Circulating anti-nuclear autoantibodies in COVID-19 survivors predict long COVID symptoms

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Circulating antinuclear autoantibodies were detected in COVID-19 patients up to 12 months post-recovery. The presence of these autoantibodies was associated with persisting symptoms and residual inflammation. https://bit.ly/3AduU02

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Abstract

Background Autoimmunity has been reported in patients with severe coronavirus disease 2019 (COVID-19). We investigated whether anti-nuclear/extractable-nuclear antibodies (ANAs/ENAs) were present up to a year after infection, and if they were associated with the development of clinically relevant post-acute sequalae of COVID-19 (PASC) symptoms.

Methods A rapid-assessment line immunoassay was used to measure circulating levels of ANAs/ENAs in 106 convalescent COVID-19 patients with varying acute phase severities at 3, 6 and 12 months post-recovery. Patient-reported fatigue, cough and dyspnoea were recorded at each time point. Multivariable logistic regression model and receiver operating curves were used to test the association of autoantibodies with patient-reported outcomes and pro-inflammatory cytokines.

Results Compared to age- and sex-matched healthy controls (n=22) and those who had other respiratory infections (n=34), patients with COVID-19 had higher detectable ANAs at 3 months post-recovery ($p<0.001$). The mean number of ANA autoreactivities per individual decreased between 3 and 12 months (from 3.99 to 1.55) with persistent positive titres associated with fatigue, dyspnoea and cough severity. Antibodies to U1-snRNP and anti-SS-B/La were both positively associated with persistent symptoms of fatigue ($p<0.028$, area under the curve (AUC) 0.86) and dyspnoea ($p<0.003$, AUC=0.81). Pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α and C-reactive protein predicted the elevated ANAs at 12 months. TNF-α, D-dimer and interleukin-1β had the strongest association with symptoms at 12 months. Regression analysis showed that TNF-α predicted fatigue ($\beta=4.65$, $p=0.004$) and general symptomaticity ($\beta=2.40$, $p=0.03$) at 12 months.

Interpretation Persistently positive ANAs at 12 months post-COVID are associated with persisting symptoms and inflammation (TNF-α) in a subset of COVID-19 survivors. This finding indicates the need for further investigation into the role of autoimmunity in PASC.

Introduction

The majority of patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus recover; however, a significant subset report persistent symptoms (*e.g.* fatigue, dyspnoea and

cognitive impairment) that do not resolve after infection [1]. This constellation of symptoms is called long COVID or post-acute sequelae of COVID-19 (PASC) and has been observed in 10–20% of convalescent patient cohorts [2]. SARS-CoV-2 infections are associated with the development of autoantibodies during the acute phase of disease, and these contribute to coronavirus disease 2019 (COVID-19) pathology [3–6]. Emerging evidence suggests that the failure to resolve these autoantibodies, or generating *de novo* pathogenic autoimmune responses post-recovery contributes to PASC with evidence of residual inflammatory cytokines [7–9]. Although it is not known if these autoantibodies are harbingers of emerging autoimmune disease, there have been many case reports of development of autoimmunity post-COVID with no prior personal or family history of autoimmunity [10]. To date, diverse autoantibody signatures including anti-nuclear/extractable-nuclear autoantibodies (ANAs/ENAs) have been reported in PASC patients as biomarkers, but there are no identified links with specific PASC symptoms [7, 8, 11, 12].

In our noninterventional, observational, longitudinal study, we utilised an extensive, clinically relevant ANA/ENA panel to serve as common rheumatological biomarkers of post-COVID trajectory of developing/sustaining PASC symptoms. We investigated circulating levels of ANAs in COVID-19 survivors with varying acute phase severities longitudinally, at 3, 6 and 12 months post-recovery. We further examined the temporal association between ANAs with COVID-19 pathology-associated inflammatory and vascular factors (*e.g.* tumor necrosis factor (TNF)-α, D-dimer), as well as commonly reported PASC symptoms of fatigue, cough and dyspnoea [1].

Methods

Study design and patient selection

This was a multicentre, multi-time-point observational study approved by the Hamilton integrated research ethics board (#11471, 13181) and the University of British Columbia clinical research ethics board (#H20-01239). Between August 2020 and September 2021, we enrolled 106 COVID-19 patients from St Joseph's Healthcare Hamilton (n=44; Hamilton, ON, Canada), Vancouver General Hospital (n=42; Vancouver, BC, Canada), and St Paul's Hospital (n=20; Vancouver, BC, Canada) for three study visits at 3, 6 and 12 months, post-recovery (deemed recovered as per public health guidelines). Consenting patients aged ⩾18 years, with a positive PCR test for SARS-CoV-2 and no previous diagnosis of autoimmune disease were recruited *via* community self-referrals, physician referrals and hospital inpatient/outpatient post-discharge follow-ups. In order to compare whether autoantibodies differed between individuals who had COVID-19 compared to other respiratory infections, we enrolled 34 individuals with respiratory symptoms consistent with COVID-19 but did not have a test or did not become seropositive between 1 and 3 months post-infection [13]. 22 age- and sex-matched non-COVID, nonvaccinated healthy adults were recruited locally from Hamilton (ON, Canada) (figure 1). Criteria for recruiting healthy volunteers during the pandemic included never having had COVID-19, not yet vaccinated for COVID-19, never-smokers, with no history of respiratory/rheumatological disease. Serum was collected at each time point and stored at −80°C within 1 h of collection for fluid phase analysis.

Symptom assessment

In addition to patient demographics, the following symptoms were recorded by study coordinators *via* analogous research protocols at all recruitment sites for each time point post-COVID recovery: fatigue (patient-reported or fatigue assessment scale), cough (patient-reported) and shortness of breath (modified Medical Research Council dyspnoea scale).

Microarray autoantibody profiling

Serum IgG and IgM antibody reactivities against 102 autoantigens were analysed using a microfluidic antigen array developed at the Microarray and Immune Phenotyping Core Facility at the University of Texas Southwestern Medical Center, as described previously [14] for the 3-month time point for participants who consented to third-party off-site exploratory analysis. Serum samples from 22 healthy controls with no previous history of autoimmune disease were used to determine the cut-off threshold for IgG and IgM autoantibody reactivity, calculated *via* median plus three standard deviations, to each of the 102 common self/autoantigens on a microarray panel (supplementary figure E1). We utilised these cut-off thresholds to determine the number of autoreactive antibodies in serum of 36 convalescent post-COVID patients at 3 months post-recovery.

Detection and quantification of ANAs/ENAs in serum

An ANA/ENA line immunoassay (IMTEC-ANA-LIA-MAXX; Human Diagnostics, Germany) targeting 18 common self-antigens was used, as described previously [15] at disease-modifying titres of 1:100. Each strip was scanned (ChemiDoc MP Imaging System; Bio-Rad, CA, USA), and images were converted into eight-bit greyscale and inverted with ImageJ analysis software. A quantitative value was derived for each

