

# Persistent Symptoms and Association With Inflammatory Cytokine Signatures in Recovered Coronavirus Disease 2019 Patients

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**Background.** The complications and sequelae of coronavirus disease 2019 (COVID-19) and their effect on long-term health are unclear, and the trajectory of associated immune dysregulation is poorly understood.

**Methods.** We conducted a prospective longitudinal multicenter cohort study at 4 public hospitals in Singapore. Patients with COVID-19 were monitored for a median of 6 months after recovery from acute infection. Clinical symptoms and radiologic data were collected, along with plasma samples for quantification of immune mediators. The relationship between clinical symptoms and immune cytokine profiles was investigated.

**Results.** Two hundred eighty-eight participants were recruited, and follow-up data were available for 183, 175, and 120 participants at days 30, 90, and 180 postsymptom onset, respectively. Symptoms related to COVID-19 were present in 31 (16.9%), 13 (7.4%), and 14 (11.7%) at days 30, 90, and 180. In a multivariable model, age >65 years, non-Chinese ethnicity, and the severity of acute infection were associated with increased likelihood of persistent symptoms. Recovered COVID-19 patients had elevated levels of proinflammatory interleukin (IL)-17A, stem cell factor, IL-12p70, and IL-1 $\beta$  and pro-angiogenic macrophage inflammatory protein 1 $\beta$ , brain-derived neurotrophic factor, and vascular endothelial growth factor at day 180 compared with healthy controls. Higher levels of monocyte chemoattractant protein-1 and platelet-derived growth factor-BB were detected in patients with persistent symptoms, versus symptom-free patients.

**Conclusions.** Approximately 10% of recovered patients had persistent symptoms 6 months after initial infection. Immune cytokine signatures of the recovered patients reflected ongoing chronic inflammation and angiogenesis. Patients with COVID-19 should be monitored closely for emerging long-term health consequences.

**Keywords.** chronic fatigue; COVID-19; cytokines; long-term; persistent symptoms.

The collective understanding of coronavirus disease 2019 (COVID-19) has evolved rapidly since its emergence, with recognition of the causative virus, evaluation of effective therapeutics, and development of multiple vaccines within the span of 1 year. However, there remain significant unanswered questions with regard to the long-term sequelae associated with COVID-19. Persistent symptoms post-COVID-19 recovery, termed “long COVID,” have been reported, describing a clinical

phenotype resembling myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), with prominent features of myalgia and fatigue [1, 2]. However, there are limited data as to the frequency of such symptoms or the pathophysiologic mechanisms driving them [3]. Studies have reported dysregulated cytokine release in patients with acute COVID-19 associated with worse disease outcomes [4, 5]. However, the dynamics of cytokine response during recovery and its association with recovery trajectories and long-term outcomes have not been well studied.

Cohort studies tracking COVID-19 patients postrecovery described a high frequency (55% to 87.6%) of persistent symptoms (most commonly fatigue and dyspnea) for up to several months, with an associated decrease in quality of life [6–9]. However, some of these are limited by their single-center study design, small sample sizes, and an absence of correlation with objective biomarkers of disease activity or immune dysregulation to explain persistent symptoms.

In this prospective multicenter cohort study, we aimed to assess the frequency of persistent symptoms up to 6 months across

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a spectrum of confirmed COVID-19 patients with varying initial disease severity. We also studied the longitudinal temporal dynamics of cytokine response, and we determined the relationship of persistent symptoms with immune cytokine profiles during the recovery course.

## METHODS

### Patient Recruitment

This was a multicenter study conducted in 4 public hospitals in Singapore that managed COVID-19 inpatients. As part of enhanced surveillance in line with national public health policy, all patients with upper respiratory tract infection or pneumonia are tested for COVID-19 with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) polymerase chain reaction (PCR). In addition, several asymptomatic individuals were tested, namely, close contacts of confirmed cases and individuals living in congregate settings (namely, migrant worker dormitories where large outbreaks occurred). Hence, confirmed diagnoses included a large proportion of mild and asymptomatic cases. In the initial phase of the outbreak, all patients with confirmed COVID-19 infection were hospitalized for initial evaluation, regardless of illness severity.

Inclusion criterion was confirmed COVID-19 via SARS-CoV-2-specific PCR and admitted in the study period from January 30 to August 14 2020. There were no predefined exclusion criteria. Patients were invited to participate; however, not all patients could be approached due to resource limitations at the peak of the outbreak. Participants were offered outpatient follow-up postdischarge at 30, 90, 180, 270, and 360 days postsymptom onset (DPSO).

### Clinical Data and Specimen Collection

Clinical data were extracted from the electronic medical record using a standardized data collection form adapted from the International Severe Acute Respiratory and Emerging Infection Consortium (ISARIC) case record form [10]. Disease severity was categorized based on worst clinical status during hospital admission: mild (no pneumonia on chest radiographs [CXR] throughout admission), moderate (pneumonia on CXR without hypoxia), and severe (pneumonia with hypoxia [oxygen saturation  $\leq 94\%$ ] requiring supplemental oxygen). Severe was further subdivided into noncritical (not requiring intensive care unit [ICU] admission) and critical (requiring ICU admission or mechanical ventilation) for the clinical analysis.

