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Early View

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

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Article Type: Original Research

Title: Circulating Anti-Nuclear Autoantibodies in COVID-19 Survivors Predict Long-COVID Symptoms

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Summary: High titers of 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.

Abstract

Background:

Autoimmunity has been reported in patients with severe COVID-19. We investigated whether antinuclear/extractable-nuclear antibodies (ANAs) 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 ANA/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 dyspnea were recorded at each timepoint. Multivariable logistic regression model and receiver-operating curves (ROC) 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 from 3 to 12 months (3.99 to 1.55) with persistent positive titers associated with fatigue, dyspnea, and cough severity. Antibodies to U1-snRNP and anti-SS-B/La were both positively associated with persistent symptoms of fatigue ($P<0.028$, AUC=0.86) and dyspnea ($P<0.003$, AUC=0.81). Pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα) and C-reactive protein predicted the elevated ANAs at 12 months. TNFα, D-dimer, and IL-1β had the strongest association with symptoms at 12 months. Regression analysis showed TNFα 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 (TNFα) 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 SARS-CoV2 virus recover; however, a significant subset report persistent symptoms (e.g., fatigue, dyspnea, and cognitive impairment) that do not resolve after infection[1]. This constellation of symptoms is called Long COVID or Post-Acute Sequalae 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 COVID-19 pathology[3-6]. Emerging evidence suggests that the failure to resolve these autoantibodies or, alternatively 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-/extractable nuclear autoantibodies (ANA/ENAs) have been reported in PASC patients as biomarkers but no identified links with specific PASC symptoms[7, 8, 11, 12].

In our non-interventional, observational, longitudinal study, we utilized an extensive, clinically relevant anti-/extractable nuclear antibody panel to serve as common rheumatologic 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 alpha (TNFα), D-dimer], as well as commonly reported PASC symptoms of fatigue, cough, and dyspnea[1].

Methods

Study Design and Patient Selection

This was a multi-center, multi-timepoint 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, Canada), Vancouver General Hospital (n=42, Vancouver, Canada), and St. Paul's Hospital (n=20, Vancouver, Canada) for three study visits at 3, 6, and 12 months, post-recovery (deemed recovered as per Public Health Guidelines). Consenting patients ≥18 years of age, 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 in/out-patient 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 who did not have either a test and did not become seropositive between 1-3 months post-recovery of their infection and/or symptoms[13]. Twenty-two age and sex matched non-COVID, non-vaccinated healthy adults were recruited locally from Hamilton (**Figure 1**). Criteria for recruiting healthy volunteers during the pandemic included never having COVID-19, not yet vaccinated for COVID-19, never smokers, with no history of respiratory/rheumatological disease. Serum was collected at each timepoint and stored at -80°C within one hour 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 timepoint post-COVID recovery: fatigue (patient reported or Fatigue Assessment Scale, FAS), cough (patient reported), and shortness of breath (modified Medical Research Council (mMRC) dyspnea scale).

Microarray Autoantibody Profiling

Serum IgG and IgM antibody reactivities against 102 autoantigens were analyzed with a microfluidic antigen array developed at the Microarray and Immune Phenotyping Core Facility at The University of Texas Southwestern Medical Center as previously described[14] for the 3

month-time point for participants who consented for 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 (**Fig. E1**). We utilized 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 Anti/Extranuclear Antibodies in Serum

An ANA/ENA line immunoassay (IMTEC-ANA-LIA-MAXX, Human Diagnostics, Germany) targeting 18 common self-antigens was used as previously described[15] at disease modifying titers of 1:100. Each strip was scanned (ChemiDoc MP Imaging System, Bio-Rad, CA, USA), and images were converted into 8-bit grayscale and inverted with ImageJ analysis software. A quantitative value was derived for each visible band and normalized to the cut-off control band to provide a mean quantitative value (MQV) with values $\geq 1{\cdot}0$ indicating positive reactivity. Validation of our quantification method was assessed using indirect immunofluorescence of HEp-2 cells (**Figure E2**).

Serum Molecular Mediator Analysis

Acute markers of inflammation (interleukin (IL)-1β, IL-6, IL-8, TNFα)[16], coagulation mediators (D-dimer, E-selectin, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1)), and C-reactive protein (CRP) were assessed and quantitated using the Ella^{TM} Automated Immunoassay System (Bio-Techne, MN, USA). Serum samples were diluted as per manufacturer's protocol for each respective mediator reported [13].

Statistical Analysis

All experimental data were analyzed 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, 2 groups), Kruskal Wallis test (nonparametric unpaired analysis, >2 groups), and Friedman test (nonparametric paired analysis with repeated measures, >2 groups), associations were determined by Spearman's rank

correlation test, and categorical variables analyzed by Chi-squared analysis. Receiver-operating curves (ROC), 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 timepoints.

Results (1174 Words)

Study Population

We recruited 106 convalescent COVID-19 patients (61 males, M / 45 females, F) with a mean age of 57 years and BMI of 27.2 kg/m^2 (**Table 1**). Twenty-six patients recovered from COVID-19 at home, 35 were admitted to the ICU, and 45 were hospitalized but not ICU-admitted. Thirty-four patients (10M/24F) with a non-COVID infection were recruited for comparison with a mean age of 46 and BMI of 22.2 kg/m^2. Of these patients, 34 recovered at home, whereas 1 was admitted to the ICU. 22 healthy volunteers (11M/11F) averaging 49 years and 26.6 kg/m^2 were enrolled as a control population.

IgG/M 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/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 **Fig E1**. While most of the healthy controls did not have IgG autoantibodies (20/22, 91%), approximately one-third of the convalescent COVID-19 group had at least one autoreactive IgG (13/36, 36%; P=0.03). Two or more autoantigens were found in 33% of COVID-19 convalescent patients (12/36, 33%; P=0.002, **Fig. 2A**). In contrast, the majority of healthy controls (21/22 (95%)) and convalescent COVID-19 patients (33/36 (92%; P>0.05)) did not have autoreactive IgM antibodies (**Fig. E1B)**. IgG autoantibodies were detected against 21 of the 102 screened antigens (21%), of which 9/21 (43%) 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's syndrome) (**Fig. 2B**). Strong significant correlations were observed between the five most prevalent autoreactivities (ACE2, MDA5, CD255, SS-B/La, and PM/Scl-75) including two against nuclear or extractable nuclear antigens (**Fig. 2C**).

ANA/ENA Autoantibodies in Patients 3 Months Post-COVID

The convalescent COVID-19 patients had higher levels (P<0.05) compared to healthy controls for 16/18 ANA/ENAs and to non-COVID infection control group for 12/18 ANAs at 3 months post-recovery (**Fig. E3**). We compared the number of positive ANAs (MQV≥1.0) at 3 months

post-recovery between healthy (**Fig. 2D**) and the non-COVID infection group (**Fig. 2E**) against convalescent COVID-19 patients recovered at home (PCI-Home; n=26, **Fig. 2F**), hospitalized non-ICU (PCI-Hosp; n=45, **Fig. 2G**), and those who were admitted in the ICU (PCI-ICU; n=35, **Fig. 2H**). The prevalence of ANAs were not different between the healthy and non-COVID respiratory infection groups. However, each had significantly fewer circulating ANA/ENAs compared to PCI-Hosp (P<0.0001) and PCI-ICU (P<0.0001) populations (**Fig. 2I, Figure E3**). The PCI-Home group patients exhibited a higher number of ANA/ENA reactivities than the infection control group (NCI, P=0.047), yet also significantly fewer reactivities than the PCI-ICU (P=0.004) (**Fig. 2I**). Patients with COVID-19 who had a more severe acute phase developed a stronger autoimmune response still evident at 3 months post-recovery (**Fig. 2J**).