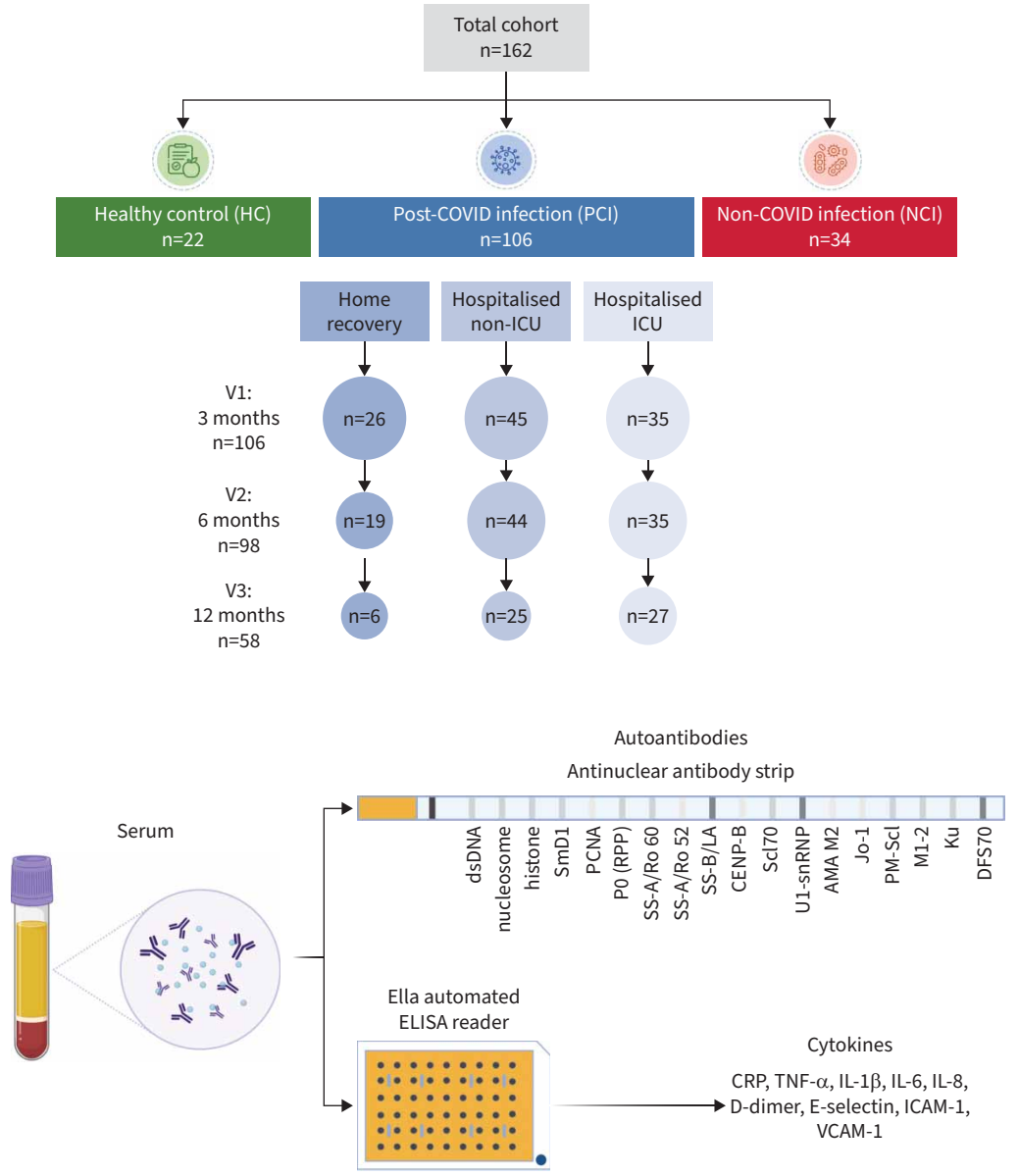

FIGURE 1 Study Consolidated Standards of Reporting Trials diagram. A total of 22 healthy controls, 34 non-coronavirus disease 2019 (COVID-19) infection controls, and 106 post-COVID-19 infection patients were enrolled in this multicentre, prospective, longitudinal study. The post-COVID-19 infection cohort were additionally stratified based on severity of their acute phase infection: recovered at home (n=26), hospitalised non-intensive care unit (ICU) (n=45) and ICU-admitted (n=35). Serum samples and symptoms were collected at 3, 6 and 12 months post-recovery for COVID-19 survivors. CRP: C-reactive protein; TNF: tumour necrosis factor; IL: interleukin; ICAM: intercellular adhesion molecule; VCAM: vascular cell adhesion molecule.

visible band and normalised to the cut-off control band to provide a mean quantitative value (MQV) with values ⩾1.0 indicating positive reactivity. Validation of our quantification method was assessed using indirect immunofluorescence of HEp-2 cells (supplementary figure E2).

Serum molecular mediator analysis

Acute markers of inflammation (interleukin (IL)-1β, IL-6, IL-8, TNF-α) [16], coagulation mediators (D-dimer, E-selectin, intercellular adhesion molecule (ICAM)-1), vascular cell adhesion protein (VCAM)-1) and C-reactive protein (CRP) were assessed and quantitated using the Ella Automated

Immunoassay System (Bio-Techne, MN, USA). Serum samples were diluted as per manufacturer's protocol for each mediator reported [13].

Statistical analysis

All experimental data were analysed and plotted using GraphPad Prism (version 9; La Jolla, CA, USA). After testing for normality, statistical comparisons between groups were performed by Mann–Whitney t-test (nonparametric unpaired analysis, two groups), Kruskal–Wallis test (nonparametric unpaired analysis, more than two groups) and Friedman test (nonparametric paired analysis with repeated measures, more than two groups); associations were determined by Spearman's rank correlation test, and categorical variables analysed using Chi-squared analysis. Receiver operating curves, multiple, and simple logistic regression using the "stats" package in the R software generated models to determine which autoantibodies or cytokines significantly predicted symptoms. p-values <0.05 were considered significant unless stated otherwise. The "pheatmap" package was used to produce the heatmaps of the estimated coefficients at the three time points.

Results

Study population

We recruited 106 convalescent COVID-19 patients (61 males, 45 females) with a mean age of 57 years and body mass index (BMI) of 27.2 $kg \cdot m^{-2}$ (table 1). 26 patients recovered from COVID-19 at home, 35 were admitted to the intensive care unit (ICU) and 45 were hospitalised, but not admitted to the ICU. 34 patients (10 male, 24 female) with a non-COVID-19 infection were recruited for comparison with a mean age of 46 years and BMI of 22.2 $kg \cdot m^{-2}$. Of these patients, 34 recovered at home, and one was admitted to the ICU. 22 healthy volunteers (11 male, 11 female) with a mean age 49 years and BMI 26.6 $kg \cdot m^{-2}$, were enrolled as a control population.

IgG/IgM autoantibodies in patients 3 months post-COVID

To determine whether circulating autoantibodies were higher in convalescent COVID-19 patients compared to uninfected controls we used an autoantigen microarray [17] that detects both IgG and IgM autoantibodies in 36 convalescent COVID-19 at the 3-month time point post-recovery and compared it to the 22 healthy donors. Heatmaps showing detected IgG and IgM autoreactivities are given in supplementary figure E1. While most of the healthy controls did not have IgG autoantibodies (20 out of 22, 91%), approximately one-third of the convalescent COVID-19 group had at least one autoreactive IgG (13 out of 36, 36%; p=0.03). Two or more autoantigens were found in 33% of COVID-19 convalescent patients (12 out of 36, 33%; p=0.002) (figure 2a). In contrast, the majority of healthy controls (21 out of

TABLE 1 Demographics of subjects in the final study cohort

	Subjects	Post-COVID-19 infection	Non-COVID-19 infection	Healthy controls
Subjects		106	34	22
Female		45 (42)	24 (71)[#]	11 (50)
Age, years		57 (20–89)	46 (20–67)[#]	49 (32–75)
BMI, $kg \cdot m^{-2}$		27.2±6.0	22.2±12	26.6±4.0
Home recovery		26	33	
Hospitalised, non-ICU		45	0	
Hospitalised, ICU		35	1	
Symptoms at 3 months				
Fatigue	89	32 (36)		0 (0)
Cough	89	19 (21)		0 (0)
Dyspnoea	98	25 (26)		0 (0)
Symptoms at 6 months				
Fatigue	47	19 (40)		
Cough	88	20 (23)		
Dyspnoea	85	24 (28)		
Symptoms at 12 months				
Fatigue	50	10 (20)		
Cough	50	11 (22)		
Dyspnoea	50	12 (24)		

Data are presented as n, n (%), mean (range) or mean±SD. COVID-19: coronavirus disease 2019; BMI: body mass index; ICU: intensive care unit. [#]: indicates group with significant variation.