Patients were followed up postdischarge at multiple time points (approximately 30, 90, and 180 DPSO). A standardized symptom questionnaire was administered at each outpatient visit, in addition to an objective assessment and physical examination by a study physician. Plasma samples were collected in cell preparation tubes during hospital admission on days 1, 3, 7, 14, 21, and 28 (if still admitted), and at each outpatient clinic visit on days 30, 90, and 180.

### Multiplex Microbead-Based Immunoassay

Plasma samples were extracted and treated with a solvent/detergent based on Triton X-100 (1%) for virus inactivation [11]. Immune mediator levels were measured using Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex Panel 1 (Thermo Fisher Scientific, Waltham, MA) (full details in [Supplementary Methods](#)). Cytokine levels were also measured in 24 healthy donor (10 males, 14 females; median age 55 years; details in [Supplementary Table S1](#)) plasma samples as baseline controls.

### Statistical Analysis

Unpaired *t*-test or Mann-Whitney *U* test was applied to ascertain significant differences in immune mediator levels between COVID-19 patients and healthy controls at different DPSO and between patients with and without persistent symptoms. A multiple linear regression analysis was conducted to examine the association between plasma cytokines and the presence of symptoms in COVID-19 patients after adjustment for age and disease severity. Two-way repeated measure analysis of variance with Tukey post hoc correction for multiple testing was used to discern the differences between various disease severity groups and timepoints. Plots were generated using GraphPad Prism, version 8 (GraphPad Software, San Diego, CA). ClustVis was used to compute hierarchical clustering and heat map on the immune mediators [12]. In the heat map presentation, the concentrations of immune mediators were scaled between 0 and 1 for visualization. Principal component analysis was performed on the logarithmically transformed concentrations using the singular value decomposition method in ClustVis.

The Mann-Whitney *U* test was used to compare continuous variables, and the Fisher's exact test was used for categorical variables. Variables that were significantly different between participants with and without persistent symptoms in univariate analysis were selected to construct a multivariable logistic regression model to identify variables independently associated with risk of developing persistent symptoms.  $P < .05$  were considered statistically significant, and all tests were 2-tailed. Analyses were performed using Stata Release 13 (StataCorp, College Station, TX).

### Patient Consent Statement

Written informed consent was obtained from all study participants. The study protocol was approved by the institutional review board (IRB) (National Healthcare Group Domain Specific Review Board, Study Reference 2012/00917). Healthy donor samples were collected under study numbers 2017/2806 and NUS IRB 04-140.

## RESULTS

### Patient Recruitment

Two hundred eighty-eight participants were recruited into the study and consented for outpatient follow-up and serial blood

sample collection (see [Supplementary Figure S1](#) for study recruitment flowchart). Median duration of follow-up in this cohort was 181 days (interquartile range [IQR], 103–191 days; range, 31–295 days). Follow-up visit data were available for 183, 175, and 120 participants at days 30, 90, and 180, respectively. Forty-five (24.6%) participants were female and median age was 44 years old (IQR, 33–56; range, 20–80). Seventy-five (41.0%) participants had at least 1 comorbidity, most commonly hypertension (28.4%) and diabetes mellitus (14.8%). Clinical features, presenting symptoms, and investigations during the index hospitalization are summarized in [Table 1](#). Patients had a range of disease severity during their index hospitalization: 30 (16.4%) asymptomatic, 51 (27.9%) mild, 47 (25.7%) moderate, and 55 (30.1%) severe as defined above.

### Persistent Symptoms and Association With Clinical Features

The number of patients with persistent symptoms at day 30, 90, and 180 were 31 (16.9%), 13 (7.4%), and 14 (11.7%), respectively. The detailed breakdown of individual symptoms is shown in [Supplementary Table S2](#). Symptoms were predominantly pulmonary, with the most common being cough and dyspnea. Participants with persistent symptoms at either day 90 or 180 were older, had a higher Charlson's comorbidity index, and were more likely to have required supplemental oxygen or ICU admission during their index hospitalization ([Table 1](#)). Ethnicity was significantly different between patients with and without persistent symptoms, although we did not capture detailed ethnicity data for those outside the 3 major categories used in Singapore.

**Table 1. Clinical Features and Outcomes During Index Hospitalization for Entire Cohort, and by Patients With and Without Persistent Symptoms at Day 90 or 180**