Changes in Circulating Levels of Anti/Extra-nuclear Antibodies Up to 12 Months Post-COVID

The majority of convalescent COVID-19 patients had ≥2 ANA/ENAs at the 3- (84/106, 79%) and 6-month (76/98, 78%) timepoints, and this was reduced to 41% by 12 months (34/58, 41%; P<0.0001; **Fig. 4A**). When stratified according to their acute phase severities, this observation was consistent within the PCI-Hosp (P<0·001, **Fig. 4C**) and PCI-ICU (P<0.0001, **Fig. 4D**) population, but absent in the PCI-Home group (**Fig. 4B**). Though we found no difference in MQVs between 3 and 6 months post-COVID for all ANA/ENAs, a significant attenuation of autoantibody levels at 12 months post-COVID was observed for 13/18 ANA/ENAs. Although the overall number of detectable ANA/ENAs declined by 12 months post-recovery (**Fig. 4E-G**), some remained detectable; anti-SmD1 (11%, **Fig. 3D**), anti-PCNA (9%, **Fig. 3E**), anti-SS-A/Ro60 (12%, **Fig. 3G**), anti-SS-B/La (21%, **Fig. 3I**), anti-U1-snRNP (30%, **Fig. 3L**), anti-PM-Scl (21%, **Fig. 3O**), anti-Ku (11%, **Fig. 3Q**), and anti-DFS70 (12%, **Fig. 3R**). 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 (**Fig. 4H**).

Relationship Between ANA/ENAs and Symptoms in Post-COVID Patients.

At 3 months post-recovery, 36% presented with persistent fatigue, 21% with cough, and 26% for dyspnea (**Table 1**). Though cough (6 months, 23%; 12 months, 22%) and dyspnea (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-score were generated from simple logistic regression analyses performed for individual ANA/ENAs per symptom at each timepoint. The two most prevalent ANA/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 dyspnea (anti-U1-snRNP: P=0.02; anti-SS-B/La: P=0.007) (**Fig. 5D-F**). Anti-U1-snRNP (P<0·007, **Fig 5G**) and anti-SS-B/La (P=0.002, **Fig. 5J**) levels were higher in patients who reported fatigue compared to those who did not. ANA/ENAs were unremarkable between patients with cough compared to those without (**Fig. 5H, K**). Though anti-U1-snRNP antibodies were slightly higher in patients with dyspnea (P=0.09, **Fig. 5I**), there was no difference in circulating anti-SS-B/La antibodies (**Fig. 5L**). There was a positive correlation between anti-SS-B/La and all three symptoms, as well as for anti-U1-snRNP with fatigue and dyspnea (**Fig. 5G-L**). The presence of either of these two ANAs at 12 months post-recovery predict fatigue (92% specificity, 70% sensitivity), dyspnea (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 (**Fig. 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 (**Table E2**).

The Relationship Between Cytokines, ANAs, and Symptoms in Patients 12 Months Post-COVID

Positive correlations were found between various ANA/ENAs and inflammatory mediators: CRP, ICAM-1, VCAM-1, IL-8, and TNFα (**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 TNFα 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 (**Table E1**).

Strong positive correlations were found between D-dimer and fatigue at 3 months (r=0·33, P=0·002**)**, TNFα and cough at 6 months (r=0·38, P=0·031), and TNFα and fatigue at 12 months (r=0·42, P=0·004) (**Table 2)**. At 6 months, TNFα, VCAM-1, and IL-6 showed the greatest association with symptoms (**Fig. 6B**). For 12 months, TNFα, D-dimer, and IL-1β had the

strongest association with symptoms **(Fig. 6C**). Multiple regression analysis for symptoms demonstrated D-dimer predicted fatigue (β=1.01, P=0·011) and dyspnea (β=0·55, P=0·024) at 3 months, ICAM-1 predicted cough at 3 months (β=1·14, P=0·028), and TNFα (β=4·65, P=0·004) predicted fatigue at 12 months (**Fig. 6D-F**). Subsequent regression analysis for general symptomaticity showed that D-dimer (β=1·08, P=0·013) and TNFα (β=2·40, P=0·03) positively predicted symptomaticity at 3 and 12 months respectively (**Fig. 6G-I**).

Discussion

We comprehensively profiled autoantibody signatures of 18 clinically relevant ANA/ENAs in 106 convalescent COVID-19 patients at 3, 6, and 12 months post-recovery. First, we demonstrated COVID-19 survivors had elevated levels of circulating ANA/ENAs compared to the healthy and non-COVID infection groups at 3 months post-recovery. Amongst the COVID-19 survivors, the number of 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 titers of circulating ANA/ENAs were maintained up to 6 months post-recovery but were significantly attenuated by 12 months. Albeit several pathogenic ANA/ENAs are still detectable in up to 30% of COVID survivors at 12 months. Further, for 12% of post-COVID patients, positive ANA/ENAs were observed at 12 months afresh, that were otherwise below the cut-off threshold at 3/6 month time-point, 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 dyspnea symptoms in COVID-19 survivors. Finally, we demonstrated that TNFα, a key cytokine associated with development/sustenance of autoimmune diseases, positively predicted the observed ANA/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 ANA/ENAs and elevated TNFα, that are 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 timepoints up to one-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 ANA/ENAs remained detectable despite their statistically significant 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 (SLE)[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 TNFα, and this delay in resolution has been hypothesized to contribute to the development of PASC symptoms [7, 35, 36]. Indeed, TNFα 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 T1 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 dyspnea (24%) were included within the five most common developed long-term symptom in over 47,000 post-COVID 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 (Aug. 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.

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 infection control cohort at matching 6- and 12-months post-infection timepoints, 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, though we managed an age-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 hospitalized 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 totaled 58 patients, compared to 106 patients at 3 months and 98 patients at 6 months. We surmise that an increased attrition rate at later timepoints may be due to alleviated symptoms in study participants, leading to an enriched, symptomatic population at 12 months. A balanced ratio of samples between timepoints 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 (NCT05459506).

In summary, anti-nuclear/extractable nuclear antibodies with known roles in autoimmune diseases were detected at elevated levels in patients at 3 and 6 months post-COVID. Attenuation in the frequency of these autoreactivities was observed by 12 months, despite anti-U1-snRNP and anti-SS-B/La antibodies remained prevalent in up to 30% of post-COVID patients. These autoreactivities strongly correlate with TNFα, and both positively predicts common PASC symptoms one 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.

Author Contributions

Conceptualization, MM; recruitment (Hamilton), SS, DMEB, IN; recruitment (Vancouver), CC; CJR; patient care and referrals, PN, TH, SW; study coordination, sample processing, database management (Hamilton), CV, RJ, ZP, BC, KR, CH, MK, KM, JS; study coordination sample processing, database management (Vancouver), AY, KL; molecular experiments, KS, RJ, KM,

ZP, KR; data analysis, KS, BS, MM; statistical analysis, AC, MM(Manan), NB, AD; microarray, QL; writing – original draft preparation, KS; writing – review and editing, CJR,TH, CC, PN, DB, IN, MM. MM takes overall guarantee for the data integrity of the manuscript. All authors have read and agreed to the submitted version of the manuscript.