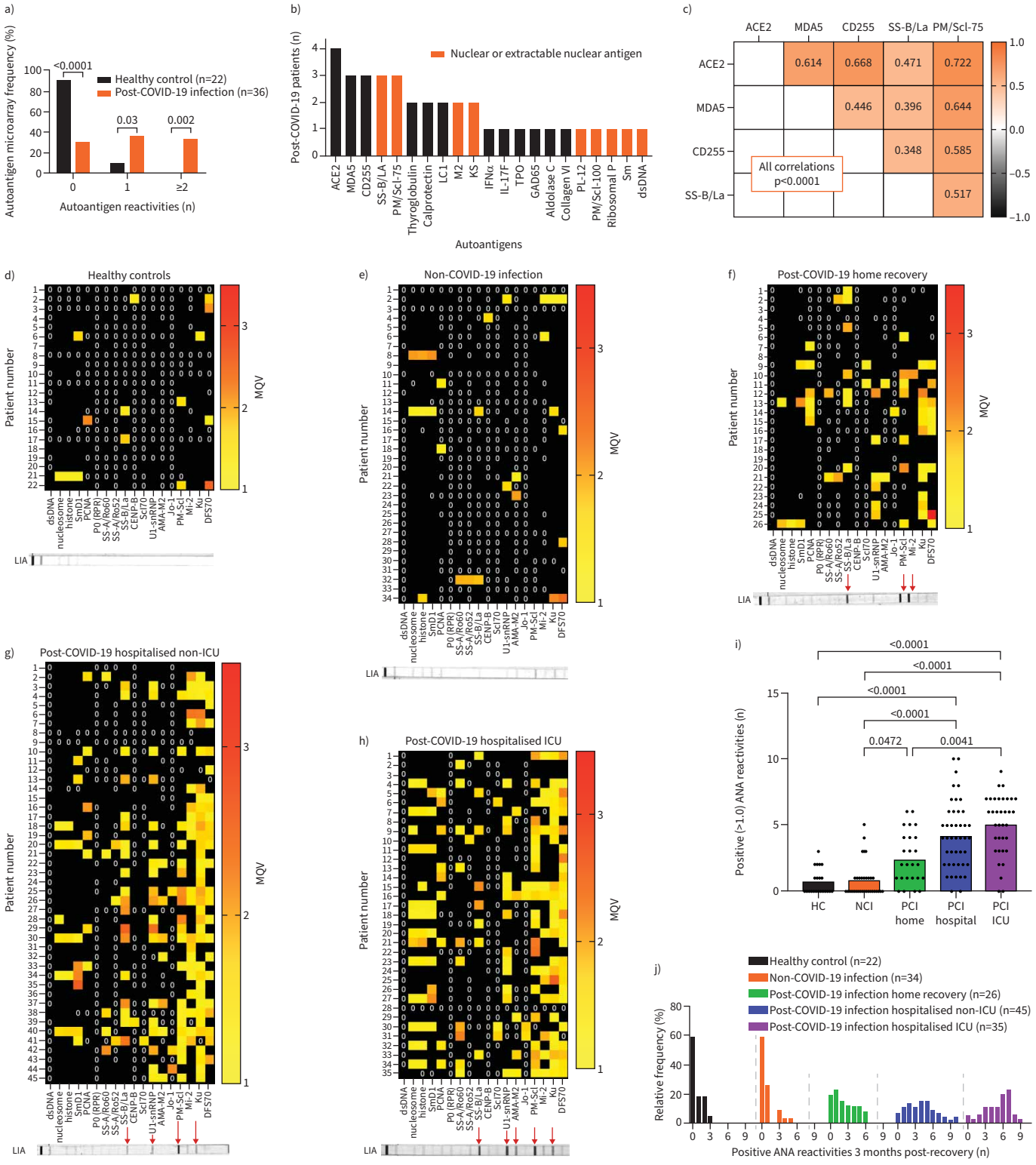

FIGURE 2 Autoantibody signatures of coronavirus disease 2019 (COVID-19) patients 3 months post-recovery. a) In our preliminary data, a higher number of autoreactivities were detected *via* microarray panel of 102 autoantigens in the first 36 post-COVID-19 infection (PCI) patients compared to healthy controls (HCs). Statistical analysis was performed by Chi-squared analysis with significance set to $p<0.05$. b) Of these autoreactivities, 43% were against nuclear antigens. c) Correlation analysis was performed between the five most prevalent autoantibodies from the autoantigen array. d–h) We evaluated anti-nuclear antibody (ANA) signatures in our control groups *versus* the post-COVID-19 cohort stratified based on severity of acute phase infection. i, j) Intensive care unit (ICU)-admitted post-COVID-19 patients demonstrated significantly more ANA reactivities compared

to home-recovered post-COVID-19 patients as well as control populations. Statistical analysis was performed with Kruskal–Wallis test with Dunn's multiple comparisons test with significance set to p<0.05. A representative ANA line immunossay (LIA) strip is given for every subgroup with arrows indicating the positive autoantigen with mean quantitative value (MQV) >1. ANA validation is shown in supplementary figure E2. NCI: non-COVID-19 infection.

22, 95%) and convalescent COVID-19 patients (33 out of 36, 92%; p>0.05) did not have autoreactive IgM antibodies (supplementary figure E1b). IgG autoantibodies were detected against 21 (21%) of the 102 screened antigens, of which nine (43%) out of 21 were against ANAs with known pathogenic roles in various autoimmune diseases (*e.g.* anti-dsDNA in systemic lupus erythematosus, anti-SS-B/La in Sjögren syndrome) (figure 2b). Strong significant correlations were observed between the five most prevalent autoreactivities (ACE2, MDA5, CD255, SS-B/La and PM/Scl-75) including two ANAs or ENAs (figure 2c).

ANAs/ENAs in patients 3 months post-COVID-19

The convalescent COVID-19 patients had higher levels (p<0.05) compared to healthy controls for 16 out of 18 ANAs/ENAs and to the non-COVID-19 infection control group for 12 out of 18 ANAs at 3 months post-recovery (supplementary figure E3). We compared the number of positive ANAs (MQV ⩾1.0) at 3 months post-recovery between the healthy (figure 2d) and the non-COVID-19 infection group (figure 2e) against convalescent COVID-19 patients recovered at home (PCI-home; n=26 (figure 2f), hospitalised non-ICU (PCI-hosp; n=45) (figure 2g) and those who were admitted to the ICU (PCI-ICU; n=35 (figure 2h). The prevalences of ANAs were not different between the healthy and non-COVID-19 respiratory infection groups. However, each had significantly fewer circulating ANAs/ENAs compared to PCI-hosp (p<0.0001) and PCI-ICU (p<0.0001) populations (figure 2i, supplementary figure E3). The PCI-home patients exhibited a higher number of ANA/ENA reactivities than the infection control group (p=0.047), yet also significantly fewer reactivities than the PCI-ICU group (p=0.004) (figure 2i). Patients with COVID-19 who had a more severe acute phase developed a stronger autoimmune response still evident at 3 months post-recovery (figure 2j).

