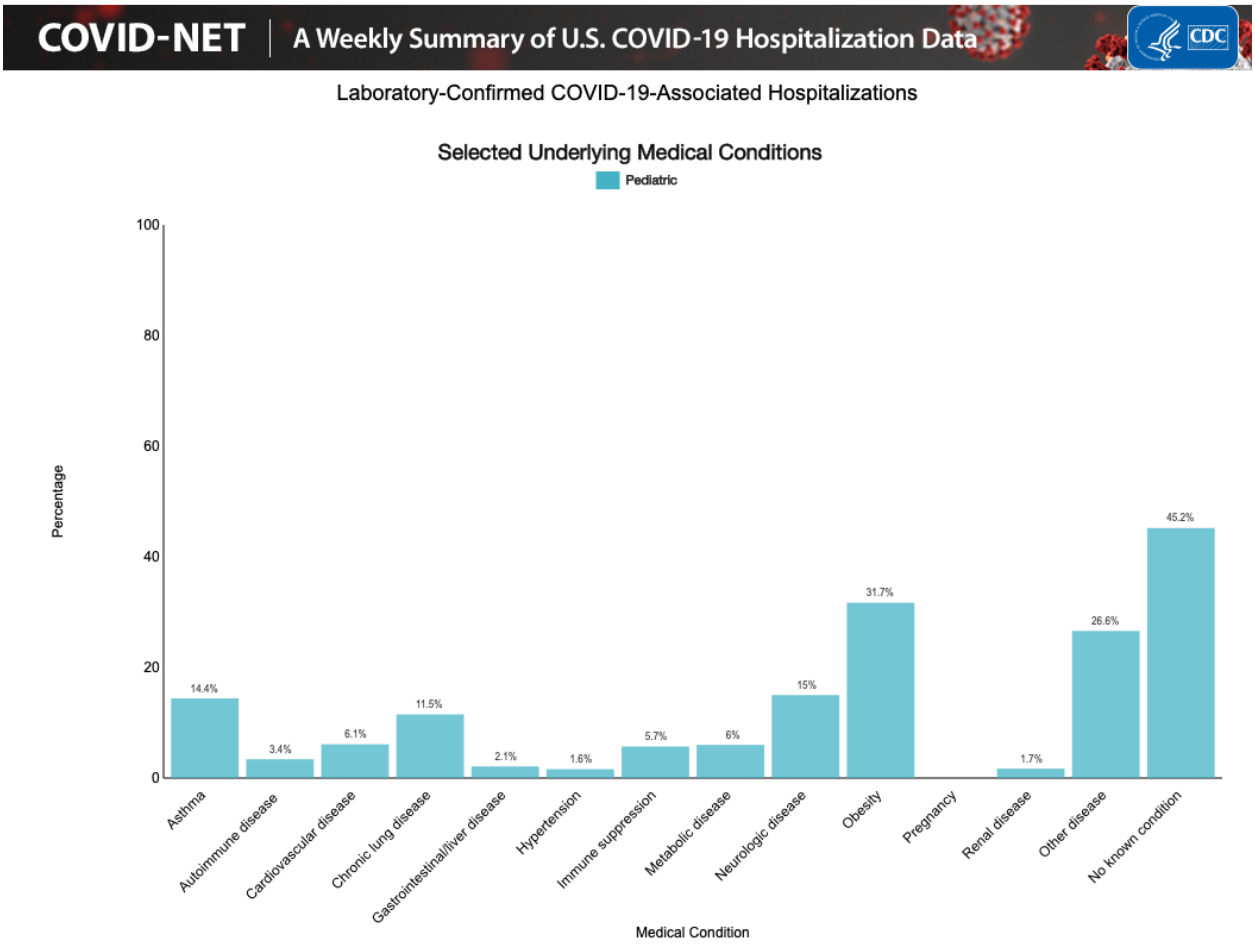

***Figure 4**: Underlying medical condition in pediatric patients. COVID-NET: COVID-19-Associated Hospitalization Surveillance Network, Centers for Disease Control and Prevention. Available at: gis.cdc.gov/grasp/COVIDNet/COVID195.html. Accessed on May 26, 2022.*

As can also be seen in the figure above (**Fig. 4**), studies on hospitalized children show that obesity is the most prevalent underlying condition. (53)

Research also suggests differences not dependent on comorbidities: there seems to be a racial disparity with higher rates of children infected by COVID-

19 and requiring hospitalization in African, American or Hispanic children (54), and a gender disparity with an increased risk of severe COVID-19 requiring hospitalization among male children. (55)

In the pediatric population, extrapulmonary involvement, such as urological manifestations and cardiac dysfunction manifesting as acute myocardial injury, myocarditis, arrhythmias and cardiomyopathy, is rare but can be severe. Such non-pulmonary findings are seen in under 5% of hospitalized children and often coexist with pulmonary disease. (56) In contrast to adult infection, clinically significant acute hepatitis is rare, though occasional case reports exist (57), as well as neurological findings, such as status epilepticus, encephalopathy, encephalitis, Guillain-Barré syndrome and acute demyelinating syndromes, which occur above all in those with pre-existing neurological conditions.

1.2.2 LABORATORY EXAMS

Typical laboratory findings in children with COVID-19 include a mild elevation of inflammatory markers (including procalcitonin), mild abnormalities in white blood cell counts (increased or decreased lymphocyte count), and a mild raise of liver enzymes. (58)

Irfan O. et al, (56) in a meta-analysis, in 66 studies with a total of 9335 children (0 to 19 years old), found:

- Elevated C-reactive protein (CRP) – 54 %;
- Elevated serum ferritin – 47 %;
- Elevated lactate dehydrogenase – 37 %;
- Elevated D-dimers – 35 %;
- Elevated procalcitonin – 21 %;
- Elevated erythrocyte sedimentation rate – 19 %;
- Elevated leukocytes – 20 %;
- Lymphocytopenia – 19 %;
- Lymphocytosis – 8 %;
- Elevated serum aminotransferases – 30 %;
- Elevated creatine kinase myocardial band – 25 %.

Several studies investigated potential markers of severe illness COVID-19-correlated. Higher inflammatory markers such as procalcitonin, CPR, D-dimer and interleukin 6 at admission or during hospitalization seem to be associated with increased gravity of the disease in children. (59-63) A lot of studies analyzed a possible association between leucopenia, lymphopenia and the disease severity (64), and discovered that increased NLR (Neutrophil Lymphocyte Ratio) is a prognostic factor which can be used independently to assess the severity and prognosis of clinical symptoms in COVID-19 pediatric patients; children with severe COVID-19 had lower lymphocyte count than those with moderate disease. Other studies showed that lymphocytopenia is correlated with a worse course of the infection in children, just like in adults: in a systematic review (65) with metanalysis including 7 studies and 2083 patients, 25% of whom had severe disease, lymphocyte counts in patients with mild disease were 30% higher than in those with severe disease thus asserting that lymphocytopenia can be considered a negative prognostic factor for severe COVID-19 progression.

1.2.3 IMAGING FINDINGS

Since COVID-19 is usually mild in children, chest imaging is not needed most of the time. However, when a moderate or severe form is suspected, therefore COVID-19 pneumonia, chest imaging come into play as a support, especially for the assessment of disease progression and prognosis.

Data on imaging features of COVID-19 in children are scarce. However, differences in chest imaging between pediatric and adult cases of COVID-19 pneumonia were reported.

While in adults the thoracic imaging is a support to the swab in the diagnosis, in children its role is a matter of debate because the imaging findings are neither specific nor sensitive so they are often unnecessary, and even worse, may carry risks of misdiagnosis. (66) Indeed, there is some overlap in the imaging presentation of COVID-19 and other entities like infections (influenza A, influenza B, or Mycoplasma pneumoniae), inflammatory processes (electronic cigarette vaping–associated lung injury or hypersensitivity pneumonitis), eosinophilic lung

disease in the pediatric population, and therefore a low COVID-19 prevalence could lead to false-positive results. (67)

A group of international experts in pediatric thoracic imaging from five continents created a consensus statement (67), following recommendations of the American College of Radiology, describing the imaging manifestations of COVID-19 in the pediatric population, and generating recommendations for the use of chest radiographs and CT in the evaluation of pediatric patients with COVID-19. They defined three key points:

- Imaging is not indicated for pediatric patients presenting with mild clinical symptoms unless the patient has risk factors for disease progression or develops worsening clinical symptoms.
- Sequential chest radiograph examinations, ordered on an as-needed clinical basis, are indicated for pediatric patients with COVID-19 to assess response to therapy, evaluate clinical deterioration, or assess the positioning of life support devices.
- Post-recovery follow-up imaging is not recommended for asymptomatic pediatric patients with a mild COVID-19 disease course; however, it may be considered in asymptomatic individuals with an initial moderate-to-severe disease course or symptomatic individuals regardless of initial disease severity depending on the level of clinical concern for long-term lung injury.

If imaging is needed, chest X-ray should be the first choice. It is even more essential in this patient population due to the increased radiation sensitivity of children and hesitancy to pursue CT, even if the results of this test may be normal in the early stage of the disease or in patients with mild disease. Both unilateral and bilateral infiltrates and opacities have been observed in pediatric COVID-19, with multifocal opacities in lower zones. There could be increased central peribronchovascular markings, but it is a sign of inflammatory lower airway disease related to viral infections in general, so chest radiographs cannot differentiate between COVID-19 and any other childhood lung infection. Furthermore, due to limited sensitivity and specificity, a negative chest radiograph does not exclude pulmonary involvement in patients with laboratory-confirmed COVID-19 nor does

indicate the absence of COVID-19 infection in cases of suspected COVID-19 infection not yet confirmed by using RT-PCR testing.

