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Age-related transcript changes in type I interferon signaling in children and adolescents with long COVID

Matteo Fracella[*1], *Enrica Mancino*[*2], *Raffaella Nenna*[2], *Chiara Virgillito*[3], *Federica Frasca*[1,3], *Alessandra D'Auria*[1], *Leonardo Sorrentino*[1], *Laura Petrarca*[2], *Domenico La Regina*[2], *Luigi Matera*[2], *Greta Di Mattia*[2], *Beniamino Caputo*[3], *Guido Antonelli*[1], *Alessandra Pierangeli*[1], *Raphael P. Viscidi*[4], *Fabio Midulla*[**2] and *Carolina Scagnolari*[**1]

[1] Department of Molecular Medicine, Laboratory of Virology, Sapienza University of Rome, Rome, Italy
[2] Department of Pediatrics and Infantile Neuropsychiatry, Sapienza University of Rome, Rome, Italy
[3] Department of Public Health and Infectious Diseases, Sapienza University of Rome, Rome, Italy
[4] Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

SARS-CoV-2 typically causes mild symptoms in children, but evidence suggests that persistent immunopathological changes may lead to long COVID (LC). To explore the interplay between LC and innate immunity, we assessed the type I interferon (IFN-I) response in children and adolescents with LC symptoms (LC; $n = 28$). This was compared with age-matched SARS-CoV-2 recovered participants without LC symptoms (MC; $n = 28$) and healthy controls (HC; $n = 18$). We measured the mRNA expression of IFN-I (IFN-α/β/ε/ω), IFN-I receptor (IFNAR1/2), and ISGs (ISG15, ISG56, MxA, IFI27, BST2, LY6E, OAS1, OAS2, OAS3, and MDA5) in PBMCs collected 3–6 months after COVID-19. LC adolescents (12–17 years) had higher transcript levels of IFN-β, IFN-ε, and IFN-ω than HC, whereas LC children (6–11 years) had lower levels than HC. In adolescents, increased levels of IFN-α, IFN-β, and IFN-ω mRNAs were found in the LC group compared with MC, while lower levels were observed in LC children than MC. Adolescents with neurological symptoms had higher IFN-α/β mRNA levels than MC. LC and MC participants showed decreased expression of ISGs and IFNAR1, but increased expression of IFNAR2, than HC. Our results show age-related changes in the expression of transcripts involved in the IFN-I signaling pathway in children and adolescents with LC.

Keywords: Long COVID · SARS-CoV-2 · Children · Adolescents · Type I interferon · ISGs · Neutralizing autoantibodies to interferon

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Although the scientific community has carried out unprecedented research into the diagnosis, treatment, and prevention of SARS-CoV-2 infection over the past 4 years, the lasting effects after the acute phase of the disease are still poorly understood. There is evidence that some symptoms and immunological changes can persist for weeks or months after virological recovery in many people who have had COVID-19 [1]. This phenomenon is collectively referred to as "long COVID" or "post-acute sequelae of SARS-

Correspondence: Prof. Carolina Scagnolari
e-mail: carolina.scagnolari@uniroma1.it

[*] Matteo Fracella and Enrica Mancino share co-first authorship.
[**] Carolina Scagnolari and Fabio Midulla share senior authorship.

CoV-2 infection" [2]. The most common symptoms are severe recurrent fatigue, dyspnea, chest tightness, cough, brain fog, and headache [3, 4]. The National Institute for Health Research has suggested that LC may reflect several clinical syndromes such as post-intensive care syndrome, chronic fatigue syndrome, and illness resulting from organ damage caused by SARS-CoV-2 infection [5]. These postinfectious syndromes can dampen an effective immune response to viral and microbial infections and impair the ability to fight pathogens [6]. COVID-19 is often asymptomatic or paucisymptomatic in children [7]. They develop a milder illness and have a better prognosis than their adult counterparts [8]. This clinical pattern contrasts in part with that of other respiratory viral infections, such as respiratory syncytial virus, influenza viruses, rhinoviruses, and metapneumoviruses, which can cause severe disease and are among the leading causes of death in children under 5 years of age [9, 10]. Proposed explanations for the limited severity of SARS-CoV-2 infection in the pediatric population include fewer comorbidities than in adults, better thymic function, robust spike-specific T-cell responses, cross-reactive immunity, and protective innate immune factors, including reduced expression of angiotensin-converting enzyme 2, and a more robust interferon (IFN) response [7, 11, 12]. Immune cell proportions change during healthy childhood and adulthood, with a notable switch from innate to adaptive immunity, leading to differences in the airway and systemic immune responses to SARS-CoV-2 infection [13]. Moreover, SARS-CoV-2, like most viruses, can evade the IFN response, to establish an effective antiviral state [14, 15]. Notably, type I IFNs (IFN-I) are known for their antiviral, antiproliferative, and immunomodulatory effects in both virus-infected and uninfected cells, by inducing transcriptional gene program that regulates multiple stages of the viral replication cycle [16]. SARS-CoV-2 has developed strategies that interfere with the upstream mediators of viral sensing, as well as strategies that bypass the signaling pathways that lead to IFN production and inhibit the activities of IFN-stimulated genes (ISGs) [17–19].