Variable	All Patients (n = 183)	No Persistent Symptoms (n = 161)	Persistent Symptoms at Day 90 or 180 (n = 22)	P Value <sup>a</sup>
<b>Demographics</b>				
Female sex	45 (24.6)	38 (23.6)	7 (31.8)	.43
Age, years	44 (33–56)	43 (31–55)	50.5 (39–66)	<b>.042</b>
Ethnicity				<b>.039</b>
Chinese	92 (50.3)	85 (52.8)	7 (31.8)	
Indian/South Asian	55 (30.1)	49 (30.4)	6 (27.3)	
Malay	14 (7.7)	10 (6.2)	4 (18.2)	
Others	22 (12.0)	17 (10.6)	5 (22.7)	
<b>Comorbidities</b>				
Charlson's comorbidity index	0 (0–1)	0 (0–0)	0 (0–1)	<b>.039</b>
Any comorbidity	75 (41.0)	63 (39.1)	12 (54.6)	.18
Hypertension	52 (28.4)	44 (37.3)	8 (36.4)	.45
Diabetes mellitus	27 (14.8)	22 (13.7)	5 (22.7)	.33
Ischemic heart disease	13 (7.1)	10 (6.2)	3 (13.6)	.19
<b>Baseline Symptoms</b>				
Duration of symptoms before hospitalization, days	3 (1–6)	2 (1–6)	4 (1–7)	.43
Fever	119 (65.8)	102 (64.2)	17 (77.3)	.34
Cough	106 (58.6)	90 (56.6)	16 (72.7)	.17
Sputum production	38 (21.0)	32 (20.1)	6 (27.3)	.41
Dyspnea	22 (12.2)	18 (11.3)	4 (18.2)	.32
Rhinorrhea	46 (25.4)	41 (25.8)	5 (22.7)	>.99
Sore throat	64 (35.4)	58 (36.5)	6 (27.3)	.48
Diarrhea	28 (15.5)	20 (12.6)	8 (36.4)	<b>.009</b>
Myalgia	32 (17.7)	27 (17.0)	5 (22.7)	.55
Asymptomatic	30 (16.4)	27 (16.8)	3 (13.6)	>.99
<b>Clinical Outcomes</b>				
CXR opacities	101 (55.2)	85 (52.8)	16 (72.7)	.11
Supplemental oxygen	54 (29.5)	42 (26.1)	12 (54.6)	<b>.011</b>
ICU admission	33 (18.0)	25 (15.5)	8 (36.4)	<b>.033</b>
Mechanical ventilation	11 (6.0)	8 (5.0)	3 (13.6)	.13
<b>Severity</b>				
Mild <sup>b</sup>	81 (44.3)	75 (46.6)	6 (27.3)	
Moderate	47 (25.7)	43 (26.7)	4 (18.2)	
Severe	55 (30.1)	43 (26.7)	12 (54.6)	

Abbreviations: CXR, chest radiograph; ICU, intensive care unit.

NOTE: Values reported as number (percentage) for categorical variables and median (interquartile range) for continuous variables. Bold text indicates *P* values < .05.

<sup>a</sup>Comparing patients without persistent symptoms against patients with persistent symptoms at day 90 or day 180.

<sup>b</sup>Including asymptomatic patients.

In the multivariable model imputing age, sex, ethnicity, Charlson's comorbidity index, and disease severity; age group >65 years old, non-Chinese ethnicity, and critical illness severity (defined as ICU admission or requiring mechanical ventilation) during the index admission were independently associated with increased odds of persistent symptoms (Table 2).

Follow-up CXRs were reviewed for all patients with an initial abnormal CXR, and the latest abnormal CXR date was recorded in relation to the symptom onset date. Median time to resolution of CXR opacities was 14 days (IQR, 9–32; range, 1–195). However, because CXRs were not systematically repeated in a protocolized manner, there was a wide range of durations between follow-up CXRs. Hence, we did not further analyze this.

#### Longitudinal Cytokine Responses in Recovered Coronavirus Disease 2019 Patients

There was a prominent increase in levels of inflammation-associated markers interleukin (IL)-6, IL-8, interferon (IFN)- $\alpha$ , IL-18, IL-1RA, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1 $\alpha$  at the acute phase of disease, which subsided significantly over the 30-day or 90-day period (Figure 1A). Compared with healthy controls, at day 90, recovered patients across all severity strata had higher levels of growth factors platelet-derived growth factor (PDGF-BB), brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF)-A, and VEGF-D, which are associated with endothelial activation. Similar trends were observed for proinflammatory mediators MIP-1 $\beta$ , stromal cell-derived factor (SDF)-1 $\alpha$ , eotaxin, IL-12p70, and IL-17A (Supplementary Figure S2). At day 180, all recovered patients, regardless of severity of initial illness, had systemic cytokine profiles distinct from healthy controls (Figure 1B), with higher

levels of multiple growth factors, cytokines, and chemokines. Recovered patients who had severe illness demonstrated higher levels of hepatocyte growth factor (HGF) compared with healthy controls (Figure 1C, Supplementary Table S3). Overall, the 180-day profile of recovered COVID-19 patients revealed signs of ongoing chronic inflammation (high levels of IL-17A, IL-12p70, stem cell factor [SCF], and IL-1 $\beta$ ) and endothelial repair and angiogenesis (high levels of BDNF, MIP-1 $\beta$ , and VEGF) (Figure 1C, Supplementary Table S3). It is notable that there were no significant differences in the levels of these immune mediators between patients with different disease severity.

The temporal changes of systemic cytokine profiles were further profiled by longitudinal analysis of plasma immune mediators from the acute phase to day 180 in 64 patients who provided blood samples across all 4 time points, stratified by initial disease severity. The levels of inflammation-associated IL-6, IP-10, IL-18, and MCP-1 significantly decreased at the second timepoint (14–75 DPSO) in patients across all severity strata (Supplementary Figure S3). Among immune mediators that remained high at day 180, levels of BDNF, MIP-1 $\beta$ , VEGF-A, and IL-12p70 were already increased in the early phase of infection, which persisted over time (Figure 2). It is notable that persistent elevation of HGF was only observed in patients with initial severe infection. Levels of VEGF-D, IL-17A, and SCF increased slowly during convalescent phase and remained high at day 180 (Figure 2).