The American College of Radiology currently recommends against using CT as a first line screening test to diagnose COVID-19 in pediatric patients and states that it should be reserved for complex cases, such as those hospitalized, possible differential diagnoses, or when there is clinical concern to assess for possible complications, especially in children with coexisting medical conditions. (66) This reluctance to use CT scans in children is justified by the fact that CT findings in COVID-19 are non-specific and resemble other lower respiratory tract infections; moreover, it often shows no obvious abnormality in quite a part of case (58, 68) and in pediatric patients an additional factor to consider is the radiation dose, which makes this modality not justified in a paucisymptomatic child. When there are CT abnormalities, the most common are bilateral peripheral and/or sub-pleural ground-glass opacities, often in the lower lobes of the lungs, with surrounding "halo" sign, a focal consolidation with a rim of surrounding ground-glass opacity.

1.3 SEQUELAE POST-INFECTION IN CHILDREN. MIS-C AND LONG COVID: TWO NEW CLINICAL IDENTITIES

Even if the acute infection with SARS-CoV-2 is generally mild in children, they may develop two more complex post-infectious syndromes, including Pediatric Multisystem Inflammatory Syndrome in Children (MIS-C), that is a life-threating condition, and Long COVID, an extremely disabling disease. Moreover, among the post-infection sequelae that children can develop, neuropsychiatric problems have also emerged (it is still debated whether these can be considered part of Long COVID).

Understanding the antibody response to study a targeted vaccination campaign in children is important not only to break down viral transmission and to avoid acute COVID-19 infection, which in itself does not worry so much, being in most cases asymptomatic or mild, but also to prevent the child from developing, after the infection, one of the two sequelae, as they can follow a clinically

insignificant acute infection, as pediatric COVID-19 is usually mild and most of the kids are previously healthy. (69, 70)

1.3.1 MIS-C OR PIMS-TS

It is variously referred to as PIMS-TS in the UK and MIS-C in the USA and by the WHO.

It is a multi-organ inflammatory condition that can cause severe damage to various organs such as lungs, heart, liver, brain.

Its pathophysiology is not fully elucidated, but it has been suggested that it may result from an abnormal immune response to the virus, with some clinical similarities to Kawasaki disease (KD) or macrophage activation syndrome (MAS), so much so that at the beginning of pandemic it wasn't identified as a specific new illness; later, based on the available studies, it turned out that MIS-C is immunologically and molecular different from KD. (71-73)

The CDC (Centers for Disease Control and Prevention) issued a Healthy Advisor on May 14, 2020, that outlines the following definition for MIS-C (74):

- An individual aged < 21 years presenting with fever > 38.0°C for ≥ 24 hours, laboratory evidence of inflammation and evidence of clinically severe illness requiring hospitalization, with multisystem (≥2) organ involvement (cardiac, renal, respiratory, hematologic, gastrointestinal, dermatologic, or neurological); AND
- No alternative plausible diagnoses; AND
- Positive for current or recent SARS-CoV-2 (COVID-19) infection by RT-PCR, serology, or antigen test; or COVID-19 exposure within the 4 weeks prior to the onset of symptoms.

Although different presentations have been described, common symptoms include Kawasaki disease-like features, such as fever, conjunctivitis, red eyes, red or swollen hands and feet, rash, red cracked lips, swollen glands. In some children, coronary artery enlargement and/or aneurysms have been described. Evidence of organ dysfunction with gastrointestinal, cardiorespiratory, renal, hematologic, dermatologic, neurologic symptoms may also be encountered.

Common laboratory findings in case reports include:

- Abnormal blood cell counts, including: lymphocytopenia, neutrophilia, mild anemia, thrombocytopenia;
- Elevated inflammatory markers;
- Elevated cardiac markers, such as troponin and BNP or N-terminal pro-BNP (NT-pro-BNP);
- Hypoalbuminemia;
- Mildly elevated liver enzymes;
- Elevated lactate dehydrogenase;
- Hypertriglyceridemia.

The usual duration between acute SARS-CoV-2 infection and onset of MIS-C symptoms is approximately two to six weeks, but rare cases of MIS-C occurring > 6 weeks have been reported. (75) In many cases, the time between acute infection and onset of MIS-C symptoms is unknown because the child was asymptomatic at the time of acute infection. It can potentially occur at any age from infancy through late adolescence, but the peak age is 9–10 years.

Moreover, Black, Hispanic and South Asian children appear to be disproportionally affected. (69) The reasons for these racial differences are unclear and may partly reflect socio-economic differences, such as access to health-care and services as well as the possibility of risks related to genetics.

1.3.2 LONG COVID

Long COVID is characterized by persistence of COVID-19 symptoms for over 3 months and occurs mostly in children aged 12 or over. (76)

It is a disabling condition, with a wide constellation of symptoms including fatigue, breathlessness, “brain fog” and depression, that hinders the patient’s ability to re-engage with normal activities, and hence carries significant long-term morbidity. (77)

Only few studies have evaluated the long-term recovery from COVID-19 in children, and common for all studies is a small sample size. A study (78) found that

0.8% of SARS-CoV-2 positive children reported symptoms lasting > 4 weeks when compared to a control group, and the most common Long COVID symptoms were fatigue, loss of smell and loss of taste, dizziness, muscle weakness, chest pain and respiratory problems. In most cases Long COVID symptoms resolve within 1-5 months. Reassuringly, a recent systematic review suggests that in most cases the prognosis is good and symptoms of Long COVID in children rarely persist beyond 8 weeks following the acute diagnosis (79), even if some children may develop long-term symptoms with a significant impact on their daily life.

Although it has been seen that adolescents and children who has had symptomatic COVID-19 have a higher probability of Long COVID (80), its symptoms also appeared in those who were non-symptomatic or just slightly symptomatic during acute SARS-CoV-2 infection.

An important side of Long COVID is the development of neuropsychiatric symptoms. Whether the neuropsychiatric symptoms widely observed in children and adolescents with Long COVID are the consequence of SARS-CoV-2 infection or are due to the tremendous stress resulting from the restrictions and the pandemics is still not clear. In both cases, psychological support can play a fundamental role in managing COVID pandemics in children. (81)

In comparison to adults, children are less likely to have persistent COVID-19 symptoms, so Long COVID seems to be less frequent. However, we must admit that post-COVID-19 symptoms have not been thoroughly evaluated in children and reports are conflicting on its prevalence, duration and impact on daily life. Establishing a clear definition for Long COVID in children and identifying objective methods for surveillance are urgent priorities.

1.4 IMMUNE RESPONSE AGAINST SARS-CoV-2

1.4.1 CHARACTERISTICS OF THE HUMORAL RESPONSE

SARS-CoV-2 infection induces the production of specific antibodies (humoral immunity) and the cell-mediated response. After the infection, the adaptive immune response confers the long-term protection. The adaptive immune response primarily comprises memory B cells, which produce different classes of

antibodies to neutralize viral particles or autologous infected cells, and memory T cells that support antibodies production and have a direct role in killing virus-infected cells. (82)

The antibody response includes an initial production of IgM NAbs in the acute phase of the infection and a subsequent production of IgG, which persist and are the indicators of the level of humoral response over time. Several studies have shown their timing of production: anti-SARS-CoV-2 IgM become detectable from day 4 onwards and IgM antibody title (b in **Fig. 5**) initially rises during the first week of infection and peaks at around 20-30 days post-symptom onset; than it gradually diminishes. Liu et al. (83) reported that mild cases have a tendency to develop a faster peak of anti-SARS-CoV-2-specific IgM responses at around 17 days, as compared to severe cases whose IgM peak around 21 days. IgG antibodies (a in **Fig. 5**) begin to rise after 10–14 days of infection and peak at around day 25. (**Fig. 5**) (83, 84)

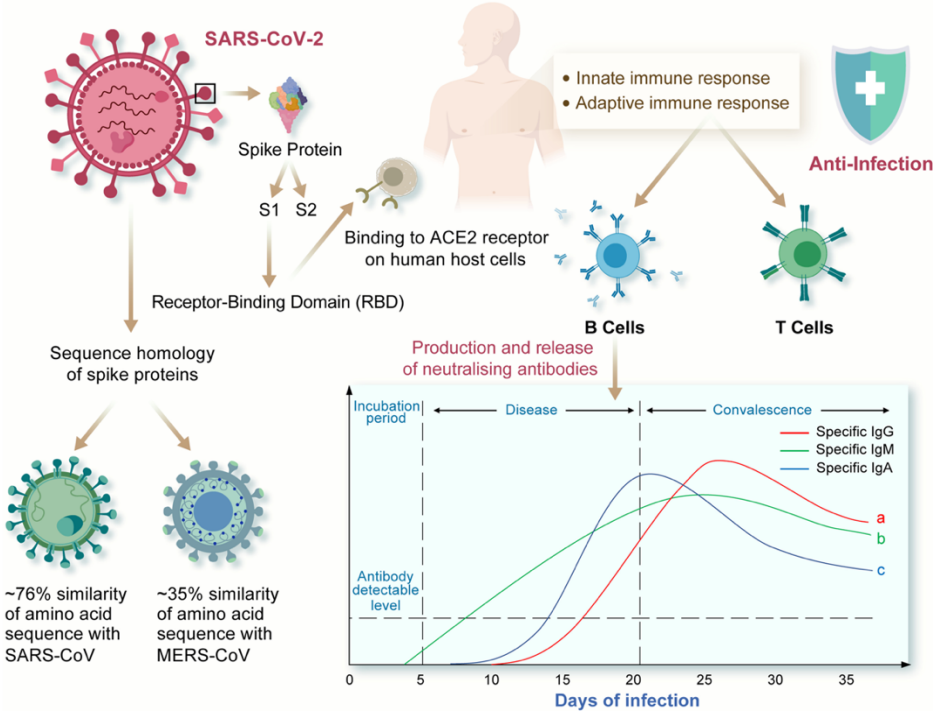

***Figure 5**: Humoral immune response (IgG, IgM, IgA) profiles of SARS-CoV-2 infections: onset and persistence of neutralizing antibodies. From study (84).*

Among these immune response mechanisms, the key neutralizing ability is played by neutralizing antibodies (NAbs). It has been shown that NAbs levels are highly predictive of infection and disease protection for several infectious diseases, representing the most reliable tool to define the quality of the host's immune response against the virus. (85) Previous studies confirm that NAbs were detected in over 90% of adults following primary infection. (86) They are a particular type of antibodies which target the receptor-binding domain of the S1 subunit (S-RBD), inactivating the virus, making it no longer able to infect host cells. Thus, NAbs interferes with the S proteins on the viral membrane, essential for the virus to enter target cells, in two ways: they prevent the binding of the S1 subunit with the ACE-2 receptor present on the target cells, or cause the S2 subunit to change conformation, preventing the virus from entering the target cell.