Changes in circulating levels of ANAs/ENAs up to 12 months post-COVID-19

The majority of convalescent COVID-19 patients had two or more ANAs/ENAs at the 3-month (84 out of 106, 79%) and 6-month (76 out of 98, 78%) time points, and this was reduced to 41% by 12 months (34 out of 58, 41%; p<0.0001) (figure 3a). When stratified according to their acute-phase severities, this observation was consistent within the PCI-hosp (p<0.001) (figure 3c) and PCI-ICU (p<0.0001) (figure 3d) populations, but absent in the PCI-home group (figure 3b). Although we found no difference in MQVs between 3 and 6 months post-COVID-19 for all ANAs/ENAs, a significant attenuation of autoantibody levels at 12 months post-COVID-19 was observed for 13 out of 18 ANAs/ENAs. Although the overall number of detectable ANAs/ENAs declined by 12 months post-recovery (figure 3e–g), some remained detectable: anti-SmD1 (11%) (figure 4d), anti-PCNA (9%) (figure 4e), anti-SS-A/Ro60 (12%) (figure 4g), anti-SS-B/La (21%) (figure 4i), anti-U1-snRNP (30%) (figure 4l), anti-PM-Scl (21%) (figure 4o), anti-Ku (11%) (figure 4q) and anti-DFS70 (12%) (figure 4r). Furthermore, 12% of the positive ANA/ENA reactivities observed at 12 months were previously below cut-off threshold, underlining a potential *de novo* autoantibody production at this time (figure 3h).

Relationship between ANAs/ENAs and symptoms in post-COVID patients

At 3 months post-recovery, 36% presented with persistent fatigue, 21% with cough and 26% for dyspnoea (table 1). Although cough (6 months, 23%; 12 months, 22%) and dyspnoea (28%, 25%) remained consistent over time, the frequency of fatigue decreased over time (6 months, 40%; 12 months, 20%). However, in individuals who had at least one symptom, the ANA/ENA frequencies remained high throughout the follow-up period (3 months, 54%; 6 months, 77%; 12 months, 50%). Heatmaps of z-scores were generated from simple logistic regression analyses performed for individual ANAs/ENAs per symptom at each time point. The two most prevalent ANAs/ENAs at 12 months, anti-U1-snRNP (p=0.028) and anti-SS-B/La (p=0.003), both positively predicted persisting symptoms of fatigue and dyspnoea (anti-U1-snRNP p=0.02; anti-SS-B/La p=0.007) (figure 5d–f). Anti-U1-snRNP (p<0.007) (figure 5g) and anti-SS-B/La (p=0.002) (figure 5j) levels were higher in patients who reported fatigue compared to those who did not. ANAs/ENAs were unremarkable between patients with cough compared to those without (figure 5h,k). Although anti-U1-snRNP antibodies were slightly higher in patients with dyspnoea (p=0.09) (figure 5i), there was no difference in circulating anti-SS-B/La antibodies (figure 5l). There was a positive correlation between anti-SS-B/La and all three symptoms, as well as for

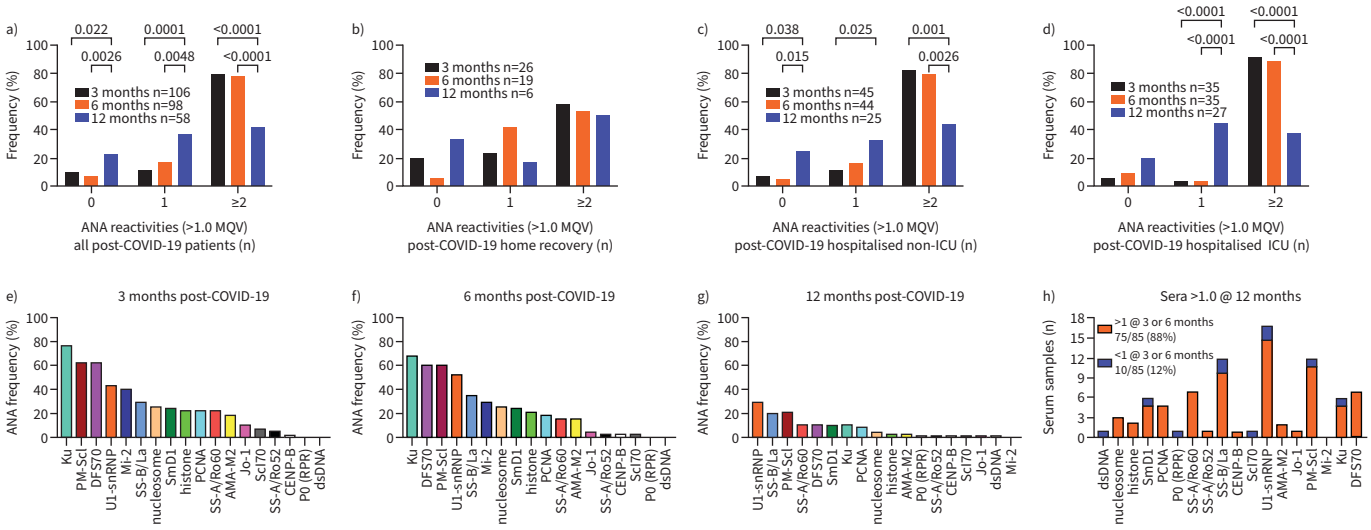

FIGURE 3 Prevalence of circulating anti-nuclear antibodies (ANAs) at 3, 6 and 12 months post-recovery. The majority of our a) total post-coronavirus disease 2019 (COVID-19) cohort had two or more ANA reactivities at 3 and 6 months post-COVID-19, but this proportion was reduced at 12 months. This attenuation is not evident in b) home-recovered post-COVID-19 patients, but observed in c) hospitalised and d) intensive care unit (ICU)-admitted COVID-19 survivors. Statistical analysis was performed with Chi-squared analysis. e–g) Histograms visualise the most prevalent ANAs for n=57 patients with ANAs assessed at all three time points. Statistical analysis was performed using the Friedman test with h) Dunn's multiple comparisons test. Out of the 85 positive ANA reactivities at 12 months post-COVID-19, 12% of samples were previously below cut-off threshold at both 3 and 6 months, indicating *de novo* autoantibody production post-COVID-19. MQV: mean quantitative value.

anti-U1-snRNP with fatigue and dyspnoea (figure 5g–l). The presence of either of these two ANAs at 12 months post-recovery predict fatigue (92% specificity, 70% sensitivity), dyspnoea (97% specificity, 58% sensitivity) and overall symptomaticity (97% specificity, 58% sensitivity). We did not observe any statistically significant sex differences for autoimmunity or symptoms in our study cohort at 3 and 6 months. However, at 12 months, a larger proportion of females presented with fatigue compared to males (supplementary figure E4). We also did not observe any differences in autoimmunity or symptoms in patients with/without comorbidities (cardiovascular, respiratory, gastrointestinal, endocrine, renal) at 12 months (supplementary table E2).

The relationship between cytokines, ANAs/ENAs and symptoms in patients 12 months post-COVID-19

Positive correlations were found between various ANAs/ENAs and inflammatory mediators: CRP, ICAM-1, VCAM-1, IL-8 and TNF-α (table 2). Multiple regression analysis was performed on all cytokines for each ANA/ENA at 12 months. At a significance of $p<0.01$, we found that TNF-α positively predicted anti-U1-snRNP and anti-anti-SS-A/Ro60 reactivity, CRP positively predicted anti-PM-Scl and anti-SmD1 autoreactivities, IL-6 positively predicted anti-PCNA and VCAM-1 positively predicted anti-Ku (supplementary table E1).

