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Translational research in severe COVID-19 and long-term symptoms post-COVID-19

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Translational research in severe COVID-19 and long-term symptoms post-COVID-19

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Editorial: Translational research in severe COVID-19 and long-term symptoms post-COVID-19

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COVID-19, long COVID-19, post-COVID-19, post-COVID-19 syndrome, SARS-CoV-2

Editorial on the Research Topic
Translational research in severe COVID-19 and long-term symptoms post-COVID-19

In addition to acute and severe symptoms of SARS-CoV-2 infection, many patients worldwide suffer persistent symptoms from post-COVID-19 syndrome (PCS).

This Research Topic aims to publish original translational research and review articles contributing to developing additional knowledge of PCS, considering the acute infection that contributes to developing post-COVID symptoms and mortality and thus trying to identify target markers for managing the affected subjects.

In this Editorial, we have summarized 13 manuscripts: 5 related to acute infection, seven concerning the role of molecular and clinical factors of PCS, and 1 *in vitro* research study about preventive therapy.

Acute SARS-CoV-2 infection could severely compromise the subject's health. There is a decreasing trend of deaths, hospitalizations, and intensive care unit (ICU) admissions, principally due to vaccination, acquired post-infection immunity, and less aggressive virus variants.

Concerning mortality, Martin-Conty et al. in a prospective, multicenter, ambulance-based ongoing study in Spain, observed long-term mortality as the primary outcome in acute patients treated by emergency medical services, and COVID-19 was observed as an independent risk factor for long-term mortality. They identified that patients who previously experienced an acute COVID-19 episode presented a mortality rate almost twice that of non-COVID-19 subjects, suggesting, with a final model adjustment, that COVID-19 was a risk factor for long-term mortality.

Due to the elevated mortality in hospitalized COVID-19 patients, there is an increased interest in finding serum biomarkers predicting mortality to adopt beneficial measures and easy protocols during the post-COVID follow-up. From Mexico, Cortes-Tellez et al. analyzed serum levels of different parameters from a routine laboratory in a cohort of severe COVID-19 hospitalized patients. They observed in a multivariate analysis that leukocytes

and neutrophils were the best biomarkers for predicting mortality risk independently of age, gender, or comorbidities. The authors concluded the importance of using them routinely.

Likewise, Pavan Kumar et al. in a comparative research study from India, elucidated the role of matrix metalloproteinases (MMPs) in the pathogenesis of pediatric COVID-19, examining the MMPs plasma levels in children with Multisystem Inflammatory Syndrome (MIS-C) and acute COVID-19 and comparing them to convalescent COVID-19 and children with other common tropical diseases. Higher levels of MMPs were observed in children needing ICU admission. Lastly, MMP levels showed a significant correlation with laboratory parameters, comprising CRP, ferritin, lymphocytes, D-dimer, and sodium levels, and the authors proposed that MMPs play a crucial role in the MIS-C and COVID-19 pathogenesis in children and may help distinguish MIS-C from other conditions with an overlapping clinical phenotype.

On the other hand, SARS-CoV-2 reinfection is also a topic because it impacts sequels and illness severity. Suljic et al. from Slovenia, showed in an observational case-control study that reinfections with the Delta variant generate fewer hospitalizations than first infection, suggesting the development of more robust immunity protection developed by infected individuals and also vaccinated individuals (hybrid immunity). This study provides additional insight into reinfection, which may allow appropriate public health measures to be taken.

Concerning the imaging study of COVID-19 pneumonia evolution during hospitalization, lung ultrasound (LUS) has been extensively used during the COVID-19 pandemic. Blair et al. from the United States, prospectively studied 244 moderate (non-ICU) and severe (ICU) COVID-19 hospitalized adults in a longitudinal cohort to evaluate the association between LUS characteristics and clinical severity. The authors described that at baseline, B-lines (edema, fibrosis, inflammation) were more prevalent in severe patients than in moderate ones. However, no significant differences were found between severe and moderate illness over time. Thus, the authors do not support the use of serial LUS to monitor the progression of disease severity.

Pulmonary fibrosis due to SARS-CoV-2 infection is a significant concern (1). A study performed on postmortem patients in Spain (Pérez-Mies et al.) documented the evolution of diffuse alveolar damage (DAD) to the fibrosing pattern and defined the transcriptional programs involved. The authors analyzed lung autopsy samples from five lobes of 33 patients with a severe and prolonged SARS-CoV-2 course. They found that progression to fibrosis in severe COVID-19 was associated with overexpression of fibrogenic pathways (PI3K-AKT) and significant expression of SPARC and CTHRC1 in exudative-fibrosing DAD compared with the control. Whereas downregulation of the Hippo pathway was observed (suggesting epithelial cell damage response), the authors did not observe any role in the epithelial–mesenchymal transition in the fibrosis process. They suggested a possible role of viral persistence in maintaining lung damage.

Concerning PCS, we know that at least 65 million individuals around the world are suffering from this multisystemic condition comprising persistent and severe symptoms lasting at least 2 months, usually after 3 months of acute SARS-CoV-2 infection that is not explained by another diagnosis (2). Different terms such as long COVID, persistent post-COVID, and post-acute COVID-19 syndrome define the same condition. Several hypotheses have been proposed to explain this syndrome. Predicting which patients will develop PCS is a challenge. In this sense, Lai et al. from Massachusetts, addressed an interesting systematic review to determine potential prognostic serum biomarkers for long COVID. They concluded that the persistence of up-regulation of IL-6, CRP, and TNF-α might present potential diagnostic biomarkers of PCS. In patients with neurological symptoms, neurofilament light chain (NFL) and glial fibrillary acidic protein (GFAP) in serum may serve as diagnostic biomarkers, and the authors proposed to evaluate IL-4, IFN-α, CCL2, ferritin, and hemoglobin too. They also suggested evaluating CXCL10, TGF-β, IFN-β, and IL-1α in patients with pulmonary symptoms.

Another interesting topic in the follow-up is the sequelae in computed tomography (CT) and their association with risk factors. Rincon-Alvarez et al. reported in their Colombian cohort that older age, male sex, and ICU admission were related to typical patterns of admission CT and that a third of patients with moderate and severe COVID had abnormal lung computed tomography at 6-month follow-up.

Concerning health-related quality of life (HRQoL) in patients with PCS, Ahmad et al. employing a multicenter cohort study in a Swedish population, explored the frequency of self-reported continued symptoms and diminished HRQoL in relation to functional exercise capacity 6 months after infection, and they also explored risk factors for COVID-19 sequelae. Hospitalization was a significant risk factor for developing persistent symptoms, reduced overall health, and post-acute COVID syndrome (PACS). They concluded that persistent symptoms and reduced HRQoL are frequent in COVID-19 survivors and that patients requiring hospitalization due to severe infection were more likely to develop PACS.

Furthermore, Al-Husinat et al. looking for the prevalence of PCS after mild-to-moderate COVID-19 in the Jordanian population, applied the Newcastle PCS Follow-up Screening Questionnaire and found that mood disturbance followed by fatigue, anxiety, and myalgia were the most frequent PCS symptoms. Female sex substantially raised the risk for multiple PCS symptoms. They concluded that PCS is highly prevalent among COVID-19 survivors, especially in female patients and patients with comorbidities, and also recommended physical and mental rehabilitation.

In contrast, Román-Montes et al. analyzed the prevalence, symptoms, and HRQoL of PCS in a retrospective cross-sectional study of 246 Mexican patients who required hospitalization because of severe infection. They determined a prevalence of 76% of PCS in patients with a median age of 55 years. It was associated with smoking, severe COVID-19, lower arterial blood oxygen saturation on admission, extensive lung involvement, and elevated fibrinogen levels. Moreover, the most frequent symptoms of PCS were difficulty concentrating (81%), dyspnea (75%), and arthralgia (71%). They suggested identifying diagnostic and therapeutic interventions to restore health and QoL in those patients.

However, no successful treatment is currently offered for managing PCS symptoms, while only rehabilitation programs are promoted, and regular drugs are prescribed for supportive

therapies (3). Concerning rehabilitation programs in PCS, Allendes et al. from Chile performed a systematic review on cardiovascular and autonomic dysfunction in PCS. They concluded that alterations in the autonomic nervous system partially mediate cardiovascular sequelae of COVID-19 infection. They hypothesized that applying new cardiovascular rehabilitation programs should allow healthcare personnel to manage the consequences of long-term COVID-19.

Dissook et al. reported on a study testing the activity of phytochemical polyphenol compounds (rosmarinic acid and luteolin) from Perilla frutescens in an *in vitro* lung cell model of SARS-CoV-2-induced inflammation. They documented that these compounds inhibited SARS-CoV-2 spike S1-induced inflammatory responses in A549 cells in a dose-dependent manner, seemingly through the JAK1/STAT3-NLRP3 inflammasome axis, at both the gene transcription and protein levels. They concluded that luteolin and P. frutescens may be potential candidates in the preventive therapeutic strategy for inflammation-related post-acute sequelae of COVID-19.

The present Research Topic contributes novel information toward a better understanding of the possible biomarkers and risk factors contributing to post-COVID symptoms, mortality, radiologic and histologic evolution, and potential preventive therapeutic plants and rehabilitation programs to improve the QoL of PCS patients.

Author contributions

VV-Z: Investigation, Methodology, Writing—original draft, Writing—review and editing. GP-R: Conceptualization, Investigation, Methodology, Validation, Writing—original draft, Writing—review and editing. LC-G: Investigation, Methodology, Writing—original draft, Writing—review and editing. IB-R: Conceptualization, Investigation, Methodology, Writing—original draft, Writing—review and editing. RF-V: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing—original draft, Writing—review and editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Post-COVID-19 syndrome symptoms after mild and moderate SARS-CoV-2 infection

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Background: Post-COVID-19 Syndrome (PCS) is characterized by residual symptoms following the initial recovery from severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection. The prevalence of PCS is known to be the highest among severe and critical forms of the disease. However, the occurrence and risk factors for PCS after mild or moderate SARS-CoV-2 infection has not been extensively investigated.

Methods: Online and offline *via* both paper or mailed questionnaires distributed among Jordan collected between 1st and 21st August 2021, including a total number of 800 respondents, of whom 495 had previous mild to moderate COVID-19 infection. The Newcastle post-COVID syndrome Follow-up Screening Questionnaire was modified, translated, and used as a standard instrument for data collection regarding psychological, medical, and socio-economic symptoms post-infection. The primary outcome was the prevalence of PCS after mild to moderate COVID-19 in Jordan. Secondary outcome was the identification of PCS risk factors.

Results: The most common PCS symptom was mood disturbance followed by fatigue, anxiety, and myalgia. Female gender significantly increased the risk for multiple PCS symptoms. Age < 30 years was found to be an independent risk factor for myalgia ($p = 0.001$).

Conclusion: PCS is highly prevalent among COVID-19 survivors in Jordan, especially in females and patients with comorbidities. Planning physical and mental rehabilitation services is recommended for those patients with PCS symptoms after mild to moderate COVID-19 infection.

KEYWORDS

post-COVID-19 syndrome, SARS-CoV-2 infection, chronic COVID-19 syndrome, mood disturbance, post-acute sequelae of SARS-CoV-2 infection

Introduction

Infection from severe acute respiratory syndrome coronavirus (COVID-19) may present different clinical presentations and degrees of severity. Clinical presentation may vary from an asymptomatic disease to an infection of the upper respiratory tract or a severe disease with potential for multiple organ involvement, high morbidity, and mortality (1). COVID-19 survivors may experience long-lasting psychological, medical, and socio-economic sequelae. Several definitions of post-COVID-19 sequelae have been proposed, including post-COVID-19 syndrome (PCS), long COVID-19, chronic COVID-19, and long haulers (2). Nevertheless, the exact definition, mechanism, and clinical impact of these symptoms are still unclear. Patients with the most severe form of the disease and requiring hospital and/or Intensive Care Unit (ICU) admission are at higher risk for developing PCS and long-term symptoms. Individuals hospitalized because of COVID-19 present high levels of disability, dyspnea, dysphagia, and dependence for both activities of daily living (ADL) and instrumental activities of daily living (IADL) (3). New illness-related fatigue was the most common reported symptom, followed by breathlessness, smelling and taste dysfunction and psychological distress (4–6). The occurrence of PCS and related symptoms after the mild to moderate COVID-19 infection remains to be elucidated. We therefore performed an online survey in Jordan with the aim to investigate the prevalence of PCS in non-hospitalized subjects with mild to moderate COVID-19 infection not requiring respiratory support. We also assessed the impact of age and gender on PCS as well as potential individual risk factors.

Materials and methods

This study was performed between 1st and 21st August 2021, in Jordan. The study protocol was approved by the International Review Board (IRB) of Yarmouk University number IRB/2022/9. Consent for participating was given by responding to the questionnaire. In the cover letter of the online-based survey, the participants were informed about the purpose of the study, ensured confidentiality, and the voluntary nature of the study; the possibility of withdrawing from the study at any time was emphasized. The questionnaire aimed to determine the pattern of PCS symptoms among mild and moderate COVID-19 survivors in Jordan and to identify subjects who may benefit from a medical and psychological multi-disciplinary assessment.

Study participants and selection criteria

A cross-sectional self-administered-online and offline-based questionnaire study involved 800 participants from all governorates of Jordan and different educational as well as governmental institutions. Inclusion criteria were previous SARS-CoV-2 infection confirmed with a positive polymerase chain reaction PCR result and currently being at least 10–12 weeks from the onset of acute illness. Patients who have been admitted to a hospital or required respiratory support of any kind were excluded from the analysis, therefore, we only analyzed outpatient survivors with history of mild to moderate illness.

Assessment procedure and material

A standard questionnaire composed of 23 questions was modified and translated into Arabic and converted into a web-based survey using Google Forms Application. A modified version of the Newcastle post-COVID syndrome Follow-up Screening Questionnaire was used as a standard instrument, it was applied as one of the long-COVID SNOMED-CT codes which were developed and released in the UK in November 2020 to support clinical care and implementation of NICE guidance (7), to be carried out 10–12 weeks after the acute illness (**Supplementary material**).

The questionnaire distribution was divided into three different parts. The first part related to demographic information, the second part was about the clinical data and the third focused on other symptoms clinically relevant for the patient.

General and neurological symptoms included myalgia, fatigue, change/loss of smell and taste, weakness, and weight loss in 3 months. Psychological symptoms included sleep disturbances, nightmares, mood problems (feeling depressed/loss of interest), and anxiety. Respiratory symptoms included shortness of breath and cough and cardiovascular symptoms included palpitations.

The questionnaire link was posted on different social media sites (Facebook®, Instagram® and WhatsApp®) to reach different clusters among the population all around Jordan and from different age groups. Moreover, it was sent *via* email to all students enrolled in Yarmouk University. A paper copy of the questionnaire was also distributed to patients in vaccination centers. To minimize errors in data collection, the respondents with any exclusion criteria characteristics could not proceed to the questions. Finally, after a total number of 23 questions, the respondents were able to submit the answers, and those answers were sent to the drive.

Data analysis and statistical methods

Descriptive and inferential statistics were used in the statistical analysis. The range, mean, and standard deviation (SD) for the continuous variables, frequencies, cross tabulation,

and odds ratio (OR) for categorical variables were calculated. Also, clustered bar charts were used for data visual examination. The chi-squared test of independence was used to investigate the relationship between the two categorical variables. Multiple binary logistic regression was conducted to assess the dependency of different symptoms on gender and age. All presented p-values were two-tailed, and p-values < 0.05 were considered as statistically significant. All statistical analyses were performed using statistical package SPSS 21.0 (SPSS Inc., Chicago, IL).

Cross tabulations of different symptoms vs. gender and age group (<30 and ≥ 30 years) are shown in Table 1. The symptoms considered in this study are mood disturbance/depression, fatigue, anxiety, changes in smelling sensation, myalgia, sleep disturbance, palpitation, residual symptoms, weakness, nightmares/flashbacks, shortness of breath, weight loss (>3 Kg), loss of smelling sensation, cough, and loss of taste sensation.

Multiple binary logistic regression has been used to analyze the relationship between predictors and a dichotomous categorical outcome variable. In this paper, multiple binary logistic regression is used to analyze the relationship between each symptom outcome (1: present, 0 absent) and the gender and age variables. The interpretation of Odds Ratio (OR) for gender is that holding the age constant, the odds of symptoms occurring (increased or decreased) by [some percent] for females compared to males.

Results

Demographic characteristics

A total of 495 subjects (from 800 overall responders) met the inclusion criteria (Figure 1). The mean (SD) age of the responders was 30.5 ± 10.9 years with a range of 14–70 years. Most of the respondents were females ($n = 329$, 66.4%) (Table 1).

Prevalence and type of post-COVID-19 syndrome symptoms

In the overall population 83% of patients had at least one PCS symptom and 33.9% had at least one residual symptom persisting after mild or moderate SARS-CoV-2 infection. The prevalence of PCS symptoms in the overall population is reported in Table 1.

In the overall population, 73.3% of patients had at least one psychological consequence of PCS. The most common symptom of PCS was mood disturbance/feeling depressed (59.4%) and fatigue was the second most common symptom (56.4%). Myalgia and weakness were detected in 42.2 and 32.5% of patients, respectively. The weight loss rate occurred in 27.3% of patients. Smelling and taste disorders were relatively rare after

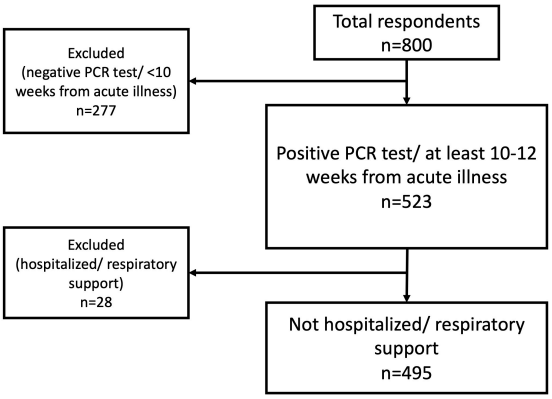

FIGURE 1
Flow chart of inclusion/exclusion process.

COVID-19 (14.7 and 5.3%, respectively). Respiratory symptoms such as breathlessness and cough were detected in 27.9 and 13.9% of patients, respectively. Palpitations were also frequent as PCS symptom in 36.8% of patients.

Prevalence and type of post-COVID-19 syndrome symptoms by gender and age

The prevalence of PCS symptoms according to gender and age is reported in Table 1. *Gender:* Female reported more PCS symptoms in comparison with male [fatigue (61 vs. 47%, $p = 0.003$), anxiety (51 vs. 40%, $p = 0.036$), palpitation (40 vs. 30%, $p = 0.018$), residual symptoms (38 vs. 13%, $p = 0.002$), weakness (36 vs. 24%, $p = 0.004$), shortness of breath (33% vs. 17, $p < 0.001$), and change in smelling sensation (50 vs. 30%, $p < 0.001$)]. Only myalgia was significantly more frequently reported in male than female (51 vs. 47%, $p = 0.007$). *Age:* No significant differences were found in prevalence of PCS between subjects aged <30 years/old and ≥ 30 years/old, except for myalgia and weakness which were more frequent in subjects aged <30 years/old in comparison with those aged ≥ 30 years/old [(51 vs. 36%, $p = 0.001$) and (38 vs. 29%, $p = 0.041$), respectively].

Clustered bar chart of different PCS symptoms according to gender and age is shown in Figure 2.

Risk factors for post-COVID-19 syndrome symptoms

Multiple binary logistic regression analysis has been performed to assess the association between different symptoms with gender and age (Table 2).

TABLE 1 The prevalence of PCS symptoms in the overall population.

Post-COVID-19 symptoms	Prevalence population (*n*, %) *n* = 495	Prevalence of PCS according to gender *n* (%)			Prevalence of PCS according to age *n* (%)		
		Female *n* = 329	Male *n* = 166	*P*-value	Age < 30 years *n* = 207	Age ≥ 30 years *n* = 288	*P*-value
Mood disturbance/ depression	294, 59.4%	204 (62%)	90 (54%)	0.059	126 (61%)	168 (58%)	0.579
Fatigue	279, 56.4%	201 (61%)	78 (47%)	**0.003**	123 (59%)	156 (54%)	0.271
Anxiety	234, 47.3%	167 (51%)	67 (40%)	**0.036**	104 (50%)	130 (45%)	0.274
Myalgia	209, 42.2%	153 (47%)	56 (51%)	**0.007**	106 (51%)	103 (36%)	**0.001**
Sleep disturbance	203, 41.1%	141 (43%)	62 (37%)	0.246	83 (40%)	120 (42%)	0.781
Change in smell	202, 40.8%	156 (50%)	46 (30%)	<**0.001**	77 (37%)	125 (47%)	0.524
Palpitation	182, 36.8%	133 (40%)	49 (30%)	**0.018**	76 (38%)	106 (37%)	1.00
Residual symptoms	168, 33.9%	126 (38%)	42 (13%)	**0.002**	72 (35%)	96 (33%)	0.773
Weakness	161, 32.5%	121 (36%)	40 (24%)	**0.004**	78 (38%)	83 (29%)	**0.041**
Nightmares/ flashbacks	138, 27.9%	95 (29%)	43 (26%)	0.525	51 (25%)	87 (30%)	0.817
Shortness of breath	138, 27.9%	110 (33%)	28 (17%)	<**0.001**	57 (28%)	81 (28%)	0.919
Weight loss (>3 Kg)	135, 27.3%	94 (29%)	41 (33%)	0.393	63 (30%)	72 (25%)	0.185
Loss of smell sensation	73, 14.7%	55 (17%)	18 (12%)	0.107	33 (16%)	40 (14%)	0.089
Cough	69, 13.9%	49 (15%)	20 (12%)	0.413	28 (14%)	41 (14%)	0.896
Loss of taste sensation	26, 5.3%	21 (6%)	5 (3%)	0.137	11 (5%)	15 (5%)	1.00

Bold values mean significant association with age/gender.

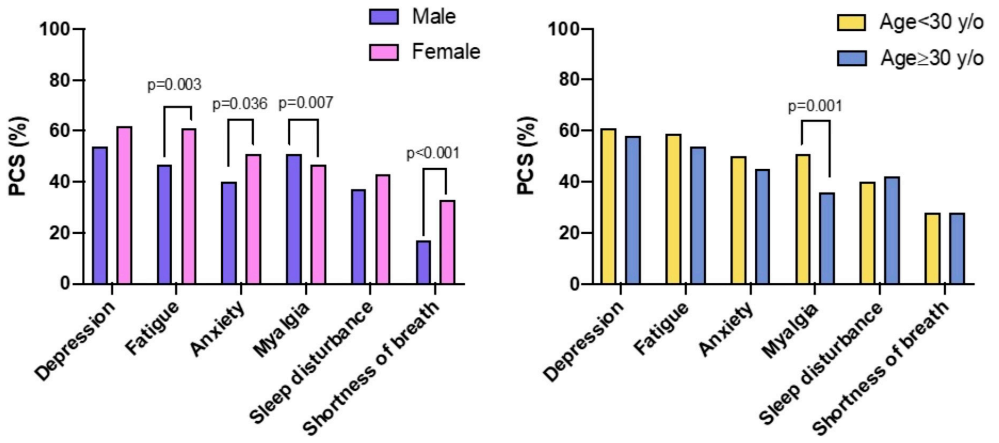

FIGURE 2
Clustered bar chart of different Post-COVID-19 Syndrome (PCS) symptoms according to gender (left chart) and age (right chart).

Gender: Fatigue ($p = 0.003$, OR = 1.768), anxiety ($p = 0.027$, OR = 1.533), palpitation ($p = 0.016$, OR = 1.633), weakness ($p = 0.005$, OR = 1.817), shortness of breath ($p < 0.001$, OR = 2.484), change in smelling sensation ($p < 0.001$, OR = 2.355), myalgia ($p = 0.009$, OR = 1.691) were significantly associated with gender.

Age: Myalgia ($p = 0.001$, OR = 1.029) and change in smelling sensation ($P = 0.024$) were significantly associated with age. The loss of baseline physical strength post infection was independently associated ($p = 0.041$) with female gender and age < 30 years.

Discussion

In the present study, conducted in Jordan among patients after mild to moderate SARS-CoV-2 infection, data indicated that: (1) PCS symptoms were frequent and mainly associated

TABLE 2 Analysis of the association between different symptoms with gender and age.

Outcome	Age			Gender		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Mood disturbance/depression	0.998	0.982–1.015	0.818	1.380	0.940–2.003	0.095
Fatigue	1.003	0.986–1.020	0.732	1.768	1.213–2.578	**0.003**
Anxiety	0.992	0.976–1.008	0.319	1.533	1.050–2.238	**0.027**
Change in smell	0.979	0.962–0.997	**0.024**	2.355	1.559–3.560	<**0.001**
Myalgia	1.029	1.012–1.046	**0.001**	1.691	1.143–2.503	**0.009**
Sleep disturbance	0.992	0.975–1.008	0.318	1.272	0.865–1.862	0.218
Palpitation	0.990	0.973–1.007	0.243	1.633	1.088–2.421	**0.016**
Residual symptoms	0.993	0.975–1.010	0.405	1.843	1.209–2.771	**0.004**
Weakness	1.016	0.998–1.033	0.075	1.817	1.192–2.770	**0.005**
Nightmares/flashbacks	0.986	0.967–1.005	0.139	1.172	0.771–1.796	0.462
Shortness of breath	0.995	0.977–1.014	0.621	2.484	1.558–3.961	<**0.001**
Weight loss (>3 Kg)	1.004	0.986–1.022	0.679	1.216	0.787–1859	0.368
Loss of smell sensation	0.996	0.973–1.019	0.714	1.655	0.929–2.899	0.082
Cough	0.992	0.969–1.017	0.533	1.284	0.734–2.239	0.380
Loss of taste sensation	0.998	0.962–1.035	0.906	2.199	0.813–5.938	0.120

CI, confidence interval; OR, Odds Ratio. Bold values mean significant association with age/gender.

with female gender; (2) psychological symptoms were prevalent; (3) age < 30 years was more likely associated with myalgia, and loss of physical strength.

To our knowledge this is the first study investigating the prevalence of PCS and its different symptoms in patients after mild to moderate SARS-CoV-2 infection in Jordan who were not hospitalized and/or required respiratory support. This was a prospective study enrolling a significant number of patients considering the overall population in the country. Further, we were able to identify risk factors for PCS in a specific geographical area with local style of life and support of patients. Currently available data reporting PCS prevalence after SARS-CoV-2 infection are related to severe infection requiring hospitalization and/or need of mechanical ventilation ranging from 85 to 87.4% (8, 9). In the present study we found that the overall prevalence of PCS symptoms is 83% which is not consistent with those previously reported in other geographical areas such as Europe (44%) and North America (31%) (10). Among different PCS symptoms, psychological (mood disturbance/feeling depressed) and fatigue symptoms were the most frequently reported in the present study in parallel with previous reports (11); this may be attributed to the fact that patients have been in quarantine for 14 days and/or feared possible worsening of infection during the time of acute illness. Premraj et al. (12) reported fatigue as the most frequent symptom of PCS followed by brain fog, sleep disturbances and memory issues. Myalgia and palpitations were also frequent in the present study which was not in agreement with previous studies [5.9% for myalgia (13) and 8.3% for palpitations (14)]. Finally, respiratory symptoms, including cough (14%) and breathlessness (28%) were less frequent than expected and in comparison, with other studies (15, 16) where respiratory symptoms are more frequent (30–50% for breathlessness).

We found that PCS symptoms, including myalgia and weakness, were highly prevalent in female gender and age < 30 years. This information may help to optimize healthcare monitoring and support after mild to moderate COVID-19 infection. On the contrary, Oronsky et al. (17), found that older age was a risk factor for PCS symptoms. Our findings were the opposite from those reported by Peghin et al. (18), who found no association between age and PCS symptoms after COVID-19. A previous study performed in the Middle East has pointed to female gender as a risk factor for PCS (19), suggesting a possible relation with Arabic culture, as females are used to take care of family members when they are infected. On the other hand, a relatively significant increase in PCS symptoms among females in UK suggests a possible biological relationship (20). Daily behaviors and environment are hypothesized to affect the probability of developing PCS symptoms. A previous observational study noted that isolation, financial status, exercise, temperature, and humidity may increase the risk of PCS symptoms (21). The presence of comorbidities is also a well-established risk factor for PCS, as multiple studies indicated that the presence of pre-existing medical conditions (P = 0.003) increases the potential of having PCS. In addition, having hypertension (odds ratio (OR) = 1.3, P = 0.018), obesity (OR = 2.31, P = 0.002), a psychiatric condition (OR = 2.32, P = 0.007), or an immunosuppressive condition (OR = 2.33,

P = 0.047) corresponded with the greatest odds of not returning to "usual health." (22). Having blood group O was associated with an increased risk of developing PCS, as group O showed a sixfold increased risk of PCS, compared to non-O (23). Smoking, low economic status, and full vaccination prior COVID-19 were also considered risk factors in patients enrolled in a single-center longitudinal study (24). Sudre et al. (25) demonstrated that 13.3% of participants reported symptoms lasting $\geq$ 28 days, 4.5% $\geq$ 8 weeks, and 2.3% $\geq$ 12 weeks. Therefore, physical, and mental rehabilitation of PCS play a relevant role to facilitate the healing process (26, 27).

In the open-ended question, people reported having the following symptoms: headache, memory problems, hair loss, joint pain and lower back pain (n = 11, 8, 6, 5, and 4, respectively), and these symptoms were major symptoms of PCS in multiple studies (28–30). Some rare manifestations of the post-viral syndrome have also been reported; according to Goërtz et al. (31) symptoms like eye problems, ear pain, red spots on toes/feet, and vomiting were noticed.

Limitations

This study has several limitations that should be addressed. First, to facilitate the survey and response rate several issues related to PCS have not been specifically addressed. Second, this survey collected data during a single time point, limiting the validity of temporal association. Third, a control of response accuracy was not feasible. Fourth, comorbid conditions were not investigated which might affect the prevalence of post-COVID symptoms. Fifth, a potential bias due to the survey strategy was encountered as it is more likely that symptomatic individuals were more prone to answer the survey than asymptomatic ones, explaining the high (83%) positivity compared to the literature available.

Conclusion

PCS is highly prevalent in COVID-19 survivors in Jordan, especially in females and patients with comorbidities. Planning physical and mental rehabilitation services is recommended for those patients with PCS symptoms after mild to moderate COVID-19.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the International Review Board (IRB) of Yarmouk University number: IRB/2022/9. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

LA-H contributed to the writing—review and editing, supervision, and project administration. MN conceived the research questions, performed statistical analysis, designed the questionnaire, and data input. HA-G performed statistical analysis and data input. AA contributed to the review, editing, and style. MS performed statistical analysis. DB and PP contributed to the writing—review and editing and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.1017257/full#supplementary-material

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Milder outcomes of SARS-CoV-2 genetically confirmed reinfections compared to primary infections with the delta variant: A retrospective case-control study

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Background: SARS-CoV-2 infection does not confer long immunity. However, studies suggest that prior infection is associated with lower risk of reinfection and milder outcomes of recurrent infections. The aims of this retrospective observational case-control study were to describe the clinical and molecular characteristics of genetically confirmed Delta reinfection cases and to assess the potential protective role of preceding infection on the severity of reinfection.

Methods: We used next generation sequencing (NGS) to explore if cases with two positive real time RT-PCR tests > 90 days apart were infected with a different SARS-CoV-2 variant. Cases with confirmed reinfection between August 1st and October 31st, 2021 (the Delta wave) in Slovenia were matched 1:4 by age, sex and timeframe (week of positive test) with individuals with primary infection. Sociodemographic and epidemiologic data, vaccination status, and data on hospitalization and outcome of infection were retrieved from several centralized and standardized national databases. Additional epidemiologic surveys were performed on a limited number of cases and controls.

Results: We identified 628 cases of genetically confirmed reinfection during the study period and matched them with 2,512 control subjects with Delta primary infection. Primary infections in individuals with reinfection were mainly caused by B.1.258.17 (51.1%), followed by B.1.1.7 (15.1%) and reinfection was detected on average 271 days after primary infection (range 101–477 days). Our results show a substantially lower probability of hospitalization

in cases with reinfection compared with controls (OR: 0.21, p = 0.017), but no significant difference was observed in intensive care unit admission and deaths. We observed a significantly lower proportion of vaccinated individuals among cases compared to controls (4.5% vs. 28.2%), suggesting that hybrid immunity leads to lower probability of reinfection. Detailed analysis of the temporal distribution of variants, responsible for reinfections, showed no significant differences in reinfection potential.

Conclusion: Reinfection with the SARS-CoV-2 Delta variant resulted in fewer hospitalizations compared to the primary Delta infection, suggesting that primary infection may, to some extent, produce at least short lasting protective immunity. This study provides additional insight into the reinfection dynamics that may allow appropriate public health measures to be taken in subsequent waves of the COVID-19 pandemic.

KEYWORDS

SARS-CoV-2, COVID-19, reinfection, Delta variant, NGS, genetically confirmed variant, protective immunity, severity

Introduction

The ongoing coronavirus disease 19 (COVID-19) pandemic has an extensive societal and economic impact (1). With each new wave of the pandemic, healthcare systems have faced large numbers of patients requiring hospitalization and intensive care unit (ICU) treatment (2) and there is growing evidence of the long-term consequences of SARS-CoV-2 infection (3). Moreover, overcoming SARS-CoV-2 infection does not provide long immunity (4). Epidemiological studies (population-based cohort studies and case-control studies) indicated that prior SARS-CoV-2 infection was associated with a significantly lower risk of reinfection over a period of 7 months to more than 1 year and for variants circulating in communities at the time of the study (5–9).

The majority of cases with reinfection had similar disease severity comparable to the first infection or had milder disease in the second episode. Cases of reinfection with SARS-CoV-2 with an adverse outcome have been described (10). The question arises regarding to what extent previously naturally acquired immunity protects against reinfection with different variants of SARS-CoV-2 and how waning immunity contributes to the frequency of reinfection (8). Different levels of exposure derived from socioeconomic determinants, occupation, living in institutional settings, differences in demographics, and comorbidities contribute to the risk of reinfection (11). The continued emergence of SARS-CoV-2 variants with higher transmissibility, immune escape, and altered pathogenicity are drivers of an increasing number of reinfections as of November 2021 (12).

Natural immunity following primary SARS-CoV-2 infection provided more sustained protection against the B.1.617.2 (Delta) variant than vaccine-mediated immunity (13). Recent studies have shown that vaccine-mediated immunity wanes after 6 months, with efficacy against the Delta variant declining rapidly after only 90 days (13, 14). Planas et al. found reduced neutralization of the SARS-CoV-2 Delta variant in comparison to previous strains (15). There was an indication toward increased severity associated with B.1.617.2 and prolonged viable viral shedding with more severe symptoms than in those infected with non-Delta variants (16, 17). Recent studies have also shown that the Delta variant was associated with an increased risk of hospitalization, ICU admission, oxygen requirement, and death (18).

In May 2021, the Delta variant was detected sporadically in Slovenia, with increasing frequency. Starting in mid-July 2021, the SARS-CoV-2 Delta variant became practically the only variant identified during national routine weekly SARS-CoV-2 genomic surveillance in Slovenia (19, 20). The increasing number of reinfections during the Delta wave provided unique opportunity to investigate the impact of pre–Delta variant SARS-CoV-2 infection on the severity of reinfection compared to primary Delta variant infections. Thus, the aims of this study were to describe the clinical and molecular characteristics of genetically confirmed Delta reinfection cases and to assess the potential protective role of preceding SARS-CoV-2 infection on the severity of reinfection.

Materials and methods

Design and eligibility criteria

We conducted a retrospective observational case-control study in residents of Slovenia with PCR-confirmed SARS-CoV-2 reinfection (cases) and primary infection (controls) between August 1st and October 31st, 2021 (the Delta wave).

Sources of data

Four national health administrative data sources collecting individual health information data were used.

National COVID-19 database

Data were extracted from the National COVID-19 Database, which is part of the National Notifiable Communicable Diseases Database. The database covers all SARS-CoV-2 cases (symptomatic and asymptomatic) in Slovenia. The National COVID-19 Database is linked to the Central Registry of Patient Data, the Central Registry of Spatial Units, and the Register of Health Workers to obtain socio-demographic and health-related data. Data extracted from National COVID-19 Database were age (in years), sex, being a healthcare worker, living in a long-term care facility, date of first confirmed infection and date of reinfection, and time interval between initial infection and reinfection (in days). For a limited number of cases, epidemiological surveys were completed with additional data available; that is, being symptomatic or asymptomatic at the time of a confirmatory real-time RT-PCR test, having an epidemiological link to a confirmed case, and which clinical symptoms were present or absent (fever, cough, sore throat, breathing difficulties, anosmia/ageusia, headache, myalgia, and arthralgia).

Inclusion criteria: Cases

A case was defined according to the following criteria: (i) two laboratory-confirmed SARS-CoV-2 episodes at least 90 days apart as registered in Slovenia, (ii) the second episode (i.e., reinfection) between August 1st and October 31st, 2021, (iii) samples of both episodes (i.e., primary infection and reinfection) were SARS-CoV-2–positive by a real-time RT-PCR assay and were available for sequencing, and (iv) genomic sequencing of paired samples was performed, yielding two distinct variants of SARS-CoV-2. These strict criteria were chosen to provide high-quality laboratory evidence of reinfection and to exclude potential long-term shedding. The study period of August–October 2021 was chosen to eliminate variant bias because Delta was the only variant circulating in Slovenia at that time. After limiting the cases to these criteria, 628 cases were identified.

Inclusion criteria: Controls

The control group consisted of individuals matched for age, sex, and timeframe (week of positive test) with real-time RT-PCR–confirmed SARS-CoV-2 Delta virus primary infection in the same period of time as reinfection occurred in cases (August 1st to October 31st, 2021). If an exact age match was not possible, a $\pm$ 2-year tolerance was allowed. When multiple controls were available, random matching was performed. Repetition of controls was not allowed. For every case, four controls were identified (i.e., 2,512 controls altogether).

National vaccination register

We obtained data on vaccination against COVID-19 from the eRCO national vaccination register (Slovenian: *Elektronski register cepljenih oseb* "Electronic Register of Vaccinated Persons"). The data extracted from the eRCO register were the date of the vaccination and the vaccine used.

Cases and controls were classified as fully vaccinated if they had received one dose of Jcovden vaccine or both doses of the two-dose schedule vaccines (mRNA vaccine: Comirnaty or Spikevax, vector vaccine: Vaxzevria) at least 14 days before reinfection (cases) or primary infection (controls). Partially vaccinated cases and controls received one dose of two-dose COVID-19 vaccines at least 14 days before confirmation of reinfection (cases) or primary SARS-CoV-2 infection (controls). Beyond that, as partially vaccinated we also counted persons that had received both doses of two-dose vector or mRNA vaccines but for whom less than 14 days had elapsed between vaccination and a positive PCR test for SARS-CoV-2.

National registry of hospitalizations

Hospitalization data were obtained from the eSBO national registry of hospitalizations (Slovenian: *Elektronski sistem bolnišničnih obravnav* "Electronic Registry of Hospitalizations"). Temporally associated admissions (14 days before and 14 days after positive PCR) to acute care hospitals were analyzed. Data collected from eSBO were main discharge and additional diagnoses, duration of hospitalization (in days), intensive care treatment (in hours), and outcome (discharge, death). By definition, COVID-19 was the cause of hospitalization if classified as the main discharge diagnosis (ICD-10 classification U07.1) or if the main discharge diagnosis was viral pneumonia caused by SARS-CoV-2.

National registry of deceased persons

The National Registry of COVID-19 Cases is regularly updated with data from the National Registry of Deceased Persons. Death is attributed to COVID-19 according to the WHO definition (i.e., death within 28 days after laboratory-confirmed SARS-CoV-2 infection).

Individual data in the national registries were linked by a unique personal identification number. The National COVID-19 Database, eRCO, eSBO, and the National Registry of

Deceased Persons are managed by the National Institute of Public Health (NIPH) of Slovenia.

GISAID repository

To determine the prevalence of SARS-CoV-2 lineages observed in the Slovenian population, we accessed the GISAID global database[1] and extracted the corresponding prevalence for each lineage detected. We calculated the percentage of the population based on the data for the entire country. This information was used to compare the prevalence of lineages detected in the primary infection of cases to the prevalence in Slovenian population. Any major deviations in prevalence could indicate a bias toward a particular lineage with regard to reinfection potential. This comparison is only possible if the assumption of representativeness of the national surveillance ability to reliably detect circulating lineages is not violated. In other words, we want to be certain (or at least know the limits of certainty) that national data on lineage presence in sequenced samples could be generalized to the entire population of Slovenia. According to the ECDC guidelines (21), our national SARS-CoV-2 surveillance strategy allowed us to detect and characterize lineages with a prevalence of less than 1%.

Laboratory analysis

Sample collection

Nasopharyngeal swab samples were collected as part of routine testing for SARS-CoV-2 at the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana and the National Laboratory of Health, Environment, and Food of the Republic of Slovenia. After the identification of possible cases of reinfection, all samples that had not already been sequenced as a part of routine weekly surveillance for SARS-CoV-2 variants were collected by laboratory personnel for retrospective sequencing.

Library preparation and next generation sequencing sequencing

RNA was extracted from 300 μl of nasopharyngeal swab samples using Maelstrom 9600 (TanBead Inc., Taoyuan, Taiwan), according to the manufacturer's instructions. PCR amplicons were prepared in accordance with the ARTIC V2 and V3 RT-PCR protocol [nCoV-2019 sequencing protocol v2 (GunIt)].[2] PCR amplicon size was inspected on 2% agarose gel. DNA concentration was measured with the Qubit dsDNA High Sensitivity assay kit on Qubit 3.0 (both Thermo Fisher Scientific, Waltham, MA, USA). We prepared NGS libraries of amplicons using the Nextera XT library preparation kit (Illumina, San Diego, CA, USA), according to the vendor's instructions. The concentrations of NGS libraries were measured using the Qubit dsDNA High Sensitivity assay on a Qubit 3.0 instrument (Thermo Fisher Scientific). The fragment sizes were analyzed using the Agilent HS DNA Kit on the Bioanalyzer 2100 (both Agilent Technologies, Santa Clara, CA, USA). Prepared samples were sequenced using the MiSeq Reagent Kit v3 (600 cycles) on the MiSeq Sequencer, the NextSeq 500/550 High Output Kit v2.5 (300 cycles) on the NextSeq 550, or NovaSeq 6000.

Bioinformatic analysis

Initially, we trimmed the raw reads obtained from the Illumina sequencers using BBDuk, which is part of the BBTools program package (22). The quality of the raw reads and the quality of the trimming procedure were evaluated with FastQC (23). We mapped the trimmed reads to the Wuhan-Hu-1 isolate reference genome (NCBI accession number NC_045512.2) using BWA-MEM with default settings (24). Mapped reads were subsequently transformed into an appropriate form using Samtools (25). This process included exporting the mapping data to bam files, sorting, mate-flagging, duplicate-marking, and indexing of the mapping data. Samtools was also used for coverage depth calculations. A consensus sequence was generated using iVAR (26). We set the minimum quality threshold to a factor of 10, the minimum depth for calling consensus to 10 reads, and the minimum frequency threshold to 0.5 (consensus was called when 50% of the reads agreed on a particular base). Lineage assignment was performed using the Phylogenetic Assignment of Named Global Outbreak Lineages (Pangolin), which implements the dynamic nomenclature of SARS-Cov-2 lineages (27). All sequences have been deposited in the GISAID repository and are available for further analyses (**Supplementary material**).

Statistical methods

Data analysis was performed using R statistical software (version 4.1.3, R Foundation for Statistical Computing, Vienna, Austria). To assess the normality of the data distributions, we used Q–Q plots and the Shapiro–Wilk test. Differences in the number of nursing home residents between groups (cases vs. controls) were evaluated using Fisher's exact test, and differences in the number of healthcare workers between groups were evaluated using Pearson's chi-squared test with Yates' continuity correction. The differences in reported symptoms and vaccination status were evaluated with a two-proportions z-test. For the effect size assessment between two proportions, we opted for Cohen's h effect size. The odds ratio between groups was calculated using Fisher's exact test for count data and, when necessary, Haldane's correction on zero values was applied. Differences in the number of asymptomatic disease courses between vaccinated and unvaccinated individuals were

1 https://www.gisaid.org

2 https://www.protocols.io/view/ncov-2019-sequencing-protocol-v2-bp2l6n26rgqe/v2?version_warning=no

evaluated with Fisher's exact test. The difference in time intervals between first infection and reinfection was assessed with ANOVA. The threshold for statistical significance was set at $p < 0.05$ in all cases.

The study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline for case-control studies.

Results

From the beginning of the COVID-19 epidemic in Slovenia (the first case was identified on March 4th, 2020) to October 31st, 2021, there were 333,959 Slovenian residents with a positive RT-PCR test (320,428 persons) or rapid antigen test (RAT) (13,531 persons). According to the national case definition, RAT was accepted as a confirmatory test for a short period of time (from December 21st, 2020 to February 12th, 2021).

The 320,428 RT-PCR–positive individuals included 318,805 individuals with single confirmed SARS-CoV-2 infection and 1,623 individuals with possible reinfection; that is, with two real-time RT-PCR–positive tests at least 90 days apart. Whole genome sequencing (WGS) confirmed two distinct variants of the SARS-CoV-2 virus in the first and second samples in 660 cases, including 32 reinfections occurring before the Delta wave. Finally, 628 persons with the first infection before the Delta wave and reinfection during the Delta wave were included in the study. In 963 cases with possible reinfection, one or both samples were unavailable for WGS, WGS was unsuccessful, or individuals were found to have a prolonged infection (the same SARS-CoV-2 variant was detected in both samples).

Demographics, source of infection, and clinical presentation

As shown in Table 1, we identified 382 (60.8%) females and 246 (39.2%) males with genetically confirmed reinfection (i.e., cases) with Delta in the study period, from 4 to 92 years of age, with the majority of cases (75.2%) in the 20–49 age group. We compared the proportion of cases with the proportion of the population to determine possible differences in reinfection potential. Initial infections in individuals with reinfection were mainly caused by B.1.258.17 (51.1%), followed by B.1.1.7 (15.1%; Table 2). The study period was selected at the beginning of the Delta wave in Slovenia, and therefore the distribution of cases (and matched controls) according to the week in which reinfection occurred was skewed to the right, as seen in Figures 1A,B. The cases and matched controls did not differ in the proportion of healthcare workers or nursing home residents (Table 1). The proportion for being asymptomatic was higher among cases ($p = 0.004$), and those cases that were symptomatic had on average statistically significant fewer symptoms compared to controls ($p = 0.02$). A comparison of clinical data showed that cases had statistically significantly lower proportions of loss of smell and taste and proportions of accompanying fever ($p = 0.005$ and $p < 0.001$, respectively) and statistically significantly higher proportions of headache and sore throat ($p = 0.03$ and $p = 0.03$, respectively) compared to controls (Table 1). Cases and controls had the same proportion of known sources of infection; however, we observed a higher proportion of infections from a family member among the controls ($p = 0.01$).

Vaccination

The vaccination status of cases and controls is presented in Table 1. Most cases were unvaccinated (575 cases, 91.6%) compared to statistically significantly fewer unvaccinated controls (1,753, 69.8%). Only 28 (4.5%) cases were fully vaccinated with Comirnaty (14 persons), Vaxzevria (4 persons) or Jcovden (10 persons). Among fully vaccinated controls 345 received Comirnaty, 191 Vaxzevria, 138 Jcovden, 32 Spikevax and 2 received a combination of Vaxzevria/Comirnaty. We observed statistically significant differences in vaccination status between groups. A significantly higher proportion of unvaccinated patients was observed among cases, with a large effect size. Furthermore, a significant difference was observed in the ratio of fully vaccinated patients between groups, with a lower proportion of cases vaccinated with both doses (Table 1). We found no statistically significant differences in asymptomatic disease course in relation to vaccination status ($p = 0.16$).

Disease severity

A statistical analysis of severity indicators (hospitalizations, ICU admissions, and death in the first 28 days after RT-PCR positivity) showed lower odds for hospitalizations in cases (OR 0.21, CI 0.05–0.86, p-value = 0.02), but not for ICU admissions and deaths (Table 3). The hospitalized cases were one male and one female, both unvaccinated. The first case had reinfection 5 months after initial infection, admitted to the ICU, and mechanically ventilated. The second case had reinfection 7 months after initial infection and hospitalized for both episodes, although no ICU or mechanical ventilation was needed. Six deaths were recorded in the control group and none in the cases with reinfection. The deceased patients were four females and two males age 51 to 92 years, who died 1 to 26 days after the RT-PCR–positive test result. Four of them died in the hospital, and only one needed ICU treatment with mechanical ventilation. The other two died after discharge, but both had severe underlying disease (cancer and diabetes, respectively).

TABLE 1 Matching and non-matching variables in cases (individuals with Delta SARS-CoV-2 reinfection) and controls (individuals with initial Delta SARS-CoV-2 infection).

	Cases, n = 628		Controls, n = 2,512		P-value	Cohen's h (effect size)
Demographics						
Sex						
Female, *n* (%)	382	(60.8)	1,528	(60.8)	–	–
Male, *n* (%)	246	(39.2)	984	(39.2)	–	–
Age (years), mean (range)	34	(4–91)	33	(4–93)	–	–
0–9, *n* (%)	10	(1.6)	40	(1.6)	–	–
10–19, *n* (%)	76	(12.1)	304	(12.1)		
20–29, *n* (%)	149	(23.7)	596	(23.7)		
30–39, *n* (%)	182	(29.0)	728	(29.0)		
40–49, *n* (%)	141	(22.5)	564	(22.5)		
50–59, *n* (%)	51	(8.1)	204	(8.1)		
60–69, *n* (%)	10	(1.6)	40	(1.6)		
70–79, *n* (%)	3	(0.5)	12	(0.5)		
80 + , *n* (%)	6	(1.0)	24	(1.0)		
Healthcare worker, *n* (%)	27	(4.3)	87	(3.5)	0.4	–
Nursing home resident, *n* (%)	5	(0.8)	10	(0.4)	0.2	–
Teachers (pre-, primary, secondary schools), *n* (%)	59	(9.4)	102	(4.1)	<0.001	0.24
EPI survey data						
Asymptomatic course, *n* (%)	27/371	(7.3)	63/1,610	(3.9)	0.004	0.15
Fever, *n* (%)	113/249	(45.4)	796/1,192	(66.8)	<0.001	0.43
Loss of taste and smell, *n* (%)	61/249	(24.5)	395/1,194	(33.1)	0.005	0.19
Sore throat, *n* (%)	75/249	(30.1)	285/1,194	(23.9)	0.02	0.14
Headache, *n* (%)	76/249	(30.5)	292/1,194	(24.5)	0.03	0.14
Muscle and joint pain, *n* (%)	47/249	(18.9)	195/1,194	(16.3)	0.4	–
Cough, *n* (%)	141/249	(56.6)	694/1,194	(58.1)	0.7	–
Difficulty breathing, *n* (%)	9/249	(3.6)	39/1,164	(3.4)	0.9	–
Shortness of breath, *n* (%)	2/249	(0.8)	21/1,175	(1.8)	0.4	–
ARDS, *n* (%)	0/249	(0)	3/1,191	(0.3)	0.9	–
No. of reported symptoms, mean/*n*, (*SD*)	2.1/249	(1.16)	2.3/1,194	(1.17)	0.02	0.15
Epi. link/Contact with a confirmed case, *n* (Yes)	165/273	(60.4)	771/1,195	(64.5)	0.2	–
Most probable source of infection: family, household, *n* (%)	92/203	(45.3)	516/942	(51.8)	0.01	0.19
Vaccination						
Unvaccinated, *n* (%)	575	(91.6)	1,753	(69.8)	<0.001	0.57
Partial, *n* (%)	25	(4.0)	47	(1.9)	0.001	0.13
Full, *n* (%)	28	(4.5)	708	(28.2)	<0.001	0.69
Boost, *n* (%)	0	(0)	4	(0.2)	0.7	–

ARDS, acute respiratory distress syndrome.

Temporal distribution of the timing of both SARS-CoV-2 episodes in cases

One can observe a heterogeneous composition of variants responsible for the first infection, or, from another perspective, the absence of any clusters that would indicate a bias toward a particular variant being more susceptible to reinfection with the Delta variant. Figure 2 shows the relational data for the cases. The time elapsed between first and second infection was a minimum of 101 days and a maximum of 477 days, on average 271 days, as presented in Figure 2. The variant distribution of the first SARS-CoV-2 episode of the cases is presented in Table 2. The frequencies of variants of cases are shown next to the population prevalence of each variant in Slovenia until August 1st, 2021 according to GISAID. The main finding is a notably lower percentage of the Alpha variant in the sample compared to the percentage in the population (15.1% vs. 42.9%). We observed an average time to reinfection of 271 days after primary infection. Figure 3 presents the distributions of time intervals between primary infection and reinfection for each of the variants that occurred in at least 10 cases. We

TABLE 2 Genomic variant composition of primary infections in individuals with the Delta variant reinfection.

Variant	*n*	Sample, %	Population, %	Ratio Sample /Population
B.1.258.17	321	51.1	38.7	1.3
B.1.1.7	95	15.1	42.9	0.4
B.1.258	64	10.2	3.6	2.8
B.1.1.70	45	7.2	3.5	2.1
B.1.160	36	5.7	4.2	1.4
B.1.149	11	1.8	1.1	1.6
B.1.1	9	1.4	1.4	1.1
B.1.146	8	1.3	0.5	2.8
C.35	8	1.3	0.7	1.8
B.1.177	6	1.0	0.5	1.9
B.1	3	0.5	1.7	0.3
B.1.236	3	0.5	0.4	1.4
B.1.1.39	2	0.3	0.0	8.1
B.1.160.14	2	0.3	0.0	32.3
B.1.221	2	0.3	0.2	1.5
C.16	2	0.3	0.1	5.4
A	1	0.2	0.0	32.3
AP.1	1	0.2	0.0	32.3
B.1.1.58	1	0.2	0.4	0.4
B.1.177.28	1	0.2	0.2	0.7
B.1.224	1	0.2	0.0	32.3
B.1.243	1	0.2	0.0	32.3
B.1.36.1	1	0.2	0.0	10.8
B.1.36.23	1	0.2	0.0	5.4
B.1.389	1	0.2	0.0	10.8
B.1.94	1	0.2	0.0	32.3
Q.1	1	0.2	0.0	32.3

The differences are expressed as ratios between percentages. A ratio lower than 1 indicates underrepresentation of the lineage in our sample (cases) in comparison to the population, and a ratio greater than 1 shows overrepresentation of the lineage in our sample in comparison to the population.

can observe that the emergence and prevalence of the variant directly correspond to the time interval. In other words, the "older" the variant, the longer the mean interval. The most prominent result is the notably shorter intervals in the Alpha variant.

Discussion

In this case-control study, we aimed to characterize the differences between individuals with SARS-CoV-2 reinfection and individuals first diagnosed with SARS-CoV-2, both infected with the Delta variant in the same calendar week. The main strength of this study is the rigorous genomic characterization of variants detected in paired samples from cases with reinfection using next-generation sequencing (NGS). This approach reinforces the comparison analysis and eliminates the false-positive bias that is introduced when accurate genomic assignment is not employed. The thorough analysis of patient metadata and genomic information is complemented by the use of national registry resources for all cases included and a fairly large control group matched by sex, age, and week of positive SARS-CoV-2 test, which ensures sufficient statistical power to differentiate between groups.

Analysis of demographic data showed no age difference between female and male cases, which may suggest that age does not play an important role in reinfection dynamics. However, female cases were overrepresented (61%). In Slovenia, there were more females with confirmed SARS-CoV-2 infection than males throughout the pandemic (53 and 47%, respectively; national data available from dashboard),[3] but not as many as in the cases included in our study. The analysis showed that more cases were employed in the education and health sectors compared to the general population (**Table 1**). About 2% of the Slovenian general population works in each sector, whereas 9% of cases are employed in the education sector and 4% in the health sector. Because women are predominantly employed in both sectors and frequent testing for SARS-CoV-2 was mandatory in both occupational groups, this could explain the female predominance. However, we did not find a statistically significant difference in the proportion of healthcare workers and nursing home residents between cases and controls. A number of studies that exclusively enrolled healthcare workers reported lower odds ratios for reinfection and less severe disease course in this profession (5, 28, 29). We were unable to replicate this finding, most likely due to the low prevalence of reinfection (range 0.1–1.1%) and the relatively small number of healthcare workers and nursing home residents in our data (5–7, 30). Similar results have been reported for nursing home residents. Although residents were among the first to be infected during the first wave, there is no evidence of a higher risk of reinfection in this group (31).

The main finding of this study is the observed statistically significant difference in the number of hospitalizations between cases and controls (two vs. 38, respectively). The calculated odds ratio of 0.2 (CI: 0.1–0.8) suggests the protective role of prior infection (approximately five times lower odds of hospitalization at reinfection in comparison to first infection). However, due to the low numbers of hospitalization events, we were confronted with a relatively broad confidence interval, which does not allow us to draw a firm conclusion about the assumed protective nature of prior infection. In addition, because of the retrospective study design, we cannot exclude survival bias, which may have contributed to this observation. Six deaths were observed among control subjects in the study and none among cases, which could also support this hypothesis,

3 https://www.nijz.si/sl/dnevno-spremljanje-okuzb-s-sars-cov-2-COVID-19

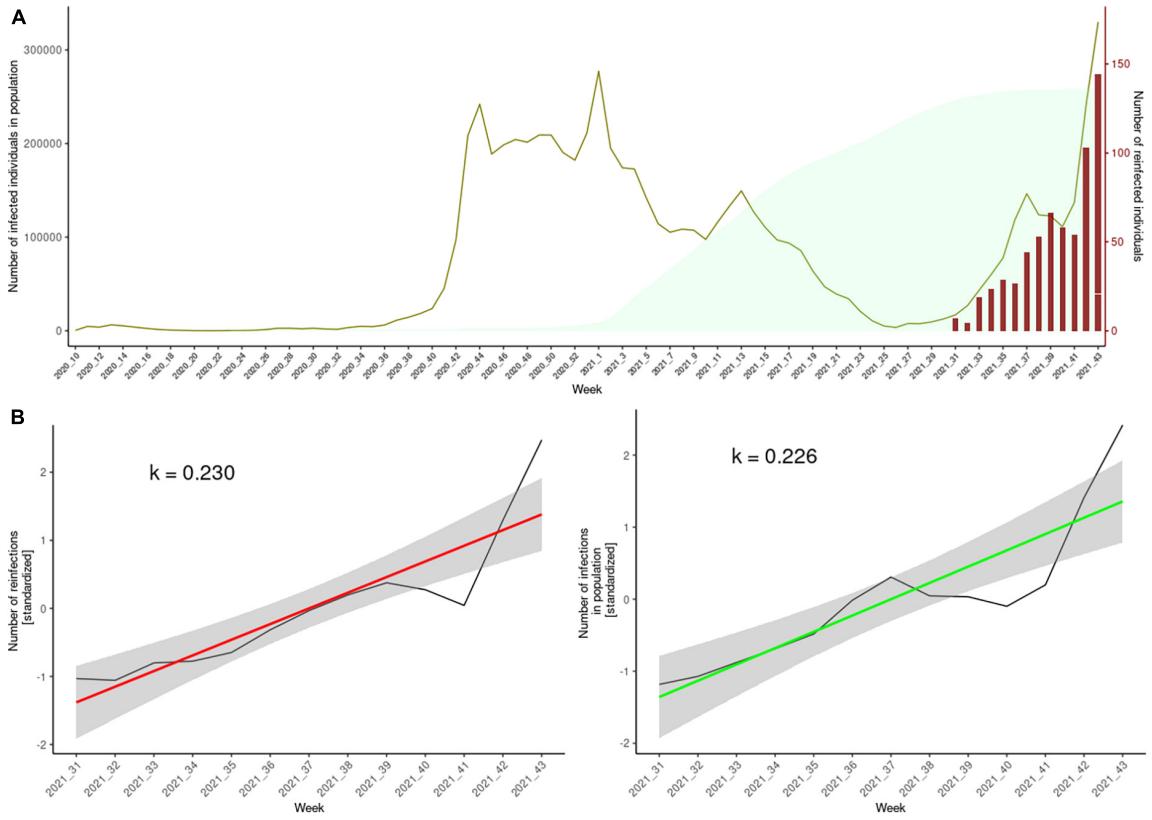

FIGURE 1
Panel **(A)** of this composite plot shows the weekly cumulative total of people eligible for reinfection (light green), the weekly number of positive SARS-CoV-2 tests times 20 (olive), and the number of confirmed reinfections (red). The y-axis on the right side of the plot corresponds to the number of reinfected individuals. The y-axis on the left side corresponds to the number of individuals eligible for reinfection and the number of weekly infections. In order to simultaneously present the two in the same plot, we multiplied the number of weekly infections by 20 (to illustrate, the peak in olive green in the first week of 2021 represent ∼ 15,000 individuals). The cumulative plot, which represents the pool of potential reinfections, is presented with a 90-day lag, according to the ECDC definition criterion for reinfection. Panel **(B)** depicts the standardized rise of weekly reinfection numbers (red regression line) and the weekly number of positive tests (green regression line).

but the numbers are too small to draw any conclusions. Data on COVID-19 hospitalization encompass the entire spectrum of causes and populations in exposure to SARS-CoV-2, however in our study, we limited the hospitalization rates specifically to "COVID-19" or "viral pneumonia" diagnoses 14 days before and 14 days after positive SARS-CoV-2 PCR. This was probably the reason for a relatively small number of hospitalization events recorded, however, there is also a possibility that we introduced a small measure of sample selection bias. Although we took some measures to prevent such eventuality (sufficiently large control group, thorough demographic characterization of chosen sample), we cannot reliably and completely eliminate sample selection bias. For a limited number of cases and controls, additional information was made available from surveys conducted by trained epidemiologists. The most prominent finding was the observed higher proportion of SARS-CoV-2 RT-PCR–positive individuals that reported having had fever on initial infection with the Delta variant compared to persons with reinfection with this variant (67% vs. 45%, respectively). Furthermore, we identified a higher proportion of reported asymptomatic cases in comparison to the control group (7% vs. 4%, respectively), and a significant lower proportion of cases reporting loss of smell and taste in comparison to the control group (24% vs. 33%, respectively). This result could indicate a protective nature of prior infection in the reinfection course. However, we also observed a significantly higher proportion of reported sore throat in the case group (30% vs. 24% in the control group) and a significantly higher proportion of headache in the case group (31% vs. 25% in the control group). The complex interplay of immune system response, virus characteristics, and the social conduct of the individuals makes the characterization of symptomatic profiles difficult (32). To address this issue would require a more focused study to further elucidate the factors that contribute to a specific symptom profile in each group.

Another noteworthy observation was a statistically significant difference in vaccination status between groups. The case group had a significantly higher proportion of

TABLE 3 Hospitalizations, ICU admissions, and deaths in cases and controls.