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Conflicts of Interest

MM (Manali Mukherjee) is supported by early investigator award from Canadian Institutes of Health Research (CIHR) and Canadian Asthma Allergy and Immunology Foundation (CAAIF). MM reports grants from CIHR, grants from Methapharm Specialty Pharmaceuticals, personal fees from AstraZeneca, GlaxoSmithKline, consultant fees from Novartis, outside the submitted work. SS reports grants from Cyclomedica, personal fees from Arrowhead Pharmaceuticals, honorarium for lectures from AZ, honorarium for lectures from Novartis, and honorarium for lectures from Polarean, outside the submitted work. SW reports grants and consulting fees from Alk Abello, grants from Canadian Allergy, Asthma, and Immunology Foundation, Aimmune, grants and consulting fees from CSL Behring, grants from Takeda, personal and consulting fees from AZ, personal and consulting fees from GSK, consulting fees from Novartis, consulting and personal fees from Sanofi, consulting and personal fees from Medexus, consulting and personal fees from Miravo Health, consulting fees from AbbVie, consulting and personal fees from Bausch Lomb, outside of the submitted work. SW reports being president for CAAIF, board of directors for Asthma Canada, and medical advisor for Food Allergy Canada. PN reports grants and personal fees from AZ, grants and personal fees from Teva, grants and personal fees from Sanofi, personal fees from GSK, personal fees from Equillium, personal fees from Arrowhead pharma, grants from Foresee, grants from Cyclomedica, outside the submitted work. DB reports grants from COVID-19 Immunity Task Force / Public Health Agency of Canada, grants from National Science and Engineering Research Council (NSERC), grants from Canadian Institutes of Health Research, personal fees from AZ Mexico, personal fees for invited presentations from academic institutions, outside the submitted work. DB reports being on the board of directors for Lung Health Foundation and being an expert witness testimony for the Government of Canada. KS, RJ, AC, Manan Mukherjee, CV, KM, KZ, ZP, BS, AY, KL, BC, KR, CH, MK, ADG, JS, QL, CR, TH, NB, IN, CC have nothing to report.

Tables

Table 1. Demographics of Subjects in Final Study Cohort

Patient Group	Post-COVID Infection (PCI)	Non-COVID Infection (NCI)	Healthy Controls (HC)
Subjects, no.	106	34	22
Female sex, no. (%)	45 (42)	24 (71)*	11 (50)
Mean age, no. (%)	57 (20-89)	46 (20-67)*	49 (32-75)
BMI (kg/m^2)	27.2 ± 6.0	22.2 ± 12	26.6 ± 4.0
Home Recovery	26	33	-
Hospitalized, Non-ICU	45	0	-
Hospitalized, ICU	35	1	-
Symptoms at 3 months			
Fatigue, no. (%), n=89	32 (36)	-	0 (0)
Cough, no. (%), n=89	19 (21)	-	0 (0)
Dyspnea, no. (%), n=98	25 (26)	-	0 (0)
Symptoms at 6 months			
Fatigue, no. (%), n=47	19 (40)	-	-
Cough, no. (%), n=88	20 (23)	-	-
Dyspnea, no. (%), n=85	24 (28)	-	-
Symptoms at 12 months			
Fatigue, no. (%), n=50	10 (20)	-	-
Cough, no. (%) n=50	11 (22)	-	-
Dyspnea, no. (%) n=50	12 (24)	-	-

Data are presented as mean ± SD or ranges (minimum-maximum). *Indicates group with significant variation.

BMI – body mass index, ICU – intensive care unit

Table 2. Inflammatory Mediators Correlate with Prevalent ANAs and Symptomaticity at 12 Months Post-COVID

Correlation Analysis: Cytokines vs. ANA/ENAs				
Cytokine	**ANA**	**Timepoint**	**r Value**	**P-Value**
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
TNFα	Nucleosome		0.34	0.018
TNFα	Histone		0.38	0.008
TNFα	SS-A/Ro60		0.31	0.031
TNFα	U1-snRNP		0.41	0.003
TNFα	PM-Scl		0.54	<0.0001
Correlation Analysis: Cytokines vs. Symptoms				
Cytokine	**Symptom**	**Timepoint**	**r Value**	**P-Value**
D-dimer	Fatigue	3 months	0.33	0.002
IL-6	Dyspnea		0.21	0.051
IL-8	Dyspnea		0.20	0.068
TNFα	Cough	6 months	0.38	0.031
CRP	Cough		0.33	0.065
IL-6	Cough		0.31	0.074
TNFα	Fatigue	12 months	0.42	0.004

All significant correlations between measured sera cytokines and ANA MQVs at 12 months post-recovery have been indicated. Further all significant correlations between measured sera cytokines and patient reported outcomes at 3, 6, and 12 months post-recovery have been indicated.

Figure Legends

Figure 1. Study Consort Diagram.

A total of 22 healthy controls, 34 non-COVID infection controls, and 106 post-COVID infection patients were enrolled in this multi-center, prospective, longitudinal study. The post-COVID infection cohort were additional stratified based on severity of their acute phase infection: recovered at home (n=26), hospitalized non-ICU (n=45), and ICU admitted (n=35). Serum samples and symptoms were collected at 3, 6, and 12 months post-recovery for COVID survivors.

Figure 2. Autoantibody Signatures of COVID 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 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 5 most prevalent autoantibodies from the autoantigen array. (D-H) We evaluated ANA signatures in our control groups versus post-COVID cohort stratified based on severity of acute phase infection. (I-J) ICU-admitted post-COVID patients demonstrated significantly more ANA reactivities compared to home recovered post-COVID patients as well as control populations. Statistical analysis was done with Kruskal-Wallis test with Dunn's multiple comparisons test with significance set to $P<0.05$. A representative ANA Lineimmunossay strip is given for every sub-group with arrows indicating the positive autoantigen with MQV>1. ANA validation is available in the online supplementary (Figure E2).

Figure 3. Longitudinal Data of Circulating ANAs at 3, 6, and 12 Months Post-Recovery

(A-R) 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 timepoints. The shaded grey 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 timepoint for all ANAs.

Figure 4. Prevalence of Circulating ANAs at 3, 6, and 12 Months Post-Recovery

The majority of our (A) total PCI cohort had ≥2 ANA reactivities at 3 and 6 months post-COVID, but this proportion was reduced at 12 months. This attenuation is not evident in (B) home recovered post-COVID patients, but observed in (C) hospitalized and (D) ICU-admitted COVID survivors. Statistical analysis was performed with Chi-squared analysis. (E-G) Histograms visualize the most prevalent ANAs for n=57 patients with ANAs assessed at all three timepoints. Statistical analysis was done with Friedman test with Dunn's multiple comparisons test (H) Of the 85 positive ANA reactivities at 12 months post-COVID, 12% of samples were previously below cut-off threshold at both 3 and 6 months, indicating de novo autoantibody production post-COVID.