As state above, IgG NAbs are the antibodies that persist over time, so they are the ones that need to be evaluated to understand the medium and long-term dynamics of antibody post COVID-19 infection. The technique used to determinate NAbs is the Plaque Reduction Neutralization Test (PRNT).

PLAQUE REDUCTION NEUTRALIZING TEST (PRNT)

The Plaque Reduction Neutralizing Test (PRNT) is a laboratory test based on the use of cell culture methods and live viruses, to determine if there are patient's NAbs, and to what extent, that can neutralize viral infection in vitro, binding the virus and thereby prevent its ability to infect cells.

It must be performed in biosafety certified laboratories designated for culturing SARS-CoV-2 infected cells (labeled as Level 3 Biocontainment Labs, BSL3) and has a turnaround time of 3–5 days. Serum sample of the patient is diluted in series and mixed with a constant viral suspension. This is then incubated for some time to allow reaction between virus and antibodies, added to a monolayer of hosts cells and covered in agar gel to prevent the viral suspension from spreading indiscriminately. The result is compared to a no-serum-added control. After some days, plaques (regions of infected cells) appear. Ability of the patient’s antibodies

to neutralize the virus can be quantified observing their effect on the plaques. The concentration of antibodies to reduce the number of plaques by 50% compared to the no-serum-added control gives the measure of how effective they are, and it is called $PRNT_{50}$. (**Fig. 6**)

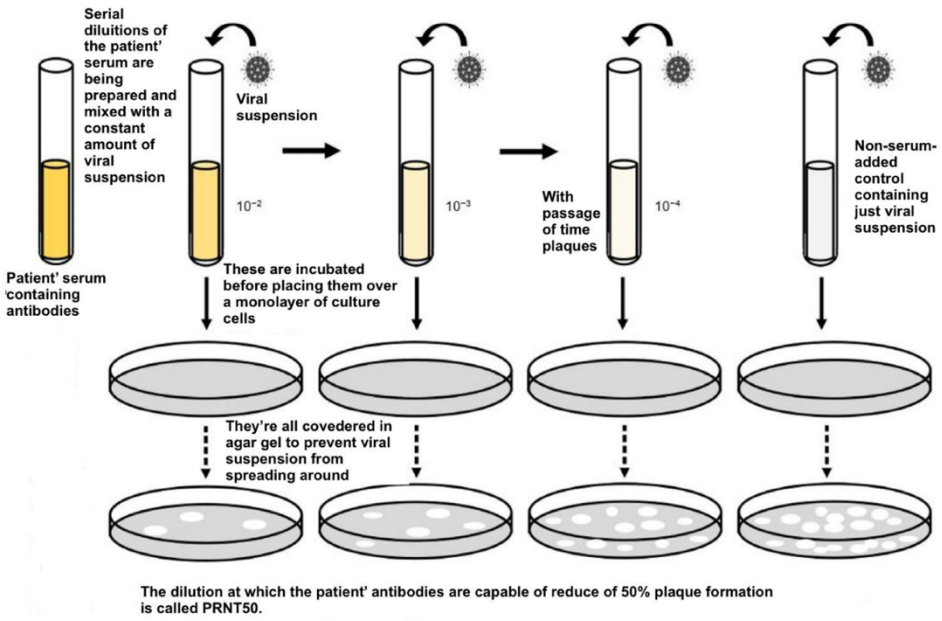

***Figure 6**: simplified description of how PRNT works. Designed with Biorender by Angelica Diaz-Basabe, University of Milan, Italy.*

However, the PRNT has many disadvantages: it is very expensive and slow to perform, and dedicated staff and specific infrastructure (biological safety level 3 laboratories, BSL-3) and equipment are needed to handle live viruses. A BSL-3 laboratory involves several problems such as having bio-safety risks, high professional ability requirements for operators, it is time-consuming, costly, and not conducive to high throughput detection. (87)

For these reasons, it became necessary to develop a cost-effective, fast and large-scale alternative NAbs detection method. Several studies have investigated the analytical and clinical performances of the SARS-CoV-2 S-RBD IgG Chemiluminescence Immunoassay (CLIA), which is a reproducible, cost-effective, fast and precise detection method of the IgG type antibodies (post infection) and IgM type antibodies (in the acute phase of infection) levels and can be run on high throughput platforms, optimizing the time and costs of the analysis. (88) It is a serological test which used immunohistochemical technique and can be used to test

a very large number of samples for the detection of COVID-19 antibodies, to know if antibodies have been created against COVID-19 or not. (88) It takes advantage of the high binding affinity between viral antigens and host antibodies. It involves the use of a plastic plate coated with one or more viral antigens that can bind and detect the corresponding antibodies present in the patient's sample. It uses a chemical reaction as probe to produce a light signal that indicates a positive signal. (89)

This alternative works because it was found that SARS-CoV-2 S-RBD IgG assay had a strong correlation with sera neutralizing activity (90), so anti-SARS-CoV-2 S-RBD IgG CLIA became a surrogate test to detect the presence of antibodies, even if with lower sensitivity and specificity compared to NAbs and PRNT. The relationship among the anti-SARS-CoV-2 S-RBD IgG and the corresponding $PRNT_{50}$ titers is shown in **Fig. 7**, panels A and B. (90)

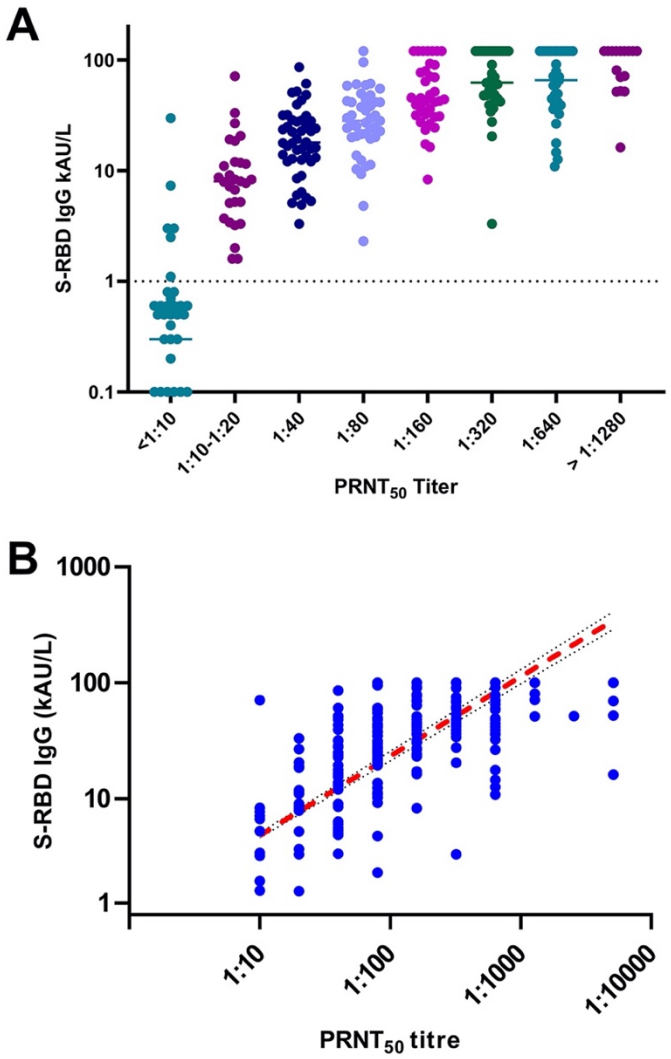

***Figure** 7: Correlation between the anti-SARS-CoV-2 S-RBD IgG CLIA results and PRNT50 titers. (A) dot plots presenting the CLIA results with respect to the different PRNT50 titers; (B) linear correlation of positive PRNT50 titers with respect to CLIA results (both in log_{10} scale). Source: (90)*

1.4.2 ANTIBODIES, SEVERITY OF THE DISEASE AND SEROLOGICAL BACKGROUND IN GENERAL POPULATION

In the last months there have been a rush among researchers to identify the effective duration of antibodies against SARS-CoV-2 and their neutralization activity. Currently, scientific knowledge investigating the long-term persistence of anti-SARS-CoV-2 Abs after the infection is mainly limited to adult patients, where a lot of evidence suggest that antibodies against SARS-CoV-2 persist over time.