Severity	Cases, *n* (%)		Controls, *n* (%)		OR (95% CI)	*P*-value	Cohen's *h* (effect size)
Hospitalization	2	(0.3)	38	(1.5)	0.21(0.05–0.86)	**0.02**	0.11
ICU	1	(0.2)	5	(0.2)	0.80(0.09–6.86)	1	–
Death	0	(0.0)	6	(0.2)	0.31(0.02–5.45)	0.6	–

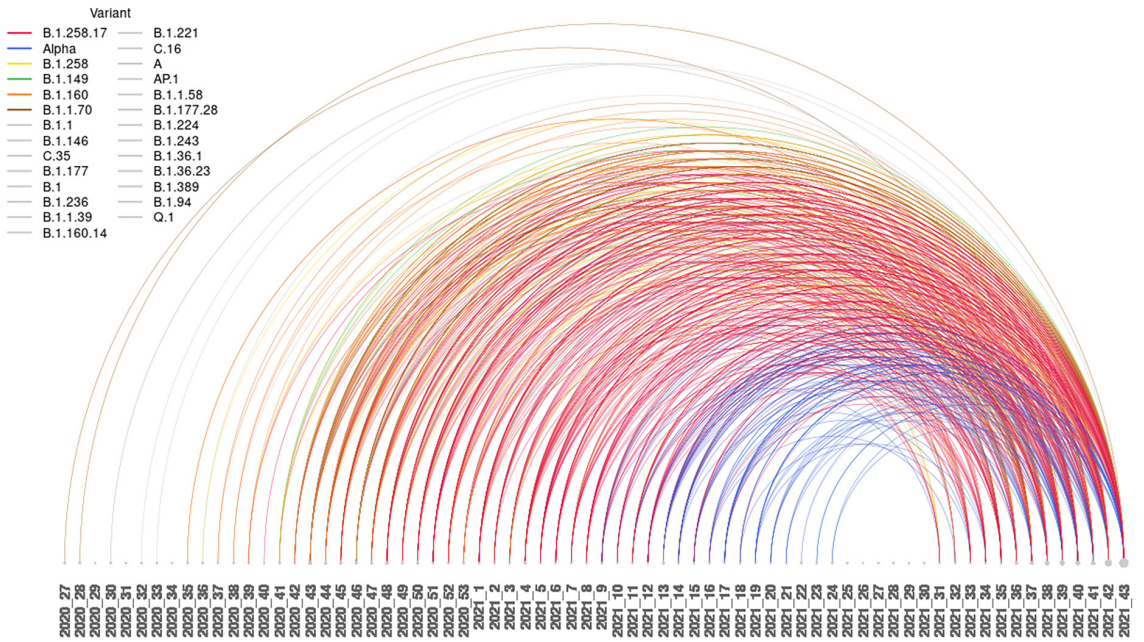

FIGURE 2
Arc plot of reinfection intervals between the first and second SARS-CoV-2 episodes. The lines are color coded for the six most represented variants.

unvaccinated individuals in comparison to the control group (91.6% vs. 69.8%, respectively). This could be explained by individuals being less inclined to vaccinate after having already experienced SARS-CoV-2 infection (33). Natural infection in combination with vaccination (i.e., hybrid immunity) offers better protection against reinfection. This finding was supported by a large observational study in Israel, which reported that SARS-CoV-2–naive individuals that received two doses of the Comirnaty vaccine were six to 13 times more likely to become infected with Delta than patients that had previously experienced infection (14). The reported vaccination imbalance lacks the background information that would set the difference in a broader perspective. We cannot pin this discrepancy to the most common demographic factors, such as age or gender, due to age and gender matched study design. On the other hand, we cannot with certainty exclude the presence or frequency of comorbidities as a confounding factor. The vaccination status is determined by the plethora of complex factors. The COVID-19 vaccine acceptance depends on perceived risk of disease, level of trust in the vaccine, in the delivery system and the recommendations given by health authorities and barriers related to geographic accessibility and, availability of vaccination services (34).

Analysis of the temporal distribution of variants causing initial infection did not reveal any obvious patterns that would suggest a bias for reinfections toward an identified variant in first infection. As expected, we observed that the variant mean time interval to reinfection directly corresponded to the emergence of the variant: the "older" the variant, the longer its mean time interval to reinfection. This is most likely the reason why we observed notably shorter intervals ($\approx$ 170 days) for the Alpha variant, a major variant that preceded the Delta wave studied here. However, the immune escape can also contribute to this effect. The lineage B.1.258.17 already harbored some specific spike mutations, such as del69_70 and N439K that have been reported to be associated with increased infectivity (35), reduction of binding affinity for the ACE2 receptor and reduced neutralizing activity of some monoclonal and polyclonal antibodies (36). Alpha exhibited additional deletion in RBD domain – del144/144, which was reported causing a fourfold reduction in neutralization titer (37). In a comparison between wild-type virus isolate harboring

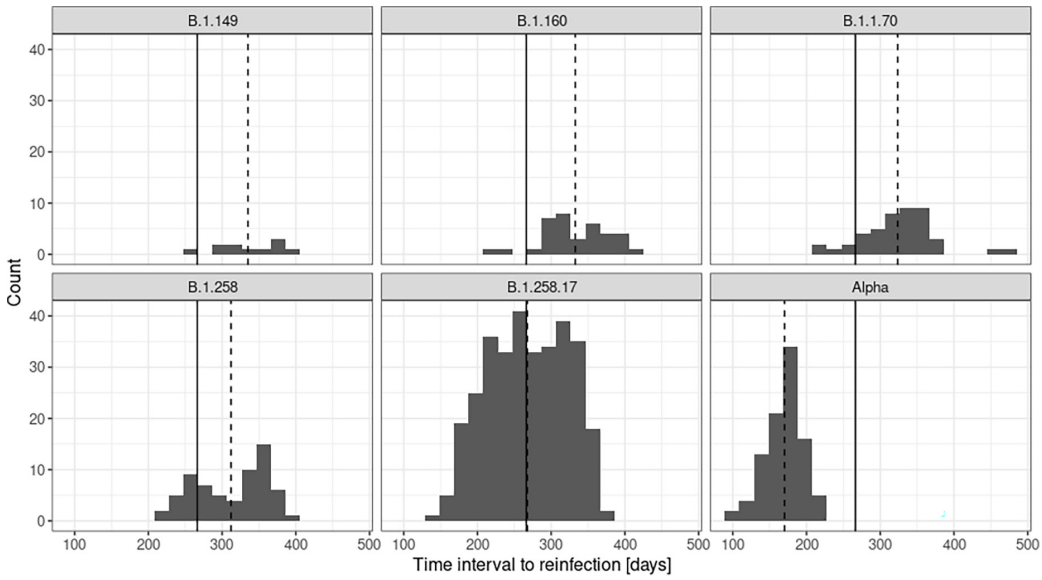

FIGURE 3
Time intervals between first infection and reinfection for the most common variants observed in primary infection in cases. The global mean of the time intervals between infections across all variants is shown as a solid line, and the mean interval for each variant is shown as a dashed line.

D614G mutation, Alpha and Delta, (38), reported that Alpha virus isolate was 2.3-fold less sensitive to the neutralizing antibodies than WT_D614G, and that Delta was 5.7-fold less sensitive to the neutralizing antibodies. On the other hand, while examining the variant composition of the SARS-CoV-2 initial episode of cases and comparing it to the prevalence of variants as indicated by GISAID until the study period, we detected a significant deviation in percentages of the Alpha variant (19, 20). It appears that the Alpha variant was underrepresented in our reinfection cases, which could indicate a higher protective capacity of the Alpha variant against Delta or could be due to a shorter time interval and immunity that was still present at the time of our study period but began to wane thereafter. Although there is a notable difference between the presence of the Alpha variant in our cases and in the population, we speculate that, if we extended the time interval to include the rest of the year 2021, we would see a rise in the percentage of Alpha as first infection in reinfection cases.

Even though this study was designed to minimize SARS-CoV-2 variant bias and set strict criteria for classification of reinfection, it has some limitations. First, cases were not randomly selected, but were chosen based on the availability of samples and finally generation of the SARS-CoV-2 genetic sequence. This eventuality exposes the results of our study to the potential of sample selection bias. However, because we included the samples from all laboratories in the country that performed SARS-CoV-2 testing, we assumed a representative sample of the population. Even though some samples were unavailable due to technical reasons, we believe that this disruption did not introduce a systemic bias.

Next, data were obtained from national repositories, but unfortunately, not all potentially interesting data were available. For example, to study the severity of the disease, it would be of great benefit if the available data were supplemented with an exhaustive medical history of the individuals investigated. This information would enable us to further refine our findings and perhaps reveal a subset of individuals with specific comorbidities that are more susceptible for reinfection. There is a higher proportion of asymptomatic cases compared to controls. However, reporting these cases was difficult because many asymptomatic infections may have been missed and underestimated, possibly because of the reluctance to screen the individuals selected here as controls. Finally, no additional testing was performed, thus we cannot completely exclude the possibility of some unrecognized reinfections in the selected controls. This could be avoided to some extent by additional serological testing, which was not performed because of the retrospective nature of this study.

Naturally acquired immunity to SARS-CoV-2 is not long-lasting and vanished within a few months, as does immunity after vaccination. The results of this study confirm that a preceding infection provides some protection against reinfection with the Delta variant and reduces the severity of the disease. The number of reinfections with SARS-CoV-2 increased during Omicron wave and is likely to increase in the future. For adapting timely and appropriate public health response, it is important to closely track the evolution of variants and the impact on previously infected individuals.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.gisaid.org/, EPI_SET_220901qg (https://epicov.org/epi3/epi_set/220901qg?main=true).

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Board at National Institute of Public Health, Slovenia (consent no. 1810-92/2021-18). Data used for the study were routinely collected for health statistics or epidemiological purposes. According to the Healthcare Databases Act, patient consent is not required to collect such data. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

MS, ML, MK, MP, and TA-Ž conceived and designed the project. MK, KP, AŠ, SJ, and TŽ-Č contributed the data. AS, MM, ML, and TZ analyzed the data. AS, MS, MM, and ML drafted the manuscript. MS, MK, MP, TA-Ž, AŠ, and KP revised the manuscript. All the authors contributed to the manuscript and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.962653/full#supplementary-material

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Progression to lung fibrosis in severe COVID-19 patients: A morphological and transcriptomic study in postmortem samples

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The development of lung fibrosis is a major concern in patients recovered from severe COVID-19 pneumonia. This study aimed to document the evolution of diffuse alveolar damage (DAD) to the fibrosing pattern and define the transcriptional programs involved. Morphological, immunohistochemical and transcriptional analysis were performed in lung samples obtained from autopsy of 33 severe COVID-19 patients (median illness duration: 36 days). Normal lung and idiopathic pulmonary fibrosis (IPF) were used for comparison. Twenty-seven patients with DAD and disease evolution of more than 2 weeks had fibrosis. Pathways and genes related with collagen biosynthesis and extracellular matrix (ECM) biosynthesis and degradation, myofibroblastic differentiation and epithelial to mesenchymal transition (EMT) were overexpressed in COVID-19. This pattern had similarities with that observed in IPF. By immunohistochemistry, pathological fibroblasts (pFBs), with CTHRC1 and SPARC expression, increased in areas of proliferative DAD and decreased in areas of mature fibrosis. Immunohistochemical analysis demonstrated constitutive expression of cadherin-11 in normal epithelial cells and a similar pattern of cadherin and catenin expression in epithelial cells from both normal and COVID-19 samples. Transcriptomic analysis revealed

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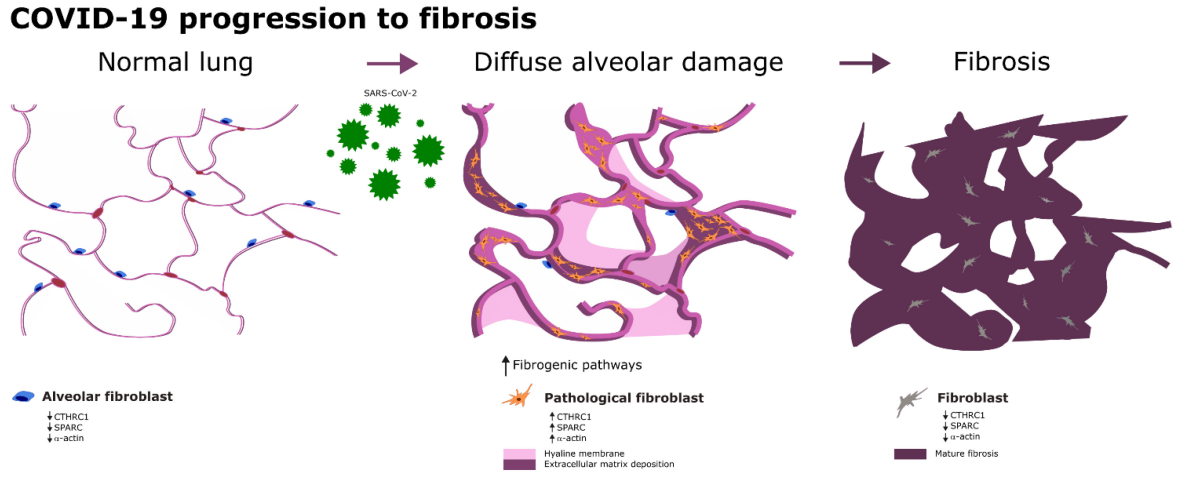

GRAPHICAL ABSTRACT

Progression to lung fibrosis in COVID-19 patients is related to an overexpression of fibrogenic pathways.

downregulation of the Hippo pathway, concordant with the observation of YAP overexpression in hyperplastic alveolar epithelial cells. Progression to fibrosis in severe COVID-19 is associated with overexpression of fibrogenic pathways and increased in CTHRC1- and SPARC-positive pFBs. Whereas the Hippo pathway seemed to be implicated in the response to epithelial cell damage, EMT was not a major process implicated in COVID-19 mediated lung fibrosis.

KEYWORDS

COVID-19, fibrosis, transcriptomic, diffuse alveolar damage (DAD), autopsy

Introduction

Autopsy studies of COVID-19 patients have demonstrated that, while SARS-CoV-2 can be detected in different organs, the primary finding associated with the cause of death is respiratory failure due to diffuse alveolar damage (DAD) in different stages of evolution. Although most patients show DAD in the exudative or organizing phase, up to 40% of autopsied patients with long term hospitalization and mechanical ventilation show fibrosing DAD (1). Whether or not this frequency of fibrosis extrapolates to recovered severe patients remains to be established in future prospective studies. However, the development of lung fibrosis is a major concern in patients that have recovered from severe COVID-19 pneumonia. It has been reported that among survivors of severe COVID-19, 20% of non-mechanically ventilated and 72% of mechanically ventilated individuals had fibrotic-like radiographic abnormalities 4 months after hospitalization. The risk factors associated with its development were greater initial severity of illness, longer duration of mechanical ventilation and shorter blood leucocyte telomeres (2). Currently, despite the efforts of the community, including different clinical trials, there are no treatments for COVID-19 induced pulmonary fibrosis (3). Thus, lung transplantation is becoming a life-saving therapeutic option for patients with end-stage lung disease due to COVID-19 (4).

At present, the cellular components and molecular mechanisms of fibrosing DAD in COVID-19 patients are poorly understood (5–7). Although it is likely that the same cells and/or pathways described in other forms of lung fibrosis also participate in SARS-CoV-2 induced DAD. Recent studies have reported that fibrosis in both idiopathic pulmonary fibrosis (IPF) and COVID-19 can be driven, at least in part, by "pathological fibroblast" (pFB), characterized by the expression of a specific set of genes (8–10). Thus, pFB, together with other types of fibroblasts, seem to be the cellular effectors of lung fibrosis. They are driven under the stimuli of several pathways, such as the transforming growth factor beta (TGF-β), WNT, HIPPO or epithelial to mesenchymal transition (EMT) (11, 12). Accordingly, a few recent reports using single cell RNAseq

technology have observed similarities in gene expression across cell lineages between end stage COVID-19 lungs and lungs of patients with pulmonary fibrosis (8, 10).

In this study, we describe the morphological, immunohistochemical and transcriptomic changes in the lungs of 33 COVID-19 patients, most with a prolonged clinical course, in order to evaluate the mechanisms of progression to fibrosis that would suggest possible new therapeutic strategies in patients with severe disease.

Materials and methods

Patients and tissue sample collection

This postmortem study included lung samples obtained from 33 COVID-19 patients. Autopsies were performed at the Hospital Universitario Ramón y Cajal (Madrid, Spain) between April 2020 and June 2021, as previously reported (13). The Research Ethics Committee approved the study (reference: Necropsias_Covid19; 355_20). Clinical, laboratory and radiological records were reviewed, and the main pathologies and treatments were recorded.

The autopsies represented about 3% of COVID-19 deaths during this period in our center. Most autopsies corresponded to patients with severe respiratory diseases and were requested by ICU staff. Consequently, the series does not represent the complete spectrum of causes of death attributable to COVID-19. All autopsies were consented by the patients' relatives and carried out according to safety protocols, in a negative pressure autopsy room, using personal protection equipment, as previously reported (13). All autopsies were performed less than 24 h after patient's death.

In the first 14 consecutive decedents, in-corpore representative sections were taken from the heart, lungs, liver, kidney, pancreas, and bone marrow. In the rest of the patients, due to improved technical training, the complete heart and lung block, left kidney, spleen and sections from the liver, pancreas and bone marrow were extracted. One autopsy was limited to the lungs.

For comparison, biopsy samples from 4 patients with typical lesions of usual interstitial pneumonia/idiopathic pulmonary fibrosis (UIP/IPF) were studied (**Supplementary Table 1**). UIP lesions met the criteria recently proposed by the 2018 ATS/ERS/JRS/ALAT Guidelines (14), including dense fibrosis with architectural distortion, predominant subpleural and/or paraseptal distribution of fibrosis, patchy involvement of lung parenchyma by fibrosis, presence of fibroblast foci and absence of features to suggest an alternate diagnosis.

In addition, 7 lung samples obtained from lung resections for different conditions other than UIP/IPF were used as normal controls. We selected areas far away from the pleura, or the specific pathological lesion in each case, that had a completely normal morphological appearance. We did not select autopsy samples as normal controls because in our institution the time of lung fixation of autopsy specimens is about 1 week, whereas lung fixation of COVID-19 specimen was between 24–72 h. Long fixation times have a negative impact on transcriptomic analysis.

Lung lesions semiquantitative evaluation

Samples from the five pulmonary lobes were taken in all patients. All histological evaluations were blinded to clinical data. The histopathological classification of the DAD lesions was performed according to Li et al. (1) as previously reported (15).

The semiquantitative evaluation of lung lesions was performed on hematoxylin and eosin (H&E) and Masson's trichrome stained sections. Ten to twenty sections were evaluated in each patient (at least 2 H&E sections from each lobe). The percentage of the section area occupied by each type of lesion was recorded. For quantitation, lesions were classified as acute/exudative, organizing/proliferating, and fibrotic. Acute/exudative lesions included massive epithelial desquamation, hyaline membranes, and intra-alveolar fibrin deposition; organizing/proliferating lesions included septal, and/or alveolar proliferation; fibrotic lesions included septal, and/or alveolar fibrosis, characterized by the presence of dense interstitial eosinophilic fibrous tissue green-stained by Massonn's trichrome. Other changes, such as alveolar edema, capillary congestion, alveolar hemorrhage, squamous metaplasia, pleural fibrosis, etc. were recorded in each patient, but not quantitated for this analysis.

Immunohistochemistry

The EnVision FLEX/HRP system was used for immunohistochemistry analysis (Agilent, Santa Clara, CA). For dual immunostainings, EnVision FLEX DAB + Chromogen (Agilent) was used to obtain the brown color and EnVision FLEX HRP Magenta Chromogen was used to obtain the magenta color. Antibodies, clones, dilutions, and providers are presented in **Supplementary Table 2**.

RNA extraction

RNA was extracted from 10 tissue sections of 5 μm obtained from representative paraffin blocks. The Recover All Total Nucleic Acid Isolation Kit was used for formalin-fixed paraffin-embedded (FFPE) tissue (Invitrogen) following the manufacturer's instructions. Quantification of RNA was measured fluorometrically with the Qubit RNA high-sensitivity assay kit (Invitrogen). RNA quality was assessed using RNA

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TABLE 1 Main clinical and laboratory data of the patients included.

Demographics and clinical characteristics			Total number of observations
Age, years	Median (IQR)	69 (13)	33
	Min, max	52, 91	
Gender, *n* (%)	Male	26 (78.8)	33
Weight, kg	Median (IQR)	76.5 (14)	28
	Min, max	53, 109	
DM, *n* (%)		4 (12.1)	33
Hypertension, *n* (%)		13 (39.4)	33
Patients admitted to ICU, *n* (%)		29 (87.9)	33
Total days	Median (IQR)	36 (17)	33
	Min, max	9, 108	
Hospitalization days	Median (IQR)	28 (16)	33
	Min, max	3, 102	
ICU days	Median (IQR)	23 (13)	29
	Min, max	12, 95	
Mechanical ventilation, *n* (%)		28 (84.5)	33
Corticosteroids use, *n* (%)		31 (93.9)	33
Immunomodulatory therapy*, *n* (%)		28 (84.8)	33
Lung pathological findings			
Patients with predominant pattern, *n* (%)			
	Normal lung	2 (6.1)	33
	Exudative DAD	6 (18.2)	33
	Proliferative/ Organizing DAD	21 (63.6)	33
	Fibrotic DAD	4 (12.1)	33
Vascular thrombi, *n* (%)		22 (66.7)	33
Endothelialitis, *n* (%)		15 (45.5)	33
Lung infections			
Acute bronchopneumonia, *n* (%)		8 (24.2)	33
Aspergillosis, *n* (%)		3 (9.1)	33
Cytomegalovirus, *n* (%)		2 (6.1)	33
Main pathological findings in other organs			
Heart			
No lesions, *n* (%)		13 (40.6)	32
Coronary artery atherosclerosis, *n* (%)		11 (34.4)	32
Left ventricle hypertrophy, *n* (%)		4 (12.5)	32
Chronic epicardial inflammation, *n* (%)		4 (12.5)	32
Chronic ischemic cardiopathy, *n* (%)		2 (6.3)	32
Myocarditis, *n* (%)		1 (3.1)	32
Senile amyloidosis, *n* (%)		1 (3.1)	32
Liver			
No lesions, *n* (%)		11 (34.4)	32
Steatosis, *n* (%)		11 (34.4)	32
Centrilobular necrosis, *n* (%)		4 (12.5)	32
Cirrhosis, *n* (%)		1 (3.1)	32
Kidney			
No lesions, *n* (%)		5 (15.6)	32
Ischemic necrosis, *n* (%)		10 (31.3)	32
Acute tubular necrosis, *n* (%)		10 (31.3)	32
Glomeruloesclerosis, *n* (%)		4 (12.5)	32
Bone marrow			
No lesions, *n* (%)		1 (3.1)	32
Hyperplasia, *n* (%)		30 (93.8)	32
Hemophagocytosis, *n* (%)		25 (78.1)	32

*Including tocilizumab and/or interferon β 1a.

Screen Tapes on a 2200 TapeStation system (Agilent, Santa Clara, CA, USA).

Gene expression analysis

The Nanostring nCounter gene expression platform was used to analyze the expression of 770 human mRNAs associated with fibrotic diseases included in the Fibrosis Panel (16). Fluorescently color-coded reporter probes and biotin-labeled capture probes were hybridized to the mRNA on a thermal cycler overnight and automatically processed and loaded to the nanoString sample cartridge in the nCounter Prep Station, in accordance with the manufacturer's protocol.

The identification of differentially expressed genes was performed with normalized data using the nSolver analysis software (nanoString technologies Inc.). Specifically, the advanced analysis tool of this software allowed us to obtain the hierarchical grouping, the scatter diagrams (volcano plots) and statistical classification of the differentially expressed genes, along with FDR corrected *p*-values.

qRT-PCR was used to validate the expression of selected genes included in the Fibrosis Panel: *COL3A1*, *LOX* (collagen biosynthesis), *SPP1* [extracellular matrix(ECM) degradation], *TGFβ1*, *SMAD3*, *TGFBR2* (TGF-β), *SNAI2* (Slug, EMT), *CXCL12* (chemokine signaling), *AMOLT1*, *MOB1B, TPJ1* (Hippo pathway). In addition, qRT-PCR was used to evaluate the expression of *CTHRC1*, *SPARC* and *YAP1,* not included in the Fibrosis Panel (**Supplementary**

Table 4). The expression of these genes was also analyzed by IHC in 20 COVID-19 lung samples, normal controls and IPF samples.

Retrotranscription was performed with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), following the manufacturer's instructions. The iTaq Universal SYBR Green Supermix Kit (BIORAD) was used following the manufacturer's instructions. Data analysis was performed by quantifying the expression levels of the indicated genes, using a relative quantification by $\Delta\Delta$Ct method. The reference gene used was *GAPDH*.

Sars-Cov-2 detection

Sars-Cov2 detection was performed as previously reported (17). Briefly, it was performed in post-mortem FFPE tissue from the lungs (all lobes), heart, liver and kidney in all patients. Genomic Sars-Cov-2 RNA (gRNA), and subgenomic viral RNA (sgRNA) were detected by RT-PCR.

Statistical analysis

Statistical analyses were performed with R3.6.2 (18). Means comparisons were tested using the Student's *t*-test and correlations using the Spearman's coefficient. A $p < 0.05$ was considered statistically significant.

Results

Some clinical, pathological and virological data from 26 patients have been previously reported (17).

Clinical and laboratory data

This series included 26 males and 7 females, ranging in age from 52 to 91 years. The median disease duration was 36 days (IQR 17). A summary of the main clinical and laboratory data is presented in Table 1.

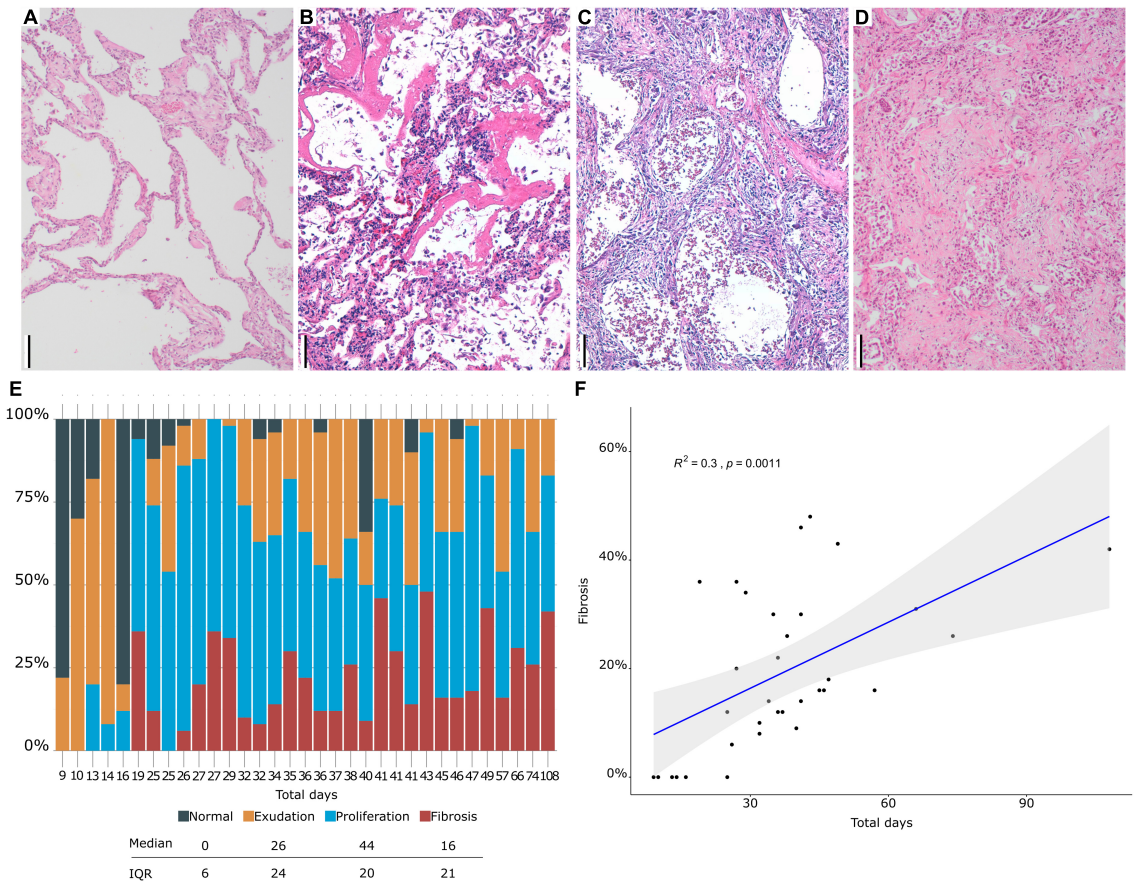

FIGURE 1
(A–D). Histological evaluation. **(A)** Normal lung. **(B)** Hyaline membranes in exudative diffuse alveolar damage (DAD). **(C)** Proliferative DAD. **(D)** Fibrosing DAD. **(E)** Proportion of different DAD patterns (y axis) in each patient (x axis). **(F)** Correlation between fibrosis and time of evolution. Scale bar: 100 μm.

Lung pathology

Macroscopic examination of the lungs showed increased consistency and poor aeration with consolidated areas in all cases, which varied in extension from patient to patient. The lungs showed a spectrum of histological lesions, both acute and chronic, consisting off desquamated pneumocytes, capillary congestion, pulmonary edema, hyaline membranes, intralveolar fibrin deposition, septal fibroblastic proliferation, intralveolar fibroblastic proliferation, septal fibrosis, intralveolar fibrosis, and squamous cell and mucinous metaplasia of the alveolar epithelium. Multiple combinations of these lesions were observed in most patients, making it difficult to assign a specific diagnosis to each patient. DAD in different evolution stages was present in all patients (Figures 1, 2). Thus, patients with a prolonged clinical course showed overlapping exudative, proliferative, and fibrotic lesions (Figure 2). The exudative pattern predominated in 6 patients (18.2%), the proliferative pattern in 21 (66.7%) and the fibrotic pattern in 4 patients (12.1%). Mean fibrosis was 16% (0–44%). Although there was a direct correlation between fibrosis and illness duration (Figure 1), the percentage of fibrosis varied among patients with a similar duration of illness (see Supplementary Table 3).

The most common inflammatory cells were CD68-positive alveolar macrophages. The proportion of these cells varied among patients, but were abundant, even in patients with a longer illness duration. In addition, all patients showed variable number of CD8-positive lymphocytes that were more abundant than CD4-positive lymphocytes.

Histological changes concordant with previous lung damage were observed, such as increased pleural fibrosis with muscularization and honeycomb changes in 3 patients (patient 12, 16, and 20), and silicosis nodules in 1 case (patient 16). None of these patients had a previous clinical history of chronic lung disease.

Sars-Cov-2 RNA detection

A detailed description of Sars-Cov2 results in lung samples is presented in Figure 3. In summary, Sars-Cov2 gRNA and sgRNA were detected in the lung of 27 and 15 patients, respectively. Sars-Cov2 gRNA was even detected in samples from the patients with a longer disease evolution (104 days). Regarding other organs, viral gRNA was detected only in the heart of Pat. 1. gRNA Cts-values in positive samples ranged from 21 to 40. sgRNA was detected in samples from 15 patients (50%).

Fibrosis-associated gene expression

Transcriptomic analysis using the NanoString platform was performed in 12 COVID-19 samples with RNA of sufficient quality. Samples originated from patients in their third to

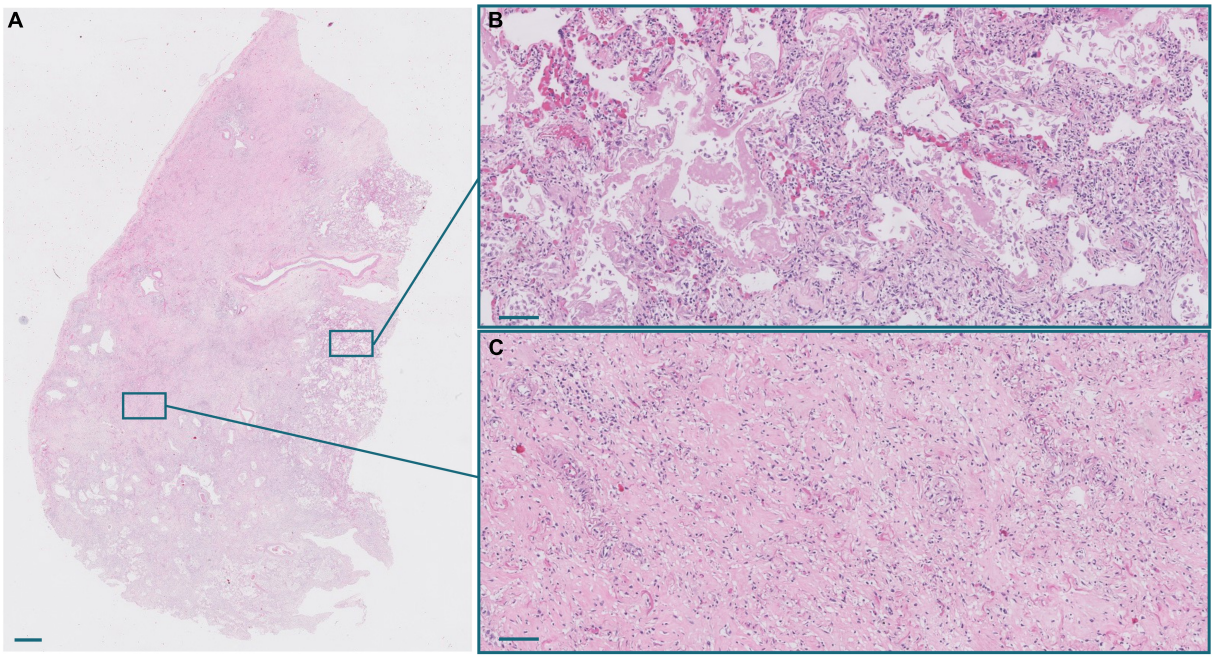

FIGURE 2
(A) Panoramic view of a lung section from a patient with a long clinical course. Scale bar: 1 mm. **(B)** Inset of an exudative area, with hyaline membranes. Scale bar: 100 μm. **(C)** Inset of a fibrotic area. Scale bar: 100 μm.

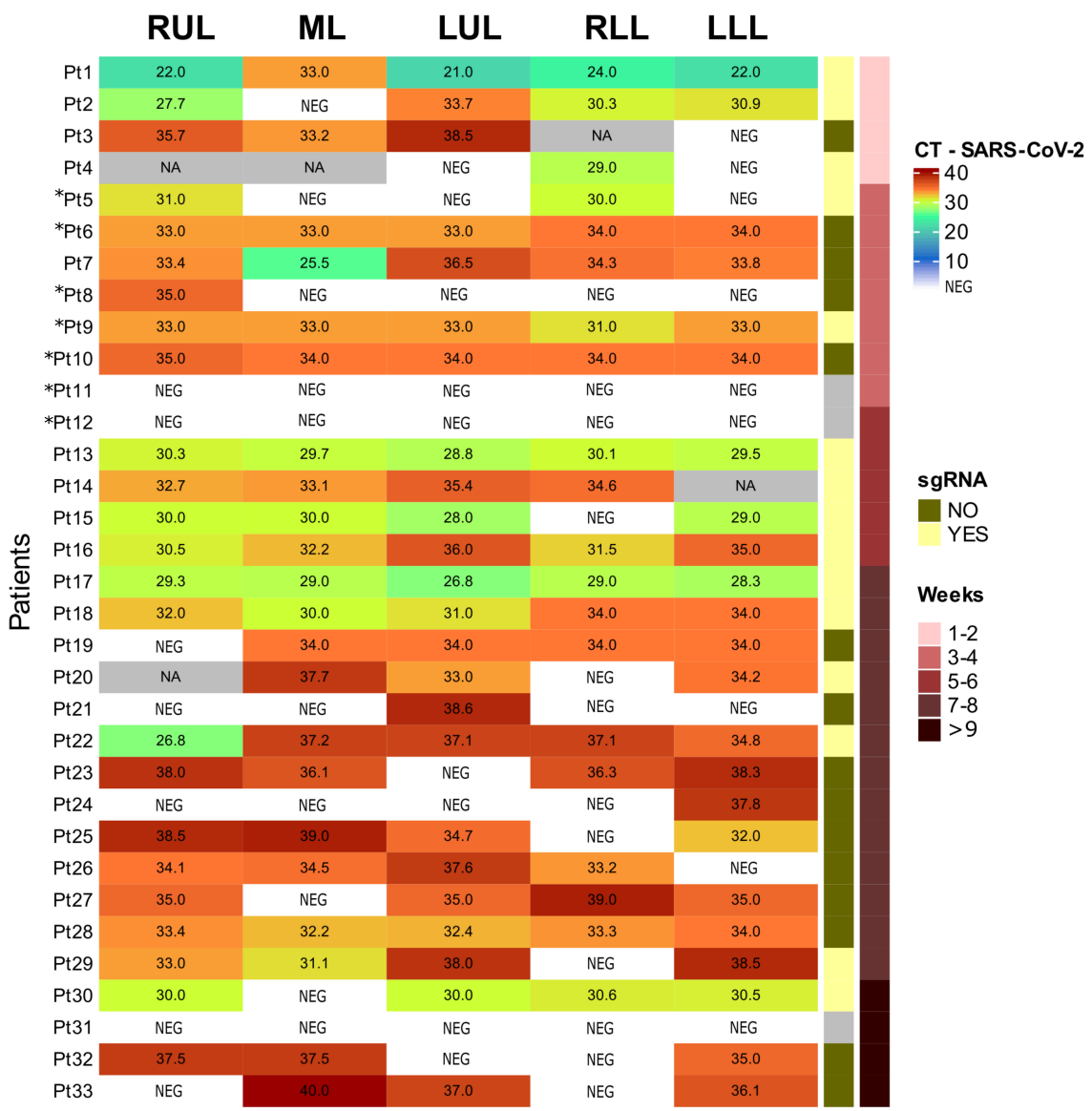

FIGURE 3
Sars-Corv-2 gRNA and sgRNA in lung samples. RUL, right upper lobe; ML, medium lobe; LUL, left upper lobe; RLL, **right lower** lobe; LLL, **left lower** lobe; CT, cycle threshold; NEG, negative; NA, non-available; sgRNA, subgenomic RNA. Patients not included in (17) are 5, 6, 8, 9, 10, 11, and 12 (with asterisks).

seventh week of disease evolution. In each case, we selected for Nanostring analysis those histological sections in which proliferative DAD predominated, assuming that it was the disease phase in which fibrogenesis was more active. This phase of DAD was also selected because previous studies on gene expression analysis have mainly focused on patients with a shorter evolution in which exudative DAD predominated, and this type of sample were underrepresented in our series. In addition, 7 normal lung controls were analyzed.

Figures 4–6, Supplementary Figure 1, and Supplementary Table 5 present the main pathways and genes differentially expressed between cases and healthy lung controls. The gene expression study confirmed the histological observation of increased fibrogenesis in COVID-19 patients, as genes related with collagen biosynthesis and ECM biosynthesis and degradation, such as *COL1A2*, *COL3A1*, *COL6A3*, *COL1A1*, *COL5A1*, *SPP1*, *MMP14*, *NCSTN*, and *LOX* were overexpressed in COVID-19 patients.

As previously mentioned, some studies have reported that fibrosis in both IPF and COVID-19 can be, at least in part, driven by pFB (also called activated fibroblasts in some articles) characterized by the expression of a set of genes, such as *CTHRC1*, *COL3A1*, and *COL1A*. Among these genes, *CTHRC1* has been proposed as one of the most specific (7). Since our

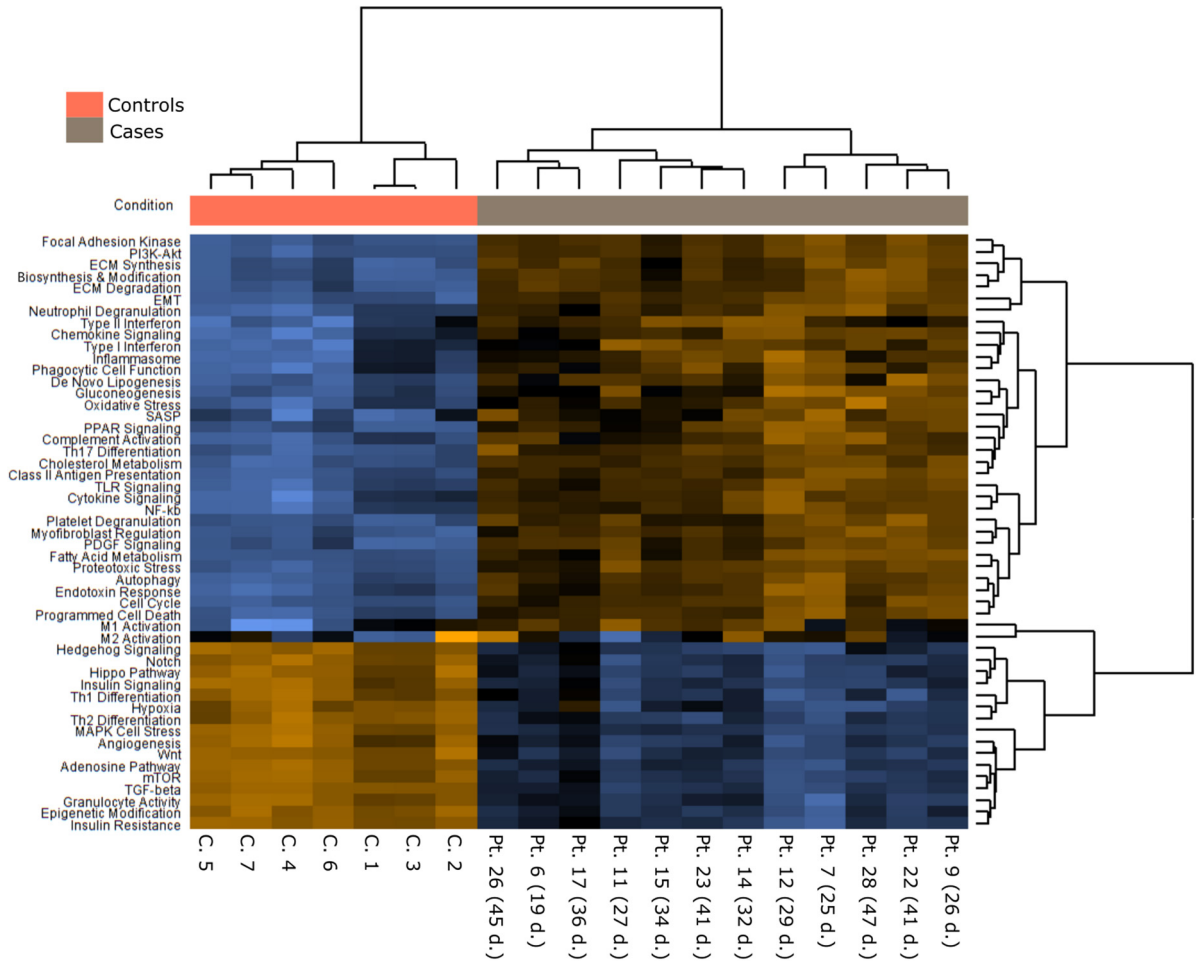

FIGURE 4
A Pathways differently expressed in normal and COVID-19 samples.

panel did not include *CTHRC1*, its expression was analyzed by both RT-PCR and IHC. There was a statistically significant increase of *CTHRC1* expression in COVID-19 samples when compared with control samples (**Figure 6**). In fact, the level of expression in normal appearing control lungs was negligible. By IHC, cells expressing CTHRC1 were not present in control lungs. In contrast, positive cells were first observed in areas of acute DAD (**Figure 7**). CTHRC1-positive cells increased in areas of fibroblastic proliferation and organization (**Figure 8**) and were scarce or absent in areas of mature fibrosis (hypocellular areas mainly composed by dense ECM) (**Figure 9**).

Melms et al. (10) observed in their single cell RNAseq analysis of COVID-19 lung samples that *SPARC* was the second most upregulated gene in pFB. This prompted us to study *SPARC* in our series by RT-PCR and immunohistochemistry, since this gene was not included in the NanoString panel. We observed a statistically significant increase of *SPARC* expression in COVID-19 samples compared with control samples. Like *CTHRC1*, the level of expression in control lungs was very low. By immunohistochemistry, we also observed that SPARC-positive fibroblasts were absent in normal lung, appeared in exudative DAD, peaked in areas of proliferative DAD and decreased in fibrotic DAD (**Figures 7**, **8**). Areas of mature fibrosis were devoid of SPARC-positive cells (**Figure 9**).

Immunostaining with α-muscle actin demonstrated that the population of positive cells were not identical to CTHRC1- and SPARC-positive cells. Thus, dual immunostaining with SPARC and α-actin in proliferative areas demonstrated a mixed population of cells expressing one or both markers at different levels (**Figure 10**). Fibroblast in areas of mature fibrosis showed no or very low α-actin expression (**Figure 9**).

An intriguing result in our study was the observation that the TGFβ pathway, a well-known fibrogenic pathway, was downregulated in our series of COVID-19 patients. However, both *TGFβ1* and its downstream target SPARC (19) were overexpressed and showed an statistically significant direct correlation (not observed between *TGFβ1* and *CTHRC1*) (**Figure 6**). Since genes such as *SMAD2*, *SMAD3*, and *SAMAD4*

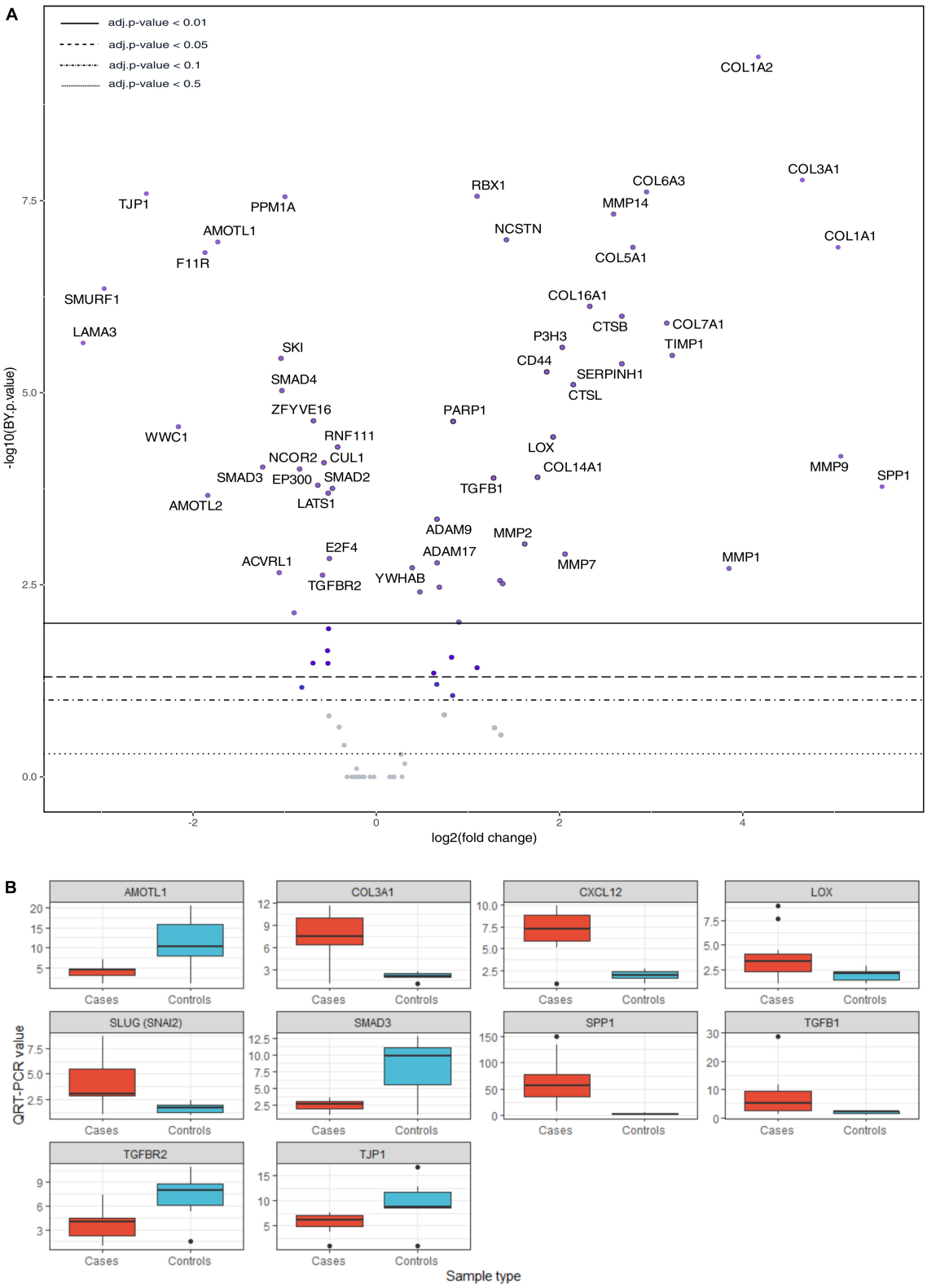

FIGURE 5
(A) Volcano plot showing differentially expressed genes of collagen biosynthesis, extracellular matrix biosynthesis and degradation, TGF-β and Hippo pathways. **(B)** Validation by qRT-PCR. *T*-test: AMOTL1 $p = 0.031$, COL3A1 $p = 0.00028$, CXCL12 $p = 3.9$ e-05, LOX $p = 0.027$, SLUG (SNAI2) $p = 0.0052$, SMAD3 $p = 0.014$, SPP1 $p = 0.00083$, TGFB1 $p = 0.043$, TGFBR2 $p = 0.029$, TJP1 $p = 0.072$. QRT-PCR units: ΔΔCt.

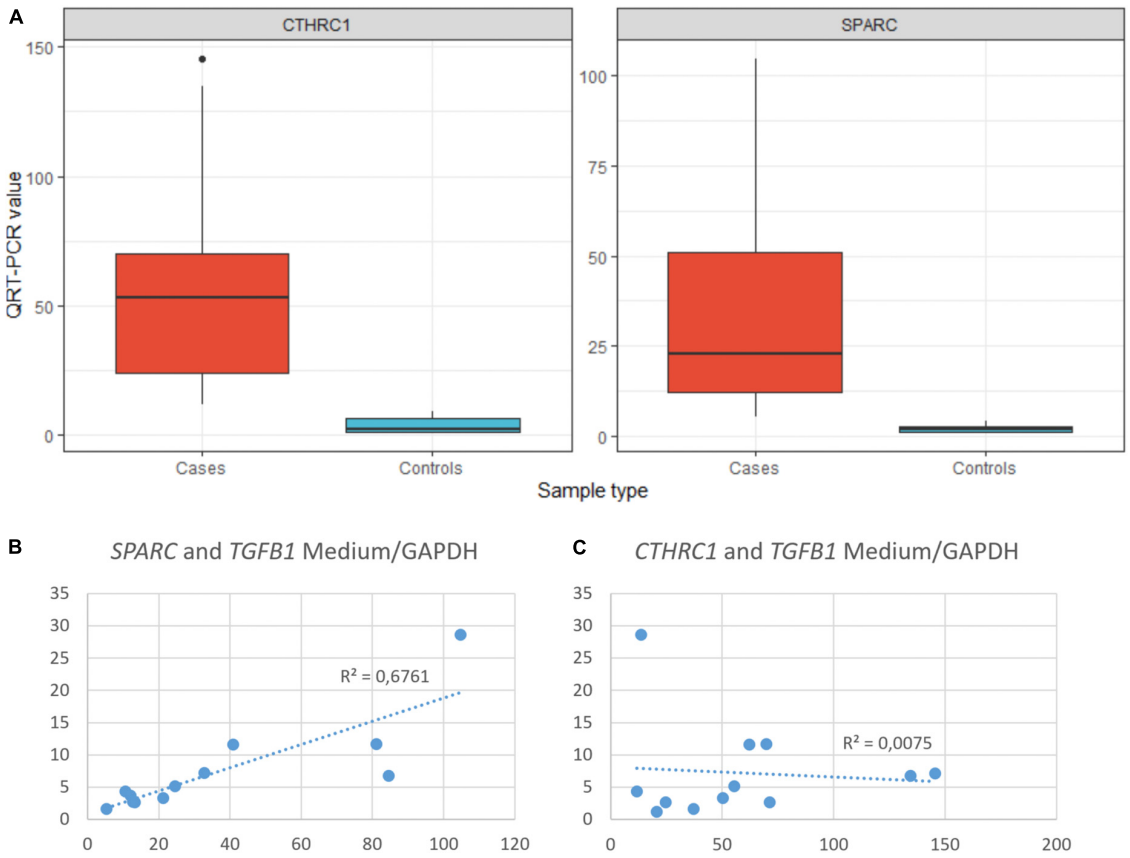

FIGURE 6
(A) Statistical differences in the expression of CTHRC1 and SPARC between COVID-19 (cases) and normal (controls) samples. *T*-test: CTHRC1 $p = 0.0012$, SPARC $p = 0.0054$. QRT-PCR units: ΔΔCt. **(B)** Correlation between the expression of SPARC and TGFβ1 in COVID-19 samples. **(C)** Correlation between the expression of CTHRC1 and TGFβ1 in COVID-19 samples.

were hypoexpressed, our results suggested non-canonical activation of the TGFβ pathway. Interestingly, the PI3K-AKT signaling pathway, a common target of the non-canonical (non-Smad2/3) TGFβ pathway (20), was activated in our series (**Figures 4**, **5** and **Supplementary Figure 1**).

COVID-19 fibrosis vs. usual interstitial pneumonia/idiopathic pulmonary fibrosis

In order to analyze possible similarities between proliferative DAD in COVID-19 patients and UIP/IPF, the transcriptomic profile of biopsies obtained in 4 UIP/IPF patients were analyzed. When compared to normal lung samples, UIP/IPF samples showed up- and down-regulation of similar pathways to those observed in COVID-19 patients. Thus, genes related with collagen biosynthesis and ECM biosynthesis and degradation were up-regulated in UIP/IPF samples (**Figure 11**). In fact, overexpression of some genes involved in these processes, such as *COL1A2* and *COL3A1*, were observed in both COVID-19 and UIP/IPF. In general, there was a higher expression in COVID-19 samples, indicating more fibrogenesis in COVID-19-associated DAD (**Supplementary Table 6**). Immunohistochemical analysis in our cohort demonstrated that CTHRC1 and SPARC expression was characteristic of pFBs in fibroblast foci (**Figure 12**).

Epithelial to mesenchymal transition in COVID-19 patients' fibrosis

EMT has been proposed as a mechanism to recruit fibroblast in some fibrogenic lung conditions (11). True EMT is a complex process that implies that epithelial cells lose their epithelial characteristics and acquire a mesenchymal phenotype (21). Our gene expression analysis indicated that the EMT process was upregulated in the lungs of COVID-19 patients (**Figures 4**, **5**). However, genes involved in EMT are usually expressed in fibroblasts and other mesenchymal cells, which were increased in number in COVID-19 lungs. To test if epithelial lung cells modified their epithelial phenotype to

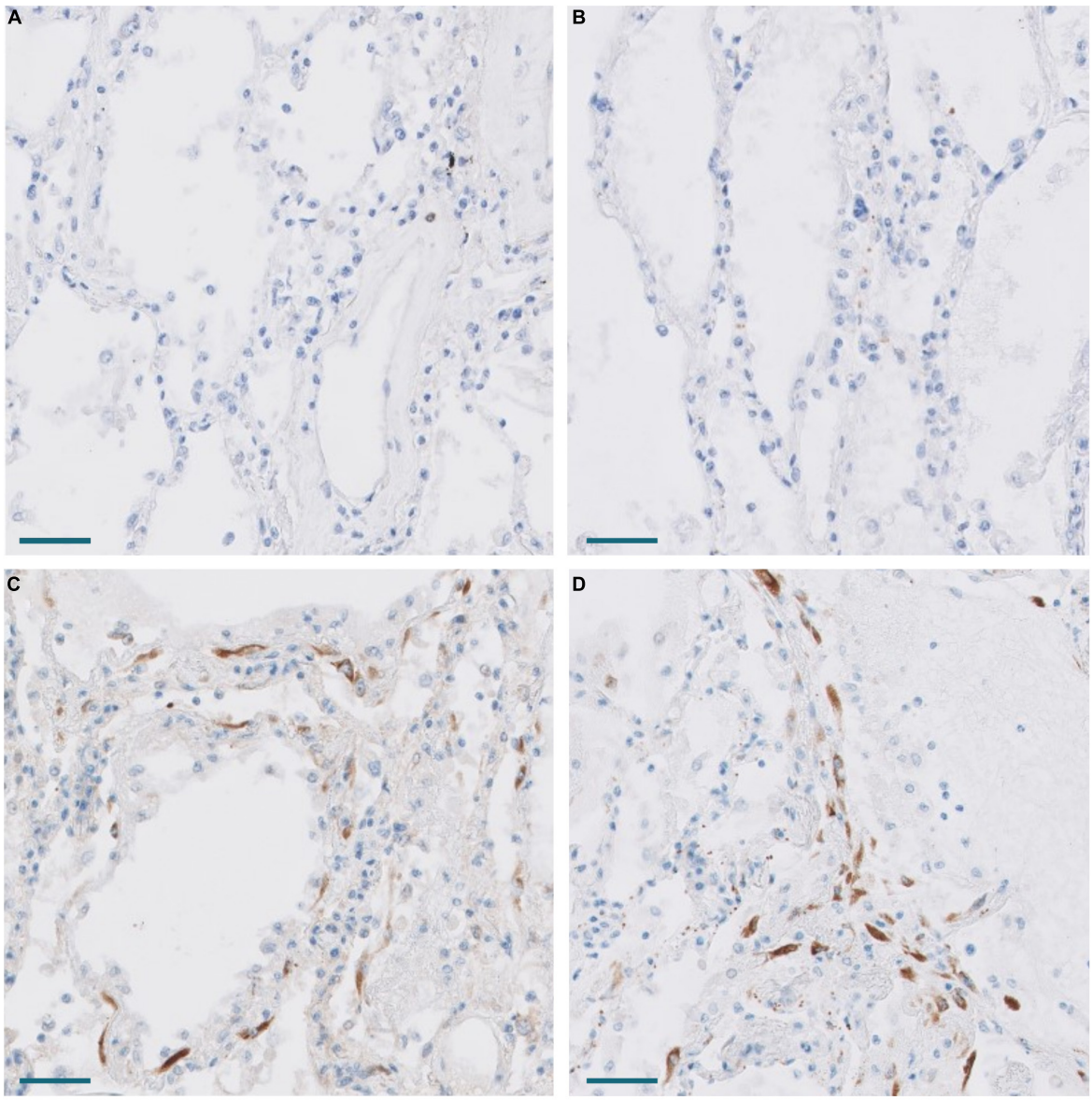

FIGURE 7
Expression of CTHRC1 and SPARC in the lung of Pt. 3 with 13 days evolution. **(A,B)** No expression of CTHRC1 and SPARC in normal lung areas. Scale bar: 100 μm. **(C,D)** CTHRC1 **(C)** and SPARC **(D)** expression in interstitial fibroblasts in areas with early exudative diffuse alveolar damage. Scale bar: 100 μm.

transform into fibroblasts, we analyzed cadherins and catenins by immunohistochemistry since they were not included in the Nanostring panel. The first step of EMT is cadherin switching, by which epithelial cells lose E-cadherin and acquire a mesenchymal cadherin (such as N-cadherin or cadherin 11), which is usually accompanied by the cytoplasmic relocation of catenin, such as p120-catenin. E-cadherin and cadherin 11 were expressed in normal bronchial, bronchiolar, and alveolar epithelial cells. In addition, they were expressed in hyperplastic epithelial alveolar cells in COVID-19 patients. Due to cell enlargement, the expression of cadherin 11 was more evident in these hyperplastic cells (Figure 13). β-catenin and p120 were expressed in epithelial cells, fibroblasts, and endothelial cells in healthy lungs. No evident changes were observed in the location of these molecules in epithelial cells of COVID-19 patients. Specifically, no nuclear expression of β-catenin was observed in any cell type and p120 was mainly expressed in the cell membrane without evident relocation into the cytoplasm (not shown). Moreover, Slug, a transcriptional repressor of E-Cadherin that was upregulated in our transcriptomic analysis (Figure 5), was expressed in some fibroblasts in COVID-19 patients but not in epithelial cells (not shown). These data argue

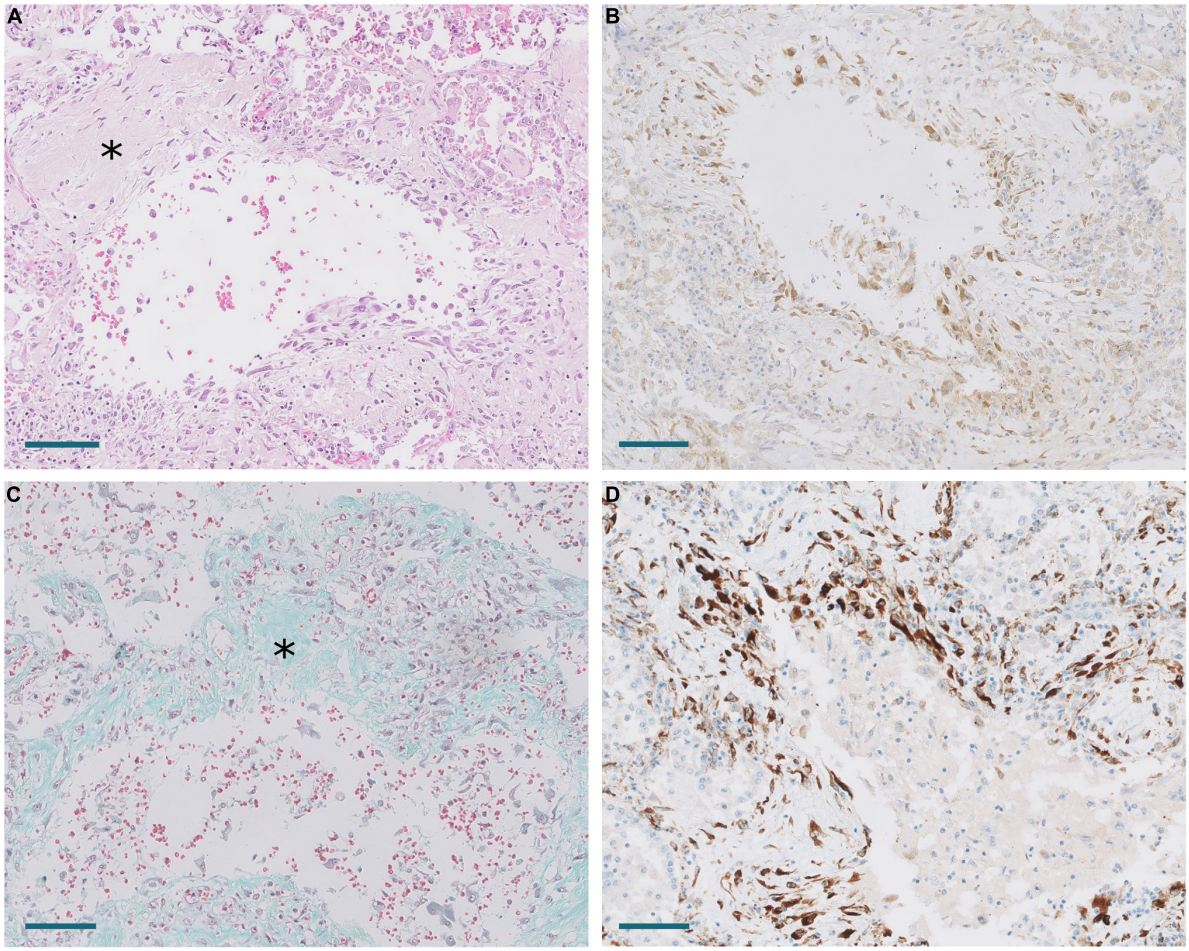

FIGURE 8
Areas of periductal proliferation/fibrosis in Pt.18 with 43 days of disease evolution. **(A)** Hematoxylin and eosin. Note deposition of collagen (*). Scale bar: 100 μm. **(B)** Expression of CTHRC1. Scale bar: 100 μm. **(C)** Masson trichrome stain. Note collagen deposition in green (*). Scale bar: 100 μm. **(D)** SPARC expression. Scale bar: 100 μm.

against a role of EMT in the process of recruiting pFB in DAD in COVID-19 patients.

Overexpression of the Hippo pathway

The Hippo pathway has been implicated in lung fibrosis (22), but it's possible role in COVID-19 lung pathology has not been analyzed. Our transcriptomic analysis indicated that the Hippo signaling pathway was downregulated in COVID-19 patients. Thus, several genes involved in the Hippo pathway were downregulated in our NanoString analysis and these results were confirmed by RT-PCR (**Figures 4**, **5**). The inhibition of the Hippo pathway leads to the activation of its effector protein YAP, which has been implicated in both normal lung development and IPF. By RT-PCR, there was a statistically significant increase of *YAP* expression in COVID-19 lung samples compared with controls. By IHC, mild YAP expression was observed in alveolar epithelial cells and in the basal cells of bronchial epithelium of normal lung samples. Increased nuclear and cytoplasmic YAP expression was mainly observed in hyperplastic pneumocytes of COVID-19 samples (**Figure 13**). These data suggest that Hippo signaling is modulated in epithelial cells in COVID-19 patients.