Strong positive correlations were found between D-dimer and fatigue at 3 months ($r=0.33$, $p=0.002$), TNF-α and cough at 6 months ($r=0.38$, $p=0.031$), and TNF-α and fatigue at 12 months ($r=0.42$, $p=0.004$) (table 2). At 6 months, TNF-α, VCAM-1 and IL-6 showed the greatest association with symptoms (figure 6b). For 12 months, TNF-α, D-dimer and IL-1β had the strongest association with symptoms (figure 6c). Multiple regression analysis for symptoms demonstrated that D-dimer predicted fatigue ($\beta=1.01$, $p=0.011$) and dyspnoea ($\beta=0.55$, $p=0.024$) at 3 months, ICAM-1 predicted cough at 3 months ($\beta=1.14$, $p=0.028$), and TNF-α ($\beta=4.65$, $p=0.004$) predicted fatigue at 12 months (figure 6d–f). Subsequent regression analysis for general symptomaticity showed that D-dimer ($\beta=1.08$, $p=0.013$) and TNF-α ($\beta=2.40$, $p=0.03$) positively predicted symptomaticity at 3 and 12 months, respectively (figure 6g–i).

Discussion

We comprehensively profiled autoantibody signatures of 18 clinically relevant ANAs/ENAs in 106 convalescent COVID-19 patients at 3, 6 and 12 months post-recovery. First, we demonstrated that COVID-19 survivors had elevated levels of circulating ANAs/ENAs compared to the healthy and non-COVID infection groups at 3 months post-recovery. Among the COVID-19 survivors, the number of

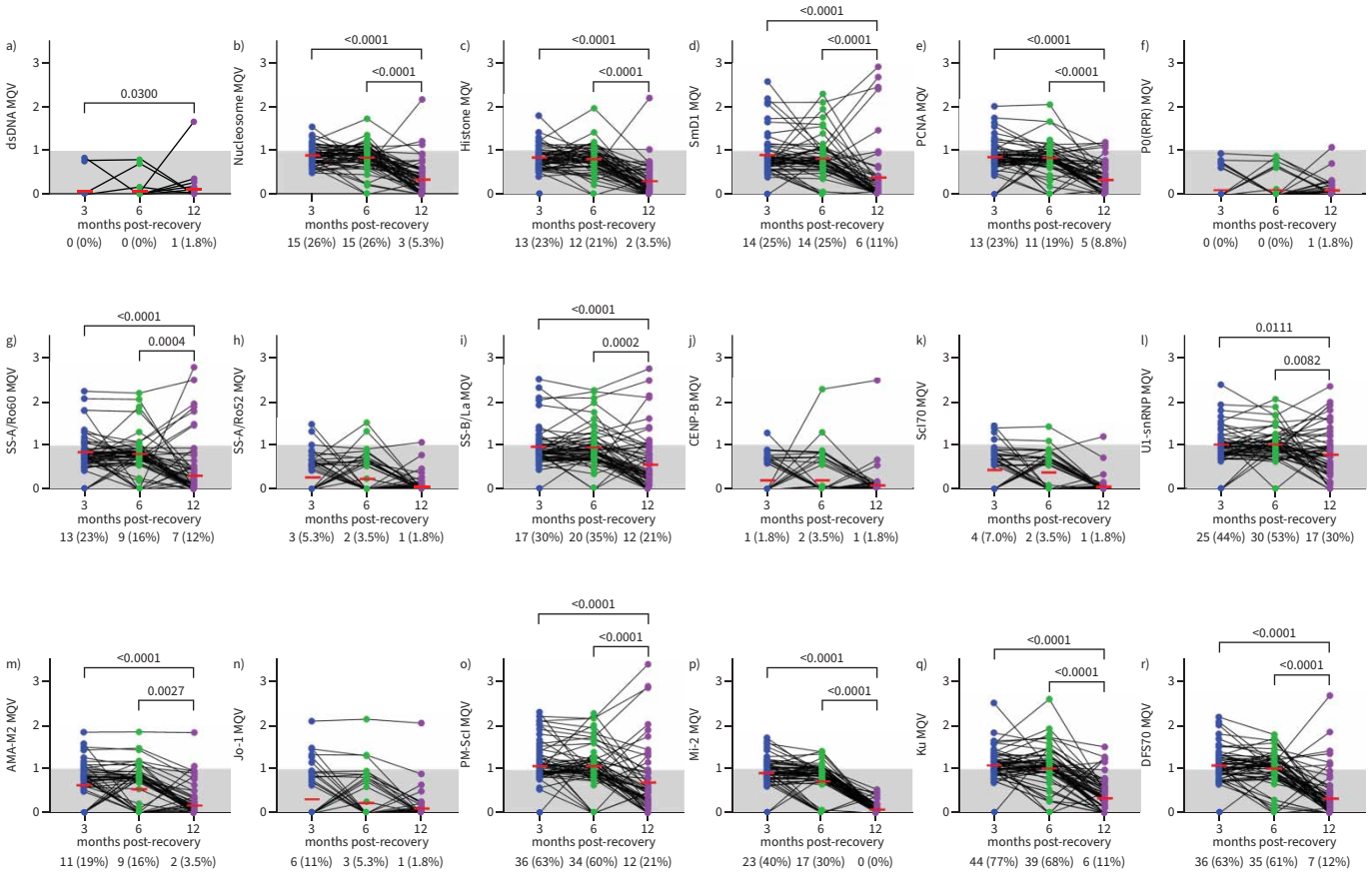

FIGURE 4 Longitudinal data of circulating anti-nuclear antibodies (ANAs) at 3, 6 and 12 months post-recovery. Longitudinal analysis was performed for each of the 18 evaluated nuclear autoantigens on the line immunoassay, and the mean quantitative value (MQV) was plotted for 57 patients with available serum samples at all three time points. The shaded region corresponds to MQV <1, indicating negative reactivity for each autoantibody. Mean MQV for each time point for each autoantibody is represented as a bold red horizontal line. The number and percentage of patients with a positive reactivity (>1:100 titer) was calculated for each time point for all ANAs.

ANA/ENA reactivities at 3 months post-recovery proportionally increased with the severity of the patient's acute phase infection; however, this correlation was absent at later time points. Second, high titres of circulating ANAs/ENAs were maintained up to 6 months post-recovery, but were significantly attenuated by 12 months, although several pathogenic ANAs/ENAs are still detectable in up to 30% of COVID survivors at 12 months. Furthermore, for 12% of post-COVID patients, positive ANAs/ENAs were observed at 12 months afresh, that were otherwise below the cut-off threshold at the 3- or 6-month time points, underlining potential *de novo* autoantibody synthesis. Two of the most prevalent autoantibodies, anti-U1-snRNP and anti-SS-B/La, positively predict both persisting fatigue and dyspnoea symptoms in COVID-19 survivors. Finally, we demonstrated that TNF-α, a key cytokine associated with development/sustenance of autoimmune diseases, positively predicted the observed ANAs/ENAs as well as symptom scores at 12 months post-recovery. Taken together, we provide evidence of an ongoing autoimmune inflammation marked by detectable circulating ANAs/ENAs and elevated TNF-α, associated with persisting symptoms at 12 months post-recovery in individuals who were otherwise healthy before contracting COVID-19.