Instead, a knowledge gap regards the pediatric population, where the antibody response seems to be different, as suggested by previous studies. (91, 92)

In adult population anti-SARS-CoV-2 S-RBD IgG persist over 12 months after the infection, regardless of disease severity. (93-101) Both these Abs seem to exhibit a biphasic decay, with RBD-binding IgG titers decreasing significantly in the first six months and remaining stable afterwards. (35, 99, 102-105) Moreover, there is a strong directly proportional correlation between SARS-CoV-2 RBD-binding IgG titer and infection severity. (106) Indeed, even if immune response against COVID-19 virus is developed in almost all infected people, there is a large inter-individual heterogeneity and in part it depends on the type of clinical course that infection has had in the subject: people who have had more severe symptoms tend to have higher antibody titers than individuals with mild symptoms or completely asymptomatic cases. Rijkers G et al. in their study (107) have compared antibody response in adult patients with mild (non-hospitalized) and sever (hospitalized) infection and emerged that the ones with severe disease developed a strong antibody response with a neutralization titer of 1:240, while between mild infection individuals only 75% developed antibodies and with low neutralization power (titer > 1:20). Similar evidence was found by Lynch KL et al. (108) where, among 52 patients, more than 80% seroconverted in 8-10 days but individuals admitted to ICU had significantly higher antibodies peak. Furthermore, the distribution and variation of antibody dynamics may be associated with the patients' age, gender, co-morbidities, viral load, and other factors that influence disease severity.

As for children, we previously proved that those recovered from mild or asymptomatic COVID-19 present an intense early NAbs production, up to 7-8 months post-infection, although with the passage of time they tend to decrease, and the magnitude of SARS-CoV-2 S-RBD IgG Abs is higher among younger children compared to older siblings and adults, at all follow-up time points, with an inversely proportional correlation between antibody titer and age groups. Moreover, we found that children aged < 3 years develop 5-fold higher levels of Abs compared to older siblings and/or adults aged >18 years and that mild and asymptomatic SARS-CoV-2 infections in family clusters elicited higher neutralizing antibodies among children. (92)

To date, longer-term kinetics of Abs in pediatric population remains to be investigated to guide public health policies and to set effective vaccines strategies in this understudied population.

2. PURPOSE OF THE STUDY

While we know the persistence of anti-SARS-CoV-2 Abs in adults well, there is a lack of knowledge as far as the pediatric population is concerned. This represents a limit in the possibility of fighting the pandemic, because children, however mildy affected, play a fundamental role in the transmission of the virus. So, knowing more about their antibody response would help formulate better strategies to stem the pandemic, first of all through vaccination.

To date, we know that, despite the decrease of antibodies over time, children recovered from asymptomatic or mild COVID-19 infection develop an intense early neutralizing antibodies (NAbs) response against SARS-CoV-2 that persists up to 7-8 months post infection and that is higher than adults. (91, 92) In particular, this same study group demonstrated that toddlers under 3 years of age have the highest titers throughout early, intermediate and late times from infection onset, so the highest long-lasting levels of NAbs compared with older siblings and/or adults. (92) Furthermore, it has also been proved that in adults anti-SARS-CoV-2 S-RBD IgG persist around 12 months after the infection, regardless of disease severity, with a decay in the first 6 months, then they remain stable (93-101). On the other side, the antibody long-term persistence and kinetics in pediatric population has not been investigated yet.

The purpose of this study is bridging this gap in the pediatric population, expanding the number of cases and follow-ups to at least 12 months after infection, compared to the previous court. (92) The goal is understanding which is the long-term persistence and kinetics of humoral response to SARS-CoV-2 in children and how it differs from that of adults. Furthermore, as explained above, since searching for NAbs through PRNT is too complex in many respects, we have chosen to use CLIA to determine anti-SARS-CoV-2 IgG, taking care also to verify that the new method confirms results in terms of NAbs titers found in the first 7-8 months.

Gaining a greater understanding of the immune response in children following SARS-CoV-2 infection has important scientific and public health implications: understanding the antibody dynamics that follow the COVID-19 infection, how the child responds not only in the short but also in the long term, we can try to predict what the antibody response to the vaccine will be and extrapolate

useful data to formulate targeted vaccination plans, fundamental to fight the pandemic.

3. MATERIAL AND METHODS

3.1 STUDY DESIGN AND DATA COLLECTION

We conducted a single-center, prospective cohort study on families including children, older siblings, and their parents attending the COVID-19 Family Cluster Follow-up Clinic (CovFC), set up at the Department of Women's and Children's Health (W&CHD) of the University Hospital of Padua (Veneto Region, Italy).

From April 1, 2020 to August 6, 2021, we enrolled 252 families four or more weeks after infection, after a referral from the Family Pediatrician (FP) or the Pediatric COVID-19 unit of the Department of Women's and Children's Health (W&CHD) of the University Hospital of Padua, if attending the following inclusion criteria:

- having children of pediatric age (< 15 years);
- and having at least one family member (e.g., mother and/or father and/or any son or daughter) with a history of COVID-19;

Exclusion criteria were:

- have received at least one dose of SARS-CoV-2 vaccine;
- or be classified as *non-COVID-19 case*.

Families were enrolled in the program through different ways:

- after being hospitalized and recovered by the Pediatric COVID-19 Unit of the W&CHD;
- and/or after being evaluated in our COVID-19 dedicated Emergency Room of the W&CHD;
- or after receiving a home-based evaluation, provided by their Family Pediatrician (FP). All FPs of the Veneto Region were informed by an institutional email (COVID.pediatrico@aopd.veneto.it) to address their patients to the COVID-19 follow-up clinic.

At enrolment, a pediatrician and/or an Infectious Diseases specialist evaluated children and relatives collecting data on demographic parameters, past and recent medical history, vaccinal status, including the SARS-CoV-2 vaccine, and performed a clinical evaluation. A blood sample was collected from all cases for characterization of the immunological response to SARS-CoV-2. Data on all patients' clinical characteristics and laboratory findings were extracted from hospital's electronic medical records and analyzed anonymously. Parents or legally authorized representatives were informed of the research proposal and provided their written consent to collect and use biological specimens and routine patient-based data for research purposes.

All patients with positive SARS-CoV-2 serology at enrolment were followed up for longitudinal clinical and serological evaluation at 1-4, 5-9 and ≥ 10 months up to 18 months after baseline. Data on new contacts with confirmed or probable COVID-19 cases and confirmed SARS-CoV-2 re-infections were collected at each visit. Follow-up was interrupted in case patients had received SARS-CoV-2 vaccine or in case of serological negativization.

Information collected at enrollment and during follow-up were anonymized and entered into a web-based database using the Research Electronic Data Capture platform (REDCap®) (Vanderbilt University, Tennessee) hosted in the server of the University of Padua.

According to the national regulation, the study protocol was communicated to the Ethical Committee (Protocol N° 0070714 of November 24, 2020; amendment N° 71779 of November 26, 2020) and approved by it.

3.2 CASE IDENTIFICATION AND DEFINITIONS

Study participants were considered *confirmed COVID-19 cases* if:

- they had a record of virological positivity for SARS-CoV-2 by real-time polymerase chain reaction (RT-PCR);
- and/or they resulted *positive* by either of the two serological tests adopted in this study, such as the quantification of SARS-CoV-2 NAbs through a

high-throughput method for Plaque Reduction Neutralization Test ($PRNT_{50}$) and/or the quantification of SARS-CoV-2 S-RBD IgG.

A confirmed SARS-CoV-2 re-infection was defined as the newly detection of positive SARS-CoV-2 virological assay at NPS, occurring after being recovered from a previous Covid-19 confirmed by negative virological assay. (109)

We specify that during the first wave of COVID-19, all enrolled family members were systematically tested for SARS-CoV-2 NAbs through PRNT, performed both at enrollment and during follow-up. However, during the second and third waves of COVID-19, many families were enrolled, leading to operational challenges and increased costs in performing PRNT on a wider scale. Therefore, considering the emerging evidence that the anti-SARS-CoV-2 S-RBD IgG assay achieves excellent analytical and clinical performances compared to PRNT titers and a strong correlation with sera neutralization activity (90), from March 26, 2021 we decided to test all patients 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 each *COVID-19 case*, a *baseline date* was defined as follows:

- for symptomatic cases: the first date chosen between the onset of symptoms or the date of first positive SARS-CoV-2 molecular assay result;
- for asymptomatic cases: the date of the first positive molecular assay result or, in those with only serologically confirmed COVID-19 infection and with negative or not performed/undetermined nasal-pharyngeal swab (NPS), by the family outbreak temporal sequence, coinciding with the date of symptoms onset in the family cluster.

Infants aged < 6 months were included in the analysis only in case of virological confirmation of SARS-CoV-2 infection, at nasal-pharyngeal swab (NPS).

The severity of COVID-19 was scored as mild, moderate, severe, critical or MIS-C following the WHO classification. (110) Subjects that were asymptomatic and had no analytical evidence of SARS-CoV-2 infection were considered *non-COVID-19 cases*.

Three periods of time or "*COVID-19 waves*" were identified and defined as follow:

- a first wave occurring from February 17, 2020 to September 18, 2020;
- a second wave from September 19, 2020 to February 18, 2021;
- a third wave from February 19, 2021 to September 20, 2021.

3.3 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 stored at -80°C and liquid nitrogen, respectively, until use.

An aliquot of serum sample was collected for the quantification of anti-SARS- CoV-2 S-RBD IgG Abs in human serum through a CLIA commercially available (Snibe Diagnostics, New Industries Biomedical Engineering Co., Ltd [Snibe], Shenzhen, China). This method, previously validated elsewhere, quantitatively determines the IgG antibodies directed against RBD portion of SARS-CoV-2 Spike protein. All analyses were performed on MAGLUMITM2000 Plus (Snibe Diagnostics), with results expressed in kilo Binding Antibody Unit (kBAU/L). Samples recording titers > 4.33 kBAU/L were considered positive.