Figure 5. Prevalent 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) dyspnea at each timepoint. A heatmap presenting the z-scores of estimates are presented with * denoting $P<0.05$, and ** $P<0.01$. The regression analysis at 12 months post-recovery is displayed in a forest plot with the odds ratio (OR) and 95% confidence intervals (CI) for (D) fatigue, (E) cough, and (F) dyspnea. The orange lines indicate significant predictors for symptoms based on 95% CI of the regression model. 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 MQVs were compared between groups for each individual symptom. Statistical analysis was done with Mann-Whitney t-test for between group comparisons. Data are expressed as mean ± SD with significance set to $P<0.05$. (G-L) Correlation analysis was also conducted for these ANAs and each respective symptom, with significance set to $P<0.05$.

Figure 6. Inflammatory Mediators Predict Symptomaticity at Post-COVID

Principle component analysis was conducted to reduce the dimensionality of our nine cytokine variable dataset. 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 timepoint (3mo, n=100; 6mo, n=75;

12mo, n=51) to determine the influence and clustering of cytokines on symptoms (A-C). Multiple regression analysis was conducted for all cytokines at each timepoint for individual symptoms. A heatmap was generated using the regression estimates for (D) fatigue, (E) cough, and (F) dyspnea, with significance marked with an asterisk was set to $P<0.05$. Subsequently, the regression analysis is displayed in a forest plot with the odds ratio (OR) and 95% confidence intervals (CI) for (G) 3 months, (H) 6 months, and (I) 12 months post-recovery. The orange lines indicate significant predictors for symptoms based on 95% CI of the regression model. CRP – C-reactive protein, ICAM – intercellular adhesion molecule, IL – interleukin, TNF – tumor necrosis factor, VCAM – vascular cell adhesion molecule.