Although previous work has demonstrated the persistence of autoantibodies in post-COVID individuals [14, 18–21], to our knowledge, the current study is the first to track specific autoantibodies with confirmed/known clinical pathogenicity with commonly reported long COVID symptoms across three time points up to 1 year post-recovery. Transient increases in autoantibodies in response to viral infections is commonly seen in weeks following recovery; however, these generally resolve [22]. Consistent with this, there was a significant reduction in the mean autoreactivities at 12 months in our post-COVID cohort for most autoantigens. That said, several ANAs/ENAs remained detectable despite their statistically significant

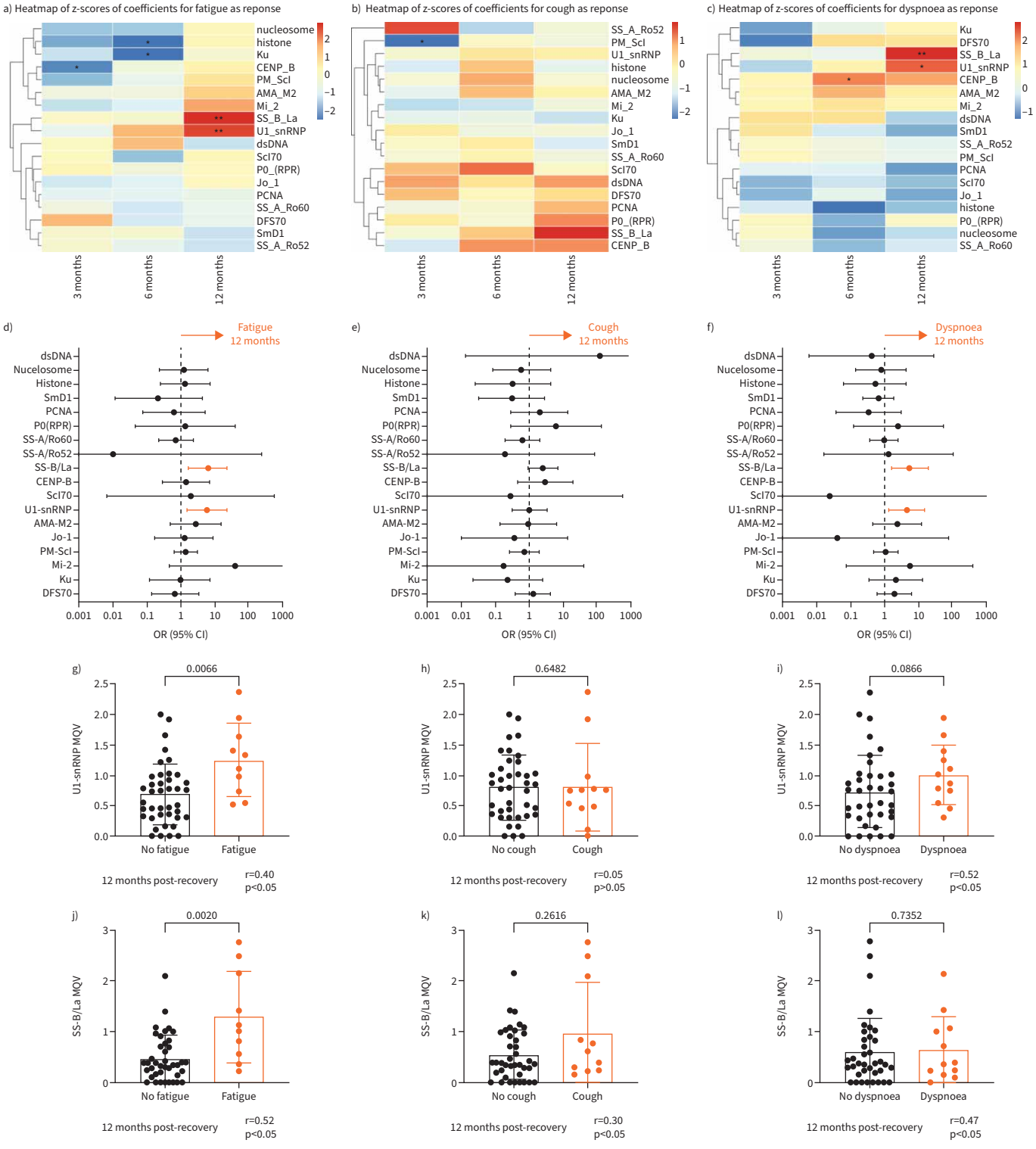

FIGURE 5 Prevalent anti-nuclear antibodies (ANAs) detected at 12 months post-recovery predict patient symptomaticity. A simple logistic regression analysis was conducted for each individual ANA assessing patient symptoms of a) fatigue, b) cough and c) dyspnoea at each time point. A heatmap presenting the z-scores of estimates are presented. The regression analysis at 12 months post-recovery is displayed in a forest plot with the odds ratio and 95% confidence intervals for d) fatigue, e) cough and f) dyspnoea. The orange lines indicate significant predictors for symptoms based on 95% confidence interval of the regression model. g–l) Patients (n=50) were then stratified by their symptoms at 12 months, and ANAs that positively predicted symptoms, g–i) anti-U1-snRNP and j–l) anti-SS-B/La mean quantitative values (MQVs) were compared between groups for each individual symptom. Statistical analysis was done with Mann–Whitney t-test for between-group comparisons. Data are presented as mean±SD with significance set to p<0.05. g–l) Correlation analysis was conducted additionally for these ANAs and each respective symptom, with significance set to p<0.05. *: p<0.05, **: p<0.01.

attenuation in some post-COVID patients, such as anti-U1-snRNP (30%), anti-SS-B/La (21%) and anti-PM-Scl (21%). Whether this is a harbinger of future autoimmunity is not known, but elevated anti-ribonucleoprotein and anti-SS autoantibodies after viral infections (*e.g.* Epstein–Barr virus, cytomegalovirus) are associated with the development of rheumatological diagnosis [23–25]. In fact, a number of cases of new-onset autoimmune diseases post-COVID have been reported including vasculitis [26, 27], arthritis [28], systemic lupus erythematosus [29] and myositis [30] in patients with no prior history of autoimmunity, irrespective of acute phase severity [31–34].

COVID-19 patients appear to have slower resolution of inflammation as evidenced by elevated IL-1β, IL-6, IL-8 and TNF-α, and this delay in resolution has been hypothesised to contribute to the development of PASC symptoms [7, 35, 36]. Indeed, TNF-α has been linked to fatigue in various diseases including chronic fatigue syndrome and rheumatoid arthritis. Thus, an incomplete mitigation of autoimmune responses/self-reactivities along with endothelial dysfunction (evident by elevated D-dimer) and residual type 1 inflammation may potentially streamline the trajectory towards persisting constitutional symptoms, chronic PASC and eventual development of rheumatological complications.

In a systemic review and meta-analysis conducted in January 2021, fatigue (58%) and dyspnoea (24%) were included within the five most commonly developed long-term symptoms in >47 000 post-COVID-19 patients [1]. We acknowledge that we did not comprehensively record all currently known long-COVID symptoms, and may have missed a subset of patients presenting with symptoms not included in the current study (*e.g.* joint pain, rashes, neurocognitive dysfunction). A significant subset of the patients was recruited at the early phase of the pandemic (August 2020) through patient referrals, community outreach and hospital recruitment; therefore, a confirmed PASC diagnosis could not be made as per the current guidelines. Symptomaticity, objectively measured, may fluctuate over time for an individual, and subject to recall bias. Hence, we have refrained from calling these patients to have confirmed PASC and aligned our analysis and conclusion with symptomaticity rather than PASC diagnosis.

TABLE 2 Inflammatory mediators correlate with prevalent anti-nuclear antibodies (ANAs) and symptomaticity at 12 months post-coronavirus disease 2019

	ANA	Symptom	Time point	r-value	p-value
Cytokines *versus* ANAs/ENAs[#]					
CRP	SS-A/Ro52		12 months	0.41	0.001
CRP	Mi-2			0.34	0.010
ICAM-1	SS-A/Ro60			0.34	0.017
VCAM-1	dsDNA			0.29	0.047
VCAM-1	U1-snRNP			0.42	0.003
VCAM-1	PM-Scl			0.39	0.006
IL-8	Nucleosome			0.31	0.034
IL-8	DFS70			0.29	0.043
TNF-α	Nucleosome			0.34	0.018
TNF-α	Histone			0.38	0.008
TNF-α	SS-A/Ro60			0.31	0.031
TNF-α	U1-snRNP			0.41	0.003
TNF-α	PM-Scl			0.54	<0.0001
Cytokines *versus* symptoms[¶]					
D-dimer		Fatigue	3 months	0.33	0.002
IL-6		Dyspnoea		0.21	0.051
IL-8		Dyspnoea		0.20	0.068
TNF-α		Cough	6 months	0.38	0.031
CRP		Cough		0.33	0.065
IL-6		Cough		0.31	0.074
TNF-α		Fatigue	12 months	0.42	0.004

ENA: extractable-nuclear antibody; CRP: C-reactive protein: ICAM: intercellular adhesion molecule; VCAM: vascular cell adhesion molecule; IL: interleukin; TNF: tumour necrosis factor. [#]: all significant correlations between measured sera cytokines and ANA mean quantitative values at 12 months post-recovery; [¶]: all significant correlations between measured sera cytokines and patient reported outcomes at 3, 6 and 12 months post-recovery.

