

2021

Six month outcomes and immune signatures of children infected with SARS-CoV-2

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**SIX MONTH OUTCOMES AND IMMUNE SIGNATURES OF
CHILDREN INFECTED WITH SARS-CoV-2**

by

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B.S., Boston College, 2019

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science

2021

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DEDICATION

I would like to dedicate this work to the children and their families that made this research possible—without their selfless commitment to our study, we would not have the foundation to learn the long-term effects of COVID-19 in children.

ACKNOWLEDGMENTS

I would like to thank all of the incredible women in medicine and science who inspire me daily. My mentor, Dr. Lael Yonker—without her guidance, encouragement and innovative ideas, this project would not have been born. To Dr. Karen Symes—without her advice and support, I would not have made it this far. And lastly, I would like to thank my family and friends for always lifting me up, even amidst my crazy pursuits, and always reminding me to ask myself: “but, are you learning something?”

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MADELEINE DELL BURNS

ABSTRACT

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a novel pathogen that emerged in December of 2019 and has since infected people of all ages around the world. Children with acute SARS-CoV-2 infection are largely spared of the severe disease seen in adults. However, a life-threatening, post-viral inflammatory condition known as Multisystem Inflammatory Syndrome – Children (MIS-C) develops in a small fraction of children four to six weeks after either past SARS-CoV-2 infection or exposure and is characterized by high fevers, significant gastrointestinal symptoms and severe cardiac complications. Little is known about the lasting immune profiles of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection in children, let alone the long-term effects of the disease in this population. This study presents clinical features and serologic immune profiles of forty-nine pediatric patients (ages 12.4 ± 6.7 years) enrolled in the Massachusetts General Hospital Pediatric COVID-19 Biorepository with previous diagnoses of SARS-CoV-2 infection or the COVID-19-related MIS-C. Thirty-two children ages 0-22 years completed a questionnaire which captured lingering clinical symptoms of COVID-19 and MIS-C at the follow-up timepoint. This questionnaire study revealed significant on-going symptoms in both cohorts, including respiratory, gastrointestinal, neurologic and cardiovascular symptoms. To characterize lasting immune responses following the acute presentation, serum antibodies to S, RBD and N proteins of

SARS-CoV-2 were quantified at the follow-up timepoint in forty-nine pediatric patients with past COVID-19 or MIS-C at a mean follow-up timepoint of 6.56 ± 1.75 months. Serologic signatures against SARS-CoV-2 in COVID-19 and severe MIS-C patients were compared at acute illness and at follow-up timepoints. Anti-SARS-CoV-2 antibodies remained elevated over time showing adequate seroconversion. Interestingly, anti-SARS-CoV-2 IgA remained elevated in the vast majority of individuals at follow-up, suggesting continued antigen exposure and mucosal inflammation. This research elucidates whether children maintain antibody levels to SARS-CoV-2 over time and speaks to the differences in antibody recovery to baseline in COVID-19 and MIS-C patients. It also highlights the lingering symptoms in both the COVID-19 and MIS-C cohorts, and suggests the need for significant long-term follow-up in children months, or even years after resolution of acute illness or disease. In total, this study addresses the substantial gap in understanding of the recovery of the adaptive immune system after SARS-CoV-2 infection in children.

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LIST OF ABBREVIATIONS

ACE2.....	Angiotensin-Converting Enzyme 2
CCS.....	Chronic COVID Syndrome
COVID-19.....	Coronavirus Infectious Disease 2019
GI.....	Gastrointestinal
IRB.....	Institutional Review Board
KD.....	Kawasaki Disease
MERS-CoV.....	Middle East Respiratory Syndrome Coronavirus
MFI.....	Mean Fluorescent Intensity
MGH.....	Massachusetts General Hospital
MIS-C.....	Multisystem Inflammatory Syndrome – Children
N.....	Nucleocapsid
RBD.....	Receptor-Binding Domain
RT-PCR.....	Real-Time Reverse Transcription Polymerase Chain Reaction
S.....	Spike
SARS-CoV.....	Severe Acute Respiratory Syndrome
SARS-CoV-2.....	Severe Acute Respiratory Syndrome Coronavirus 2
SD.....	Standard Deviation

INTRODUCTION

Origin of SARS-CoV-2

Since the discovery of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in Wuhan, China in December of 2019, the Coronavirus Infectious Disease 2019 (COVID-19) has spread rapidly throughout the world^{1,2}. SARS-CoV-2, a novel pathogen composed of an enveloped single-stranded, positive-sense RNA virus, spreads primarily by airborne droplets^{1,3,4}. It has shown high transmission rates amongst humans and can lead to multi-organ disease, marking it as an overwhelming threat to global public health⁴. SARS-CoV-2 shares typical characteristics of coronaviruses, and belongs to the betacoronavirus 2B lineage⁵. Genetic sequencing of patients infected with SARS-CoV-2 suggest that bats may have been the original reservoir and host of SARS-CoV-2⁵. Moreover, strains of SARS-CoV-2 prevalent amongst human infections show a 96% sequence identity to the bat SARS-like coronavirus strain, BatCoV RaTG13⁵. This high sequence similarity BatCoV RaTG13 suggests an evolutionary linkage between SARS-CoV-2 and BatCoV RaTG13⁵.

SARS-CoV-2 is not the first coronavirus to infect humans⁶. Indeed, two other highly pathogenic coronaviruses, known as severe acute respiratory syndrome coronavirus 1 (SARS-CoV) and Middle East Respiratory Syndrome Coronavirus (MERS-CoV), emerged in the early 2000s and caused a similar respiratory illness as SARS-CoV-2⁶. Also

believed to be derived from bats, SARS-CoV and MERS-CoV infect cells of the lungs, resulting in clinical symptoms that mimic the viral pneumonia seen in COVID-19^{6,7}. While SARS-CoV-2 appears to cause less fatalities than SARS-CoV and MERS-CoV, the rapid and expansive geographical breadth of infectious spread caused by SARS-CoV-2 has far surpassed those of SARS-CoV and MERS-CoV, raising many alarms for the health implications that SARS-CoV-2 may cause⁶.

SARS-CoV-2 Transmission

SARS-CoV-2 spreads mainly through droplets originating from individuals in close, unprotected contact to one another⁵. However, it can also indirectly infect through contaminated hands, which allows for individuals to unknowingly touch the mucous membranes of the mouth, nose and eyes⁵. SARS-CoV-2 gains entry to cells through the exploitation of human angiotensin-converting enzyme 2 (ACE2) found on the surface of cells in the nasopharynx^{1,3,8}. The spike protein of SARS-CoV-2 interacts with ACE2 on respiratory epithelial cells using its receptor-binding domain, initiating the infection that results clinically in COVID-19^{1,3,4,8}.