#### Comparison of Cytokine Responses Between Patients With and Without Persistent Symptoms

We next examined the relationship between systemic cytokine profiles and persistent symptoms. At day 30, there was a significantly higher level of MCP-1 in patients with persistent

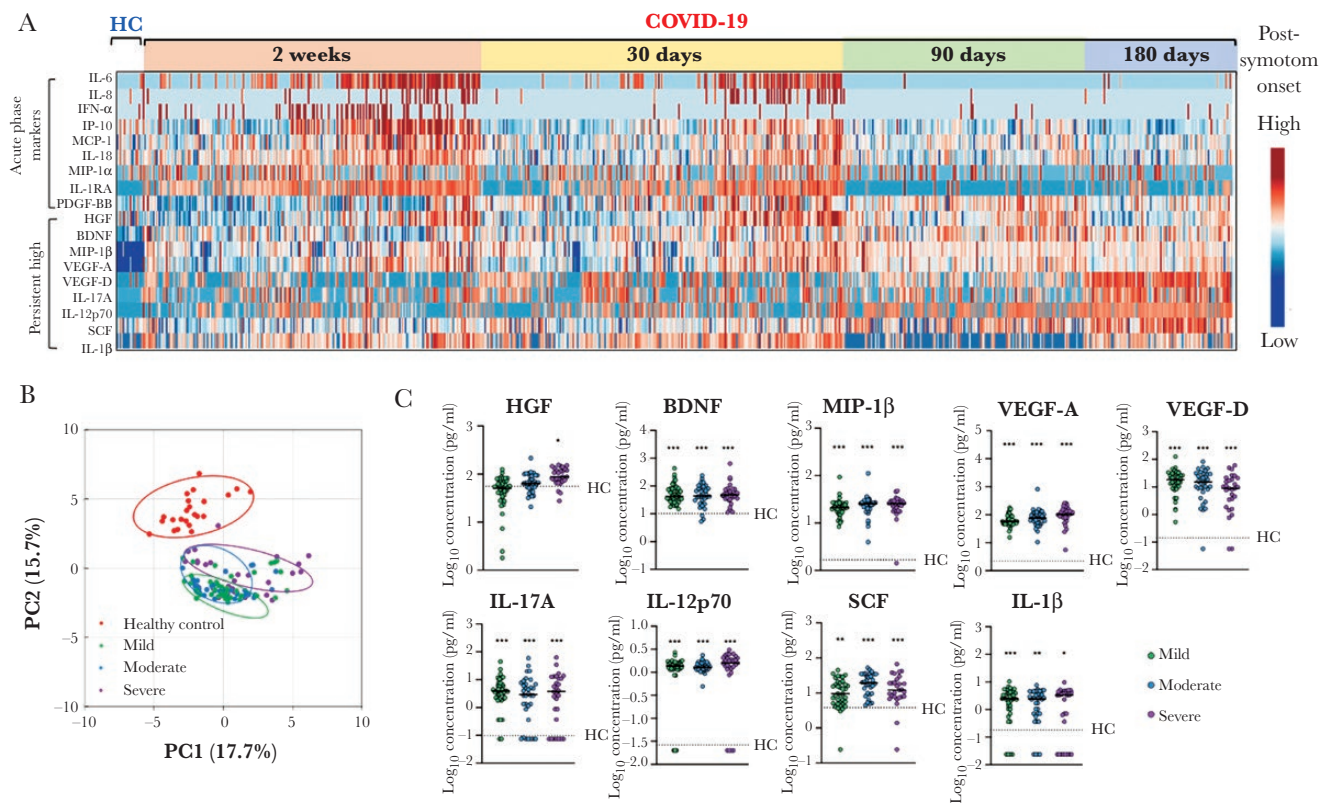
**Table 2. Logistic Regression Analysis of Variables Associated With Persistent Symptoms at 90 or 180 days**

Variable	Univariable Model		Multivariable Model	
	Odds Ratio (95% CI)	P Value	Adjusted Odds Ratio (95% CI)	P Value
<b>Age Group</b>				
<45 years old	Ref	N.A.	Ref	N.A.
45–65 years old	1.25 (.44–3.51)	.67	1.66 (.49–5.60)	.41
>65 years old	6.67 (1.89–23.57)	<b>.003</b>	8.75 (1.68–48.34)	<b>.013</b>
Female sex	1.52 (.57–4.01)	.40	3.05 (.88–10.56)	.078
<b>Ethnicity</b>				
Chinese	Ref	N.A.	Ref	N.A.
Indian/South Asian	1.62 (.51–5.11)	.41	5.49 (1.15–26.14)	<b>.033</b>
Malay	4.74 (1.18–19.09)	<b>.028</b>	5.23 (.94–29.22)	.059
Others	3.71 (1.04–13.15)	<b>.043</b>	17.14 (2.99–98.16)	<b>.001</b>
Charlson's comorbidity index $\geq$ 1	2.25 (.89–5.69)	.087	1.48 (.46–4.76)	.51
<b>Severity</b>				
Mild	Ref	N.A.	Ref	N.A.
Moderate	1.05 (.28–3.95)	.94	1.23 (.28–5.32)	.78
Severe	3.24 (1.13–9.28)	<b>.029</b>	3.10 (.91–10.62)	<b>.072<sup>a</sup></b>

Abbreviations: CI, confidence interval; N.A., not applicable; Ref, reference variable.