Discussion

This series included 33 COVID-19 patients with a prolonged illness (median 36 days), hospitalization (median 28 days) and mechanical ventilation (median 23 days). We demonstrated that, although proliferating DAD predominated, some degree of fibrosing DAD was present in 27 patients with more than 2 weeks of disease evolution, being the predominant pattern in 4 patients (12%). Few autopsy series have analyzed the evolution to fibrosis in COVID-19 patients. Li et al. (1) analyzed a cohort of 30 minimally invasive autopsies with a mean illness duration

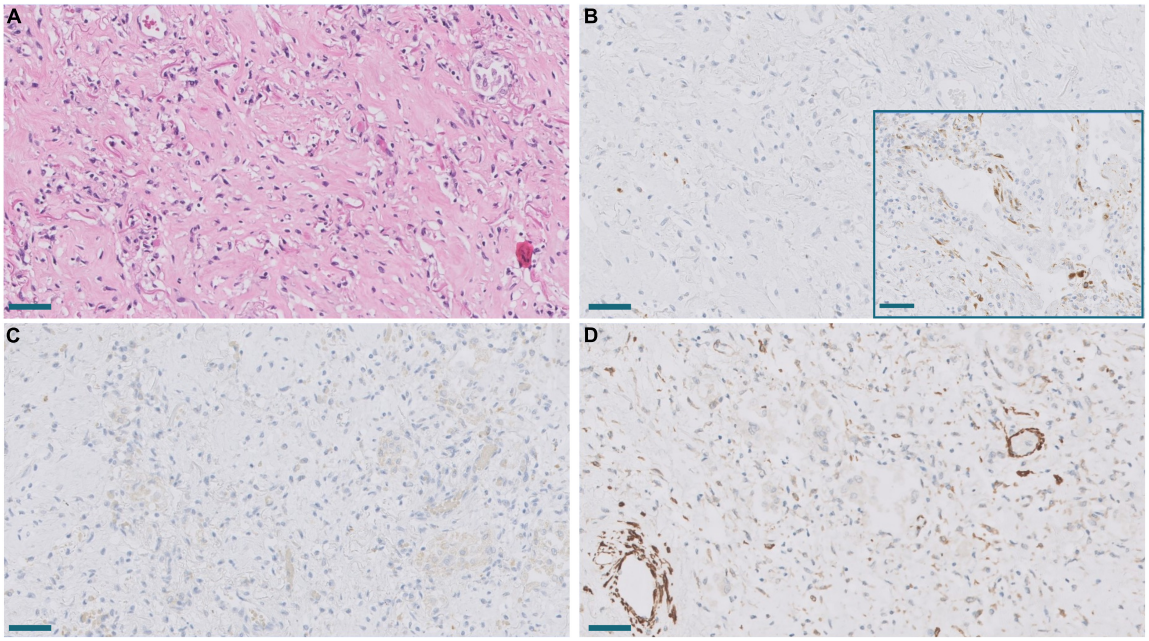

FIGURE 9
(A) Established area of mature fibrosis (HE). Scale bar: 100 μm. **(B)** Lack of expression of SPARC in mature fibrosis in contrast to moderate SPARC expression in an active proliferative area (inset). Scale bar: 100 μm. **(C,D)**. Lack of CTHRC1 expression **(C)** and diminished α-muscle actin expression **(D)** in the same fibrotic area. Scale bar: 100 μm.

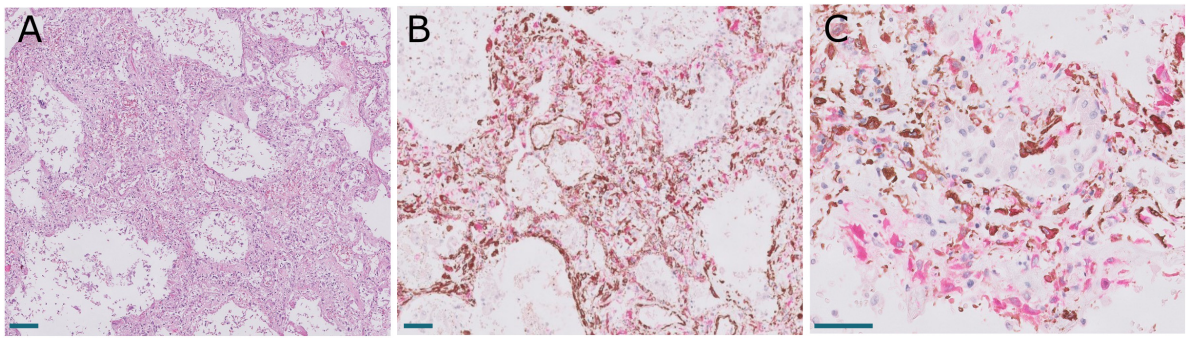

FIGURE 10
Lung lesions in Pt. 25 with 41 days of evolution. **(A)** Hematoxylin and eosin showing interstitial enlargement. Scale bar: 100 μm. **(B,C)**. Dual immunostaining with SPARC (magenta) and α-muscle actin showing a heterogeneous population of fibroblast expressing different levels of both proteins. Scale bar: 100 μm.

of 43 days and observed that fibrosis was the predominant pattern in 43% of the patients. However, this high incidence might be an overestimation since the tissue was obtained by ultrasound-guided core biopsy of selected areas. Regarding fibrosis extension, this reached a maximum of 48%. Similarly, Melms et al. (10) analyzed a series of 16 autopsies with a mean illness duration of 28 days and observed a maximum of 30% of fibrosis in some patients.

Although the presence of fibrosis correlated with the duration of illness, hospitalization and ICU stay, important differences were observed among patients with a similar evolution time. These differences were probably related to different factors, such as SARS-CoV-2 persistence, previous lung conditions, superimposed infections and the ventilatory management of each patient. In this sense, we must stress that exudative DAD was present in most patients, even in those with prolonged evolution. Thus, indicating the existence of persistent lung injury in most patients.

The detection of Sars-Cov2 RNA in most lung samples, but not in other organs in this series (17), suggests a role of viral persistence in the maintenance of lung injury in COVID-19 patients. It is reasonable to speculate that viral persistence

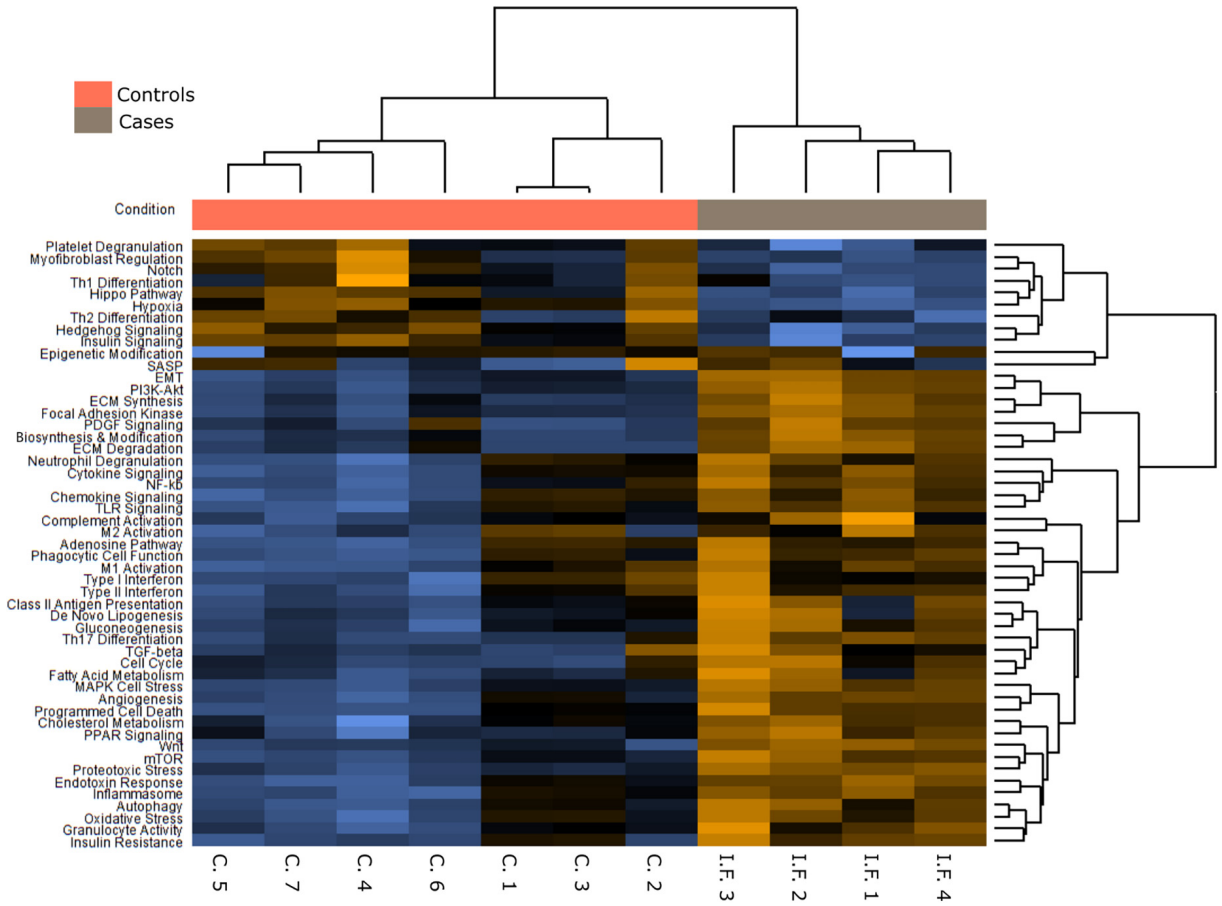

FIGURE 11
Pathways differentially expressed in idiopathic pulmonary fibrosis (IPF) in comparison with normal lung.

could be partially due to a defective viral clearance by impaired host immunity in this cohort of aged patients, most of them with lymphopenia and subjected to immunosuppressive therapy such as corticoid.

In order to gain an insight into the mechanisms involved in the progression of lung lesions, we performed a transcriptomic and immunohistochemical analysis of lung samples. Concordant with our morphological observations, we observed overexpression of genes involved in the fibrogenic processes such as collagen biosynthesis and ECM remodeling in COVID-19 samples. Lung fibroblasts represent a heterogenous population of cells whose diversity is being elucidated by sc-RNA seq analysis. Tsukui et al. (9) identified a subpopulation of pFB characterized by the expression of *COL1A1* and *COLA31* in IPF samples (two of the genes highly overexpressed in our NanoString analysis in COVID-19 samples) and other ECM genes such as *SPP1* (also overexpressed in our COVID-19 samples) or *TNC* (not included in our panel). Among ECM genes, *CTHRC1* was the most specific for pFB. An enrichment of *COLA31* + */CTHRC1* + pFB has also been observed in some COVID-19 samples (8, 10, 23). However, the temporo-spatial expression of CTHRC1 + cells has not been previously described in these patients. We observed a progressive increase of CTHRC1 + cells from normal lungs, in which they were absent, to proliferative DAD. Their increased frequency during this period suggested that CTHRC1 + cells were pFBs promoting rapidly evolving lung fibrosis in individuals with COVID-19. CTHRC1 + cells were initially located in alveolar septa and then distributed in areas of septal and periductal fibrosis. As previously suggested in IPF, CTHRC1 + cells seemed to respond to alveolar injury by migrating into injured areas where they participated in tissue fibrosis by producing excess quantities of ECM (9).

Our study also suggested an important role of the fibrogenic factor SPARC in COVID-associated lung fibrosis. SPARC is a matricellular component of ECM. It has been demonstrated that lung fibroblasts isolated from IPF patients constitutively express more SPARC than those derived from subjects without IPF (24). Moreover, SPARC-null mice display a diminished degree of pulmonary fibrosis compared with control mice after exposure to bleomycin (19). In addition, it has been

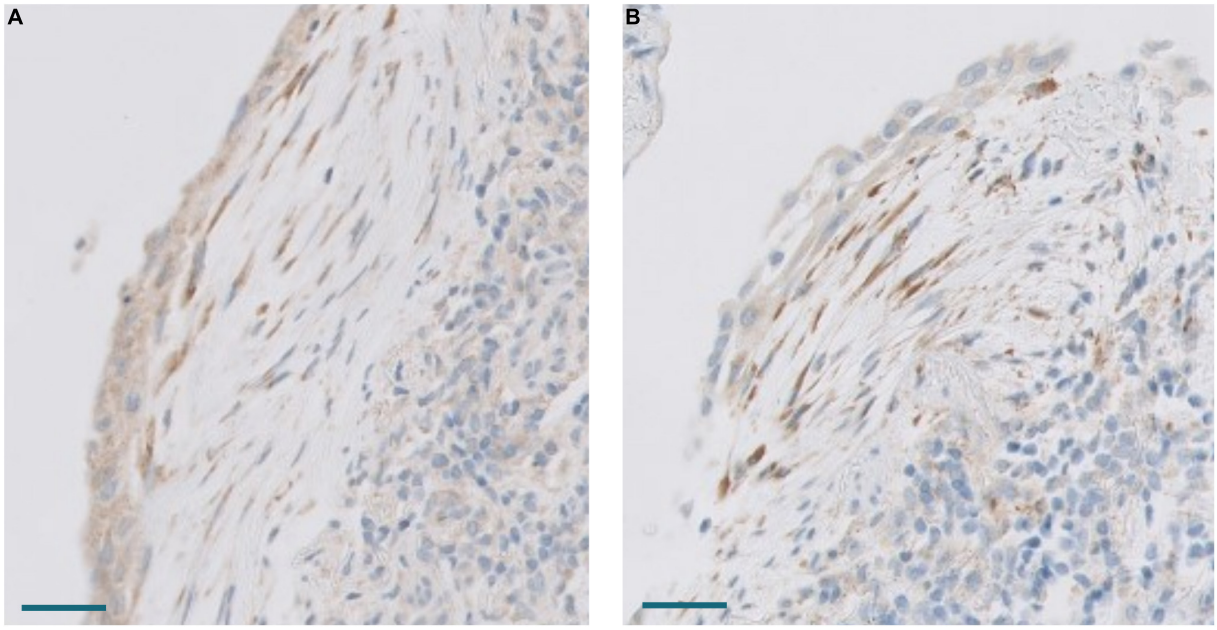

FIGURE 12
(A) Expression of CTHRC1 in a fibroblastic focus. Scale bar: 100 μm. **(B)** Expression of SPARC in the same fibroblastic focus. Scale bar: 100 μm.

demonstrated that SPARC secreted by IPF fibroblasts acts as a paracrine signal promoting persistent alveolar epithelial activation, thus, preventing normal epithelial repair responses and restoration of tissue homeostasis (24). Whereas these previous observations clearly demonstrated that SPARC is involved in the development of IPF, its possible role in other pulmonary lesions, including COVID-19 DAD, was not previously evaluated in deep. Although the expression of SPARC was consistently increased in some sc-RNA-Seq datasets of COVID-19 patients (10), no previous studies have analyzed its expression during the different phases of DAD nor its tissue distribution. Our dual immunostaining study with an anti-α-actin antibody indicated that some, but not all, SPARC-positive fibroblast also expressed different amounts of α-muscle actin, suggesting different functional states among the population of pathological (or activated) fibroblast. Our study also demonstrated that areas of mature fibrosis were devoid of cells expressing CTHRC1, SPARC or α-actin, suggesting that loss a "pathological" or "activated" fibroblast phenotype was associated with fibrosis maturation, as previously reported during heart infarct scar maturation (25).

We compared the transcriptomic profile of proliferative DAD in COVID-19 with that observed in a group of UIP/IPF lesions and found up- and down-regulation of similar pathways in both conditions. Thus, genes related with collagen biosynthesis and ECM biosynthesis and degradation were up-regulated in both COVID-19 and UIP/IPF. In addition, a population of CHTR1 + and SPARC + fibroblasts were present in both active proliferative areas of DAD and in fibroblastic foci of UIP/IPF. These results indicate that, despite differences in etiology, time of evolution and morphology of lesions, the fibrotic process in both entities is associated with a similar transcriptomic program and with the activation of a similar population of fibroblasts. Accordingly, some studies have suggested the use in COVID-19 patients of anti-fibrotic drugs currently approved for the treatment of IPF (26).

EMT has been implicated in lung pathologies as a mechanism to promote fibrosis (11, 27). The study of cadherins and catenins in this series seems to exclude EMT as an mechanism of epithelial transdifferentiaton to fibroblasts in COVID-19 DAD, as also suggested in IPF (11, 28). Cadherin 11 is a mesenchymal cadherin whose expression in epithelial cell is usually related with EMT. An interesting observation in our study was the constitutively co-expression of cadherin 11 with E-cadherin in normal epithelial lung cells, which has not been previously reported. Accordingly, the expression of cadherin 11 in hyperplastic COVID-19 alveolar epithelial cells does not represent the cadherin switching process that initiates EMT. Both E-cadherin and cadherin 11, together with β-catenin and p120 showed a normal expression pattern in alveolar epithelial cells and other epithelial cells in COVID-19 patients. Whereas these data seemed to exclude the acquisition of a mesenchymal (fibroblastic) phenotype by epithelial cells, this does not preclude a paracrine role of epithelial cells in the promotion of fibrosis through the production of growth factors after the activation of EMT program (11, 27). The immunohistochemical analysis of β-catenin supported our transcriptomic results suggesting a minor role of the canonical

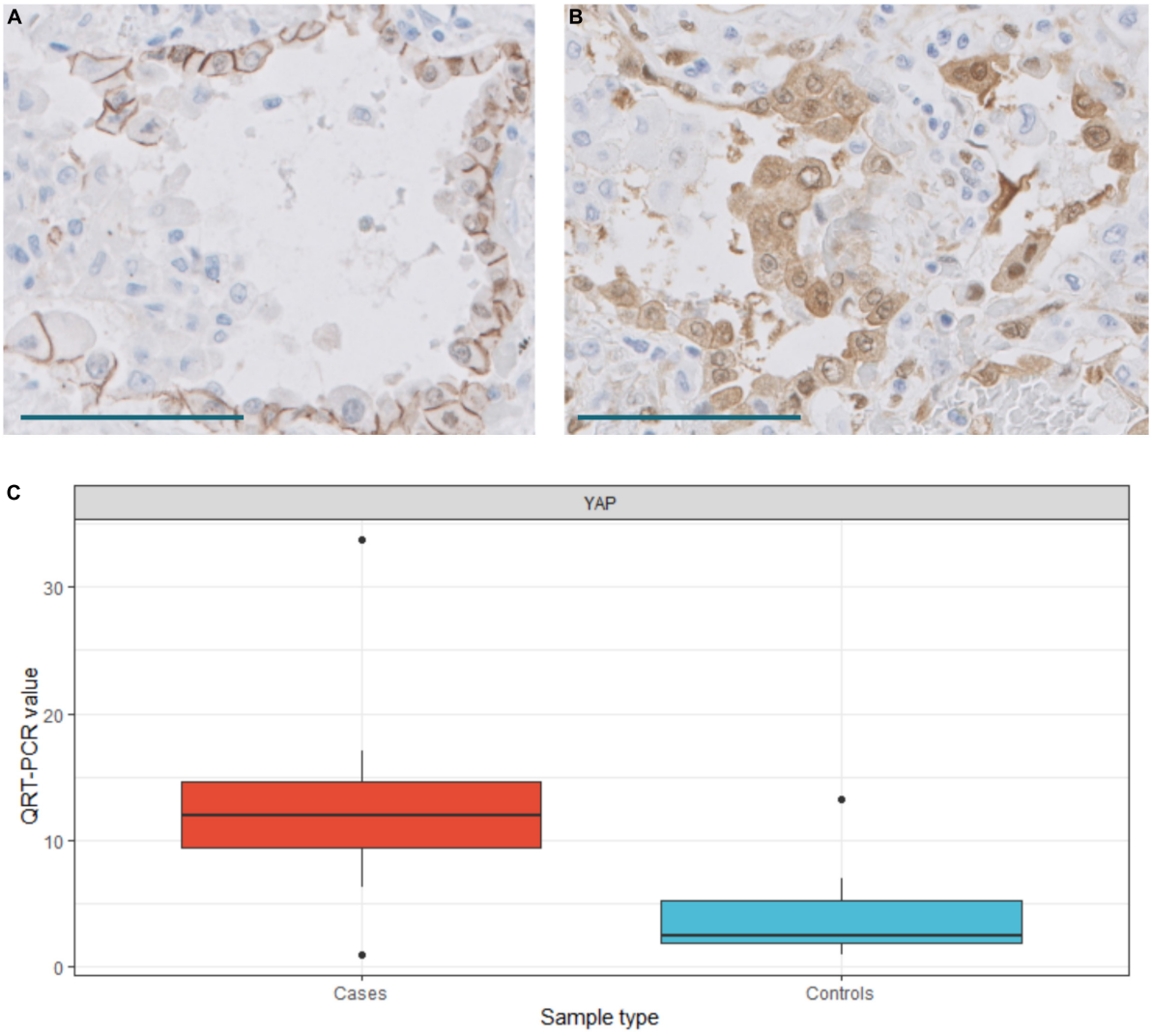

FIGURE 13
(A) Cadherin 11 expression in hyperplastic pneumocytes. Scale bar: 100 μm. **(B)** YAP expression in hyperplastic pneumocytes. Scale bar: 100 μm. **(C)** Differences in YAP expression between normal (control) and COVID-19 (case) samples. *T*-test, $p = 0.0076$. QRT-PCR units: ΔΔCt.

WNT pathway in fibrogenesis, since no nuclear β-catenin expression was observed in any cell population. Our data are in accordance with those reported by Chilosi et al. (29), where nuclear β-catenin was not observed in pulmonary diseases such as DAD, organizing pneumonia, non-specific interstitial pneumonia and desquamative interstitial pneumonia.

In our transcriptomic study, we observed down-regulation of the Hippo pathway. To gain insights into the main cellular component implicated in the modulation of the Hippo pathway, we performed an IHC analysis of YAP expression, one of the main effectors of this pathway. We observed overexpression of YAP mainly in hyperplastic epithelial alveolar cells, suggesting a role of the Hippo pathway in mediating the epithelial lung response after SARS-COV2 infection. Our results confirm and expand previous observations indicating that the Hippo signaling modulates alveolar regeneration after acute lung injury (30). During embryogenesis, YAP is expressed in progenitor basal cells and controls airway epithelial differentiation (31, 32). When these progenitor cells are subjected to acute injury, YAP localization shifts from the cytoplasm to the nucleus and proper differentiation does not occur, resulting in epithelial hyperplasia and stratification (33). The Hippo signaling pathway is also modulated in epithelial cells in IPF, where nuclear YAP is expressed in epithelial cells (22). Abnormal regulation of the Hippo pathway has been observed during infection with a variety of viruses such as HBV, HCV, MCV, ZIKV, EBV, KSHV, HPV, and MuPyV (34). Recently, it has been reported that several proteins involved in the Hippo pathway, including YAP, can be targeted by the SARS-CoV-2 protease $3CL^{pro}$ (35). Although the exact role of SARS-COV2 in the modulation of

the Hippo pathway remains to be established, our study suggests that deregulation of the Hippo pathway may contribute to the dramatically altered cell morphology in SARS-CoV-2–infected epithelial cells in the lungs of COVID-19 patients.

In summary, our study shows that progression to fibrosis in severe COVID-19 was associated with overexpression of fibrogenic pathways and increased in CTHRC1- and SPARC-positive pFB. Whereas the Hippo pathway seemed to be implicated in the response to epithelial cell damage, EMT was not a major process involved in COVID-19 mediated lung fibrosis. A possible role of viral persistence in the maintenance of lung damage is therefore suggested.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE206788.

Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committee, Ramón y Cajal University Hospital, approved the study (reference: Necropsias_Covid19; 355_20). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

JP and BP-M: study design. TB, JR-B, RP, and DP: clinical data collection. BP-M, JP, IC-B, AB, MG-C, IG-G, and MG-C: pathological evaluation. TC-C, DP, and YR: transcriptomic analysis. MR and EC: tissue processing. JG: viral analysis. IC-B, DP, and TC-C: statistical analysis. JP, BP-M, and IC-B: drafting the manuscript. All authors: discussion and final approval of manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.976759/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(A) Volcano plot. Genes differentially expressed between COVID-19 and normal samples. **(B)** Bar graphs of some of differentially expressed pathways between COVID-19 and controls: collagen biosynthesis and modification, extracellular matrix (ECM) degradation, epithelial to mesenchymal transition (EMT), PI3K-Akt, Hippo pathway and TGF-β.

SUPPLEMENTARY TABLE 1
Clinicopathological characteristics of the controls used. Four controls were taken from IPF biopsies and 7 control from normal lung present in segmentectomies due to pneumothorax.

SUPPLEMENTARY TABLE 2
Antibodies, clones, dilutions, and providers.

SUPPLEMENTARY TABLE 3
Detailed information of the cases in the study.

SUPPLEMENTARY TABLE 4
Primers design for validation.

SUPPLEMENTARY TABLE 5
Results of Nanostring analysis, comparing COVID-19 samples and controls.

SUPPLEMENTARY TABLE 6
Common genes differentially expressed in COVID-19 and IPF/UIP samples when compared with normal samples.

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Dynamic inflammatory response among routine laboratory biomarkers and their predictive ability for mortality in patients with severe COVID-19

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Background: The severity of coronavirus disease 2019 (COVID-19) is related to several factors, including age, sex, and comorbidities (obesity, type 2 diabetes, and hypertension). However, systemic inflammation plays a fundamental role in COVID-19 pathophysiology. Several studies have described this association employing specific biomarkers that are not routinely used in clinical practice. On the other hand, very few reports in the literature focused on the analysis of the routine laboratory biomarkers to predict the outcome of severe COVID-19 patients.

Objective: We aimed to analyze the dynamic inflammatory response using routine laboratory biomarkers to predict in-hospital mortality in Mexican patients with severe COVID-19.

Methods: This is a cohort study including patients with severe COVID-19. Demographic characteristics were retrieved from medical charts and biochemical parameters were measured at hospital admission and subsequently on days 3, 5, 7, 10, 14, and 21 during the hospital stay; measurements were stopped when patients were discharged from the hospital (alive or death).

Results: A total of 250 patients were included in the study, 40.8% of patients died. The analyzed routine laboratory parameters, such as serum levels of neutrophil-to-lymphocyte ratio, C-reactive protein, and D-dimer remained elevated in hospitalized patients who did not survive, whereas eosinophil and platelets were maintained at lower levels. In the multivariate analysis,

leukocytes, and neutrophils were the best biomarkers for predicting mortality risk and were independent of age, gender, or comorbidities.

Conclusion: Our results support the use of routine laboratory biomarkers as predictors of mortality in Mexican hospitalized patients with severe COVID-19.

KEYWORDS

SARS-CoV-2, Mexican population, leukocytes, neutrophils, CRP, D-dimer

Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, has been spreading worldwide with 625,740,449 confirmed cases of COVID-19, including 6.563.667 deaths by 26 October 2022 (1). COVID-19 disease is clinically heterogeneous, the most common symptoms are fever (55–65%), cough (45–50%) and respiratory distress (27.5%), whereas diarrhea (9.5%) and vomiting (6.5%) are less common (2). In severe cases, the disease can cause systemic inflammation, conditioning multiple organ failure, and the appearance of complications such as acute respiratory distress syndrome (ARDS), acute cardiac injury, acute kidney injury, acute liver injury, sepsis, shock, coagulopathy, and pulmonary embolism, that require hospitalization (3–5). Vaccination decreased the rate of hospitalized patients need to be transferred to the intensive care unit (ICU), moving from 20.6% to around 2% (6). Latin America became one of the most affected regions, and Mexico ranked as one of the first places regarding the number of deaths (7). This situation in Mexico was related to the high prevalence of different comorbidities and conditions such as obesity, high blood pressure (HBP), and type 2 diabetes (T2D). In fact, these factors increase the risk of death up to 7.7 times (8). Furthermore, the presence of these abnormalities is not the only factor in COVID-19 pathogenesis. These pathologies are related to an increased proinflammatory state, which plays a fundamental role in the evolution and severity of COVID-19 (9). This response enhances the activation of the immune system, promoting an increase in the neutrophil count with a substantial reduction of CD4(+) T cell and CD8(+) T cell counts (effector T cells) altogether with the release of proinflammatory markers such as C-reactive protein (CRP), D-dimer, and several cytokines (8–11). The secretion of proinflammatory cells leads to an aberrant inflammatory response, named "cytokine storm," a potentially fatal immune disease, considered to be the main cause of disease severity and death in patients with COVID-19 (9, 12). There is little information about the role of inflammation on the severity and mortality of COVID-19 in the Mexican population. One study showed that serum biomarkers such as albumin (OR 3.76 [CI 95% 1.56–9.07], $P = 0.003$), lactate dehydrogenase (OR 5.45 [CI 95%2.36–12.57], $P < 0.001$) and neutrophil-to-lymphocyte (N/L) ratio (OR 4.64 [CI 95% 2.05–10.53], $P < 0.001$) were independent risk factors associated with mortality in COVID-19 patients (13). Another study showed that proinflammatory cytokine interlukin-6 (HR 1.01 [CI 95% 1.003–1.020], $P < 0.011$) is a risk factor associated with mortality in Mexican individuals (14). Nonetheless, information is limited in low-income countries, and further research is needed. Prognostic predictors obtained from routine laboratory biomarkers during the early phase of the disease might be useful to make timely treatment decisions; as well as predicting clinical progression and disease severity. The aim of the present study was to determine the dynamic inflammatory response among routine laboratory biomarkers and mortality in Mexican patients with severe COVID-19.

Materials and methods

Study design

A cohort study among patients attended in the COVID specialty unit between October 2020 and April 2021, whose fulfilled World Health Organization (WHO) diagnostic criteria for severe COVID-19 (15) and confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) for SARS-CoV-2. The study was approved by the Ethics Committee of the Hospital Regional de Alta Especialidad de la Península de Yucatán (2020-023), and it was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline (16).

Study population

Selection criteria of patients were as follows, inclusion criteria included: clinical signs of pneumonia (fever, cough, dyspnea, fast breathing) and respiratory rate >30 breaths/min; severe respiratory distress or O_2 saturation <90% on room air; meanwhile exclusion criteria were: patients diagnosed with critical COVID-19 according to WHO criteria (15) or any other

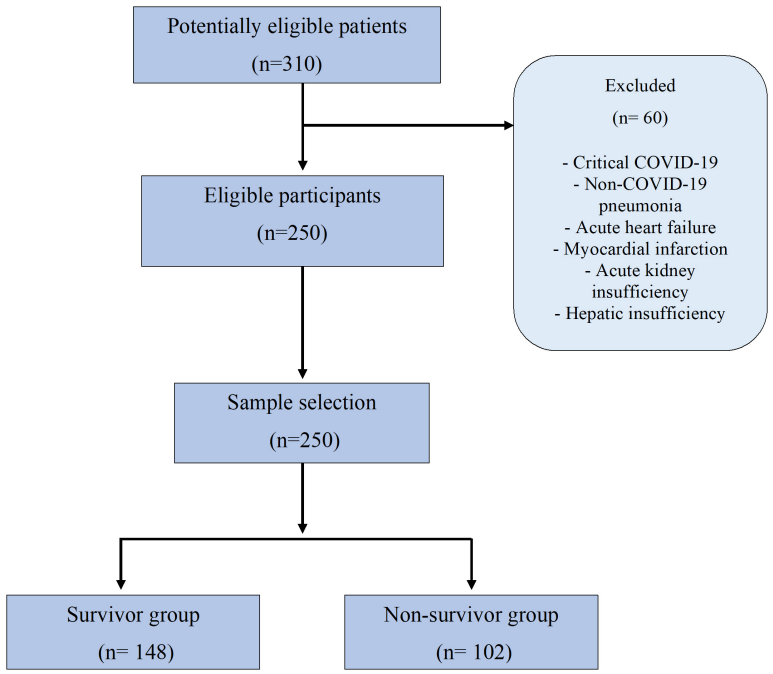

FIGURE 1
Flow chart.

TABLE 1 Comparative analysis of routine biochemical parameters of population according to survival status (n = 250).

	Survivors N = 148	Non-Survivors N = 102	P-value
Leukocytes ($\times 10^3$ cells/μL)	9.5 [7.3, 12.7]	13.1 [10.3, 17.8]	< 0.001
Neutrophils ($\times 10^3$ cells/μL)	7.5 [7.3, 7.9]	11.9 [11.3,12.5]	< 0.001
Lymphocytes ($\times 10^3$ cells/μL)	1.3 [1.2, 1.5]	0.9 [0.8, 1.0]	0.157
Eosinophil ($\times 10^3$ cells/μL)	0.07 [0.02, 0.18]	0.02 [0.01, 0.08]	< 0.001
Platelets ($\times 10^3$ cells/μL)	371 [350, 419.4]	291 [286.0, 293.5]	0.014
CRP (mg/mL)	76.5 [71.7, 81.6]	170 [150, 207]	< 0.001
D-dimer (ng/mL)	975 [757, 1516]	2035 [1600, 2517]	< 0.001
ERS (mm/h)	31 [26.5, 34]	33.7 [30.2, 40.2]	0.001
N/L ratio	5.63 [5.50, 6.11]	13.9 [12.2, 14.5]	< 0.001

Data are presented as median and Quartile 1, Quartile 3 [Q1, Q3]. Statistical analysis was performance by the Mann–Whitney U test. All results were considered statistically significant at $P < 0.05$. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; N/L ratio, neutrophil-to-lymphocyte ratio.

alternative diagnosis: non-COVID-19 pneumonia, acute heart failure, myocardial infarction, acute kidney insufficiency, and hepatic insufficiency.

Data collection: Clinical characteristics, blood sample collection, and routine laboratory biomarkers

Data were collected at admission, including sex, age, and self-reported comorbidities like HBP, T2D, and obesity (BMI > 30kg/m^2). Moreover, clinical characteristics were documented, such as oxygen saturation (O2 saturation), among others. Blood samples were individually collected at hospital admission and every 72 h for 21 days. Measurements were stopped at any time if either of the following: (1) patients were discharge alive or (2) death in-hospital. Biochemical parameters such as complete blood count (CBC), CRP, D-dimer were included, and the N/L ratio was calculated. The concentration of CRP was measured by immunoturbidimetric test (CRPL3®), D-dimer was evaluated by automated latex enhanced immunoassay f (Hemosil® D-dimer HS 500) and CBC by automatic flow cytometry using a semiconductor laser for leukocyte analysis (XT-2000i®).

Statistical analyses

Continuous variables were evaluated using the Kolmogorov–Smirnov test to analyze the type of distribution. The patients were classified as groups of survivors or non-survivors. Data are presented as the mean ± standard deviation (SD) or medians and 95% confidence interval [CI 95%]. Categorical variables are reported as frequencies. Differences between groups were performed with the Mann–Whitney U test for quantitative variables. The proportions were analyzed through the chi-squared statistical test. The risk of death by COVID-19 was analyzed considering the behavior of the variables from the time the participants entered the hospital until their discharge. The cut-off values of routine laboratory biomarkers were determined, and survival analysis was performed using the Kaplan–Meier method. Random-effects parametric survival-time model analysis was performed and generated a model unadjusted and adjusted by sex, age, and comorbidities. Data from participants with at least two measurements were considered for inclusion in the analysis. A value of $P < 0.05$ was considered significant. Data were analyzed using Stata V15.1.

Results

General characteristics of the study population

A total of 250 patients were included in the study, the mean age was 54.3 ± 15.3 years, and 68% were male (Figure 1). Mean of period of hospitalization was 9.42 ± 8.23 days and the most frequent comorbidities in the population included were obesity (51.6%), followed by HBP (33.2%) and T2D (30.4%). Baseline levels of different routine laboratory biomarkers were included, highlighting eosinophils (0.05 ± 0.10 × 10^3 cells/μl), CRP (174.63 ± 123.76 ng/ml), D-dimer (2010 ± 3584 ng/dl), and N/L ratio (11.9 ± 10.8) (Supplementary Table 1).

TABLE 2 Analysis of a survival-time model of the risk factors associated with mortality in severe COVID-19 patients.

	HR [CI 95%]	*P*-value
Leukocytes (×10^3 cells/μL)	1.31 [0.87, 1.98]	0.19
Neutrophils (×10^3 cells/μL)	2.47 [1.64, 3.72]	<0.001
Lymphocytes (×10^3 cells/μL)	0.49 [0.33, 0.72]	<0.001
Eosinophil (×10^3 cells/μL)	0.40 [0.22, 0.75]	0.004
Platelets (×10^3 cells/μL)	0.56 [0.35, 0.89]	0.010
CRP (mg/mL)	3.04 [2.03, 4.56]	<0.001
D-dimer (ng/mL)	2.46 [1.67, 3.64]	<0.001
ESR (mm/h)	1.13 [0.76, 1.68]	0.52
N/L ratio	2.68 [1.78, 4.05]	<0.001

Data are presented as median and 95% confidence interval [CI 95%]. All results were considered statistically significant at $P < 0.05$. CRP, C-reactive protein; ESR, Erythrocyte sedimentation rate, N/L ratio, neutrophil-to-lymphocyte ratio.

Comparison of clinical characteristics between populations according to survival status

Overall, we registered a mortality of 40.8% (n = 102). The comparisons according to survival status are summarized in Table 1. The presence of comorbidities such as HBP, T2D, obesity, chronic obstructive pulmonary disease (COPD), cardiopathy, chronic kidney disease (CKD), hepatopathy, human immunodeficiency virus (HIV), and dyslipidemia did not show significant differences according to the survival status in the patients (Supplementary Table 2).

Main risk factors of mortality in Mexican severe COVID-19 patients

The routine laboratory biomarkers were evaluated as far as 21 days. Our results showed that at baseline, the non-survivors group presented higher levels of neutrophils (12.9 ± 6.3 vs. 8.5 ± 4.9 × 10^3 cells/μl, $P < 0.0001$), CRP (220 ± 133 vs. 143 ± 106 mg/ml, $P < 0.0001$), N/L ratio (12.3 ± 9.82 vs. 6.79 ± 8.02 $P < 0.05$) and D-dimer (3105 ± 4668 vs. 1267 ± 2347 ng/ml, $P < 0.0001$) and N/L ratio (12.3 ± 9.82 vs. 6.79 ± 8.02 P < 0.05) (Figures 2A–D) compared with survivor group. Meanwhile, the survivor group showed higher levels of platelets (318 ± 185 vs. 283 ± 109 × 10^3 cells/μl, $P = 0.015$) and eosinophils (0.06 ± 0.10 vs. 0.04 ± 0.09 × 10^3 cells/μl, $P = 0.039$) compared with the non-survivor group (Figures 2E,F). Dynamical changes in the levels of immune response in the non-survivor group showed that on day 3 of hospital stay, the highest level of CRP (244 ± 137 mg/ml) was observed (Figure 2B), at day 7 the highest concentration of D-dimer (4335 ± 7615 ng/ml) was reported (Figure 2C). Seven days later, the highest concentration was observed in the levels of neutrophils (16.0 ± 7.5 × 10^3 cells/μl) (Figure 1A). On the contrary, in this group, it was observed that the lowest values for eosinophils (0.10 ± 0.13 × 10^3 cells/μl) were at day 10 (Figure 2F) and platelets (285.3 ± 153.9 × 10^3 cells/μl) at day 14 (Figure 2E). Over the period of analysis of the routine laboratory biomarkers, we evaluated the median of these variables throughout the follow-up of the participants. Results showed that the group of non-survivors showed an increase in the levels of leukocytes, neutrophils, CRP, D-dimer, ERS, and N/L ratio, and a decrease in the levels of eosinophil and platelets respect to the survivor group (Table 1).

To evaluate the impact generated by the modifications in the levels of routine parameters, cut-off points were determined for the study population (Supplementary Table 3) with model

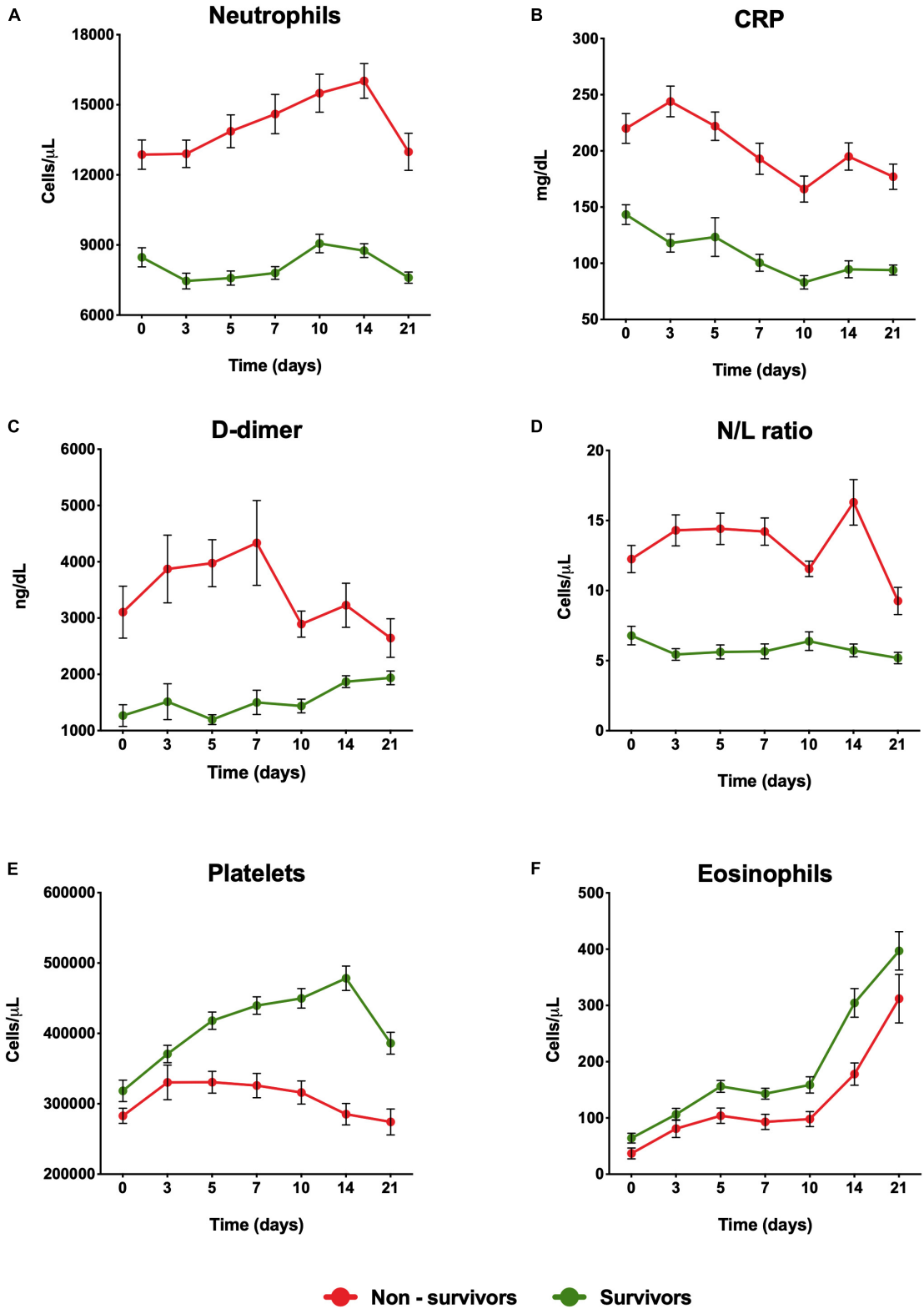

FIGURE 2
Dynamical changes in the levels of risk factors in severe COVID-19 patients during hospitalization. **(A)** Neutrophil, **(B)** CRP, **(C)** D-Dimer, **(D)** N/L ratio, **(E)** Platelets, **(F)** Eosinophils in patients within 21 days from admission onset. The patients were stratified into a non-survival group and a survival group. Data represents mean ± SD. CRP, C-reactive protein; N/L ratio, neutrophil-to-lymphocyte ratio.

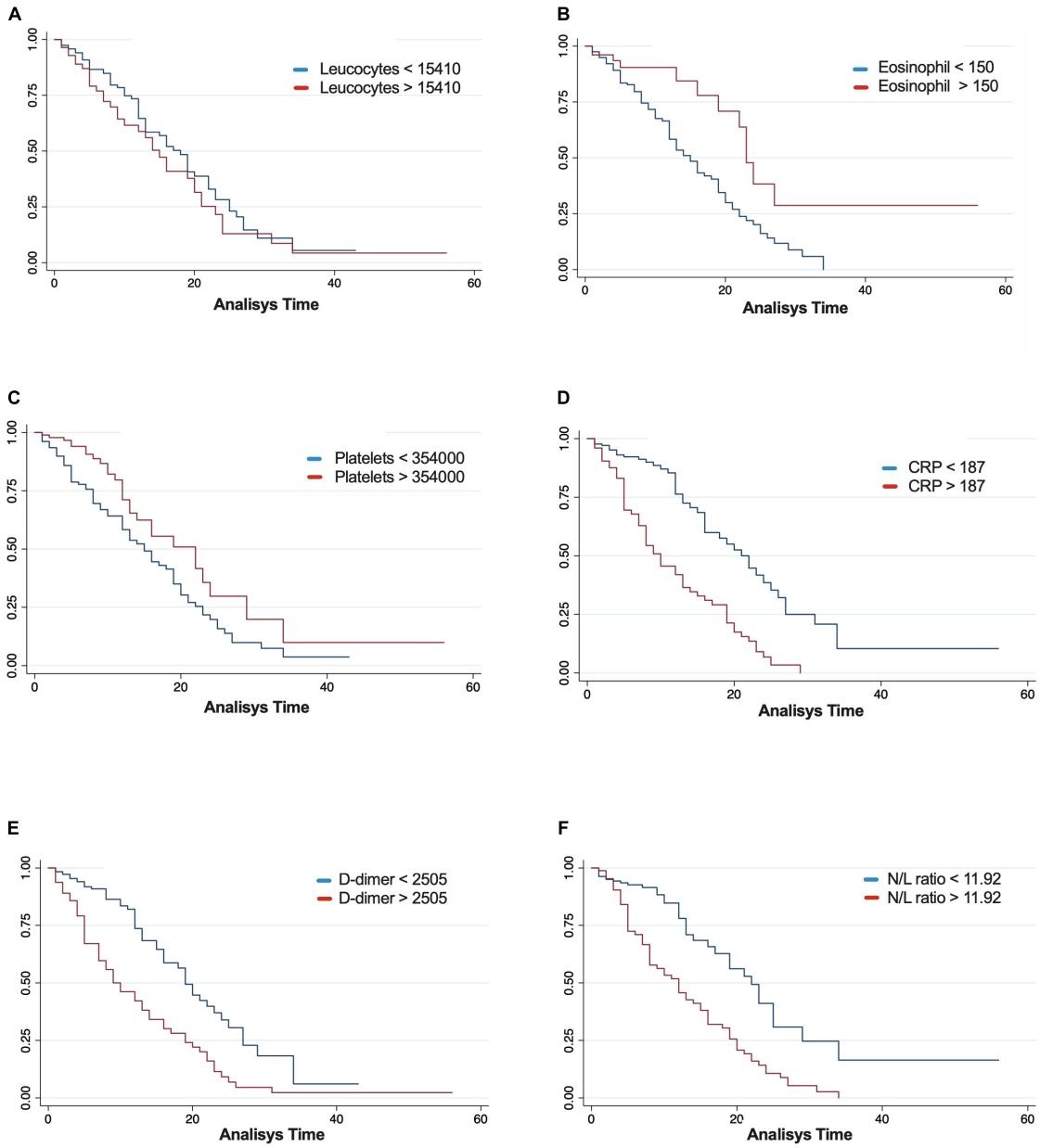

FIGURE 3
Kaplan–Meier survival estimates for leukocytes **(A)**, eosinophil **(B)**, platelets **(C)**, C-reactive protein (CRP) **(D)**, D-dimer **(E)**, and neutrophil-to-lymphocyte ratio (N/L ratio) **(F)**.

unadjusted and adjusted by sex, age, and comorbidities to determine the mortality risk for each parameter. Our results showed that CRP (HR 3.04, CI 95% 2.03–4.56), N/L ratio (HR 2.68, CI 95% 1.78–4.05), and D-dimer (HR 2.46, CI 95% 1.67–3.64) were the highest predictive variables for mortality ($P < 0.001$), whereas the levels of eosinophils, lymphocytes and platelets showed an inverse association for mortality, as lower the level the higher risk for an adverse outcome (Table 2 and Figures 3A–F).

Interestedly, inflammatory markers such as neutrophils, CRP, D-dimer, and N/L ratio considerably increased the risk of death (Table 2). On the other hand, the levels of lymphocytes, eosinophils, and platelets were associated with improving the survival of the patients (Table 2). After adjusting the analysis by covariates (age, sex, and comorbidities), leukocytes (HR 0.9996 [0.99, 0.99], $P = 0.03$) and neutrophils (HR 1.0003 [CI 95% 1.00, 1.00], $P = 0.007$) showed a significant association with mortality (Table 3).

TABLE 3 Analysis of risk factors associated with mortality in severe COVID-19 patients by adjusted model.

Characteristic	HR [CI 95%]	*P*-value
Leukocytes ($\times 10^3$ cells/μl)	0.9996 [0.99–0.99]	0.030
Neutrophils ($\times 10^3$ cells/μl)	1.0003 [1.00–1.00]	0.007

Statistical analysis was performance by random-effects parametric survival-time model with adjusted by sex, age, and comorbidities. Log likelihood = −180.82; $P < 0.0001$. All results were considered statistically significant at $P < 0.05$.

Discussion

In this study, the baseline, and subsequent changes of the routine laboratory biomarkers among the non−survival group were characterized by significantly higher levels of leukocytes, neutrophil count, N/L ratio, and inflammatory as well as coagulation markers such as CRP and D-dimer, while lymphocytes, eosinophils, and platelets count were significantly decreased. Besides, after estimating cut-off values, it was found that CRP, N/L ratio, neutrophils, and D-dimer are better predictors of mortality among the population. Our results strengthened the association between inflammatory response and mortality in patients with severe COVID-19. The proinflammatory biomarkers related to the Th-1 pathway such as leukocytes, neutrophils, CRP, and D-dimer were significantly associated with mortality in patients with severe COVID-19. During COVID-19 disease, there is a dysregulation of the inflammatory immune response and an increase of proinflammatory marker production. This alteration results in a dynamic process associated with tissue damage and multiple organ failure (17). Some studies identify the role of proinflammatory markers such as CRP, interleukine-6, and neutrophil count in the prediction of a worse prognosis in patients with COVID-19. Our results were accordingly with these findings, and introduce the concept of dynamic change in biomarkers as another factor associated with severity, whereas patients with mild disease are characterized by the presence of anti-inflammatory, prophagocytic, and antigen-presenting macrophages in the lungs, severe, and critical COVID-19 leads to an enrichment of hyperinflammatory macrophages with aberrant response in cytokines and inflammatory gene expressions (18). Previous reports highlighted that the increase of serum cytokines was related with an unbalance ratio with a decrease in lymphocytes and an increase neutrophils levels among patients with severe COVID-19 (19). This is consistent with previous studies, which showed that an excessively elevated neutrophil count released neutrophil DNA, and associated proteins that led to tissue damage, severe pneumonia, and death (19). High CRP levels are also linked to severe shock, systemic inflammatory response syndrome, and multiple organ dysfunction syndrome (20). In fact, a previous study showed that the cutoff point of CRP (≥40 mg/L) performed well in predicting mortality in patients with COVID-19 (21). Thus, the dynamic profile of CRP, has clinical relevance due to our results showed that CRP (>187 mg/L) increased 3.04 times the mortality risk in the population analyzed. This strengthened the suggestion of monitoring levels of CRP in these patients to identify a timely effective treatment to improve the prognosis of COVID−19. The latter was confirmed in hospitalized patients with hypoxia (SpO2 < 92%) in whom the addition of tocilizumab reduced the mortality rate by 15% at 28 days of follow-up whenever they had evidence of systemic inflammation (CRP ≥ 75mg/L) (22).

Another factor that contributes to the poor prognosis of the pathology is D-dimer, our results showed that D-dimer is a predictive variables for mortality. This is according with other studies that has been show similar results. In fact, a study reported that D-dimer levels increased in 36–43% of COVID-19 patients (23). Other study in the Turkish population showed that not only was D-dimer elevation common among patients diagnosed with COVID-19, but that this increase was associated with disease severity and mortality (24). A recent systematic review concluded that D-dimer is an independent predictor of COVID-19 mortality, even after subgroup analysis (countries, sample size, study design) (25). Mortality in COVID-19 patients has also been associated with changes in lymphocytes and platelets. In fact, it has been reported that in non-survivors, there is a decrease in levels of both parameters (26). Eosinophils are less well characterized in relation to COVID-19, thus some reports have found an association with the severity of the disease (27). In this sense, we found an inverse association of eosinophil levels with mortality. Lower levels of eosinophils might facilitate an alternative route of hyperinflammation, mainly due to Th-1. Additionally, the severity of the immuno-inflammatory state and dysregulation of the immune response are also related to alterations in levels of neutrophils and lymphocytes, which results in an increase in the N/L ratio. However, little number of studies consider the cost-benefit of this type of marker as an early indicator of severity in COVID-19. A meta-analysis showed that increased N/L ratio level have been associated with enhanced inflammation and a poor prognosis in COVID-19 patients, which is similar to our results (28).

This is the first evidence of the impact of routine laboratory biomarkers for mortality prediction in Mexican population. Because the Mexican health system continues to face challenges and has limited resources, the use of these routine laboratory biomarkers, particularly the N/L ratio and absolute neutrophil levels, and possibly special attention to eosinophils, could have an impact on the treatment of COVID-19 patients.

Our study has some limitations; firstly, only patients admitted to the hospital with severe COVID-19 were included, which leads to selection bias and therefore limits the overall applicability of the results. Besides, we were not able to measure specific molecules such as cytokines to reinforce our findings. However, to reduce the bias toward having heterogeneous disease conditions, critical illness cases (patients who required

non-invasive or invasive mechanical ventilation) were not included in our study.

Conclusion

The prompt dynamic analysis of biomarkers should be used for the early and appropriate detection of risk in patients with COVID-19 and making interventions accordingly. Our results suggest that leukocytes, neutrophil count, N/L ratio, CRP, and D-dimer values predicted mortality in patients with severe COVID-19 admitted to our hospital. These results may be used in the development of early strategies, which can also assist in the better management of patients.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

This study was approved by the Ethics Committee of the Hospital Regional de Alta Especialidad de la Península de Yucatán (2020-023), and it was conducted in accordance with the Declaration of Helsinki.

Author contributions

AC-T, VÁ-S, and AA-N designed the study and wrote the manuscript. AC-T, VÁ-S, and RL performed the experiments. AC-T, AG-G, and AA-N analyzed the data. All authors contributed to the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Do worsening lung ultrasound scans identify severe COVID-19 trajectories?

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Background: While point-of-care ultrasound (POCUS) has been used to track worsening COVID-19 disease it is unclear if there are dynamic differences between severity trajectories.

Methods: We studied 12-lung zone protocol scans from 244 participants [with repeat scans obtained in 3 days (N = 114), 7 days (N = 53), and weekly (N = 9)] $\geq$ 18 years of age hospitalized for COVID-19 pneumonia. Differences in mean lung ultrasound (LUS) scores and percent of lung fields with A-lines over time were compared between peak severity levels (as defined by the WHO clinical progression scale) using linear mixed-effects models.

Results: Mean LUS scores were elevated by 0.19 (p = 0.035) and A-lines were present in 14.7% fewer lung fields (p = 0.02) among those with ICU-level or fatal peak illness compared to less severe hospitalized illness, regardless of duration of illness. There were no differences between severity groups in the trajectories of mean LUS score 0.19 (p = 0.66) or percent A-lines (p = 0.40).

Discussion: Our results do not support the use of serial LUS scans to monitor COVID-19 disease progression among hospitalized adults.

KEYWORDS

lung ultrasound, point-of-care lung ultrasound, COVID-19, severe COVID-19, cohort study

Background

Serial point-of-care lung ultrasound (LUS) provides actionable results at the point-of-care without ionizing radiation. LUS has been an essential tool in evaluating patients with COVID-19 pneumonia, albeit with heterogenous uptake based on center expertise. In contrast to serial chest X-rays, which are no longer standard of care, serial LUS scans are performed without radiation exposure, are more sensitive for detecting lung pathology, and therefore may be more useful as a daily measurement.

While multiple studies (1–5) have assessed the prognostic value of LUS, few (6, 7) have assessed changes over time in a methodical manner. In addition to identifying resolving of severe pulmonary disease, changes in LUS findings could help monitor patient trajectories. However, the variability or trends over time have not been well-described. In the present study we evaluate the association between LUS characteristics (i.e., A-lines, B-lines, consolidations, pleural effusions, pleural line thickening, and a composite score averaged across lung zones) clinical severity among adults hospitalized with COVID-19.

Methods

We conducted a prospective enrollment of adults age ≥ 18 who were admitted to Johns Hopkins University Hospital and tested positive for SARS-CoV-2, in a larger COVID-19 prospective cohort (ClinicalTrials.gov number, NCT04496466), from April 2020 to September 2021. This protocol was approved by the Johns Hopkins University Institutional Review Board (IRB00245545). After screening SARS-CoV-2 RT-PCR positive patients, a convenience sample of 264 patients were enrolled depending on LUS-trained research staff availability as previously described (1). After enrollment, study visits including LUS scans occurred until hospital discharge on study days 3, 7, and weekly for up to 90 days from enrollment. To evaluate the value of serial scans, our analysis was restricted to 244 participants (413 scanning encounters) after excluding those with an initial scan at >28 days of symptom onset, only one scan, or those with subsequent scans > 7 days of the preceding scan (**Figure 1**).

As described (1), lung images were collected using 6-s clips from 12 zones using a Lumify S4 phase array probe (Philips, Amsterdam, Netherlands). Study personnel were subsequently masked to clinical information and provided reads identifying and characterizing A-lines (an indicator of normal lung), B-lines (an indicator of edema, fibrosis, or inflammation), consolidations, pleural effusions, and pleural line thickening. Due to a high number of incomplete studies given the hospitalized and intubated status of many patients, a minority (N = 58; 23.8%) had at least one complete LUS scan with all 12 zones. Therefore, the mean LUS (mLUS) score was calculated, including those with less than a full 12-zone exam. This composite score (ranging from 0 to 3 with a higher score signifying higher severity) is an average across zones with 1 point for discrete B-lines, 2 points for coalescent B-lines, and 3 points for lung consolidation as previously described (1). Mean number of lung zones scanned across all participants and visits was 5.6.

Participants were grouped by the highest severity reached at the peak of their illness (i.e., moderate or severe) with severe disease defined by requiring high flow nasal cannula, mechanical ventilation, or fatal cases based on the WHO clinical progression scale (8). Disease worsening was considered moving from a moderate level to a severe level and resolving disease was consider moving from a severe level to a moderate level of disease. Summary statistics were calculated for each week post-symptom onset. For individuals with multiple time points during a given week post-symptom onset, a time point was selected at random for each week post-symptom onset to decrease risk of confounding for summary statistics. Differences in mLUS score or percent A-lines changes over time (days post-symptom onset) between peak severity groups were evaluated using linear mixed-effects models. As a sensitivity analysis, models were also run using generalized estimating equations (GEE) and using severity as an ordinal covariate (moderate or severe). Data were analyzed using statistical analytical software Stata version 15.0 (StataCorp LLC, College Station, TX, USA) and figures were created in R (v3.6.3) using the "ggplot" (v3.3.3) package (v1.0.7).

Results

The cohort included 244 total participants [mean age of 58.2 (SD 15.0) years, and 55.7% female] with 199 participants at 0–14 days post-symptom onset at baseline scan and 45 participants at 15–28 days post-symptom onset. The median LUS time was 9.1 days (IQR: 5.0, 12.7 days) from symptom onset. The median time to peak illness was 11.0 days (IQR: 6.2, 18.3 days) from symptom onset among those that developed severe disease.

The distributions of lung zones with A-lines (normal), B-lines (abnormal), and pleural line abnormalities increased by each level peak severity at baseline (**Table 1**). At the baseline scan, a widely variable percentage of lung fields contained B-lines (median 58.3%, IQR: 33.3, 0.0%) in patients with moderate COVID-19 but a high degree of B-line changes was consistently present among those with severe peak severity (median 83.3%, IQR: 66.7, 100.0%) (**Supplementary Table 1**). Conversely, participants in the severe group had a lower percent of A-lines (median 63.6%, IQR: 33.3, 85.7%) compared to the moderate group (median 85.7%, IQR: 66.7, 100.0%). The mLUS score median was 1.0 (IQR: 0.5, 1.3) for the overall cohort.

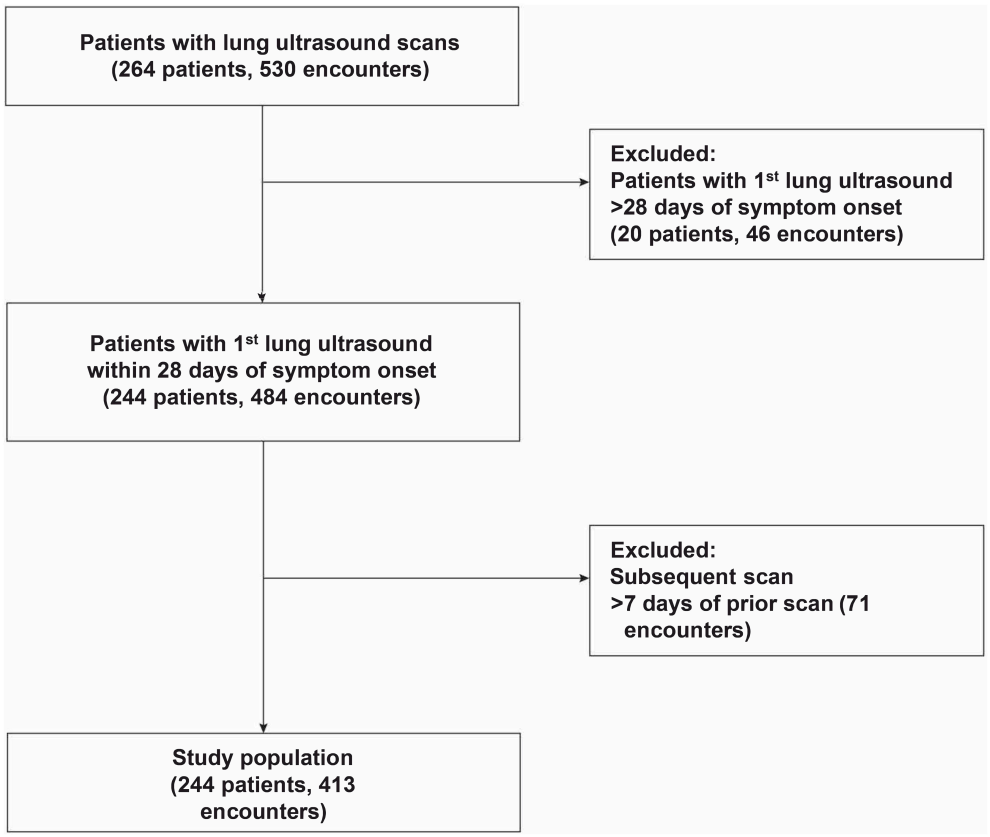

FIGURE 1
Flow diagram of study population.

TABLE 1 Lung ultrasound findings by week of illness among adult participants hospitalized with COVID-19 stratified by peak severity.

Variables–median (IQR)	Week of illness			
	0–7 days (*N* = 92)	8–14 days (*N* = 140)	15–21 days (*N* = 55)	22–29 days (*N* = 25)
Moderate disease–no.	74	75	33	12
mLUSS	0.750 (0.250, 1.125)	0.875 (0.333, 1.250)	0.667 (0.500, 1.000)	1.000 (0.000, 1.310)
A lines,%	90.9 (75.0, 100.0)	90.9 (66.7, 100.0)	100.0 (60.0, 100.0)	82.9 (63.3, 100.0)
Any B lines,%	46.4 (25.0, 80.0)	62.5 (33.3, 83.3)	58.3 (33.3, 87.5)	72.2 (0.0, 94.4)
Confluent B lines,%	0.0 (0.0, 20.0)	8.3 (0.0, 25.0)	0.0 (0.0, 0.0)	0.0 (0.0, 18.3)
Consolidations,%	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 7.1)
Pleural line abnormalities,%	0.0 (0.0, 8.3)	0.0 (0.0, 12.5)	0.0 (0.0, 0.0)	0.0 (0.0, 10.6)
Pleural effusions,%	0.0 (0.0, 8.3)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)
Severe disease–no.	18	55	37	13
mLUSS	1.000 (0.667, 1.500)	1.000 (0.667, 1.500)	1.250 (1.000, 1.600)	1.400 (1.000, 1.667)
A lines, %	69.0 (42.9, 100.0)	66.7 (50.0, 100.0)	66.7 (16.7, 83.3)	37.5 (25.0, 75.0)
Any B lines, %	73.2 (50.0, 100.0)	75.0 (50.0, 100.0)	100.0 (62.5, 100.0)	100.0 (750, 100.0)
Confluent B lines, %	0.0 (0.0, 25.0)	0.0 (0.0, 33.3)	0.0 (0.0, 40.0)	28.6 (0.0, 50.0)
Consolidations, %	0.0 (0.0, 25.0)	0.0 (0.0, 22.2)	0.0 (0.0, 25.0)	0.0 (0.0, 25.0)
Pleural line abnormalities, %	0.0 (0.0, 14.3)	0.0 (0.0, 16.7)	0.0 (0.0, 37.5)	60.0 (0.0, 75.0)
Pleural effusions, %	0.0 (0.0, 12.5)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	25.0 (0.0, 50.0)

mLUSS, mean lung ultrasound score; IQR, interquartile range.