A high-throughput method for $PRNT_{50}$ was used to quantify NAbs in plasma samples for a subgroup of patients infected by SARS-CoV-2 within the first and second waves. Samples were heat-inactivated by incubation at 56°C for 30 minutes 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 hours 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 hours at 37°C in a 5% CO_2 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 26 hours of incubation, cells were fixed with a 4% paraformaldehyde (PFA) solution. Visualization of plaques was obtained with an immunocytochemical staining methos using an anti-dsRNA monoclonal antibody (J2, 1:10,000; Scicons) for 1 hour, followed by 1 hour of incubation with peroxidase-labeled goat anti-mouse antibodies (1:1000; DAKO) and 7 minutes of incubation with the True Blue® (KPL) peroxidase substrate. FFUs were counted after acquisition of pictures on a flatbed scanner.

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 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. (111)

3.4 STATISTICAL ANALYSES

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

Subjects enrolled in the study were included in the statistical analysis if:

- being *COVID-19 cases*;
- and having a defined *baseline date*;
- and having at least one serological assay performed during the study time, and before SARS-CoV-2 vaccination, for those vaccinated.

The Abs titers response was assessed by comparing the median and the interquartile range (IQR) of anti-SARS-CoV-2 S-RBD IgG values in the overall dataset, including both independent and subject-paired samples, and stratified by age classes (age < 3 years, 3 ≤ age < 6 years, 6 ≤ age < 12 years, 12 ≤ age < 18 years, and age ≥ 18 years), and by the time between serological sampling and baseline, categorizing patients into three intervals, namely 1-4, 5-9 and ≥ 10 months.

In a sub-cohort of COVID-19 cases, a further analysis was performed comparing the median of percentage of decrease between two consecutive samples for each patients, stratified for age classes (age < 6 years, 6≤age≤18 and ≥ 18 years).

In addition, the Kruskal-Wallis test and the Wilcoxon rank-sum test were performed, where appropriate. They are both methods used to verify whether statistical samples come from the same population (or from populations with the same median).

A linear regression analysis was used to assess the association between anti-SARS-CoV-2 S-RBD IgG and NAbs, using the Log_2 of both variables given data skew. Despite the transformation of the variables into logarithm, the strength of associations between variables was assessed by 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 confidence intervals considerably in the unadjusted analyses, so correlation structures were omitted from all analyses.

Analyses were performed using the Statistical Analysis System software (version 9.4; SAS Institute, Cary, North Carolina). Statistical significance was set at the 0.05 level. All P-values were 2-sided.

Graphs were made using GraphPad Prism version 9.2 (GraphPad Software, San Diego, California USA). Our manuscript was structured in accordance with Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline for cohort studies.

4. RESULTS

4.1 PATIENTS CHARACTERISTICS

From April 1, 2020 to August 6, 2021, at the COVID-19 Family Cluster Follow-up Clinic (CovFC) of the Department of Women's and Children's Health (W&CHD), University of Padua (Veneto Region, Italy), we prospectively evaluated 252 family clusters of COVID-19.

A total of 902 subjects were recruited and had a serological assessment performed during the first follow-up. Among these, we excluded from the analyses a total of 205 individuals, because: (**Fig. 8**)

- 25 (2,8%) subjects of these had received at least one dose of SARS-CoV-2 vaccine before the first serological follow-up;
- and 180 (20%) subjects of these have been defined as *non-COVID-19 cases*.

Five-hundred and seventy-five (63.7%) subjects who tested positive for SARS-CoV-2 by RT-PCR, together with 122 (17.5%) subjects that had no record of virological positivity but showed evidence of seropositivity by either one of the two serological tests adopted in this study, were considered *COVID-19 cases* and were included in the analysis. (**Fig. 8**)

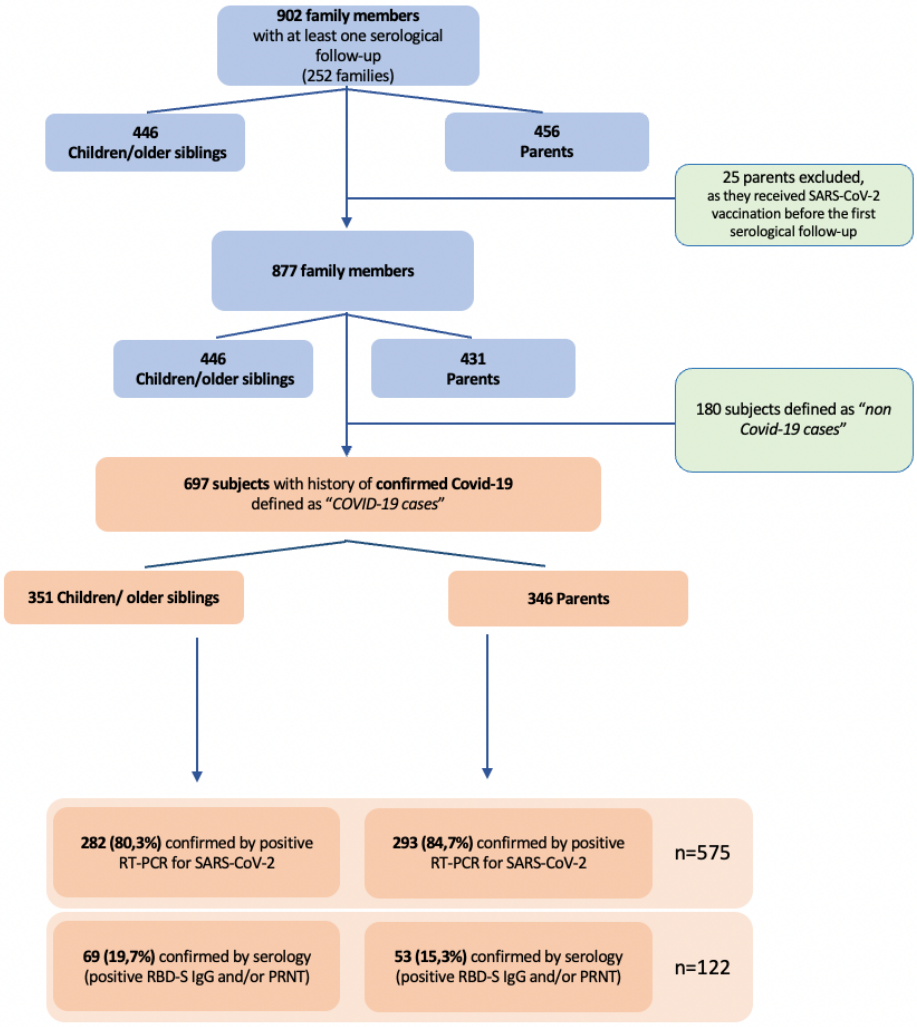

***Figure 8**: Flow chart of family clusters of COVID-19 observed from April 1, 2020 to August 6, 2021, at the COVID-19 follow-up clinic of the Pediatric Department, Department of Women's and Children's Health, University of Padua. Blue: whole cohort of enrolled subjects; green: individuals excluded from the analysis; orange: confirmed COVID-19 cases.*

As a result, 697 confirmed *COVID-19 case* were studied. Among these, 321 (46%) were female, 351 children/older siblings, and 346 parents, with a median age of 8,6±5,1 and 42,5±7,1 years, respectively.

Among COVID-19 positive children (n=351), 241 (68,6%) were symptomatic, and 231 (65,3%) developed a mild COVID-19. Only one child developed pneumonia, and 9 (2,5%) presented with MIS-C. Comorbidities were detected in 61/351 (17,4%) children, with asthma as the most frequent one. Among parents, 299 (86,4%) were symptomatic, 285 (82,4%) developed a mild COVID-19, 13 (3,8%) a moderate/severe infection. (**Tab. I**)

The infection onset or *baseline time* was determined for each confirmed COVID-19 case, and additional information on baseline identification is provided in **Fig. 9**. Among 697 confirmed COVID-19 cases, only for 67 (9,7%) asymptomatic cases with negative or not performed NPS, the baseline was identified as symptom onset of the first symptomatic family member. None of the patients reported exposure to other COVID-19 patients, developed symptoms of COVID.19, nor was reinfected after recovery.

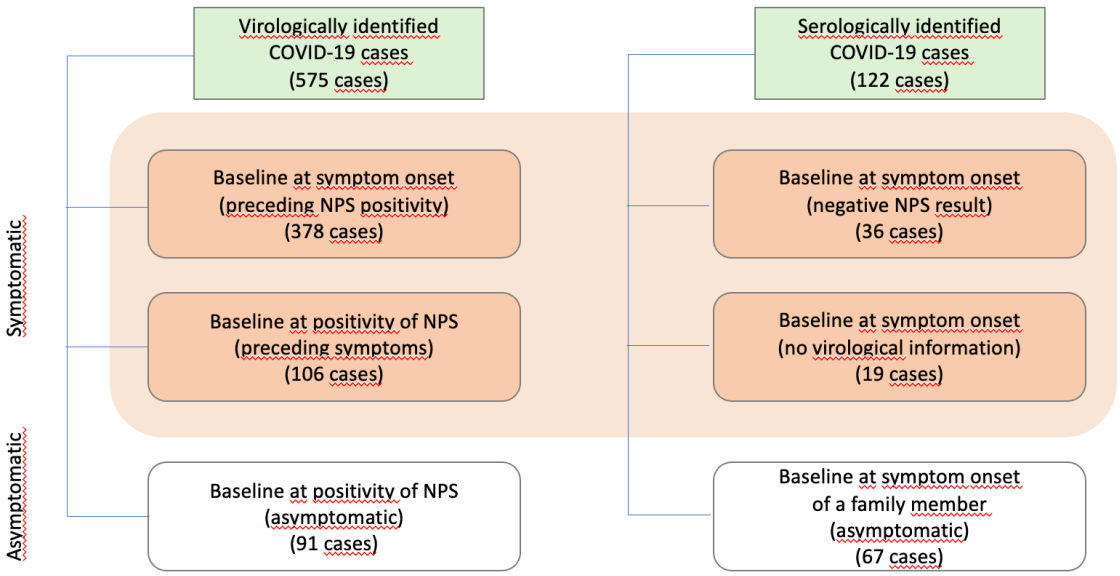

***Figure 9**: Criteria for the definition of the baseline time for COVID-19 cases.*

Subjects who were asymptomatic and had no analytical evidence of SARS-CoV-2 infection were considered *non-COVID-19 cases.*