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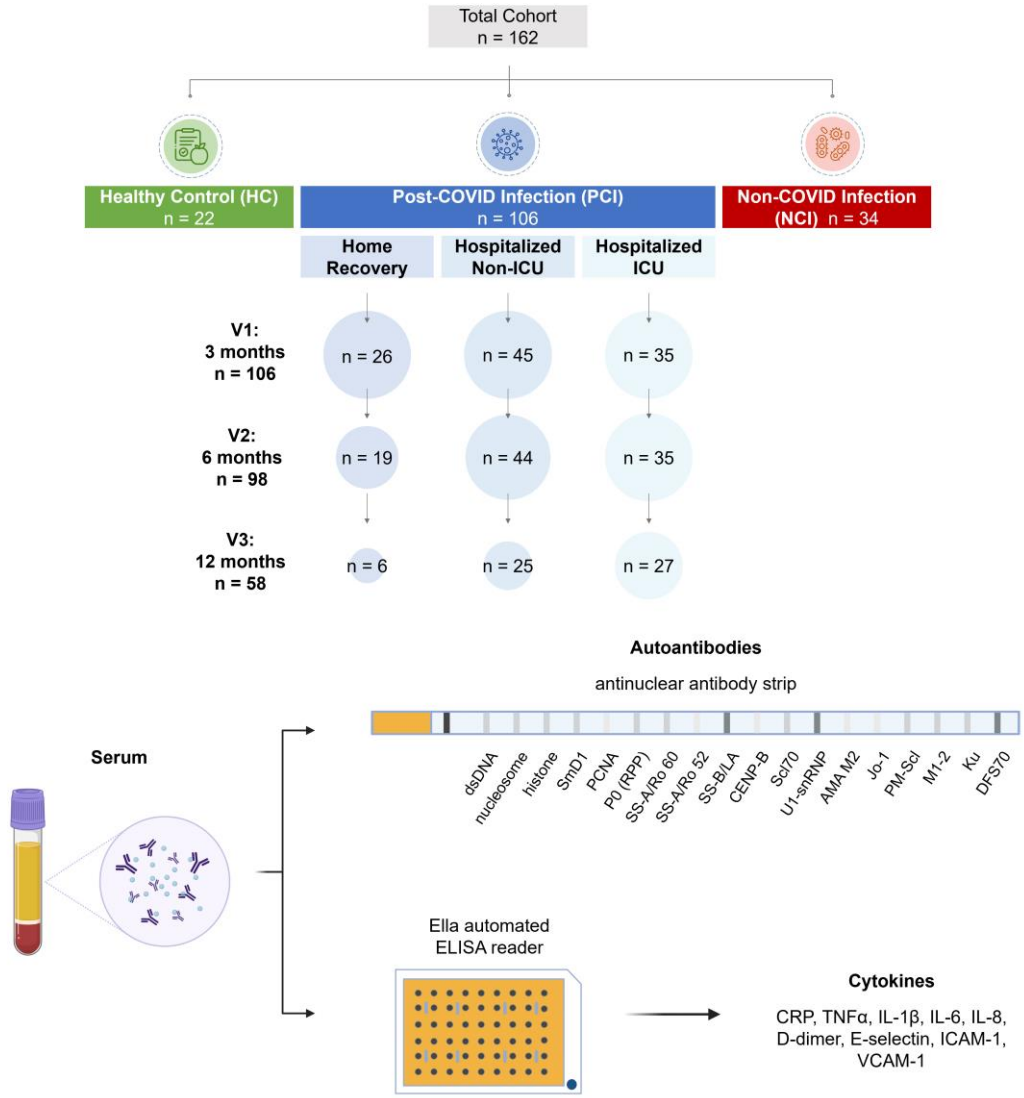
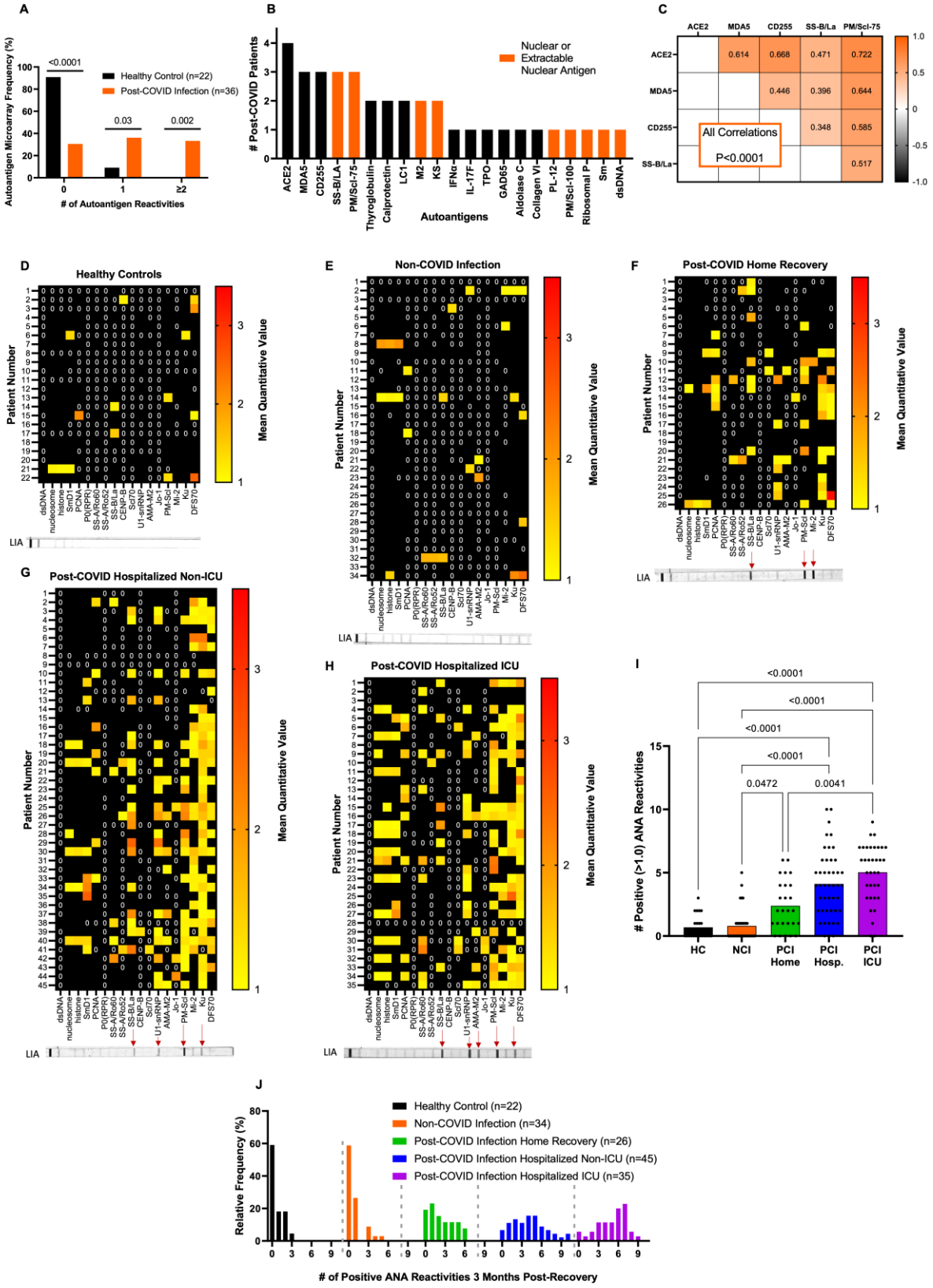