Stratifying by these peak severity groups, the mLUS score remained higher in severe disease than in moderate disease (Figure 2 and Supplementary Table 1). Regardless of week of illness, more pervasive B-lines were higher in the severe group than in moderate illness and A-lines were lower in severe illness (Supplementary Table 1). Severe COVID-19 patients had consistently higher percent lung zones with B-lines throughout the first month of illness than moderate COVID-19 (Figure 2 and Table 1). Most lung zones were unaffected by pleural line abnormalities in either severity group. A-lines became less prevalent over time among those with moderate or severe disease (Figure 2 and Supplementary Table 1), and B-lines became more prevalent over time among participants that remained hospitalized during their third and fourth week of illness. Consolidations, pleural line abnormalities, and pleural effusions were uncommon throughout illness in both moderate and severe groups (Supplementary Table 1). The percent of lung zones with pleural line abnormalities or pleural effusions was higher in severe disease than moderate at 22–29 days of illness, but sample size was limited to 13 and 12, respectively (Figure 2 and Table 1).

In the linear mixed-effects model, mLUS scores were elevated by 0.19 (p = 0.035) in the severe group compared to the moderate group regardless of duration of illness. However, there was no difference in mLUS trajectory (i.e., change over time) between severity groups (p = 0.66). A sensitivity analysis using study visits instead of duration of symptoms and another analysis using GEE resulted in the same qualitative conclusion (data not shown). Similarly, A-lines were in 14.7% fewer lung fields (p = 0.02; Figure 2) and B-lines were in 17.8% more lung fields among those with severe disease compared to moderate disease regardless of duration of illness. However, the trajectory

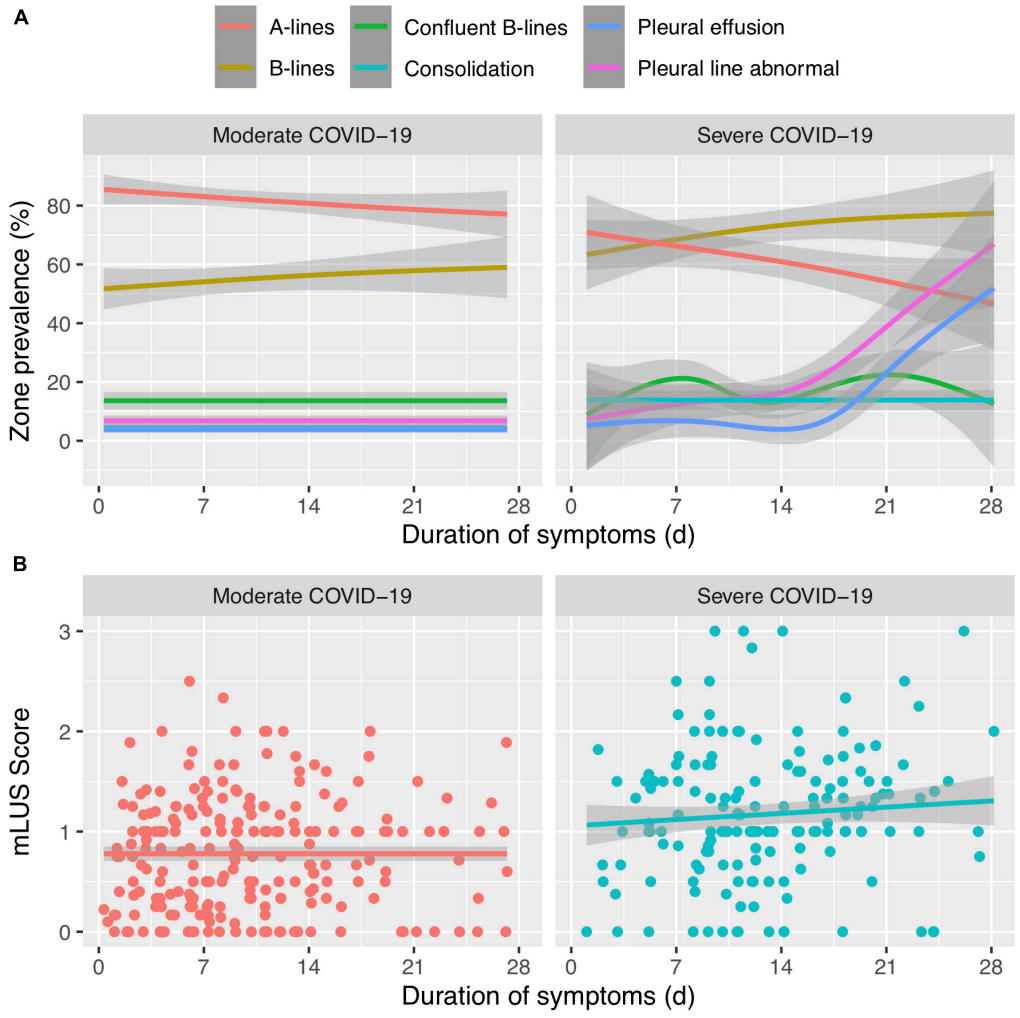

FIGURE 2
(A) Trends of lung ultrasound lung zone involvement (%) over time of different ultrasound artifacts or abnormalities between moderate and severe COVID-19. **(B)** The composite mean LUS (mLUS) score over time between moderate and severe COVID-19. Line is fitted with generalized additive model and the gray line represents standard error.

of percent A-lines did not differ significantly between peak severity levels (p = 0.40) and the trajectory of percent B-lines did not differ (p = 0.83).

Discussion

Our study found no significant differences in LUS findings over time between those with severe (i.e., ICU-level or fatal cases) and moderate (non-ICU) peak illness among adults hospitalized with COVID-19. Lung ultrasound abnormalities became increasingly prevalent among the minority of those that remained hospitalized during the third or fourth week of illness. However, the slope of that increase did not differ between moderate or severe disease. While the benefits of portability and bedside results of LUS are appealing, our results did not reveal differences in the composite mLUS scores or percent of zones with A-lines over the patients' clinical course. Baseline LUS or LUS after a prolonged stay may be more informative than dynamic LUS changes among those hospitalized with COVID-19 pneumonia.

This represents, to our knowledge, the largest study with serial COVID-19 LUS. Prior research has identified similar findings of persistent abnormalities (7) but has not evaluated for changes as potential indicators of a severe disease trajectory. The dearth of differences may be related to persistent architectural changes that may lag clinical improvement similar to that which may occur with chest X-rays or computed tomography (CT) scans in which residual disease observed on medical imaging does not reflect the relative recovery in clinical condition.

There are limitations to our study. Participants were initially scanned after admission to the hospital and earlier changes in lung ultrasound findings may not have been observed. Mean LUS score was used to mitigate the effect of incomplete lung scans of <12 zones. While this may introduce bias, it reflects the real-world application of ultrasound in the clinical care of patients with moderate and severe illness. The small sample size within the third and fourth weeks of illness due to attrition from discharges could have resulted in an inability to detect more subtle differences and included those that were more ill. As participants were not scanned after discharge, our findings during late illness are likely representative of the course for individuals that remain hospitalized for illness due to COVID-19 rather than individuals that are hospitalized for COVID-19 in general. Scans were collected in the pre-omicron era; however, imaging is expected to be similar among those hospitalized with severe COVID-19 due to omicron variants. Differences or sensitivity analyses based on ventilator settings (e.g., positive end-expiratory pressure) were not assessed due to data availability. Further studies are needed in the ambulatory setting to improve understanding of diagnostic accuracy among non-hospitalized individuals with COVID-19.

Mean LUS scores correlated with clinical severity among hospitalized adults when assessed cross-sectionally; however, mLUS score did not change or differ between peak severity levels over the time course of hospitalization. These results do not support serial LUS scans to monitor progression of disease severity. While future studies may identify other potential applications for serial LUS, LUS remains an important tool for clinical care in detecting and diagnosing various lung pathologies.

Data availability statement

The raw data supporting the conclusions of this article will be made available upon reasonable request to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Johns Hopkins University Institutional Review Board (IRB00245545). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

The Clinical Characterization Protocol for Severe Infectious Diseases (CCPSEI) research team members

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Author contributions

PB wrote the manuscript and performed the statistical analysis. JH and JP performed the statistical analysis and edited the manuscript. TF edited the manuscript and was involved in concept development. EC and CC provided the statistical support and oversight. PH provided the operational support for data acquisition. GL was involved in data acquisition. TS was part of concept development and data acquisition. DC contributed to the support and concept development. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Role of matrix metalloproteinases in multi-system inflammatory syndrome and acute COVID-19 in children

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Introduction: Multisystem Inflammatory Syndrome in children (MIS-C) is a serious inflammatory sequela of SARS-CoV2 infection. The pathogenesis of MIS-C is vague and matrix metalloproteinases (MMPs) may have an important role. Matrix metalloproteinases (MMPs) are known drivers of lung pathology in many diseases.

Methods: To elucidate the role of MMPs in pathogenesis of pediatric COVID-19, we examined their plasma levels in MIS-C and acute COVID-19 children and compared them to convalescent COVID-19 and children with other common tropical diseases (with overlapping clinical manifestations).

Results: Children with MIS-C had elevated levels of MMPs ($P < 0.005$ statistically significant) in comparison to acute COVID-19, other tropical diseases (Dengue fever, typhoid fever, and scrub typhus fever) and convalescent COVID-19 children. PCA and ROC analysis (sensitivity 84–100% and specificity 80–100%) showed that MMP-8, 12, 13 could help distinguish MIS-C from acute COVID-19 and other tropical diseases with high sensitivity and specificity. Among MIS-C children, elevated levels of MMPs were seen in children requiring intensive care unit admission as compared to children not needing intensive care. Similar findings were noted when children with severe/moderate COVID-19 were compared to children with

mild COVID-19. Finally, MMP levels exhibited significant correlation with laboratory parameters, including lymphocyte counts, CRP, D-dimer, Ferritin and Sodium levels.

Discussion: Our findings suggest that MMPs play a pivotal role in the pathogenesis of MIS-C and COVID-19 in children and may help distinguish MIS-C from other conditions with overlapping clinical presentation.

KEYWORDS

MIS-C, COVID-19, seropositive, MMPs, biomarker

Introduction

Children mostly have either mild or no symptoms of SARS-CoV2 infection but may rarely manifest with an inflammatory condition 1–2 months after acute infection, now classified as multisystem inflammatory syndrome in children (MIS-C) (1–4). Symptoms of MIS-C such as fever, multi-organ dysfunction and elevated inflammatory markers are largely similar to acute COVID-19 of adults or Kawasaki disease in children (5, 6). However, MIS-C has distinct immunological features that are different from adult COVID-19 or Kawasaki disease with respect to cytokine profiles and immune cell compartments (7–9). Although we are yet to fully understand the pathogenesis of MIS-C, the presence of auto-antibodies including those directed at the casein kinase family of proteins have been reported (8, 9). The exact pathogenesis of MIS-C remains vague, with virus-induced post-infective immune dysregulation appearing to play a leading role. Previously published data has reported that overall MIS-C prognosis is good and reported mortality rates are 0–4% (10).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent extracellular matrix remodeling enzymes that have the capacity to degrade almost every component of the extracellular matrix (11). Alterations in MMP levels can lead abnormal degradation of the extracellular matrix and result in pathology in most tissues (12). MMPs are implicated in all lung pathologies (13) and also involved in inflammation, modulating the synthesis and the release of cytokines and chemokines, and in cell growth, proliferation, and remodeling (14). Hence, we hypothesized that plasma levels of MMPs would be reflective of pathology in MIS-C. Studies from animal models have shown that an increase in MMP8, MMP9, and MMP14 levels in the lungs post SARS-CoV-2 infection was associated with degradation of lung extra-cellular matrix components, suggesting that MMP proteolytic activity in SARS-CoV-2 infection may be a potential target for COVID-19 treatment (15). Subsequently another study also reported that improper expression of several MMPs was correlated to lung disease of SARS-CoV-2 infection and the main findings from this study also reveals that MMPs are emerging as an important component of COVID-19 immunopathogenesis (16). Moreover, MMPs could potentially serve as biomarkers of MIS-C and enable its discrimination from acute COVID-19 as well as multiple other inflammatory and/or infectious conditions in children. To this end, we studied the association of a large panel of circulating MMPs in MIS-C, acute COVID-19 and children with other diseases in well-defined clinical cohort. We report that MIS-C and acute COVID-19 are characterized by heightened levels of MMPs (which also reflects disease severity) and that certain MMPs can help distinguish MIS-C from acute COVID-19 and other diseases.

Materials and methods

Ethics statement

Informed consent was obtained from parent/guardians of all children along with assent where appropriate. The Internal Ethics Committee (IEC) of the participating institutes approved the study. The study was also registered at Clinical Trials Registry India (CTRI/2021/01/030605). The study was also registered with Clinical Trials registry clinicaltrials.gov (No: NCT04844242).

Study population and procedures

We prospectively enrolled children admitted to Kanchi Kamakoti CHILDS Trust Hospital (KKCTH), Chennai, India Institute of Child Health, Dr Mehta's Children Hospital, Rainbow Children's Hospital, from 1 June 2020 to 30 September 2020 with MIS-C, acute COVID-19, other diseases with include other infectious diseases (dengue, scrub typhus. Salmonella typhi infection [enteric fever]) or non-infectious diseases (e.g., systemic lupus erythematosus, diabetic ketoacidosis, Kawasaki's disease), convalescent COVID-19 and control children. The study population and the enrolment criteria have been previously described (17). Briefly, we included children of either sex between 1 and 18 years of age who or whose parents were

willing to provide informed consent/assent. Blood collection was performed prior to any immunomodulatory medication. Plasma was isolated and used for measuring multiple immune parameters. The demographic, epidemiological, medical and laboratory data have been previously reported (17) and are described in **Tables 1**, **2**. COVID-19 disease severity was defined according to the Ministry of Health and Family Welfare (MOHFW) guidelines (18) issued by Government of India and children with MIS-C were diagnosed according to the World Health Organisation (WHO) case definition (19) and all the enrolled MIS-C cases have no other microbial or viral inflammatory focus. Children who were both SARS-CoV-2 RT-PCR negative and seronegative and who presented to the hospital for elective surgery were used as controls. They had no other co-morbid conditions and no history of contact with anyone with COVID-19. SARS-CoV-2 real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was performed by Indian Council of Medical Research (ICMR) approved laboratories. The inclusion for convalescent COVID was determined by serology using the iFlash® SARS-CoV-2 IgG chemiluminescence antibody assay (CLIA) (YHLO Biotechnology Corporation, Shenzhen, China) according to the manufacturer's instructions. An IgG antibody titre of $\geq$ 10 AU/ml was considered positive (**Table 1**).

For analyses, children were classified into five groups: MIS-C (n = 65), acute COVID-19 (n = 56), other diseases (n = 43), convalescent (n = 47) and control (n = 21). There is no published evidence concerning these research objectives at the beginning of this study. Therefore, the formal sample size for the study was not calculated and a convenient sample was obtained. Blood was collected in EDTA tubes (BD Biosciences) and heparin tubes and processed within 4 h of collection at the National Institute for Research in Tuberculosis (NIRT), Chennai. Sampling in all children was done prior to receiving any immunomodulatory treatment. To avoid the measurement bias and to increase the precision of the estimates for the accuracy of the assay, the study staff involved in immunological assays were blinded to any clinical data.

Measurement of matrix metalloproteinases

Circulating plasma levels of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12 and MMP-13 were determined using a multiplex enzyme-linked immunosorbent assay system using the Luminex Magpix Multiplex Assay system; Bio-Rad. MMP level were measured using a commercially available kit (Luminex Human Magnetic Assay kit 8 Plex from R&D Systems). All the samples were tested in duplicates and averages were used for the analysis. The lowest detection limits were as follows: MMP-1, 115.8 pg/ml; MMP-2, 809 pg/ml; MMP-3, 199.2 pg/ml; MMP-7, 27.7 pg/ml; MMP-8, 31.7 pg/ml; MMP-9, 257.5 pg/ml; MMP-10, 78.4 pg/ml; MMP-12, 18.5 pg/ml; MMP-13, 32.9 pg/ml.

Statistical analysis

Geometric means (GM) were used for measurements of central tendency. Statistically significant differences between MIS-C, acute COVID, children with other diseases, convalescent COVID and control children were analysed using the Kruskal-Wallis test with Dunn's multiple comparisons. The Mann–Whitney test was used to compare the levels of MMPs between MIS-C children with pediatric intensive care unit (PICU) versus non-PICU admission as well as between COVID-19 children with mild disease versus moderate/severe disease. Multiple linear regression analyses using Spearman rank correlation coefficients were used to determine correlations between variables. $P \leq 0.05$ was considered statistically significant and all tests were two sided. Analyses were performed using Graph-Pad PRISM Version 9.0 (GraphPad Software, San Diego, CA, USA). CombiROC analysis was performed using the online site http://combiroc.eu. The optimal cut-offs were determined from the best out of the Youden index, distance and absolute difference methods for all possible cut-off points derived from a classifier (i.e., logistic regression). Principle Component Analysis (PCA) was performed to seek linear combinations of the biomarkers that separate out different clusters corresponding to each biomarker that best explain the variance in the data using the R studio.

Results

Baseline characteristics of the study cohort

As shown in **Table 1**, we included 229 children in the study cohort (65 MIS-C, 56 acute COVID-19, 40 other diseases, 47 convalescent COVID-19 and 21 control children). The median age was 5 years (range 1–17 years) and overall, the genders were equally distributed among the groups. All the enrolled acute COVID-19 children were SARS-CoV-2 RT-PCR positive among which 18% children were asymptomatic, 67% presented with mild symptoms, 9% had moderate symptoms and 6% had severe symptoms needing PICU care. Next, all the MIS-C children were seropositive (IgG) among which 52% had severe disease needing PICU care.

Children with other diseases (n = 40) include Dengue fever = 3, Typhoid fever = 3, Scrub typhus = 5, other etiology = 8, Diabetic Ketoacidosis = 6, Kawasaki's Disease = 3, Juvenile idiopathic arthritis (JIA) = 1, Guillain-Barre syndrome (GBS) = 3, Systemic Lupus Erythematosus (SLE) = 4 and chronic renal failure = 3 (**Table 2**). Seropositive children (n = 47) were included as Convalescent COVID-19 controls, some of

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TABLE 1 Characteristics of study population.

	COVID-19 (*n* = 56)	MIS-C (*n* = 65)	Other diseases (*n* = 40)	Convalescent COVID-19 (*n* = 47)
Age median (years, IQR)	5.5 (1–17)	6.4 (1–14)	5.8 (1–12)	4.4 years (1–17)
Male n (%)	29 (52%)	30 (46%)	22 (55%)	35 (74%)
RT-PCR positive n (%)	56% (100%)	0	0	0
Serology IgG positive n (%)	0	65 (100%)	5 (13%)	47 (100%)
Underlying conditions n (%)	10 (18%)	4 (6%)	22 (55%)	11 (23%)
Co-existing infections n (%)	4 (7%)	5 (8%)	NA	6 (13%)
Median duration since proven or suspected COVID illness or contact (weeks, range)	NA	3 w (10 d–4 w)	NA	3.2 w (10 d–5 w)
COVID-19 symptoms *n* (%)				
Fever	47 (84%)	65 (100%)	20 (50%)	17 (36%)
Gastrointestinal	20 (36%)	52 (80%)	22 (55%)	15 (32%)
Respiratory	15 (27%)	14 (22%)	11 (28%)	16 (34%)
Mucocutaneous	0	49 (75%)	5 (13%)	0
Asymptomatic	8 (14%)	0	0	30 (64%)
Cardiovascular symptoms/signs				
Hypotension	1 (2%)	34 (52%)	9 (23%)	0
Shock	1 (2%)	21 (32%)	NA	2
Coronary artery dilatation	0	5 (8%)	0	0
Mycocardial dysfunction	0	34 (52%)	9 (23%)	0
Laboratory parameters				
CRP (<3 mg/L)	7.2 (<3–181)	101 (3.5–473)	21.3 (<3–78)	5 (<5–181)
WBC 10^3 cells/ul *Geo Mean/Range*	5.319 (3.020–8.390) (*n* = 14)	4.284 (2.77–8.870) (*n* = 21)	8.283 (1.060–29.83)	NA
Hb (g/dl) *Geo Mean/Range*	11.23 (8.70–13.09) (*n* = 14)	11.05 (9.20–14.65) (*n* = 21)	9.82 (4.68–15.23)	NA
Lymphocyte(/mm3) (1,500–4,000) median (IQR)	3,873 (650–12,000)	1,343 (330–6,270)	7,800 (2,400–28,600)	3,890 (650–12,000)
Neutrophils (/mm3) (1,500–7,000) median (IQR)	3,716 (120–13,160)	11,179 (8,500–15,900)	6,100 (810–9,490)	6,300 (120–13,160)
Platelets (200–450) × 10^9/L median (IQR)	271 (116–435)	107 (58–255)	290 (100–810)	327 (100–540)
Sodium (135–145mmol/l) median (IQR)	137 (135–145)	133 (124–139)	136 (131–148)	138 (135–148)
Ferritin (ng/ml) (7 to 140) median (IQR)	NA	1,348 (306–5,377)	527 (13–7,200)	NA
Median duration of stay	3.5 (1–9)	5 (3–18)	8 (1–21)	3 (1–10)
IVIG	0	44 (68%)	3 (8%)	0
Steroids	2 (5%)	47 (72%)	8 (20%)	2 (4%)
PICU admission	4 (9%)	34 (52%)	20 (50%)	6 (11%)
Antibiotics	13 (30%)	60 (92%)	33 (83%)	16 (31%)
Tocilizumab (8 mg/kg)	0	3 (5%)	0	0
Respiratory support *n* (%)				
Mechanical ventilation	0	1(2%)	11 (28%)	0
HHFNC	2 (5%)	5 (8%)	5 (13%)	2 (4%)
Oxygen	3 (7%)	9 (14%)	1 (3%)	5 (10%)
Cardiovascular support *n* (%)				
Inotropes	0	34 (52%)	9 (23%)	0
Fluid Bolus	2 (5%)	43 (66%)	6 (15%)	2 (4%)

whom had previous history of symptomatic COVID-19. Control children (n = 21) were both SARS-CoV-2 RT-PCR negative and seronegative with a similar median age (6 years, IQR: 1–15 years) and sex (male: 52%, 11/21) (Table 1).

Heightened plasma levels of matrix metalloproteinases in multisystem inflammatory syndrome in children in the study cohort

To determine whether MMPs are able to differentiate the different clinical phenotypes of SARS-CoV-2 infection in children, we measured the plasma levels of MMPs-1, 2, 3, 7, 8, 9, 12 and 13. As illustrated in Figure 1, MIS-C children exhibited significantly heightened levels of MMPs-1, 2, 3, 8, 9, 12 and 13 when compared with acute COVID-19 and/or children with other diseases and/or convalescent COVID-19 and/or control groups. Thus, heightened plasma levels of MMPs are associated with MIS-C in children.

Principle component analysis and receiver operating characteristic analysis of matrix metalloproteinases clearly differentiates multisystem inflammatory syndrome in children from COVID-19 and control groups

To evaluate whether MMPs can help determine the differences between MIS-C and the other groups, we performed PCA (principal component analysis) between the groups on the whole data set and envisaged the clustering pattern of MMPs in above mentioned group of children. We excluded factors with commonalities as low as 0.5 and assessed MMP-8, MMP-12 and MMP-13. As described in Figure 2A, PCA analysis showed that MMPs clusters clearly differentiated MIS-C from acute COVID-19, children with other diseases, convalescent COVID-19 and control group of children.

We performed area under the receiver operating characteristic (ROC) curve (Figure 2B) analysis to assess whether the best possible combination of MMPs is able to discriminate MIS-C from acute COVID-19, children with other diseases, convalescent COVID-19 and controls. A combination of 3 MMPs (MMP-8, MMP-12 and MMP-13) produced an area under the curve (AUC) of 0.89–1, indicating that these MMPs could distinguish MIS-C from acute COVID-19 with 83–92% sensitivity and 80–85% specificity. Next, we performed CombiROC between MIS-C and children with other diseases groups to assess if MMPs could distinguish between them and demonstrated that a combination of 3 MMPs (MMP-8, MMP-12 and MMP-13) produced an area under the curve (AUC) of 0.96–0.99, indicating that these MMPs could distinguish MIS-C from other diseases groups with 83–98% sensitivity and 89–97% specificity. In addition, we also performed CombiROC between MIS-C and convalescent COVID-19 children to assess if MMPs could distinguish between them and demonstrated that a combination of 3 MMPs (MMP-8, MMP-12 and MMP-13) produced an area under the curve (AUC) of 0.95–1, indicating that these MMPs could distinguish MIS-C from convalescent children with 88–100% sensitivity and 90–100% specificity. Finally, we also performed CombiROC between MIS-C and control children to assess if MMPs could distinguish between them and demonstrated that a combination of 3 MMPs (MMP-8, MMP-12, and MMP-13), produced an area under the curve (AUC) of 1, indicating that these MMPs could distinguish MIS-C from other diseases groups with 100% sensitivity and 100% specificity.

TABLE 2 Additional features of children with other diseases.

Total (n)	**40**
Male n (%)	22 (55%)
Age (Median, IQR)	5.8 y (1–12 years)
Diagnosis	n (%)
Dengue fever	3 (8%)
Scrub typhus	5 (13%)
Typhoid	3 (8%)
Acinetobacter sepsis	2 (5%)
Urinary tract infection	3 (8%)
No microorganism isolated	3 (8%)
Underlying diagnosis	n (%)
Diabetic ketoacidosis	6 (15%)
Kawasaki's disease	3 (8%)
Juvenile idiopathic arthritis	1 (3%)
Guillain-Barre syndrome	3 (8%)
Systemic Lupus Erythematosus	4 (10%)
Chronic renal failure	3 (8%)
Hypothyroidism	1 (3%)
Clinical symptoms	
Fever	20 (50%)
Respiratory	11 (28%)
Gastrointestinal	22 (55%)
Mucocutaneous	5 (13%)
Neuromuscular (Headache, abnormal gait, etc.)	6 (15%)
Renal	5 (13%)
Underlying conditions (n = 1)	
Neurodevelopmental delay	1

Bold values are subdivision of the clinical characteristics.

Heightened plasma matrix metalloproteinase levels are associated with disease severity in multisystem inflammatory syndrome in children and COVID-19

To determine whether MMPs could be used to reflect disease severity in MIS-C and acute COVID-19, we compared the plasma levels of MMPs-1, 2, 3, 7, 8, 9, 12 and 13 between

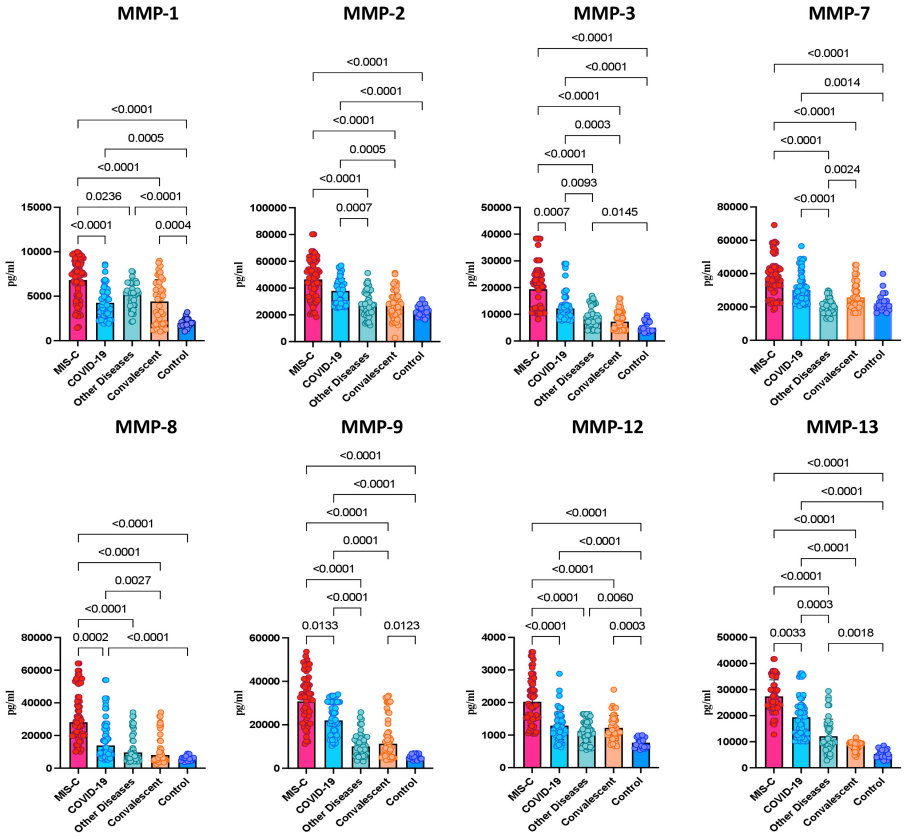

FIGURE 1
Heightened plasma levels of matrix metalloproteinases (MMPs) in multisystem inflammatory syndrome in children (MIS-C) and acute COVID-19 children. The plasma levels of MMP-1, 2, 3, 7, 8, 9, 12 and 13 were measured in MIS-C (n = 65), acute COVID-19 (n = 56), children with other diseases (n = 40), convalescent COVID-19 (n = 47) and control children (n = 21). The data are represented as scatter plots with each circle representing a single individual. p values were calculated using the Kruskal-Wallis test with Dunn's *post hoc* for multiple comparisons.

MISC children requiring PICU (pediatric intensive care unit) (denoting severe disease) and children who did not need PICU care (denoting mild/moderate disease). As illustrated in **Figure 3**, MIS-C children with PICU admission exhibited significantly elevated baseline levels of MMPs-1, 2, 3, 7, 8, 9, 12 and 13 compared to children with no PICU care implying that MMPs are associated with disease severity in MIS-C (**Figure 3A**) Finally, we examined if the MMPs levels may be used to reflect disease severity in acute COVID-19 children. As shown in **Figure 3B**, moderate/severe COVID-19 children exhibited significantly elevated baseline levels of MMP-1, 2, 3, 7, 8, 9, 12 and 13 compared to mild COVID-19 children, demonstrating that MMPs are associated with disease severity in COVID-19.

Correlation between matrix metalloproteinase levels and other clinical laboratory parameters

We wanted to examine the relationship between MMPs and other laboratory parameters (Lymphocyte, CRP, D-Dimer, Ferritin, LDH, Sodium) in MIS-C, acute, acute COVID-19, children with other diseases, convalescent COVID-19 and control children As illustrated in **Figure 4**, a multiparametric scatter plot matrix correlation plot exhibited a positive correlation of CRP with MMP 8 and 12, D-Dimer with MMP 12 and 13 and negative correlation of Lymphocytes with MMP 1, 2, 8, 9 and 13, Sodium with MMP 1, 2, 3, 8, 9, 12 and 13 indicating that these clinical laboratory parameters are associated the MIS-C disease status.

Discussion

COVID-19 in children manifests with a wide range of clinical symptoms ranging from asymptomatic SARS-CoV2 infection; mild, moderate and severe acute COVID-19; convalescent COVID-19 to MIS-C (20). MIS-C is related temporally to SARS-CoV2 infection of unknown pathogenesis that possibly occurs due to the delayed immunological reaction of children to the virus (3, 21). Both MIS-C and COVID-19 can involve multiple organ systems in children. Apart from the respiratory symptoms, children can

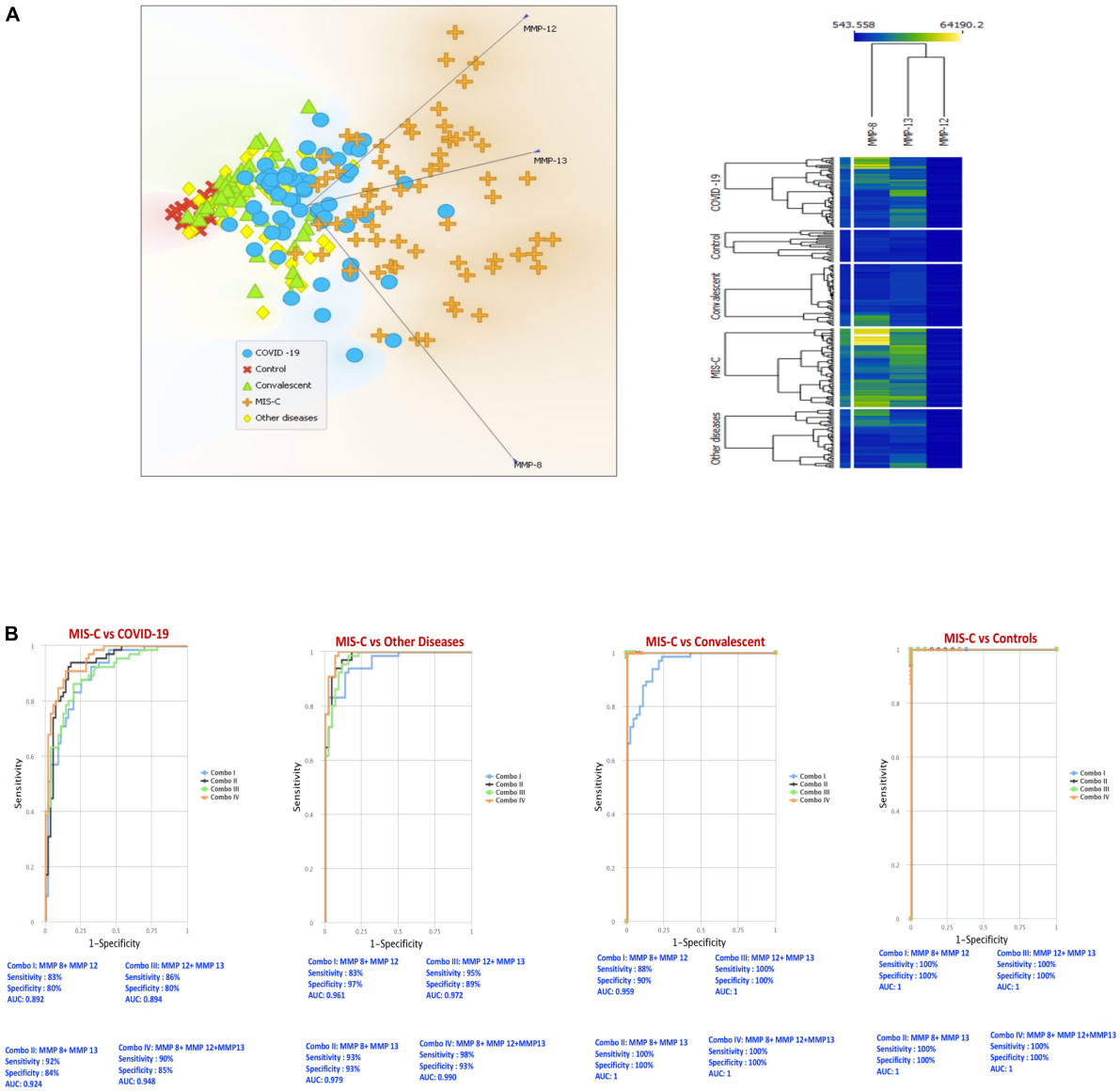

FIGURE 2
PCA and ROC reveals the trend of matrix metalloproteinases (MMPs) among multisystem inflammatory syndrome in children (MIS-C), COVID-19 and other groups. **(A)** Principal component analysis (PCA) was performed to show the distribution of data from the combination of five groups: MIS-C (brown), acute COVID-19 (blue), children with other diseases (yellow color), convalescent COVID-19 (green) and control (red) children depicted using normalized data from plasma levels of MMP-8, 12 and 13. **(B)** CombiROC analysis was performed to determine the role of MMP 8, 12 and 13 in distinguishing between MIS-C vs. acute COVID-19, MIS-C vs. other diseases, MIS-C vs. convalescent and MIS-C vs. Controls.

present with gastro-intestinal, cardiac and muco-cutaneous manifestations as seen in MIS-C and rarely with neurological symptoms (22). The symptoms of MIS-C are most typically similar to Kawasaki disease and/or macrophage activation syndrome (23–26). The immune profile of MIS-C shows changes in innate immune response with elevated pro-inflammatory cytokines, chemokines and enhanced neutrophil and monocyte/macrophage activation, while the changes in adaptive immune response are seen as T cell, B cell and NK cell lymphopenias, enhanced anti-viral antibody responses and elevated circulating autoantibodies (7, 8, 27–30). Our study involves a large number of different COVID-19 groups including acute and convalescent as well as MIS-C and in addition, children with other diseases, who have been very well characterized clinically. As shown in **Table 1**, we have performed extensive clinical, laboratory and demographic characterization of these children, which adds considerable value to our study findings. In addition, power calculations were carried out to determine the ability of MMPs (MMP8, MMP12, MMP13) to distinguish MIS-C from acute COVID-19 and other tropical

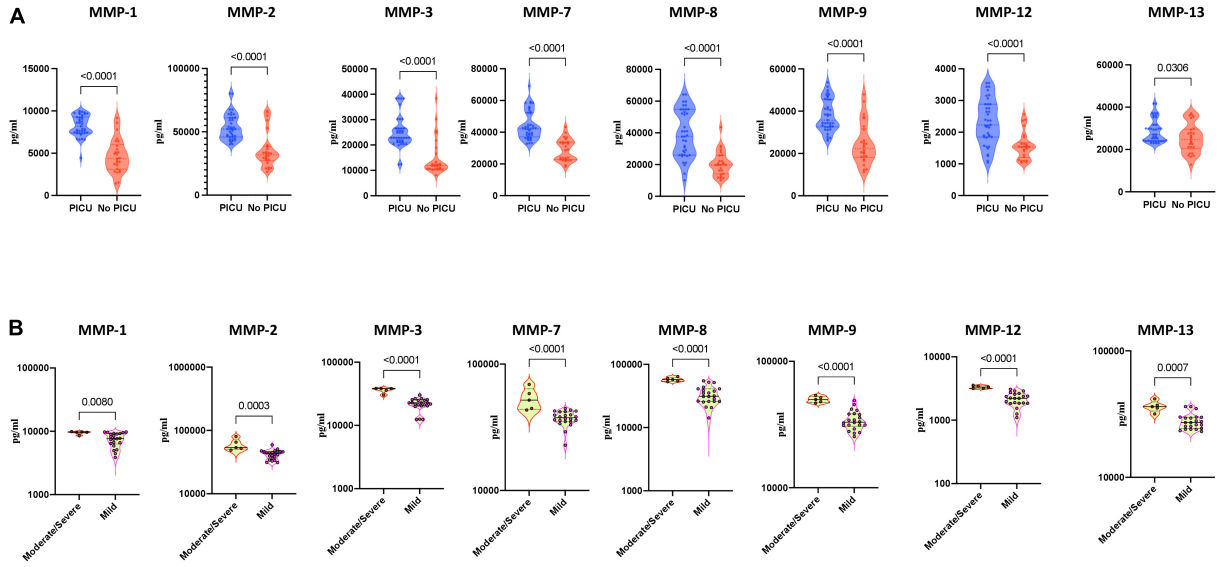

FIGURE 3
Heightened plasma matrix metalloproteinases (MMP) levels are associated with disease severity in multisystem inflammatory syndrome in children (MIS-C) and COVID-19. **(A)** The plasma levels of MMPs-1, 2, 3, 7, 8, 9, 12 and 13 were measured in MIS-C children with PICU care (n = 39) and MIS-C children no PICU care (n = 26). **(B)** The plasma levels of MMPs-1, 2, 3, 7, 8, 9, 12 and 13 were measured in COVID-19 children with moderate to severe (n = 5) and children with mild (n = 22) disease. The data are represented as scatter violin plots with each circle representing a single individual. P values were calculated using the Mann–Whitney test with Holm's correction for multiple comparisons.

diseases. These MMPs differ with an effect of −0.5 to −2.5 and have a power of >90%.

It has been postulated that release of MMPs by neutrophils, macrophages and other cells mediate the extracellular remodeling and tissue pathology in various organ systems that underlie pathology in COVID-19 (31). Since, MIS-C is also a multi-system disease, MMPs may have a major role in the pathogenesis (32). Under steady-state conditions, MMPs are poorly expressed in tissues. However, upon injury, inflammation, extracellular matrix turnover, and repair, their expression is enhanced (33). It has been also reported that overactivation of MMPs may contribute to the dengue pathogenesis and disease severity. In addition, few other studies reported that MMPs might significantly impact the pathogenesis of respiratory viral infections including MIS-C and COVID-19 (33). In addition, studies in adult COVID-19 have reported that circulating levels of MMP-7 are potential biomarkers of disease severity in patients requiring invasive mechanical ventilation (34). Consistent with this hypothesis, our study clearly shows that MIS-C is characterized by elevated levels of MMP-1, 2, 3, 8, 9, 12 and 13. Similarly, acute COVID-19 is also characterized by elevated levels of MMP-3, 7 and 9 in comparison to convalescent COVID-19 and other diseases controls. The PCA analyses substantiates the role of MMPs by displaying the clear distinction of four groups with or without SARS-CoV2 related manifestations. In addition, by using the ROC analysis, we observed that MMPs may serve as significant biomarkers to distinguish MIS-C from acute COVID-19 and other diseases.

Previous studies have examined and identified biomarkers that could differentiate MIS-C from acute and convalescent COVID-19 and healthy control children (17, 35, 36). But very little information is available about the systemic parameters in MIS-C and acute COVID-19 in comparison to children with other diseases, especially those with considerable overlapping clinical presentations and routine laboratory parameters. We fill this knowledge gap by demonstrating major differences in plasma levels of MIS-C from children with other diseases. These diseases include both those of infectious etiology (such as Dengue, Scrub Typhus and Typhoid), which are endemic in India and those of non-infectious etiology (including Kawasaki disease, JIA, SLE), which have a common clinical picture and often confound diagnosis. Our study further highlights the observation that heightened levels of MMPs are an important characteristic of MIS-C and Acute COVID-19 in contrast to children suffering from other illnesses. Our study innovates by including a group of children with other diseases, that despite varied etiologies, have common clinical manifestations that make the final diagnosis of MIS-C very difficult in a country where these diseases are very common.

We also observed that MMPs of children with severe MIS-C and COVID-19 were highly elevated, signifying that MMPs may also be helpful in stratifying the disease severity. The blood sampling in our cohort of children was performed at

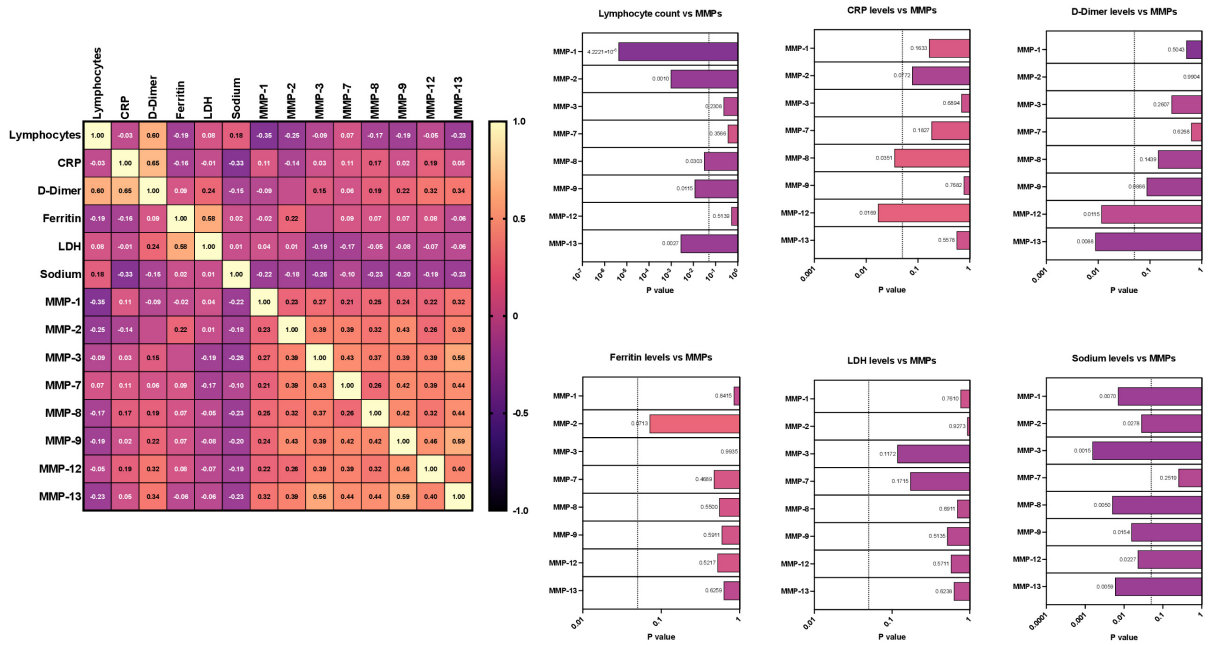

FIGURE 4
Correlation between matrix metalloproteinases (MMP) levels and other laboratory parameters. Multiparametric matrix correlation plot of MMPs-1, 2, 3, 7, 8, 9, 12 and 13 and laboratory parameters (Lymphocyte, CRP, D-Dimer, Ferritin, LDH, Sodium) with MIS-C and COVID-19. Spearman's correlation coefficients are visualized by color intensity. *P* values and spearman *r* values are ordered by hierarchical clustering in the study cohort.

hospital admission; prior to receiving any immunomodulatory treatment, suggesting that MMPs may be potentially used as baseline biomarkers for predicting disease severity in our population. Finally, we also observed that MMP levels correlate well with laboratory parameters such as CRP, D-Dimer, Ferritin, LDH and Sodium reinforcing the potential contribution of MMPs to pathogenesis of MIS-C and COVID-19. In addition, published studies and meta-analysis have revealed that inflammatory markers, especially WBC, platelets, CRP, ferritin, D-dimer and LDH levels, were correlated with MIS-C and also measurement of these clinical parameters are important for dynamic monitoring of MIS-C and might assist pediatricians to effectively evaluate and manage children and adolescents with MIS-C (37). Findings from this study reveals that MMPs are immune markers of MIS-C and COVID-19 in children.

Our study suffers from the limitation of examining children from a single city and not having any follow up samples to analyse and also other infection group are not homogeneous Despite limitations, our data suggest that MMPs might play a pivotal role in the pathogenesis of MIS-C and COVID-19 in children and may serve as a novel biomarker of both disease severity and biomarker to distinguish MIS-C from other syndromes of overlapping clinical and laboratory presentation. Future studies to corroborate our findings should serve to confirm the role of MMPs as both biomarker and pathogenetic factor of disease in MIS-C and COVID-19 in children.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by NIRT-IEC. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

SB and NP designed the study. AN and AR conducted the experiments. AV, AR, and NP acquired the data. NP and KT analyzed the data. SB, UR, TN, and BS contributed the reagents and also revised subsequent drafts of the manuscript. AV, PV, ES, TS, RS, AT, SN, GR, SP, KS, BS, and SH contributed the enrolement of the participants. AV, PV, SN, BS, and SH

contributed to acquisition and interpretation of clinical data. SB and NP wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Luteolin-rich fraction from *Perilla frutescens* seed meal inhibits spike glycoprotein S1 of SARS-CoV-2-induced NLRP3 inflammasome lung cell inflammation *via* regulation of JAK1/STAT3 pathway: A potential anti-inflammatory compound against inflammation-induced long-COVID

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Objective: The multi-systemic inflammation as a result of COVID-19 can persevere long after the initial symptoms of the illness have subsided. These effects are referred to as Long-COVID. Our research focused on the contribution of the Spike protein S1 subunit of SARS-CoV-2 (Spike S1) on the lung inflammation mediated by NLRP3 inflammasome machinery and the cytokine releases, interleukin 6 (IL-6), IL-1beta, and IL-18, in lung epithelial cells. This study has attempted to identify the naturally- occurring agents that act against inflammation-related long-COVID. The seed meal of *Perilla frutescens* (*P. frutescens*), which contains two major dietary polyphenols (rosmarinic acid and luteolin), has been reported to exhibit anti-inflammation activities. Therefore, we have established the ethyl acetate fraction of *P. frutescens* seed meal (PFEA) and determined its anti-inflammatory effects on Spike S1 exposure in A549 lung cells.

Methods: PFEA was established using solvent-partitioned extraction. Rosmarinic acid (Ra) and luteolin (Lu) in PFEA were identified using the HPLC technique. The inhibitory effects of PFEA and its active compounds against Spike S1-induced inflammatory response in A549 cells were determined by RT-PCR and ELISA. The mechanistic study of anti-inflammatory properties of PFEA and Lu were determined using western blot technique.

Results: PFEA was found to contain Ra (388.70 ± 11.12 mg/g extract) and Lu (248.82 ± 12.34 mg/g extract) as its major polyphenols. Accordingly, A549 lung cells were pre-treated with PFEA (12.5-100 μg/mL) and its two major compounds (2.5-20 μg/mL) prior to the Spike S1 exposure at 100 ng/mL. PFEA dose-dependently exhibited anti-inflammatory properties upon Spike S1-exposed A549 cells through *IL-6*, IL-1β, *IL-18*, and *NLRP3* gene suppressions, as well as IL-6, IL-1β, and IL-18 cytokine releases with statistical significance ($p < 0.05$). Importantly, Lu possesses superior anti-inflammatory properties when compared with Ra ($p < 0.01$). Mechanistically, PFEA and Lu effectively attenuated a Spike S1-induced inflammatory response through downregulation of the JAK1/STAT3-inflammasome-dependent inflammatory pathway as evidenced by the downregulation of NLRP3, ASC, and cleaved-caspase-1 of the NLRP3 inflammasome components and by modulating the phosphorylation of JAK1 and STAT3 proteins ($p < 0.05$).

Conclusion: The findings suggested that luteolin and PFEA can modulate the signaling cascades that regulate Spike S1-induced lung inflammation during the incidence of Long-COVID. Consequently, luteolin and *P. frutescens* may be introduced as potential candidates in the preventive therapeutic strategy for inflammation-related post-acute sequelae of COVID-19.

KEYWORDS

***Perilla frutescens* extract, luteolin (Lu), spike glycoprotein S1, long-COVID, anti-inflammation, lung inflammation, NLRP3 inflammasome pathway, JAK1/STAT3 pathway**

Introduction

The SARS-CoV-2 or severe acute respiratory syndrome coronavirus-2 infection is a highly transmissible infectious respiratory disease with more than 270 million confirmed cases and approximately 5.3 million deaths were recorded at the end of the year 2021 (1). The influences of SARS-CoV-2 infection was observed not only at the acute phase of the disease, but it was also found that about 30% of COVID-19 survivors may develop long-COVID or post-COVID-19 syndrome, which can be characterized by long-term symptoms lasting for more than 3 months after experiencing COVID-19 infection (2). The long-COVID symptoms can vary between patients with relative intensity of the symptoms and within different organs (3). Oxidative stress and inflammation of the cells play imperative roles in prolonged disease conditions, including lung injury, adult respiratory distress syndrome (ARDS), sepsis, hyperoxia, and chronic obstructive pulmonary disease (COPD) (4).

With regard to respiratory system pathologies, long-COVID patients can suffer a range of symptoms including sore throats and coughing, dyspnea (breathing difficulties), chest pains, and chronic lung inflammation. The prevalence and incident rate of long-COVID symptoms has been reported at approximately 87% of hospitalized patients (5). In severe cases, patients can display pulmonary distress, lung inflammation and multi-systemic inflammation. The origin of this inflammation-related long-COVID was found in the lung's alveoli where pro-inflammatory cytokines production continued and the cytokines were released into the surrounding tissue and blood circulation, triggering the inflammation (6, 7). According to longitudinal cohort Post-hospitalization COVID-19 (PHOSP-COVID) studies in adults aged ≥18 years ($n = 626$ participants) across the UK, the inflammatory profiles associated with the increase in inflammatory cytokines, such as IL-6 concentrations, were observed in the plasma of both the very severe and the moderate SARS-CoV-2 infected patients at the 5-month

visit after recovery (8). Additionally, in pulmonary parenchyma damage, cases were associated with the release of the NLRP3 (the Nod-like receptor proteins family, pyrin domain-containing 3) inflammasome-related cytokines such as IL-6, IL-1β, and IL-18. Many articles have reported that the SARS-CoV-2 infection can stimulate NLRP3-mediated COVID-19 inflammation, which has been associated with severity in long-COVID patients (4, 9, 10). The NLRP3 inflammasome is an intracellular complex molecule, and its function of them is to maintain the homeostasis of cytokine production and initiate cytolysis. Regarding the inflammasome assembly process, initially, the assembly is facilitated by the pattern recognition receptor (PRR), resulting in the recruitment of the adaptor molecule called an apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) to the NLRP3 molecule. Then, caspase-1 is activated and it proteolytically cleavage the pro- IL-1β and pro-IL-18 to IL-1β and IL-18 and release them outside the cell (11, 12).

It has been well documented that SARS-CoV-2 can enter the lung and immune cells through the binding of angiotensin-converting enzyme 2 receptor (ACE2 receptor) (13). Many studies have suggested that the persistent chronic inflammation of long-COVID patients could partially be associated with SARS-CoV-2 spike glycoprotein S1 which is the structural protein of SARS-CoV-2 virus. In addition to the viral attachment to the host cell, the SARS-CoV-2 uses the spike protein to bind the infected cells and activate different pathways including the inflammatory pathways. Spike protein can activate the cells *via* the interaction with the toll-like receptors (TLRs) resulting in the pro-inflammatory cytokines production (14, 15). Currently, no effective therapy is available for the management of long-COVID symptoms, while only common drugs have been prescribed for supportive therapies (16). Therefore, our attention has been drawn to the search for novel plant and active compounds with potential preventive therapeutic effects that could reduce lung inflammation and relieve long-COVID symptoms.

At present, the anti-inflammatory nutraceutical or pharmaceutical compounds derived from natural products, especially phytochemicals, have been increasingly recognized as having beneficial effects with regard to COVID-19 outbreaks and the long-COVID phenomenon due to the lesser adverse effects (17, 18). Hesperetin from *Clerodendrum petasites* S. Moore, Cyanidin-3-O-glucoside and Peonidin-3-O-glucoside anthocyanins from black rice germ and bran have been reported to exhibit an anti-inflammatory effects by inhibition of the Spike S1-exposed lung epithelial (A549) and Macrophages (THP-1) (13, 19). *Perilla frutescens* (*P. frutescens*) has long been recognized as a health promoting herb and is popular garnishes in many Asian countries. Extracts of P.F. appears to exhibit strong anti-inflammation activities as it can inhibit the histamine release in the mast cells, inhibit lipoxygenase activity, and serve as a potent antioxidant (19, 20). Different parts of P.F. have been reported for medicinal effects. Briefly, the stalks of P.F. have traditionally been used as an analgesic and an anti-abortive agent (21, 22). The leaves are helpful in treating respiratory problems such as asthma, colds and the flu, while the seeds can be employed for dyspnea, cough relief, and bowel relaxation (23–25). Interestingly, the seed meal part of P.F. has frequently been used in Asia countries such as Japan, China, and Thailand as food colorants. In our previous report, the ethyl acetate fractions of *P. frutescens* seed meal exhibited strong antioxidant effects and anti-inflammatory activities by downregulating the receptor activator of the NF-κB ligand (RANKL)-induced NF-κB and AP-1 activities in RAW264.7 macrophages (24). It was reported that rosmarinic acid (Ra) as well as luteolin (Lu) were the two dominant phytochemical compounds found in the *P. frutescens* extract. Lu (3′,4′,5,7-tetrahydroxyflavone) and Ra (4-coumaroyl-4′-hydroxyphenyllactic acid) have been reported as possessing anti-inflammatory properties for various diseases such as asthma, allergic dermatitis and colitis (20, 26, 27). However, at present, there is no available information on their anti-inflammatory properties against inflammation-related long-COVID nor on the inhibition of the spike glycoprotein S1 of SARS-CoV-2-induced inflammatory condition.

In this study, we proposed to determine the responsible molecular mechanisms underlying the potential anti-inflammation properties of the ethyl acetate fraction of *P. frutescens* seed meal (PFEA) against lung inflammation through the use of a cellular model of spike glycoprotein S1-induced inflammation. Our objectives were to explore the anti-inflammation properties of PFEA together with its active flavonoid compounds through the inhibition of Spike S1-induced inflammatory gene expressions (*IL-6*, *IL-1β*, *IL-18*, and *NLRP3*) and cytokine releases (IL-6, IL-1β, and IL-18), as well as to determine the responsible anti-inflammation signaling pathway using the *in vitro* lung model (A549 cells). Our findings demonstrate that Lu and PFEA inhibited Spike S1-induced inflammatory responses in A549 lung cells, apparently through the modulation of the JAK1/STAT3-NLRP3 inflammasome-axis. Accordingly, our findings could urge the use of a naturally occurring plant and its bioactive compounds against inflammation-related long-COVID.

Materials and methods

Chemical and reagents

The standard compounds including Ra, Lu, gallic acid, caffeic acid, and apigenin were obtained from MedChemExpress company (Monmouth Junction, NJ, USA). A recombinant human coronavirus SARS-CoV-2 Spike Glycoprotein S1 (ab273068) was purchased from Abcam company (Cambridge, UK). Dulbecco's Modified Eagle Medium (DMEM) was

purchased from Gibco company (Grand Island, NY, USA). The fetal bovine serum was purchased from Thermo Scientific company (Waltham, MA USA). The MTT or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye and mouse anti-beta-actin primary antibody were purchased from Sigma-Aldrich company (St. Louis, MO, USA). The TRI reagent® was purchased from Merck Millipore company (Billerica, MA, USA.). The ReverTra Ace® qPCR Master Mix was purchased from Toyobo Co., Ltd. (Osaka, Japan). The SensiFAST SYBR Lo-ROX Kit was purchased from Meridian Bioscience® company (Cincinnati, OH, USA). rabbit anti-NLRP3 primary antibody, anti-ASC primary antibody, anti-caspase-1 (p50 and p20) primary antibody, anti-p-JAK1 primary antibody, and anti-p-STAT3 primary antibody and goat horseradish peroxidase-conjugated anti-mouse- or anti-rabbit-IgG were obtained from Cell Signaling Technology company (Danvers, MA, USA).

Herb sample and solvent-partitioned extraction

Perilla frutescens (Nga-Mon) seed meals were collected from a local farm in Nan province, Thailand in 2021. The voucher specimen number of *Perilla frutescens* (QSBG-K2) was accredited from the Queen Sirikit Botanic Garden Herbarium, Chiang Mai, Thailand, which. The *P. frutescens* seed meal, a by-product from cold-press oil extraction was used to prepare *P. Frutescens* extract in this study. The *P. frutescens* seed meal ethyl acetate fraction was prepared as previously described protocol (24, 25). *P. frutescens* seed meal dried material start 500 g was soaked in 70% ethanol and were mixed at 256 rpm using digital stirrer (IKA® ^{RW} 20, Staufen, Germany). After 24 h, the extracted aqueous was collected (first aqueous), refill 70% ethanol into the same *P. frutescens* seed meal and mixed for 24 h again. The second aqueous was harvest and mixed with first aqueous, ethanolic extract (EtOH) was partitioned with hexane (EtOH: hexane, 1:2) and evaporated. Next, the extract was partitioned with dichloromethane (1:1, 2 times), collected, evaporated and lyophilized (dichloromethane fraction, PFD). Then, PFD was partitioned with ethyl acetate (1:1, 2 time), collected, evaporated and lyophilized (ethyl acetate fraction, PFEA), and residue water (water fraction, PFW), respectively. The PFEA were kept at −20°C for further experiment and resuspended in dimethyl sulfoxide (DMSO) before conducting the experiment.

Total phenolic contents

The total polyphenol or phenolic contents of PFEA were examined by the Folin-Ciocalteu assay and was modified from previously described protocol (28). Briefly, various concentration of PFEA (400 μL) were mixed with Folin-Ciocalteu reagent (300 μL) and incubated in room temperature for 3 min. Next, 7.5% sodium carbonate (Na_2CO_3) (300 μL) were added to the mixture. After 30 min incubation, the mixture was examined by microplate reader (TECAN®, Sunrise™ Absorbance Reader, Männedorf, Switzerland) at the absorbance of 765 nm. The total phenolic content was calculated by comparing with gallic acid (standard phenolic compound) and expressed as mg GAE/g extract.

Total flavonoid contents

Total flavonoid contents in PFEA were determined using the $AlCl_3$ colorimetric assay with minor modifications (24). Various concentrations of the PFEA (250 μL) were mixed with 5% $NaNO_2$ (125 μL) and incubated for 5 min. Next, 10% $AlCl_3$ (125 μL) was added to the mixture. After 5 min of incubation, NaOH (1.0 mL) was added and incubated for next 15 min at room temperature. The mixture was measured for the absorbance at 510 nm using microplate reader. The total flavonoid contents were calculated by comparing with catechin (standard flavonoid compound) and expressed as mg CE/g extract.

Determination of active compounds in PFEA using HPLC technique

Polyphenol compounds (Ra, Lu, Gallic acid, Caffeic acid, Apigenin, Kaempferol) were selected according to the polyphenol compounds in *P. Frutescens* that have previously reported (20, 24, 26, 27) and were quantitatively determined using reversed-phase HPLC (Infinity 1260, Agilent Technologies, Santa Clara, CA, USA) with Zorbax Eclipse Plus C18 (250 mm × 4.6 mm, 5 μm, Agilent Technologies) and Zorbax Eclipse Plus C18 pre-column (12.5 mm × 4.6 mm, 5 μm, Agilent Technologies). The mobile phase condition and wavelength detection were modified from previously described protocol (24). Briefly, the mobile phase was comprised of 0.1% acetic acid in Acetonitrile (mobile phase A) and 0.1% acetic acid in water (mobile phase B) under isocratic conditions (30:70). The flow rate was set to 1.0 mL/min for 60 min. The detection wavelength was at 325 and 350 nm. The area under peak was calculated and compared with respective polyphenol standards to establish the concentration for each detected compound and displayed as mg/g extract.

Cell cultures

The human lung epithelial cell line (type II pneumocytes), A549 cells (CCL-185™) was purchased from American Type

Culture Collection (ATCC, Manassas, VA, USA). The A549 lung cells were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were maintained in a 5% CO_2 humidified incubator at 37°C.

Cell cytotoxicity assay

The cytotoxicity of the PFEA and its active compounds (Ra and Lu) on A549 cells was determined using MTT assay as has been previously described (24). Briefly, A549 cells (4×10^3 cells/well) were seeded into a 96-well plate and incubated at 37°C in 5% CO_2 overnight. After that, the A549 cells were treated with the increasing concentration PFEA (0–200 μg/mL) or its active compounds, Ra and Lu (0–20 μg/mL) for 24 and 48 h. After incubation, 100 μL of culture medium was removed and then MTT dye (15 μL) was added and incubated at 37°C for next 4 h. The formazan crystal was dissolved with a DMSO (200 μL), and the absorbance was measured at 540 and 620 nm using a microplate reader. Cells viability was calculated by comparing with control and interpreted as the% of control.

Determination of inflammatory cytokine releases by ELISA

The cytokine secretions including IL-1β, IL-18, and IL-6 in cultured medium were examined using the ELISA kit (Biolegend, San Diego, CA, USA). The detection protocol was followed according to the manufacturer instruction. The A549 cells (3×10^5 cells/well) were seeded in a 6-well-plate and incubated overnight. Next, the A549 cells were pre-treated with increasing concentrations of PFEA (0–100 μg/mL) or active compounds, Ra and Lu (0–20 μg/mL) for 24 h. Then, the cells were induced by spike glycoprotein S1 of SARS-CoV-2 (Spike S1) at concentration 100 ng/mL for further 3 h. The cultured medium was collected. The cytokine releases were determined and calculated by comparing with standard curve for each cytokine.

Determination of *IL-1β*, *IL-18*, *IL-6*, and *NLRP3* gene expressions by RT- qPCR analysis

To determine the inflammatory gene expressions, the A549 cells were pre-treated with increasing concentrations of PFEA (0–100 μg/mL) or active compounds, Ra and Lu (0–20 μg/mL) for 24 h. After that, the cells were exposed to 100 ng/mL of Spike S1 for 3 h. Then, the total mRNA was isolated using TRI reagent®. The total RNA concentration and purity were determined using NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher Scientific, Waltham, MA, USA). The ratio of A260 to A280 (A260/A280) higher than 1.8 were used to indicating RNA purity. The total RNA was converted to cDNA by reverse transcription using a Mastercycler® nexus gradient machine (Eppendorf, GA, Germany). Then, quantitative real-time PCR technique was determined using a qRT-PCR ABITM 7500 Fast and 7500 Real-Time PCR machine (Thermo Fisher Scientific, Waltham, MA, USA). Gene expressions were analyzed using QuantStudio 6 Flex real-time PCR system software (Applied Biosystems, USA).