NOTE: Patients who only had data up to 30 days were excluded from logistic regression analysis. Bold text indicates P values < .05.

<sup>a</sup>Further stratification into noncritical severe (not requiring intensive care unit [ICU] admission) and critical (requiring ICU admission or mechanical ventilation) showed critical severity was independently associated with persistent symptoms in the multivariable model (adjusted OR, 4.23; 95% CI, 1.02–17.56; P = .047).



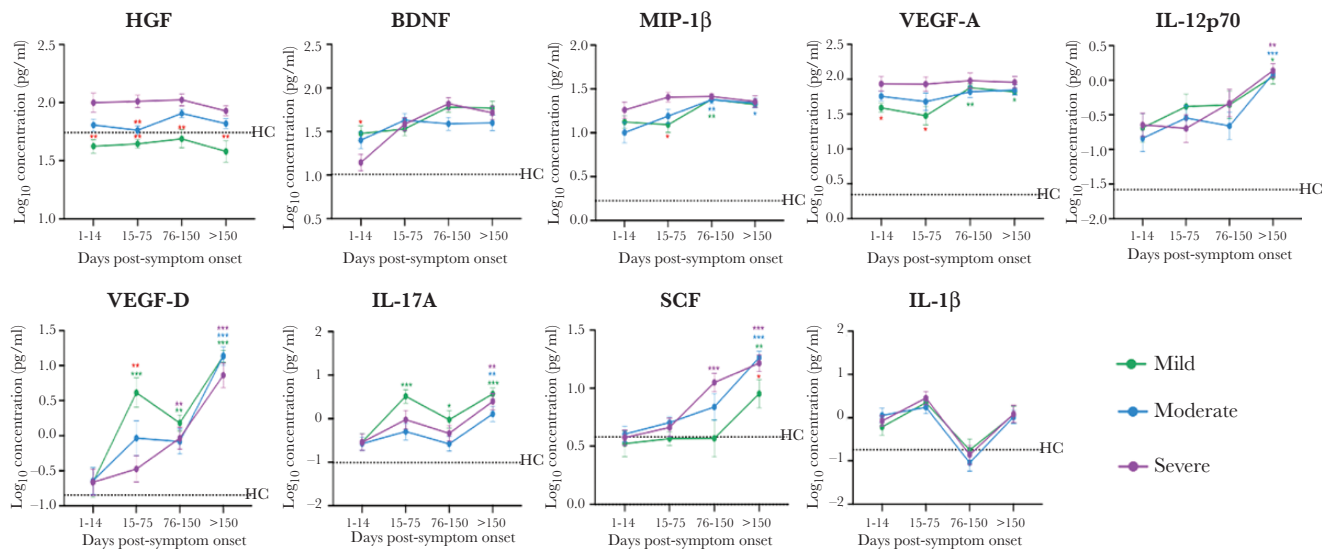
**Figure 1.** Plasma immune mediator levels of coronavirus disease (COVID-19) patients at 90 and 180 days postsymptom onset. Concentrations of 45 immune mediators were quantified using a 45-plex microbead-based immunoassay. (A) Heatmap of immune mediator levels in plasma samples of COVID-19 patients at first 2 weeks, 30 days, 90 days, and 180 days postsymptom onset. Each color represents the relative concentration of a particular analyte. Blue and red indicates low and high concentration, respectively. (B) Principal component analysis of 45 immune mediator levels analyzed in 101 COVID-19 patients (mild,  $n = 38$ ; moderate,  $n = 34$ ; severe,  $n = 29$ ) at 180 days postsymptom onset and healthy controls (HC) ( $n = 24$ ). PC1 explains 17.7% of the variation, whereas PC2 explains 15.7% of the variation; color denotes different groups of patients and healthy donors. (C) Profiles of immune mediators that are significantly higher in COVID-19 patients at 180 days postsymptom onset compared with HCs are illustrated as scatter plots. Immune mediator levels in plasma fraction samples of COVID-19 patients collected during 180-day follow up (median 186 days postillness onset) were compared with the levels in HCs. Unpaired  $t$  test was performed on the logarithmically transformed concentration ( $*P < .05$ ;  $**P < .01$ ;  $***P < .0001$ ). Immune mediator levels for HCs are indicated by the black dotted line. Patient samples with concentration out of measurement range are presented as the value of logarithm transformation of limit of quantification. BDNF, brain-derived neurotrophic factor; HGF, hepatocyte growth factor; IL, interleukin; MIP, macrophage inflammatory protein; SCF, stem cell factor; VEGF, vascular endothelial growth factor.