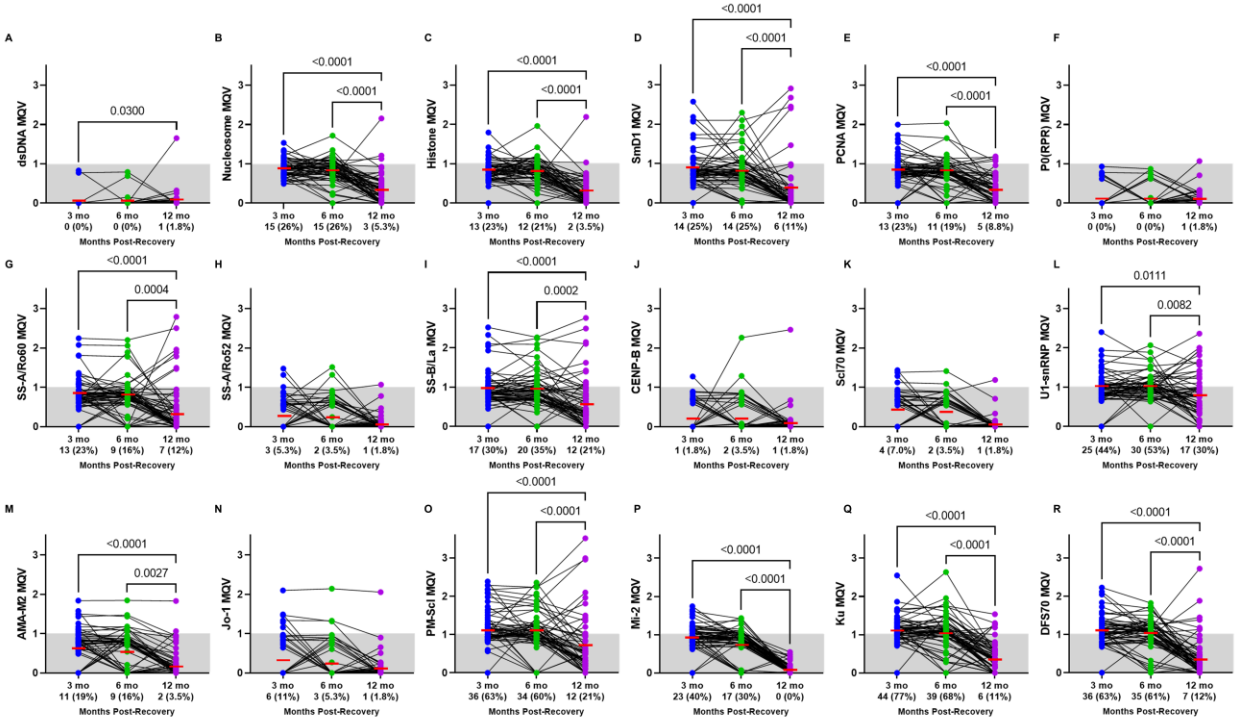
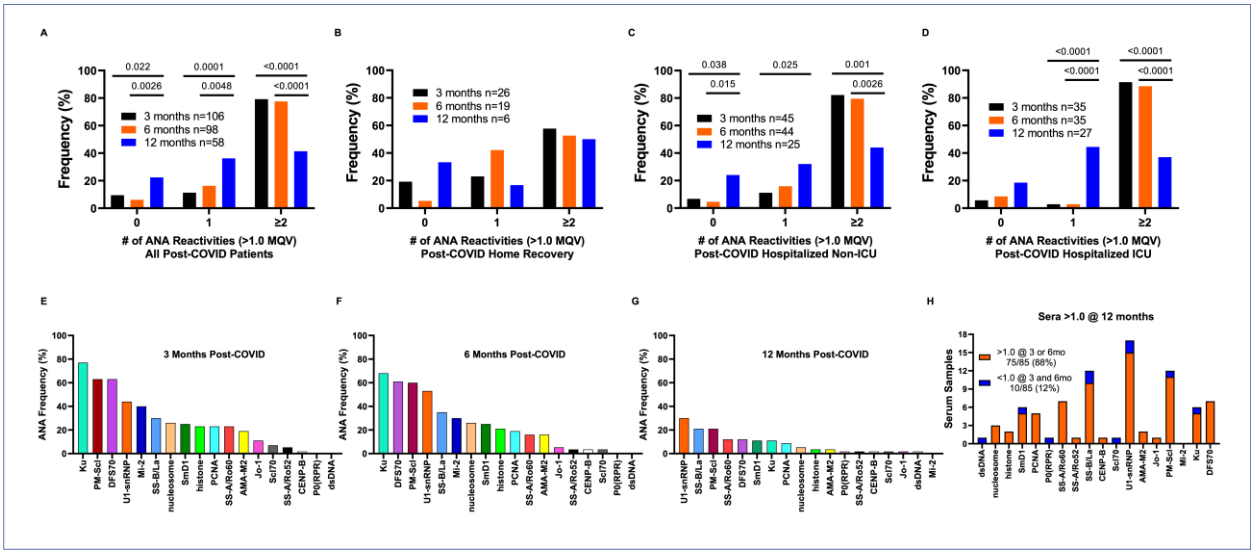
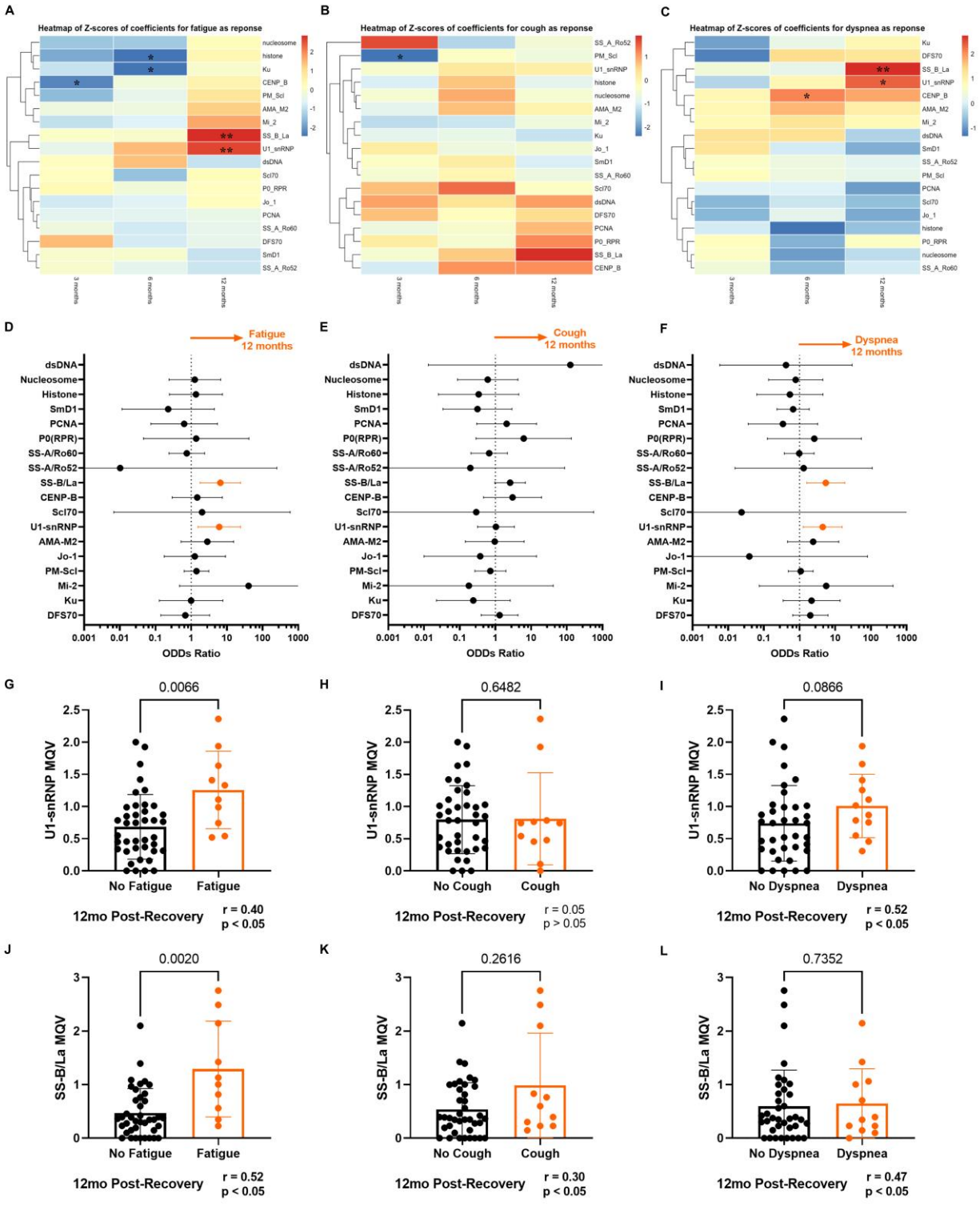
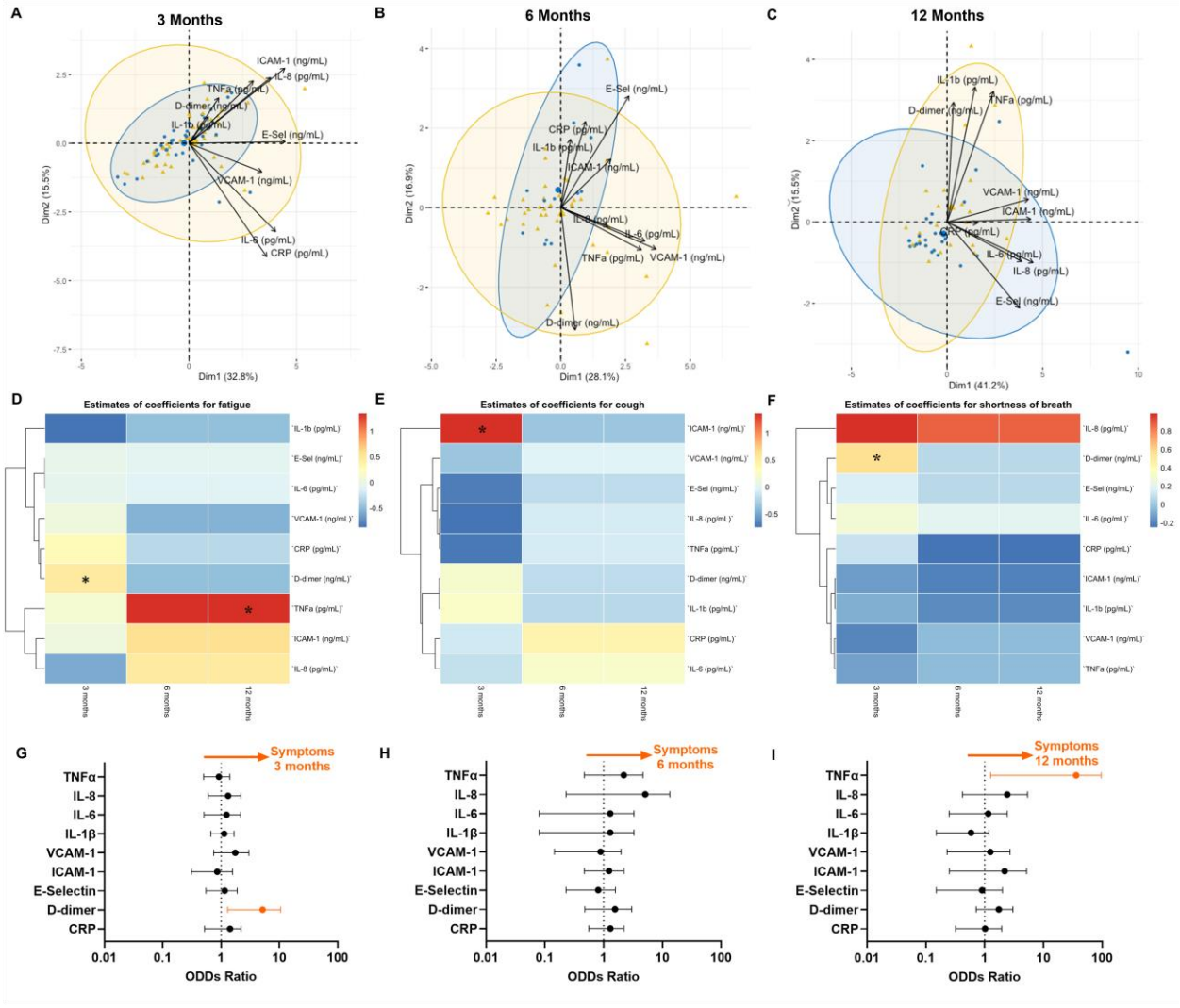