All primer sequences used in this study were as follows: IL-6 forward, 5′-ATG AAC TCC TTC ACA AGC-3′, reverse, 5′-GTT TTC TGC CAG TGC CTC TTT G-3′; IL-1β forward, 5′- ATG ATG GCT TAT TAC AGT GGC AA-3′, reverse, 5′-GTC GGA GAT TCG TAG CTG GA-3′; IL-18 forward, 5′-AAA CTA TTT GTC GCA GGA ATA AAG AT-3′ reverse, 5′-GCT TGC CAA AGT AAT CTG ATT CC-3′; NLRP3 forward, 5′- AAG GGC CAT GGA CTA TTT CC-3′ reverse, 5′- GAC TCC ACC CGA TGA CAG TT-3′ and GAPDH forward, 5′-TCA ACA GCG ACA CCC AC-3′ reverse, 5′- GGG TCT CTC TCT TCC TCT TGT G-3′ (Humanizing Genomics Macrogen, Geumcheon-gu, Seoul, Republic of Korea) (29). The $2^{-\Delta\Delta CT}$ method with normalization to GAPDH and controls were used for calculation of results.

Western blot analysis

In order to investigate the inhibitory mechanism of PFEA and Lu on Spike S1-induced inflammation in A549 cells, NLRP3 inflammasome machinery proteins and protein involved in JAK/STAT pathway were determined for their expression using western blot analysis. The A549 cells were pre-treated with increasing concentrations of PFEA (0–100 μg/mL) or Lu (0–20 μg/mL) for 24 h. Then, the A549 cells were exposed to 100 ng/mL of Spike S1 for 3 h.

RIPA buffer was used to collect and lyse the A549 cells. Then, the Bradford method was used to determine the protein concentration. The whole-cell lysate was subjected to 8 or 12% SDS-PAGE. Separated proteins were transferred into nitrocellulose membranes. Membranes were incubated with 5% bovine serum albumin (BSA) in 0.5% TBS-Tween for 1 h at room temperature. Then, membranes were incubated with the primary antibody overnight at 4°C. Next, the membranes were washed with 0.5% TBS-Tween to the removed unbound primary antibody.

After that, the membraned was incubated with horseradish peroxidase-conjugated anti-mouse or rabbit-IgG depending on the primary antibody for 2 h at room temperature. The membrane bound antibodies were detected using the chemiluminescent detection system and then exposed to X-ray film (G.E. Healthcare Ltd., Little Chalfont, UK). Each membrane was stripped and re-probed with anti-β-actin antibody to

confirm equal values of protein loading. The band densities were determined using IMAGE J 1.410 software.

Statistical analysis

All experiments were carried out in three independent experiments and the data were presented as mean ± standard deviation (mean ± S.D.) values. Prism version 8.0 software was used for statistical analysis using an independent *t*-test and one-way ANOVA with Dunnett's or Tukey's *post-hoc* test. Statistical significance was determined at the p-value < 0.05, 0.01, and 0.001.

Results

Phytochemical characteristics and identification of active compounds of PFEA

To study the anti-inflammation properties of *P. frutescens* seed meal upon Spike S1 induction, we first established the ethyl acetate fraction of *P. frutescens* seed meal (PFEA) using the solvent-partitioned extraction technique and determined its active compounds using the HPLC technique. The PFEA was found to contain high amounts of total phenolic compounds (605.94 ± 15.70 mg GA/g extract) as well as total flavonoid compounds (567.51 ± 5.51 mg CE/g extract). Moreover, in this study, Ra, Lu, apigenin, kaempferol, caffeic acid, and gallic acid in PFEA were identified using the HPLC technique. The results demonstrated that Ra was found to be the major compound in PFEA at a concentration of 388.70 ± 11.12 mg/g extract followed by Lu that was found at 248.82 ± 12.34 mg/g extract as shown in Table 1. Apigenin, kaempferol, caffeic acid, and gallic acid were found at much lower amounts when compared with the former two major compounds. Overall, it can be concluded that the PEFA obtained from the solvent partition extraction technique of *P. frutescens* seed meal contained two major compounds, Ra and Lu, that will be used in the further experiments together with PFEA in deeper investigations of their relevant anti-inflammation properties against Spike S1-exposed A549 lung cells.

TABLE 1 Phytochemical characteristics and the identification of the active compounds in PFEA.

Ethyl acetate fraction of *P. frutescens* seed meal (PFEA)	mg/g extract (mean ± S.D.)
Total phenolic contents	605.94 ± 15.70
Total flavonoid contents	567.51 ± 5.51
Rosmarinic acid	388.70 ± 11.12
Luteolin	248.82 ± 12.34
Apigenin	88.22 ± 12.70
Kaempferol	70.13 ± 10.50
Caffeic acid	9.22 ± 3.18
Gallic acid	8.02 ± 3.82

Data are presented as mean ± S.D. values of three independent experiments.

Cytotoxicity of PFEA and its active compounds on A549 cells

Before the determination of the anti-inflammation properties of PFEA, the effects of PFEA and its major compounds, Ra and Lu, on the A549 cells cytotoxicity were determined using the MTT assay. After 24- and 48 h of incubation, PFEA at concentrations of 0–200 μg/mL exhibited no significant cytotoxicity effects on A549 cells (Figure 1A). Lu and Ra at concentration of 0–20 μg/mL displayed at least 80% cell survival after both 24 and 48 h of incubation (Figures 1B, C). Overall, it can be summarized that PFEA and its major active compounds, Ra and Lu, exhibited no cytotoxic effects against A549 cells. Therefore, in accordance with the exposure time in the further experiments, A549 cells were pre-treated with PFEA, or its active compounds for 24 h followed by Spike S1 induction for another 3 h before the cells and supernatants were harvested. Non-toxic concentrations (with % of cells survival of more than 80%) of PFEA (0–100 μg/mL) and its active compounds, Ra and Lu, (0–20 μg/mL) were selected for further investigations of their related anti-inflammatory properties on Spike S1-exposed A549 cells.

Effect of PFEA and its major active compounds on the inhibition of pro-inflammation cytokine releases in Spike S1-exposed A549 cells

To investigate the anti-inflammation effects of PFEA and its major active compounds (Ra and Lu), the cytokine release into the culture supernatant of Spike S1-exposed A549 cells was examined by ELISA testing. The IL-6, IL-1β, and IL-18 cytokine releases under the Spike S1-exposed condition in A549 cells were significantly increased when compared with the non-Spike S1 control group ($p < 0.001$), as is shown in Figure 2. The IL-6, IL-1β, and IL-18 releases from Spike S1-exposed A549 cells were significantly diminished in a dose-dependent manner ($p < 0.001$) by PFEA treatments, as it is shown in Figures 2A–C. With regard to the active compounds, the IL-6, IL-1β, and IL-18 releases from Spike S1-exposed A549 cells were significantly decreased in a dose-dependent manner ($p < 0.01$) only by Lu treatment. The Ra treatment exhibited no inhibitory effects on the IL-6, IL-1β, and IL-18 releases in Spike S1-exposed A549 cells, as it is shown in Figures 2D–F. When we compared

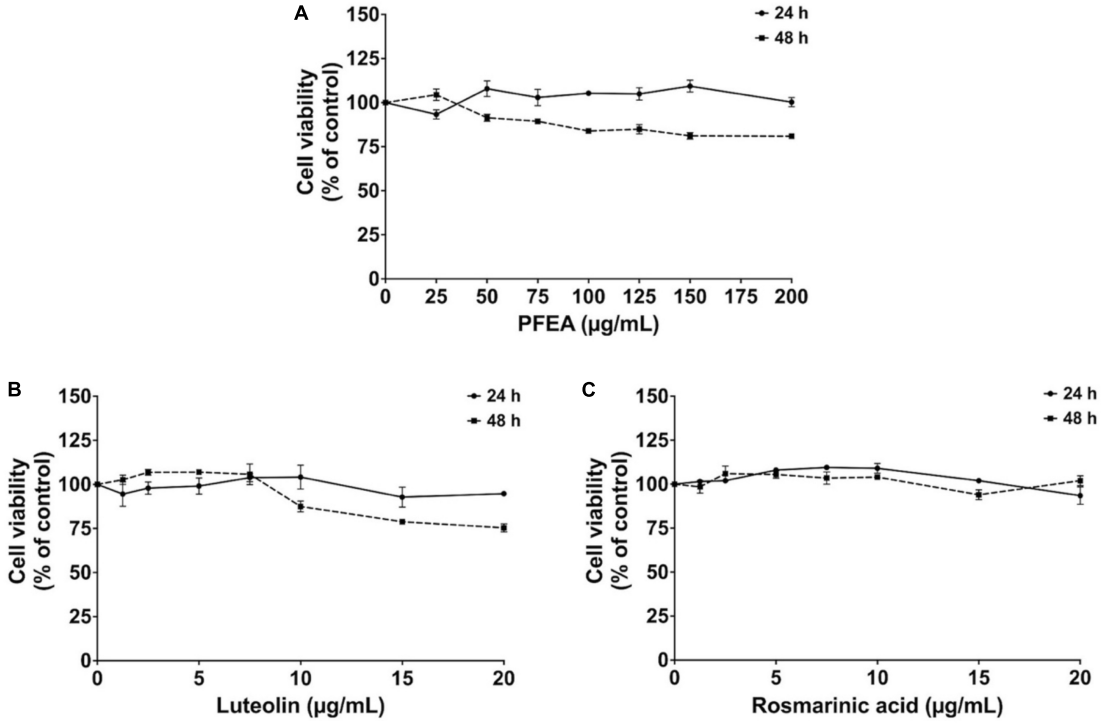

FIGURE 1
Cytotoxicity of PFEA and its active compounds on A549 cells. The cells were treated with PFEA **(A)**, luteolin **(B)**, and rosmarinic acid **(C)** for 24 and 48 h. Cell survival was determined using the MTT assay. Data are presented as mean ± S.D. values of three independent experiments. Data are presented as mean ± S.D. values of three independent experiments.

the inhibitory effects of Lu and Ra on the pro-inflammation cytokine secretion, we found that Lu had significantly greater inhibitory effect on IL-6, IL-1β, and IL-18 cytokine releases from Spike S1-exposed A549 cells than that of Ra ($p < 0.05$). Taken together, it can be summarized that PFEA and Lu significantly exhibited anti-inflammatory properties upon Spike S1-exposed A549 cells by suppressing the IL-6, IL-1β, and IL-18 cytokine releases.

Effect of PFEA and its major bioactive compounds on inhibition of *IL-6*, *IL-1*β, *IL-18*, and *NLRP3* gene expressions in Spike S1-exposed A549 cells

The effects of the PFEA, as well as its major active compounds, Ra and Lu, on *IL-6*, *IL-1*β, *IL-18*, and *NLRP3* mRNA expressions in Spike S1-exposed A549 cells were examined using RT-qPCR. As is shown in **Figure 3**, the *IL-6*, *IL-1*β, *IL-18*, and *NLRP3* mRNA levels were significantly increased in the Spike S1-exposed A549 cells treatment group when compared with the control, non-Spike S1 group ($p < 0.001$). The PFEA and Lu treatments significantly decreased *IL-6*, *IL-1*β, *IL-18*, and *NLRP3* mRNA levels in Spike S1-exposed A549 cells in a dose-dependent manner as shown in **Figures 3A–D**. However, Ra treatment exhibited no inhibitory effects on the *IL-6*, *IL-1*β, *IL-18*, and *NLRP3* mRNA expressions in Spike S1-exposed A549 cells (**Figures 3E–H**). When compared the inhibitory effects of Lu and Ra on the Spike S1 induced-inflammatory gene expressions, it was found that Lu significantly exhibited more potent inhibitory effects of *IL-6*, *IL-1*β, *IL-18*, and *NLRP3* mRNA levels than those of Ra ($p < 0.05$). All above the results, it can be summarized that Lu is a key active compound in PFEA and exhibited anti-inflammation properties upon Spike S1 induction through a significant reduction in *IL-6*, *IL-1*β, *IL-18*, and *NLRP3* gene expressions, as well as the release of IL-6, IL-1β, and IL-18 cytokines in A549 cells. Consequently, PFEA and Lu were used in further experiments to investigate the relevant inhibitory mechanism *via* the NLRP3 inflammasome pathway in Spike S1-exposed A549 cells.

Inhibition effects of PFEA and luteolin on the NLRP3 inflammasome pathway in Spike S1-exposed A549 cells

The NLRP3 inflammasome component is comprised of NLRP3, ASC, and pro-caspase-1 (p50). To activate the NLRP3

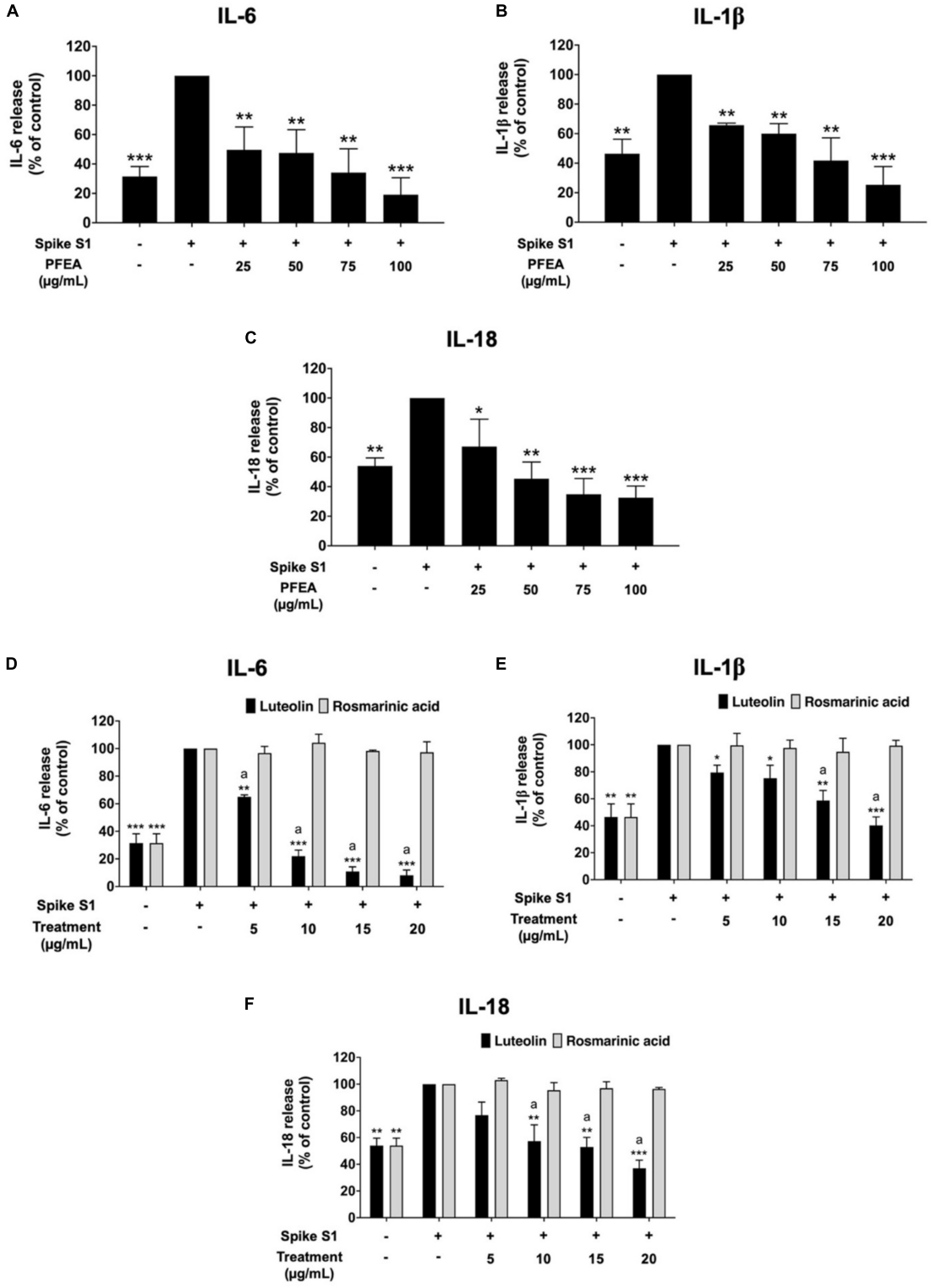

FIGURE 2
Anti-inflammatory properties of PFEA and its active compounds on cytokines releases upon Spike S1-induced inflammation in A549 cells. A549 cells were pre-treated with PFEA **(A–C)**, at the concentration of 0–100 μg/mL or active compounds **(D–F)**, rosmarinic acid and luteolin, at the concentration of 0–20 μg/mL for 24 h. The cells were then exposed to Spike S1 (100 ng/mL) for 3 h. The IL-6, IL-1beta, and IL-18 releases into the culture supernatant were examined by ELISA. The Spike S1-exposed A549 cells are presented as 100%. Data are presented as mean ± S.D. values of three independent experiments, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ vs. the Spike-exposed control group. $^{a}p < 0.05$ vs. rosmarinic acid at the same concentration.

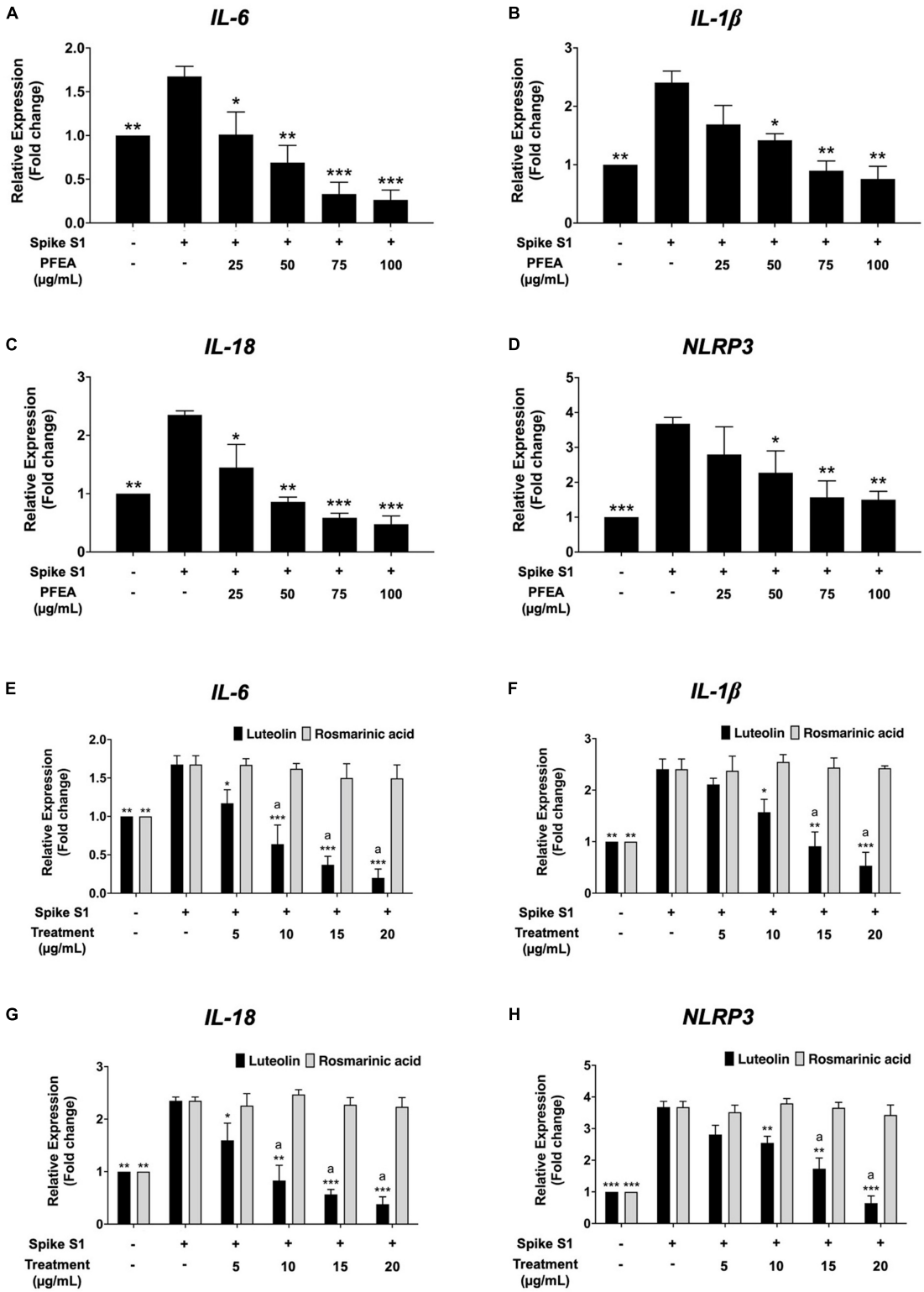

FIGURE 3
Anti-inflammatory properties of PFEA and its active compounds on *IL-6*, *IL-1β*, *IL-18*, and *NLRP3* gene expressions upon Spike S1-exposed A549 cells. A549 cells were pre-treated with PFEA **(A–D)** at concentration of 0–100 μg/mL and or active compounds **(E–H)**, rosmarinic acid and luteolin, at concentration of 0–20 μg/mL for 24 h. The cells were then exposed to Spike S1 (100 ng/mL) for 3 h. The mRNA expressions were determined using RT-qPCR. Data are presented as mean ± S.D. values of three independent experiments, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ vs. the Spike S1-exposed A549 cells. $^{a}p < 0.05$ vs. rosmarinic acid at the same concentration.

inflammasome, protein–protein interaction between NLRP3 and ASC causes the ASC to associate with pro-caspase-1 (p50). Then, pro-caspase-1 was activated to cleaved-caspase-1 (p20) followed by the release of IL-1β and IL-18 cytokines (11, 12). Therefore, inhibition of the NLRP3 inflammasome complex or inflammasome component could potentially be the targeted pathway for Spike S1-induced inflammation. Accordingly, the inhibitory effects of PFEA and Lu on NLRP3 inflammasome machinery proteins in Spike S1-exposed A549 cells were determined by western blot analysis. As it is shown in Figure 4, the NLRP3, ASC, and pro-caspase-1 (p50) protein expressions were significantly increased ($p < 0.01$) in Spike S1-exposed A549 cells when compared with the control, non-Spike S1 group. Meanwhile, the NLRP3, ASC, and pro-caspase-1 (p50) protein expressions in Spike S1-exposed A549 cells were significantly decreased in a dose-dependent manner of PFEA (0–100 μg/mL) and Lu (0–20 μg/mL) treatments as it is shown in Figures 4A, B. Moreover, the cleaved-caspase-1 (p20) expression was significantly increased in Spike S1-exposed A549 cells when compared with the control, non-Spike S1 group as is shown in Figures 4A, B. When we treated the cells with PFEA and Lu, the cleaved-caspase-1 expression in Spike S1-exposed A549 cells was significantly decreased in a dose-dependent manner. Overall, it can be concluded that PFEA and Lu were partially responsible for the anti-inflammatory properties upon Spike S1-exposed A549 cells *via* inhibition of the expressions of NLRP3, ASC and pro-caspase-1 (p50) and the cleavage form of caspase-1 (p20), which would then lead to a decrease in pro-inflammatory cytokine releases (IL-1β and IL-18) at both the gene and protein levels.

Inhibitory effects of PFEA and luteolin on the JAK1/STAT3 signaling pathway in Spike S1-exposed A549 cells

To examine the upstream regulatory pathway which is responsible for the anti-inflammatory properties of PFEA and Lu upon Spike S1-induced NLRP3 inflammasome in A549 cells, the protein expressions of the JAK1/STAT3 signaling pathway

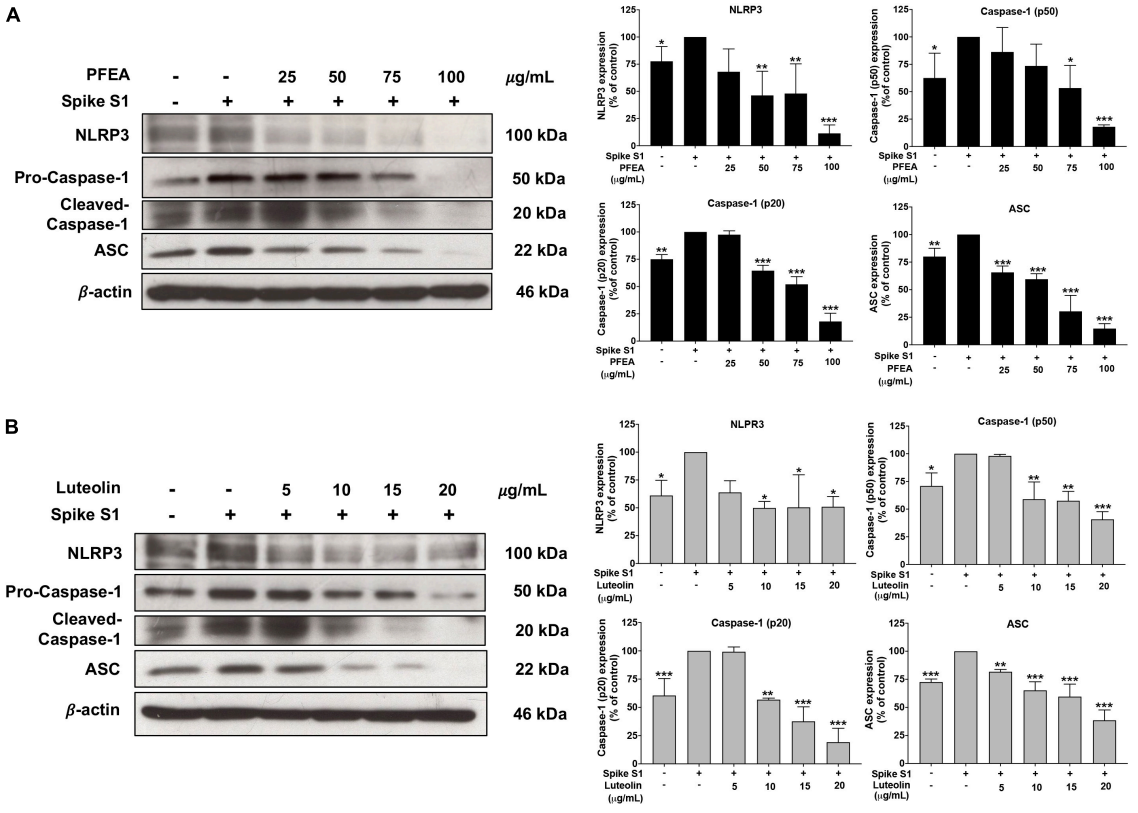

FIGURE 4
Effects of PFEA and luteolin on the NLRP3 inflammasome pathway inhibition in Spike S1-exposed A549 lung cells. A549 lung cells were pre-treated with the PFEA at concentration of 0–100 μg/mL or luteolin at concentration of 0–20 μg/mL for 24 h, and then exposed to Spike S1 (100 ng/mL) for 3 h. The inhibitory effects of PFEA **(A)** and luteolin **(B)** on the expression of NLRP3, ASC, caspase-1 (p50), and the cleaved caspase-1 (p20) in the cell lysate of A549 cells were determined by western blot. The band density was measured using Image J software. Spike S1-exposed A549 cells are presented as 100% of control. Data are presented as mean ± S.D. values of three independent experiments, $*p < 0.05$, $**p < 0.0,1$ and $***p < 0.001$ vs. the Spike S1-exposed A549 cells.

were examined using the western blot technique. The results indicate that, Spike S1 induction significantly increased the phosphorylation of JAK1 and STAT3 proteins in A549 cells when compared with the non-Spike S1 group, as is shown in Figure 5 ($p < 0.05$, band density measurements). The results also indicate that the PFEA and Lu treatments significantly reduced the phosphorylation of JAK1 and STAT3 proteins in a dose-dependent manner when compared with the Spike S1-exposed A549 cells group, as is shown in Figures 5A, B ($p < 0.05$, band density measurement). Taken together, it can be indicated that the PFEA and Lu treatments could attenuate the Spike S1-induced IL-6 release and NLRP3 inflammasome activation through the inhibition of the JAK1/STAT3 axis resulting in a suppression of inflammatory cytokine releases, including those of IL-6, IL-1β, and IL-18. The conclude mechanism of PFEA and Lu on the inhibition of Spike S1-induced inflammatory responses in A549 cells is shown in Figure 6.

Discussion

The SARS-CoV-2, or COVID-19 outbreak, has developed into a severe public health crisis world-wide. SARS-CoV-2 affects the human respiratory tract and causes severely inflamed responses, and the later variants are reported to be easily spread (30–32). Since a large number of the world's population has been infected. The virus is likely to become endemic in the near future, the consequences of the SARS-CoV-2 infection have drawn the attention of medical practitioners and researchers worldwide. The most prominent systemic effects of post-acute COVID condition commonly known as long-COVID, are the systemic inflammatory triggers in the body (8, 32). Previous studies have reported that the SARS-CoV-2 spike glycoprotein can infect human mucosal cells and alveolar cells in the respiratory tract *via* the spike glycoprotein, that cleavage into S1 and S2 protein subunits. Subsequently, S1 spike protein subunit directly attaches to the ACE2 receptor of the respiratory cells. It can penetrate the lung cell membrane and stimulate of lung damage by inducting a cascade of inflammation (7, 33). The S1 spike protein subunit of SARS-CoV-2 (Spike S1) was found to activate an inflammatory reaction in epithelial lung cells and immune cells (34, 35). Briefly, the A549 cells that were exposed to culture supernatants from spike-exposed alveolar macrophages caused inflammatory cytokine releases (IL-1β, IL-6, IL-8, and TNF-α) with the involvement of NF-κB, AP-1, and STATs transcription factor activation resulting in the driver of inflammation. In PBMC and THP-1 macrophages,

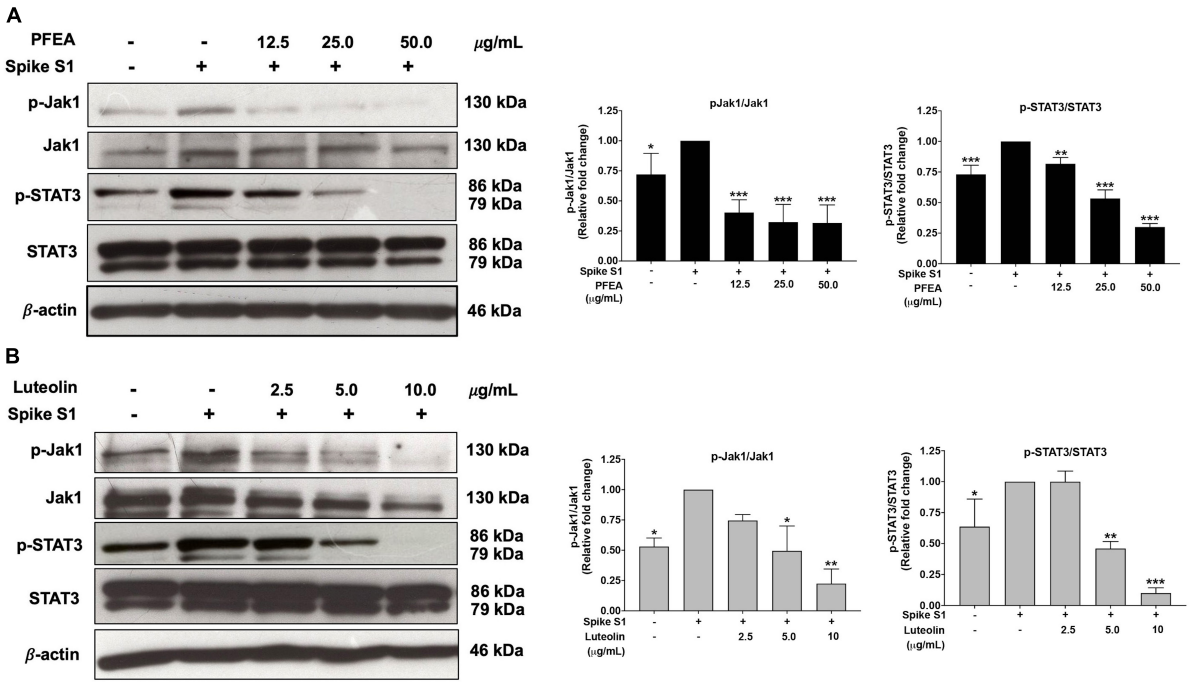

FIGURE 5
PFEA and luteolin inactivated the JAK1/STAT3 signaling pathway in Spike-S1-exposed A549 cells. A549 lung cells were pre-treated with the PFEA at concentration of 0–50 μg/mL or luteolin at concentration of 0–10 μg/mL for 24 h, and then exposed to Spike S1 for 3 h. The inhibitory effects of PFEA **(A)** and luteolin **(B)** on the phosphorylation of the JAK1, and STAT3 proteins in A549 cells were displayed in western blot and band density measurements. The Spike S1-exposed A549 cells are presented as 100% of the control. Data are presented as mean ± S.D. values of three independent experiments, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ vs. the Spike S1-exposed A549 cells.

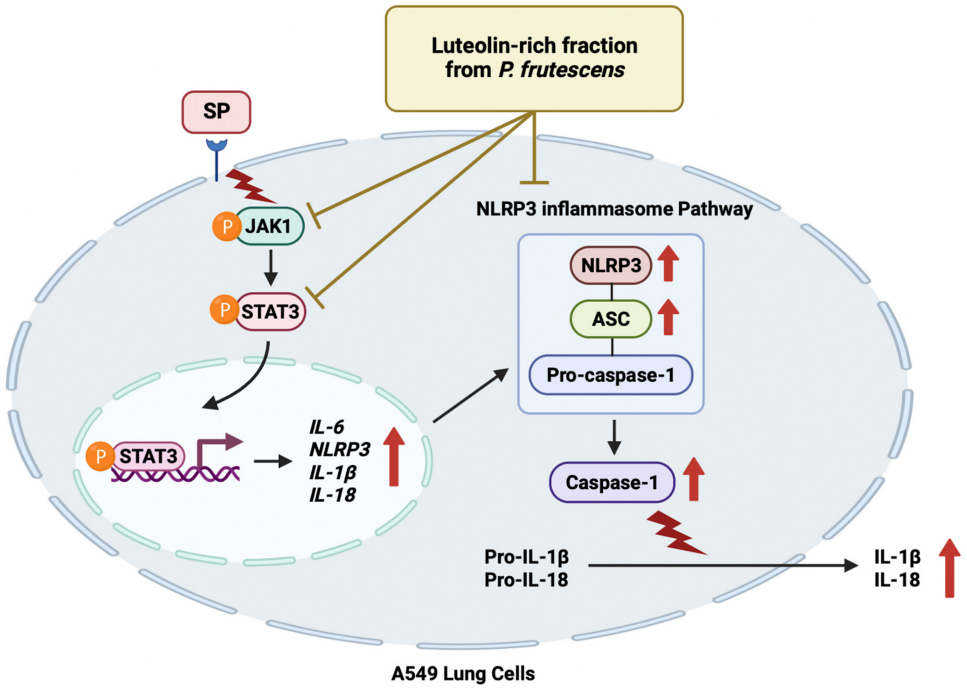

FIGURE 6
Schematic conclude mechanism of luteolin-enriched fraction from *P. frutescens* seed meal (PFEA) attenuated Spike S1-induced NLRP3 inflammasome inflammation through the inactivation of the JAK1/STAT3 signaling pathway in A549 cells.

biochemical studies revealed that the S1 protein of SARS-CoV-2 triggered inflammation through stimulation of the NF-κB pathway in the toll-like receptor 2 (TLR2)-MyD88-dependent manner and induced the IL-6, TNF-α, and IL-1β cytokine expressions. In this study, we employed the cellular model of Spike S1-induced inflammation directly against A549 epithelial cells as representative of the inflammatory responses upon the SARS-CoV-2 spike protein induction. This model has also been used in other research studies and in our previously published studies (29, 34–36).

As of today, no specific therapies have become available for the treating inflammation-related long-COVID complications. Accordingly, most treatments have been developed for the acute COVID-19 for anti-viral purposes (37, 38), and some of which have been found to be associated with the emergence of new variants with prolonged infection (39). As pro-inflammatory cytokines cascade, inflammation is induced by active phospholipase A2 and cyclooxygenase enzymes. Non-steroidal anti-inflammatory drugs (NSAIDs) can cause inhibition of cyclooxygenases. These enzymes are associated with the arachidonic acid biosynthesis cascade, results in a decrease in the prostaglandins production (40). Therefore, NSAIDs, such as paracetamol, ibuprofen, and aspirin, have been prescribed to both acute and long-COVID patients for antipyretic and anti-inflammatory purposes (41, 42). Nevertheless, due to the long-term use side effects, such as gastritis, gastric ulcers, and renal disorders, alternative therapeutic strategies for long-COVID related-inflammation intervention should be encouraged. Accordingly, the naturally occurring compounds from either plants or functional foods could be applied suitably for home-based therapeutic nutraceuticals or preventive medicine to attenuate the inflammatory responses in the long-COVID syndromes (43, 44). In this study, we investigated the preventive roles as anti-inflammatory properties of the ethyl acetate fraction of the seed meal part of *P. frutescens*, which contained high amounts of Ra and Lu phytochemical compounds, in the Spike S1 protein induction model in A549 cells and evaluated their responsible anti-inflammatory mechanism.

To attain the most benefit from *P. frutescens* seed meal, the ethyl acetate fraction of *P. frutescens* seed meal (PFEA) was used in this study by employing the solvent-partitioned extraction technique to obtain this fraction. In agreement with our previous study, this ethyl acetate fraction contained significantly high amounts of polyphenol compounds, including Ra, Lu, apigenin, kaempferol, etc., as is shown in **Table 1**. The PFEA obtained from this study displayed a similar phytochemical profile compared with the previously reported ethyl acetate fraction obtained from *P. frutescens* seed meal (24). Regarding the phytochemical compounds found in PFEA, Ra and Lu were recognized for their potent medicinal effects, including their anti-inflammatory activities in various

stimulation models (18, 45, 46). Briefly, Ra (IC_{50} = 14.25 μM or 5.13 μg/mL) attenuated LPS-induced nitric oxide (NO) production in RAW 264.7 mouse macrophage cells and repressed the pro-inflammatory cytokine expression, including iNOS, MCP-1, IFN-β, IL-6, IL-1β, and IL-10 together with NF-κB activation (47). Additionally, *P. frutescens* seed meal ethyl acetate fraction (at 6.25–50 μg/mL), which contained high Ra content, exhibited osteoclastogenic protection through its anti-inflammatory activities by downregulating RANKL-induced NF-κB and AP-1 activation (24). Ra derived from *Rosmarinus officinalis* possessed *in vitro* antioxidant activities and exhibited *in vivo* anti-inflammatory effects in carrageenan-induced paw edema in the rat model (48). In contrast, in our study, Ra displayed no potent anti-inflammatory properties upon Spike S1 exposure in A549 cells, as evidenced by a non-significant reduction in pro-inflammatory cytokines (IL-1β, IL-18, and IL-6) upon Spike S1 stimulation at both the gene and protein levels. The reasons for this discrepancy might involve the differences in inflammatory stimuli or the fact that different cell lines used in other studies could have produced diverse anti-inflammatory effects. Moreover, as phytochemical compounds with bioactive properties could have strong biological effects even at small amount. We indeed determined the anti-inflammatory properties of the non-major compounds in PFEA at their concentrations that are found in the plant. We found that neither apigenin, kaempferol, caffeic acid, nor gallic acid was able to significantly inhibit the cytokine releases upon Spike S1 exposure (data not shown). On the other hand, in our study, Lu displayed remarkable anti-inflammatory properties upon Spike S1 induction in A549 cells, as observed by significant suppression of pro-inflammatory cytokines (IL-1β, IL-18, and IL-6) at both of the transcript and protein levels.

Lu has been well known to commonly present in many plants. Accordingly, plants with a high Lu content have been used pharmacologically to treat inflammatory diseases. Lu isolated and extracted methods for obtained Lu from plants have been reported using various models and have displayed anti-inflammatory effects (49). Briefly, Lu at 3–10 μM (0.86–2.9 μg/mL) attenuated the expression level of TNF-α and IL-6, IL-1β, and IL-8 mRNA in THP-1 macrophage cells (50). Luteolin at 14.3 and 28.6 μg/mL decreased the total levels of phosphorylated-JAK-1 and phosphorylated-STAT-1 in cytokine-stimulated HT-29 intestinal cells and resulting in a significant inhibition in IL-8 production, as well as inducible nitric oxide synthase (iNOS), nitric oxide (·NO) and COX-2 and expression overproduction (51). In accordance with our studies, the ethyl acetate fraction of *P. frutescens* seed meal and its bioactive compound, Lu, showed anti-inflammatory properties against Spike S1 exposed-A549 cells by the attenuation of pro-inflammatory cytokines including IL-6, IL-1β , and IL-18.

With regard to Spike S1 subunit induced-pulmonary inflammation, the most well-known inflammatory mechanism that seemed to link with inflammation-related long-COVID was the induction of the NLRP3 inflammasome pathway. Briefly, the lung epithelial cells can express the NLRP3, which is known to be uncontrolled and one of the most detrimental inflammatory pathways in pulmonary inflammatory statuses referred to as inflammasome (52, 53). When the NLRP3 inflammasome component (comprised of ASC, NLRP3, and caspase-1) is triggered, the protein complex is assembled, and the inflammasomes cleaved caspase-1. Subsequently, the matured caspase-1 proteolytically cleaves pro-IL-1β and pro-IL-18, resulting in the functional mature IL-1β and IL-18 production, which are subsequently secreted (6, 53, 54). In this study, we demonstrated that A549 cells could successfully be induced with the Spike S1 protein resulting in an increase in the expression of those NLRP3 inflammasome machinery proteins (NLRP3, ASC, and caspase-1), confirming the mechanisms mentioned above. Remarkably, upon investigating Spike S1-exposed A549 cellular model, we also observed the upregulation of IL-6 cytokine at both the gene and protein levels upon Spike S1 induction. From the molecular biology of inflammation perspective, IL-6 cytokine has mostly been recognized for being involved in cytokine receptors and the JAKs/STATs signaling pathway (55). The molecular connection between inflammation and viral infection is complex, and multiple mechanisms might be involved. One proven mechanism is the JAK/STAT signaling pathway, which is central to the production of many cytokines and has been linked to inflammatory induction. Moreover, it has been well-established that the JAK/STAT signaling pathway constitutes the crucial role of a defensive mechanism against viral and bacterial infections (56), including COVID-19 (57–59). Accordingly, several studies have indicated that JAK1/STAT3 is associated with pulmonary inflammation (60–62). Furthermore, numerous receptors have been verified for the collaboration of the SARS-CoV-2 spike protein and the host cells, including ACE2, P2×7, and the IL-6 receptor (63). IL-6 is a key inflammatory mediator associated with COVID-19 severity and inflammation-related COVID (64). The IL-6 can bind to its receptor and promotes dimerization with gp130, which lead to the activation of TYK2, JAK1, and JAK2 (55). Phosphorylated of JAKs and gp130 resulted in the recruitment of SH2 containing STAT1 and STAT3 then these molecules become phosphorylated. Within several of cells, STAT1 and STAT3 assemble either hetero- or homo dimers that are affected as transcription factors to control the expression of multiple inflammation-related genes (65). In previous studies, the JAKs/STAT3 for signal transduction can activate pro-inflammatory gene expressions and facilitate the NLRP3 inflammasome to secrete IL-1β and IL-18 during the pathogenesis of COVID-19-associated neurodegenerative diseases (63, 66, 67). Therefore, a pure compound from natural products targeting inflammasome and the JAK/STAT pathway can be considered a potential anti-inflammatory agent against inflammation-related long-COVID.

Our results evidently show that PFEA and Lu, in a concentration-dependent manner, effectively inhibit Spike S1-induced main inflammatory mediators, including IL-6, IL-1β, and IL-18. Accordingly, this indicates a significant anti-inflammatory effect of PFEA and Lu against Spike S1-induced inflammation in A549 lung cells. Consequently, JAK1 and STAT3 phosphorylation was suppressed, which suggests that PFEA and Lu can inhibit the JAK/STAT cascade, specifically during the step of JAK1 and STAT3 phosphorylation. Consistent with the previous report (68), this resulted in the downregulation of the NLRP3, ASC, and caspase-1 of the inflammasome machinery proteins. Notably, the primary active compound responsible for the effect of PFEA is Lu. Our study is the first to have displayed the efficacy of PFEA and Lu on the inhibition of Spike S1-induced inflammation through the targeting of NLRP3 inflammasome and JAK1/STAT3 pathway. This study enlightens important and unexplored mechanisms by which Lu may suppress SARS-CoV-2-related inflammation, allowing the development of *P. frutescens* seed meal extracts and Lu as a strategic preventive therapy to limit inflammatory progressions.

In conclusion, this study elucidated the significance of preventing the after-effects of SARS-CoV-2 spike protein S1-induction by suppressing the NLRP3 inflammasome complex and its upstream signaling. Furthermore, the outcomes of this study highlight the applicability of Lu and Lu-rich fraction from the *P. frutescens* plant as a potential medicinal plant and bioactive product in the development of nutraceuticals for supportive home-based therapeutic compounds in preventive medicine. Previous studies have concluded that Lu is considered as a non-toxic agent, as determined by the LD_{50} data obtained from animal acute toxicity testing (69–71). *P. frutescens* seed meal and its bioactive compounds could possibly be utilized in preventive home-based therapeutic nutraceuticals by interacting with the involved cytokines throughout SARS-CoV-2-induced inflammation in long-COVID.

Nonetheless, additional studies on the anti-inflammatory properties of *P. frutescens* seed meal extracts and Lu, in animal lung tissues and in clinical settings, should be further investigated to establish the efficacy of *P. frutescens* and Lu. The data gathered from this research could be an invaluable source of biological evidence to strengthen the direction of preventive approaches in COVID-19-related inflammation.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

SD and SU: primary draft of the manuscript, performed the experiment, and data collection and analysis. SM and WS: experimental design, performed the experiments, data analysis, and revised the manuscript. PA and KS: performed the experiments. PD: review and critical appraisal of manuscripts, obtained grant funding, and made the final decisions in the manuscript preparation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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COVID-19 as a risk factor for long-term mortality in patients managed by the emergency medical system: A prospective, multicenter, ambulance-based cohort study

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Introduction: COVID-19 has initially been studied in terms of an acute-phase disease, although recently more attention has been given to the long-term consequences. In this study, we examined COVID-19 as an independent risk factor for long-term mortality in patients with acute illness treated by EMS (emergency medical services) who have previously had the disease against those who have not had the disease.

Methods: A prospective, multicenter, ambulance-based, ongoing study was performed with adult patients with acute disease managed by EMS and transferred with high priority to the emergency department (ED) as study subjects. The study involved six advanced life support units, 38 basic life support units, and five emergency departments from Spain. Sociodemographic inputs, baseline vital signs, pre-hospital blood tests, and comorbidities, including COVID-19, were collected. The main outcome was long-term mortality, which was classified into 1-year all-cause mortality and 1-year in- and out-of-hospital mortality. To compare both the patients with COVID-19 vs. patients without COVID-19 and to compare survival vs non-survival, two main statistical analyses were performed, namely, a longitudinal analysis (Cox regression) and a logistic regression analysis.

Results: Between 12 March 2020 and 30 September 2021, a total of 3,107 patients were included in the study, with 2,594 patients without COVID-19 and 513 patients previously suffering from COVID-19. The mortality rate was higher in patients with COVID-19

than in patients without COVID-19 (31.8 vs. 17.9%). A logistic regression showed that patients previously diagnosed with COVID-19 presented higher rates of nursing home residency, a higher number of breaths per minute, and suffering from connective disease, dementia, and congestive heart failure. The longitudinal analysis showed that COVID-19 was a risk factor for mortality [hazard ratio 1.33 (1.10–1.61); $p < 0.001$].

Conclusion: The COVID-19 group presented an almost double mortality rate compared with the non-COVID-19 group. The final model adjusted for confusion factors suggested that COVID-19 was a risk factor for long-term mortality.

KEYWORDS

clinical decision rules, COVID-19, emergency medical services, long-term mortality, pre-hospital care

Introduction

The current coronavirus disease 2019 (COVID-19) pandemic has been described as a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a disease condition at the beginning characterized by a massive number of cases, leading to unplanned intensive care unit (ICU) admissions, pneumonia with the multiorgan disease, and related mortality (particularly before mass vaccination programs) (1).

At the peak of the pandemic, a drop in notifications to emergency call centers for life-threatening diseases was observed, with a significant decrease in incidents attended by the emergency medical services (EMS) and the emergency department (ED) (2). A marked decrease in cases of acute myocardial infarction, stroke, or traffic accidents has also been reported (3–5), prioritizing COVID-19 (2). EMS were called upon to respond to biohazard medical emergencies, monopolizing patients with COVID-19 and virtually all ambulance transfers. Pre-hospital care was initially provided under unfavorable circumstances, e.g., the use of personal protective equipment, excessive evacuation delays, and, above all, a general unawareness concerning the transmission of the virus (6, 7).

With rapid tests, vaccinations, and effective therapies, the current pandemic has been kept under control, and health systems have managed to deal with COVID-19. We hypothesize that COVID-19 has likely been one of the factors, but not the unique one, of the exacerbation of chronic pathologies and of the observed over-mortality compared to the historical time series. This excess of mortality may result from the lack of appropriate and timely attention to life-threatening diseases, excess mortality due to COVID-19, or a combination of both circumstances (8).

Over the course of the outbreak, health systems have changed from assisting patients with COVID-19 and focusing all efforts on controlling the virus to assisting patients with diseases associated with COVID-19. In other words, COVID-19 has changed from being the primary disease to being treated for a patient in need of urgent care to being part of the full set of pathologies that may negatively affect the prognosis of the patient as a whole (9).

The objective of the present study was to compare long-term mortality (1-year mortality by all-cause and in- and out-of-hospital) in cases managed by EMS and subsequently transferred with high priority to ED in the following two contrasting prospective cohorts: cases with the acute disease without past COVID-19 vs. cases with the acute disease after COVID-19.

Methods

Study design and settings

The present prospective, multicenter, ambulance-based, ongoing study included adult patients with acute disease managed by EMS and transferred with high priority to the ED, collected from two back-to-back prospective studies carried out under the same operative guideline from 12 March 2020 to 30 September 2021.

The study was carried out in four Spanish provinces, i.e., Burgos, Salamanca, Segovia, and Valladolid, covering 24/7 urban, suburban, and rural areas with a reference population of 1,166,746 inhabitants, involving the coordination center 1-1-2, six advanced life support units (ALSU), 38 basic life support units (BLSU), and five EDs, resources managed by the regional public health system (SACYL).

The study protocol was registered in the WHO International Clinical Trials Registry Platform (ISRCTN48326533 and ISRCTN49321933), was approved by the institutional review board of public health (reference: PI-049-19/PI-GR-19-1258), and followed the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) (Supplementary material) (10). Written informed consent was obtained from all the study

participants at the EMS attendance. Patients without informed consent were excluded.

Population

In this study, two prospective cohorts were established. Cohort #01 included acute disease cases with no prior history of COVID-19. Cohort #2 was composed of acute disease cases who had been previously infected by COVID-19.

Adult patients (≥18 years) with acute disease, assisted consecutively by an ALSU and evacuated to ED by ALSU or BLSU, with a 1-year follow-up period were included. Those patients who present the following exclusion criteria were not considered in the study: patients with active COVID-19 cases (this exclusion criterion was selected to avoid the effect of acute infection and to focus on the long-term effects of the previous infection), patients aged <18 years, patients who had cardiorespiratory arrest (on the scene or *en route*), patients who were terminally ill (documented condition), pregnant women, cases discharged *in situ*, and patients with <1-year follow-up. The sample size was based on an opportunity sample method, i.e., selecting all the patients who met the criteria during the study time.

Outcome

The main outcome was long-term mortality, which was classified into 1-year all-cause mortality and 1-year in- and out-of-hospital mortality after the ambulance transfer. The 1-year follow-up period was in line with comparable studies (11, 12). The principal outcome was blinded to the clinical researchers responsible for collecting the data. As the electronic health record is linked to the community mortality registry, all deaths, even those that occurred out-of-hospital, were included in the study. The outcome was retrieved at the end of the study follow-up.

Measures

Sociodemographic inputs (sex, age, urban/rural area, nursing home residence, and evacuation way to the hospital) were collected by an ALSU emergency medical technician. Baseline vital signs (respiratory rate—number of breaths per minute, oxygen saturation, pulse oximetry saturation/fraction of inspired oxygen ratio, blood pressure, heart rate, temperature, and Glasgow Coma Scale) and pre-hospital blood tests (glucose, lactate, and creatinine) were picked up and recorded by the ALSU emergency registered nurse during the first contact with the patient, either at the scene or *en route*. Oxygen saturation, blood pressure (systolic, diastolic, and mean), and heart rate was obtained using LifePAK® 15 monitor-defibrillator (Physio-Control, Inc., Redmond, USA), and temperature using ThermoScan® PRO 6000 thermometer (Welch Allyn, Inc., Skaneateles Falls, USA). The analytical blood test was carried out using point-of-care testing epoc® Blood Analysis System (Siemens Healthcare GmbH, Erlangen, Germany). Finally, the ALSU physician compiled the pre-hospital advanced life support special follow-up procedures, namely, non-invasive respiratory support, invasive respiratory support, and/or use of vasoactive medications (norepinephrine), as well as the pre-hospital presumptive diagnosis, updated based on the 11th revision of the International Classification of Diseases.

To correctly match EMS and the electronic medical record of a hospital patient, we required the exact linkage of at least 5 identifiers, including date, admission time in ED, age, sex, ambulance code, name and surname, and/or healthcare card number. Upon data de-screening, an exact linkage failed with at least five identifiers out of 39 cases, which were excluded from the final analysis.

To assess in-hospital variables, an associate investigator assigned to each hospital (with pre-hospital care records blinded) captured the following at the end of follow-up: SARS-CoV-2 positives (polymerase chain reaction and/or rapid antigen test), 17 categories of comorbidities required to calculate the age-adjusted Charlson comorbidity index (aCCI) (myocardial infarction, congestive heart failure, peripheral vascular disease, stroke or transient ischemic attack, dementia, chronic obstructive pulmonary disease, connective tissue disease, peptic ulcer disease, mild liver disease, uncomplicated diabetes mellitus, hemiplegia, moderate to severe chronic kidney disease, diabetes mellitus with end-organ damage, localized solid tumor, leukemia, lymphoma, moderate to severe liver disease, metastatic solid tumor, and AIDS), hospitalization, ICU admission, and 1-year mortality (all-cause and in- and out-of-hospital). Finally, a data manager calculated the modified sequential organ failure assessment (mSOFA) (13) and aCCI scores (14).

Statistical analysis

Percentages were used to represent categorical variables, and the mean and standard deviation was used as continuous variables. All the comparisons followed the same procedure: first, a univariate comparison, followed by a multivariate regression using those variables with a p-value of <0.001. In particular, two main factors were used to compare groups: patients who had COVID-19 or patients without COVID-19 and mortality. This comparison was performed by considering the whole cohort, selecting only those patients who died or selecting those patients who previously suffered from COVID-19.

A comparison between patients with COVID-19 and patients without COVID-19 for the whole cohort and for those

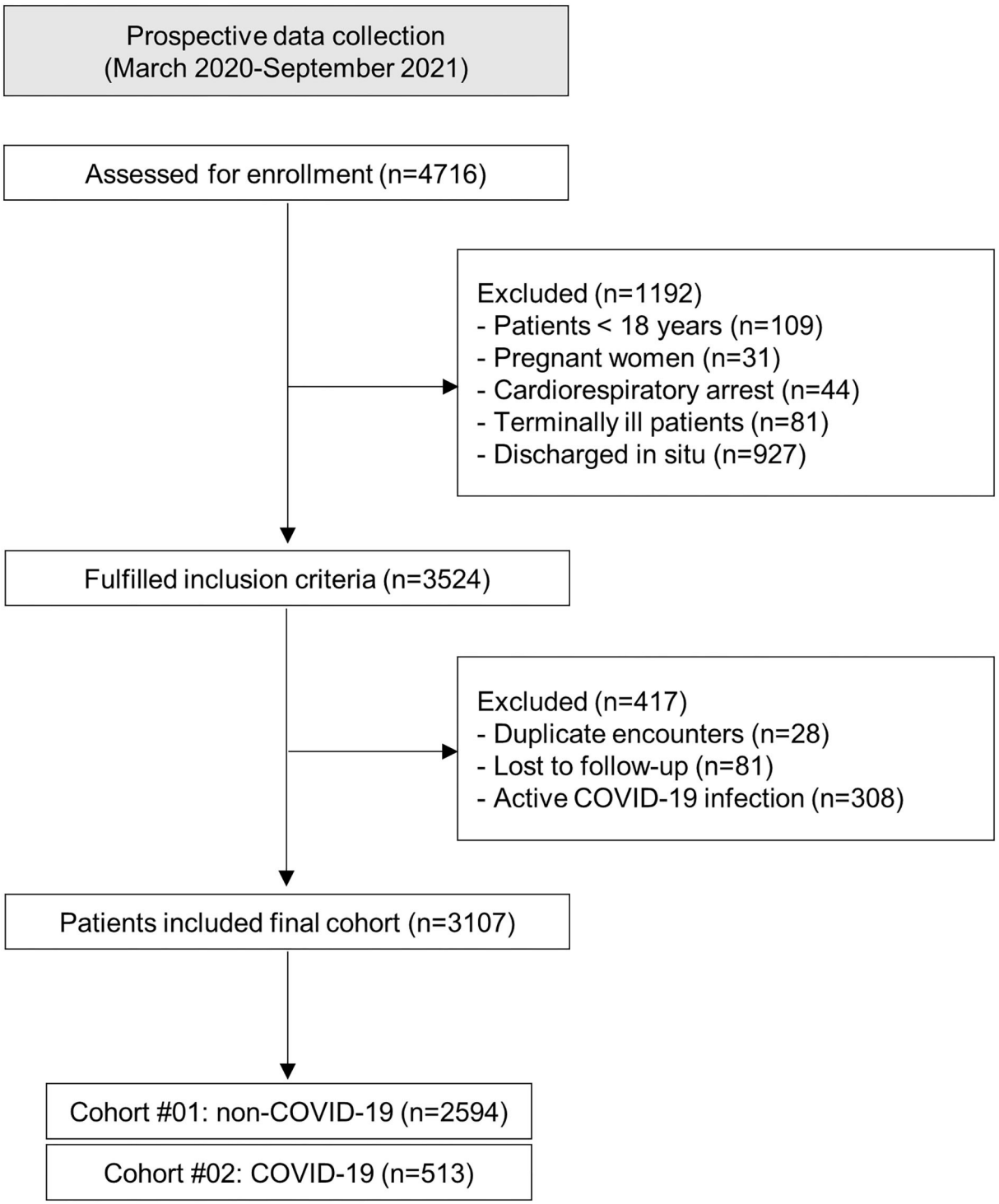

FIGURE 1
Study flowchart. COVID-19, coronavirus disease 2019.

who died within the follow-up time was performed using the Mann-Whitney U test, *T*-test, or chi-squared test, when appropriate, followed by logistic regression with a forward and backward stepwise variable selection. The comparison for mortality was performed by the log rank followed by Cox regression. Furthermore, the survival according to patients with

COVID-19 or patients without COVID-19 was obtained using the Kaplan-Meier method (KM).

Data were analyzed using our own codes and basic functions in R, version 4.2.1 (http://www.R-project.org; the R Foundation for Statistical Computing, Vienna, Austria).

Results

A total of 3,107 patients with acute disease managed by pre-hospital care and referred to the ED were included in the final evaluation: 2,594 in cohort #01 (non-COVID-19) and 513 in cohort #02 (COVID-19). We excluded 308 confirmed active COVID-19 cases in ED (Figure 1).

The median age was 67 years (IQR (interquartile range): 50–81 years), with 41.8% women (1,299 cases). Demographic characterization by COVID-19 cohort included older adults evacuated by ALSU mainly from urban areas to ED and derived to a large extent from nursing homes, with a significant number of comorbidities (especially congestive heart failure, myocardial infarction, dementia, connective disease, and severe chronic kidney disease). The non-COVID-19 cohort exhibited a similar median age, with more middle-aged cases, significantly reduced comorbidities, and a lower nursing home origin. Clinically, both groups reported the same qSOFA, and a similar percentage of pre-hospital advanced life support special procedures, with comparable hospitalization and ICU admission rates (Tables 1, 2).

The overall 1-year mortality was 20.3% (629 cases). Comparing both cohorts, the mortality rate in the COVID-19 group was 13.9 points higher than the one in the non-COVID-19 group (31.8 vs. 17.9%). Cumulative mortality by time points, respectively, 1, 2, 7, 30, 90, 180, and 365 days, in the COVID-19 cohort increased consistently over all time points, exhibiting about double the cumulative mortality vs. the non-COVID-19 cohort for all the time points (Table 3). This result was corroborated by the KM curve (Figure 2); as can be observed, both groups remained parallel throughout the follow-up.

When considering the whole cohort (Table 4A) or only those with 1-year mortality (Table 4B), the logistic multivariate analysis of COVID-19 vs. non-COVID-19 showed that the main characteristics of patients with COVID-19 were being in a nursing home and suffering from dementia or congestive heart failure. Additionally, when considering the whole cohort (Table 4A), patients with COVID-19 suffered from connective disease, presented a higher number of breaths per minute, and had higher 1-year mortality.

Similar to the comparison between patients with COVID-19 and patients without COVID-19, the longitudinal analysis of mortality for the whole cohort (Table 5) showed that factors associated with mortality included (results from Cox regression) age, respiratory support both invasive and non-invasive, noradrenaline administration, hospital admission, and hospital stay duration. The diagnosis groups that stood out as risk factors were respiratory, digestive, infection, and trauma and injury. Pathologies associated with mortality were a metastatic solid tumor, leukemia, and congestive heart failure. Those patients with COVID-19 presented a higher risk of mortality, a variable that remains statistically significant despite the high number of confounding factors. Finally, the mSOFA score was higher in those patients with a higher risk of mortality, suggesting its reliability in predicting clinical worsening even at long-term follow-ups. Further details of the results from this analysis can be found in Supplementary Table S1.

To determine the factors critical for mortality for patients with COVID-19, the same procedure applied in the previous analysis was used for the cohort of patients with COVID-19 (Table 6); further details of these results can be found in Supplementary Table S2. Again, age, mSOFA, respiratory disease, metastatic solid tumor, leukemia, and congestive heart failure were risk factors for mortality. This more detailed analysis showed that hemiplegia, high aCCI, diastolic blood pressure, and FiO_2 were critical factors for mortality within the COVID-19 group.

Discussion

The massive caseload caused by SARS-CoV-2 has consequently led to an increase in mortality rates, associated both with the pandemic and with the suboptimal support provided to non-COVID-19 disease at the start of the outbreak.

Patients treated by pre-hospital care without COVID-19 (cases with an acute disease that did not present the previous COVID-19) showed a 1-year mortality rate close to 18%. According to our results, 1-year mortality for those from the COVID-19 group (cases formerly infected by COVID-19) was 13.9 points higher. A longitudinal analysis showed that presenting COVID-19 as an antecedent is a risk factor for long-term mortality.

Chronic preexisting health conditions are well-documented to play a key role in long-term survival; the greater the number of pathologies, the lower the likelihood of survival and the higher the likelihood of in-patient hospitalization, rehospitalization, and ICU admission rates (15, 16). The number of pathologies was observed as a key factor for short-, medium-, and long-term related mortality since the beginning of the pandemic (17, 18). Different studies examined long-term mortality in post-COVID-19 patients (19–21), but to the best of our knowledge, no research has analyzed the impact of COVID-19 as a previous condition among acute disease patients managed in pre-hospital care.

This over-mortality, according to our study, appears to have a multi-causal explanation. The cases included were multi-pathological patients, such as cardiovascular and neurologic diseases or trauma and injury. Pre-hospital care was

TABLE 1 Demographic characteristics.