	OVERALL					CHILDREN/OLDER SIBLINGS					PARENTS				
	COVID-19 negative (n=180)		COVID-19 positive (n=697)		*p-value* §	COVID-19 negative (n=94)		COVID-19 positive (n=351)		*p-value* §	COVID-19 negative (n=85)		COVID-19 positive (n=346)		*p-value* §
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	(68.6)	<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)	61	(17.4)		13 (15.3)		61	(17.6)	

***Table I**: Descriptive Analysis of the 252 Families Observed at the Department of Women's and Children's Health of the University Hospital of Padua (Italy). Considering the total number of study participants (902), to whom 25 individuals have been remove as they received SARS-CoV-2 vaccination before the first serological follow-up (Fig. 8), in the table they are shown as Overall (n=877) and stratified by familiar status as Children or Older siblings (n=446) and Parents (n=431), later divided between positive and negative COVID-19 cases. *WHO, World Health Organization; MIS-C, Multisystem Inflammatory Syndrome in Children. **The following co-morbidities were found among 61 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), immune-deficiency (n=2), metabolic disease (n=1), kidney/ureteral disease (n=4), endocrinological disease (n=2), gastrointestinal disease (n=4).*

4.2 KINETICS AND LONG-TERM PERSISTENCE OF ANTI-SARS-CoV-2 S-RBD IgG

We assessed the production and long-term persistence of anti-SARS-CoV-2 S-RBD IgG Abs up to 18 months following infection. Among 697 confirmed COVID-19 cases, a total of 659 subjects had at least one anti-SARS-CoV-2 S-RBD IgG titer performed after infection. During follow-up, 99.7% of subjects still

recorded positive titers, while 2/659 (0,3%) patients with confirmed COVID-19 negativized, after 64 and 556 days from baseline, respectively. (**Tab. II**, **Fig. 10, 11**)

During follow-up visits, none of the patients reported either exposure to other COVID-19 patients, nor developed COVID-19 symptoms or re-infection. However, we recorded for 17 subjects an unexpected increase in S-RBD IgG titer. Considering the possibility of an unknown exposure to a confirmed COVID-19 case that may impact on our results, the last time-point sera of these 17 subjects were excluded from the analysis.

To better assess the impact of age on the immunological response to SARS-CoV-2 infection, we analyzed 769 samples, collected at 1-4 (529 samples), 5-9 (161 samples), and ≥ 10 months (79 samples) from *baseline*, stratifying among five classes of age (<3, ≥3-<6, ≥6-<12, ≥12-<18, ≥18 years of age). (**Tab. II**, **Fig. 10, 11**) We observed that the S-RBD IgG titers differ among age classes ($p<0.0001$) (**Tab. II**, **Fig. 12**). Overall, higher levels of Abs were observed among younger children compared to older children, adolescents and adults, with a median S-RBD IgG titer presenting a 44.29% decrease from < 3 years of age to ≥ 3-<6 years (304.83 [139-519.6] versus 169.3 [103.1-277.1] kBAU/L), 25.5% from ≥3-<6 to ≥6-<12 years (169.3 [103.1-277.1] versus 126.2 [74-207.8] kBAU/L), 22.2% from ≥6-<12 to ≥12-<18years (126.2 [74-207.8] versus 98.2 [44.7–169] kBAU/L), and 43.4% from ≥12-<18 to ≥18 years (98.2 [44.7–169] versus 55.6 [24.2-136] kBAU/L). (**Tab. II**) Differences in S-RBD IgG titers among all age classes, with younger children presenting significantly higher levels of Abs, were also observed when samples where stratified by time of collection, so at 1-4 ($p<0.0001$), 5-9 ($p<0.0001$), and ≥ 10 months ($p=0.0237$) from infection. (**Tab. II**)

All data, irrespective of onset						
Age Classes (yr.)	**< 3 yr. (n=67) Median (IQR)**	**3 ≤ yr. < 6 (n=62) Median (IQR)**	**6 ≤ yr. < 12 (n=156) Median (IQR)**	**12 ≤ yr. < 18 (n=109) Median (IQR)**	**≥ 18 yr. (n=375) Median (IQR)**	**p-value ł**
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=49) Median (IQR)**	**3 ≤ yr. < 6 (n=36) Median (IQR)**	**6 ≤ yr. < 12 (n=109) Median (IQR)**	**12 ≤ yr. < 18 (n=74) Median (IQR)**	**≥ 18 yr. (n=262) Median (IQR)**	**p-value ł**
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) Median (IQR)**	**3 ≤ yr. < 6 (n=19) Median (IQR)**	**6 ≤ yr. < 12 (n=32) Median (IQR)**	**12 ≤ yr. < 18 (n=23) Median (IQR)**	**≥ 18 yr. (n=77) Median (IQR)**	**p-value ł**
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) Median (IQR)**	**3 ≤ yr. < 6 (n=7) Median (IQR)**	**6 ≤ yr. < 12 (n=15) Median (IQR)**	**12 ≤ yr. < 18 (n=12) Median (IQR)**	**≥ 18 yr. (n=36) Median (IQR)**	**p-value ł**
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

***Table II**: Serological data of 769 sera samples obtained from 659 confirmed COVID-19 cases among age classes, overall and stratified by time from baseline. As for 17 people had the last RBD-S IgG titer higher than the previous ones, their sera at the last time-point were excluded from the analysis. ł Kruskal-Wallis Test.*

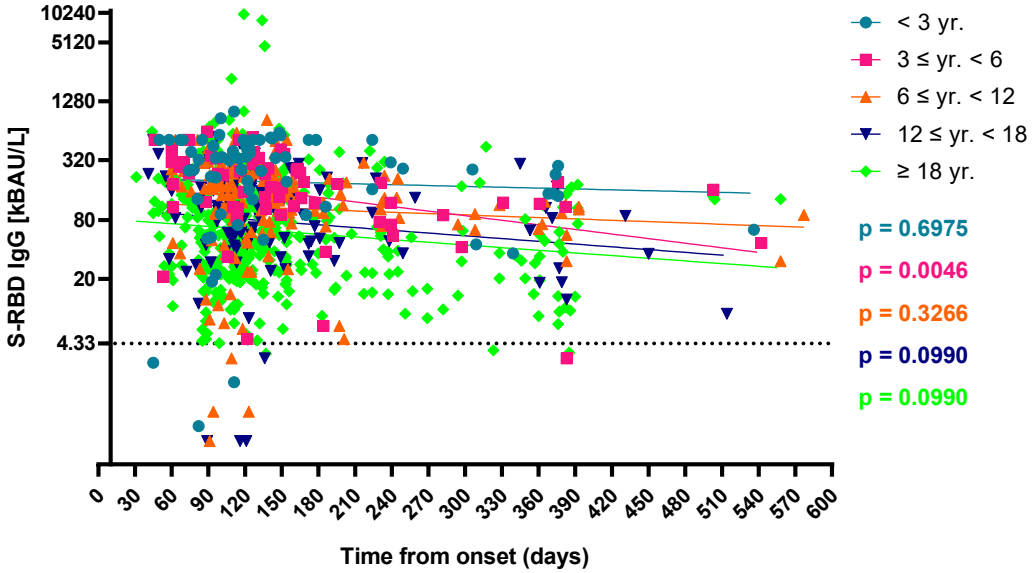

***Figure 10**: Distribution of S-RBD IgG samples according to time of collection and age classes (n=769). Younger patients presented higher levels of Abs across all time points of samples collection. S-RBD IgG levels are reported in log2 scales. The dotted lines at 4.33 kBAU/L correspond to the assay cut-off for discriminating positive from negative samples.*

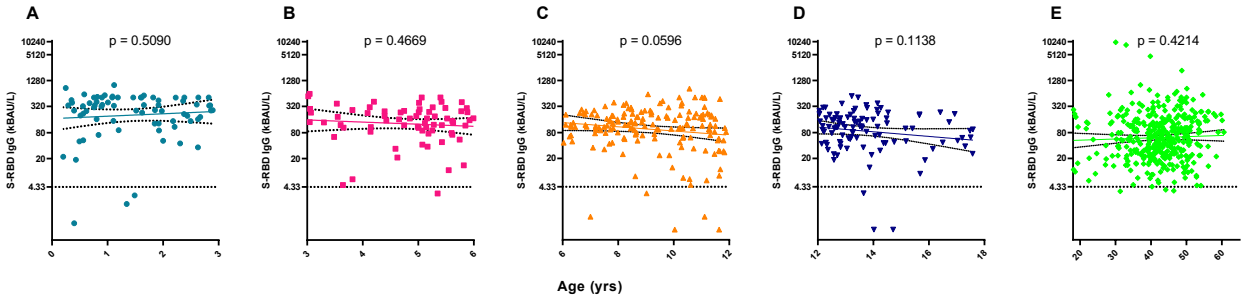

***Figure 11**: Distribution of S-RBD IgG samples according to age classes (A-E). Younger children presented a significantly higher levels of Abs than adults. S-RBD IgG levels are reported in log2 scales. The dotted lines at 4.33 kBAU/L correspond to the assay cut-off for discriminatin positive from negative samples.*