Despite the mild presentation of COVID-19 in the pediatric population, debilitating symptoms can develop months after the acute phase of SARS-CoV-2 infection [20, 21]. The causes of LC are poorly understood and there are no reports on immunological processes, including IFN response, associated with clinical manifestations of LC in childhood. However, recent evidence has shown that LC and associated neurocognitive symptoms in adults are associated with reduced circulating serotonin levels whose depletion might be mediated by viral RNA-induced IFN-I [22].

Therefore, to understand the role of IFN during LC in children and adolescents, we measured the mRNA expression levels of genes encoding for IFN-I (IFN-α/β/ε/ω), IFN-I receptor subunits (IFNAR1/2), and several ISGs, including genes that have been shown to act as restriction factors for SARS-CoV-2 (BST2, LY6E, ISG15, IFI27, OAS1-3) [23–25]. Gene expression was measured in peripheral blood mononuclear cells (PBMCs) from children and adolescents who developed LC symptoms from 3–6 months after SARS-CoV-2 infection. Protein expression and functional activity of IFN-I were also assessed in plasma samples from the participants.

Results

Cohort characteristics

The baseline demographic and clinical characteristics of the participants, divided into children (6–11 years) and adolescents (12–17 years), are reported in [Table 1]. The mean age of children was 7.8, 9.1, and 9.8 years for healthy controls (HC), matched controls (MC), and LC, respectively. The mean age of the adolescents was 13.8, 14.2, and 14.4 years for HC, MC, and LC, respectively. The MC and LC groups had almost equal numbers of males and females, whereas the HC group had a majority of males (16/18 [88%]; 8/10 for HC children [6–11 years], and 8/8 for HC adolescents [12–17 years]). On average, blood samples were collected 4 months after SARS-CoV-2 infection for participants in the MC and LC groups (i.e. 126 days for MC children, 126 days for LC children, 129 days for MC adolescents, and 134 days for LC adolescents). The most commonly reported comorbid conditions for MC and LC were a history of bronchitis, conjunctivitis, and inhalant allergy. Most MC and LC participants had respiratory, gastrointestinal, neurological, and skeletal muscle symptoms during the early acute phase of SARS-CoV-2 infection. At 1 month and 3–6 months after COVID-19, LC participants had at least one symptom, with the most common being neurological- (children: 1 month [40%], 3–6 months [70%]; adolescents: 1-month [77.7%], 3–6 months [72.22%]) and skeletal muscle (children: 1-month [50%], 3–6 months [50%]; adolescents: 1-month [66.66%], 3–6 months [44.44%]) symptoms. Distinct neurological symptoms, including cephalalgy, ageusia, anosmia, amnesia, sleep-wake disturbances, and difficulty concentrating, have been reported in children and adolescents during LC phase and their frequency is reported in (Supporting information Table S1).

Neutralizing autoantibodies (NAB) to IFN-I, have been found in severe COVID-19 patients and are associated with a defective IFN-I/ISGs response [26–29]. Thus, to avoid confounding factors that could interfere with gene and protein expression analysis, we tested for the presence of anti-IFN NAB in plasma samples from participants. All plasma samples were negative for anti-IFN-α/β/ω NAB (<10 TRU/mL).

Gene expression of IFN-I in children/adolescents after SARS-CoV-2 infection compared with healthy controls and in relation to the development of LC symptoms

Generalized linear mixed models (GLMMs) were used to account for confounding parameters, that is, age and gender of the participants. All models indicated that gender did not influence the differences in mRNA expression levels between the groups studied.

Table 1. Demographic and clinical characteristics of SARS-CoV-2-matched controls and long COVID children and adolescents.