Variable	Total	Associated comorbidity		Standardized difference[b]	*p*-value[c]
		COVID-19	Non-COVID-19		
No. (%) with data[a]	3,107	513 (16.5)	2,594 (83.5)	N.A.	N.A.
Age, year	67 (50–81)	74 (56–83)	66 (50–80)	0.264	<0.001
Age groups, year[d]				0.219	<0.001
18–49	726 (23.4)	92 (17.9)	634 (24.4)		
50–74	1,203 (38.7)	183 (35.7)	1,020 (39.3)		
>75	1,178 (37.9)	238 (46.4)	940 (36.2)		
Sex, women	1,299 (41.8)	237 (46.2)	1,062 (40.9)	0.106	0.027
ALS	1,992 (64.1)	302 (58.9)	1,690 (65.2)	0.13	0.007
Urban area resident	2,233 (71.9)	396 (77.2)	1,837 (70.8)	0.146	0.003
Nursing homes resident	305 (9.8)	99 (19.3)	206 (7.9)	0.335	<0.001
Basal vital signs					
RR, number of breaths/min	17 (14–23)	19 (15–26)	17 (14–22)	0.29	<0.001
SpO_2, %	96 (94–98)	96 (92–98)	97 (94–98)	0.254	<0.001
FiO_2, %	0.21 (0.21–0.21)	0.21 (0.21–0.21)	0.21 (0.21–0.21)	0.019	0.689
SaFi	457 (443–467)	452 (429–467)	457 (443–467)	0.179	<0.001
SBP, mmHg	133 (113–151)	131 (107–151)	133 (114–151)	0.104	0.035
DBP, mmHg	78 (65–90)	77 (61–88)	78 (65–90)	0.14	0.004
MBP, mmHg	96 (83–109)	94 (79–108)	97 (83–110)	0.132	0.007
HR, number of beats/min	84 (70–103)	87 (70–105)	83 (70–102)	0.1	0.043
Temperature, °C	36.1 (35.9–36.6)	36.1 (35.8–36.7)	36.1 (35.9–36.6)	0.028	0.589
GCS, points	15 (15–15)	15 (14–15)	15 (15–15)	0.101	0.044
Glucose, mg/dL	130 (106–164)	135 (109–180)	128 (105–160)	0.111	0.022
Creatinine, mg/dL	0.92 (0.76–1.22)	0.98 (0.77–1.41)	0.91 (0.76–1.18)	0.149	0.002
Lactate, mmol/L	2.08 (1.23–3.21)	2.33 (1.36–3.32)	2.07 (1.21–3.18)	0.095	0.041
Outcomes					
mSOFA, points	1 (0–3)	1 (0–4)	1 (0–3)	0.159	0.002
NIRS	92 (3)	27 (5.3)	65 (2.5)	0.143	0.001
IRS	191 (6.1)	33 (6.4)	158 (6.1)	0.014	0.768
Noradrenaline use	82 (2.6)	22 (4.3)	60 (2.3)	0.111	0.011
Hospital-inpatient	1,701 (54.7)	308 (60)	1,393 (53.7)	0.128	0.008
Hospitalization-day	2 (0–8)	2 (0–8)	2 (0–7)	0.07	0.158
ICU-admission	329 (10.6)	57 (11.1)	272 (10.5)	0.02	0.674
1-year mortality	629 (20.3)	163 (31.8)	466 (17.9)	0.323	<0.001

NA, not applicable; ALSn, advanced life support requirement; RR, respiratory rate: SPO_2, oxygen saturation; FiO_2, fraction of inspired oxygen; SaFi, pulse oximetry saturation/fraction of inspired oxygen ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure {MBP = [(2 × DBP) + SBP] / 3}; HR, heart rate; GCS, Glasgow Coma Scale; mSOFA, modified sequential organ failure assessment; NIRS, non-invasive respiratory support; IRS, invasive respiratory support; hospital-inpatient (admission to hospital); hospitalization-day (days of hospitalization); ICU, intensive care unit; COVID-19, coronavirus disease.

[a]Values expressed as total number (fraction) and medians [25 percentile-75 percentile], as appropriate.

[b]The Cohen's d-test was used to estimate the effect size.

[c]The Mann–Whitney U test, T-test, or chi-squared test were used as appropriate.

[d]The age group selection was based on both epidemiological and statistical criteria, i.e., our distribution of patients across groups.

TABLE 2 Comorbidities of baseline patients and diagnosis group.

Variable	Total	Associated comorbidity		Standardized difference[b]	*p*-value[c]
		COVID-19	Non-COVID-19		
No. (%) with data[a]	3,107	513 (16.5)	2,594 (83.5)	N.A.	N.A.
Diagnosis group				0.046	0.349
Cardiovascular	1,149 (37)	187 (36.5)	962 (37.1)		
Neurology	562 (18.1)	80 (15.6)	482 (18.6)		
Respiratory	211 (6.8)	56 (10.9)	155 (6)		
Digestive	131 (4.2)	30 (5.8)	101 (3.9)		
Infection	183 (5.9)	48 (9.4)	135 (5.2)		
Trauma and injury	541 (17.4)	71 (13.8)	470 (18.1)		
Poisoning	250 (8)	26 (5.1)	224 (8.6)		
Others[d]	80 (2.6)	15 (2.9)	65 (2.5)		
aCCI (points)	2 (0–5)	3 (1–6)	1 (0–4)	0.252	<0.001
AIDS	38 (1.2)	12 (2.3)	26 (1)	0.105	0.012
Solid tumor metastatic	118 (3.8)	25 (4.9)	93 (3.6)	0.064	0.063
Liver disease severe	112 (3.6)	29 (5.7)	83 (3.2)	0.119	0.06
Lymphoma	35 (1.1)	4 (0.8)	31 (1.2)	0.042	0.416
Leukemia	31 (1)	7 (1.4)	24 (0.9)	0.041	0.360
Solid tumor localized	498 (16)	97 (18.9)	401 (15.5)	0.091	0.052
DM end organ damage	316 (10.2)	58 (11.3)	258 (9.9)	0.044	0.352
Severe CKD	304 (9.8)	70 (13.6)	234 (9)	0.146	0.001
Hemiplegia	129 (4.2)	34 (6.6)	95 (3.7)	0.134	0.002
DM uncomplicated	376 (12.1)	77 (15)	299 (11.5)	0.103	0.027
Liver disease mild	105 (3.4)	25 (4.9)	80 (3.1)	0.092	0.040
Peptic ulcer disease	276 (8.9)	61 (11.9)	215 (8.3)	0.12	0.009
Connective disease	187 (6)	51 (9.9)	136 (5.2)	0.178	<0.001
COPD	643 (20.7)	123 (24)	520 (20)	0.095	0.045
Dementia	287 (9.2)	84 (16.4)	203 (7.8)	0.264	<0.001
Cerebrovascular disease	287 (9.2)	56 (10.9)	231 (8.9)	0.067	0.151
Peripheral vascular disease	319 (10.3)	60 (11.7)	259 (10)	0.055	0.243
Congestive heart failure	441 (14.2)	130 (25.3)	311 (12)	0.348	<0.001
Myocardial infarction	582 (18.7)	117 (22.8)	465 (17.9)	0.121	0.010

NA, not applicable; aCCI, age-adjusted Charlson comorbidity index; AIDS, acquired immunodeficiency syndrome; DM, diabetes mellitus; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; COVID-19, coronavirus disease.

[a]Values expressed as total number (fraction) and medians [25 percentile-75 percentile], as appropriate.

[b]The Cohen's d-test was used to estimate the effect size.

[c]The Mann–Whitney U test, T-test, or chi-squared test were used as appropriate.

[d]Other pathology: endocrine, genitourinary, diseases of the blood, and the immune system.

homogeneous among both cohorts in terms of assessment using the mSOFA (13) (pulse saturation/inspired oxygen fraction ratio, mean arterial pressure, Glasgow Coma Scale, creatinine, and lactate), although some advanced life support techniques were preferred in COVID-19 cohort, e.g., non-invasive mechanical ventilation and noradrenaline use (22). The rates of hospital-inpatient, hospitalization-day, and ICU admission were statistically equivalent.

TABLE 3 Outcomes of long-term mortality patients.

Variable	1-year mortality		Standardized difference[b]	*p*-value[c]
	COVID-19	Non-COVID-19		
Cumulative mortality[a]			N.A.	N.A.
1-day	30 (5.8)	75 (2.8)	0.061	<0.001
2-day	45 (8.7)	104 (4.1)	0.122	<0.001
7-day	65 (12.6)	159 (6.1)	0.124	<0.001
30-day	96 (18.7)	239 (9.2)	0.153	<0.001
90-day	123 (23.9)	343 (13.2)	0.042	<0.001
180-day	139 (27.1)	399 (15.3)	0.01	<0.001
In-hospital	88 (54)	234 (50.2)	0.075	0.407
Out-hospital	75 (46)	232 (49.8)	0.075	0.407
Age, year	81 (71–88)	79 (66–86)	0.203	0.023
Age groups, year[e]			0.132	0.146
18–49	11 (6.7)	28 (6)		
50–74	38 (23.3)	153 (33.8)		
>75	114 (69.9)	285 (61.2)		
Sex, female	72 (44.2)	171 (36.7)	0.152	0.092
ALS	111 (68.1)	325 (69.7)	0.035	0.696
Urban area resident	124 (76.1)	349 (74.9)	0.027	0.764
Nursing homes resident	59 (36.2)	84 (18)	0.417	<0.001
Pre-hospital outcomes				
mSOFA, points	4 (2–7)	4 (2–6)	0.065	0.479
NIRS	18 (11)	40 (8.6)	0.083	0.351
IRS	25 (15.3)	90 (19.3)	0.105	0.259
Noradrenaline use	20 (12.3)	41 (8.8)	0.113	0.198
Diagnosis group			0.035	0.697
Cardiovascular	57 (35)	132 (28.3)		
Neurology	20 (12.3)	99 (21.2)		
Respiratory	27 (16.6)	67 (14.4)		
Digestive	9 (5.5)	28 (6)		
Infection	24 (14.7)	53 (11.4)		
Trauma and injury	18 (11)	59 (12.7)		
Poisoning	2 (1.2)	11 (2.4)		
Others[d]	6 (3.7)	17 (3.6)		
aCCI (points)	5 (3–9)	4 (2–8)	0.162	0.013
Hospital-inpatient	135 (82.8)	394 (84.5)	0.047	0.604
Hospitalization-day	4 (1–9)	5 (1–12)	0.133	0.172
ICU-admission	27 (16.6)	109 (23.4)	0.171	0.069

NA, not applicable; ALS, advanced life support requirement; mSOFA, modified sequential organ failure assessment; NIRS, non-invasive respiratory support; IRS, invasive respiratory support; aCCI, age-adjusted Charlson comorbidity index; Hospital-inpatient, admission to hospital; Hospitalization-day, days of hospitalization; ICU, intensive care unit; COVID-19, coronavirus disease.

[a]Values expressed as total number (fraction) and medians [25 percentile-75 percentile], as appropriate.

[b]The Cohen's d-test was used to estimate the effect size.

[c]The Mann–Whitney U test, T-test, or chi-squared test were used as appropriate.

[d]Other pathology: endocrine, genitourinary, diseases of the blood, and the immune system.

[e]The age group selection was based on both epidemiological and statistical criteria, i.e., our distribution of patients across groups.

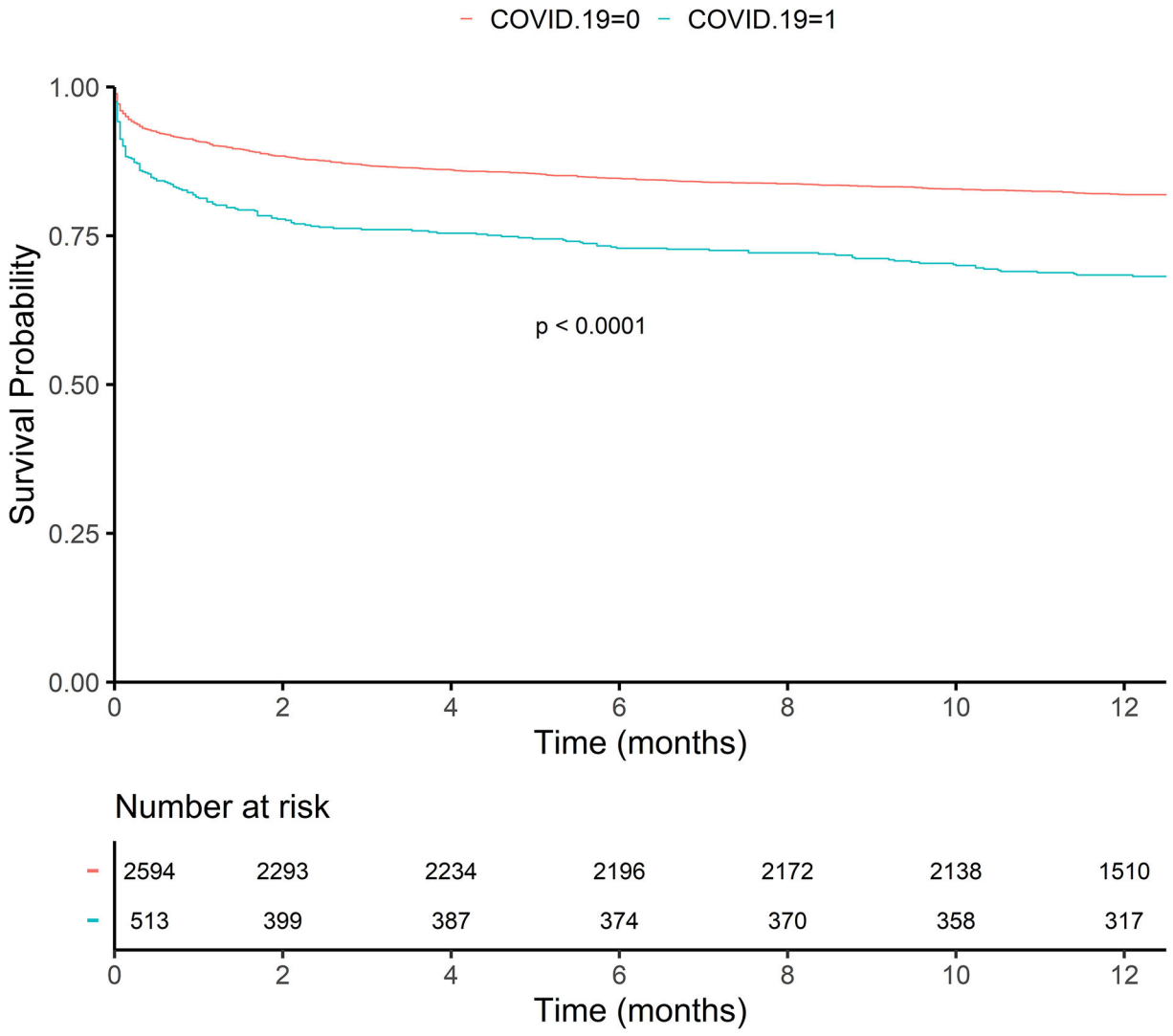

FIGURE 2
The Kaplan–Meier curve showing the survival probability for patients with and without COVID-19. The red line represents patients without COVID-19. The blue line represents patients with COVID-19.

The above results reinforce the argument that over-mortality could be caused by a combination of variables. Chronological age is an unquestionable biological factor. In addition, chronological age plays a pivotal role in chronic diseases, so the older the age, the increased the comorbidities (23, 24). Despite age showing significant differences between groups, we believe that comorbidity burden was the most decisive factor since age was not statistically significant in the multivariate logistic regression. The COVID-19 cohort exhibited a median aCCI of 3 points vs. 1 point in the non-COVID-19 cohort. A detailed analysis highlighted an increase observed in cardiovascular pathology (congestive heart failure and myocardial infarction) and dementia in the COVID-19 cohort, with data in line with similar studies (25–27), since those conditions are associated with common exacerbations, hospital-inpatient, and ultimately poor long-term outcomes. Other pulmonary diseases, such as chronic obstructive pulmonary disease, were not related to a significant increase in 1-year mortality with similar results in both cohorts (28).

Nursing home affiliation was a critical factor directly involved in the mortality of the COVID-19 cohort. Remarkably, at the start of the outbreak, unacceptable mortality rates associated with nursing homes were observed. Admittedly, patients are multi-pathological, with multiple comorbidities, and generally of elderly age, but the over-mortality described in nursing homes should give us a wake-up call to reconsider this fact as a healthcare system (29, 30). Nursing home mortality was two times as high as in the COVID-19 cohort

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TABLE 4 Multivariate logistic regression for patients with COVID-19 vs. patients without COVID-19.

Variable	Odds ratio (95%CI)	*p*-value
(A) Whole cohort		
Nursing homes resident	1.74 [1.35–2.24]	<0.001
RR, number of breaths/min	1.02 [1.01–1.03]	<0.001
Connective disease	1.63 [1.21–2.18]	<0.001
Dementia	1.44 [1.10–1.87]	<0.001
Congestive heart failure	1.80 [1.46–2.22]	<0.001
1-year mortality	1.45 [1.19–1.76]	<0.001
(B) Selecting those patients with 1-year mortality		
Nursing homes resident	2.04 [1.41–2.95]	0.001
Dementia	1.55 [1.05–2.28]	0.06
Congestive heart failure	1.91 [1.38–2.64]	<0.001

RR, respiratory rate; 95% CI, 95% confidence interval.

compared to patients managed by EMS due to acute disease without COVID-19; this irrefutable observation flags nursing home affiliation as a critical factor underlying poor long-term outcomes (31).

The above-mentioned results suggest that COVID-19 plays an important role in this long-term mortality, and three main reasons could be argued for the importance of COVID-19 in long-term mortality: First, in the selection of patients, all the patients were selected based on an opportunity sample method, i.e., selecting all the patients who accomplished criteria during the study time. The difference between the COVID-19 and non-COVID-19 groups regarding age or comorbidities was due to chance rather than a consequence of having suffered from COVID-19. When using the above-mentioned confusion factors in the Cox regression (Table 5), none of them (and the other confusion factors) exclude COVID-19 as a risk factor for mortality. In addition, when all statistically significant factors (including age and aCCI) were adjusted in a regression model to determine the final model that described the difference between the COVID-19 and non-COVID-19 groups (Table 4), age and aCCI were automatically (by the regression algorithm) excluded from the final model, and only a few comorbidities alone were included. Epidemiological studies have shown an excess of mortality in patients with COVID-19 compared to analogous historical series (32, 33). Even though mortality also increased in patients without COVID-19 in the early stages of the pandemic, this trend has gradually normalized to previous levels as healthcare returned to pre-pandemic attention levels and due to the improvement of COVID-19 handling (34). Therefore, as the pandemic evolved, one should expect a reduction in mortality, which was not the case according to our results.

Our study is not free of limitations. First, a pure convenience sample was collected consecutively. To control for potential bias, data input was gathered 24/7 non-stop throughout the study period in ambulance stations located in urban, suburban, and rural areas, patients transferred to ED of different hospitals, and hospitals with different clinical qualifications, attempting to be a true cross-section of the analyzed population. Second, the data extractors were not blinded. To avoid cross-contamination, the EMS staff was unaware of the scores being estimated and interpreted, and as a double fail-safe, the research associates from each hospital were unaware of the pre-hospital parameters as well. Only the data manager and the principal investigator could access the master database. Third, confirmed cases of COVID-19 were taken as patients with a positive polymerase chain reaction and/or rapid antigen test, but an underestimation is possible. Currently, some people skip screening or do not report self-test results. At the onset of the outbreak, the availability of test kits was limited, even though the incidence rates should be treated with caution. In this sense, antibody tests for the non-COVID-19 group were not available, so it cannot be completely ruled out that they did not have COVID-19. Fourth, the study was carried out across different provinces, all of which comprise the same health system. To validate the findings, multicenter studies in different regions involving several institutions should be carried out. Fifth, in the present study, we did not consider all the patients who could present long-term mortality; this is because patients could reach the emergency department by their means without requiring assistance from EMS. However, this study aimed to focus on patients who required pre-hospital emergency care. Sixth, since this study has been developed in the pre-hospital scenario, critical factors related to the long-term consequences of COVID-19 have not been considered due to the impossibility to achieve them, for instance: the date of infection (hampering determining the time between infection and the EMS attendance), the severity of COVID-19, the treatment the COVID-19 (whether it required intensive therapy or invasive mechanical ventilation), and the treatment after COVID-19 hospitalization. Seventh, the duration or diagnostic time of comorbidities was not available; however, despite being important information regarding the status of the patients, it is not included in the commonly used comorbidity-based scores.

Conclusion

According to our results, the COVID-19 group presented a higher mortality rate than the non-COVID-19 group. The predictive model, when adjusted by confusion factors, showed COVID-19 as a relevant risk factor for mortality.

TABLE 5 Factors associated with mortality (univariate and multivariate by Cox regression) for the whole cohort.

	Univariate (log-rank)		Multivariate (Cox regression)	
Variable	Hazard ratio (95%CI)	*p*-value	Hazard ratio (95%CI)	*p*-value
Age, year	1.04 [1.04;1.05]	<0.001	1.03 [1.01–1.05]	<0.001
mSOFA, points	1.40 [1.37;1.43]	<0.001	1.28 [1.18–1.38]	<0.001
NIRS	4.85 [3.70;6.35]	<0.001	2.06 [1.46–2.91]	<0.001
IRS	5.79 [4.73;7.09]	<0.001	2.93 [2.03–4.23]	<0.001
Noradrenaline use	9.46 [7.25;12.3]	<0.001	1.70 [1.21–2.39]	0.002
Respiratory (diagnosis group)	3.13 [2.45;4.01]	<0.001	2.06 [1.52–2.78]	<0.001
Digestive (diagnosis group)	1.79 [1.26;2.55]	0.001	1.87 [1.28–2.73]	0.001
Infection (diagnosis group)	3.04 [2.33;3.96]	<0.001	1.96 [1.43–2.68]	<0.001
Trauma and injury (diagnosis group)	0.87 [0.67;1.14]	0.313	1.65 [1.22–2.22]	0.001
Solid tumor metastatic	4.41 [3.46;5.63]	<0.001	4.28 [3.20–5.73]	<0.001
Leukemia	3.34 [2.03;5.49]	<0.001	4.21 [2.49–7.11]	<0.001
Congestive heart failure	2.85 [2.40;3.39]	<0.001	1.52 [1.25–1.85]	<0.001
Hospital-inpatient	5.16 [4.17;6.39]	<0.001	2.43 [1.90–3.10]	<0.001
Hospitalization-day	1.01 [1.01;1.02]	<0.001	0.97 [0.96–0.98]	<0.001
COVID-19	1.93 [1.62;2.31]	<0.001	1.33 [1.10–1.61]	<0.001

Only statistically significant variables or categories of variables are shown.
95% CI, 95% confidence interval; mSOFA, modified sequential organ failure assessment; NIRS, non-invasive respiratory support; IRS, invasive respiratory support; Hospital-inpatient, admission to hospital; Hospitalization-day, days of hospitalization; COVID-19, coronavirus disease.

TABLE 6 Factors associated with mortality (univariate and multivariate by Cox regression) for those patients with COVID-19.

	Univariate (log-rank)		Multivariate (Cox regression)	
Variable	Hazard ratio (95%CI)	*p*-value	Hazard ratio (95%CI)	*p*-value
Age, year	1.04 [1.03;1.06]	<0.001	1.06 [1.02–1.11]	0.001
FiO_2, %	10.5 [3.52;31.5]	<0.001	0.01 [0.00–0.50]	0.020
DBP, mmHg	0.98 [0.98;0.99]	<0.001	1.01 [1.00–1.02]	0.025
Outcomes				
mSOFA, points	1.39 [1.33;1.45]	<0.001	1.40 [1.17–1.68]	<0.001
Noradrenaline use	10.5 [6.50;17.0]	<0.001	2.88 [1.37–6.06]	0.005
Respiratory (diagnosis group)	1.77 [1.12;2.80]	0.014	2.41 [1.30–4.45]	0.004
aCCI (points)				
0	Ref.	Ref.		
1	0.80 [0.30;2.16]	0.659	0.25 [0.07–0.84]	0.025
2	2.63 [1.24;5.56]	0.012	0.28 [0.08–0.98]	0.048
3	2.85 [1.45;5.59]	0.002	0.20 [0.04–0.88]	0.033
Solid tumor metastatic	2.90 [1.76;4.80]	<0.001	3.73 [1.95–7.12]	<0.001
Leukemia	7.59 [3.53;16.3]	<0.001	10.0 [3.86–26.0]	<0.001
Hemiplegia	3.00 [1.93;4.67]	<0.001	1.75 [1.00–3.04]	0.046
Congestive heart failure	2.45 [1.79;3.35]	<0.001	2.02 [1.33–3.05]	<0.001

Only statistically significant variables or categories of variables are shown.
95% CI, 95% confidence interval; Ref, reference category for hazard ratio calculation; FiO_2, fraction of inspired oxygen; DBP, diastolic blood pressure; mSOFA, modified sequential organ failure assessment; aCCI, age-adjusted Charlson comorbidity index.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Research Review Board of each health area (reference: PI-049-19/PI-GR-19-1258). The patients/participants provided their written informed consent to participate in this study.

Author contributions

FM-R conceptualized the project, managed and coordinated the project, assisted with the design of methodology, analyzed data, and prepared the initial and final drafts of the manuscript. AS-G takes responsibility for the data and their analysis. LM-M, JB-J, RC-S, BP-L, CP, MC, and JM-C assisted with the management and coordination of the project, assisted with the design of methodology, and helped review the manuscript. RL-I conceptualized the project and helped review and comment on the initial and final drafts of the manuscript. All authors performed a critical review and approved the final manuscript for interpretation of the data and important intellectual input.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.1076627/full#supplementary-material

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Cardiovascular and autonomic dysfunction in long-COVID syndrome and the potential role of non-invasive therapeutic strategies on cardiovascular outcomes

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A significant percentage of COVID-19 survivors develop long-lasting cardiovascular sequelae linked to autonomic nervous system dysfunction, including fatigue, arrhythmias, and hypertension. This post-COVID-19 cardiovascular syndrome is one facet of "long-COVID," generally defined as long-term health problems persisting/appearing after the typical recovery period of COVID-19. Despite the fact that this syndrome is not fully understood, it is urgent to develop strategies for diagnosing/managing long-COVID due to the immense potential for future disease burden. New diagnostic/therapeutic tools should provide health personnel with the ability to manage the consequences of long-COVID and preserve/improve patient quality of life. It has been shown that cardiovascular rehabilitation programs (CRPs) stimulate the parasympathetic nervous system, improve cardiorespiratory fitness (CRF), and reduce cardiovascular risk factors, hospitalization rates, and cognitive impairment in patients suffering from cardiovascular diseases. Given their efficacy in improving patient outcomes, CRPs may have salutary potential for the treatment of cardiovascular sequelae of long-COVID. Indeed, there are several public and private initiatives testing the potential of CRPs in treating fatigue and dysautonomia in long-COVID subjects. The application of these established rehabilitation techniques to COVID-19 cardiovascular syndrome represents a promising approach to improving functional capacity and quality of life. In this brief review, we will focus on the long-lasting cardiovascular and autonomic sequelae occurring after COVID-19 infection, as well as exploring the potential of classic and

novel CRPs for managing COVID-19 cardiovascular syndrome. Finally, we expect this review will encourage health care professionals and private/public health organizations to evaluate/implement non-invasive techniques for the management of COVID-19 cardiovascular sequalae.

KEYWORDS

COVID-19, long-COVID, cardiovascular dysfunction, autonomic impairment, therapeutic strategy, cardiovascular outcomes, autonomic dysfunction

1. Introduction

The acute phase of the COVID-19 pandemic has tested health systems around the world. While respiratory aspects of COVID-19 have rightfully taken a primary focus in patient management due to their critical nature, it is worth emphasizing that COVID-19 also has potentially profound effects on cardiovascular, hepatic, renal, gastrointestinal, neurological, and metabolic function. Recent studies and meta-analyses show that there are sequelae of this disease that persist beyond the typical post-viral recovery period (1–3). According to the WHO, this "long form" of COVID-19 disease is defined as a "*condition that occurs in individuals with a history of probable or confirmed SARS-CoV-2 infection, usually 3 months from the onset of COVID-19 with symptoms that last for at least 2 months and cannot be explained by an alternative diagnosis. Common symptoms include fatigue, shortness of breath, and cognitive dysfunction, but may include others and are generally associated with an adverse impact on everyday function. Symptoms may be new onset following initial recovery from an acute COVID-19 episode or persist from the initial illness. Symptoms may also fluctuate or relapse over time*" (4). As a result of its relative recency, long COVID is not well-defined epidemiologically nor is the pathophysiology understood. In the years to come long COVID will impose new burdens on healthcare systems, making it urgent to develop new tools to manage the multiple dimensions of the disease. Underscoring the importance of this challenge, the Spanish Society of Cardiopulmonary Rehabilitation foresees an eventual collapse of its care systems due to the management of the cardiovascular sequelae of COVID-19 (5). A recent study of 5 million patients revealed that COVID-19 survivors experienced a significant increase (up to 2,000%) in the risk of suffering from cardiovascular (infarction, arrhythmias), pulmonary (hypoxemia, dyspnea), metabolic (diabetes, dyslipidemia) and neurological (cognitive impairment, sleep disorders, cerebral infarction) conditions from 1 to 6 months post-infection (1), with the highest risk observed in patients who were critical, followed by hospitalized and asymptomatic patients (3). Subsequent studies have shown a relationship between cardiovascular sequelae of COVID-19 and development of dysautonomia (6), often a product of chronic systemic inflammation that increases sympathetic nerve activity (6–8). This dysautonomia is a component of "post-COVID Guillan-Barré syndrome" (PCGBS) which is the most recurrent type of neurological post-COVID disorder (observed in 15% of patients) (8–11) and has been linked to the neuro-psychological sequelae of long COVID, such as anxiety, depression, and cognitive impairment (9, 10). Despite the fact that long COVID has not yet been fully characterized, dysautonomia is thought to play an important role in the pathophysiology of the syndrome (11, 12), especially with respect to the cardiovascular and neurological aspects. Accordingly, interventions intended to restore normal sympathovagal function could improve the cardiovascular and neurological complications of long-COVID (13, 14). With this in mind, we propose that cardiovascular rehabilitation programs (CRPs) are a feasible tool already established in clinical practice which may be applied to treatment of cardiovascular and neurological sequelae of long COVID.

Cardiovascular rehabilitation programs (CRPs) are interdisciplinary and multidimensional interventions that are defined by: (i) the recurrent execution of simple and well-tolerated exercises that stimulate parasympathetic activity and reduce sympathetic activity, (ii) a family education-based program of exercise, self-care and healthy habits promotion, (iii) an accompaniment program for patients and their caregivers (13). The benefits of CRPs for improving cardiorespiratory fitness has been consistently shown in large cohort studies, and they are first-line therapies for rehabilitation after myocardial infarction and stroke, as well as in the management of elder people with elevated cardiovascular risk (13–19). Given the effectiveness and feasibility of CRPs in clinical contexts, they have great promise as an approach for managing cardiovascular sequelae of long-COVID.

Therefore, we aimed to summarize post-COVID-19 cardiovascular consequences and to encourage health care professionals and private/public health organizations to evaluate/implement non-invasive techniques for the management of COVID-19 cardiovascular sequelae. For that, we selected clinical studies from 2020 to 2022 using the keywords "long-COVID," "cardiovascular," "autonomic/dysautonomia," and other publications from 2009 to 2022 regarding to

"cardiorespiratory fitness," "cardiovascular rehabilitation," "dysautonomia." Finally, these studies were filtered according to their pertinence for long-COVID cardiovascular sequelae epidemiology and non-invasive strategies to improve cardiovascular and autonomic outcomes of long-COVID syndrome, as well as their potential feasibility in clinical contexts. This resulted in the selection of 54 publications, including epidemiological studies, clinical trials, scientific papers, and reviews.

2. Long-COVID syndrome pathophysiology

COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Most of the patients affected by the acute form of the disease (i.e., acute COVID-19) develop mild symptoms such as anosmia, fever, headache, cough, fatigue, and muscle aches (**Figure 1** and **Table 1**; 20). However, a more susceptible population can also develop a severe pneumonia, with the most severe cases progressing to respiratory failure and death (21, 22). Even though SARS-CoV-2 is considered a respiratory virus, COVID-19 disease is a complex inflammatory syndrome that causes diffuse peripheral organ damage that has been shown to adversely affect myocardial, renal, gastrointestinal, and neurological function. In approximately 30% of the cases, neurological dysfunction may include demyelination, encephalitis, encephalopathies, hallucinations, and general behavioral alterations (**Figure 1** and **Table 1**; 23–25). Strikingly, current evidence indicates that up to 50% of COVID-19 patients could develop a post-acute syndrome after the original SARS-CoV-2 infection (26, 27), while one study indicates that 87% of patients continue expressing at least one sign of the disease over 2 months after the first infection (28). Other common symptoms of long COVID include memory loss, alteration of taste and smell, muscle pain and sleep disorders, along with signs specifically associated with autonomic nervous system-dysfunction and related cardiovascular abnormalities (i.e., tachycardia, palpitations, chest pain, thromboembolism, myocardial fibrosis, inflammatory heart disease, and cerebrovascular disorders) (**Figure 1** and **Table 1**; 2, 25, 27, 29).

Patients suffering long COVID do not necessarily test positive for SARS-CoV-2 *via* PCR detection, even in the early first infection phase, and it seems that the risk for developing long-COVID does not correlate with the severity of the acute phase of the virus (30, 31). Although the etiology(ies) of the long-COVID syndrome is still undetermined, there are several reports indicating the presence of chronic cardiorespiratory impairment (i.e., a chronic decrease in lung blood flow) and a hyperinflammatory state (32–34).

Three primary non-exclusive hypotheses have emerged as the possible causes of long-COVID:

1. The first revolves around end-organ vascular endothelial dysfunction and related hypercoagulability leading to microvascular thromboses and local ischemia. Evidence from lung blood flow measurements suggest a chronic impairment in lung vessels due to the presence of small blood clots in pulmonary capillaries and arterioles, leading to hypoperfusion, V/Q mismatch, hypoxemia, and the consequent chronic dyspnea observed in the long-COVID syndrome (28, 35).
2. The second hypothesis theorizes that the persistence of SARS-CoV-2 viral particles either embedded in organ tissue or spread systemically *via* extracellular vesicles stimulates a persistent or intermittent inflammatory response ultimately promoting thromboses and local organ dysfunction. The presence of SARS-CoV-2 viral particles in the lung, brain and heart in post-mortem tissue analysis has been observed (35, 36). Unfortunately, most of this data comes from studies that did not differentiate between acute and long-COVID diseases. However, a recent report indicates the presence of viral particles more than 6 months after the first mild acute-COVID-19 manifestation in long-COVID patients (36, 37). In the study, 70% of the individuals suffering inflammatory bowel disease, presented RNA and proteins of the virus in the gut tissue. Although the causal relationship between these "lingering" viral particles and the development of long-COVID symptoms remains to be elucidated, this evidence invites speculation on whether these viral molecules are responsible for the hyperinflammatory response present in the chronic form of the disease (32, 36).
3. Finally, several studies report that the uncontrolled inflammatory response broadly described in COVID-19 patients (i.e., systemic cytokine storm) (32, 34) might be linked to a hyperactive immune system response, which can be altered even up to 8 months after the initial infection (**Table 1**). Relatedly, there is some thought that acute COVID-19 might spur long-term autoimmune dysfunction such as PCGBS leading to neural degeneration, autonomic dysregulation, and related organ system dysfunction.

One, or more likely, a combination of these hypotheses could explain the causes of this chronic form of COVID-19. Further research is still necessary to determine the origin and the precise mechanisms underlying long-COVID syndrome.

3. Cardiovascular and autonomic consequences of long-COVID

It has been proposed that long-COVID emerges as a consequence of remnant viral particles after acute COVID infection that drives a sustained systemic inflammatory

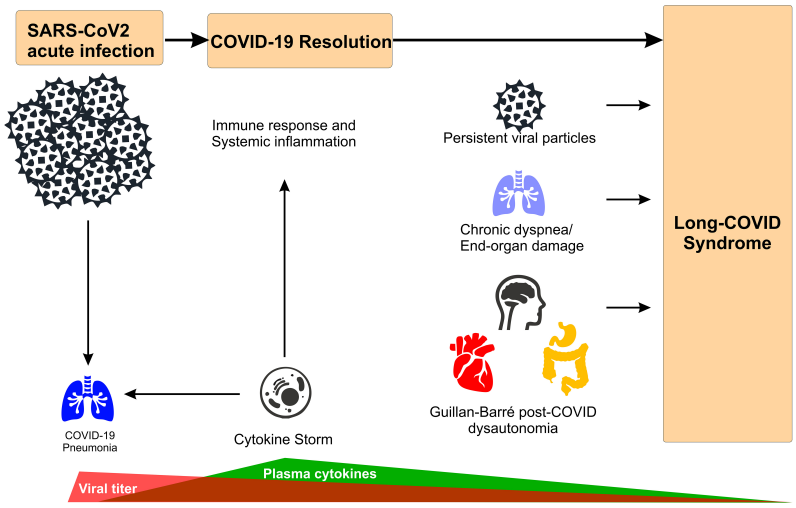

FIGURE 1
Main mechanism of long-COVID syndrome. Acute SARS-CoV-2 infections leads to broad inflammatory response (i.e., cytokine storm) to combat viral infection that causes COVID-19 pneumonia. After infection resolution, potential mechanisms underlying long-COVID syndrome include: (i) remanent viral particles in several tissues/organs, (ii) chronic dyspnea associated with lung function impairment (i.e., hypoperfusion, focal ischemia), and (iii) Guillan-Barré-like dysautonomia post-COVID, characterized by depression/anxiety behavior, excessive daytime fatigue, cardiac arrhythmogenesis, orthostatic hypotension and digestive system complications.

response, which in turn drives cardiovascular, respiratory, neurological, and/or metabolic sequelae (11). Importantly, not every patient experiences the same long-COVID syndrome, depending on their particular inflammatory response after SARS-CoV-2 infection.

One study revealed higher risk of developing cardiovascular diseases up to 12 months post COVID-19 infection compared to contemporary controls (a cohort of more than 5 million people with no evidence of SARS-CoV-2 infection during the study period) and historical controls (additional cohort of ∼6 million people during 2017) (2). These cardiovascular diseases included cerebrovascular diseases (stroke and transient ischemic attacks), dysrhythmias (atrial fibrillation, sinus tachycardia/bradycardia, ventricular arrhythmias, and atrial flutter), cardiac inflammatory disease (pericarditis and myocarditis), ischemic heart disease (acute coronary disease, ischemic cardiomyopathy, and myocardial infarction), heart failure, thromboembolic disorders, and non-ischemic cardiomyopathy; independent of pre-existing cardiovascular morbidities (2). The results showed that the adjusted incident rate ratios of cardiovascular outcomes in the post-COVID-19 exposure period were significantly higher than those in the pre-exposure period and exhibited a graded increase by severity of the acute phase of the disease, and importantly, vaccination significantly reduced the risk of developing myocarditis and pericarditis, supporting the notion that cardiovascular consequences of SARS-CoV-2 infection are dependent on viral infection *per se* rather than pre-existing comorbidities (2).

In the context of cardiovascular and autonomic consequences of long-COVID, it has been reported that around 13% of acute COVID and long-COVID infected patients show a specific type of dysautonomia termed "post-COVID Guillan-Barré syndrome" (PCGBS) (3), described as a microinflammation exclusively occurring in autonomic nerve fibers (vs. autonomic and motor fibers in "traditional" Guillan-Barré syndrome). This localized inflammation drives nerve constriction and augments basal autonomic fiber activity promoting chronic activation of the sympathetic nervous system. This in turn leads to arrhythmogenesis, orthostatic hypotension, altered peristalsis and/or cognitive decline (11). Also, PCGBS has been reported among the fatal complications of long-COVID (30). The reasons why PCGBS is purely autonomic are not known, but it is accepted that dysautonomia (especially chronic sympathetic activation) could be a central focus in the management of long-COVID patients, given the role of the autonomic nervous system in cardiac, respiratory, and metabolic function (3). Therefore, the application of strategies aiming to restore normal autonomic nervous system activity, such as CRP, could have a positive impact on cardiovascular and sympathetic consequences of long-COVID.

4. Diagnostic approaches to long-COVID dysautonomia

There are several direct and indirect clinical tools for diagnosing dysautonomia, including measurements of plasma catecholamines, heart rate variability analysis (HRV), spontaneous baroreflex sensitivity analysis, skin sympathetic nerve activity, skeletal muscle microneurography, and the

COMPASS-31 survey, among others (2, 26). Measurement of plasma catecholamines would be considered a gold standard direct measurement of autonomic activation however this requires a blood draw and additional laboratory testing. The COMPASS-31 survey can easily be applied to patients, but it has several notable limitations, most importantly that it does not generate a quantitative score and that it is dependent on patient recall and honesty. For these reasons its value and application in clinical contexts has been questioned. HRV is a more suitable tool for diagnosing post-COVID dysautonomia compared to COMPASS-31 given that it generates a quantitative score, it is non-invasive, its application is independent of consciousness or cognitive function, it does not rely on patient recall or honesty, and it has been robustly validated in clinical practice as indicator of autonomic function (38–40). In fact, a pilot study has validated HRV analysis as a predictor for the inflammatory and autonomic state of post-COVID patients by using short ECG recordings and AI-processing, making it a potentially powerful tool for diagnosing long-COVID dysautonomia and predicting related cardiovascular dysfunction (41).

TABLE 1 Main characteristics of acute-COVID and long-COVID syndrome*.

	Acute COVID-19	Long-COVID syndrome
Time course window	1 to 4 weeks	4 weeks to > 6 months
Viral detection by PCR	Positive	Negative
Common respiratory symptoms	Dyspnea Cough Sore throat	Dyspnea Cough Oxygen requirement
Major non-respiratory symptoms	Fever Fatigue, muscle/Body aches Headache Loss of taste and/or smell	Fatigue, muscle weakness, joint pain Sleep disturbances Cognitive impairment ("brain fog") Tachycardia, palpitations, chest pain, inflammatory heart disease Chronic kidney failure Inflammatory bowel disease
Causes (major leading hypothesis)	SARS-CoV2 infection	Lung hypoperfusion (blood cloths) Persistent viral proteins/RNA Persistent inflammation

*Based on Nalbandian et al. (27) Nat Med 27:601–615; Couzin-Frankel (36) science, 376:1261–5, and World Health Organization and Mayo Clinic data base on COVID-19 disease.

5. Cardiac rehabilitation programs as a therapeutic adjunct in treatment of long-COVID

Recently CRP has emerged as a potential tool for managing cardiorespiratory and autonomic dysfunction associated with long-COVID. In a pilot study undertaken in Japan (n = 50, 65–74 years of age), a CRP program including easy cardiovascular rehabilitation exercises, education, and individual psychosocial support program, resulted in 90% adherence, a significant reduction in anxiety, improved patient autonomy, and a positive impact on patient quality of life (42). A recent case study examined the efficacy of a personalized CRP in a patient with confirmed PCGBS, and found dramatic improvements in dyspnea, fatigue, muscular strength, autonomy, and functional state (43). These early studies suggest that the application of personalized CRP in patients with long-COVID is a feasible and potentially effective approach to managing autonomic and cardiovascular sequelae of long-COVID.

6. Clinical management of long-COVID-associated cardiovascular dysfunction

As previously discussed, several cardiovascular complications have been described in patients with COVID; however, there is still much to discover about these complications in post-COVID patients, and even more in those patients who have long-COVID. The WHO and the Long-COVID Forum Group have declared the importance of studying and clinically characterizing long-COVID patients to be able to create care and management strategies for these patients in the future (2). Healthcare organizations have stated that research priorities should aim to identify characteristics of long-COVID however given the diffuse and varied presentation of this condition this will undoubtedly be a challenging task (44). What is known at the present time is that there is a wide range of cardiovascular manifestations associated with long-COVID (i.e., those directly related to COVID-19 infection such as pericarditis and myocarditis; and the other ones plausibly related to systemic inflammation and PCGBS, including dysautonomia, arrhythmias, fatigue), and therefore a wide range of potential treatments. In order to tailor potential treatments continuous observation of cardiac biomarkers could be used to fine-tune treatment strategies to the specific manifestation of long-COVID in any given cohort (44). In support of this notion, an expert panel recently convened by the American College of Cardiology recommended that all patients who have had COVID-19 should be tested for abnormal cardiac function especially those with known immunosuppression and older adults at risk for suffering adverse cardiovascular events associated with long-COVID (45). Whether alterations in cardiac function cause or result from impaired cardiac autonomic regulation is still not known. However, the NICE guidelines recommend the use of β-blockers for angina,

coronary syndromes, and cardiac arrhythmias, suggesting that controlling for cardiac sympathoexcitation in long-COVID may offer therapeutic potential in this population (44). An important factor to consider when designing an intervention in long-COVID patients is age. There is evidence that, depending on the age of those involved, COVID-19 infection can result in significant cardiovascular events such as subclinical myocarditis (46). Due to the large number of factors that could mask symptoms in long-COVID patients, it is imperative to develop effective screening, and specialized care and treatment programs. There are clinical studies that have tested different treatment regimens for long-COVID, such as medications, dietary supplements, and even the use of hyperbaric oxygen, but the appropriate design(s) for clinical management still is undetermined (47). There is ample evidence that exercise training improves cardiovascular and autonomic function in clinical populations with underlying cardiac dysfunction (48–50). With this in mind, we are enthusiastic that exercise may represent a complementary therapeutic strategy that may be beneficial to long-COVID patients with dysautonomia and cardiovascular dysfunction (47). These benefits may accrue through a variety of pathways including improvements in vascular endothelial function, autonomic function, and direct effects on myocardial function (51). Focused future studies are needed to provide compelling and comprehensive evidence that support incorporation of exercise programs in the treatment of autonomic and cardiovascular dysfunction associated with long-COVID.

7. Long-COVID syndrome and children

Most investigations of acute and long-COVID infection have been focused on older adults due to their high vulnerability to adverse events. Few studies have analyzed the pediatric COVID-19 population (52). It is recognized that available data on the pediatric population should be interpreted with caution since it's still incomplete and/or missing adjusted values according to several confounding factors (52). Long-COVID-like syndrome has been reported in children and adolescents from 4 to 15 years old (53). Children with SARS-CoV2 history present identical symptoms to those present in Kawasaki disease, cytokine storm, or toxic shock syndrome. Initially, this new set of symptoms were named "Kawashocky" or "pediatric COVID-19 associated inflammatory disorder" (53). Later, The Royal College of Pediatrics and Child Health defined it as "pediatric inflammatory multisystem syndrome temporally associated with SARS-CoV-2" (MIS-C) (54). Interestingly, the underlying mechanisms of MIS-C and adult long-COVID are similar, involving systemic inflammation, cytokine storm, and oxidative stress. MIS-C symptoms have been observed to start from 2 to 6 weeks after acute SARS-CoV2 infection and include cardiac dysfunction and dyspnea (52–54); however, no data from autonomic sequelae in infants/adolescents are currently available. Furthermore, no comprehensive follow-up studies have been done in the pediatric population after COVID infection. This precludes any definite conclusion about any mechanisms that may be involved in cardiovascular/autonomic sequelae of long-COVID in this population. Accordingly, there is an urgent need for studies in children/adolescents to fully characterize long-term sequelae of COVID-infection in order to provide clinical management strategies specially designed for this population that may help to improve long-term outcomes.

8. Conclusion

In summary, long-lasting cardiovascular sequelae of COVID-19 infection are partially mediated by alterations in the autonomic nervous system. Accordingly, the application of new cardiovascular rehabilitation programs to the clinical management of long-COVID patients should provide healthcare personnel with the ability to manage the consequences of long-COVID and may help to reduce future disease burden.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

FA wrote first draft. HD, FO, NM, RQ, NI, and RDR contributed to manuscript formulation and revision. All authors have read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Biomarkers in long COVID-19: A systematic review

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Purpose: Long COVID, also known as post-acute sequelae of COVID-19, refers to the constellation of long-term symptoms experienced by people suffering persistent symptoms for one or more months after SARS-CoV-2 infection. Blood biomarkers can be altered in long COVID patients; however, biomarkers associated with long COVID symptoms and their roles in disease progression remain undetermined. This study aims to systematically evaluate blood biomarkers that may act as indicators or therapeutic targets for long COVID.

Methods: A systematic literature review in PubMed, Embase, and CINAHL was performed on 18 August 2022. The search keywords long COVID-19 symptoms and biomarkers were used to filter out the eligible studies, which were then carefully evaluated.

Results: Identified from 28 studies and representing six biological classifications, 113 biomarkers were significantly associated with long COVID: (1) Cytokine/Chemokine (38, 33.6%); (2) Biochemical markers (24, 21.2%); (3) Vascular markers (20, 17.7%); (4) Neurological markers (6, 5.3%); (5) Acute phase protein (5, 4.4%); and (6) Others (20, 17.7%). Compared with healthy control or recovered patients without long COVID symptoms, 79 biomarkers were increased, 29 were decreased, and 5 required further determination in the long COVID patients. Of these, up-regulated Interleukin 6, C-reactive protein, and tumor necrosis factor alpha might serve as the potential diagnostic biomarkers for long COVID. Moreover, long COVID patients with neurological symptoms exhibited higher levels of neurofilament light chain and glial fibrillary acidic protein whereas those with pulmonary symptoms exhibited a higher level of transforming growth factor beta.

Conclusion: Long COVID patients present elevated inflammatory biomarkers after initial infection. Our study found significant associations between specific biomarkers and long COVID symptoms. Further investigations are warranted to identify a core set of blood biomarkers that can be used to diagnose and manage long COVID patients in clinical practice.

KEYWORDS

biomarker, long COVID, IL-6, CRP, TNF-α

Introduction

The coronavirus disease (COVID-19) was defined as an infectious disease caused by the SARS-CoV-2 virus (1). While the majority of people recovered fully from COVID-19, 45% of COVID survivors might suffer from a variety of unresolved symptoms, which persisted for nearly 4 months after SARS-CoV-2 infection and are referred to as long COVID (2). Older adults might be less likely to experience long COVID than younger adults (3). Additionally, the incidence of experiencing long COVID symptoms post-infection is significantly greater among women versus men (4).

Long COVID manifests as a complex set of symptoms, including neurological, neuropsychiatric, cardiopulmonary, and gastrointestinal (3). Across the studies that have reported the prevalence of long COVID symptoms, among the neurological and neuropsychiatric symptoms more frequently associated with long COVID are fatigue (29–58%), headache (10–44%), and anxiety or depression (22–28%) (5–8). Shortness of breath or difficulty breathing (21–24%) and loss of taste or smell (12–15%) are also frequently reported by long COVID patients with pulmonary symptoms (7, 8). Interestingly, in patients who experienced long COVID syndrome, neurological, neuropsychiatric, cardiopulmonary, and gastrointestinal, and other complications (primarily rheumatological complications) were significantly more likely observed in female than in male patients (9). Furthermore, persistent pulmonary or neurological manifestations seen in long COVID may affect an individual's ability to perform their work, as well as routine daily living activities, such as household chores (6).

One urgent public health question is how to monitor and relieve these long COVID symptoms (3). In this regard, it is desirable to have access to non- or minimally invasive biomarkers, such as those that are often measured in readily available patient blood samples. Clinically-relevant circulating biomarkers may serve as valuable indicators of patients' normal physiological conditions or disease severity. For example, the up-regulated levels of neurofilament light chain (NFL) and glial fibrillary acidic protein (GFAP) in serum may indicate neuronal damage in the progression of neurodegenerative diseases, such as Alzheimer's disease (10) or Parkinson's disease (11). In addition, Interleukin (IL) 6 was not only identified as a prognostic biomarker for disease monitoring in cancer patients with severe COVID-19 (12) but also served as a target for treating COVID-19-related systemic inflammation, such as acute respiratory distress syndrome and cytokine release syndrome (13, 14). While the literature on this topic is evolving fast, to-date diagnostic biomarkers for long COVID remain unclear. This study aims to systematically evaluate the published peer-reviewed literature with the goal of identifying blood biomarkers that may serve as indicators or therapeutic targets for long COVID.

Materials and methods

Search strategy

A systematic literature review in PubMed, Embase, and CINAHL was performed on 18 August 2022. The publication time limit for this search was not specified in order to capture all relevant literature. The search keywords included two broad categories of (1) long COVID-19 symptoms and (2) biomarkers, each with a defined yet broad subset of keywords, as documented in **Supplementary Table 1**. Duplicate records retrieved from these three databases were removed. Then, all relevant articles from reference lists were identified. Articles must be available in full text. After screening the titles and abstracts, final eligibility is determined based on the full content.

Eligibility criteria

The inclusion criteria for this systematic review were as follows: (1) Types of study: the primary source of quantitative studies in a peer-reviewed journal published in English. All original studies, including randomized or non-randomized controlled clinical trials, case reports/case series, and correspondences, were included. For mixed-method studies, if quantitative data could be extracted separately, the studies were included. (2) Types of participants: adult long COVID patients were allowed. No restrictions were imposed on the participants' sex, ethnicity, and clinical symptoms. (3) Types of outcome measures: biomarker data were reported. Articles that did not provide biomarkers or did not have statistically significant data were excluded. Unpublished theses, dissertations, review articles, conference proceedings, and studies using animal models were also excluded.

Data extraction

According to the inclusion and exclusion criteria, data extraction was completed by two authors (SM and S-HL) and verified by another author (Y-JL). The following headlines were extracted from the articles: authors, study location, number of total patients, patients age (median/mean), long COVID timeframe, comparison groups, types of symptoms, biomarker measurements, and conclusions (**Supplementary Table 2**).

Quality assessment

The quality of the studies was assessed using the modified REporting recommendations for tumor MARKers prognostic studies (REMARK), which provides a valuable reference when reporting or analyzing medical studies related to diseases markers or prognostic markers (12, 15, 16). Two independent reviewers (S-HL and T-AL) verified the total scores. The percentages of studies reviewed that met the criteria for methodological quality are shown in **Supplementary Table 3**, and outcomes were summarized in the respective section of results.

Results

Characteristics of the studies

Through the database search, 574 studies from PubMed, CINAHL, and Embase were identified. The search process adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (17) flow diagram, as shown in **Figure 1**. After screening each article's title and abstract, 133 full-text articles were assessed for eligibility. Twenty-eight studies met the eligibility

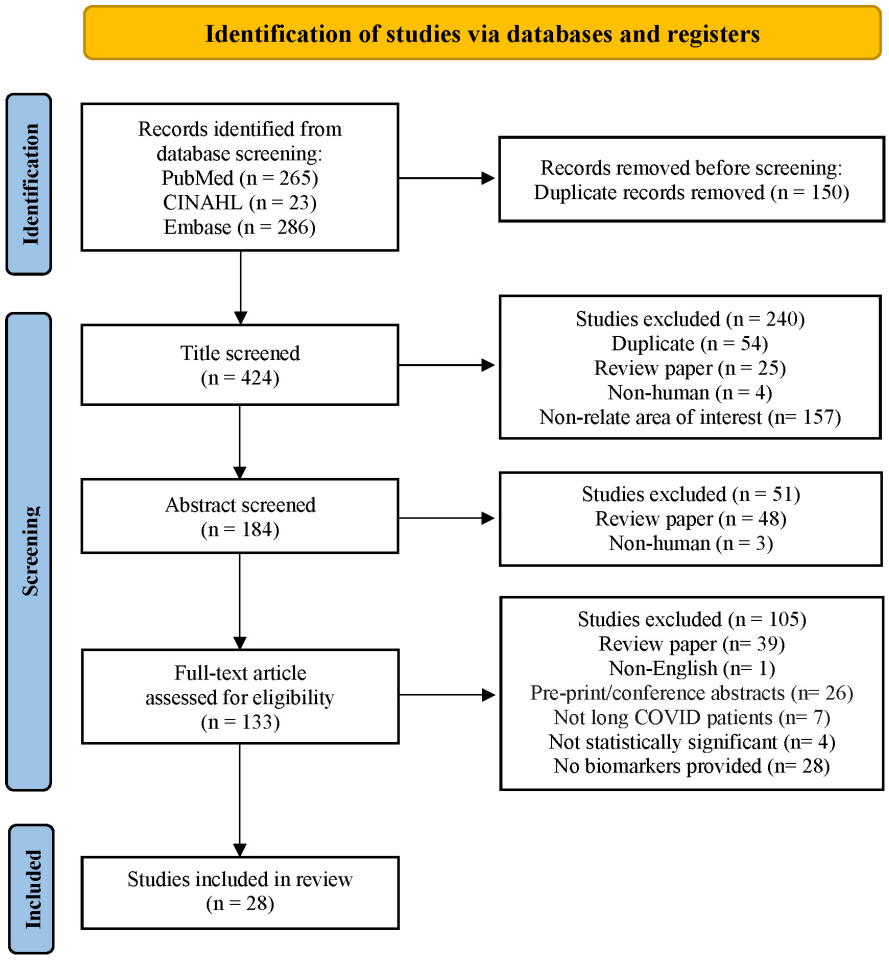

FIGURE 1
Flow diagram of the literature search. Adapted from Page et al. (17).

criteria and were included in this systematic review. The 28 articles listed in ascending alphabetical order (Supplementary Table 2) were published between 2021 and 2022. The eligible studies were conducted in the United States (9, 32.1%), Spain (4, 14.3%), Italy (3, 10.7%), Germany (2, 7.1%), and 1 (3.6%) each in Australia, Brazil, Colombia, Costa Rica, Egypt, India, Ireland, Mexico, Singapore, and Turkey. Among 3,374 participants, 1,569 (46.5%) were long COVID patients, 1,419 (42.1%) were participants who completely recovered from COVID-19, 255 (7.6%) were healthy participants (vaccinated and unvaccinated), and 104 (3.1%) were patients with COVID-19. Of 193 biomarkers tested in the 28 studies, 113 (58.5%) were significantly associated with long COVID symptoms. Long COVID timeframe was defined according to definitions used in the reviewed articles: less than 3 months after SARS-CoV-2 infection in 8 (28.6%) studies, 3–6 months in 9 (32.1%) studies, and 6 or more months in 3 (10.7%) studies. There is one (3.6%) study with a various range (22 to 322 days), and the rest 7 (25%) studies did not provide the definition.

Methodological assessment

All the eligible studies were further evaluated by the modified REMARK questionnaire (18). As shown in Supplementary Table 3 and Figure 2, the majority of studies used a prospective design (96.4%), provided a rationale for the sample sizes (96.4%), described the characteristics of the study population (96.4%), and provided information on the measurement of biomarkers (82.1%). 67.9% defined clinical outcomes, 60.7% provided a list of candidate variables, and 57.1% defined patients' enrollment period. Few articles blinded the measurements of biomarkers to patient outcomes (10.7%).

Biomarker findings

Biomarkers related to biological functions

Among 113 biomarkers, 69.9% (79 of 113) biomarkers were significantly increased, 25.7% (29 of 113) biomarkers were decreased, and 4.4% (5 of 113) biomarkers required further determination in long COVID patients. To facilitate the understanding of biological mechanisms in long COVID related biomarkers, the biomarkers were divided into six categories based on their biological function: (1) Cytokines/Chemokines (38, 33.6%); (2) Biochemical markers (24, 21.2%); (3) Vascular markers (20, 17.7%); (4) Neurological markers (6, 5.3%); (5) Acute phase protein (5, 4.4%); and (6) Others (20, 17.7%) (Table 1). With respect to immune response, long COVID patients exhibited higher levels of pro-inflammatory

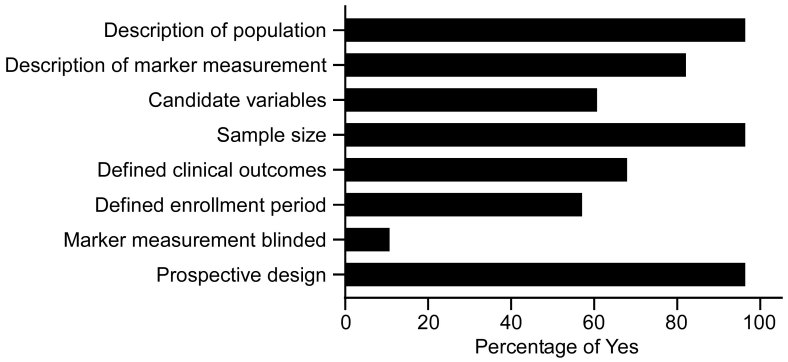

FIGURE 2
Study quality of the 28 articles in the systematic review assessed by the modified REMARK questionnaire (**Supplementary Table 3**).

TABLE 1 Categories of biomarkers significantly associated with long COVID symptoms.

Category	Biomarker	References
Acute phase protein	Albumin, C5b-9, CRP, Ferritin, Fibrinogen	(20, 22, 40–45)
Biochemical marker	1-Methylnicotinamide, 2-Phenylphenol, 3,5-Dihydroxybenzoic acid, ADA, ALT, AST, β-glucan, CPA3, Glutamine/Glutamate ratio, Indole-3-lactic acid, L-Cystein, LDH, L-Glutamine, L-Methionine, Ornithine, Pipecolic acid, Quinolinic acid, Quinolinic acid/Tryptophan, Sarcosine, S-Sulfocysteine, ST1A1, Taurine, Tryptase, uPA	(22, 42, 46–51)
Cytokine/chemokine	CCL2, CCL3, CCL4, CCL5, CCL7, CCL19, CCL20, CCL23, CXCL1, CXCL9, CXCL10, CXCL11, Flt3L, G-CSF, GM-CSF, IFN-α, IFN-β, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-10, IL-10Rβ, IL-12β, IL-13, IL-17, IL-18, IL-33, IP-10, M-CSF, SCF, TGF-α, TGF-β, TNF-α, TNF-β	(19, 21, 22, 40, 43, 47, 48, 52–59)
Neurological marker	GDNF, GFAP, NGF-β, NFL, NT-3, pGFAP/pNFL	(19, 20, 48, 60)
Vascular marker	Ang-2, Col1A2, Col3A1, D-dimer, ESR, ET-1, Factor VIII:C, Hemoglobin, MMP-1, MMP-9, MPO, NO, PDGF-BB, sICAM-1, sTM, sVEGFR, sVCAM-1, VEGF, VWF:Ag, VWF:pp	(21, 42, 44, 46, 48, 52, 56, 58, 59, 61–63)
Others	Ab, ARTN, α-SMA, AXIN, CASP-8, CST-5, Cystatin C, Hs TnT, IGFBP-4, LBP, miRNA21, MRP8/14, NGAL, NT-proBNP/NT-BNP, OPG, OSM, SIRT2, STAMBP, TNFRSF9, Zonulin	(21, 48, 49, 51, 54, 58, 59, 63)

A detailed list of abbreviations in **Table 1** can be found in **Supplementary Table 2**.

cytokines/chemokines [IL-6, tumor necrosis factor alpha (TNF-α), IL-17, IL-4, and C-C motif chemokine ligand (CCL) 2] and acute phase proteins [C-reactive protein (CRP) and ferritin]. For biochemical markers associated with metabolism, COVID-19 patients with elevated levels of lactate dehydrogenase (LDH) tended to experience long COVID symptoms. Furthermore, in terms of neurological and vascular markers, patients with increased NFL and vascular endothelial growth factor (VEGF) plus decreased hemoglobin showed worse long COVID symptoms.

Biomarkers for long COVID patients

Among 28 studies, 20 (71.4%) studies reported biomarkers between long COVID and completely recovered patients, and 12 (42.9%) studies demonstrated biomarkers between long COVID patients and healthy participants (**Table 2**). Compared with recovered COVID patients, long COVID patients showed higher levels of IL-6 (6 of 20, 30%), CRP (3 of 20, 15%), and TNF-α (3 of 20, 15%); lower levels of hemoglobin (2 of 20, 10%). Notably, cytokine/chemokine and biochemical markers accounted for 23.9% (11 of 46) and 39.1% (18 of 46), respectively. Moreover, matched with healthy participants, increased levels of IL-6 (4 of 12, 33.3%), TNF-α (2 of 12, 16.7%), IL-17 (2 of 12, 16.7%), and CCL3 (2 of 12, 16.7%) were associated with long COVID patients. In particular, 44.2% (38 of 86) are cytokine/chemokine, and 20.9% (18 of 86) are vascular markers. The Venn diagram comparison analysis of the differently regulated biomarkers among various groups revealed that IL-6, CRP, and TNF-α remain up-regulated in long COVID patients and may be important indicators of long COVID syndrome (**Figure 3**).