Individuals of all ages are susceptible to SARS-CoV-2 infection, but the clinical appearance varies with age^{3,9,10}. As of March 2021, more than 28 million cases were reported in the United States, with children \leq 18 years old representing 11.3% of those cases¹¹. However, this likely under-represents the true prevalence of disease in children,

given that infection in children may be unrecognized as most children remain paucisymptomatic or asymptomatic, or families with children may choose to forgo COVID-19 testing in children and opt for quarantine instead¹². Regardless, it is now evident that children are not spared from SARS-CoV-2 infection.

Clinical Profiles of Pediatric SARS-CoV-2 Infection

Epidemiological data suggest that children with COVID-19 are largely spared of the severe sequelae of symptoms often associated with adult-onset of illness^{3,10,13,14}. Pediatric disease is characterized by mild cold-like symptoms, such as fever, cough and nasal discharge, resulting in lower hospitalization and mortality rates compared to adults^{3,10,15,16}. Many children with SARS-CoV-2 infection lack symptoms altogether, which exacerbates the spread of SARS-CoV-2 since they are highly infectious during the asymptomatic or mild period of disease, and are known to carry equally high viral loads and often higher SARS-CoV-2-specific antibody responses than adults^{3,5,8,15-18}. Children have fewer ACE2 receptors, which could partially explain the disparate clinical courses across the age spectrum^{3,18}. However, more research is needed to define the clinical and immunologic responses to acute COVID-19 in children.

A Post-Viral Inflammatory Syndrome in Children

While the majority of children have favorable outcomes after SARS-CoV-2 infections, in some rare cases a severe, life-threatening, hyperinflammatory response develops four to six weeks after SARS-CoV-2 exposure or infection^{3,19-22}. Resembling the features of Kawasaki Disease (KD) in children, this post-infectious condition, now known as Multisystem Inflammatory Syndrome in Children (MIS-C), causes high fever, prominent gastrointestinal symptoms such as abdominal pain, nausea or diarrhea, systemic inflammation and severe cardiac complications including ventricular failure, coronary artery aneurysms and shock^{21,23-26}. These children also exhibit an overwhelming cytokine storm, monocyte-driven innate immune responses with hyperphagocytosis and activation, and exaggerated humoral responses^{13,20,27}. While other post-infectious syndromes such as rheumatic fever have been shown to have long term effects such as heart failure, valve disease, atrial fibrillation, endocarditis and stroke, the long-term implications of this severe inflammatory syndrome in children are not yet recognized^{28,29}.

Immune Profiles of Pediatric SARS-CoV-2 Infection

Significant advances have been made to define immune profiles of children with acute COVID-19 and in MIS-C with a particular focus on the humoral response to the super-antigen spike (S) protein, which promotes viral entry into host cells, the receptor binding domain (RBD) component of the S protein, and the nucleocapsid (N) protein,

which aids viral replication^{3,13,14,30}. At onset of illness, children display similar immune responses to those of mildly affected adults, with anti-SARS-CoV-2 IgM predominating early in the infection^{3,13,14}. Children with MIS-C, however, mimic severely-ill adults and exhibit a maturing immune response with a profound, expansive immunoglobulin profile where anti-SARS-CoV-2 IgG predominates^{3,13}. This is further proven when comparing within the MIS-C cohort, where severe cases display heightened IgM and IgG in comparison to their milder counterpart³.

However, in adults, SARS-CoV-2-specific antibodies wane over time³¹⁻³³. Lasting immune responses would be needed for lasting immunity, and amidst the continuing global pandemic, this decline in immune response raises the possibility of vulnerability to re-infection over time. The longevity of SARS-CoV-2 antibodies in children has not been studied, leaving substantial gaps in our understanding of their response to SARS-CoV-2 infection over time. A clear picture of the long-term immune responses in children must be defined.

Long-Term Symptoms Following COVID-19 in Adults

As we surpass one year since the first cases of COVID-19 emerged, many long-term follow-up studies have reported prolonged symptoms of COVID-19 in adults who should have long since recovered from SARS-CoV-2 infection³⁴⁻³⁶. Now termed “chronic COVID syndrome” (CCS), individuals with this post-viral illness present most often with lingering malaise and fatigue³⁶⁻³⁸. Those who recovered from severe cases of COVID-19

show concerning follow-up imaging and biomarkers consistent with long-term pulmonary and cardiac disease and continued inflammation—damage which could be due to intensive care interventions such as intubation, but also due to SARS-CoV-2 itself^{35,36}. However, even adults who were asymptomatic or paucisymptomatic during acute illness showed elevated inflammatory biomarkers over one month after acute illness³⁵.

Little is known of the underlying pathogenic mechanism resulting in CCS and its prolonged organ effects. The stratification of long-term risk in adults is still being explored, but previous reports suggest that individuals with past history of pneumonia are at risk of developing cardiovascular disease a decade later³⁶. Therefore, it is crucial to understand the symptoms, immunological origins and complications of CCS in adults in order to appreciate its relevance and application in children.

Need for Pediatric Follow-Up Studies

Due to the rapid spread of SARS-CoV-2 amongst adults and children, substantial follow-up studies are required to inform the role that the adaptive immune system plays in both the pathogenesis of disease, as well as prolonged symptoms many months after onset of illness^{13,39}. Preliminary follow-up studies show that children have a much more tempered response one month after SARS-CoV-2 infection in comparison to adults, as seen by slower seroconversion rates after one-month post-acute infection^{10,40}. Further, antibodies in children remain relatively fixed at a lower level in the months after SARS-CoV-2 infection in comparison to adults¹⁴. Clearly, there is a significant gap in

understanding of the clinical recovery and serologic maintenance of immune protection against SARS-CoV-2 in children. Additional study of the relationship between humoral antibody response, symptomatology and predictive inflammatory markers will guide treatment of children with SARS-CoV-2 infection and MIS-C^{3,13,20}.

This study characterizes clinical symptomatology and serology of pediatric patients with SARS-CoV-2 infection and children with MIS-C, comparing acute presentation and six-month follow-up. This investigation endeavors to understand the long-term immune responses in convalescent children, ascertaining post-infectious symptoms that may warrant longer-term follow up, despite indications of a stabilized immune response. We hope that the resulting data will lay the groundwork for a new infrastructure of interdisciplinary pediatric follow-up care, and inform unforeseen outcomes that may result from the long-lasting systemic involvement after acute SARS-CoV-2 infection in children.

SPECIFIC AIMS

This study presents the clinical features and serology of forty-nine children with past SARS-CoV-2 infection or MIS-C diagnoses at six-months follow-up from acute illness. SARS-CoV-2 is a novel, highly transmissible pathogen composed of a single-stranded, positive-sense RNA virus that spreads primarily by airborne droplets, especially when individuals are in unprotected, close contact to each other^{1,3-5}. SARS-CoV-2 enters the body through the mucous membranes of the mouth, nose and eyes, and then invades cells through the exploitation of human angiotensin-converting enzyme 2 found on the surface of cells in the nasopharynx^{1,3,4,8}. This results clinically in COVID-19.