Items	Children (6–11 years)				Adolescents (12–17 years)			
	HC (n = 10)	MC (n = 13)	LC (n = 10)	p-value	HC (n = 8)	MC (n = 15)	LC (n = 18)	p-value
Features								
Age (years) mean (95% CI)	7.8 (7.5–8.2)	9.1 (8.1–10.3)	9.8 (7.8–10.4)	0.300 (a)	13.8 (13–14.5)	14.2 (13.5–14.9)	14.4 (13.9–15)	0.500 (a)
Male, *n* (%)	8 (80)	6 (46.15)	7 (70)	0.278 (b)	8 (100)	9 (60)	6 (33.33)	0.003 (b)
Mean days between SARS-CoV-2 swab and blood sampling (IQR)	NA	126 (110–129)	126 (113–138)	0.500 (c)	NA	129 (112–141)	134 (121–147)	0.500 (c)
History of comorbidities								
Bronchitis, *n* (%)	NA	6/13 (46.15)	3/10 (30)	0.943 (b)	NA	5/15 (33.33)	3/18 (16.66)	0.221 (b)
Conjunctivitis, *n* (%)	NA	4/13 (30.76)	3/10 (30)		NA	6/15 (40)	8/18 (44.44)	
Atopic Dermatitis, *n* (%)	NA	2/13 (15.38)	3/10 (30)		NA	5/15 (33.33)	1/18 (5.55)	
Asthma, *n* (%)	NA	2/13 (15.38)	2/10 (20)		NA	0/15 (0)	2/18 (11.11)	
Inhalant allergies, *n* (%)	NA	6/13 (46.15)	4/10 (40)		NA	6/15 (40)	9/18 (50)	
Acute phase symptoms								
Respiratory, *n* (%)	NA	4/13 (30.76)	6/10 (60)	0.485 (b)	NA	9/15 (60)	8/18 (44.44)	0.543 (b)
Gastrointestinal, *n* (%)	NA	3/13 (23.07)	1/10 (10)		NA	3/15 (20)	8/18 (44.44)	
Neurological, *n* (%)	NA	8/13 (61.53)	4/10 (40)		NA	12/15 (80)	16/18 (88.88)	
Skeletal muscle, *n* (%)	NA	6/13 (46.15)	7/10 (70)		NA	7/15 (46.66)	13/18 (72.22)	
Post-COVID symptoms (1 month from infection)								
Respiratory, *n* (%)	NA	NA	2/10 (20)	0.400 (d)	NA	NA	0/18 (0)	0.468 (d)
Gastrointestinal, *n* (%)	NA	NA	1/10 (10)		NA	NA	1/18 (5.55)	
Neurological, *n* (%)	NA	NA	4/10 (40)		NA	NA	14/18 (77.70)	
Skeletal muscle, *n* (%)	NA	NA	5/10 (50)		NA	NA	12/18 (66.66)	
Long COVID symptoms (3–6 months from infection)								
Respiratory, *n* (%)	NA	NA	0/10 (0)	0.692 (d)	NA	NA	0/18 (0)	0.468 (d)
Gastrointestinal, *n* (%)	NA	NA	0/10 (0)		NA	NA	1/18 (5.55)	
Neurological, *n* (%)	NA	NA	7/10 (70)		NA	NA	13/18 (72.22)	
Skeletal muscle, *n* (%)	NA	NA	5/10 (50)		NA	NA	8/18 (44.44)	

Note: Data are expressed as number (frequency) or mean. Participants and their parents were asked to provide information about symptoms developed during the acute phase of COVID-19 and whether each symptom persisted and/or new symptoms developed at 1 month (post-COVID-19 stage) and at a range of 3–6 months after SARS-CoV-2 infection (long COVID stage). p-values were calculated using: (a) Kruskal–Wallis rank sum test; (b) Fisher's exact test; (c) Mann–Whitney U test; (d) Wilcoxon rank sum exact test.

Abbreviations: HC, healthy controls; LC, long COVID participants; MC, SARS-CoV-2 recovered participants who did not develop LC symptoms; NA, not applicable.

All parameters (age group, IFN-I mRNA levels, and ISGs mRNA levels) did not follow a normal distribution (Shapiro–Wilk test; $p < 0.001$).

Using multivariate logistic regression to compare LC and HC groups, IFN-β, IFN-ε, and IFN-ω expression was found to change with age (the Akaike Information Criteria [AIC] in the models with age as a fixed effect was lower than in models with age as a random effect). In an exploratory analysis, we grouped the participants into children (age group 6–11 years) and adolescents (age group 12–17 years), according to the categories formulated by the US Center for Disease Control's National Center on Birth Defects and Developmental Disabilities to inform parents about certain birth defects, disabilities, mental, emotional, developmental, and blood disorders that may affect children [30]. The median values of IFN-β, IFN-ε, and IFN-ω mRNA in the age group 6–11 years were lower in the LC group compared with the HC group, whereas the values of IFN-β, IFN-ε, and IFN-ω in the age group 12–17 years were higher in the LC group compared with the HC group. IFN-α did not change with age (Table 2; Supporting information Fig. S1 [panel A]). In the sensitivity analysis, we found similar results when the age of the participants was not used as a categorical variable (age group 6–11 years and age group 12–17

Table 2. Logistic regression models of IFN-I gene expression levels.

Variable	LC vs. HC OR (95% CI)	*p*-value	LC vs. MC OR (95% CI)	*p*-value
IFN-α (age group 6–11 years)	0.69 (0.46–1.03)	0.070	0.74 (0.57–0.95)	**0.018**
IFN-β (age group 6–11 years)	0.81 (0.65–0.99)	**0.045**	0.78 (0.63–0.96)	**0.019**
IFN-ε (age group 6–11 years)	0.91 (0.84–0.99)	**0.030**	0.98 (0.91–1.06)	0.670
IFN-ω (age group 6–11 years)	0.75 (0.56–0.98)	**0.040**	0.81 (0.66–0.98)	**0.050**
IFN-α (age group 12–17 years)	1.44 (0.97–2.16)	0.070	1.36 (1.05–1.75)	**0.018**
IFN-β (age group 12–17 years)	1.24 (1.01–1.54)	**0.045**	1.29 (1.04–1.60)	**0.019**
IFN-ε (age group 12–17 years)	1.10 (1.01–1.20)	**0.030**	1.02 (0.94–1.10)	0.670
IFN-ω (age group 12–17 years)	1.34 (1.01–1.79)	**0.040**	1.23 (1.01–1.52)	**0.050**

Note: Data are expressed as odds ratio (OR) (exponentiated betas, 95% confidence interval [CI]) of associations between the main outcome (LC vs. HC and LC vs. MC) and IFN-I mRNA levels in logistic regression models adjusted for age group (categorized as age group 6–11 years [children and age group 12–17 years [adolescents]) and gender of participants. Age was evaluated as a categorical variable (fixed effect) in interaction with IFN-I gene expression levels. Statistically significant values (p-value < 0.05) calculated with generalized linear mixed models (GLMMs) are shown in bold. Abbreviations: HC, healthy controls; LC, long COVID participants; MC, SARS-CoV-2 recovered participants who did not develop LC symptoms.

years) but as a continuous variable (Supporting information Table S2; Supporting information Fig. S2 [panels A–C]).