Biomarkers in long COVID-19 symptoms

Among 28 studies, 57.1% (16 of 28) of the studies reported biomarkers in patients with multiple symptoms, followed by 39.3% (11 of 28) with neurological symptoms and 17.9% (5 of 28) with pulmonary symptoms (**Table 3**). A total of 113 blood biomarkers that were related to long COVID symptoms after SARS-CoV-2 infection, 92 (81.4%), 22 (19.5%), and 15 (13.3%) biomarkers, respectively, showed a significant association with multiple, neurological, and pulmonary symptoms. The major classification of biomarkers was cytokines/chemokines: 35.9% (33 of 92) in multiple symptoms, 22.7% (5 of 22) in neurological symptoms, and 33.3% (5 of 15) in pulmonary symptoms. As shown in **Figure 4**, through the Venn diagram comparative analysis of these biomarkers, increased CRP was found to be a significant indicator of multiple, neurological, and pulmonary long COVID symptoms. Additionally, several up-regulated vascular biomarkers associated with angiogenesis [VEGF or Platelet derived growth factor BB (PDGF-BB)] and coagulation [D-dimer, von Willebrand factor antigen (VWF:Ag), von Willebrand factor propeptide (VWF:pp), soluble thrombomodulin (sTM), or

TABLE 2 Biomarkers significantly associated with different comparison groups.

Comparison groups	Categories of biomarkers						References
	Acute phase protein	Biochemical marker	Cytokine/Chemokine	Neurological marker	Vascular marker	Others	
Long COVID vs recovered	Albumin, CRP, Ferritin, Fibrinogen	1-Methylnicotinamide, 2-Phenylphenol, 3,5-Dihydroxybenzoic acid, ALT, AST, β-glucan, Indole-3-lactic acid, LDH, L-Cystein, L-Glutamine, L-Methionine, Ornithine, Pipecolic acid, Quinolinic acid, Quinolinic acid/Tryptophan, Sarcosine, S-Sulfocysteine, Tryptase	CCL2, CXCL10, GM-CSF, IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17, IP-10, TNF-α	GFAP, NFL, pGFAP/pNFL	ET-1, Hemoglobin, NO, PDGF-BB, VEGF	Ab, Hs TnT, LBP, NT-proBNP/NT-BNP, Zonulin	(19, 20, 40, 41, 43–49, 51, 53, 54, 56–58, 60, 62, 63)
Long COVID vs healthy	Albumin, C5b-9, CRP, Ferritin	ADA, CPA3, Glutamine/Glutamate ratio, LDH, ST1A1, Taurine, Tryptase, uPA	CCL2, CCL3, CCL4, CCL5, CCL7, CCL19, CCL20, CCL23, CXCL1, CXCL9, CXCL10, CXCL11, Flt3L, G-CSF, GM-CSF, IFN-α, IFN-β, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-10, IL-10Rβ, IL-12β, IL-13, IL-17, IL-18, IL-33, IP-10, M-CSF, SCF, TGF-α, TGF-β, TNF-α, TNF-β	GDNF, NGF-β, NT-3	Ang-2, Col1A2, Col3A1, D-dimer, ESR, ET-1, Factor VIII:C, Hemoglobin, MMP-1, MMP-9, MPO, sICAM-1, sTM, sVCAM-1, sVEGFR, VEGF, VWF:Ag, VWF:pp ARTN, α-SMA, AXIN, CASP-8, CST-5, Cystatin-C, IGFBP-4, miRNA21, MRP8/14, NGAL, OPG, OSM, SIRT2, STAMBP	ARTN, α-SMA, AXIN, CASP-8, CST-5, Cystatin-C, IGFBP-4, miRNA21, MRP8/14, NGAL, OPG, OSM, SIRT2, STAMBP, TNFRSF9	(21, 22, 42, 47, 48, 50, 52, 54, 55, 59, 61, 62)
Long COVID vs active infected	Albumin, CRP, Feritin		G-CSF, IFN-α, IL-1β, IL-6, IL-13, IL-17, IP-10, TNF-α		D-dimer, ESR, Hemoglobin		(55)

A detailed list of abbreviations in Table 2 can be found in Supplementary Table 2.

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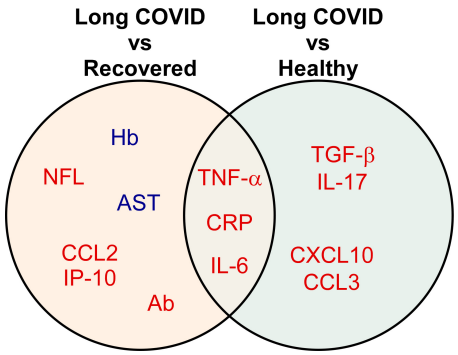

FIGURE 3
Biomarkers significantly associated with different comparison groups. The Venn diagram presents the 13 biomarkers that were reported by two or more eligible studies. Red indicates up-regulated, while blue refers to down-regulated biomarkers. A detailed list of abbreviations in **Figure 3** can be found in **Supplementary Table 2**.

Factor VIII:C] were reported in patients with multiple symptoms. Elevated neurological biomarkers related to nerve injuries, such as NFL and GFAP, may serve as diagnostic biomarkers for long COVID neurological symptoms, especially for long COVID headaches (19, 20). Moreover, in long COVID pulmonary symptoms, compared with healthy control, long COVID patients with pulmonary fibrosis exhibited higher Transforming growth factor beta (TGF-β) (21, 22). As a result, these biomarkers may serve as indicators of distinct long COVID symptoms.

Discussion

Of 193 putative biomarkers tested, 113 were found in this review to be statistically significantly associated with long COVID. To provide a functional view of the biomarkers, we divided the 113 biomarkers into six categories based on their biological function: cytokine/chemokine, biochemical markers, vascular markers, neurological markers, acute phase protein, and others. Through a comprehensive evidence synthesis of biomarkers in long COVID, the up-regulated IL-6, CRP, and TNF-α were found to be a potential core set of biomarkers for long COVID.

Role of circulating biomarkers in long COVID-associated neurological dysfunction

Severe systemic inflammation and substantial tissue damage in acute COVID are accelerated by pro-inflammatory cytokines/chemokines (23), which involve many pathophysiological mechanisms, like leukocyte trafficking (24), cytokine storm (25), and normal tissue necroptosis (26). Systemic inflammatory markers, such as IL-6 and CRP, were associated with disease severity and mortality among COVID-19 patients (27). Moreover, consistent with our findings (**Figures 3**, **4**), the prolonged IL-6, TNF-α, and CRP were also implicated in systemic and neurological long COVID sequelae (28).

Neurological symptoms are the most common long COVID clinical manifestations (7). NFL and GFAP are skeleton proteins that maintain the stability of neuron axons and astrocytes. The expression of these neural peptides in circulation may serve as biomarkers associated with neuronal degeneration and damage (29, 30). Long COVID patients with elevated serum NFL and GFAP showed worse headaches and persistent neuropathic pain (19, 20). Furthermore, Peluso et al. reported that the serum levels of NFL and GFAP in post-acute COVID patients are positively correlated with IL-6, TNF-α, and CCL2 (19) that may induce immune cells and activate detrimental neuroinflammation (31). This indirect mechanism demonstrates that pro-inflammatory cytokines/chemokines may exacerbate substantial neuronal damage.

Role of circulating biomarkers in long COVID-associated pulmonary fibrosis

Pulmonary fibrosis is one of the complications of severe COVID cases (32). Similar to the long COVID patients with pulmonary symptoms, elevated levels of IL-6, CRP, and TGF-β were identified in patients at increased risk of developing pulmonary fibrosis after SARS-CoV-2 infection (**Figure 4**) (33, 34). TGF-β is a multifunctional cytokine that plays a crucial role in tissue repair after injury. Upon a pulmonary viral infection, epithelial cell injury may induce the activation of M2 macrophages to secrete TGF-β, stimulating fibroblast proliferation and collagen synthesis and leading to fibrosis (22, 35). Recently, Zhou et al. demonstrated that Pirfenidone, an Food and Drug Administration (FDA)-approved TGF-β/collagen-targeted drug, attenuated the post-COVID-19 pulmonary fibrosis manifestation (36). Hence, a combination therapy targeting the anti-inflammatory (such as IL-6 blockades) (13) and anti-fibrotic pathways (such as Pirfenidone) (36) may be a potential therapeutical strategy for long COVID with pulmonary fibrosis.

Future directions toward the use of biomarkers

In this review, we have evaluated and summarized the long COVID-related biomarkers. However, because of the heterogeneity of long COVID, no laboratory test could definitively distinguish long COVID from other diseases. A panel of markers may effectively differentiate long COVID cases from others and serve as potential biomarkers for early detection of long COVID. As shown in **Figures 3**, **4**, in addition to the use of a core set of biomarkers (IL-6, CRP, and TNF-α), IL-4, Interferon (IFN) gamma, CCL2, Ferritin, Hemoglobin, NFL, and GFAP may be added in the penal of long COVID patients with neurological symptoms. Likewise, C-X-C motif chemokine ligand 10 (CXCL10), TGF-β, IFN-β, and IL-1α may be included in the panel in patients with pulmonary symptoms. Holistic patient-centered care and the improved management of long COVID may require the integration of symptom management approaches, the current panel of long COVID biomarkers, as well as additional more specific biomarkers that are yet to be identified. Furthermore, some biomarkers may also be affected by participants' existing clinical conditions. For example, NFL may serve as not only a biomarker for long COVID neurological symptoms in this study but also a biomarker for neurodegenerative diseases, such as Alzheimer's disease (10) and Parkinson's disease (11). Therefore, future application of this panel of long COVID biomarkers may

TABLE 3 Biomarkers significantly associated with long COVID Symptoms.

Long COVID symptoms	Categories of biomarkers						References
	Acute phase protein	Biochemical marker	Cytokine/Chemokine	Neurological marker	Vascular marker	Others	
Neurological symptoms	Albumin, CRP, Ferritin, Fibrinogen	β-glucan, S-Sulfocysteine	CCL2, IFN-γ, IL-4, IL-6, TNF-α	GFAP, NFL, pGFAP/pNFL	ET-1, Hemoglobin, sICAM-1, sVCAM-1, sVEGFR	Ab, Hs TnT, IGFBP-4	(19, 20, 43–46, 49, 54, 59, 60, 62)
Pulmonary symptoms	C5b-9, CRP	ALT, AST, LDH	CXCL10, IFN-β, IL-1α, IL-6, TGF-β		Col1A2, Col3A1	α-SMA, miRNA21, NT-proBNP	(21, 22, 40, 46, 51)
Multiple symptoms	Albumin, CRP, Ferritin,	1-Methylnicotinamide, 2-Phenylphenol, 3,5-Dihydroxybenzoic acid, ADA, β-glucan, CPA3, Glutamine/Glutamate ratio, Indole-3-lactic acid, L-Cystein, LDH, L-Glutamine, L-Methionine, Ornithine, Pipecolic acid, Quinolinic acid, Quinolinic acid/Tryptophan ratio, Sarcosine, ST1A1, S-Sulfocysteine, Taurine, Tryptase, uPA	CCL2, CCL3, CCL4, CCL5, CCL7, CCL19, CCL20, CCL23, CXCL1, CXCL9, CXCL10, CXCL11, Flt3L, GM-CSF, IFN-γ, IL-1α, IL-2, IL-4, IL-6, IL-7, IL-10, IL-10Rβ, IL-12β, IL-17, IL-18, IL-33, IP-10, M-CSF, SCF, TGF-α, TGF-β, TNF-α, TNF-β	GDNF, NGF-β, NT-3	Ang-2, D-dimer, ESR, ET-1, Factor VIII:C, Hemoglobin, MMP-1, MMP-9, MPO, PDGF-BB, sVCAM-1, sTM, sVEGFR, VEGF, VWF:Ag, VWF:pp	Ab, ARTN, AXIN, CASP-8, CST-5, Cystatin C, LBP, MRP8/14, NGAL, OPG, OSM, SIRT2, STAMBP, TNFRSF9, Zonulin	(40–42, 47–50, 52, 53, 55–59, 61, 62)
Cardiac symptoms					NO	NT-BNP	(63)

A detailed list of abbreviations in Table 3 can be found in Supplementary Table 2.

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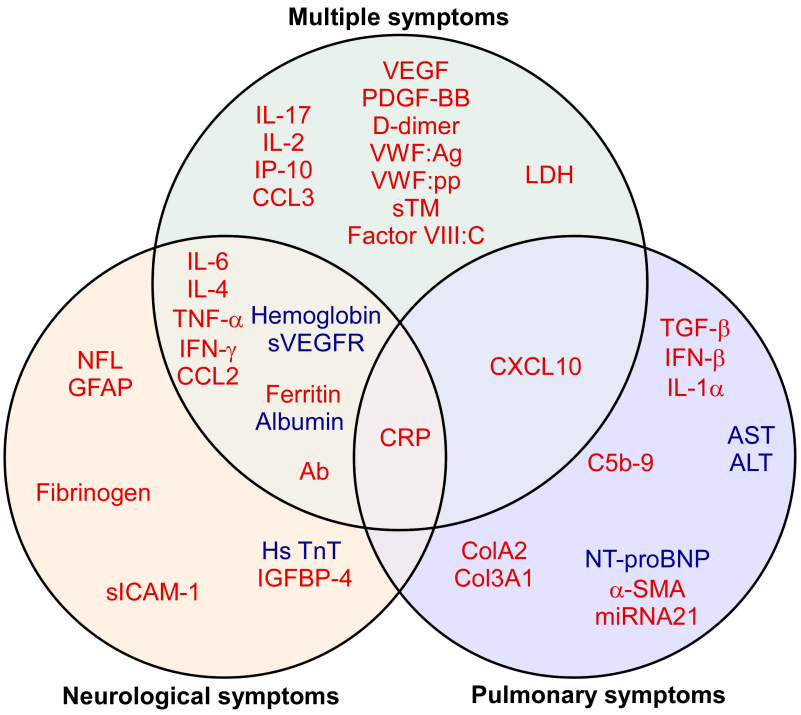

FIGURE 4
Biomarkers of long COVID symptoms. The Venn diagram presents the 41 biomarkers that were reported by two or more eligible studies. Red indicates up-regulated, while blue refers to down-regulated biomarkers. A detailed list of abbreviations in **Figure 4** can be found in **Supplementary Table 2**.

need to consider the patient's clinical history to avoid concomitant pathologies of other diseases.

Strengths and weaknesses of the research

Our study highlights the first systematic review to synthesize unique expression patterns of inflammatory biomarkers in long COVID, and assess whether they can serve as diagnostic or prognostic markers. Categorization of the biomarkers into six categories based on biological function may further inform our understanding of the clinicopathology of long COVID. The findings may guide and help clinicians to identify a core set of blood biomarkers that can be used to monitor and manage long COVID in clinical practice. Nevertheless, there are several limitations to our approach. First, as shown in the quality assessment (**Supplementary Table 3**) of the manuscript, 96.4% (27 of 28) of the eligible articles provided different sampling criteria to exclude participants with some existing disease conditions based on the clinical history of patients. Moreover, most of the biomarkers in the eligible studies were measured after the onset of the long COVID symptoms. Therefore, the main biomarkers found to be overexpressed in long COVID, such as IL-6, CRP, and TNF-α, although important in COVID, likely lack specificity to serve as predictors for long COVID. The identified biomarkers are real and reflect the biology of viral infections which do activate the inflammasome, leading to the production of important cytokines/chemokines (IL-1β, TNF-α, IL-6, etc.) (37, 38). This may be the main reason why existing studies predominantly measured only these main inflammatory biomarkers. Unfortunately, many other stressors, such as viruses, bacteria, inhaled nano/particles, industrial toxins, etc., do share common mechanistic features that involve inflammation via inflammasome activation. For this reason, it is necessary that future studies on long COVID employ broader screening platforms that are likely to yield unique and likely specific biomarkers for SARS-CoV-2. For example, a recent study proposed that IL-26 may be a COVID-19-specific biomarker, but none of the studies to date have measured IL-26 (39). Furthermore, there is a lack of consistency on specific long COVID symptoms. 57.1% of the eligible studies examined biomarkers for patients with multiple symptoms of long COVID. Additionally, the duration of facing persistent long COVID symptoms varied within and across studies. Incongruent with long COVID symptoms and timeframes may contribute to distinct biomarkers. Finally, vaccination may affect patients' physiological variables and the levels of biomarkers in serum. However, there is no sufficient evidence to know the effect of vaccination because only one of the 28 eligible studies separated the vaccinated participants from the unvaccinated ones. Such issues should be addressed in future longitudinal studies on biomarker expression among long COVID patients to understand the causal relationship between long COVID symptom development and acute COVID inflammation.

Summary

Long COVID patients present elevated inflammatory biomarkers after initial infection. Our study found that people with higher levels of IL-6, CRP, and TNF-α after SARS-CoV-2 infection for one or more months may experience long-term COVID symptoms. This systematic review could identify a panel of blood biomarkers that can be used to manage long COVID patients in clinical practice.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

Y-JL supervised the entire project, designed, analyzed manuscripts and wrote manuscript. S-HL and SM designed, analyzed manuscripts, and wrote manuscript. T-AL analyzed manuscripts and wrote manuscript. C-TK analyzed manuscripts. DB provided scientific input and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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High prevalence of persistent symptoms and reduced health-related quality of life 6 months after COVID-19

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Background: The long-term sequelae after COVID-19 constitute a challenge to public health and increased knowledge is needed. We investigated the prevalence of self-reported persistent symptoms and reduced health-related quality of life (HRQoL) in relation to functional exercise capacity, 6 months after infection, and explored risk factors for COVID-19 sequalae.

Methods: This was a prospective, multicenter, cohort study including 434 patients. At 6 months, physical exercise capacity was assessed by a 1-minute sit-to-stand test (1MSTST) and persistent symptoms were reported and HRQoL was evaluated through the EuroQol 5-level 5-dimension (EQ-5D-5L) questionnaire. Patients with both persistent symptoms and reduced HRQoL were classified into a new definition of post-acute COVID syndrome, PACS+. Risk factors for developing persistent symptoms, reduced HRQoL and PACS+ were identified by multivariable Poisson regression.

Results: Persistent symptoms were experienced by 79% of hospitalized, and 59% of non-hospitalized patients at 6 months. Hospitalized patients had a higher prevalence of self-assessed reduced overall health (28 vs. 12%) and PACS+ (31 vs. 11%). PACS+ was associated with reduced exercise capacity but not with abnormal pulse/desaturation during 1MSTST. Hospitalization was the most important independent risk factor for developing persistent symptoms, reduced overall health and PACS+.

Conclusion: Persistent symptoms and reduced HRQoL are common among COVID-19 survivors, but abnormal pulse and peripheral saturation during exercise could not distinguish patients with PACS+. Patients with severe infection requiring hospitalization were more likely to develop PACS+, hence these patients should be prioritized for clinical follow-up after COVID-19.

KEYWORDS

COVID-19, PACS, long-COVID, post-acute COVID syndrome (PACS), EQ-5D, SARS-CoV-2, Post COVID-19 condition (PCC)

1. Introduction

Since the start of the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, there has been a growing interest in the long-term health consequences of Coronavirus disease 2019 (COVID-19). Previous studies have shown that 49–68% of hospitalized COVID-19 survivors experience persistent symptoms 6–12 months post infection, with fatigue, dyspnea, muscle weakness, and anxiety/depression as the most commonly reported persistent symptoms (1–3). Studies have also reported reduced physical performance in 22–33% of hospitalized COVID-19 survivors, assessed by the one-minute sit-stand test (1MSTST) and six-minute walk test (1, 2), as well as peripheral oxygen desaturation (3). Few studies of COVID-19 sequalae in non-hospitalized patients have so far been published. In a recent large cohort study, 13% of participants experienced persistent symptoms attributable to the infection (4), while another study reported persistent symptoms in 84% of study participants (5). Varying terminology has been used to describe the persistent symptoms and long-term health consequences after COVID-19, such as long-COVID and post-acute COVID-19 syndrome (PACS). The current definition adopted by WHO suggests that "Post-COVID-19 condition occurs in individuals with a history of probable or confirmed SARS-CoV-2 infection, usually 3 months from the onset, with symptoms that last for at least 2 months" (6).

Health-related quality of life (HRQoL) is a term used to describe to what extent different diseases and their treatments affect the physical, emotional, and social health of an individual (7). The existing studies show that a large proportion of COVID-19 patients experience a reduced HRQoL up to 1 year after infection, due to disabilities affecting everyday life (1, 8).

Little attention has been paid to sequalae in patients with mild disease, and the risk factors for long-term health consequences are still largely unknown. Mild COVID-19 is by far the most common disease manifestation and thus generates the majority of PACS cases. In addition, there is paucity of knowledge regarding the benefit of functional exercise tests during clinical follow-ups. The aim of this study was to investigate the prevalence of self-reported persistent symptoms, abnormal physical performance during exercise, and reduced HRQoL, among hospitalized and non-hospitalized COVID-19 patients 6 months after infection. A secondary aim was to explore risk factors for developing long-term sequelae after COVID-19.

Abbreviations: 1MSTST, one-minute sit-to-stand test; BMI, Body mass index; CCI, Charlson Comorbidities Index Score; CI, confidence interval; COVID-19, coronavirus disease 2019; CT, computed tomography; EQ-5D-5L, EuroQol 5-dimension 5-level; EQ-VAS, EuroQol visual analog scale; HRQoL, health-related quality of life; HFNC, high-flow nasal canula; ICU, intensive care unit; IMV, invasive mechanical ventilation; IQR, interquartile range; mMRC, modified Medical Research Council; NIV, non-invasive ventilation; PACS, Post-acute COVID-19 syndrome; PCR, polymerase chain reaction; RR, relative risk ratio; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SD, standard deviation; WHO, World Health Organization.

2. Materials and methods

2.1. Study design and study cohort

Data was retrieved from a prospective multicenter cohort study (CoVUm, clinicaltrials.gov ID: NCT04368013) coordinated by Örebro University hospital and University hospital of Umeå, Sweden. Patients were prospectively enrolled between April 2020 and June 2021 from study sites in Örebro, Umeå, Västerås and Karlstad. Hospitalized (≥18 years of age) and non-hospitalized (≥15 years) patients with a positive PCR test for SARS-CoV-2 were eligible for enrolment. Patients hospitalized due to acute COVID-19 infection were enrolled at the Departments of Infectious Diseases and the intensive care units (ICU) at Örebro University Hospital, University Hospital of Umeå, Västerås Central Hospital and Karlstad Central Hospital. Non-hospitalized patients fulfilling the inclusion criteria, were prospectively enrolled using convenience sampling at the infectious diseases' outpatient clinic at University Hospital of Umeå. Exclusion criteria were inability to provide informed consent and inability to read and communicate in Swedish. At Västerås and Karlstad sites, patients hospitalized due to COVID-19 were enrolled at a follow-up visit within 6 months from discharge.

2.2. Data collection

Data on disease severity, level of care, clinical and laboratory parameters, and baseline characteristics including comorbidities and medication was collected for each patient. Mortality risk and comorbidity-based disease burden was calculated using the Charlson Comorbidity Index (CCI) (1). Study data was collected and managed using REDCap electronic data capture tools hosted at Umeå University (2).

Follow-up visits were conducted at 2 weeks, 4 weeks, 2 months, 3 months, and 6 months after discharge from hospital or enrolment for hospitalized/non-hospitalized patients, respectively. Patients enrolled at Karlstad and Västerås attended the follow-up protocol from 6 months and onwards. Data was exported from the database on February 20th, 2022.

2.3. Outcome measures

Persistent symptoms were assessed with a custom questionnaire containing 15 different symptoms: Cough, dizziness, headache, hyposmia/dysgeusia, experienced impaired memory function, difficulties finding words, mental fatigue, panic attacks, concentration difficulties, sleeping difficulties, nightmares, myalgia, physical fatigue, restless legs and upset stomach, at follow-up visits from 4 weeks until 6 months. In addition, experienced dyspnea was assessed at all follow-up visits with the modified Medical Research Council (mMRC). Dyspnea Scale, ranging from 0 to 4, were 0 corresponds to "dyspnea only during strenuous exercise", and 4 to "too dyspneic to leave the house or breathless when getting dressed" (3).

Health-related quality of life was measured with the generic health status instrument EuroQol 5-dimension 5-level questionnaire (EQ-5D-5L), covering five dimensions; mobility, usual activities, self-care, pain/discomfort, and anxiety/depression. For each dimension, five levels are presented, ranging from "no problems" to "extreme

problems". EQ-5D-5L also includes the EuroQol Visual Analog Scale (EQ-VAS), which is a visual analog scale for the patients self-assessed overall health, ranging from 0 to 100. The endpoints on the scale are marked as "the worst health you can imagine" and "the best health you can imagine" (4). EQ-5D-5L assessment was added to the follow-up protocol in December 2020, resulting in missing data for the patients enrolled before July 2020.

Due to lack of data on patients' premorbid HRQoL and dyspnea, the following questions were also posed to the study participants: (1) How has your view of the future changed since before your illness? (2) How physically active have you been the past week, compared to before your illness? (3) How has your breathing been the past week, compared to before your illness (Data Sheet 1)?

2.4. Functional test of exercise capacity

A 1-minute sit-to-stand test (1MSTST) (5) was performed at each follow-up visit from 4 weeks after enrolment or discharge from hospital. A pulse oximeter was used to record oxygen saturation and heart rate, before and after the test. A decrease in saturation with more than four percent units, and a post-test change of the heart rate with $\leq$0, or more than two standard deviations (SD) from the mean, were considered pathological (6).

2.5. Definitions

Disease severity was defined as: Mild (non-hospitalized patients, corresponding to WHO clinical progression scale 1–3 B) and (Severe: hospitalized patients, corresponding to WHO clinical progression scale 4–9) (7).

Impairment in the EQ-5D-5L dimensions was defined as at least moderate difficulties (score $\geq$ 3) in each dimension. Histogram analysis of the distribution of EQ-VAS-scores was performed, revealing a bimodal distribution with one peak below the score value of 60. We therefore defined a reduced overall health as an EQ-VAS-score $\leq$ 60. The cut-off for breathlessness was set at mMRC $\geq$ 1.

We incorporated persistent symptoms and reduced HRQoL into a new definition: PACS+, to enable analysis of the group of patients who, in addition to persistent symptoms, also experienced significant negative consequences in their daily life and/or in their overall health. PACS+ was defined as the prevalence of $\geq$1 symptom at the 6-month follow-up, together with either moderate (score $\geq$ 3) difficulties in $\geq$2 dimensions of EQ-5D-5L and/or self-assessed overall health $\leq$ 60 in EQ-VAS.

2.6. Statistical analysis

Statistical analyses were performed using IBM SPSS statistics (Version 25, IBM Corp., NY, USA), Jamovi (version 2.2.5), GraphPad Prism (version 9.3.1, GraphPad Software, San Diego, CA, USA) and STATA release 17. Groups were compared with X^2-test or Fisher's exact test for categorical variables, and unpaired T-test or Mann-Whitney U-test for continuous variables, depending on the normality. The normality of continuous variables was tested using the Shapiro Wilks test.

Multivariable Poisson regression with robust standard errors was performed to explore risk factors for developing persistent symptoms, reduced HRQoL and PACS+ and to compare the hospitalized vs. non-hospitalized groups. All analysis comparing the groups were adjusted for age, sex, WHO classified body mass index (BMI), CCI (0 none, 1–2 mild, $\geq$3 moderate/severe) and smoking status. Effect sizes are presented as relative risk ratios (RR) with 95% confidence intervals (CI). Significance level was set at the 5% level (p-value $<$ 0.05). Non-response analyses were performed for the participants who did not complete the EQ-5D-5L questionnaire.

2.7. Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients. Ethical approval for the study was granted from Swedish Ethical Review Authority, Uppsala (approval number: 2020-01557).

3. Results

3.1. Study cohort

At data export, on February 20, 2022, the study cohort comprised 543 patients. Before the 6-month follow up, 55 patients had dropped out of the study and five patients had died. Forty-six patients were lost to follow-up. In total, 434 patients with COVID-19, of which 151 had been hospitalized, attended the 6-month follow-up visit. Data on EQ-5D-5L was available for 295 patients (Figure 1).

3.2. Baseline characteristics of the study cohort

Baseline characteristics of the study cohort are presented in Table 1. The median number of days from disease onset to the 6-month follow-up visit for all patients was 192 days. The median age was lower in the non-hospitalized group compared to hospitalized patients (45 vs. 58 years), and a larger proportion of non-hospitalized patients were women (56 vs. 35%). The median BMI of non-hospitalized patients was lower than that for hospitalized patients (25 vs. 30). None of the patients had received any SARS-CoV2 vaccine dose $>$ 14 days before enrolment. All participants were thus considered unvaccinated. Diabetes, hypertension, and cardiovascular disease were significantly more common among hospitalized patients, and a larger proportion were former smokers.

Twenty-seven patients (27/151, 18% of hospitalized patients) were admitted to the intensive care unit (ICU) during hospitalization. Of all hospitalized patients, 68% received respiratory support with either non-invasive ventilation, high-flow nasal oxygen or invasive mechanical ventilation, and 9% received conventional oxygen therapy only.

The non-response analysis showed that among those who did not complete the EQ-5D-5L questionnaire (N = 139), there was a larger proportion of former smokers (Supplementary Table 1). No other significant differences were found in the baseline data.

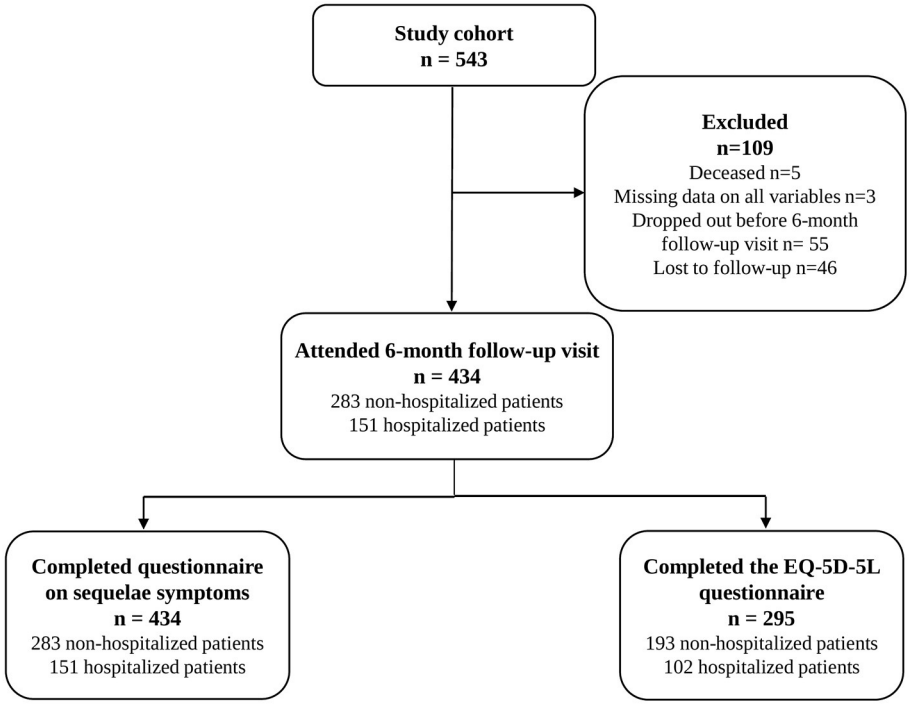

FIGURE 1
Flowchart of included patients.

3.3. A majority of both hospitalized and non-hospitalized patients experienced persistent symptoms at 6 months

At 6-month follow-up, persistent symptoms (any symptom) were reported by a majority of both non-hospitalized and hospitalized patients, however it was significantly more common in hospitalized patients (59 of vs. 79%). The most common symptoms overall were physical fatigue, mental fatigue, hyposmia/dysgeusia, and concentration difficulties (Table 2). The most common symptom among non-hospitalized patients was hyposmia/dysgeusia (29%), which was equally common among hospitalized patients (23%).

Hospitalization was independently associated with neuropsychiatric symptoms, i.e., the experience of impaired memory function, difficulties finding words, mental fatigue, concentration difficulties, panic attacks, and headache. Hospitalization was also associated with an increased risk of dyspnea (mMRC $\geq$ 1), restless legs, and physical fatigue (Table 2).

3.4. Problems with daily usual activities, physical activity, and pain/discomfort were more common in hospitalized patients

Problems with usual activities, mobility, and pain/discomfort, measured by the EQ-5D-5L questionnaire, were more common in the hospitalized group. However, pain/discomfort and usual activities were the only two EQ-5D-5L dimensions that were independently associated with hospitalization (Table 3). A large proportion of patients reported that they were able to perform less physical activity than prior COVID-19; 30% of non-hospitalized and 55% of hospitalized patients ($p = 0.005$). Of the non-hospitalized patients, 4% experienced moderate to severe breathing impairment compared to before their illness, vs. 23% in the hospitalized group ($p < 0.001$). A large proportion of patients experienced a more negative view of the future compared to before illness (14 vs. 21% of non-hospitalized/hospitalized).

3.5. Hospitalization was the main risk factor for persistent symptoms, reduced overall health, and PACS+

Hospitalization was the main risk factor for experiencing several symptoms and a reduced HRQoL 6 months after infection. Female sex increased the risk of experiencing at least one symptom, especially dyspnea and neuropsychiatric symptoms, such as mental fatigue, concentration difficulties, and experience of impaired memory function (Table 4). Female patients were also at greater risk of experiencing long-term hyposmia/dysgeusia, dyspnea and physical fatigue. Among specific comorbidities, cardiovascular disease was associated with mental fatigue and malignancy with at least one persisting symptom and dyspnea (Table 4).

Out of the 295 patients that completed the EQ-5D-5L questionnaire, 54 (18%) met the criteria for PACS+ including at least one persisting symptom and reduced HRQoL. Hospitalization was the only variable associated with an increased risk of developing PACS+ with a relative risk ratio of 2.77 (95% CI 1.36–5.65, Table 4).

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TABLE 1 Demographic and baseline characteristics of all hospitalized and non-hospitalized patients that attended the 6-month follow-up visit.

	Total (n = 434)	Non-hospitalized patients (n = 283)	Hospitalized patients (n = 151)	p-value
Age in years—median (IQR)	48 (36–60)	45 (29–55)	58 (47–65)	**<0.001***
Sex—n (%)				
Women	212 (48.8)	159 (56.2)	53 (35.1)	**<0.001•**
BMI—n	420	275	145	
BMI—median (IQR)	26.3 (23.4–30.1)	24.8 (22.6–27.5)	30.0 (26.9–33.2)	**<0.001***
<25 underweight/normal	160 (38.1)	147 (53.5)	13 (9.0)	
25–29 overweight	151 (40.0)	92 (33.4)	59 (40.7)	
≥30 obese	109 (35.9)	36 (13.1)	73 (50.3)	
Comorbidities—n (%)				
Diabetes	25 (5.8)	7 (2.5)	18 (11.9)	**<0.001•**
Hypertension	86 (19.8)	35 (12.4)	51 (33.8)	**<0.001•**
Cardiovascular disease[a]	31 (7.1)	13 (4.6)	18 (11.9)	**0.005•**
Chronic lung disease[b]	74 (17.1)	41 (14.5)	33 (21.8)	0.052•
Asthma	67 (15.4)	38 (13.4)	29 (19.2)	0.113•
Autoimmune disease[c]	24 (5.5)	13 (4.6)	11 (7.3)	0.243•
Immunocompromised[d]	10 (2.3)	5 (1.8)	5 (3.3)	0.327◦
Malignancy[e]	9 (2.1)	3 (1.1)	6 (4.0)	0.071◦
CCI—median (IQR)	0 (0–0)	0 (0–0)	0 (0–1)	**<0.001***
0	328 (75.6)	233 (82.3)	95 (62.9)	
1–2 mild	100 (23.0)	49 (17.3)	51 (33.8)	
≥3 moderate/severe	6 (1.4)	1 (0.4)	5 (3.3)	
Smoking status—n (%)				**0.001•**
Non-smoker	309 (71.2)	218 (77.0)	91 (60.3)	
Current smoker	9 (2.1)	6 (2.1)	3 (2.0)	
Former smoker	116 (26.7)	59 (20.8)	57 (37.7)	
Snuff—n (%)	70 (16.1)	48 (17.0)	22 (14.6)	0.519•
Level of education[f]–n (%)				0.186•
Lower	33 (7.9)	18 (6.4)	15 (10.9)	
Medium	191 (45.5)	127 (44.9)	64 (46.7)	
Higher	196 (46.6)	138 (48.8)	58 (42.3)	
Symptoms at onset				0.436◦
≥1 symptom	418 (96.3)	274 (96.8)	144 (95.4)	
Asymptomatic	16 (3.7)	9 (3.2)	7 (4.6)	
Respiratory support				**<0.001•**
No respiratory support	318 (73)	283 (100)	35 (23.2)	
Conventional oxygen therapy only	13 (3)	0 (0)	13 (8.6)	
NIV, HFNC or IMV	103 (23.7)	0 (0)	103 (68.2)	

[a]Ischemic heart disease, congestive heart failure, arrythmias, aortic disease, valvular heart disease, or peripheral arterial insufficiency.
[b]Chronic obstructive pulmonary disease and asthma.
[c]Including rheumatic diseases.
[d]Immune deficiency diseases or immunosuppressive/immunomodulatory medication.
[e]Solid localized tumor, lymphoma, or leukemia.
[f]Level of education missing in 14 patients. The analysis is based on 420 patients. Lower: <3 years beyond Swedish compulsory school. Medium: 3 years beyond Swedish compulsory school, but no college or university degree. Higher: University or college degree.
*Mann-Whitney U-test.
• X^2-test.
◦Fischer's exact test.
Significant p-values are written in bold.
n, number of patients; BMI, body mass index; CCI, Charlson Comorbidities Index; HFNC, high-flow nasal cannula; IMV, invasive mechanical ventilation; IQR, interquartile range; NIV, non-invasive ventilation.

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TABLE 2 Number of hospitalized and non-hospitalized patients (%) with different persistent symptoms.

	Non-hospitalized patients ($n = 283$)	Hospitalized patients ($n = 151$)	Adj. RR ($n = 420$)	p-value
At least one symptom—*n* (%)	168 (59.4)	119 (78.8)	1.23 (1.05–1.43)	**0.011**
Respiratory symptoms—*n* (%)				
Cough	19 (6.7)	25 (16.6)	1.64 (0.83–3.23)	0.152
Dyspnea (mMRC ≥ 1)—***n (%)***	$n = 236$ 32 (13.6)	$n = 130$ 75 (57.7)	3.83 (2.50–5.86)	**<0.001**
Neurological symptoms—*n* (%)				
Dizziness	31 (11.0)	24 (15.9)	1.48 (0.75–2.91)	0.255
Headache	37 (13.1)	37 (24.5)	1.85 (1.06–3.23)	**0.031**
Hyposmia/dysgeusia	81 (28.6)	34 (22.5)	0.71 (0.45–1.12)	0.144
Impaired memory function	50 (17.7)	58 (38.4)	1.92 (1.29–2.85)	**0.001**
Difficulties finding words	57 (20.1)	53 (35.1)	1.50 (1.01–2.25)	**0.045**
Mental fatigue	62 (21.9)	62 (41.1)	1.74 (1.22–2.48)	**0.002**
Psychiatric symptoms—*n* (%)				
Panic attacks	31 (11.0)	35 (23.2)	2.54 (1.39–4.65)	**0.002**
Concentration difficulties	54 (19.1)	58 (38.4)	1.95 (1.32–2.90)	**<0.001**
Sleeping difficulties	54 (19.1)	42 (27.8)	1.27 (0.82–1.97)	0.284
Nightmares	24 (8.5)	24 (15.9)	1.60 (0.77–3.32)	0.209
Other—*n* (%)				
Myalgia	23 (8.1)	31 (20.5)	1.54 (0.81–2.94)	0.186
Physical fatigue	55 (19.4)	71 (47.0)	1.98 (1.38–2.84)	**<0.001**
Restless legs	17 (6.0)	31 (20.5)	3.00 (1.47–6.11)	**0.002**
Upset stomach	33 (11.7)	24 (15.9)	0.99 (0.54–1.83)	**0**.984

Relative risk ratios and p-values have been adjusted for age, sex, BMI, CCI, and smoking status. Significant p-values are written in bold.
Adj., adjusted; RR, relative risk ratio; EQ-5D-5L, EuroQol 5-level 5-dimension questionnaire; EQ-VAS, EuroQol Visual Analog Scale.

3.6. PACS+ was associated with a lower exercise performance, but not with desaturation or abnormal pulse reaction

Among the 295 patients that completed EQ-5D-5L questionnaire at 6 months follow up, and thus could be defined as PACS+ ($n = 54$) or non-PACS+ ($n = 241$), 289 (100/289, 35% hospitalized) performed a 1MSTST between 4 weeks and 6 months after study enrolment. Out of these, 12 patients discontinued the test before 60 s, mainly due to pain and or discomfort in lower extremity/extremities. PACS+ patients performed significantly fewer elevations compared to non-PACS+ patients, both at 30 s (14 vs. 17, $p = 0.021$) (Figure 2A), and 60 s (26 vs. 33, $p = 0.005$) (Figure 2B). Forty-two patients (42/289, 15%) had a pathological decrease in oxygen saturation during at least one 1MSTST between 4 weeks and 6 months after COVID-19, but among these only 6 (14%) had PACS+ (Figures 2C, D). Twenty-four patients (24/289, 8%) showed an abnormal change in heart rate during at least one 1MSTST. Out of these, three patients (13%) had PACS+ (Figures 2E, F). There were no significant differences with regards to age, sex, or proportion hospitalized between patients with or without desaturation or pathological heart rate during 1MSTST.

4. Discussion

We found that neuropsychiatric symptoms are more common than respiratory symptoms 6 months after COVID-19. Although most patients experienced persistent symptoms, less than one fifth had a significant impact on their HRQoL, with hospitalization being the most important risk factor for long-term sequelae. While numerous studies of symptoms after COVID-19 have been published, most studies either have a small sample size, include only non-hospitalized or hospitalized patients, or lack assessment of HRQoL and/or physical exercise capacity (8). The CoVUm cohort is unique in its design, including both hospitalized patients with severe COVID-19 and a large proportion of non-hospitalized patients with mild disease. Moreover a very low fraction of patients was lost to follow-up, and a detailed follow-up protocol was used, including objective tests of functional exercise capacity. This enabled in-depth analysis of risk factors for persistent symptoms, reduced HRQoL as well as abnormal physical reactions during exercise. Finally, a major strength of the present study is the prospective design which enables a more correct assessment of the long-term effects of this disease in contrast to studies enrolling patients after presentation of suspected PACS-related symptoms.

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TABLE 3 Results from the multivariable regression analysis on the variables from the EQ-5D-5L questionnaire and the three additional questions added by the research group.

	Non-hospitalized patients ($n = 193$)	Hospitalized patients ($n = 102$)	Adj. RR	p-value
EQ-5D-5L dimensions—n (%)				
Mobility: problems with walking around	5 (2.6)	15 (14.7)	3.26 (0.92–11.52)	0.067
Personal care: problems with washing or dishing	2 (1.0)	4 (3.9)	1.59 (0.10–24.48)	0.738
Usual activities: problems with usual activity	14 (4.7)	21 (20.6)	**2.31 (1.04–5.17)**	**0.041**
Pain or discomfort	20 (10.4)	26 (25.5)	**2.23 (1.07–4.67)**	**0.033**
Anxiety or depression	16 (8.3) $n = 191$	14 (13.7) $n = 101$	2.10 (0.58–7.59)	0.256
EQ-VAS $\leq$ 60—n (%)	22 (11.5)	28 (27.7)	**2.11 (1.04–4.29)**	**0.038**
EQ-VAS—mean (±SD)	78 (±15)	72 (±19)	−5.1 (−10.2 to 0.0)[a]	0.052
Additional questions—n (%)				
Future outlook: changed negatively compared to before illness	27 (14.1)	21 (20.8)	1.43 (0.67–3.02)	0.355
Physical activity: can do less than before illness	57 (29.8)	56 (54.9)	**1.71 (1.18–2.49)**	**0.005**
Breathing: moderately to severely worsened compared to before illness	7 (3.7)	$n = 100$ 23 (23.0)	**7.78 (2.94–20.64)**	**<0.001**
Capacity to work: reduced compared to before illness	$n = 176$ 19 (10.8)	$n = 40$ 20 (20.0)	0.94 (0.49–1.78)	0.840
PACS+–n (%)	22 (11.4)	32 (31.4)	**2.59 (1.30–5.17)**	**0.007**

Relative risk ratios and p-value have been adjusted for age, sex, BMI, CCI, and smoking status. Significant p-values are written in bold.

[a]Analyzed with linear regression which gives mean difference with 95% CI as association measure.

Adj., adjusted; RR, relative risk ratio; mMRC, modified Medical Research Council.

The high prevalence of neuropsychiatric symptoms in both hospitalized and non-hospitalized patients 6 months after infection was the most striking finding, in particular since the non-hospitalized group was a generally healthy patient cohort upon enrolment. Although previous studies have also shown that a large proportion of COVID-19 patients report long-term problems with memory loss, insomnia, and mental slowness (9, 10), the evidence is conflicting as to whether the symptoms are preexisting, related to the infection, severe disease in general, or to indirect effects of the COVID-19 pandemic (11). Our study cannot establish causality, but the data suggest that neuropsychiatric symptoms occur in all patient groups, even though these potentially disabling symptoms are much more common in hospitalized patients. Recent studies have started to provide possible mechanistic explanations to the impact of COVID-19 on the central nervous system. Douaud et al. revealed important differences in brain structure between COVID-19 patients and matched controls; COVID-19 patients exhibited a reduction in gray matter and global brain size, compared to before illness (12). Rau et al. showed that white matter changes and signs of vasogenic oedema are associated with cognitive impairment during the subacute phase of COVID-19 (13). Recently, white-matter-selective microglial reactivity and increased levels of the proinflammatory chemokine CCL11 leading to impaired hippocampal neurogenesis was suggested as pathophysiological mechanisms behind cognitive impairment (14). Perceived hyposmia/dysgeusia and dizziness were the only neuropsychiatric symptoms that were equally common among non-hospitalized in our study. This finding is consistent with that of previous research, as many studies have reported a high prevalence of experienced hyposmia/dysgeusia (15).

We also show that women had a higher risk for experiencing long-term symptoms in general and, in particular, physical fatigue, mental fatigue, hyposmia/dysgeusia, concentration difficulties, and experience of impaired memory function. These findings are in congruence with previous research both within (16) and outside the COVID-19 research field (17). Multiple explanations, biological and sociocultural, to why women report symptoms at a higher frequency than men, have been proposed. In the case of COVID-19 female sex hormones seem to have an impact on the disease phenotype. Studies investigating this sex-related difference in disease outcome have elucidated that female sex hormones, such as estrogen, may play an important role in protection against severe disease (18). However, in contrast to the protective effect in the acute phase, female sex hormones may partly contribute to the increased risk of persistent symptoms post-infection (19). Further studies on subgroups of patients with different sex hormone levels and outcomes are needed to investigate the biological background to differences in disease phenotype.

Importantly, our study also included assessment of the patients' HRQoL. Since self-reported persisting symptoms are common after COVID-19, 66% in our cohort, the current definition of PACS (at

TABLE 4 Risk factors for developing the most commonly occurring persistent symptoms, reduced overall health (≤60 in EQ-VAS), and PACS.

	Outcomes								
	At least one symptom ($n = 420$)	Dyspnea (mMRC ≥ 1) ($n = 357$)	Physical fatigue ($n = 420$)	Hyposmia/ dysgeusia ($n = 420$)	Mental fatigue ($n = 420$)	Concentration difficulties ($n = 420$)	Impaired memory function ($n = 420$)	Reduced overall health[b] ($n = 278$)	PACS+ ($n = 281$)
	Adj. RR (95% CI)	Adj. RR (95% CI)	Adj. RR (95% CI)	Adj. RR (95% CI)	Adj. RR (95% CI)	Adj. RR (95% CI)	Adj. RR (95% CI)	Adj. RR (95% CI)	Adj. RR (95% CI)
Hospitalization	**1.23 (1.05–1.44)**	**3.82 (2.49–5.86)**	**1.98 (1.37–2.86)**	0.72 (0.46–1.12)	**1.72 (1.21–2.45)**	**1.94 (1.32–2.86)**	**1.93 (1.30–2.86)**	**2.38 (1.16–4.90)**	**2.77 (1.36–5.65)**
Age	1.00 (0.99–1.01)	1.01 (0.99–1.02)	1.01 (0.99–1.02)	1.00 (0.99–1.01)	1.01 (0.99–1.02)	1.01 (0.98–1.02)	1.01 (0.99–1.02)	1.00 (0.98–1.02)	0.99 (0.97–1.01)
Female sex	**1.21 (1.05–1.39)**	**1.46 (1.06–1.99)**	**1.36 (1.01–1.83)**	**1.43 (1.01–2.04)**	**1.72 (1.27–2.32)**	**1.47 (1.05–2.05)**	**1.60 (1.15–2.22)**	1.43 (0.84–2.44)	1.37 (0.82–2.28)
Comorbidities									
Diabetes	1.12 (0.90–1.39)	0.94 (0.54–1.64)	1.12 (0.70–1.78)	1.03 (0.50–2.08)	1.49 (0.92–2.42)	1.33 (0.79–2.26)	1.29 (0.76–2.20)	1.79 (0.89–3.60)	1.38 (0.68–2.79)
Hypertension	0.99 (0.83–1.18)	1.01 (0.69–1.49)	1.21 (0.84–1.74)	0.92 (0.57–1.48)	0.85 (0.57–1.27)	0.79 (0.49–1.27)	0.91 (0.57–1.43)	1.36 (0.68–2.73)	1.49 (0.76–2.94)
Cardiovascular disease	1.17 (0.98–1.39)	1.05 (0.68–1.63)	1.38 (0.91–2.08)	1.41 (0.83–2.39)	**1.75 (1.13–2.71)**	1.52 (0.92–2.50)	1.30 (0.77–2.21)	0.87 (0.28–2.69)	1.05 (0.39–2.79)
Chronic lung disease	1.00 (0.65–1.53)	1.40 (0.56–3.52)	0.96 (0.28–3.22)	1.52 (0.65–3.56)	1.75 (0.79–3.90)	1.20 (0.41–3.50)	1.58 (0.71–3.50)	2.29 (0.32–16.4)	1.66 (0.18–14.8)
Asthma	1.24 (0.81–1.90)	1.06 (0.40–2.82)	1.56 (0.46–5.29)	0.64 (0.26–1.59)	0.97 (0.44–2.16)	1.33 (0.44–3.97)	1.21 (0.53–2.77)	0.61 (0.08–4.57)	0.88 (0.09–8.12)
Autoimmune disease	1.02 (0.80–1.28)	1.48 (0.97–2.26)	0.75 (0.40–1.38)	1.36 (0.80–2.30)	1.19 (0.73–1.94)	1.10 (0.57–2.11)	1.04 (0.55–1.97)	2.13 (0.98–4.63)	2.14 (0.92–5.00)
Malignancy	**1.31 (1.14–1.50)**	**2.24 (1.27–3.98)**	1.30 (0.58–2.90)	1.12 (0.36–3.48)	0.94 (0.43–2.08)	1.37 (0.70–2.65)	1.00 (0.42–2.37)	1.36 (0.28–6.55)	1.33 (0.36–4.90)
Smoking[a]	**1.18 (1.03–1.36)**	1.18 (0.86–1.62)	0.86 (0.62–1.20)	1.07 (0.74–1.55)	0.93 (0.66–1.32)	1.04 (0.72–1.48)	1.17 (0.80–1.71)	1.05 (0.59–1.88)	0.98 (0.57–1.70)
BMI									
<25 underw/normal	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
25–29 overweight	0.96 (0.80–1.15)	0.79 (0.49–1.26)	1.07 (0.71–1.60)	1.19 (0.81–1.73)	1.09 (0.75–1.57)	1.24 (0.81–1.88)	1.39 (0.90–2.15)	0.81 (0.37–1.79)	1.24 (0.57–2.69)
≥30 obese	1.02 (0.83–1.25)	1.04 (0.65–1.67)	1.20 (0.79–1.82)	1.12 (0.69–1.81)	0.90 (0.60–1.35)	0.91 (0.57–1.46)	1.00 (0.61–1.63)	0.94 (0.42–2.08)	1.06 (0.46–2.44)

Significant relative risk ratios with a p-value < 0.05 are written in bold.

[a]Current or former smoker.

[b]≤60 in the EQ-VAS.

Adj., adjusted; RR, relative risk ratio; mMRC, modified Medical Research Council; PACS, post-acute COVID-19 syndrome.

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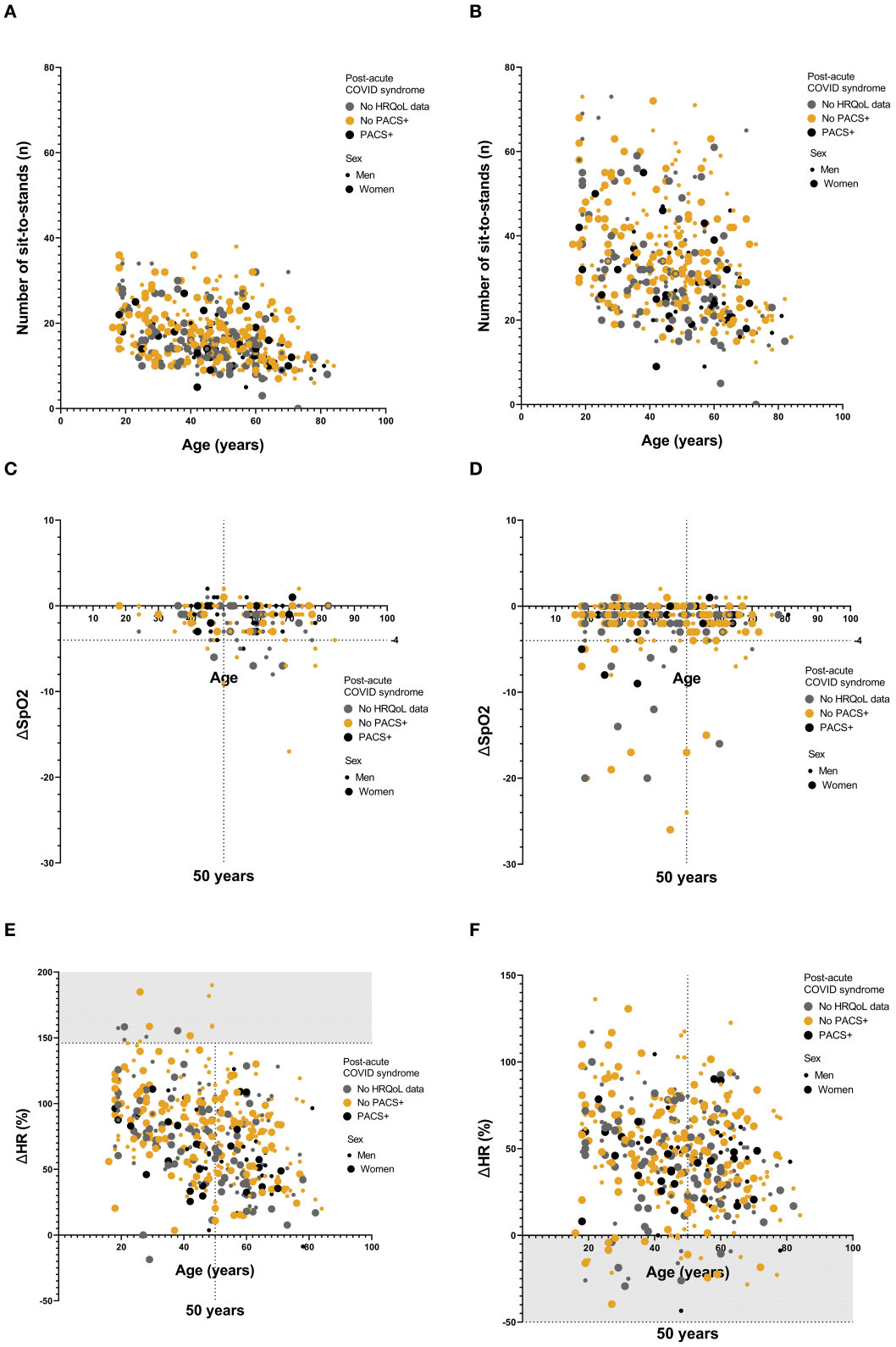

FIGURE 2
(A–F) Results from 1MSTST presented by number of sit-to-stands, oxygen saturation and heart rate. **(A)** Number of sit-to-stands at 30 s for the whole cohort. Age in years presented on the *x*-axis. Numbers presented are the minimum values observed for each study participant between 4 weeks and 6 months. PACS+, black, non-PACS+, yellow and values for patients with no recorded HRQoL data, in gray. Women, large dot; men, small dot. Median values were 14 (range: 5–27) for PACS+, vs. 17 (range: 6–38) for non-PACS+, $p = 0.021$ (Mann-Whitney *U*-test). **(B)** Number of sit-to-stands at 60 s for the whole cohort. Age in years presented on the *x*-axis. Numbers presented are the minimum values observed for each study participant between 4 weeks and 6 months. PACS+, black, non-PACS+, yellow and values for patients with no recorded HRQoL data, in gray. Women, large dot; Men, small dot. Medians: 26 (range: 9–55) for PACS, vs. 33 (range: 10–73) for non-PACS+, $p = 0.005$ (Mann-Whitney *U*-test). **(C, D)** Values of delta-SpO2 after 1MSTST, divided by hospitalized **(C)** and non-hospitalized **(D)**. PACS+, black, non-PACS+, yellow and values for patients with no recorded HRQoL data, in gray.
(Continued)

FIGURE 2 (Continued)
Women, large dot; men, small dot. Age in years presented at *x*-axis, with a dotted line at 50 years. Another dotted line at delta-SpO2 −4, with values on or below this line regarded as pathological. Forty-two participants (15%) had a pathological decrease in oxygen saturation during the test, of which six participants (14%) were also defined as PACS+, ($p = 0.664$) (Fisher's exact test). **(E, F)** Values of delta-Heart rate in percent after 1MSTST, presented as maximum values **(E)** and minimum values **(F)** observed for each study participant between 4 weeks and 6 months. PACS, black, non-PACS+, yellow values for patients with no recorded HRQoL data, in gray. Women, large dot; men, small dot. Age in years presented at x-axis, with a dotted line at 50 years. The Gray dotted areas in the graphs represent pathological values. The limit is set at 0% for minimum values and at +2SD from the mean at maximum values, 146%. Twenty-four participants (8%) had a pathological change in heart rate. Of these, three participants (13%) were also defined as PACS ($p = 0.779$) (Fisher's exact test).

least on persisting symptom) is of limited use to identify patients in need of resource demanding clinical follow-ups and rehabilitation efforts. We therefore added reduced HRQoL to our definition, PACS+, which applied to 18% of the cohort. Hospitalization was the single most important risk factor for PACS+, which indicates that these patients should be prioritized for clinical assessment post-infection. Reduced overall health and problems with pain/discomfort and usual activities were more common among patients with severe disease, which is not uncommon after critical disease regardless of cause (20). In critical care, Thiolliere et al. recently demonstrated that there was no difference in self-reported HRQoL between COVID-19 patients and non-COVID-19 patients 6 months after ICU discharge (11). We used validated instruments, EQ-5D-5L and EQ-VAS, for HRQoL assessment, and our results may be compared to previous Swedish cohort studies. Here, COVID-19 has a negative impact also in the group with mild disease. In a study from 2001 consisting of a cohort with similar age distribution as ours, mean self-rated overall health was 85, compared to 72 and 78 for hospitalized and non-hospitalized patients, respectively, in our study (21). Another more recent Swedish study, where the mean age of the cohort was noticeably higher (64 years), reported a mean EQ-VAS of 76 (22). The impact on HRQoL in mild COVID-19 is supported by recently published data (9).

The inclusion of a validated physical exercise test enabled us to assess abnormal physical reactions to exercise in patients with and without PACS+. A group of patients presented with an abnormal pulse response and/or oxygen desaturation during the 1MSTST. These test results are indicative of autonomic dysfunction, a phenomenon that has previously been described after COVID-19 (23), but to our knowledge not in relation to reduced HRQoL. In the present study, these physical signs were not significantly associated with PACS+, nor with hospitalization or a certain sex. However, autonomic dysfunction has also been associated with objective functional limitations, not with subjective symptoms or limitations (24). We argue that this may be a reason as to why pulse and oxygen saturation reaction, consistent with autonomic dysfunction, were not associated with PACS+ as defined here. A large proportion of patients reported that they had a lower physical performance level after COVID-19 compared to before the illness. This indicates that exercise capacity may be affected, but not perceived as reduced HRQoL by the patient. The underlying pathophysiology and implication of autonomic dysfunction needs to be studied further to distinguish any characterizing factors. We acknowledge that our study has a number of limitations, including the lack of participants' baseline data of HRQoL, symptoms and physical exercise capacity, lack of information on the number of patients that declined participation, and the exclusion of patients with pronounced cognitive dysfunction, and/or inability to read and communicate in Swedish. These patients potentially differ from others in terms of comorbidities and socioeconomic status, which are factors that may affect the long-term health outcomes. In our cohort, 46 patients were lost to follow-up and 139 did not complete the EQ-5D-5L questionnaire, which may result in a minor bias. In addition, our study was not powered to study effects of interventions, for example early antiviral treatment or specific rehabilitation programs, on incidence and severity of long-term health sequalae after COVID-19.

In conclusion, long-term symptoms after COVID-19 were commonly reported in this longitudinal, prospective, multicenter COVID-19 study. We identified that hospitalization due to severe COVID-19 was the single most important independent risk factor for developing clinically relevant long-term health consequences. Less than a third of the hospitalized patients experienced a significantly reduced quality of life. Our aim was to identify patients who experience significant negative consequences in their daily life and need to be prioritized for follow-up. Thus, we suggest an adjustment of the definition of PACS by adding low HRQoL measured by EQ-5D-5L or EQ-VAS.

Our findings, and the suggested PACS+ definition, may hopefully guide optimization of algorithms for clinical long-term follow-up after COVID-19.

Data availability statement

The datasets presented in this article are not readily available because ethical approval does not support publication of the entire dataset of the study cohort. Requests to access the datasets should be directed to johan.normark@umu.se.

Ethics statement

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants involved in the study.

Author contributions

AE, CA, JN, SC, AK, and AM: conceptualization and methodology. AK, I-LP, SC, EM, ST, and IM: data collection. JN, SC, and AE: supervision. AE, CA, JN, MF, SC, ST, EM, and IM: resources. AE, IA, LK, CG, SC, and AM: formal analysis. AE, IA, LK, CG, and SC: data curation. IA and SC: writing—original draft.

AE, IA, LK, CG, AL, CA, JN, JS, MF, SC, ST, AK, I-LP, EM, IM, and AM: writing—review and editing and visualization. CA, JN, MF, SC, ST, and EM: funding acquisition. AL, JN, SC, ST, and EM: project administration. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1104267/full#supplementary-material

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Risk factors for persistent tomographic abnormalities at 6 months of follow-up in a cohort of hospitalized patients with moderate and severe COVID-19 living at high altitude

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Introduction: After COVID-19, functional and tomographic lung alterations may occur, but there are no studies at high altitude where, due to lower barometric pressure, there are lower levels of arterial oxygen pressure and saturation in both normal subjects and patients with respiratory disease. In this study, we evaluated the computed tomographic (CT), clinical, and functional involvement at 3 and 6 months post-hospitalization in survivors with moderate-severe COVID-19, as well the risk factors associated with abnormal lung computed tomography (ALCT) at 6 months of follow-up.

Materials and methods: Prospective cohort, after hospitalization for COVID-19, of patients older than 18 years residing at high altitude. Follow-up at 3 and 6 months with lung CT, spirometry, diffusing capacity of the lung for carbon monoxide (DLCO), six-minute walk test (6MWT), and oxygen saturation (SpO_2). Comparisons between ALCT and normal lung computed tomography (NLCT) groups with X^2 and Mann–Whitney U test, and paired test for changes between 3 and 6 months. A multivariate analysis was performed to evaluate the variables associated with ALCT at 6-month follow-up.

Results: We included 158 patients, 22.2% hospitalized in intensive care unit (ICU), 92.4% with typical COVID CT scan (peripheral, bilateral, or multifocal ground glass, with or without consolidation or findings of organizing pneumonia), and median hospitalization of 7 days. At 6 months, 53 patients (33.5%) had ALCT. There were no differences between ALCT and NLCT groups in symptoms or comorbidities on admission. ALCT patients were older and more frequently men, smokers and hospitalized in ICU. At 3 months, ALCT patients had more frequently a reduced forced vital capacity (< 80%), and lower meters walked (6MWT) and SpO_2. At 6 months, all

patients improved lung function with no differences between groups, but there were more dyspnea and lower exercise SpO_2 in ALCT group. The variables associated with ALCT at 6 months were age, sex, ICU stay, and typical CT scan.