symptoms versus symptom-free patients (Figure 3A). Patients with persistent symptoms also had high levels of PDGF-BB at 90 and 180 days (Figure 3A). Both PDGF-BB and MCP-1 may be associated with respiratory manifestations, because patients with persistent respiratory symptoms showed higher levels of MCP-1 and PDGF-BB, particularly at days 90 and 180 (Supplementary Figure S4). After adjustment for age, ethnicity, and disease severity by multiple linear regression analysis, levels of MCP-1 and PDGF-BB were significantly associated with persistent symptoms (Supplementary Table S4) or respiratory symptoms (Supplementary Table S5) at day 30 and day 90, respectively. Longitudinal profiling of MCP-1 and PDGF-BB in patients with persistent symptoms revealed upregulation of MCP-1 at the early stage of disease (first 2 weeks of infection) with gradual increase of PDGF-BB at the convalescent phase (90 DPSO) (Figure 3B). Other cytokines evaluated were not significantly different between patients with and without persistent

symptoms (Supplementary Table S6). Overall, persistent symptoms in recovered COVID-19 patients might be associated with elevated levels of MCP-1 in the early convalescent phase and PDGF-BB at 30-day assessment or later.

## DISCUSSION

In this multicenter cohort study, COVID-19 was associated with a significant but overall low frequency of persistent symptoms after resolution of the acute illness: 7.4% and 11.7% at 90 and 180 days, respectively. This is lower compared with other similar cohort studies in Italy, France, the United Kingdom, and China, which reported persistent symptoms in 55% to 87.6% of patients [6–9]. Despite the proportion of severe initial infections being similar in our cohort compared with these cohorts (18% admitted to ICU compared with 12.6%, 20%, 32%, and 4%, respectively) [6–9], we showed that cytokine expression



1-14 DPSO versus 15-75 DPSO or 76-150 DPSO or >150 DPSO in mild patients;

1-14 DPSO versus 15-75 DPSO or 76-150 DPSO or >150 DPSO in moderate patients;

1-14 DPSO versus 15-75 DPSO or 76-150 DPSO or >150 DPSO in severe patients;

\* mild or moderate patients versus the severe patients

**Figure 2.** Dynamic changes of plasma immune mediator levels in coronavirus disease (COVID-19) patients across 4 time periods, including 1 to 14 days (2 weeks), 15 to 75 days, 76 to 150 days, and  $\geq 150$  days postsymptom onset. Longitudinal plasma samples were collected across 4 timepoints up to 180 days postsymptom onset from 64 COVID-19 patients with different disease severity outcomes (mild,  $n = 18$ ; moderate,  $n = 23$ ; severe,  $n = 23$ ). Concentrations of 45 immune mediators were quantified using a 45-plex microbead-based immunoassay. The levels of immune mediators that persist high in COVID-19 patients at 180 days postsymptom onset at various timepoints were shown as mean  $\pm$  standard error of the mean. Two-way repeated measure analysis of variance with Tukey post hoc correction for multiple testing was used to discern the differences between various disease severity groups and timepoints ( $*P < .05$ ;  $**P < .01$ ;  $***P < .0001$ ). Mean levels of immune mediators for healthy controls are indicated by the black dotted line. Patient samples with concentration out of measurement range are presented as the value of logarithm transformation of limit of quantification. BDNF, brain-derived neurotrophic factor; HGF, hepatocyte growth factor; IL, interleukin; MIP, macrophage inflammatory protein; SCF, stem cell factor; VEGF, vascular endothelial growth factor.

for proinflammatory cytokines was only marginally different in follow-up samples for those with initial severe disease compared with asymptomatic and mildly infected individuals, suggesting that other reasons are important for the development of persistent symptoms.