The logistic regression models showed age-dependent differences in IFN-α, IFN-β, and IFN-ω expression between the LC and MC groups, therefore, age was considered as a fixed effect in the interaction with mRNA levels. The median value of IFN-α, IFN-β, and IFN-ω mRNA in the age group 6–11 years was higher in MC cases compared with LC cases, whereas the opposite was observed in the age group 12–17 years, where IFN-α, IFN-β, and IFN-ω were higher in LC cases compared with MC cases (Table 2, Supporting information Fig. S1 [panel B]). Sensitivity analysis showed similar results when the age of participants was used as a continuous variable (Supporting information Fig. S3 [panels A–C]).

The results of logistic regression models (i.e. age group as a random or fixed effect) indicated that age did not influence differences in mRNA expression levels between MC and HC participants (AIC was lower in models with age as a random effect than in models with age as a fixed effect; data not shown).

Gene expression of ISGs and IFNAR1/2 in children/adolescents after SARS-CoV-2 infection compared with healthy controls and in relation to the development of LC symptoms

In all logistic regression models, the differences in expression of ISGs and IFN-I receptor subunits (IFNAR1/2) between groups were not influenced by the age of the participants, so age was introduced as a random effect (AIC was lower in the models with age as a random effect compared with those with age as a fixed effect). Most of the ISGs (BST2, IFI27, ISG15, MxA, LY6E, OAS2, MDA5) and IFNAR1/2 differentiated the HC group from the MC and LC groups, whereas there were no statistically significant differences between the MC and LC groups. All the ISGs and IFNAR1 were expressed at lower levels in the LC and MC groups compared with the HC group, while IFNAR2 expression was higher in the LC and MC groups compared with the HC group (Table 3; Supporting information Fig. S4 [panels A, B]).

Transcriptional dysregulation of IFN-I in LC participants with neurological symptoms

To investigate the effect of LC symptoms on IFN gene expression, we compared LC participants with neurological symptoms (n = 20; 7/10 LC children, 13/18 LC adolescents) with the MC group ($n = 28$; 13 MC children, 15 MC adolescents) using multivariate logistic regression. In this context, IFN-α and IFN-β expression was found to change with age (AIC in models with age as a fixed effect was lower than in models with age as a random effect). In particular, the median value of IFN-α and IFN-β expression in the 6–11 age group was lower in the LC group with neurological symptoms ($n = 7$) compared with the MC group ($n = 13$), whereas the opposite was true in the 12–17 age-group, where IFN-α and IFN-β expression was higher in the LC group ($n = 13$) compared with the MC group ($n = 15$) (Table 4; Supporting information Fig. S5).

No statistically significant differences in the expression of ISG and IFNAR1/2 were found between the LC and MC groups (data not shown). Moreover, no differences in IFN-I expression were observed between the LC participants without neurological symptoms ($n = 8$) and the MC group ($n = 28$) (Supporting information Table S3). LC participants who developed skeletal muscle symptoms ($n = 13$) showed no differences in IFN gene expression compared with the MC group ($n = 28$) (Supporting information Table S4).

Protein expression and functional activity of IFN in children/adolescents after SARS-CoV-2 infection compared with healthy controls and in relation to the development of LC symptoms

Plasma samples were tested for IFN activity using a bioassay based on the inhibition of the cytopathic effect (CPE) caused by Encephalomyocarditis virus (EMCV) [31]. No IFN activity was detected in any of the plasma samples (≤4 IU/mL of IFN). Furthermore, IFN-α, IFN-β, IFN-ε, and IFN-ω protein levels were determined by different enzyme-linked immunosorbent assay

Table 3. Logistic regression models adjusted for participant age group and gender (random effect) of gene expression levels of ISGs and IFN-I receptor subunits.