Total Cohort
n = 162
Healthy Control (HC)
n = 22
Post-COVID Infection (PCI)
n = 106
Non-COVID Infection (NCI) n = 34
Home Recovery
Hospitalized Non-ICU
Hospitalized ICU
V1:
3 months
n = 106
n = 26
n = 45
n = 35
V2:
6 months
n = 98
n = 19
n = 44
n = 35
V3:
12 months
n = 58
n = 6
n = 25
n = 27
Autoantibodies
antinuclear antibody strip
dsDNA
nucleosome
histone
SmD1
PCNA
P0 (RPP)
SS-A/Ro 60
SS-A/Ro 52
SS-B/LA
CENP-B
Scl70
U1-snRNP
AMA M2
Jo-1
PM-Scl
M1-2
Ku
DFS70
Serum
Ella automated
ELISA reader
Cytokines
CRP, TNFα, IL-1β, IL-6, IL-8,
D-dimer, E-selectin, ICAM-1,
VCAM-1


A
Autoantigen Microarray Frequency (%)
<0.0001
0.03
0.002
Healthy Control (n=22)
Post-COVID Infection (n=36)
0
1
≥2
# of Autoantigen Reactivities
B
Post-COVID Patients
Nuclear or Extractable Nuclear Antigen
ACE2
MDA5
CD255
SS-B/LA
PM/Scl-75
Thyroglobulin
Calprotectin
LC1
M2
KS
IFNα
IL-17F
TPO
GAD65
Aldolase C
Collagen VI
PL-12
PM/Scl-100
Ribosomal P
Sm
dsDNA
Autoantigens
C
ACE2
MDA5
CD255
SS-B/La
PM/Scl-75
0.614
0.668
0.471
0.722
0.446
0.396
0.644
0.348
0.585
0.517
All Correlations
P<0.0001
1.0
0.5
0
-0.5
-1.0
D
Healthy Controls
Patient Number
Mean Quantitative Value
LIA
E
Non-COVID Infection
F
Post-COVID Home Recovery
G
Post-COVID Hospitalized Non-ICU
H
Post-COVID Hospitalized ICU
I
Positive (>1.0) ANA Reactivities
<0.0001
0.0472
0.0041
HC
NCI
PCI Home
PCI Hosp.
PCI ICU
J
Relative Frequency (%)
Healthy Control (n=22)
Non-COVID Infection (n=34)
Post-COVID Infection Home Recovery (n=26)
Post-COVID Infection Hospitalized Non-ICU (n=45)
Post-COVID Infection Hospitalized ICU (n=35)
# of Positive ANA Reactivities 3 Months Post-Recovery


A
dsDNA MQV
0.0300
3 mo 0 (0%)
6 mo 0 (0%)
12 mo 1 (1.8%)
Months Post-Recovery
B
Nucleosome MQV
<0.0001
<0.0001
3 mo 15 (26%)
6 mo 15 (26%)
12 mo 3 (5.3%)
Months Post-Recovery
C
Histone MQV
<0.0001
<0.0001
3 mo 13 (23%)
6 mo 12 (21%)
12 mo 2 (3.5%)
Months Post-Recovery
D
SmD1 MQV
<0.0001
<0.0001
3 mo 14 (25%)
6 mo 14 (25%)
12 mo 6 (11%)
Months Post-Recovery
E
PCNA MQV
<0.0001
<0.0001
3 mo 13 (23%)
6 mo 11 (19%)
12 mo 5 (8.8%)
Months Post-Recovery
F
P0(RPR) MQV
3 mo 0 (0%)
6 mo 0 (0%)
12 mo 1 (1.8%)
Months Post-Recovery
G
SS-A/Ro60 MQV
<0.0001
0.0004
3 mo 13 (23%)
6 mo 9 (16%)
12 mo 7 (12%)
Months Post-Recovery
H
SS-A/Ro52 MQV
3 mo 3 (5.3%)
6 mo 2 (3.5%)
12 mo 1 (1.8%)
Months Post-Recovery
I
SS-B/La MQV
<0.0001
0.0002
3 mo 17 (30%)
6 mo 20 (35%)
12 mo 12 (21%)
Months Post-Recovery
J
CENP-B MQV
3 mo 1 (1.8%)
6 mo 2 (3.5%)
12 mo 1 (1.8%)
Months Post-Recovery
K
Scl70 MQV
3 mo 4 (7.0%)
6 mo 2 (3.5%)
12 mo 1 (1.8%)
Months Post-Recovery
L
U1-snRNP MQV
0.0111
0.0082
3 mo 25 (44%)
6 mo 30 (53%)
12 mo 17 (30%)
Months Post-Recovery
M
AMA-M2 MQV
<0.0001
0.0027
3 mo 11 (19%)
6 mo 9 (16%)
12 mo 2 (3.5%)
Months Post-Recovery
N
Jo-1 MQV
3 mo 6 (11%)
6 mo 3 (5.3%)
12 mo 1 (1.8%)
Months Post-Recovery
O
PM-Scl MQV
<0.0001
<0.0001
3 mo 36 (63%)
6 mo 34 (60%)
12 mo 12 (21%)
Months Post-Recovery
P
Mi-2 MQV
<0.0001
<0.0001
3 mo 23 (40%)
6 mo 17 (30%)
12 mo 0 (0%)
Months Post-Recovery
Q
Ku MQV
<0.0001
<0.0001
3 mo 44 (77%)
6 mo 39 (68%)
12 mo 6 (11%)
Months Post-Recovery
R
DFS70 MQV
<0.0001
<0.0001
3 mo 36 (63%)
6 mo 35 (61%)
12 mo 7 (12%)
Months Post-Recovery


A
Frequency (%)
0.022
0.0026
0.0001
0.0048
<0.0001
<0.0001
3 months n=106
6 months n=98
12 months n=58
0
1
≥2
# of ANA Reactivities (>1.0 MQV)
All Post-COVID Patients
B
3 months n=26
6 months n=19
12 months n=6
# of ANA Reactivities (>1.0 MQV)
Post-COVID Home Recovery
C
0.038
0.015
0.025
0.001
0.0026
3 months n=45
6 months n=44
12 months n=25
# of ANA Reactivities (>1.0 MQV)
Post-COVID Hospitalized Non-ICU
D
<0.0001
<0.0001
<0.0001
<0.0001
3 months n=35
6 months n=35
12 months n=27
# of ANA Reactivities (>1.0 MQV)
Post-COVID Hospitalized ICU
E
3 Months Post-COVID
ANA Frequency (%)
Ku
PM-Scl
DFS70
U1-snRNP
Mi-2
SS-B/La
nucleosome
SmD1
histone
PCNA
SS-A/Ro60
AMA-M2
Jo-1
Scl70
SS-A/Ro52
CENP-B
P0(RPR)
dsDNA
F
6 Months Post-COVID
ANA Frequency (%)
Ku
DFS70
PM-Scl
U1-snRNP
SS-B/La
Mi-2
nucleosome
SmD1
histone
PCNA
SS-A/Ro60
AMA-M2
Jo-1
SS-A/Ro52
CENP-B
Scl70
P0(RPR)
dsDNA
G
12 Months Post-COVID
ANA Frequency (%)
U1-snRNP
SS-B/La
PM-Scl
SS-A/Ro60
DFS70
SmD1
Ku
PCNA
nucleosome
histone
AMA-M2
P0(RPR)
SS-A/Ro52
CENP-B
Scl70
Jo-1
dsDNA
Mi-2
H
Sera >1.0 @ 12 months
Serum Samples
>1.0 @ 3 or 6mo
75/85 (88%)
<1.0 @ 3 and 6mo
10/85 (12%)
dsDNA
nucleosome
histone
SmD1
PCNA
P0(RPR)
SS-A/Ro60
SS-A/Ro52
SS-B/La
CENP-B
Scl70
U1-snRNP
AMA-M2
Jo-1
PM-Scl
Mi-2
Ku
DFS70