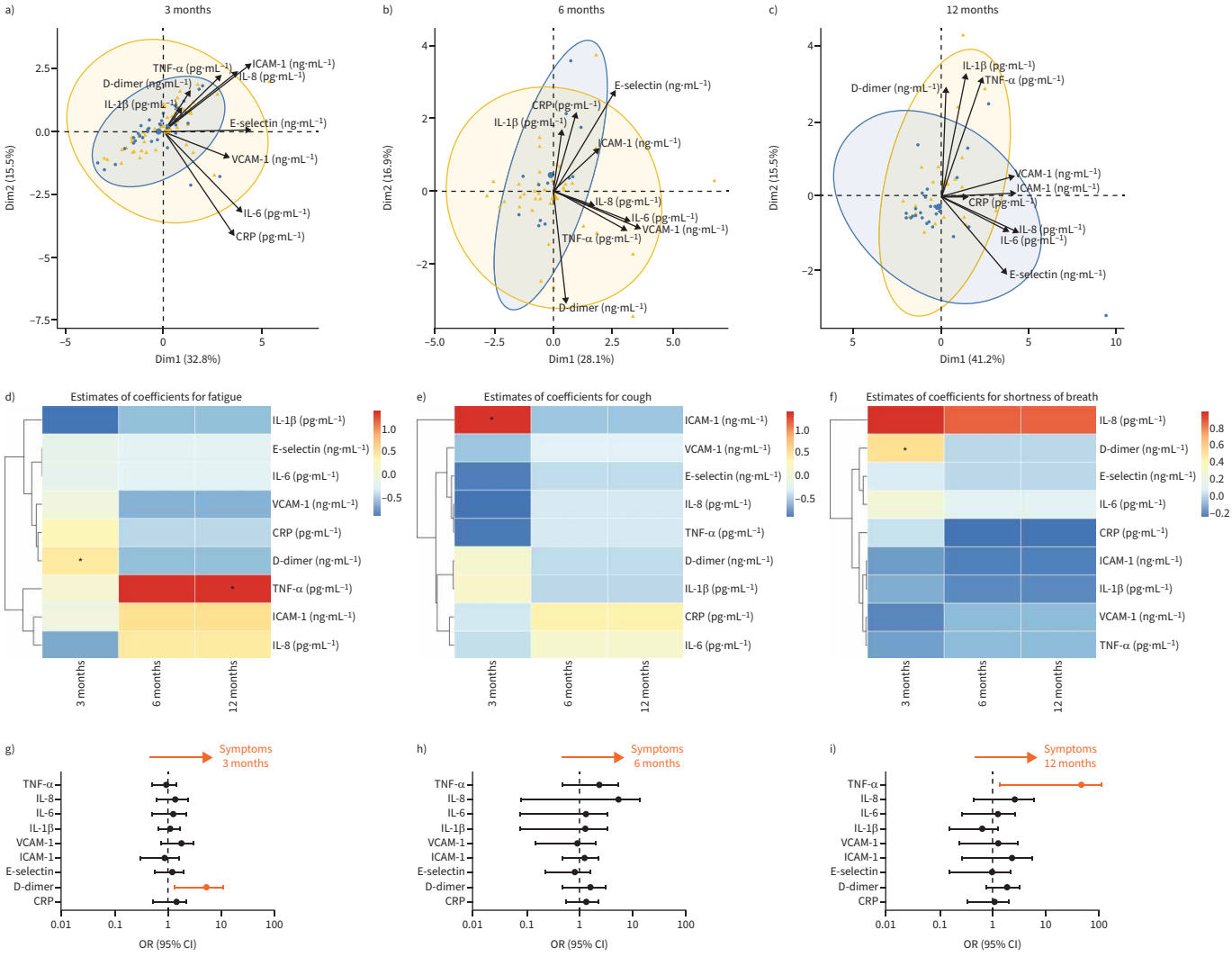

FIGURE 6 Inflammatory mediators predict symptomaticity post-coronavirus disease 2019. Principal component analysis was conducted to reduce the dimensionality of our nine cytokine variable dataset. a–c) The patient cytokine data transformed onto two dimensions with the highest contribution to variability were plotted, and a 95% confidence ellipsoid was determined for both the symptomatic and asymptomatic populations at each time point (3 months n=100, 6 months n=75, 12 months n=51) to determine the influence and clustering of cytokines on symptoms. Multiple regression analysis was conducted for all cytokines at each time point for individual symptoms. A heatmap was generated using the regression estimates for d) fatigue, e) cough and f) dyspnoea. Subsequently, the regression analysis is displayed in a forest plot with the odds ratio and 95% confidence intervals for g) 3 months, h) 6 months and i) 12 months post-recovery. The orange lines indicate significant predictors for symptoms based on 95% confidence intervals of the regression model. CRP: C-reactive protein, ICAM: intercellular adhesion molecule, IL: interleukin, TNF: tumour necrosis factor, VCAM: vascular cell adhesion molecule. *: p<0.05.

A few limitations of our study merit consideration. First, given the study's focus on longitudinal observations, it would have been ideal to collect samples and symptoms from our non-COVID-19 infection control cohort at matching 6- and 12-months post-infection time points, similar to our post-COVID-19 population. The ever-changing pandemic landscape made it logistically difficult to allow the longitudinal recruitment of the non-COVID-19 participants. The reluctance of non-COVID-19 participants (infection control and healthy cohorts) to come into the hospital during the pandemic impacted study recruitment. Indeed, although we managed an age- and sex-matched cohort for the healthy participants, we could not do so for the infection control group. In addition, this mismatch was further impaired by the exclusion of five PCR-negative older individuals due to pre-existing rheumatological complications. A more proportional comparison including adequate hospitalised and ICU-admitted controls would shed more light on whether the development of autoantibodies is specific to SARS-CoV-2 infection or due to a general pathogenicity associated with severe viral infection. In addition, our convalescent COVID-19 patient

sample size at 12 months totalled 58 patients, compared to 106 patients at 3 months and 98 patients at 6 months. We surmise that an increased attrition rate at later time points may be due to alleviated symptoms in study participants, leading to an enriched symptomatic population at 12 months. A balanced ratio of samples between time points could result in better statistical power for detecting relevant associations between output variables. However, given the topical scenario, we found merit in reporting our observations promptly. Finally, as we do not have pre-pandemic ANA values, we are currently unable to assess if the observed autoimmunity was prevalent pre-COVID, and whether causality exists with the observed symptoms. Though this is currently beyond the scope of the present study, a mechanistic investigation is underway in our ongoing longitudinal long-COVID trial (clinicaltrials.gov identifier NCT05459506).

In summary, ANAs/ENAs with known roles in autoimmune diseases were detected at elevated levels in patients at 3 and 6 months post-COVID-19. Attenuation in the frequency of these autoreactivities was observed by 12 months, despite anti-U1-snRNP and anti-SS-B/La antibodies remaining prevalent in up to 30% of post-COVID-19 patients. These autoreactivities strongly correlate with TNF-α, and both positively predict common PASC symptoms 1 year post-infection. The incomplete attenuation of clinically relevant autoreactivities 12 months post-COVID in one third of patients, associated with persisting symptoms and residual inflammation warrant long-term investigation of autoimmunity in PASC patients.