Variable	LC vs. HC OR (95% CI)	*p*-value	MC vs. HC OR (95% CI)	*p*-value
ISG15	0.55 (0.38–0.78)	**0.002**	0.55 (0.37–0.80)	**0.001**
ISG56	0.36 (0.10–1.32)	0.127	0.29 (0.08–1.09)	0.060
IFI27	0.78 (0.64–0.94)	**0.008**	0.86 (0.76–0.98)	**0.021**
MxA	0.99 (0.80–1.00)	0.060	0.93 (0.88–0.98)	**0.004**
BST2	0.75 (0.47–1.21)	0.238	0.83 (0.54–1.29)	0.414
LY6E	0.59 (0.41–0.84)	**0.004**	0.67 (0.53–0.84)	**0.001**
OAS1	0.59 (0.27–1.27)	0.181	0.75 (0.39–1.44)	0.388
OAS2	0.98 (0.96–0.99)	**0.004**	0.98 (0.96–0.99)	**0.002**
OAS3	0.47 (0.22–1.00)	0.054	0.41 (0.18–0.95)	**0.034**
MDA5	0.83 (0.72–0.96)	**0.013**	0.77 (0.62–0.95)	**0.014**
IFNAR1	0.83 (0.73–0.95)	**0.008**	0.82 (0.71–0.95)	**0.015**
IFNAR2	1.05 (1.02–1.08)	**0.001**	1.05 (1.02–1.09)	**0.001**

Note: Data are expressed as odds ratio (OR) (exponentiated betas, 95% confidence interval [CI]) of associations between the main outcome (LC vs. HC, and MC vs. HC groups) and ISGs and IFN-I receptor subunits (IFNAR1/2) mRNA levels in logistic regression models adjusted for age group and gender of participants. Age was evaluated as a random effect in interaction with gene expression levels of ISGs and IFNAR1/2. Statistically significant values (p-value < 0.05) calculated with generalized linear mixed models (GLMMs) are shown in bold.
Abbreviations: HC, healthy controls; LC, long COVID participants; MC, SARS-CoV-2 recovered participants who did not develop LC symptoms.

(ELISA). IFN-I protein levels were below the detection limit of the ELISA tests (IFN-α [3.2 pg/mL], IFN-β [50 pg/mL], IFN-ω [1.5 pg/mL], and IFN-ε [3.9 pg/mL]).

Discussion

The LC syndrome and associated immunopathological changes have not yet been studied in detail [32]. To date, researchers have mainly focused on studying the acute or early phase of SARS-CoV-2 infection. In this regard, several transcriptomic analyses have shown that a preactivated innate immunity leads to a more effective anti-SARS-CoV-2 response upon infection in children [33, 34]. By contrast, unbalanced IFN-I signatures and the protective or detrimental role of IFN-I in both local and systemic responses have been described in adults with COVID-19, with significant implications for antiviral defense against SARS-CoV-2 and disease severity [35, 36]. However, many children and adolescents continue to experience debilitating symptoms after recovery [21]. Hence, the present study aims to characterize the interplay between clinical manifestations associated with LC and factors involved in the innate immune response such as IFN-I.

Table 4. Logistic regression models of IFN-I gene expression levels in relation to neurological LC symptoms.

Variable	LC vs. MC OR (95% CI)	*p*-value
IFN-α (age group 6–11 years)	0.73 (0.55–0.97)	**0.029**
IFN-β (age group 6–11 years)	0.75 (0.58–0.96)	**0.022**
IFN-ε (age group 6–11 years)	0.90 (0.79–1.04)	0.160
IFN-ω (age group 6–11 years)	0.83 (0.64–1.08)	0.163
IFN-α (age group 12–17 years)	1.38 (1.03–1.83)	**0.029**
IFN-β (age group 12–17 years)	1.34 (1.04–1.72)	**0.022**
IFN-ε (age group 12–17 years)	1.11 (0.96–1.27)	0.160
IFN-ω (age group 12–17 years)	1.20 (0.93–1.55)	0.163

Note: Data are expressed as odds ratio (OR) (exponentiated betas, 95% confidence interval [CI]) of associations between the main outcome (LC who have developed neurological symptoms vs. MC) and IFN-I mRNA levels in logistic regression models adjusted for age group (categorized as age group 6–11 years [children] and age group 12–17 years [adolescents]) and gender of participants. Age was evaluated as a categorical variable (fixed effect) in interaction with IFN-I gene expression levels. Statistically significant values (p-value < 0.05) calculated with generalized linear mixed models (GLMMs) are shown in bold.
Abbreviations: LC, long COVID participants; MC, SARS-CoV-2 recovered participants who did not develop LC symptoms.

Although no detectable protein levels or functional activity of IFN-I were found in plasma samples from any of the participants, an alteration in IFN-I mRNA levels was observed. To investigate age-related differences in IFN gene expression during LC syndrome, we distinguished between children (age group 6–11 years) and adolescents (age group 12–17 years). In comparisons of cases with LC, defined as persistent symptoms more than 3 months after acute illness, and cases whose symptoms resolved within 1 month after infection (MC) with healthy uninfected donors (HC), we found IFN-I mRNA levels differed between the groups studied depending on age (i.e. LC vs. MC, LC vs. HC). Notably, IFN-β, IFN-ε, and IFN-ω mRNA levels were higher in LC cases compared with HC in adolescents, whereas the opposite (lower levels of IFN-β, IFN-ε, and IFN-ω mRNA in LC cases compared with HC) was observed in children aged 11 years or younger. Possible explanations for the lower IFN-I mRNA levels in LC children could be a more rapid decline of IFN-I during LC, as observed during the acute phase of the SARS-CoV-2 infection when children mount a more transient innate antiviral response than adults [37], or a functional impairment of plasmacytoid dendritic cells during infection, which are a major source of IFN-I expression [38]. Furthermore, differences in IFN-I expression could be influenced by immune cell proportions and epigenetic signatures, which differ between children, adolescents, and adults [39–41]. Our findings in LC adolescents may be

consistent with what has been described in LC adults, where one study showed elevated serum IFN-β protein concentrations 4 months after SARS-CoV-2 infection compared with unexposed healthy donors [42]. Of note, we observed activation of IFN-ε and IFN-ω genes, as well as IFN-β, which may be a distinguishing feature of LC adolescents compared with LC adults. These IFN subtypes may play a critical role during LC, as suggested by our previous study performed in respiratory mucosal cells of SARS-CoV-2 positive children (up to 16 years) and young adults (17–40 years) [33].