Individuals of all ages are susceptible to SARS-CoV-2 infection^{3,9,10}. However, the clinical markers of disease vary with an individual's age, comorbidities and sex¹². This study addresses the longitudinal progression of pediatric COVID-19 disease. Studies of adults and children at onset of acute illness speak to the high transmissibility of SARS-CoV-2 amongst humans⁴. Adults are known to display more severe symptoms than children, and are at higher risk for complications, namely viral pneumonia and death (3,34,35). Children present with much milder symptoms than adults, the most notable being congestion, cough and fevers³. Rare cases of a severe, hyperinflammatory response has been reported in children who are four to six weeks after SARS-CoV-2 exposure or infection^{3,19-22}. This life-threatening condition, known as MIS-C, causes high fever, prominent gastrointestinal symptoms such as abdominal pain, nausea or diarrhea, systemic inflammation and severe cardiac complications including ventricular failure, coronary

artery aneurysms and shock^{21,23-26}. However, the long-term effects of COVID-19 and MIS-C in children have not yet been explored.

The acute and long-term symptomatology data outlined in this study suggest that follow-up care may be needed to address lingering respiratory, gastrointestinal, cardiovascular, and neurological symptoms that have persisted in children months after onset of illness. While advances have been made to outline the immune signatures of children with acute COVID-19 and MIS-C, little is known of the long-term effects of disease in this population^{3,13,14,30}. This study also shows that Anti-S IgG and IgA following pediatric SARS-CoV-2 infection and MIS-C remain elevated in the majority of children. These persistent immunoglobulin levels suggest ongoing mucosal inflammation due to possible continued antigen exposure. The symptoms and serology reported in this pediatric study provide substantial evidence for the need for new treatment plans and follow-up protocols for children recovering from COVID-19 and MIS-C.

METHODS

Patient Selection

Pediatric patients ≤ 22 years of age who were previously enrolled in the Institutional Review Board (IRB)-approved Massachusetts General Hospital (MGH) Pediatric COVID-19 Biorepository (#2020P000955) with COVID-19 or severe MIS-C diagnoses and who were at least one-month post-SARS-CoV-2 infection or exposure were approached for follow-up. Informed consent, and where appropriate, assent, was verbally obtained from patients and/or parent/guardian in accordance with IRB guidelines for blood specimen collection and questionnaire administration.

Study Definitions

Patients were categorized into two groups: COVID-19 and MIS-C. Individuals within the COVID-19 cohort had a previous nasopharyngeal swab sample positive for SARS-CoV-2 tested by real-time reverse transcription polymerase chain reaction (RT-PCR) testing using SARS-CoV-2 specific primers and probes obtained by a hospital laboratory. MIS-C was defined per the Centers for Disease Control and Prevention criteria: fever $> 38^{\circ}\text{C}$ for >24 hours, laboratory evidence of inflammation, ≥ 2 organs involved, and no alternative plausible diagnoses and a positive SARS-CoV-2 test by RT-PCR, serology or antigen test, or exposure to an individual with COVID-19 within 4 weeks before the

onset of symptoms. Only children with severe cases of MIS-C, defined as children with hypotension or cardiac involvement requiring treatment such as steroids, intravenous immunoglobulin and anakinra, were recruited for the serology study. Mild cases of MIS-C, which did not require significant interventions and merely general hospital care, were excluded from this study. The follow-up timepoint was determined by the number of months passed between date of initial COVID-19 or MIS-C evaluation or diagnosis and follow-up blood specimen collection. Samples were considered matched if the participant provided a blood specimen during acute illness, as well as at follow-up. Unmatched samples were from participants who had past COVID-19 or MIS-C diagnoses, but only provided blood specimens at follow-up. Four children with negative SARS-CoV-2 RT PCR testing were used as negative controls, depicted by the dotted threshold line in **Figures 1-3**. Chart analyses were performed to determine if participants re-presented to a clinic, emergency department, hospital or pediatric specialist with recurrent or persistent symptoms between acute illness and follow-up. Pediatric specialists included gastroenterologists, pulmonologists, cardiologists, rheumatologists and neurologists. Recurrent symptoms were defined by presenting symptoms, and included cough, congestion, fever, chest pain, abdominal pain and diarrhea.

Data Collection

Medical records were reviewed to assess demographic and clinical factors, including date of birth, age, sex, race, ethnicity, laboratory testing, presenting features at

time of acute illness or diagnosis, and instances of re-presentation to a pediatric specialist or the hospital for recurrence of symptoms. At the follow-up time point, consented participants were invited to donate a second blood specimen for serum antibody quantification. Venipuncture was performed by trained phlebotomists; plasma and serum were collected and immediately stored at -80°C. Follow-up participants were also asked to complete a structured, qualitative questionnaire that inquired about lingering symptoms (Appendix A). Data were stored in a REDcap database.

IgG1, IgM and IgA1 Titers Measured by Luminex

Sera from blood specimens were obtained for each participant. SARS-CoV-2-S, SARS-CoV-2-RBD and SARS-CoV-2-N specific antibody isotypes were analyzed by Luminex multiplexing as described in Brown – immune methods⁴¹. The antigens were carboxy-coupled to Luminex microspheres provided by Luminex Corp, Austin, Texas, and were then incubated with IgG1, IgM, and IgA1 polyclonal plasma samples. A second antibody tagged with fluorophore was used to probe the SARS-CoV-2-specific isotypes. Relative concentrations were analyzed by flow cytometry, given by mean fluorescent intensity (MFI).

Statistical Analyses

The Fisher exact test was used for statistical significance of all categorical comparisons. The Mann-Whitney U test assessed statistical significance between acute and follow-up timepoints for each cohort. The ordinary one-way ANOVA test assessed the statistical significance between three groups at different timepoints. Prism software (Prism 8, Graphpad Software, San Diego, California) was used to analyze and graph data.

RESULTS

All children enrolled in the pediatric COVID-19 biorepository follow-up study were approached about providing blood specimens for research. A total of forty-nine children (mean age, 12.4 ± 6.7 years) with past COVID-19 or severe MIS-C were enrolled in this follow-up study, of which thirty samples were matched from acute illness to follow-up. The demographics of the enrolled participants are summarized in **Table 1**. Of the forty-nine children enrolled in the follow-up study, a subset of thirty-two children participated in the questionnaire study, whose results are shown in **Table 2**.

Follow-Up Participant Demographics

Children ranging from 0-22 years old were enrolled in this study (**Table 1**). The average age at follow up for the COVID-19 cohort was 13.9 ± 6.5 years. The average age for the MIS-C cohort was 6.5 ± 3.5 years. Sex was distributed equally between children in the COVID-19 and MIS-C cohorts. Latino/Hispanic children were most highly represented in both the COVID-19 (23 [59.0%]) and MIS-C (6 [60.0%]) groups. Children who identified racially as White comprised 28.2% of the COVID-19 cohort and 20.0% of the MIS-C cohort.