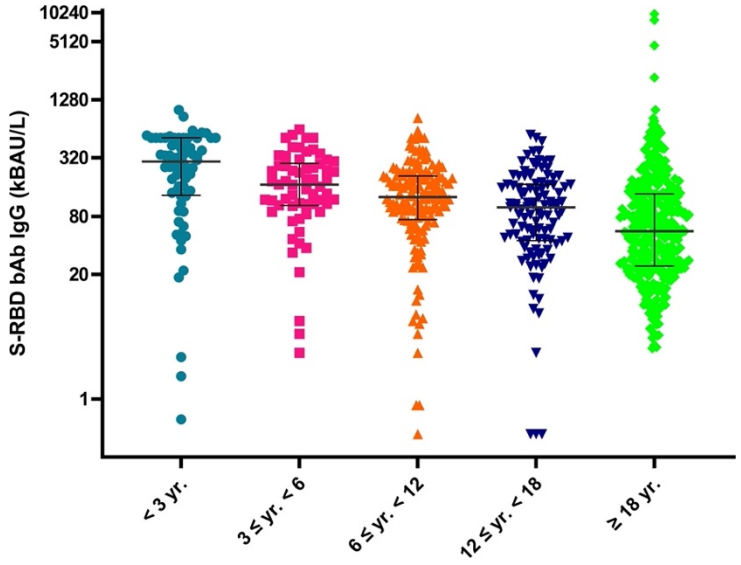

***Figure 12**: Distribution of S-RBD IgG titers according to age classes (n=769). Note the progressive decrease of median Abs titers from children <3 years of age to adults (≥18 years).*

A longitudinal analysis was conducted on subject-paired plasma from a sub-cohort of 56 COVID-19 cases tested at least twice for S-RBD IgG titers, with the first sample collected at 1-4 months from baseline. A first analysis was conducted on 31 subjects who were sampled at 89.2 (STD, ± 38.6) and 199.2 (STD, ± 30.3) days from baseline, while a second analysis was conducted on 40 subjects 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. (**Tab. III**) Twenty-two patients were tested three times, thus contributing to both above-mentioned subgroups of patients. Both analyses were stratified by three age subgroups: young children aged < 6 years, older children and adolescents aged 6 to 18 years, and adults > 18 years. (**Tab. III**)

All three age groups exhibited persistence of anti-S-RBD IgG titers at both intervals. 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. (**Tab. III**)

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

***Table III**: Subject-paired serological data of 56 subjects 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. § Wilcoxon Signed-Rank Test.*

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 anti-S-RBD IgG titers, regardless of the time of the first serum collection. A total of 194 samples were analyzed according to time from baseline. The current analysis was also stratified among three subgroups of age (<6, >6-<18, >18 years). Tracing a theroetical 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 6 years showed an apparently faster early waning of Abs titers. In addition, Abs titer remained detectable for 18 months. (**Fig. 13**)

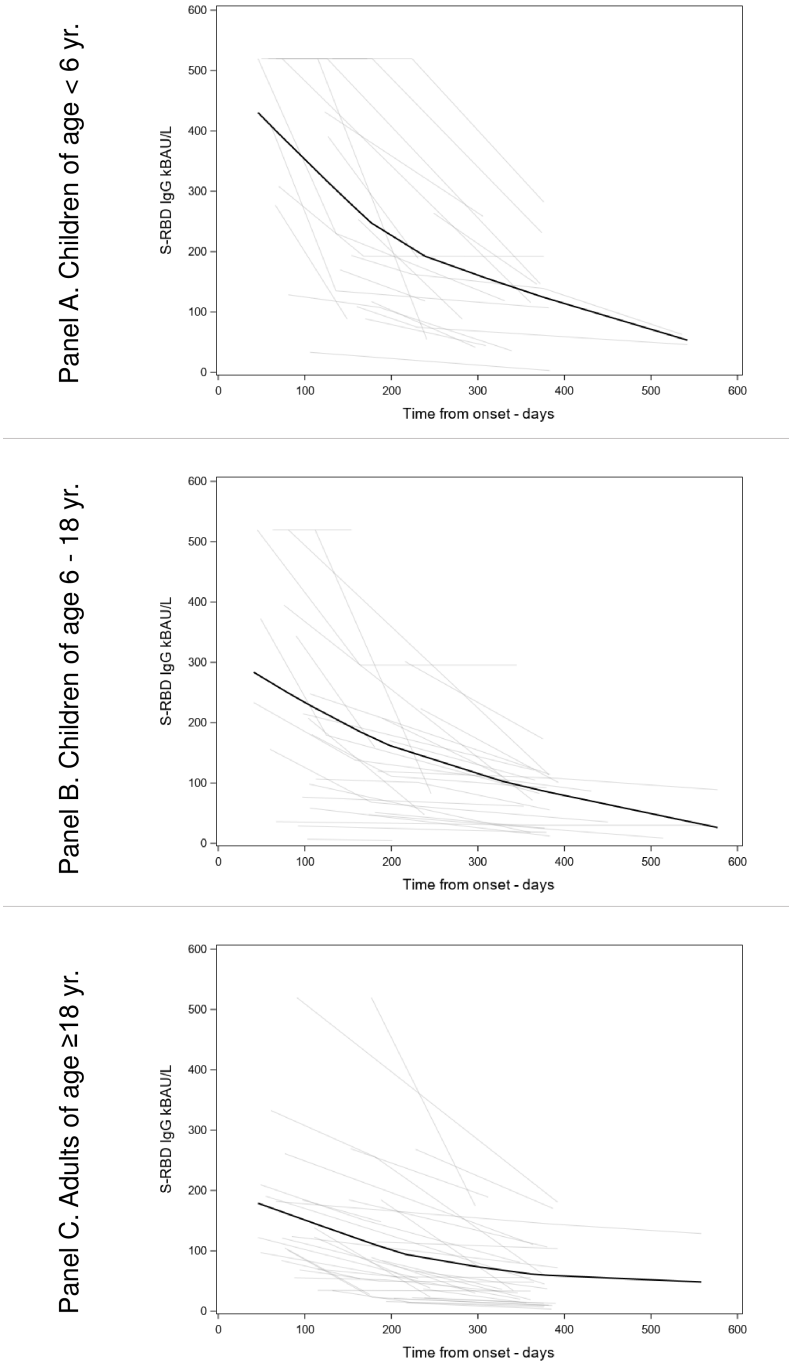

***Figure 13**: Individual kinetics of S-RBD IgG titers in subjects with at least two time points of follow-up, regardless of the time of the first serum collection, according to age classes and time of collection (n=194). The black lines represent the estimated Abs titer kinetics.*

4.3 CORRELATION BETWEEN NAbs AND ANTI-SARS-CoV-2 S-RBD IgG

Considering the 139 individuals who were tested in parallel for both serological 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 CLIA immunoassay and $PRNT_{50}$, respectively.

Overall, in the linear regression model, a positive correlation was found between $PRNT_{50}$ log titers and log_2 S-RBD IgG titers (correlation coefficient R^2 0.47; Spearman coefficient 0.73, $p<0.0001$) (**Fig.14**). A similar correlation between $PRNT_{50}$ log titers and log_2 S-RBD IgG was observed when samples were stratified according to follow-up time points and age classes (data not shown).

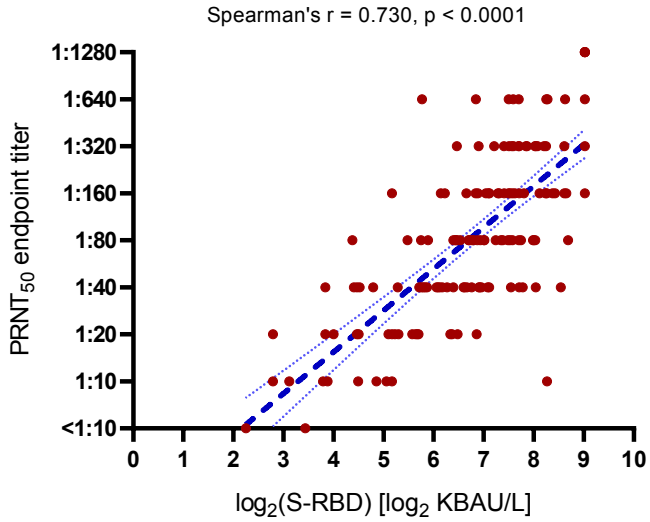

***Figure 14**: Correlation between NAbs and S-RBD IgG titers in 139 patients analyzed simultaneously with both methods.*

5. DISCUSSION

We evaluated the dynamic changes of the SARS-CoV-2 binding Abs titers in a prospective cohort of family clusters mostly affected by asymptomatic or mild COVID-19, up to 12 months after the infection. We proved that anti-SARS-CoV-2 S-RBD IgG persist over a year from infection in all age groups, with a peak Abs titer that was inversely related with age and different longitudinal Abs kinetics according to age classes.

Since April 2020, we started a novel program to provide a longitudinal clinical and serological follow-up for families affected by COVID-19. Through our COVID-19 Family Cluster Follow-up Clinic, to date, we recruited 252 families, representing, to our knowledge, the largest cohort of COVID19 family clusters followed prospectively for up to 12 months from infection onset. The unicity of a cohort allows for evaluating the modifications of the immunological responses related to different ages. Furthermore, analyzing family clusters permitted to define the onset of infection of those asymptomatic serologically confirmed COVID-19 cases with negative/undetermined NPS by the family outbreak temporal sequence, coinciding with the date of symptoms onset in the family cluster. Moreover, being composed of more than 95% of individuals with mildly symptomatic COVID-19, our cohort reflected the epidemiological situation in the whole European Union/European Economic Area (EU/EEA), where among overall COVID-19 cases, only 0.9% developed a severe disease (112).