Age-related differences in IFN-I gene expression were also found between LC and MC cases. We observed lower levels of IFN-α, IFN-β, and IFN-ω in the LC group compared with the MC group in children. This leads us to hypothesize that SARS-CoV-2 infection and the subsequent development of LC symptoms could have influenced the IFN mRNA signature. Contrastingly, increased levels of IFN-α, IFN-β, and IFN-ω mRNA were found in LC cases compared with MC cases among adolescents. LC syndrome is mainly characterized by neuropsychiatric disorders, including sleep abnormalities, chronic headaches, "brain fog", and memory and mood disturbances [43]. Sustained IFN-I production has been implicated in neuroinflammation in adulthood [44, 45] and may explain the long-term effects of COVID-19 in adolescents. Consistently, our investigations showed that the levels of IFN-α and IFN-β mRNA increased in LC adolescents with neurological symptoms compared with MC adolescents. However, LC children with neurological symptoms had decreased levels of IFN-α and IFN-β compared with the children in the MC group. The marked differences in IFN-I expression in children and adolescents may be due to developmental changes in innate and adaptive immunity [46–48] and merit further investigation. Notably, despite significant differences in the IFN transcript levels between the LC, MC, and HC groups, no detectable protein levels and functional activity of IFN-I were observed in plasma samples from all children/adolescents. Otherwise, the measurement of IFN proteins has proven to be particularly challenging. Even in clinically established IFN-driven diseases, IFN protein levels are often below the detection limit of conventional ELISA assays [49]. However, they can be quantified using more sensitive approaches such as single-molecule array (Simoa) digital ELISA technology [49–51]. This highlights the importance of using highly sensitive immunoassays when directly analyzing IFN protein from clinical samples.

Our findings also revealed that differences between study groups (LC vs. HC; MC vs. HC) in the expression of several ISGs (ISG15, IFI27, MxA, LY6E, OAS2, OAS3, MDA5), many of which have been identified as SARS-CoV-2 restriction factors [23–25], and IFN-I receptor subunits (IFNAR1/2), were not influenced by age. We observed that ISGs and IFNAR1 mRNA levels decreased in the LC and MC groups compared with HC, while the opposite was found for the IFNAR2 gene. The production of ISGs after SARS-CoV-2 infection appears to be more complex than other respiratory viruses and could be regulated by different pathways, including the IFN response factor (IRF) family [52]. IFN-I classically activates JAK kinase (Tyk-2 and JAK1) and STAT proteins (STAT-1 and 2) through interaction with their specific membrane receptor [53]. However, abnormalities in the IFN-signaling cascade have been reported in SARS-CoV-2 infection, which may lead to the failure of ISG activation [23, 53-55]. Nsp5 has been shown to inhibit ISG induction by cleaving histone deacetylases [54], Nsp6 blocks IRF3 activation and inhibits STAT-1 and 2 tyrosine phosphorylation, while Nsp12 inhibits IRF3 nuclear translocation. In addition, Nsp13 blocks STAT-1 and 2 tyrosine phosphorylation [53]. Moreover, SARS-CoV-2 antagonizes the ISG15-dependent activation of MDA5 through direct de-ISGylation mediated by the protease Nsp3, while BST2 is antagonized by the SARS-CoV-2 accessory protein Orf7a [23]. An additional explanation could be provided by the prolonged IFN-desensitized state, established by regulatory proteins, including inhibitors of activated STAT proteins, IFN-stimulated suppressors of cytokine signaling, ubiquitin-specific peptidase 18, induced by SARS-CoV-2 infection [53]. These proteins collectively modulate the JAK-STAT pathway, which might limit the expression of ISGs even several months after SARS-CoV-2 infection [55]. A recent study also confirmed that changes in the blood transcriptome due to SARS-CoV-2 infection and immune evasion persist in adults with LC. Consistent with our findings, that study showed decreased expression of several IFN-I-inducible genes, including MxA and OAS3 [56].

Regarding IFN signaling, IFN-I amplifies and propagates the antiviral response by interacting with their receptors, IFNAR1 and IFNAR2. Notably, IFNAR1 is correlated to desensitization of IFN-α in mature DCs and its expression is lower than that of IFNAR2 [57]. IFNAR2 can repress transcription of the IFN-stimulated response element reporter, thus modulating gene transcription of IFN pathways [58]. In line with our findings, in vitro analysis of the JAK-STAT pathway showed that SARS-CoV-2 interferes with the proximal signaling elements, including IFNAR1, leading to the inhibition of IFN-induced STAT phosphorylation [59]. Moreover, the IFNAR2 gene has been associated with the severity of COVID-19 [60]. Our results show, for the first time in children and adolescents, that IFNAR2 expression is still elevated, even 3–6 months after SARS-CoV-2 infection, regardless of LC symptoms.