Table 1: Follow-Up Patient Characteristics (n = 49) of Children with COVID-19 or Severe MIS-C. Patient were approached at an average follow-up time point of six months since acute illness. Data was obtained by chart review.

Follow-Up Patient Characteristics	COVID-19 (n = 39)	MIS-C (n = 10)
Age, years, mean (SD)	13.9 (6.5)	6.5 (3.5)
Male sex, number (%)	24 (61.5)	7 (70.0)
Race, number (%)		
American Indian/Alaska Native	0 (0)	0 (0)
Asian	1 (2.6)	1 (10.0)
Black or African American	3 (7.7)	2 (20.0)
Native Hawaiian/Pacific Islander	0 (0)	0 (0)
White	11 (28.2)	2 (20.0)
Other	24 (61.5)	5 (50.0)
Ethnicity, number (%)		
Latino/Hispanic	23 (59.0)	6 (60.0)
Non-Latino/Non-Hispanic	15 (38.5)	4 (40.0)
Unknown	1 (2.6)	0 (0)

Questionnaire Cohort Symptomology

Acute Symptoms of children with COVID-19 and MIS-C

Of the 19 children with COVID-19 enrolled in the questionnaire follow-up study, 16 (84.2%) were symptomatic at acute illness, whereas 100% of 13 MIS-C patients enrolled were symptomatic at presentation to care (**Table 2**). Presenting symptoms including gastrointestinal, cardiovascular, respiratory, mucocutaneous, neurological and general symptoms were quantified and compared between the two cohorts (COVID-19 and MIS-C) at acute onset of disease. A statistically significant difference between children in the COVID-19 and MIS-C cohorts was seen for gastrointestinal symptoms, cardiovascular symptoms, and mucocutaneous symptoms reported when presenting to care, with MIS-C predominating in each of these symptom categories ($p \leq 0.02$). Respiratory symptoms reported were elevated in COVID-19 and MIS-C (15 [79.0%] vs. 9 [69.2%]), but did not differ significantly between groups, which is consistent with reported data in current literature^{3,13}. Interestingly, both COVID-19 and MIS-C reported similarly in neurological (3 [15.8%] vs. 3 [23.1%]) and general symptoms (6 [31.6%] vs. 6 [46.2%]).

Lingering Symptoms at Follow-Up

More children with MIS-C reported lingering symptoms at the follow-up timepoint than children with past SARS-CoV-2 infection (4 [21.1%] vs. 10 [77.0%]; $p \leq 0.0033$) (**Table 2**). Overall, both children with past COVID-19 and MIS-C endorsed persistent symptoms in each category. However, a significant difference was seen in lingering

respiratory (8 [61.5%] vs. 4 [21.1%]; $p < 0.03$) and general symptoms (7 [53.8%] vs. 3 [15.8%]; $p < 0.05$) between the COVID-19 and MIS-C groups. While respiratory symptoms in the COVID-19 cohort, including dyspnea, cough, loss of smell or taste, sore throat and congestion, seemed to improve from acute illness to follow-up (15 [79.0%] in acute illness vs. 4 [21.1%] at follow up; $p \leq 0.0009$), respiratory symptoms in MIS-C seemed to persist (9 [69.2%] vs. 8 [61.5%]), especially congestion. Of note, both the COVID-19 and MIS-C cohorts reported increased neurological symptoms from time of presentation to care to follow-up (3 [15.8%] vs. 9 [47.4%] for COVID-19 vs. 3 [23.1%] vs. 10 [77.0%]; $p \leq 0.01$ for MIS-C), a finding that deserves significant consideration given the average ages of these cohorts. Additionally, almost 50% of the MIS-C cohort endorsed unresolved, lingering gastrointestinal symptoms at follow-up, which is of interest given findings in many studies that suggest persistent shedding of SARS-CoV-2 in the stool months after acute illness⁴². Lastly, while a significant difference was not found, 3 (23.1%) of MIS-C patients reported cardiovascular symptoms at six-months follow-up, which is noteworthy given the multi-organ involvement seen in severe MIS-C. **Table 2a-c** summarizes symptoms reported at both acute and follow up visits.

Table 2a-c: Symptomatology at Acute Illness and Follow-Up of Children with COVID-19 and MIS-C. Symptom subgroups include gastrointestinal, cardiovascular, respiratory, mucocutaneous, neurological and general symptoms. The average follow-up time point was six months since acute illness. Participants filled out a printed questionnaire. Parents of participants filled out the questionnaire if children were under the age of five years old. If participants did not report symptoms on the questionnaire at the follow-up timepoint, they were considered asymptomatic. Categories of symptoms within each time point (acute or follow up) were analyzed using Fisher's exact test. * $P < 0.05$, ns not significant.

Table 2a: Gastrointestinal and Cardiovascular Symptomatology at Acute Illness and Follow-Up of Children with COVID-19 and MIS-C. * P<0.05, ns not significant.

Symptoms Reported, number (%)	Acute Presentation		Follow-Up		p
	COVID-19 (n = 19)	MIS-C (n = 13)	COVID-19 (n = 19)	MIS-C (n = 13)	
Symptomatic, number (%)	16 (84.2)	13 (100)	4 (21.1)	10 (77.0)	**
Gastrointestinal symptoms	5 (26.3)	10 (77.0)	4 (21.1)	6 (46.2)	ns
Ageusia	1 (5.3)	0 (0)	0 (0)	1 (7.7)	
Abdominal pain	3 (15.8)	5 (38.5)	2 (10.5)	2 (15.4)	
Anorexia	2 (10.5)	4 (30.8)	Not asked	Not asked	
Diarrhea	1 (5.3)	5 (38.5)	0 (0)	2 (15.4)	
Nausea/vomiting	1 (5.3)	6 (46.2)	2 (10.5)	1 (7.7)	
<i>Cardiovascular symptoms</i>	0 (0)	4 (30.8)	1 (5.3)	3 (23.1)	ns
Persistent chest pain/pressure	0 (0)	0 (0)	1 (5.3)	2 (15.4)	
Heart palpitations	0 (0)	3 (23.1)	0 (0)	1 (7.7)	
Syncope	0 (0)	0 (0)	0 (0)	0 (0)	

Table 2b: Respiratory and Mucocutaneous Symptomatology at Acute Illness and Follow-Up of Children with COVID-19 and MIS-C. * P<0.05, ns not significant.