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References

1 Lopez-Leon S, Wegman-Ostrosky T, Perelman C, *et al.* More than 50 long-term effects of COVID-19: a systematic review and meta-analysis. *Sci Rep* 2021; 11: 16144.
2 Nalbandian A, Sehgal K, Gupta A, *et al.* Post-acute COVID-19 syndrome. *Nat Med* 2021; 27: 601–615.
3 Bastard P, Rosen LB, Zhang Q, *et al.* Autoantibodies against type I IFNs in patients with life-threatening COVID-19. *Science* 2020; 370: eabd4585.
4 Zuo Y, Estes SK, Ali RA, *et al.* Prothrombotic autoantibodies in serum from patients hospitalized with COVID-19. *Sci Transl Med* 2020; 12: eabd3876.
5 Hasan Ali O, Bomze D, Risch L, *et al.* Severe coronavirus disease 2019 (COVID-19) is associated with elevated serum immunoglobulin (Ig)A and antiphospholipid IgA-antibodies. *Clin Infect Dis* 2021; 73: e2860–e2874.
6 Gatto M, Perricone C, Tonello M, *et al.* Frequency and clinical correlates of antiphospholipid antibodies arising in patients with SARS-CoV-2 infection: findings from a multicentre study on 122 cases. *Clin Exp Rheumatol* 2020; 38: 754–759.
7 Proal AD, VanElzakker MB. Long COVID or post-acute sequelae of COVID-19 (PASC): an overview of biological factors that may contribute to persistent symptoms. *Front Microbiol* 2021; 12: 698169.
8 Douaud G, Lee S, Alfaro-Almagro F, *et al.* SARS-CoV-2 is associated with changes in brain structure in UK Biobank. *Nature* 2022; 604: 697–707.
9 Su Y, Yuan D, Chen DG, *et al.* Multiple early factors anticipate post-acute COVID-19 sequelae. *Cell* 2022; 185: 881–895.
10 Gracia-Ramos AE, Martin-Nares E, Hernández-Molina G. New onset of autoimmune diseases following COVID-19 diagnosis. *Cells* 2021; 10: 3592.
11 Cabral-Marques O, Halpert G, Schimke LF, *et al.* Autoantibodies targeting GPCRs and RAS-related molecules associate with COVID-19 severity. *Nat Commun* 2022; 13: 1220.
12 Richter AG, Shields AM, Karim A, *et al.* Establishing the prevalence of common tissue-specific autoantibodies following severe acute respiratory syndrome coronavirus 2 infection. *Clin Exp Immunol* 2021; 205: 99–105.
13 Kennedy AE, Cook L, Breznik JA, *et al.* Lasting changes to circulating leukocytes in people with mild SARS-CoV-2 infections. *Viruses* 2021; 13: 2239.
14 Rojas M, Rodríguez Y, Acosta-Ampudia Y, *et al.* Autoimmunity is a hallmark of post-COVID syndrome. *J Transl Med* 2022; 20: 129.
15 Mukherjee M, Bulir DC, Radford K, *et al.* Sputum autoantibodies in patients with severe eosinophilic asthma. *J Allergy Clin Immunol* 2018; 141: 1269–1279.
16 Del Valle DM, Kim-Schulze S, Huang HH, *et al.* An inflammatory cytokine signature predicts COVID-19 severity and survival. *Nat Med* 2020; 26: 1636–1643.
17 Mukherjee M, Thomas SR, Radford K, *et al.* Sputum antineutrophil cytoplasmic antibodies in serum antineutrophil cytoplasmic antibody-negative eosinophilic granulomatosis with polyangiitis. *Am J Respir Crit Care Med* 2019; 199: 158–170.
18 Wallukat G, Hohberger B, Wenzel K, *et al.* Functional autoantibodies against G-protein coupled receptors in patients with persistent long-COVID-19 symptoms. *J Transl Autoimmun* 2021; 4: 100100.
19 Bertin D, Kaphan E, Weber S, *et al.* Persistent IgG anticardiolipin autoantibodies are associated with post-COVID syndrome. *Int J Infect Dis* 2021; 113: 23–25.
20 Liu Y, Ebinger JE, Mostafa R, *et al.* Paradoxical sex-specific patterns of autoantibody response to SARS-CoV-2 infection. *J Transl Med* 2021; 19: 524.
21 Taeschler P, Cervia C, Zurbuchen Y, *et al.* Autoantibodies in COVID-19 correlate with antiviral humoral responses and distinct immune signatures. *Allergy* 2022; 77: 2415–2430.
22 Smatti MK, Cyprian FS, Nasrallah GK, *et al.* Viruses and autoimmunity: a review on the potential interaction and molecular mechanisms. *Viruses* 2019; 11: 762.
23 Newkirk MM, van Venrooij WJ, Marshall GS. Autoimmune response to U1 small nuclear ribonucleoprotein (U1 snRNP) associated with cytomegalovirus infection. *Arthritis Res* 2001; 3: 253–258.
24 Liu Z, Chu A. Sjögren's syndrome and viral infections. *Rheumatol Ther* 2021; 8: 1051–1059.
25 Didier K, Bolko L, Giusti D, *et al.* Autoantibodies associated with connective tissue diseases: what meaning for clinicians? *Front Immunol* 2018; 9: 541.
26 Izci Duran T, Turkmen E, Dilek M, *et al.* ANCA-associated vasculitis after COVID-19. *Rheumatol Int* 2021; 41: 1523–1529.
27 Oda R, Inagaki T, Ishikane M, *et al.* Case of adult large vessel vasculitis after SARS-CoV-2 infection. *Ann Rheum Dis* 2020; in press [https://doi.org/annrheumdis-2020-218440].
28 Baimukhamedov C, Barskova T, Matucci-Cerinic M. Arthritis after SARS-CoV-2 infection. *Lancet Rheumatol* 2021; 3: e324–e325.
29 Slimani Y, Abbassi R, El Fatoiki F-Z, *et al.* Systemic lupus erythematosus and varicella-like rash following COVID-19 in a previously healthy patient. *J Med Virol* 2021; 93: 1184–1187.
30 Beydon M, Chevalier K, Al Tabaa O, *et al.* Myositis as a manifestation of SARS-CoV-2. *Ann Rheum Dis* 2021; 80: e42.

31 Zamani B, Moeini Taba SM, Shayestehpour M. Systemic lupus erythematosus manifestation following COVID-19: a case report. *J Med Case Rep* 2021; 15: 29.

32 Gigli GL, Vogrig A, Nilo A, *et al.* HLA and immunological features of SARS-CoV-2-induced Guillain-Barré syndrome. *Neurol Sci* 2020; 41: 3391–3394.

33 de Ruijter NS, Kramer G, Gons RAR, *et al.* Neuromyelitis optica spectrum disorder after presumed coronavirus (COVID-19) infection: a case report. *Mult Scler Relat Disord* 2020; 46: 102474.

34 Fineschi S. Case report: systemic sclerosis after Covid-19 infection. *Front Immunol* 2021; 12: 686699.

35 Hirschenberger M, Hunszinger V, Sparrer KMJ. Implications of innate immunity in post-acute sequelae of non-persistent viral infections. *Cells* 2021; 10: 2134.

36 Peluso MJ, Lu S, Tang AF, *et al.* Markers of immune activation and inflammation in individuals with postacute sequelae of severe acute respiratory syndrome coronavirus 2 infection. *J Infect Dis* 2021; 224: 1839–1848.