Data limitations and perspectives

Several limitations should be discussed. First, we did not characterize longitudinal IFN-I gene expression in LC and MC participants, including sampling during the acute phase of SARS-CoV-2 infection. We were unable to perform bulk RNA sequencing on a selected set of PBMCs to obtain a comprehensive landscape of ISGs (known to be >500 genes) due to insufficient biological samples. The implementation of a more sensitive technology (i.e. single-molecule array [Simoa] digital ELISA) could improve the quantification of IFN-I protein levels, and data on IFN-I mRNA and protein levels in infants, preschool children, and adults could help us to further confirm the age-dependent role of IFN-I pathways in LC syndrome and support our observations. Finally, our hypothesis that impaired IFN-I expression in children months after SARS-CoV-2 infection may be caused by viral escape strategies (e.g. epigenetic regulation) and may be involved in LC manifestations,

requires in-depth investigation. On the other hand, a strength of our study was the ability to perform a comprehensive analysis of the IFN-I pathway in an age-homogeneous group of children (age group 6–11 years) and adolescents (age group 12–17 years), who had not yet been vaccinated against SARS-CoV-2 and who had or had not experienced LC symptoms after SARS-CoV-2 infection.

Conclusion

Our findings lead to the hypothesis that the IFN-I pathway is involved in immunopathological changes in children and adolescents 3–6 months after recovery from symptomatic SARS-CoV-2 infection. We also found age-related differences in IFN-I gene expression, with upregulation of IFN-I genes in adolescents and downregulation of expression in children. Adolescents with LC have an immune landscape similar to that reported in adults, where IFN-I transcript activation appears to play a role in the development of LC manifestations, particularly neurological symptoms. On the other hand, the IFN-I mRNA signature in children with LC differs from that of adolescents and adults and is characterized by a downregulation of IFN genes. These findings suggest that LC in children is the result of as-yet-unknown immunological mechanisms. In conclusion, our study provides new insights into the immunopathogenesis of pediatric LC and may help to provide the basis for the development of immunotherapeutic strategies to reduce the impact of COVID-19 on children's health.

Materials and Methods

Participants and study design

From February to December 2021, children and adolescents with SARS-CoV-2 infection confirmed by molecular methods underwent routine follow-up visits at the Pediatric department of Sapienza University Hospital "Policlinico Umberto I" in Rome, Italy. Participants and their parents were asked to provide information about symptoms during the acute phase of COVID-19 and whether symptoms persisted and/or new symptoms developed at 1 month (post-COVID-19 stage) and at 3–6 months after infection (LC stage). Data on symptoms potentially associated with COVID-19 and LC, including respiratory, neurological, gastrointestinal, and skeletal muscle symptoms, were obtained from 226 children and adolescents using a standardized questionnaire.

At the 3–6 month visit after COVID-19 diagnosis, 34/226 (15%) participants with one or more respiratory, gastrointestinal, neurological, or skeletal muscle symptoms were defined as LC cases. Blood samples were collected from 28 LC cases who were age-matched to 28 participants who had confirmed COVID-19 but did not develop LC symptoms 3–6 months after SARS-COV-2 infection: MC. In addition, blood samples were collected at enrolment from 18 children and adolescents with a negative molecular test for SARS-CoV-2-RNA and no previous history of SARS-CoV-2 infection were obtained as HC. None of the participants had been previously vaccinated against SARS-CoV-2. All SARS-CoV-2 infected children and adolescents were symptomatic during the acute phase of the infection, but none were hospitalized. There were very few infants or preschool children, probably because of the low incidence of SARS-CoV-2 infection in this age group, and they were not included in this study due to the lack of age-matched controls. Ethical approval for this study was obtained from the Sapienza University of Rome, Italy (Ethics Committee - RIF. CE 0399/2021, Sapienza University of Rome, University Hospital "Policlinico Umberto I"). Informed consent was obtained from the parents or guardians of all participants.

TaqMan-based real-time RT-PCR assays for mRNA expression

Expression levels of genes for IFN-I (protein/gene name: IFN-α/IFN-α2, IFN-β/IFN-β1, IFN-ε/IFN-ε1, and IFN-ω/IFN-ω1), the receptor subunits IFNAR1 and IFNAR2, and selected ISGs (ISG15, ISG56, MxA, IFI27, BST2, LY6E, OAS1, OAS2, OAS3, and MDA5/IFIH1) were measured by quantitative RT (real-time)-PCR assays, using the LightCycler-480II sequence detector (Roche) [33, 61, 62]. Primer and probe sequences are listed in (Supporting information Table S5). Briefly, PBMCs were isolated from fresh blood by Ficoll–Hypaque density gradient centrifugation (Sigma–Aldrich), and the dry pellets were stored at −80°C. Total RNA was extracted from PBMCs using the TRIzol reagent (Invitrogen) and reverse transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems), according to the manufacturer's protocol. Primers and probes for each gene were added to the Probes Master Mix (Roche) at 500 and 250 nM, respectively, in a final volume of 20 μL. The housekeeping gene β-glucuronidase was used as an internal control. Gene expression levels were calculated using the comparative cycle threshold value (Ct) method ($2^{-\Delta Ct}$). All RT-PCR reactions were performed in duplicate.