Symptoms Reported, number (%)	Acute Presentation			Follow-Up		
	COVID-19 (n = 19)	MIS-C (n = 13)	p	COVID-19 (n = 19)	MIS-C (n = 13)	p
<i>Respiratory symptoms</i>	15 (79.0)	9 (69.2)	ns	4 (21.1)	8 (61.5)	*
Dyspnea	2 (10.5)	3 (23.1)		1 (5.3)	2 (15.4)	
Chronic cough	6 (31.6)	6 (46.2)		0 (0)	0 (0)	
Anosmia/hyposmia	2 (10.5)	1 (7.7)		1 (5.3)	1 (7.7)	
Sore throat	6 (31.6)	3 (23.1)		1 (5.3)	0 (0)	
Congestion/rhinorhea	7 (36.8)	1 (7.7)		1 (5.3)	5 (38.5)	
<i>Mucocutaneous symptoms</i>	0 (0)	4 (30.8)	*	2 (10.5)	4 (30.8)	ns
Rash	0 (0)	4 (30.8)		2 (10.5)	3 (23.1)	
Eye redness	0 (0)	0 (0)		0 (0)	1 (7.7)	

Table 2c: Neurological and General Symptomatology at Acute Illness and Follow-Up of Children with COVID-19 and MIS-C. * P<0.05, ns not significant.

Symptoms Reported, number (%)	Acute Presentation		p	Follow-Up		p
	COVID-19 (n = 19)	MIS-C (n = 13)		COVID-19 (n = 19)	MIS-C (n = 13)	
<i>Neurological symptoms</i>	3 (15.8)	3 (23.1)	ns	9 (47.4)	10 (77.0)	ns
Loss of concentration	0 (0)	0 (0)		2 (10.5)	1 (7.7)	
Headache	3 (15.8)	2 (15.4)		1 (5.3)	4 (30.8)	
Difficulty sleeping	0 (0)	0 (0)		1 (5.3)	1 (7.7)	
Dizziness	0 (0)	1 (7.7)		1 (5.3)	1 (7.7)	
Feeling anxious, depressed	0 (0)	0 (0)		2 (10.5)	2 (15.4)	
Altered mental status	0 (0)	0 (0)		2 (10.5)	1 (7.7)	
<i>General symptoms</i>	6 (31.6)	6 (46.2)	ns	3 (15.8)	7 (53.8)	*
Inability to exercise/be active	0 (0)	0 (0)		0 (0)	1 (7.7)	
Myalgia/arthritis	5 (26.3)	3 (23.1)		1 (5.3)	3 (23.1)	
Fatigue	3 (15.8)	2 (15.4)		2 (10.5)	3 (23.1)	
Chills	1 (5.3)	1 (7.7)		Not asked	Not asked	

Follow-Up Care Required Post-SARS-CoV-2 Infection or MIS-C Diagnosis

The follow-up care required as a result of past SARS-CoV-2 infection or MIS-C diagnosis were quantified (**Table 3**). As expected, all children with MIS-C required follow-up care with pediatric cardiologists due to the cardiac involvement seen in MIS-C. These visits included consults, electrocardiograms and echocardiograms to rule out continued or recurrent cardiomyopathy within this subcohort. Only 7.7% of children with past COVID-19 followed-up with a pediatric cardiologist. Almost 50% in both cohorts re-presented to their primary care physicians with complaints of lingering symptoms as a result of past illness. These persistent symptoms included congestion, rhinorrhea, abdominal pain, cough and diarrhea. 25.6% of children with COVID-19 sought follow-up care from pulmonologists and 23.1% from gastroenterologists, which is consistent possible persistent mucosal inflammation in both the lungs and gut. A small subset in both cohorts re-presented to the emergency department or urgent care with recurrent symptoms at follow-up, and a similar fraction of the cohort were hospitalized during follow-up for lingering symptoms such as fever, shortness of breath, pancreatitis and abdominal pain. Interestingly, 38.5% of the COVID-19 cohort and 20% of the MIS-C cohort re-presented for COVID-19 testing, despite having positive infection in the past.

Table 3: Follow-Up Care Required in Children Post-COVID-19 and Severe MIS-C. This data was obtained by chart review. The follow-up time point was defined as at least one month after presentation to care for acute illness. Data obtained for re-presentation for SARS-CoV-2 RT-PCR testing was limited to Partners-affiliated hospitals. *Reasons for hospitalization were fever, shortness of breath, pancreatitis and abdominal pain.

Follow-Up Care, number (%)	COVID-19 (n = 39)	MIS-C (n = 10)
<i>Re-Presented for Follow-Up SARS-CoV-2 RT-PCR Testing, number (%)</i>	15 (38.5)	2 (20.0)
<i>Re-Presented for Follow-Up Medical Evaluation</i>		
Primary Care Pediatrician	19 (48.7)	5 (50.0)
Gastroenterologist	9 (23.1)	0 (0)
Pulmonologist	10 (25.6)	1 (10.0)
Cardiologist	3 (7.7)	10 (100.0)
Rheumatologist	1 (2.6)	2 (20.0)
Neurologist	2 (5.1)	0 (0)
Emergency Department / Urgent Care	5 (12.8)	3 (30.0)
Re-Admitted / Hospitalized*	2 (5.1)	2 (20.0)

SARS-CoV-2 Antibody Response at Follow-Up

To characterize lasting immune responses following the acute presentation, serum antibodies to S, RBD and N proteins of SARS-CoV-2 were quantified at the follow-up timepoint in forty-nine pediatric patients with past COVID-19 or MIS-C. Median serum antibody levels for each immunoglobulin isotype within a sub-cohort is designated by the horizontal line on each graph. Plasma from never infected, SARS-CoV-2 (-) individuals (negative control), which are negative for any antibody responses, represent the threshold for seroconversion.

SARS-CoV-2- Specific IgG Response

Children with acute COVID-19 already had elevated levels of anti-S-IgG at acute presentation (**Figure 1A**). Because the magnitude of serologic responses to infection correlate with exposure and symptom onset and seroconversion to SARS-CoV-2 is similar to other viruses, IgG would be expected to begin this rise 7-14 days into the infection^{13,39,40}. However, since these children are presenting after only 14 days of symptoms, this suggests that these children may have had a longer asymptomatic, infectious period allowing IgG response to mount before presentation.

SARS-CoV-2-IgG levels are normally elevated for about three months after acute infection, suggesting brief protective immunity in children. Soon thereafter, Anti-S-IgG levels in children with COVID-19 are seen to decline over time, consistent with recovery from infection and decreased inflammation (**Figure 1A**). Even so, a fair number of

individuals with COVID-19 do not mount an antibody response, which may represent technical limitations of this assay, or may reveal a subgroup of infected individuals that do not mount a humoral response to SARS-CoV-2 (**Figure 1A**).

Children with severe MIS-C, on the other hand, appear to separate into two groups at acute presentation, those with elevated IgG and those with low IgG (**Figure 1A**). Severe MIS-C, defined as children with acute MIS-C with hypotension or cardiac abnormalities requiring therapeutic intervention such as steroids, intravenous immunoglobulin and/or anakinra, had more elevated IgG1-S levels. At follow-up, anti-spike IgG remains elevated, suggesting a plateau in response that persists months after initial infection.