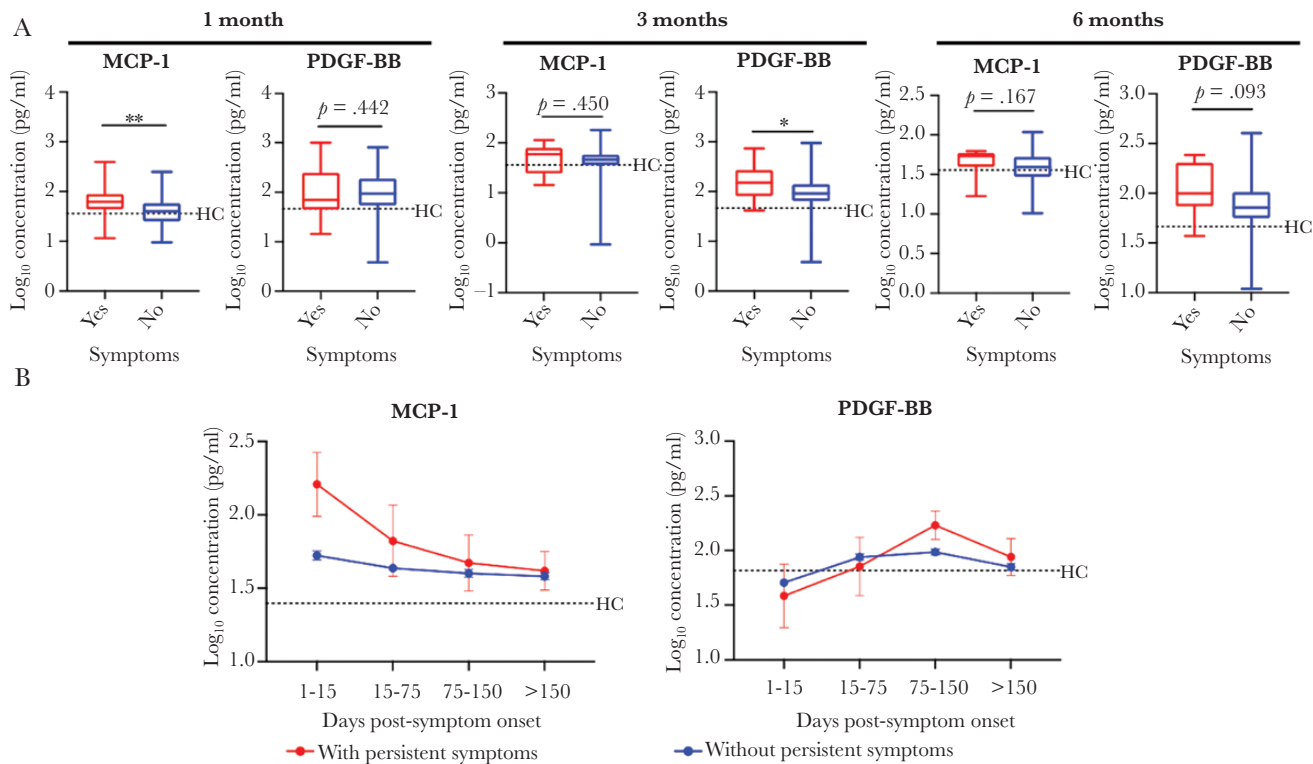
For example, differences in demographics and ethnicity may explain this discordance in the frequency of persistent symptoms. Epidemiologic studies of ME/CFS demonstrated differences in incidence rates across ethnic groups [13], which may be explained by genetic differences because specific human leukocyte antigen alleles were associated with ME/CFS [14]. Indeed, in our cohort, there were significant differences in the frequency of persistent symptoms across ethnic groups (with higher proportions in the “Others” category, which included those of European descent), which merits further investigation. Although Huang et al [9] found a high proportion (76%) of persistent symptoms in a Chinese cohort, they had a much higher proportion of patients with severe disease (75% requiring supplemental oxygen), which may explain the difference between our cohorts.

Furthermore, we had a higher proportion of patients (16.4%) with asymptomatic index infection. This may result in an underestimation of the frequency of persistent symptoms at follow-up for patients with symptomatic index infection.

Nevertheless, the finding that 3 of these asymptomatic patients went on to develop persistent symptoms later on, coupled with cytokine analysis showing abnormal cytokine profiles even in asymptomatic patients, is novel and stresses the importance of disregarding patients with asymptomatic index infection.

We found that COVID-19 patients had distinct systemic cytokine profiles at up to 6 months postsymptom onset compared with healthy controls, regardless of initial severity of illness or persistent symptoms. Levels of proinflammatory T cell-associated cytokines such as IL-17A, IL-12p70, IL-1 $\beta$ , and SCF were elevated and increased postdischarge. Interleukin-12p70, SCF, and IL-17A have been found to be associated with chronic inflammatory diseases such as ME/CFS and inflammatory spondyloarthritis [15, 16]. Liu et al [17] found that convalescent individuals still experienced enhanced CD4 and CD8 T-cell activation and proliferation, along with a contraction of NKT-like cells 3 months after resolving SARS-CoV-2 infection, whereas Bergamaschi et al [18] observed that persistent inflammation correlates with activation of cell metabolic pathways. This sustained immune and metabolic dysregulation may be a driver of chronic inflammation in recovered COVID-19 patients.

Our longitudinal analyses of cytokine responses in recovered patients revealed subclinical changes potentially underpinning



**Figure 3.** Plasma immune mediator levels in coronavirus disease (COVID-19) patients with symptoms at 1 month, 3 months, and 6 months postsymptom onset. (A) Profiles of immune mediators that are significantly higher in COVID-19 patients with symptoms at 30 days ( $n = 28$ ), 90 days ( $n = 12$ ), and 180 days ( $n = 8$ ) postsymptom onset are illustrated as box plots. Immune mediator levels in plasma fraction samples of COVID-19 patients with respiratory symptoms collected during 30, 90, and 180-day follow-up were compared with the levels in those without respiratory symptoms (30 days,  $n = 72$ ; 90 days,  $n = 155$ ; 180 days,  $n = 87$ ). Mann-Whitney  $U$  test was performed on the logarithmically transformed concentration ( $*P < .05$ ;  $**P < .01$ ). Median levels of immune mediator in healthy controls (HC) are indicated by the black dotted line. Patient samples with concentration out of measurement range are presented as the value of logarithm transformation of limit of quantification. (B) Dynamic changes of plasma monocyte chemoattractant protein (MCP)-1 and platelet-derived growth factor (PDGF)-BB in COVID-19 patients with ( $n = 4$ ) and without persistent symptoms ( $n = 77$ ) across 4 time periods, including 1 to 14 days, 15 to 75 days, 16 to 150 days, and >150 days postsymptom onset.

the development of post-COVID-19 cardiovascular complications even in those who did not have persistent symptoms. Regulators of endothelial activation such as VEGF, BDNF, and MIP-1 $\beta$  could promote atherosclerosis and potentially result in silent coronary artery disease [19–22]. Convalescent patients were found to have pronounced endothelial activation hallmarks, abnormal cardiovascular imaging findings, and ongoing myocardial inflammation after COVID-19 recovery [23, 24]. Our findings of persistent elevation of pro-angiogenic factors warrants long-term monitoring and assessment of potential cardiovascular effects.