To assess the humoral response of our cohort, we performed seriate serological analyses, which have undergone a methodological modification over time. As we previously stated, the higher number of subjects that we recruited in the second and third waves imposed us the implementation of a more cost-effective and rapid serological assay, transitioning from PRNT to SARS-CoV-2 RBD IgG CLIA, which demonstrated a strongly correlation with viral neutralization power (90, 114-117), in both adults and children, for more than six months after SARS-CoV-2 infection (90, 117, 118). Thus, what could have represented a limitation of our study revealed to be a precious source of novel scientific findings, with an immediate implication in daily clinical practice. The high number of samples

(n=172) tested with both serological methods allowed us to define a linear correlation model for estimating the neutralizing power of patients' sera starting from their S-RBD IgG titer. If confirmed on a larger scale, our linear correlation model could open the possibility to identify a conversion method between the two methodologies of analysis, representing a promising "open-access" tool for estimation of serum's neutralizing power.

This study strengthens and expands what we observed previously about the medium-term humoral response after COVID-19 in family clusters (92). Analyzing preliminary data on the first 57 families affected by mild COVID-19 enrolled in our cohort, we have already demonstrated that children produce more NAbs than adults (92). In this study, similarly to PRNT evaluation, we found that the magnitude of SARS-CoV-2 S-RBD IgG Abs is higher among younger children than older siblings and adults at all follow-up time points. Considering the two ends of the age spectrum of our cohort, children aged < 3 years and adults aged > 18 years, the S-RBD IgG median titer experienced a 5-fold decrease. A similar age classes-related Abs response was presented during the whole follow-up.

These results align with previous studies using PRNT and surrogate-neutralization based-assays describing higher Abs titer and neutralizing ability in children than adults, especially in the first months after infection (91, 118).

Showing a different Abs titer among mildly affected age groups, these findings confirm the hypothesis of the key role of not yet well-explored factors at the basis of a high variation in the magnitude of the immune response between individuals, and not attributable only to the different clinical severity of the disease. The high Abs titer measured in some mildly COVID-19 adults, despite it is known that neutralizing responses are proportional to the infection's severity and duration (119), already led scientists to think that genetic, environmental, and stochastic factors could influence the immune response induced by SARS-CoV-2. Our cohort, including households, supports this hypothesis, giving greater importance to both genetic and environmental factors. First, a repeating exposure to previous endemic HCoVs may impair the humoral response to SARS-CoV-2 in adults compared to children, targeting mainly conserved epitopes and less against novel immunodominant proteins, blunting the neutralizing power. A recently published

paper (44) reinforces our supposition, proving 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. On the other hand, as our patients were exposed to the same environmental background among their own family cluster, we may suppose that genetic factors contribute to different potency and durability of humoral responses.

However, two studies contrast with our findings, reporting no differences in the expression of specific Abs between age classes or, surprisingly, showing an even lower neutralizing activity in children compared to adults (120-122). However, in the study by Marquez et al. et al. (120), samples were collected around three weeks after infection, possibly representing a bias as the IgG peak occurs not before 7-8 weeks from baseline. In addition, 40% of pediatric patients presented the virus jointly with cancer, implying a probable state of immunosuppression that may have altered the humoral response to infection in these selected children. In the second and third studies (121, 122), authors compared children with mildly affected adults previously selected as plasma donors, might have recruited only hyper-immune adults.

Our study showed the persistence of anti-S-RBD Abs one year after infection in children and adolescents for the first time. This result is in line with recent studies concerning adult subjects (93-99, 100, 102-105). Furthermore, anti-S-RBD Abs have recently been shown to persist for up to 12 months in mildly COVID-19 adults, showing a significant decline in the first 6-8 months after infection and then reaching a subsequent plateau in their concentration (95).

The long-term persistence of anti-SARS-CoV-2 Abs in adults could be expected from the analyses of humoral response to previous HCoVs after two and three years. It could be explained by the presence of the long-lived plasma cells in the bone marrow that produce lower but detectable levels of pathogen-specific Abs, providing serological memory for years after the pathogen has been cleared. In light of this, it was recently observed that anti-S Abs titer correlates with the frequency

of S-specific plasma cells in bone marrow aspirates from adults who had recovered from mild COVID-19 at 7 to 8 months after infection (95).

This work reinforces what has already been demonstrated about the 6-month anti-SARS-CoV-2 Abs persistency in the pediatric convalescent population (91, 92, 123).

Stratifying individuals by age groups, we demonstrated that both children and adults experienced a decrease in anti-S-RBD IgG levels during follow-up, mostly during the first 200-300 days from infection. Interestingly, children younger than 6 years showed a faster waning of Abs titers in contrast to the others, and then reached a plateau without negativization.

This finding differs slightly from what we have shown previously (92). Analyzing NAbs trends at 1-3, 4-6, 7-9 months from infection across different age groups, we found that mildly affected children under six years displayed increasing NAbs levels over 236 days from infection, children aged 6-15 years plateaued around the same period, and adults showed a significant decline in NAbs, recording a 40% decrease between 3 and 7 months from infection.

Observing the Abs titer at up to 10 months from baseline, children < 6 years showed a mean S-RBD IgG titer 1.8-fold higher than children/adolescents aged 6-18 years and 2.8-fold higher than adults. Thus, assuming that long-term immune response could be influenced by Abs peak, as well as from specific humoral response, specific cellular response (124), and presence of long-term S-specific plasma cells (95), a higher titer is expected in the young child in all time-points of follow-up.

On the other hand, Bloise et al. (125) observed a higher titer of Abs in parents than in children six months after baseline. However, including 25% of hospitalized adults versus only mild/asymptomatic children and analyzing without the possibility of discrimination both N than S-specific Abs, this study may have overestimated the parents' Abs titer. In fact, in addition to the correlation with disease's severity, adults tend to have more N-specific Abs than children, especially in the first weeks after infection, in line with the presence of potentially cross-reactive Abs to HCoVs.

Compared to what Sananez et al. (126) recently observed, namely that severe COVID-19 pediatric cases have fewer Abs than mildly infected children, it is possible that the high number of Abs detected in our individuals is related to the mildness/asymptomaticity of the infection.

In addition, the persistence of a *satisfactory* S-RBD IgG titer more than ten months after infection was observed in all age groups, regardless of whether they declined over time. 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. Moreover, only two subjects evaluated at >10 months from infection achieved complete sera Abs negativization. Recent studies have estimated that the correlate of 50% protection from re-infection and from severe infection were 20% and 3% respectively (127). Relying on these findings, Lau et al. (128) 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). It was estimated that $PRNT_{50}$ will drop to this threshold 990 (95% CI lower bound 441) days after symptom onset in symptomatic patients. As previously estimated by Padoan et al. (90), an S- RBD IgG titer >70 kBAU/L is assumed to correspond to $PRNT_{50}$ titer >1:20. In line with these findings, our data showed that children <6 years might be protected from re-infection up to 1 year. Moreover, none of the subjects evaluated at up to 10 months from infection achieved complete sera Abs negativization.

In conclusion, in our unique family cluster cohort, we confirmed the different kinetics of the COVID-19 humoral response across several age groups of mildly COVID-19 cases. Furthermore, understanding the longevity of humoral immunity to SARS-CoV-2, this study provides 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.

Recent studies have demonstrated that adults with prior SARS-CoV-2 infection had increased levels of anti-SARS-CoV-2 S-RBD and NAbs after just a single dose of vaccine compared with individuals with no previous infection (129, 130). Moreover, Fraley et al. (131) recently observed a longer Abs half-life at seven months after vaccination in individuals with prior COVID-19 before vaccination compared with individuals with no infection history.

In this view, assuming that children have a high specific Abs titer even more than ten months after infection, a single dose vaccine schedule could be considered for pediatric patients with a history of SARS-CoV-2 infection.

Furthermore, considering that children aged <6 years demonstrated a more intense long-term resilience of their immune response starting to decline significantly only after ten months from infection, a unique dose COVID-19 vaccine campaign might be hypothesized in this age class.

However, in the absence of correlates of protection for anti-S-RBD IgG and NAbs acquired after infection, it is not advisable to translate our data into predictions of a superior immunity of children to re-infection. Moreover, as emerging of different virus variants, the level of protective immunity may be compromised.

To better understand the long-term persistence of immune protection against new emerging SARS-CoV-2 variants, future studies should include the evaluation of the longevity of B and T cells which has been shown to play a key role in the global human infection's immune response. In fact, whilst we focused on the Abs responses to infection in this analysis, cellular immune responses are also likely to play an important role in protection against SARS-CoV-2 re-infection, as we and others have previously shown (124). Children presented a higher absolute number of circulating T cells and a high proportion of naïve T cells than adults, thus enabling an efficient adaptive immune response to previously unrecognized microbial antigens, which persist until six months after infection (132).

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 quote of them, an intermediate follow-up was missing. However, 659 over 697 COVID-19 cases had tested at least twice, allowing us to estimate the median Abs titer in a time-scale extended up to more than ten months from the baseline. Secondly, identifying the baseline of infection for COVID-19 cases with no virological record of positivity through the only temporal reference to infection of the first symptomatic household, may be susceptible to a minimal temporal error. However, the initial temporal discrepancy, which may alter the evaluation of the acute phase

of humoral response, was minimized instead during long-term follow-up analysis, which was the main purpose of our study.

6. CONCLUSIONS

In one of the largest available COVID-19 family cluster cohorts, comprehensive of 697 confirmed COVID-19 cases, we evaluated the dynamic changes of the SARS-CoV-2 Abs titers up to 12 months after infection. We demonstrated that anti- SARS-CoV-2 IgG persisted until 12 months after infection in all age groups, showing significant higher Abs peaks for younger individuals at every follow-up time point.

We provided novel insights into the long-term kinetics of the humoral response to COVID-19 for different age classes that could help in optimizing future COVID-19 vaccination strategies and prevention policies. Our work confers further evidence of a robust and sustained immune response in children following primary SARS-CoV-2 infection.

Further studies are needed to assess protection against emerging variants of concern.

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