SARS-CoV-2-Specific IgM Response

A sharp rise in SARS-CoV-2-S-IgM is seen in the first days of acute COVID-19, followed by a decline to low, if not undetectable levels at as early as twenty-one days follow-up, consistent with seroconversion to more specific antibodies such as IgG-S and IgA-S. Most individuals in this study followed this waning response at follow-up, however, a subgroup of individuals continued to have elevated IgM-S for months after initial infection (**Figure 1B**). This is also seen in severe MIS-C, where the IgM-S levels in some children remain elevated at both acute presentation and follow-up. This is interesting because in MIS-C, the anti-S-IgM response should be fully developed 2-6 weeks after initial infection, and at follow-up, one would expect nonreactive levels of IgM in children with MIS-C due to resolution of infection and delayed onset of illness.

SARS-CoV-2-Specific IgA Response

Anti-S-IgA1 remains elevated in the vast majority of individuals throughout follow-up. This elevation in IgA months after initial infection reflects an immune response to mucosal surfaces such as the airway or gut even after resolution of illness (**Figure 1C**). This elevation suggests ongoing antigen presence at mucosal surfaces months after acute infection, and has implications for persistent inflammation in the respiratory and gastrointestinal tracts in children even after recovery from COVID-19 or MIS-C.

Anti-RBD and N Immunoglobulin Response

Immunoglobulin responses to the RBD component of the Spike protein (**Figure 2**) and the nucleocapsid protein (**Figure 3**) displayed similar patterns to anti-S responses. Continued elevation of anti-RBD and anti-N IgM was shown at follow-up in both COVID-19 and severe MIS-C. Only anti-N IgA1 showed a mild elevation at follow-up, whereas anti-RBD IgA1 returned to baseline levels. IgG1 showed a minimal response to the RBD component in both COVID-19 and MIS-C at acute presentation and follow-up, and anti-N IgG1 demonstrated a decline in response (approaching baseline) between acute illness and follow-up in both groups. In total, this humoral response quantified in this follow-up study may speak to chronic, low-grade inflammation after disease.

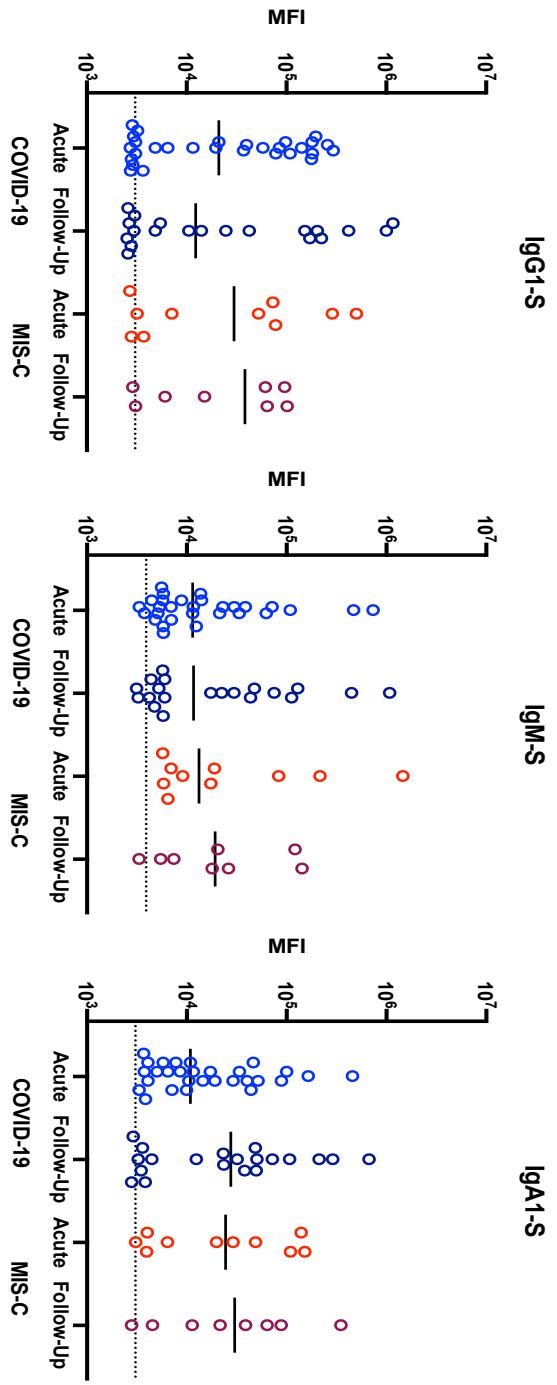


Figure 1: Anti-SARS-CoV-2-S Antibody Responses within Cohorts at Acute and Follow-Up Timepoints. Solid line represents median value within each group. Dashed line represents SARS-CoV-2 negative control.

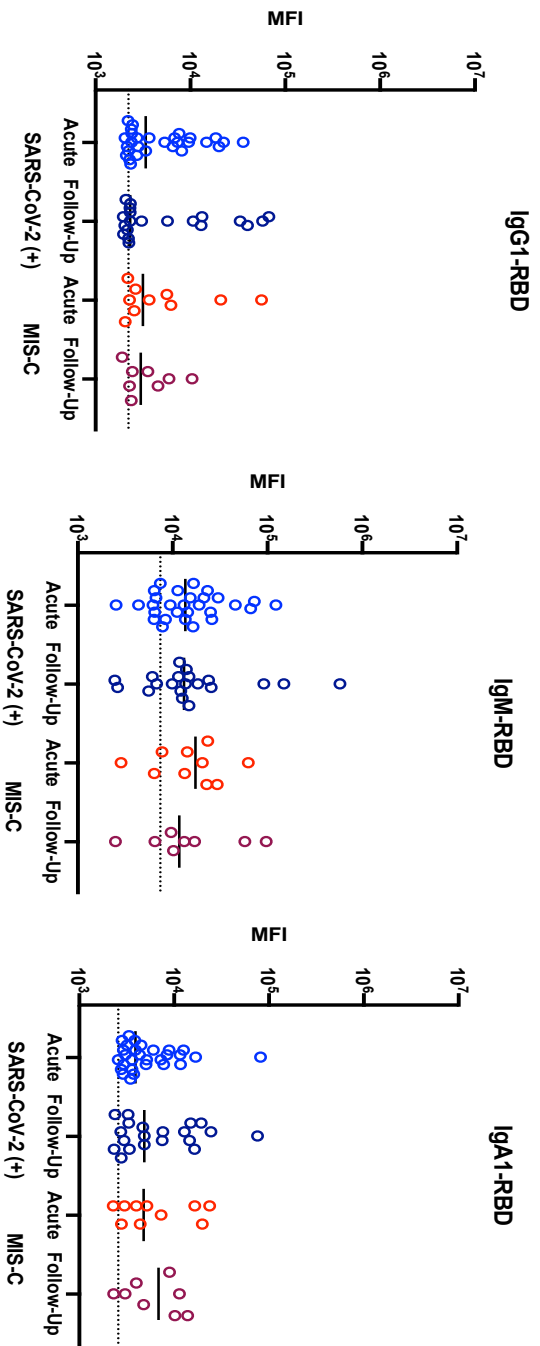


Figure 2: Anti-SARS-CoV-2-RBD Antibody Responses within Cohorts at Acute and Follow-Up Timepoints. Solid line represents median value within each group. Dashed line represents SARS-CoV-2 negative control.