Platelet-derived growth factor-BB, a potent chemotactic growth factor that stimulates the growth and migration of lung fibroblasts, has been implicated in the pathogenesis of pulmonary fibrosis [25], whereas elevated levels of MCP-1 in patients with chronic obstructive pulmonary disease play a role in inducing emphysema [26]. Increased levels of PDGF-BB and MCP-1 in patients with persistent respiratory symptoms suggest a mechanism for chronic lung disease, and therapeutic strategies targeting these immune mediators could be beneficial

to ameliorate the progression of pulmonary symptoms in recovered COVID-19 patients [27, 28].

There are several limitations to our study. First, although we correlated the presence of persistent symptoms with cytokine features, we did not systematically evaluate end-organ involvement using functional assessments such as lung function tests, exercise tolerance scales, or detailed imaging. Second, symptom questionnaires were qualitative, and we did not utilize quantitative indices to measure impact on quality-of-life or functional status; thus, there may be intersubject variability and subjectivity. Third, we did not have a control group to compare the frequency of persistent symptoms following other respiratory viruses to determine whether COVID-19 is associated with an increased risk of persistent symptoms. We also cannot be certain whether reported symptoms are due to COVID-19 or other conditions. Finally, a significant proportion of patients were lost to follow-up, which may introduce an element of selection bias. However, it is likely that those who defaulted further follow-up were more likely to not have persistent symptoms, because those with persistent symptoms would be expected to have continued

seeking medical care, and as such selection bias is more likely to have overestimated the frequency of persistent symptoms.

Differences among study cohorts make comparison of persistent COVID-19 symptoms difficult, and a consensus case definition should be developed to standardize diagnosis and data collection to better understand this condition. The relatively nonspecific and subjective nature of persistent symptoms makes the clinical picture harder to define. Establishing a causal link between COVID-19 and persistent symptoms remains challenging in the absence of a control group of non-COVID-19 patients. The presence of persistent symptoms posthospitalization has been described in many conditions, including community-acquired pneumonia [29], severe acute respiratory syndrome (SARS) [30, 31], Middle East respiratory syndrome [32], and a general “post-ICU syndrome” [33, 34].

Nevertheless, the persistent symptoms of COVID-19 should not be overlooked, given (1) the significant impact on quality-of-life and functional status and (2) a potentially huge impact on costs and resource utilization. It is clear that there are long-lasting immune changes triggered by COVID-19 infection, and although we could not establish a strong association between these immune signatures and persistent symptoms in our study, it is an intriguing signal that requires further study.

Further questions remain regarding persistent COVID-19. Although we have demonstrated differences in the immune signatures of recovered COVID-19 patients, the mechanism behind this remains unclear. Prolonged viral shedding of SARS-CoV-2 nucleic acid has been observed from the respiratory tract, although multiple studies have shown that these are largely nonviable genetic material [4, 35]. A postmortem pathologic study showed evidence of persistent viral infection of pneumocytes and endothelial cells in patients who died of severe COVID-19, even later in the illness up to 30–40 days postinfection [36]. The persistently elevated levels of cytokines in our cohort involved in endothelial repair and angiogenesis (MIP-1 $\beta$ , BDNF, and VEGF-A) are consistent with a hypothesis of active endothelial inflammation even in the absence of persistent symptoms. Viral persistence in sanctuary sites could elicit an ongoing immune response, providing a pathophysiologic basis for endothelial dysfunction and some of the symptoms observed in persistent COVID-19.

## CONCLUSIONS

In conclusion, we have shown that there remain durable changes in immune signatures in recovered COVID-19 patients, and this is seen across the entire spectrum of disease severity at index presentation. Some of these dysregulated immune changes may underlie the pathophysiologic mechanisms of persistent COVID-19 symptoms in a subset of recovered patients. Further large multicenter and international cohort studies are needed to better characterize the clinical

features, risk factors, and pathophysiology behind persistent COVID-19.

## Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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**Author contributions.** S. W. X. O. and S.-W. F. have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. B. E. Y., Y.-S. L., and D. C. L. designed the study protocol. S. W. X. O., B. E. Y., P. T., S. P., S. Y. T., and Y. D. collected the data. S. W. F., Y.-H. C., B. L., S. N. A., R. S.-L. C., N. K.-W. Y., L. R., and L. F. P. N. conducted the laboratory investigations. S. W. X. O. and S.-W. F. conducted the data analysis and drafted the manuscript. L. R., Y.-S. L., L. F. P. N., and D. C. L. provided overall supervision. All authors read and approved the final manuscript.

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