Conclusion: At 6-month follow-up, 33.5% of patients with moderate and severe COVID had ALCT. These patients had more dyspnea and lower SpO_2 in exercise. Regardless of the persistence of tomographic abnormalities, lung function and 6MWT improved. We identified the variables associated with ALCT.

KEYWORDS

COVID-19, altitude, six-minute walk test, respiratory function tests, computed tomography, dyspnea

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes coronavirus disease 2019 (COVID-19), can compromise the lower respiratory tract and cause pneumonia (1). COVID-19 was declared a pandemic on 6 March 2020, and has caused 606,795,204 reported cases and 6,507,435 deaths in the world (2). In Colombia, as of 1st September 2022, 6,302,809 cases and 142,259 deaths have been reported (2).

A significant proportion of patients with COVID-19, particularly the more severe cases, can develop long-term functional and radiographic abnormalities (3). These findings have been previously described in patients with coronavirus infections that developed severe acute respiratory syndrome (SARS-CoV) (4, 5). In survivors of middle east respiratory syndrome coronavirus (MERS-CoV), at 6 weeks of follow-up radiographic abnormalities were found including pulmonary fibrosis, the presence of ground glass, and pleural thickening (6, 7). Respiratory sequelae after recovery from COVID-19 infection have not been fully reported and have become a cause for concern, not only because it is today one of the main reasons for consultation, but also because of the injuries that can be found in the long term.

Some studies, with a follow-up of 3–6 months, have reported the presence of post-infection symptoms such as fatigue, muscle weakness, anxiety, and, in patients with a more critical condition, alterations in the diffusing capacity of the lung for carbon monoxide (DLCO) and abnormalities in the chest tomography [computed tomographic (CT) scan] (8). In a study with a follow-up of up to 1 year, improvement in forced vital capacity (FVC) and the six-minute walk test (6MWT) has been reported, although with alterations in DLCO and persistence of abnormalities in the CT scan (9).

At altitude, due to lower barometric pressure and lower inspired pressure of oxygen, there are lower levels of arterial oxygen pressure and saturation in both normal subjects and patients with respiratory disease (10, 11). More than 80 million people in the world live 2,500 m above sea level, mainly in Latin America and the Andean region (12). So far, some of the effects of the altitude on the severity of the acute presentation of COVID-19 disease are known, given by lower oxygenation indices upon admission to the intensive care unit (ICU) and the requirement for invasive mechanical ventilation (13), however, the medium-term effects of this disease are not known.

Our objective is to describe respiratory symptoms, lung function, and chest CT findings at 3 and 6 months after discharge of hospitalization for COVID-19-associated pneumonia in a population of patients older than 18 years living in a high-altitude city (Bogotá). In addition, to describe the risk factors that were associated with abnormal lung computed tomography (ALCT) at 6 months of follow-up.

Materials and methods

Design and participants: a prospective cohort study with patients older than 18 years who required hospitalization due to a diagnosis of COVID-19 confirmed by PCR of nasopharyngeal secretion and lower respiratory tract involvement by clinical findings and CT. The participants consulted the emergency and outpatient services of the Fundación Cardioinfantil and Fundación Neumológica Colombiana between August 2020 and May 2021. All had to be residents of Bogotá, a city located 2,640 m above sea level, and complete an outpatient follow-up of up to 6 months.

Patients who died during the hospital stay and those with interstitial abnormalities on CT scan before COVID-19 were excluded. All subjects included signed informed consent and the study was approved by the Ethics Committee of the Fundación Neumológica Colombiana (approval number 202007-25702).

Procedures

At hospital admission, sociodemographic variables, respiratory symptoms (cough, dyspnea, and chest pain), smoking habit, comorbidities, blood count, D-dimer, lactate dehydrogenase (LDH), ferritin, electrolytes, and arterial blood gases (ABG) were recorded. Dyspnea was assessed by modified medical research council (mMRC) score. In phase II (follow-up at 3 and 6 months after hospital discharge), clinical evaluation and respiratory function tests were performed, including spirometry, DLCO, ABG, 6MWT, and oxygen saturation (SpO_2). CT scan was performed at admission and at 3-month follow-up in all patients, and at 6-month follow-up in those with ALCT at 3-month follow-up.

Pulmonary function tests were performed in a V-MAX Encore (CareFusion, Yorba Linda, CA, USA) in the pulmonary function laboratory of the Fundación Neumológica Colombiana according to the recommendations of the American Thoracic Society (ATS) and the European Respiratory Society (ERS), and Crapo reference equations were used (14–16). The 6MWT was performed according to ATS and ERS recommendations (17).

The CT scan was performed according to the technical recommendations of the American College of Radiology (18) in a Somaton Definition Edge equipment (Siemens). The interpretation was performed by a certified radiologist with chest experience as recommended by the Radiological Society of North America (RSNA) (19). The CT findings were classified into (1) Typical appearance: peripheral, bilateral, ground glass opacity (GGO) with or without consolidation or visible intralobular lines ("crazy-paving"); multifocal GGO of rounded morphology with or without consolidation or visible intralobular lines ("crazy-paving"); reverse halo sign or other findings of organizing pneumonia. (2) Indeterminate appearance: absence of typical features and presence of multifocal, diffuse, perihilar, or unilateral GGO with or without consolidation lacking a specific distribution and are non-rounded or non-peripheral; few very small GGO with a non-rounded and non-peripheral distribution. (3) Atypical appearance: absence of typical or indeterminate features and presence of: isolated lobar or segmental consolidation without GGO; discrete small nodules (centrilobular, "tree-in-bud"); lung cavitation; smooth interlobular septal thickening with pleural effusion. (4) Negative for pneumonia: no CT features to suggest pneumonia (19).

Abnormal lung computed tomography was defined as the persistence of pulmonary infiltrates in the CT scan at follow-up at 6 months, and normal lung computed tomography (NLCT) as the absence of infiltrates on the CT scan. All information was collected by REDCap software to minimize missing entries and allow data validation.

Statistical analysis

The qualitative variables were described in relative and absolute frequencies, and the quantitative variables in measures of central tendency and dispersion according to the assumption of normality. For the comparison between qualitative variables between the ALCT and NLCT groups in the follow-up at 3 and 6 months, the X^2 test or Fisher's exact test was used. For non-parametric quantitative variables, the Mann–Whitney U test for independent samples was used. For comparisons between quantitative variables in the follow-up at 3 and 6 months, the paired Mann–Whitney U test was used.

A multivariable logistic regression model was performed to determine the variables associated with the persistence of tomographic abnormalities at 6 months of follow-up. Variables with a p-value < 0.25 in the initial bivariate analysis were included in the multivariate model. The model was evaluated in terms of the AUROC curve. The goodness of fit was evaluated using the Hosmer–Lemeshow test. For the analyses, the Stata 16 and R studio statistical

TABLE 1 Baseline characteristics of the study population.

	Total N = 158	NLCT N = 105	ALCT N = 53	p
Age, years	55.0 (46.0–62.0)	51.0 (41.0–60.0)	59.0 (52.0–65.0)	< 0.001
Male sex	91 (57.6)	53 (50.5)	38 (71.7)	0.011
BMI, kg/m^2	26.5 (24.1–30.3)	26.7 (24.6–31.6)	26.0 (23.8–30.2)	0.287
Smoker/former smoking	41 (26.0)	21 (20.0)	20 (37.7)	0.016
Obesity	47 (29.8)	33 (31.4)	14 (26.4)	0.515
Asthma	7 (4.4)	6 (5.7)	1 (1.9)	0.270
COPD	4 (2.5)	2 (1.9)	2 (3.8)	0.480
Hypertension	51 (32.3)	31 (29.5)	20 (37.7)	0.297
Type 2 diabetes	19 (12.0)	13 (12.4)	6 (11.3)	0.847
CKD	3 (1.9)	2 (1.9)	1 (1.9)	0.994
Chest pain	30 (19.0)	23 (21.9)	7 (13.2)	0.188
Cough	122 (77.2)	80 (76.2)	42 (79.3)	0.666
mMRC 0	56 (35.4)	36 (34.3)	20 (37.7)	0.669
mMRC $\geq$ 1	102 (64.6)	69 (65.7)	33 (62.3)	
LDH, U/L	398 (304–497)	388 (291–497)	428 (327–496)	0.157
D dimer, mg/L	1.0 (0.6–1.6)	0.9 (0.6–1.3)	1.4 (0.7–2.0)	0.017
Ferritin ng/ml	801 (402–1,781)	691 (359–1,529)	1,005 (564–2,351)	0.022
Chest CT on admission				
Typical appearance	146 (92.4)	94 (89.5)	52 (98.1)	
Atypical appearance	2 (1.3)	2 (1.9)	0 (0.0)	0.151
Indeterminate appearance	10 (6.3)	9 (8.6)	1 (1.9)	
ICU admission	35 (22.2)	16 (15.2)	19 (35.9)	0.003
ICU length of stay, days	7.0 (4.0–13.0)	8.5 (5.0–13.0)	5.0 (3.0–13.0)	0.443

NLCT, normal lung computed tomography; ALCT, abnormal lung computed tomography; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; mMRC, modified medical research council score; LDH, lactate dehydrogenase; CT, computed tomography; ICU, intensive care unit. Data are presented as median (p25–p75) or n (%). p: normal vs. abnormal CT.

programs were used, the tests were two-tailed, and a value of $p < 0.05$ was considered statistically significant.

Results

Demographics and characteristics at admission

A total of 158 patients were included, 57.6% men, with a median age of 55 years. At the six-month follow-up, 53 patients (33.5%) had ALCT. At admission, 26.0% of the subjects had a history of smoking and the main comorbidities were arterial hypertension (32.3%), obesity (29.8%), and diabetes (12.0%). Cough was the main symptom (77.2%), followed by dyspnea in 64.6% (Table 1).

In the ALCT group, there were more men, smokers, and older people in the NLCT group (Table 1). ALCT group also had higher levels of D-dimer [1.4 (0.7–2.0) vs. 0.9 (0.6–1.3), $p = 0.017$] and ferritin [1,005.0 (564.0–2,350.7) vs. 691.0 (359.0–1,529.0), $p = 0.022$]. Of the total group, 22.2% of the patients were admitted to the ICU. These patients had lower PaO_2/FiO_2 ($p < 0.001$), higher levels of LDH ($p = 0.036$), ferritin ($p = 0.008$), leukocytes ($p = 0.013$), and more ALCT at 6 months after hospital discharge than those of the group that did not enter the ICU ($p = 0.003$) (Table 2). There were no significant differences in the total days of hospitalization between NLT and ALCT groups [7.0 (5.0–10.0) vs. 8.0 (6.0–12.0), $p = 0.064$].

Pulmonary function tests and symptoms

At the 3-month follow-up, FVC was similar in both groups, but a higher % of patients had FVC < 80% of predicted than in the ALCT group compared to NLCT (17.7 vs. 2.7%, $p = 0.003$). At the 6-month follow-up, there was no difference in FVC between ALCT and NLCT. Also, there were no differences in DLCO between the ALCT and NLCT groups at 3 or 6 months follow-up. At the 6-month follow-up, in both groups, there was an increase in FVC and DLCO, but 9.8% in NLCT and 14.3% in ALCT had FVC < 80% of predicted, and 14.6% in NLCT and 14.7% in NLCT had DLCO < of 70% of predicted.

In the 6MWT there were no differences between the NLCT and ALCT groups in the meters walked at follow-up at 3 ($p = 0.029$) or 6 months ($p = 0.667$), although in both groups, the meters walked increased at 6 months ($p < 0.001$). The SpO_2 at rest at the 3 months of follow-up was significantly lower in the ALCT group than in the NLCT group ($p = 0.007$) and also during exercise at the 3 ($p < 0.001$) and the 6 months of the follow ($p = 0.031$) (Figure 1). The PaO_2/FiO_2 ratio was lower in the ALCT group than in the NLCT at the 3-month

TABLE 2 Baseline characteristics according to ICU admission (ICU and non-ICU patients).

	Total *N* = 158	No ICU *N* = 123	ICU *N* = 35	*p*
Age, years	55.0 (46.0–62.0)	53.0 (44.0–62.0)	58.0 (49.0–63.0)	0.208
Male, *n* (%)	91 (57.6)	65 (52.9)	26 (74.3)	0.024
BMI, kg/m^2	26.5 (24.1–30.3)	26.7 (24.3–30.3)	25.8 (23.2–31.3)	0.459
Smoker/former smoker	41 (26)	29 (23.6)	12 (34.3)	0.202
Obesity	47 (29.8)	35 (28.5)	12 (34.3)	0.506
Asthma	7 (4.4)	6 (4.9)	1 (2.9)	0.608
COPD	4 (2.5)	4 (3.3)	0 (0.0)	0.280
Hypertension	51 (32.3)	37 (30.1)	14 (40.0)	0.268
Type 2 diabetes	19 (12.0)	12 (9.8)	7 (20)	0.100
CKD	3 (1.9)	3 (2.4)	0 (0.0)	0.351
Chest pain	30 (19.0)	27 (22.0)	3 (8.6)	0.075
Cough	122 (77.2)	94 (76.4)	28 (80.0)	0.656
mMRC 0	56 (35.4)	47 (38.2)	9 (25.7)	
mMRC ≥ 1	102 (64.6)	76 (61.8)	26 (74.3)	0.173
LDH, U/L	398 (304–497)	388 (295–484)	436 (266–549)	0.036
D dimer, mg/L	1.0 (0.6–1.6)	0.9 (0.6–1.5)	1.1 (0.7–1.9)	0.459
Ferritin ng/ml	801 (402–1,781)	838 (381–1,420)	1,120 (809–2,359)	0.008
White blood cell count, ×109/L	7,470 (5,490–9,935)	7,090 (5,230–9,660)	8,750 (6,520–12,000)	0.013
Lymphocyte count, ×109/L	1,040 (720–1,405)	1,050 (750–1,450)	870 (670–1,240)	0.136
PaO_2/FiO_2 ratio	245.4 (208.5–274.0)	249.0 (216.1–285.7)	207.5 (145.6–259.6)	< 0.001
Chest CT on admission				
Typical appearance	146 (92.4)	113 (91.9)	33 (94.3)	
Atypical appearance	2 (1.3)	1 (0.8)	1 (2.9)	0.412
Indeterminate appearance	10 (6.3)	9 (7.3)	1 (2.9)	
ALCT at 3 months	85 (53.8)	58 (47.2)	27 (77.1)	0.002
ALCT at 6 months	53 (33.5)	34 (27.6)	19 (54.3)	0.003

NLCT, normal lung computed tomography; ALCT, abnormal lung computed tomography; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; mMRC, modified medical research council; LDH, lactate dehydrogenase; CT, computed tomography; ICU, intensive care unit. Data are presented as median (p25–p75) or *n* (%). *p*: normal vs. abnormal CT.

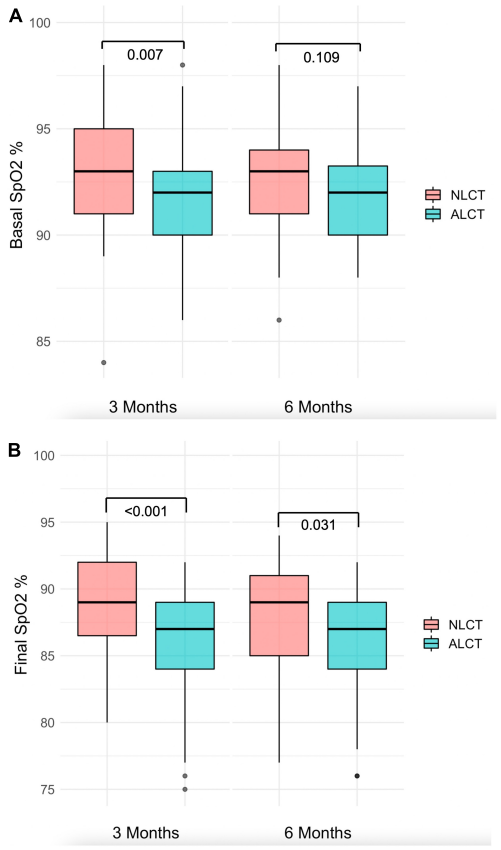

FIGURE 1
Basal and final SpO_2 in 6MWT in NLCT and NLCT groups. **(A)** In the ALCT group, baseline SpO_2 was lower than in NLCT both at 3 and 6 months of follow-up. **(B)** In the ALCT group, final SpO_2 was lower than in NLCT at 6 months of follow-up. SpO_2, oxygen saturación; 6MWT, six-minute walk test; NLCT, normal lung computed tomography; ALCT, abnormal lung computed tomography.

follow-up (p = 0.016), with no difference at 6 months (p = 0.479) (Table 3).

There were no differences between groups in the presence of cough, chest pain, or dyspnea on admission to hospitalization or at 3-month follow-up. At the 6-month follow-up, patients with ALCT had more dyspnea (mMRC $\geq$ 1) than those with NLCT (32.1 vs. 15.8%; p = 0.021).

CT scan findings

On admission, 92.4% of the patients had CT scans typical findings of SARS-CoV-2 infection, with no differences between the NLCT and ALCT groups (p = 0.412). In the follow-up 6 months after hospital discharge, the most frequent pattern of alteration in the ALCT group was the GGO (31.7%), followed by the reticular pattern in 2.5%, and no patient presented findings of traction bronchiectasis or honeycomb (Figure 2).

In the multivariate analysis, the variables associated with the persistence of infiltrates on CT at 6 months were older age, male sex, stay in the ICU, and the typical pattern on admission CT, test de Hosmer–Lemeshow p = 0.816 and the AUROC curve was 0.768 (Table 4).

Discussion

In this cohort of patients with moderate to severe COVID-19 disease, evaluated at a fourth-level hospital in the city of Bogotá, it was shown that 33.5% had ALCT at 6 months of follow-up. Although there were no differences in FVC and DLCO between ALCT and NLCT, those with ALCT had more dyspnea and lower SpO_2 during exercise than patients with NLCT. The factors associated with the persistence of infiltrates in the CT scan at 6 months were older age, male gender, presence in the ICU, and the typical pattern in the CT scan at hospital admission.

In our cohort, the main abnormality on the CT scan at 6 months of follow-up was the presence of GGO. This finding has been described as occurring in 80% of patients with COVID-19 at 2–3 months after admission to the hospital, and in 40% at 6–7 months of follow-up (8). In a study that included 114 survivors of severe COVID-19, the lesions found at 6 months of follow-up were GGO, interstitial thickening, and fibrotic lesions (traction bronchiectasis and parenchymal bands) in 27, 35, and 9%, respectively (20). Another study in 83 patients in the city of Wuhan, showed that the GGO was the main finding in the CT scan at 3 months of follow-up in 78% of the patients, and at 6 months in 46%, without complete resolution at 9 months of follow-up (9). Recently, a cohort from Spain of 284 patients with a 1-year follow-up reported alterations in tomography in 123 patients, with the presence of GGO in 47%, reticulation in 19%, and the presence of parenchymal bands in 22% (21).

The risk factors for the presence of ALCT at 6 months of follow-up in our cohort were older age, male sex, stay in the ICU, and the presence of a typical pattern in the chest CT on hospital admission. These findings are similar to those described in the 1-year follow-up in the COVID-FIBROTIC study team cohort from Spain, where they found that initial radiological compromise on admission was associated with the persistence of persistent tomographic lesions (21).

Another of the Wuhan cohorts, with follow-up at 1 year, described that ICU admission was associated with the presence of GGO at 1 year of follow-up (22). In the 7-month follow-up of tomographic sequelae in another cohort from China, the risk factors for the presence of these ALCTs were older age, longer hospital stay, and the need for ICU hospitalization (23), similar to that described in our cohort. Although there are few works that describe the presence of symptoms after SARS-CoV-2 infection differentiated between men and women, systematic reviews describe more severe diseases in men and greater residual respiratory symptoms in women (24), similar to our results.

The characteristics of populations infected with SARS-CoV-2 vary according to their geographic distribution and epidemiological characteristics at the time of infection. In our cohort, there were more men, and among the comorbidities that were found the most were smoking, arterial hypertension, obesity, and type 2 diabetes, as previously described in cohorts from Latin America, Wuhan, Italy, and New York (25–27). In Latin America, different risk factors for COVID-19 infection that are associated with the severity of the disease have been reported (28). In Colombia, it has been described that age, male gender, and the presence of comorbidities are associated with admission to the ICU (29), and in Bogotá, older age, lower PaO_2/FiO_2 ratio, and higher LDH at admission were associated with higher mortality (13). Similarly, the patients in our cohort that were admitted to ICU were more frequently male and with lower PaO_2/FiO_2 and higher levels of LDH.

TABLE 3 Pulmonary function test in follow-up at 3 and 6 months.

	Follow-up 3 months*N* = 158			Follow up 6 months*N* = 158				
	NLCT *N* = 73	ALCT *N* = 85	*p*	NLCT *N* = 105	ALCT *N* = 53	*p*	NLCT *	ALCT **
FVC,% of predicted	96.3 (91.0–107.4)	96.3 (85.3–111.0)	0.664	99.5 (91.1–108.9)	97.0 (84.7–109.9)	0.434	0.043	< 0.001
FVC, < 80% of predicted	2 (2.7)	15 (17.7)	0.003	12 (9.8)	5 (14.3)	0.445	0.563	0.008
FEV_1, % of predicted	96.6 (88.1–107.3)	97.0 (86.9–107.5)	0.962	98.3 (90.0–107.8)	98.7 (86.1–111.3)	0.837	0.640	0.005
FEV_1, < 80% of predicted	6 (8.2)	12 (14.1)	0.245	14 (11.4)	4 (11.4)	0.994	1.000	0.058
DLCO, % of predicted	90.7 (80.9–100.7)	86.3 (73.2–101.1)	0.156	93.1 (81.1–101.4)	88.5 (75.9–97.5)	0.155	0.008	0.001
DLCO, < 70% of predicted	7 (9.6)	16 (19.1)	0.095	18 (14.6)	5 (14.7)	0.992	0.102	0.179
6MWT								
Meters	582.5 (531–636.5)	559.0 (494–614)	0.229	598.0 (527.0–649.0)	587.0 (526.0–646.0)	0.667	< 0.001	< 0.001
Basal SpO_2, %	93.0 (91.0–95.0)	92.0 (90.0–93.0)	0.007	93.0 (91.0–94.0)	92.0 (90.0–93.5)	0.109	0.899	0.894
Final SpO_2, %	89.0 (86.0–92.0)	87.0 (84.0–89.0)	< 0.001	89.0 (85.0–91.0)	87.0 (84.0–89.0)	0.031	0.154	0.266
PaO_2/FiO_2 ratio	310.5 (288.1–338.1)	295.7 (275.7–316.9)	0.016	300.5 (282.9–316.2)	295.0 (275.0–319.0)	0.479	0.315	0.370

NLCT, normal lung computed tomography; ALCT, abnormal lung computed tomography; FVC, forced vital capacity; FEV_1, forced expiratory volume in the first 1 s; DLCO, carbon monoxide diffusion capacity; 6MWT, six-minute walk test; SpO_2, oxygen saturation; PaO_2, partial pressure of arterial oxygen; FiO_2, fraction of inspired oxygen. Data are presented as median (p25–p75), or *n* (%). *p*: NLCT vs. ALCT at 3 and 6 months. **p*: differences between 3 and 6 months in NLCT group; ***p*: differences between 3 and 6 months in ALCT.

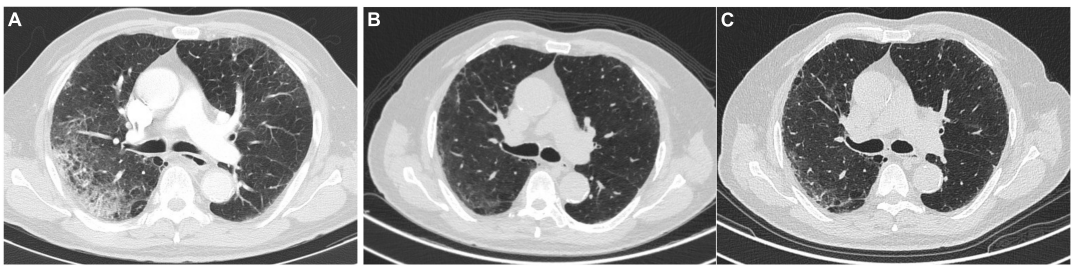

FIGURE 2
Changes in the CT scan in a patient with COVID pneumonia. **(A)** Upon admission. **(B)** At 3 months of follow-up. **(C)** At six-month follow-up.

Although smoking has also been associated with tomographic and functional abnormalities and greater severity of symptoms in patients with COVID-19 (21, 22), in our study it was not associated with ALCT in the multivariate analysis, similar to that described in other studies with follow-ups of 6 and 12 months (8, 23).

In our cohort, it occurred in less than 25%, and in the multivariate analysis, it was not associated with ALCT, similar to that described in follow-up cohorts of more than 6 and 12 months.

In the follow-up of lung function in SARS-CoV-2, restrictive and obstructive alterations in spirometry and a decrease in DLCO have been found (30). Several studies have shown that the reduction of DLCO in combination with restrictive patterns was the most frequent parameter in the follow-up of these patients (31, 32). At 12 months post-COVID, Huang et al. found that spirometry values were normal, and there was a decrease in DLCO (< 80% of predicted) in 23% of patients with moderate disease and 31% with severe disease (22). In another study with a 6-month follow-up, the reduction in DLCO occurred between 22 and 56% of the patients and was associated with the severity of COVID-19 and the need for hospitalization (8). Strikingly in our cohort, there were no differences between the ALCT and NLCT groups in FVC or DLCO at 6-month follow-up, but about 10% of patients had FVC < 80% predicted, and 15% of patients had DLCO < 70% of predicted.

TABLE 4 Multivariate analysis of risk factors associated with ALCT in follow-up at 6 months.

	Univariate		Multivariate	
	OR (95% CI)	*p*	OR (95% CI)	*p*
Age, years	1.056 (1.024–1.088)	< 0.001	1.068 (1.031–1.107)	< 0.001
Sex, male	2.485 (1.222–5.053)	0.012	2.692 (1.223–5.925)	0.014
CT scan typical appearance	6.085 (0.764–48.462)	0.088	9.738 (1.108–85.564)	0.040
ICU hospitalization	3.108 (1.434–6.737)	0.004	2.516 (1.077–5.873)	0.033

ALCT, abnormal lung computed tomography; ICU, intensive care unit. Test de Hosmer–Lemeshow $p = 0.816$.

It has been described that patients infected by SARS-CoV-2 have less exercise capacity after 6 months of infection, compared to the population without infection (33). In 2005, Hui et al. followed up 110 SARS survivors, noting that decreased DLCO was the most frequent finding of impaired lung function, and that those who had been admitted to the ICU walked fewer meters in the 6MWT (5). At sea level, in patients with SARS-CoV-2 pneumonia, the average number of meters walked reported in the 6MWT at 3 months was 539 ± 102.8 m, which increased in the year of follow-up to 556 ± 92 m (34). In Spain, in a prospective study carried out at sea level, the average number of meters walked at 2 months of follow-up was 524 m, at 6 months 521 m and at 12 months 519 m (21). In Latin America, a Mexican group located at sea level in the Yucatán Peninsula described that patients with mild to severe COVID-19 disease followed by persistent dyspnea walked an average of 493 ± 7 m (35). In our cohort of moderate to severe COVID survivors, there were no differences between the ALCT and NLCT groups in meters walked at 3 or 6 month follow-up, with values higher than those reported in previous studies (21, 33–35).

Despite more meters walked, the saturation values during exercise in our cohort were lower than those reported at sea level (30, 31, 36), even in severe pneumonia cohorts followed up only 2 months after symptom onset with average saturations greater than 97% (36). These differences in saturation can be explained by altitude. Due to Bogotá's location at 2,640 m above sea level, PaO_2 is around 60 mmHg and SaO_2 is 90% in normal patients, with significant desaturations during exercise in patients with interstitial lung disease (11). Despite these lower saturations at altitude, different studies suggest that this does not represent a negative impact on the mortality of patients with COVID-19 residing at high altitudes (37).

At the 6-month follow-up, dyspnea was the main symptom in patients with ALCT, which occurred significantly more frequently than in the NLCT group. These findings are consistent with the new definitions of the long-term effects of COVID-19 or Long-COVID-19, in which dyspnea occurs in up to 61% of patients suspected of having this syndrome (38). In addition, the presence of dyspnea has been reported in more than half of the patients during physical activity, even without having abnormalities in the pulmonary function tests (39), similar to the results of our cohort (40).

In this study, with a significant number of patients, we show the medium-term behavior of patients with moderate and severe COVID-19 pneumonia at high altitude. We emphasize that clinical follow-up was achieved up to 6 months after hospitalization with symptoms, functional evaluation with spirometry, DLCO, 6MWT, and CT scan. Among the limitations of the study, we highlight that it was carried out in a single center in the city of Bogotá, and the patients included were younger and had fewer comorbidities than those included in other cohorts with COVID-19. The study was carried out before vaccination against COVID and during the first and second epidemiological peaks in the country, which could determine that the population had a more severe disease with greater alterations in the follow-up computed tomography. We also did not record the type of ventilatory support in patients admitted to the ICU and the number of patients with pulmonary embolism as a complication of COVID-19 during hospitalization, although none of the patients had anticoagulant treatment at post-hospitalization follow-up visits. Another point of improvement was that we did not evaluate the fatigue symptom since we focused only on the presence of cough, chest pain and dyspnea. Finally, the 6-month evaluation period, although longer than most studies, is shorter than other studies with post-COVID follow-up of up to 1 year.

Conclusion

Our study, conducted in a high-altitude city, showed that one-third of patients hospitalized for moderate and severe COVID have persistent chest tomography abnormalities 6 months after discharge, with lower exercise saturation and more dyspnea than those without persistent pulmonary infiltrates.

Data availability statement

The original contributions presented in this study are included in this article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Fundación Neumológica Colombiana (approval number 202007-25702). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

ER-A, MG-G, CT-D, CP, WR, MC, AM, and PP-T: conception and design. NP and ER-A: analysis and interpretation. MG-G, LG-C, ER-A, MG-G, CT-D, AA-M, and AC: drafting the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Post-COVID-19 syndrome and quality of life impairment in severe COVID-19 Mexican patients

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Introduction: Post-COVID-19 syndrome (PCS) usually occurs 3 months after the onset of COVID-19 with a symptom duration of at least 2 months without an alternative diagnosis.

Objective: This study aimed to describe the prevalence, characteristics, and impact on the quality of life (QoL) of post-COVID-19 syndrome in patients with a history of hospitalization for COVID-19.

Materials and methods: We conducted a cross-sectional study. Patients who required hospitalization due to COVID-19 between March 2020 and October 2021 were invited to answer a PCS questionnaire and the EQ-5D instrument. A total of 246 patients were included: 187 (76%) met the definition of PCS and 54% were men, with a median age of 50 years (IQR 41–63).

Results: From 187 patients with PCS, the median time to symptom onset after hospital discharge was 1 day (IQR 1–20), and the median symptom duration was 150 days (IQR 90–225). A total of 27 different symptoms were reported; the most frequent were difficulty concentrating (81%), dyspnea (75%), arthralgia (71%), fatigue (68%), and hair loss (60%). Some symptoms, such as difficulty concentrating, arthralgia/myalgia, and hair loss, were more prevalent in women with PCS. Patients with PCS had a higher frequency of tobacco smoking (37 vs. 4%, $p = 0.02$) and increased severity of lung involvement in the initial chest tomography (75 vs. 58%, $p = 0.01$) than those without PCS. Patients with PCS were less likely to receive antivirals (15.5 vs. 27%, $p = 0.04$). No difference between ICU admission, mechanical ventilation, and length of hospital stay was found. Patients with PCS had a lower visual analog scale result for EQ-5D vs. those without (80 [IQR 70–90] vs. 89.5 [IQR 75–90], $p = 0.05$). All five QoL dimensions were affected in PCS patients, showing increased pain/discomfort (67 vs. 39%, $p = <0.001$), difficulties in performing usual activities (39.2 vs. 20.3%, $p = 0.03$), and anxiety/depression (57.5 vs. 37%, $p = 0.02$).

Conclusion: PCS occurred in 76% of hospitalized patients with prolonged duration and QoL impairment. Neurological symptoms such as difficulty concentrating were the most frequent symptoms. Timely diagnostic and therapeutic interventions are required.

KEYWORDS

post-COVID-19 syndrome, long-COVID-19, quality of life, chronic COVID-19 syndrome, severe COVID-19, Mexican

Introduction

Coronavirus disease-19 (COVID-19) is still a critical comorbidity and mortality cause worldwide. SARS-CoV-2 infection no longer carries the same risks of adverse outcomes as it did in the early months of the pandemic because of the vaccines and the new subvariants of SARS-CoV-2 with a diverse rate of transmission and virulence (1). Post-COVID-19 syndrome (PCS) was defined by the World Health Organization (WHO) in late 2021 as symptoms occurring in individuals within 3 months of a history of probable or confirmed SARS-CoV-2 infection, with at least 2 months that cannot be explained by an alternative diagnosis (2). Common symptoms of PCS include shortness of breath, fatigue, difficulty thinking or concentrating (referred to as "brain fog"), changes in smell and taste, sleep problems, and hair loss. Before this definition, different terms such as long COVID syndrome, persistent post-COVID syndrome, and post-acute COVID-19 syndrome were used. Symptoms may be new onset following initial recovery or persistent since the initial COVID-19 episode. Symptoms may also fluctuate or relapse over time.

A meta-analysis reported a high prevalence of up to 80% (95% CI 65–92%) (3). Other cohorts report a lower prevalence, such as 32.6% in Michigan, USA, with limitations such as the study date and lack of PCS definition (4). Another study in Wuhan, China, using questionnaires, physical examination, 6-min walk tests (6MWT), laboratory tests, pulmonary function tests (PFTs), and high-resolution computed tomography described that 76% had at least one symptom. Lopez-León et al. described more than 50 symptoms as part of PCS; among them, the most frequent were fatigue (58%), headache (44%), attention disorder (27%), hair loss (25%), and dyspnea (24%) (3, 5). Other reviews and meta-analyses in the UK also found fatigue (37%) as the most prevalent, followed by dyspnea (6).

The quality of life (QoL) definition encompasses individual perceptions of their position in life in the context of the culture and value systems concerning goals, expectations, standards, and worries (7). Hence, QoL is used as a general predictor of health and is essential to understand the repercussions of COVID-19 on physical health status, social restrictions, and psychological states. Several tools measure the QoL; some are generic, such as SF-36 (36-item Short-Form Health Survey) and EQ-5D (EuroQol-5 Dimension), and they are used to assess multiple domains of the health and wellbeing of the patient. Specific tools are also used in diseases such as rheumatoid arthritis and metabolic disorders (8).

In other pandemic scenarios, such as influenza, QoL impairment was described (9). After the COVID-19 pandemic, it has been published that survivors' QoL is generally affected, particularly in patients with PCS (10–12).

In this study, we aimed to describe the prevalence of PCS, the frequency of symptoms, and the impact on the QoL of patients with an initial episode of severe or critical COVID-19.

Methods

Patients and data collection

A retrospective, cross-sectional study was done. We found 1,379 patients ≥18 years of age hospitalized with COVID-19 between 1st July 2020 and 31st December 2021. Among these, 317 who had been hospitalized 3, 6, 9, and 12 months before were randomly selected and invited to answer an adapted questionnaire to identify the presence of PCS and EQ-5D. Among 312 patients who were invited to participate, only 246 patients were included who then answered both the questionnaires (Figure 1).

Research tools and instruments

The content of the PCS questionnaire was adapted from the questionnaire used by Huang et al. on a group of Chinese patients in 2020. A committee comprising a rheumatologist and an infectious diseases specialist performed a translation and adaptation to suit Mexican patients from the original version. The latter is a questionnaire that has been validated in multiple populations worldwide and showed good psychometric properties in Mexico (13); the first part is a visual analog scale, and the second part corresponds to five domains: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression, with three possible response options: no problems, some problems, or extreme problems. The visual analog scale was reported as 0–100, where 0 represents the worst imaginable health and 100 the best imaginable health; the five domains were dichotomized into not affected (answer "no problems") and affected (answers "some problems," or "extreme problems") (14). All reported symptoms, duration, and impact on QoL were recorded.

Definitions

The WHO definition of PCS was used to classify patients into two groups: PCS and no-PCS for comparison (2). Regarding COVID-19 acute episode characteristics, we described severity using the NIH classification. We also describe the clinical, laboratory, and computed tomography (CT) characteristics and the treatment received. Definitions of the compatible or indeterminate chest CT were according to the Radiology Society of North America Expert Consensus (15). In the vaccination record, we considered only those who had a first full scheme before the COVID-19 episode.

Statistics

Sample size calculation estimated 246 subjects considering a prevalence of 80% (2). All analyses were performed in STATA v 14.0 (StataCorp., College Station, TX, USA). Baseline characteristics are reported with descriptive non-parametric statistics, bivariate

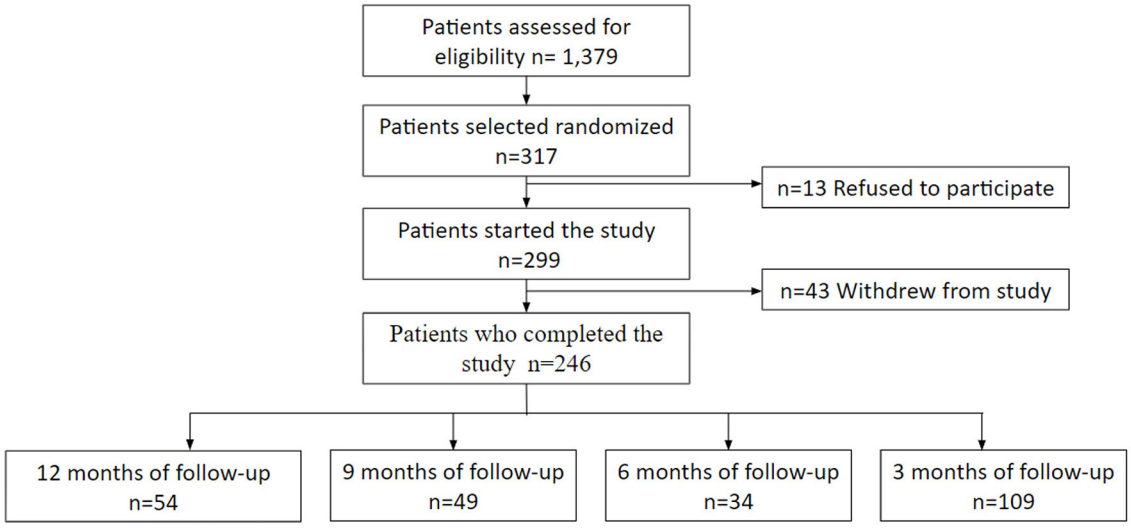

FIGURE 1
Pacients flowchart. Comments: Patients assessed for eligibility were patients who were hospitalized in the chosen period and who were discharged home.

comparisons were made with the $\mathbf{X}^2$ test, Student's *T*-test, or Mann–Whitney's *U*-test, as appropriate, and a two-tailed *p*-value of <0.05 was considered significant.

Ethics

The protocol was approved by the local research and ethics committee (Local Ref Nr. 3692). Participants' data privacy was preserved during the study. Digital informed consent was signed and kept for record.

Results

The prevalence of PCS in hospitalized patients with severe or critical COVID-19 was 76% (n = 187). Table 1 shows the demographic and clinical characteristics of the initial COVID-19 episode among groups. Patients with PCS had a median age of 55 years (IQR 41–63), and 54% (n = 101) were men. We found no statistical differences in obesity and overweight in both groups, and BMI at the time of acute COVID-19 was a median of 27.74 kg/m^2 (IQR 25.31–32.39) vs. 29.41 kg/m^2 (IQR 26.12–34.6). Smoking was more frequently reported in the PCS group (19.7 vs. 6.7%, RR 1.23; 95% CI 1.08–1.40, p = 0.02). Other comorbidities were not statistically different between the groups.

Table 2 shows the characteristics of the index hospitalization. More than 90% in both groups had a chest CT scan compatible with COVID-19 (PCS 97% (181/187) vs. without PCS 92% (54/59), p = 0.08). The remaining percentage in both groups was described as indeterminate for COVID-19.

During the initial episode of COVID-19, PCS patients had low room air SatO2 levels (oxygen saturation) (82% IQR 74–86 vs. 85% IQR 82–88, p = 0.002), and their chest CT scans had severe lung involvement more frequently (75 vs. 58%, p = 0.01). Furthermore, 97% (182/187) of PCS patients had severe COVID-19 as per NIH classification; 33% (n = 62) of patients in the PCS group were admitted to the intensive care unit (ICU), and 32% (n = 59) required mechanical ventilation at any time during their hospitalization, with no statistical differences between groups.

The most frequent symptoms of PCS were difficulty concentrating in 81% (n = 152), dyspnea in 75% (n = 141), arthralgias in 71% (n = 132), weakness in 69.5% (n = 130), fatigue in 68% (n = 127), hair loss in 60% (n = 112), myalgia in 53% (n = 99), sleep disturbances in 52% (n = 97), dizziness in 47% (n = 88), and palpitations in 41% (n = 76). A total of 27 different symptoms were described. We decided to classify them into clusters by system (Figure 2). The median time between hospital discharge and symptom onset was 1 day (IQR 1–20 days), and the median symptom duration was 150 days (IQR 90–225 days). When comparing smoking with respiratory symptoms such as dyspnea, we do not find differences, and only 18% of patients with dyspnea are smokers (p = 0.42). Among female patients with PCS, difficulty concentrating (87 vs. 76%, p = 0.05), arthralgias (79 vs.63%, p = 0.02), hair loss (82.5 vs. 40%, $p \leq 0.0001$), myalgia (63 vs. 44.5%, p = 0.01), and dizziness (56 vs. 39%, p = 0.02) were more frequent compared to their male counterparts (Table 3).

Regarding health status and QoL, 63% (n = 117) of PCS patients described their health status as "worse" than before COVID-19 (OR 9.2, 95% CI 4.1–22.6, $p \leq 0.0001$). In the EQ-5D instrument, we found disturbances in all five domains; the pain and discomfort domain was the most affected in PCS at 65.5 vs. 39% without PCS ($p < 0.001$). Also, patients with PCS were referred to a worse QoL (visual analog scale) compared to those without PCS [80 IQR (70–90) vs. 89.5 (75–90), p = 0.05]. Affected domains are described in Table 4 and Figure 3.

TABLE 1 Demographic and clinical characteristics of patients with and without post-COVID-19 syndrome.

Characteristics	General *n* = 246 (100%)	PCS *n* = 187 (76%)	Without PCS *n* = 59 (24%)	*p* bivariate
Male sex	135 (54.87)	101 (54)	34 (58)	0.62
Age, median (IQR)	52.5 (41–64)	55 (41–63)	50 (39–69)	0.55
Obesity	106 (43)	86 (46)	20 (34)	0.10
Overweight	93(38)	66 (35)	27 (45)	0.14
Hypertension	82 (33)	61 (33)	21 (35.5)	0.67
Type 2 diabetes	56 (23)	42 (22)	14 (24)	0.83
Chronic kidney disease	15 (6)	9 (4.8)	6 (10.1)	0.13
Rheumatic disease	16 (6.5)	13 (7)	3 (5)	0.61
Solid cancer	6 (2.4)	4 (2)	2 (3)	0.06
Hematologic cancer	2 (0.8)	1 (0.5)	1 (2)	0.42
COPD	4 (2)	4 (2)	0	0.57
Asthma	7 (3)	6 (3)	1 (2)	0.54
Immunosuppression	23 (9)	15 (8)	8 (13.5)	0.20
HIV infection	4 (2)	2 (1)	2 (3)	0.24
Ischemic cardiopathy	10 (4)	8 (4.2)	2 (3.3)	0.76
Smoking	41 (17)	37 (20)	4 (7)	**0.02**
COVID-19 vaccine	23 (9)	18 (10)	5 (8)	0.79

PCS, Post-COVID-19 syndrome; IQR, interquartile range; COPD, chronic obstructive pulmonary disease; HIV, human immunodeficiency virus.

TABLE 2 Index hospitalization for COVID-19 among patients with and without PCS.

Characteristics	General *n* = 246 (100%)	PCS *n* = 187 (76%)	Without PCS *n* = 59 (24%)	*p* bivariate
COVID-19 compatible chest CT	235 (96)	181 (97)	54 (92)	0.08
Severe lung involvement in chest CT	174 (71)	140 (75)	34 (58)	**0.01**
SatO2 (%), median (IQR)	83 (75–87)	82 (74–86)	85 (82–88)	**0.002**
PaO2/FiO2 ratio	164.75 (92.13–251)	155.32 (92.44–251)	177.81 (89.11–252.38)	0.90
Intensive care unit admission	76 (31)	62 (33)	14 (24)	0.17
Invasive mechanical ventilation	73 (30)	59 (32)	14 (24)	0.25
Steroids for COVID-19	239 (97)	183 (98)	56 (95)	0.23
Antiviral for COVID-19	45(18)	29(15.5)	16(27)	**0.04**
Empirical antibiotic	48 (20)	36 (19)	12 (20)	0.81
Hospital length stay (days) median (IQR)	10 (6–20)	10 (6–21)	9 (5–19)	0.20
C Reactive protein, (mg/dL), median (IQR)	11.13 (6.01–18.8)	11.91 (6.66–19.26)	9.45 (5.07–17.21)	0.26
Leucocytes (x $10^3/\mu L$), median (IQR)	8350 (5900–12300)	8600 (6100–12600)	7800 (5300–10800)	0.12
Lymphopenia ($<1.0 \times 10^3/\mu L$)	176 (71.5)	131 (70)	45 (76)	0.35
D dimer (ng/mL), median (IQR)	699 (446–1191)	704.5 (438–1168)	682 (523–1245)	0.33
Ferritin (ng/mL), median (IQR)	566.05 (260.95–1060.1)	592.75 (280.8–1088)	519.8 (192–879)	0.15
CPK (U/L), median (IQR)	84 (43.5–161)	79 (37–159)	101 (54–190)	0.11
Lactic dehydrogenase (U/L), median (IQR)	329 (262–437)	327 (262–438)	333.5 (265–420.5)	0.97
Fibrinogen (mg/dL), median (IQR)	614 (462–767)	635 (479–776)	490.5 (429–710)	**0.006**

PCS, post-COVID-19 syndrome; CT, computed tomography; IQR, interquartile range; $SatO^2$, oxygen saturation; PaO^2, partial pressure of arterial oxygen; FiO^2, fraction of inspired oxygen; CPK, creatine phosphokinase. Bold values are values <0.05, which means that they are statistically significant.

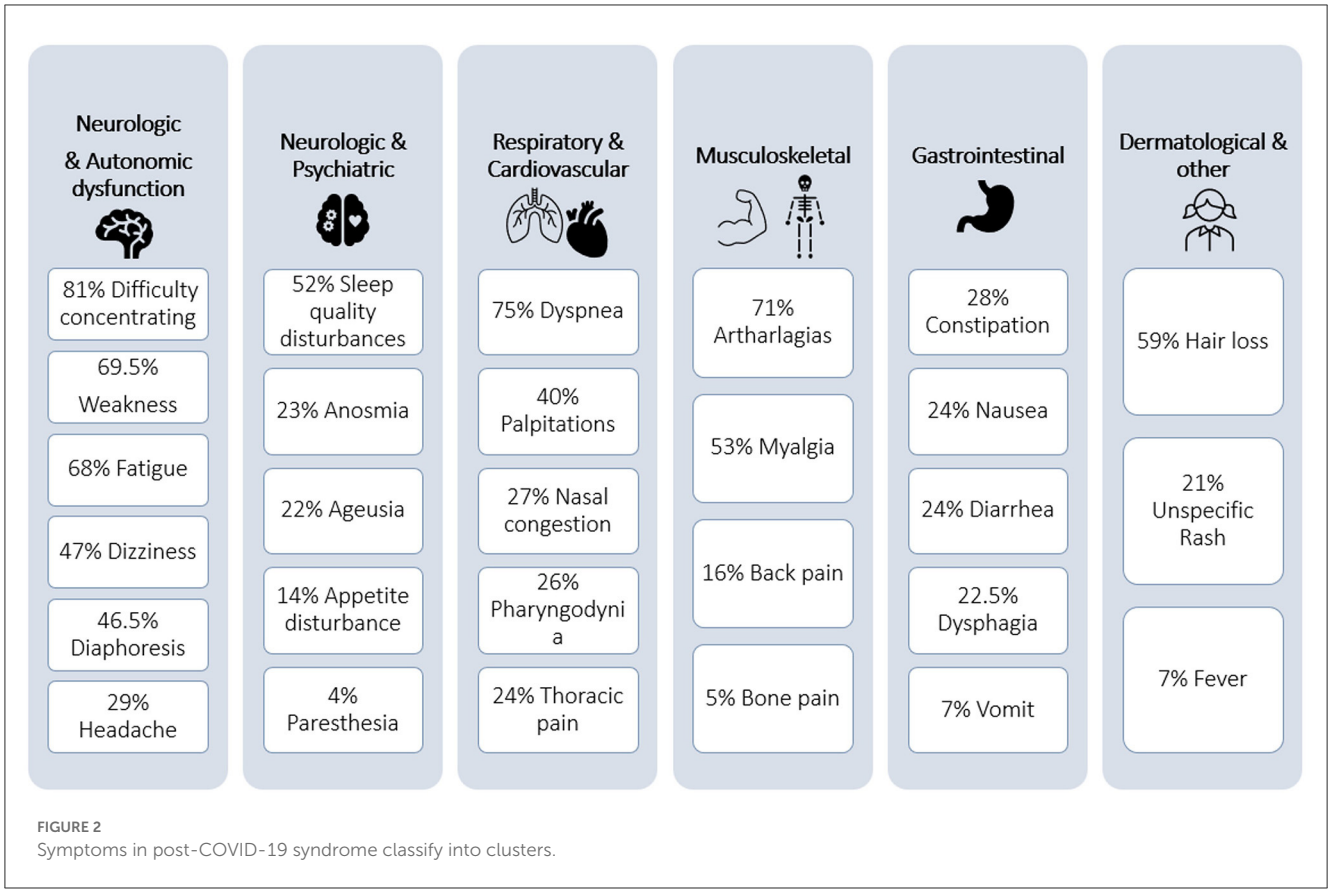

FIGURE 2
Symptoms in post-COVID-19 syndrome classify into clusters.

TABLE 3 Frequency and differences of PCS symptoms between men and women.

Symptoms	General PCS *N* = 187 (100%)	Males with PCS *N* = 101 (54%)	Females with PCS *N* = 86 (46%)	*p* bivariate
Difficulty concentrating	152 (81)	77 (76)	75 (87)	**0.05**
Dyspnea	140 (75)	70 (69)	70 (81)	0.06
Arthralgias	132 (71)	64 (63)	68 (79)	**0.02**
Weakness	130 (69.5)	67 (66)	63 (73)	0.30
Fatigue	126 (67)	64 (63)	62 (72)	0.20
Hair loss	111 (59)	40 (40)	71 (82.5)	**<0.0001**
Myalgia	99 (53)	45 (44.5)	54 (63)	**0.01**
Sleep disturbances	98 (52)	50 (49.5)	48 (56)	0.39
Dizziness	87 (46.5)	39 (39)	48 (56)	**0.02**
Palpitations	75 (40)	35 (35)	40 (46.5)	0.09

Bold values are values <0.05, which means that they are statistically significant.

Discussion

We found a 76% prevalence of post-COVID-19 syndrome in patients hospitalized for severe or critical SARS-CoV-2 infection. This rate is comparable to a systematic review describing over 50 symptoms but lower. Of note, the WHO definition we used had not been published at that time (2, 3). The definition used in the systematic review considered symptoms, signs, or abnormal clinical parameters persisting 2 or more weeks after COVID-19 onset that do not return to a healthy baseline; but given our prevalence, the defining factor does not have much impact. According to the literature, the prevalence of PCS ranges between 5 and 50%. Some extensive surveys describe a prevalence of 39%, including infections by different SARS-CoV-2 variants (16). Therefore, the variability is due to various factors: the definition of PCS, the hospitalized and non-hospitalized population, the

TABLE 4 Distribution of the VAS and dimensions of the EQ-5D among patients with and without PCS.

Dimensions in EQ5D	General *n* = 246 (100%)	PCS *n* = 187 (76%)	Without PCS *n* = 59 (24%)	*p* bivariate
Visual analog scale, median (IQR)	80(70–90)	80 (70–89)	89.5 (75–90)	**0.05**
Mobility				0.5
-No problems	168 (69)	124 (67)	44 (75)	
-Some problems	73 (30)	59 (32)	14 (24)	
-Confined to bed	4 (2)	3 (2)	1 (2)	
Self-care				0.3
-No problems	211 (86)	157 (84)	54 (91.5)	
-Some problems	32 (13)	27 (14.5)	5 (8.5)	
-Unable to	2 (0.8)	2 (1.0)	0	
Usual activities				**0.03**
-No problems	161 (66)	114 (61)	47 (80)	
-Some problems	82 (33.5)	70 (38)	12 (20)	
-Unable to	2 (0.8)	2 (1)	0	
Pain/discomfort				**<0.001**
-None	99 (40)	63 (34)	36 (61)	
-Moderate	133 (54)	115 (61.5)	18 (30.5)	
-Extreme	14 (6)	9 (5)	5 (8.5)	
Anxiety/depression				**0.02**
-None	115 (47)	78 (42)	37 (63)	
-Moderate	120 (49)	100 (54)	20 (34)	
-Extreme	10 (4)	8 (4)	2 (3)	

PCS, post-COVID-19 syndrome; EQ-5D, EuroQol-5 Dimensions; IQR, interquartile range. Bold values are values <0.05, which means that they are statistically significant.

variants, and even other additional factors such as vaccination or the treatments received.

In addition, we found 27 different symptoms as well as slightly more than half of those described in other research may be due to the strategies of searching for or questioning the presence of symptoms; the questionnaire and the interview strategy can influence the finding of more or fewer symptoms.

A few publications on PCS prevalence in Mexico were found. In a study from Guanajuato, Mexico, Muñoz-Corona et al. reported 75.9% of persistent symptoms in COVID-19 patients at 90 days of hospital discharge (17). In a study from Zacatecas, Mexico, long-term symptoms were found in 85% of patients; however, it was carried out in 2020 with a different PCS definition (18).

Some studies have associated older age and women with a higher risk for PCS (19, 20), but we did not find significant differences in age between groups nor in the proportion between sexes; this last finding may be associated with our hospitalized cohort comprising more men, with the male sex being at higher risk of severe COVID-19. However, we found some differences in the frequency of symptoms according to sex; difficulty concentrating, arthralgia, myalgia, dizziness, and hair loss are more frequent and with statistical significance in women. Fernandez DPC et al. also described that some PCS symptoms are more frequent in women such as fatigue, dyspnea, hair loss, ocular problems, depression, and poor sleep quality (20).

Fatigue is one of the predominant symptoms in PCS (21); however, respiratory manifestations, such as dyspnea, persistent cough, and chest pain, remain frequent and presumably associated with the lungs as the primary site of infection (3). Our patients referred to difficulties with concentration and attention, the so-called "brain fog," as the main neurological complaint, with a higher than reported frequency. This has been linked to direct viral damage to the limbic system after entering through the nasal sensory cells (22). Other theories explaining cognitive abnormalities include direct neuronal infection and autoimmune/inflammatory CSF and brain tissue abnormalities (23). Ongoing studies, at our institution, found a prevalence of psychopathological PCS manifestations, memory complaint, and mild cognitive impairment a year after the acute COVID-19 episode, in 42, 45, and 30%, respectively (unpublished data, personal communication from Flores-Silva F.) The frequency of neurological symptoms during acute COVID-19 might explain the high prevalence of these symptoms. Another study from our center found that up to 65% of patients hospitalized for COVID-19 had neurological symptoms on admission, and 15% developed some neurological event, such as seizures, delirium, altered alertness, or weakness during hospitalization (24). Moreover, Wong-Chew et al.

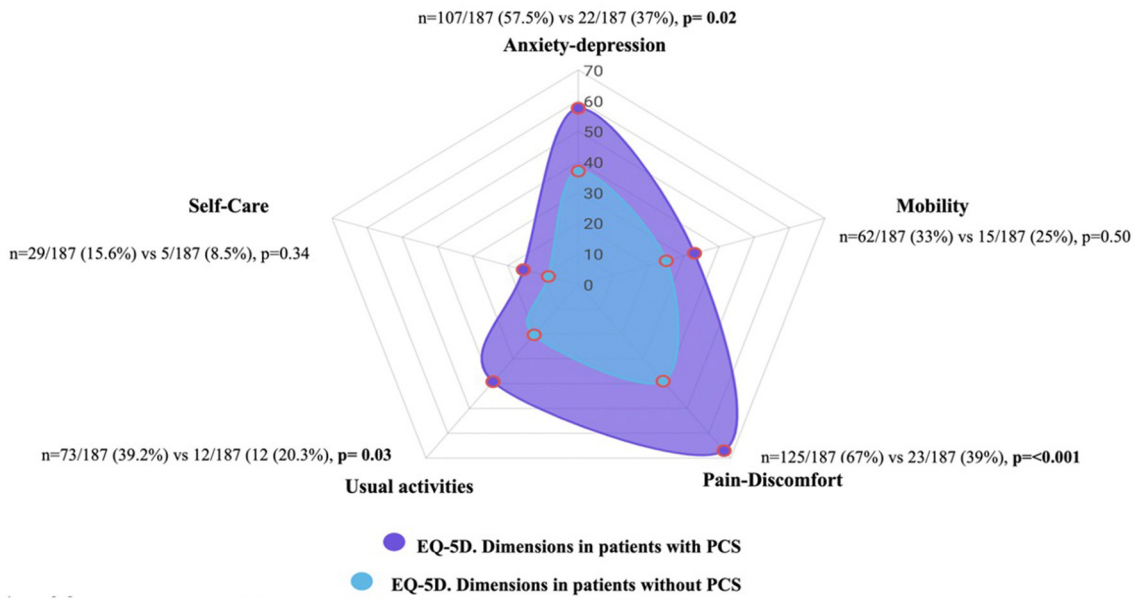

FIGURE 3
Distribution of the dimensions affected in EQ-5D.

showed that the most frequent post-COVID-19 symptoms were neurological (25).

Interestingly, some overlapping with the ME/CFS (myalgic encephalomyelitis/chronic fatigue syndrome) pathogenesis has been found. Other hypotheses explaining PCS vary from changes in host-microbiome diversity leading to dysbiosis and persistent autoimmune or inflammatory stimuli, the persistence of viral reservoirs in specific tissues, alterations in the coagulation cascade, or a complex combination of multiple mechanisms (26).

In our study, any degree of hair loss was reported by 60% of PCS patients. The most accepted mechanism for alopecia is telogen effluvium (TE), which is associated with systemic stress, although other mechanisms have been described (27). It has been reported in different proportions in post-COVID patients, and some studies report a higher prevalence in women than in men, as we found, even in the sequelae initially described in patients from Wuhan, China, at the beginning of the pandemic (28, 29). Of note, arthralgias and myalgias were frequent. A physical examination and inflammatory marker determination would clarify whether arthritis occurs as a PCS manifestation or whether another condition may be unmasked. The descriptive nature of this study is a major limitation.

Our patients with PCS reported smoking more frequently, which has been described in a study from France, where smoking was the main factor associated with tachycardia or hypertension 2 months post-COVID-19 (30). Studies from the UK and Turkey found smoking was more frequently reported in patients with post-COVID-19 symptoms (31, 32). These findings underscore the importance of focusing on strategies to quit tobacco consumption among vulnerable patients as a measure to reduce PCS.

We did not find other previously described differences between groups, such as female sex, obesity, or older age. However, this study involved more than 600,000 people and included both hospitalized and outpatients, perhaps leading to a mix in various extents of viral damage, symptom duration, and baseline clinical features (31).

Regarding COVID-19 vaccination, we found no differences between our groups. However, the proportion of vaccinated patients was small at the time of the study. A systematic review by Notarte et al. (33) showed a reduction of PCS after vaccination, although this finding remains controversial with a lack of evidence to make conclusions.

A high proportion of our patients with PCS had a severe acute COVID-19 episode, although ICU stay was not associated with increased PCS. This has been inconsistently seen in studies. Kamal et al. observed that the severity of COVID-19 was related to post-COVID-19 manifestations, although 80% of patients with PCS had mild COVID-19 (5, 34, 35).

The only inflammatory marker that we found associated with PCS was a higher fibrinogen level. Fibrin amyloid micro-clotting and platelet dysfunction have been demonstrated in PCS models, unveiling a possible association between coagulation dysregulation and chronic COVID-19 symptoms (36).

A much-anticipated effect of antivirals, such as remdesivir, is the ability to protect from or ameliorate symptoms of PCS. A prospective cohort showed a 35.9% reduction of PCS at a 6-month follow-up in patients receiving remdesivir (37). Antivirals may halt the cytokine response and inflammatory cascade that activate clotting and fibrosing factors playing a role in the pathogenesis of PCS. In addition, tissue damage inflicted by SARS-CoV-2 has been linked to chronic sequelae and manifestations of organ dysfunction even months after resolution (26).

Quality of life was significantly affected in patients with PCS. This finding is consistent with Muñoz-Corona et al., who found that 75.9% of PCS patients had the lowest scores in the roles of physical dimension and general health dimension (SF-36

questionnaire) studied 90 days after discharge (17); Tobada et al. showed a decrease in QoL measured with the EQ-5D 6 months after the acute infection with moderate-to-severe disturbances in the following domains: 56% in mobility, 48% in pain/discomfort, 46% in anxiety/depression, 37% in usual activities, and 13% in self-care; these findings are similar to ours (38). In addition, a systematic review confirmed that QoL in PCS patients was significantly affected, regardless of time elapsed since discharge or recovery, although the tools applied to measure QoL were heterogeneous (39, 40). Thiolliere et al. (41) compared the QoL of older patients with COVID-19 who required ICU with other ICU patients and found no differences between the EQ-5D scores or autonomy at day 180. This finding supports that in older people, the deterioration of QoL is more likely linked to the infection per se and not to the ICU stay (41).

Our study has various limitations: The cross-sectional design does not allow for follow-up data at different times. However, the survey was conducted at different times after the acute COVID-19 episodes. Although memory bias was likely present, the survey strategy was the most efficacious approach to surpass the decline in COVID-19 cases over time, for reasons such as the circulation of variants and the current use of vaccines. In addition, none of the patients had a QoL assessment before their acute COVID-19, so direct comparisons cannot establish a strong causal relationship with SARS-CoV-2 infection. On the other hand, the patient's clinical characteristics and comorbidities were fully analyzable, which gives our results and study strength when compared with other works. Finally, a prospective approach with clinical evaluation and intervention is undoubtedly required for these patients, from the moment they are diagnosed with COVID-19, to assess the development of PCS. We consider the descriptive nature of our approach to be a limitation since we did not have any studies or interventions in those who had PCS. During the pandemic, our institution focused solely on caring for patients with COVID-19. However, due to these findings, we are currently developing multidisciplinary care for patients with PCS.

Regarding the diagnosis and management of PCS, it took not long for the scientific community to understand the complexity of PCS. Thus, since early 2021, several multidisciplinary programs and ambulatory rehabilitation clinics and projects have been launched, whether virtual or hybrid, to be able to cope with this ever-growing population. Diagnostic criteria have been set through a Delphi consensus (2). However, treatment strategies are still under investigation, mainly to ascertain the best type and duration of therapy necessary for a patient suffering from PCS to restore health and QoL (42).

Although in the future, the prevalence and severity of PCS will be modified by factors such as more robust vaccination schemes, antivirals, or anticoagulants. Furthermore, infections with new viral strains and host-derived factors may impact PCS incidence (43). Comprehensive multidisciplinary studies are needed to set the ground for better understanding and managing this disease.

Conclusion

A high prevalence of PCS in previously hospitalized patients with COVID-19 was found. Smoking, severe COVID-19, lower SatO2 on admission, increased lung involvement, and elevated fibrinogen levels were associated with increased frequency of PCS. Some symptoms, such as difficulty concentrating, arthralgia/myalgia, and hair loss, were more prevalent in women with PCS. A significant QoL impairment was evident in PCS.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics and Research Committee, Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán". The patients/participants provided their written informed consent to participate in this study.

Author contributions

YF-S conducted the surveys. CR-M, YF-S, and GG-B performed the clinical data search and capture. CR-M, YF-S, GG-B, KT-T, and MG-L aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript. MG-L, JS-O, and AL supervised the research, provided critical feedback, and helped shape the research, analysis, and manuscript. All authors participated in the idea, general objectives, and design of the study.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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