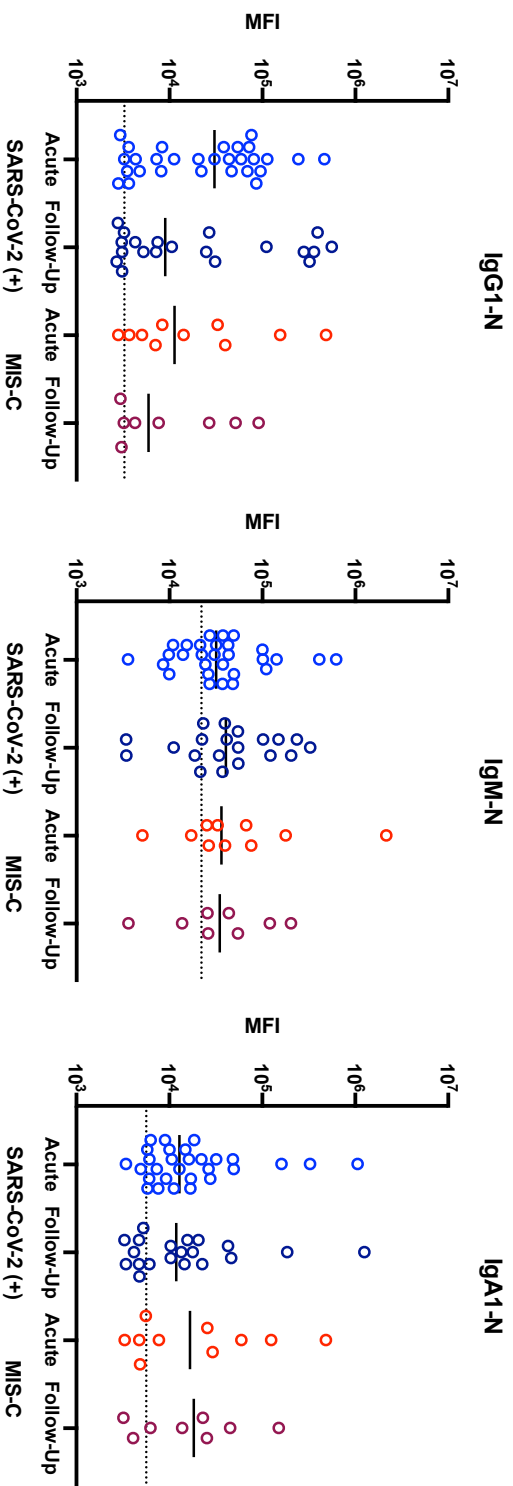


Figure 3: Anti-SARS-CoV-2-N Antibody Responses within Cohorts at Acute and Follow-Up Timepoints. Solid line represents median value within each group. Dashed line represents SARS-CoV-2 negative control.

DISCUSSION

Over one year has passed since the first cases of SARS-CoV-2 infection in children, and little is known about the immune system's recovery in the months after resolution of acute illness. This study presents findings from the first long-term follow-up pediatric COVID-19 biospecimen repository, describing symptomatology and serology as they relate to children with past SARS-CoV-2 infection and MIS-C. The data presented here provide insight on the long-term risks in children after SARS-CoV-2 infection, and provide compelling evidence for the need for interdisciplinary follow-up in the months, and potentially years, after children recover from COVID-19 or MIS-C.

Post-COVID-19 mucosal inflammation drives many lingering symptoms in children

Increased respiratory symptoms in children following recovery from SARS-CoV-2 infection

As expected, respiratory symptoms, albeit mild in children, predominate in acute SARS-CoV-2 infection³. Clearance of virus from the respiratory tract is seen between 1-2 weeks after symptom onset, confirmed by negative RT-PCR tests of nasopharyngeal swabs³. However, children with MIS-C, presenting weeks after their initial upper respiratory tract infection with SARS-CoV-2 infection resolved, demonstrated higher levels of respiratory symptoms than expected given the time lag. Further, nearly a quarter of children who had acute COVID-19 and over 60% of children with MIS-C had on-going respiratory symptoms at follow-up. In adults, it is now known that COVID-19 can result

in significant functional impairment and radiographic abnormalities of the lungs⁴³. The lasting effect of COVID-19 on pulmonary function in children is not well established and these findings highlight that much is unknown about the long-term impact of SARS-CoV-2 infection on the lungs of children. A standing, respiratory follow-up structure would be worthwhile since patients may warrant further testing during recovery.

Increased post-infectious gastrointestinal symptoms in children

Over a quarter of children with acute COVID-19 presented with gastrointestinal (GI) symptoms, while the vast majority of children with MIS-C reported prominent GI symptoms. At follow up, nearly a quarter of children with COVID-19 and half of the children with MIS-C still reported ongoing GI symptoms. There is an increased recognition of shedding of SARS-CoV-2 in the stool months after acute illness in both children and adults, suggesting persistent antigen exposure in the gastrointestinal tract even months after resolution of disease⁴². This ongoing antigen exposure at the mucosal surfaces of the gut may explain these persistent gastrointestinal symptoms seen in children previously infected with SARS-CoV-2. It is unclear as to whether this virus in the gut is live or dead, and further characterization of SARS-CoV-2 in the gut of children is needed. Regardless, post-infectious GI symptoms are amongst the more common symptoms, especially in children who have MIS-C, and the impact of the virus on the gut warrants further research.

Rising levels of anti-SARS-CoV-2 IgA reveal active mucosal immune responses in children

While a clear relationship cannot be determined from our follow-up data, it is interesting that anti-SARS-CoV-2 IgA titers remain elevated in COVID-19. Typically, the removal of an antigen from the mucosal surface will result in a decline in IgA production and most viruses have a decline in IgA over time⁴⁴⁻⁴⁶. In contrast, children with prior COVID-19 show a trending increase in anti-S-IgA1, which may be related to persistent SARS-CoV-2 in the gastrointestinal tract during the months following disease. In adults with severe COVID-19, IgA serves as a potent activator of the innate immune system; its role in the pediatric response to COVID-19 and MIS-C needs to be further defined^{13,46}. Future studies of IgA recovery are required to fully outline the relationship between persistent symptoms reported in this cohort and the adaptive immune response and the health implications of ongoing mucosal inflammation in children.