Detection of anti-IFN-I neutralizing autoantibodies

Plasma samples collected from participants were tested for neutralizing autoantibodies to IFN-α2 subtype (IFN-α2a, Intron; Schering-Plough), IFN-β (Rebif, Serono), and IFN-ω (PBL Interferon Source) in a bioassay based on IFN-mediated inhibition of EMCV-induced CPE on A549 cells as previously described [26, 27]. Titers were calculated using Kawade's method, and titers were expressed as tenfold reduction units (TRU)/mL, where one TRU is the plasma dilution capable of reducing IFN titer from 10 to 1 IU/mL [63].

Detection of IFN activity

Plasma samples collected from LC, MC, and HC participants were evaluated for IFN activity using a bioassay based on the inhibition

of EMCV-induced CPE on A549 cells [31]. Briefly, threefold serial dilutions of plasma were added to duplicate monolayers of A549 cells in 96-well microtiter plates. After 24 h, the cells were challenged with EMCV (MOI = 0.01 $TCID_{50}$/cell) and incubated at 37°C for 24 h. Controls included a titration of the IFN-α2 subtype (IFN-α2a, Intron; Schering-Plough). The detection limit for IFN activity was 4 IU/mL.

Detection of IFN-I proteins

All plasma samples were analyzed for human IFN-α, IFN-β, IFN-ε, and IFN-ω protein levels using different ELISA assays (human IFN alpha [cat #BMS216], human IFN beta [cat #414101], and human IFN omega [cat #BMS233] ELISA kits - Invitrogen, Thermo Fisher Scientific Inc.; human IFN epsilon [CSB-EL011049HU] ELISA kit - Cusabio) according to the manufacturer's protocol. Detection limit: IFN-α (3.2 pg/mL), IFN-β (50 pg/mL), IFN-ω (1.5 pg/mL), and IFN-ε (3.9 pg/mL).

Statistical analysis

To account for multiple and potentially confounding variables the analysis was performed using multivariate logistic regression models (i.e. GLMMs) adjusted for age and gender. The models were run separately for each gene, and separately for each comparison: LC vs. HC, LC vs. MC, and MC vs. HC. Odds ratios (OR) between groups within each comparison were estimated by logistic regression. Hence, in GLMMs we included the groups as Bernoulli dependent variable, mRNA expression levels as an independent variable, and gender (due to gender imbalance between groups; [Table 1]) and age (categorized into two groups: age group 6–11 years [children] and age group 12–17 years [adolescents]) as adjusted parameters. Due to the gender imbalance in the HC group, gender was included as an a priori random effect, whereas age group was included as (1) a random effect or (2) a fixed effect. We decided whether to include age group as a random or fixed effect by comparing the models by the AIC. When age group was considered as a fixed effect (age group 6–11 years and age group 12–17 years) in the regression model an interaction term was considered (IFN genes/age group) because of possible changes in IFN mRNA expression between age groups, especially before and during the adolescence age. To test the statistical strength of our results a sensitivity analysis was performed using age as a continuous variable without assigning a "age group" label (categorical variable) to each participant for the models in which a significant interaction term (between age group and IFN-I mRNA levels) was found. The continuous covariates were standardized to improve the numerical stability of the GLMMs. All parameters were tested using the Shapiro–Wilk test to assess normal distribution. All the statistical analyses were performed with R version 4.1.3 (R Studio Team (2020); RStudio: Integrated Development for R. RStudio, PBC) open-source software using the following packages: tidyverse, FSA, ggplot2, and lme4.

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Abbreviations: **AIC**: Akaike Information Criteria · **CPE**: cytopathic effect · **BST2**: bone marrow stromal cell antigen 2 · **EMCV**: Encephalomyocarditis virus · **GLMMs**: generalized linear mixed models · **HC**: healthy controls · **IFI27**: interferon alpha inducible protein 27 · **IFNAR1/2**: interferon alpha and beta receptor subunit 1/2 · **IFN-I**: type I interferons · **IRF**: IFN response factor · **ISG15**: interferon-stimulated gene 15 · **ISG56**: interferon-stimulated gene 56 · **ISGs**: interferon-stimulated genes · **LC**: long COVID · **LY6E**: lymphocyte antigen 6 family member E · **MC**: matched controls · **MDA5**: melanoma differentiation-associated protein 5 · **MxA**: myxovirus resistance gene A · **NAB**: neutralizing autoantibodies · **OAS1/2/3**: 2'-5'-oligoadenylate synthetase 1/2/3 · **PBMCs**: peripheral blood mononuclear cells

Full correspondence: Prof. Carolina Scagnolari, Department of Molecular Medicine, Laboratory of Virology, Sapienza University of Rome, Viale di Porta Tiburtina n° 28, 00185 Rome, Italy.
Email: carolina.scagnolari@uniroma1.it

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