High rates of neurological symptoms are seen post-COVID-19 in children

Children with acute COVID-19 and especially those with MIS-C have higher rates of neurological symptoms than expected. Few studies report neurological complaints amongst children at disease onset, and little is known about the long-term effect of SARS-CoV-2 infection on the mental wellbeing of children and their families. Our data suggest a positive relationship between neurological symptoms and months of follow-up in both children with past SARS-CoV-2 infections and MIS-C. Interestingly, none of the children followed in this study presented with any neurological complications at presentation to

care, and yet over 50% of children in the COVID-19 cohort and 77% in the MIS-C cohort reported symptoms at follow-up. This is corroborated by accounts from current literature, which report lingering neurological symptoms such as unresolved headache, fatigue and altered mental status^{2,47}. However, it is also important to note the possibility that children and their families may have reported neurological symptoms within the context of the pandemic as a whole, rather than as a consequence of SARS-CoV-2 infection. Regardless, the negative implications of the pandemic on children are undeniable. Given the age of these children, many of whom are struggling to adjust to online or hybrid learning amidst the COVID-19 pandemic, it is important to recognize how these augmented symptoms may impact cognitive development and mental health of children. Furthermore, while many recognize the respiratory, cardiovascular and gastrointestinal complications associated with COVID-19 and MIS-C, it is clear that long-term neurological effects of SARS-CoV-2 infection are prevalent amongst children. These long-lasting symptoms must be addressed, given their widespread implications and impact on the general wellbeing of children.

Severe cardiac complications can arise following SARS-CoV-2 infection in children

Lastly, there is a clear connection between children with MIS-C and cardiac involvement. Early studies show that immunoglobulins could perhaps play a role in the development of autoantibodies against cardiac tissue or activation of antibodies against tissue-specific macrophages which resultingly attack the myocardium^{13,20}. This study

highlights that children with past SARS-CoV-2 infection, and not just MIS-C, present with persistent cardiac symptoms. The long-term consequences of COVID-19 and MIS-C in children need to be understood, especially given increasing evidence that children with COVID-19 develop post-infection myocarditis. Continued follow-up in children is necessary to inform long-term outcomes and potential complications months after acute illness.

Conclusion

In summary, the symptomatology and serology findings presented in this study call for significant follow-up of children as they recover from SARS-CoV-2 infection or MIS-C. Lingering respiratory, cardiovascular, gastrointestinal and neurological symptoms in both cohorts present compelling evidence for the need for future, system-specific studies with large sample sizes in children. The humoral immune responses at follow-up in children with past-SARS-CoV-2 infection and MIS-C suggest ongoing mucosal inflammation. Despite the high rates of ongoing symptoms and complications in children presented in this study, there are not clear treatment guidelines for follow-up. Many children and their families are not seeking follow-up care because of a lack of infrastructure and advanced planning to meet the needs of those recovered from acute illness. As more data emerges on the spectrum of COVID-19 and MIS-C phenomena in children, it is crucial to continue to develop the signature picture of immune responses following SARS-CoV-2 infection as it pertains to recovery from disease, protective immunity and enduring

symptoms during follow-up. The Pediatric COVID-19 Biorepository at Massachusetts General Hospital has enrolled over 900 children with COVID-19 or COVID-related illness since the onset of the pandemic. This follow-up study has successfully enrolled 49 children with past COVID-19 or MIS-C and represents the first step in understanding the lasting humoral responses and long-term implications of this disease in children.

APPENDIX

Appendix A. Symptomatology Questionnaire

Six Month Follow-Up Questionnaire

1. What symptoms are you/your child currently experiencing that have not resolved since your/their COVID-19 infection? Select all that apply.

Respiratory symptoms:

- a. Shortness of breath or difficulty breathing
- b. Chronic cough

GI symptoms:

- a. Diarrhea
- b. Nausea/vomiting
- c. Abdominal pain

Cardiovascular symptoms:

- a. Persistent chest pain or pressure
- b. Heart palpitations

General symptoms:

- a. Fatigue/excessive tiredness
- b. Muscle/body aches
- c. Rash
- d. Eye redness
- e. Sore throat
- f. Partial or complete loss of sense of smell
- g. Partial or complete loss of sense of taste
- h. Congested or runny nose
- i. Difficulty concentrating or focusing
- j. Inability to exercise or be active
- k. Headache
- l. Syncope/fainting
- m. Difficulty sleeping
- n. Feeling anxious, feeling depressed/sad or hopeless
- o. Dizziness
- p. None of the above

LIST OF JOURNAL ABBREVIATIONS

<i>Acta Paediatr Oslo Nor</i>	<i>Acta Paediatrica (Oslo, Norway: 1992)</i>
<i>AM J Otolaryngol</i>	<i>American Journal of Otolaryngology</i>
<i>Anatol J Cardiol</i>	<i>Anatolian Journal of Cardiology</i>
<i>Cell Mol Immunol</i>	<i>Cellular & Molecular Immunology</i>
<i>Clin Infect Dis</i>	<i>Clinical Infectious Diseases</i>
<i>Emerg Infect Dis</i>	<i>Emerging Infectious Diseases</i>
<i>Eur J Pediatr</i>	<i>European Journal of Pediatrics</i>
<i>Eur Respir J</i>	<i>European Respiratory Journal</i>
<i>Front Immunol</i>	<i>Frontiers in Immunology</i>
<i>Front Med</i>	<i>Frontiers in Medicine</i>
<i>J Immunol Methods</i>	<i>Journal of Immunological Methods</i>
<i>J Med Virol</i>	<i>Journal of Medical Virology</i>
<i>J Pediatr</i>	<i>Journal of Pediatrics</i>
<i>JAMA</i>	<i>JAMA: The Journal of the American Medical Association</i>
<i>JAMA Pediatr</i>	<i>The Journal of the American Medical Association: Pediatrics</i>
<i>MMWR Morb Mortal Wkly Rep</i>	<i>Morbidity and Mortality Weekly Report</i>
<i>N Engl J Med</i>	<i>New England Journal of Medicine</i>
<i>Nat Immunol</i>	<i>Nature Immunology</i>
<i>Nat Med</i>	<i>Nature Medicine</i>
<i>Nat Rev Dis Primer</i>	<i>Nature Reviews Disease Primers</i>

Nat Rev Immunol

Nat Rev Rheumatol

Neurosci Lett

Pediatr Res

Poult Sci

Sci Rep

Trends Microbiol

Nature Reviews. Immunology

Nature Reviews. Rheumatology

Neuroscience Letters

Pediatric research

Poultry Science

Scientific Reports

Trends in Microbiology

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