A
Heatmap of Z-scores of coefficients for fatigue as reponse
nucleosome
histone
Ku
CENP_B
PM_Scl
AMA_M2
Mi_2
SS_B_La
U1_snRNP
dsDNA
Scl70
P0_RPR
Jo_1
PCNA
SS_A_Ro60
DFS70
SmD1
SS_A_Ro52
3 months
6 months
12 months
B
Heatmap of Z-scores of coefficients for cough as reponse
SS_A_Ro52
PM_Scl
U1_snRNP
histone
nucleosome
AMA_M2
Mi_2
Ku
Jo_1
SmD1
SS_A_Ro60
Scl70
dsDNA
DFS70
PCNA
P0_RPR
SS_B_La
CENP_B
3 months
6 months
12 months
C
Heatmap of Z-scores of coefficients for dyspnea as reponse
Ku
DFS70
SS_B_La
U1_snRNP
CENP_B
AMA_M2
Mi_2
dsDNA
SmD1
SS_A_Ro52
PM_Scl
PCNA
Scl70
Jo_1
histone
P0_RPR
nucleosome
SS_A_Ro60
3 months
6 months
12 months
D
Fatigue
12 months
dsDNA
Nucleosome
Histone
SmD1
PCNA
P0(RPR)
SS-A/Ro60
SS-A/Ro52
SS-B/La
CENP-B
Scl70
U1-snRNP
AMA-M2
Jo-1
PM-Scl
Mi-2
Ku
DFS70
0.001
0.01
0.1
1
10
100
1000
ODDs Ratio
E
Cough
12 months
dsDNA
Nucleosome
Histone
SmD1
PCNA
P0(RPR)
SS-A/Ro60
SS-A/Ro52
SS-B/La
CENP-B
Scl70
U1-snRNP
AMA-M2
Jo-1
PM-Scl
Mi-2
Ku
DFS70
0.001
0.01
0.1
1
10
100
1000
ODDs Ratio
F
Dyspnea
12 months
dsDNA
Nucleosome
Histone
SmD1
PCNA
P0(RPR)
SS-A/Ro60
SS-A/Ro52
SS-B/La
CENP-B
Scl70
U1-snRNP
AMA-M2
Jo-1
PM-Scl
Mi-2
Ku
DFS70
0.001
0.01
0.1
1
10
100
1000
ODDs Ratio
G
0.0066
U1-snRNP MQV
2.5
2.0
1.5
1.0
0.5
0.0
No Fatigue
Fatigue
12mo Post-Recovery
r = 0.40
p < 0.05
H
0.6482
U1-snRNP MQV
2.5
2.0
1.5
1.0
0.5
0.0
No Cough
Cough
12mo Post-Recovery
r = 0.05
p > 0.05
I
0.0866
U1-snRNP MQV
2.5
2.0
1.5
1.0
0.5
0.0
No Dyspnea
Dyspnea
12mo Post-Recovery
r = 0.52
p < 0.05
J
0.0020
SS-B/La MQV
3
2
1
0
No Fatigue
Fatigue
12mo Post-Recovery
r = 0.52
p < 0.05
K
0.2616
SS-B/La MQV
3
2
1
0
No Cough
Cough
12mo Post-Recovery
r = 0.30
p < 0.05
L
0.7352
SS-B/La MQV
3
2
1
0
No Dyspnea
Dyspnea
12mo Post-Recovery
r = 0.47
p < 0.05


A
3 Months
ICAM-1 (ng/mL)
IL-8 (pg/mL)
TNFa (pg/mL)
D-dimer (ng/mL)
IL-1b (pg/mL)
E-Sel (ng/mL)
VCAM-1 (ng/mL)
IL-6 (pg/mL)
CRP (pg/mL)
Dim2 (15.5%)
Dim1 (32.8%)
B
6 Months
E-Sel (ng/mL)
CRP (pg/mL)
IL-1b (pg/mL)
ICAM-1 (ng/mL)
IL-8 (pg/mL)
IL-6 (pg/mL)
TNFa (pg/mL)
VCAM-1 (ng/mL)
D-dimer (ng/mL)
Dim2 (16.9%)
Dim1 (28.1%)
C
12 Months
IL-1b (pg/mL)
TNFa (pg/mL)
D-dimer (ng/mL)
VCAM-1 (ng/mL)
ICAM-1 (ng/mL)
CRP (pg/mL)
IL-6 (pg/mL)
IL-8 (pg/mL)
E-Sel (ng/mL)
Dim2 (15.5%)
Dim1 (41.2%)
D
Estimates of coefficients for fatigue
'IL-1b (pg/mL)'
'E-Sel (ng/mL)'
'IL-6 (pg/mL)'
'VCAM-1 (ng/mL)'
'CRP (pg/mL)'
'D-dimer (ng/mL)'
'TNFa (pg/mL)'
'ICAM-1 (ng/mL)'
'IL-8 (pg/mL)'
3 months
6 months
12 months
E
Estimates of coefficients for cough
'ICAM-1 (ng/mL)'
'VCAM-1 (ng/mL)'
'E-Sel (ng/mL)'
'IL-8 (pg/mL)'
'TNFa (pg/mL)'
'D-dimer (ng/mL)'
'IL-1b (pg/mL)'
'CRP (pg/mL)'
'IL-6 (pg/mL)'
3 months
6 months
12 months
F
Estimates of coefficients for shortness of breath
'IL-8 (pg/mL)'
'D-dimer (ng/mL)'
'E-Sel (ng/mL)'
'IL-6 (pg/mL)'
'CRP (pg/mL)'
'ICAM-1 (ng/mL)'
'IL-1b (pg/mL)'
'VCAM-1 (ng/mL)'
'TNFa (pg/mL)'
3 months
6 months
12 months
G
Symptoms 3 months
TNFα
IL-8
IL-6
IL-1β
VCAM-1
ICAM-1
E-Selectin
D-dimer
CRP
0.01
0.1
1
10
100
ODDs Ratio
H
Symptoms 6 months
TNFα
IL-8
IL-6
IL-1β
VCAM-1
ICAM-1
E-Selectin
D-dimer
CRP
0.01
0.1
1
10
100
ODDs Ratio
I
Symptoms 12 months
TNFα
IL-8
IL-6
IL-1β
VCAM-1
ICAM-1
E-Selectin
D-dimer
CRP
0.01
0.1
1
10
100
ODDs Ratio

Online supplementary figures

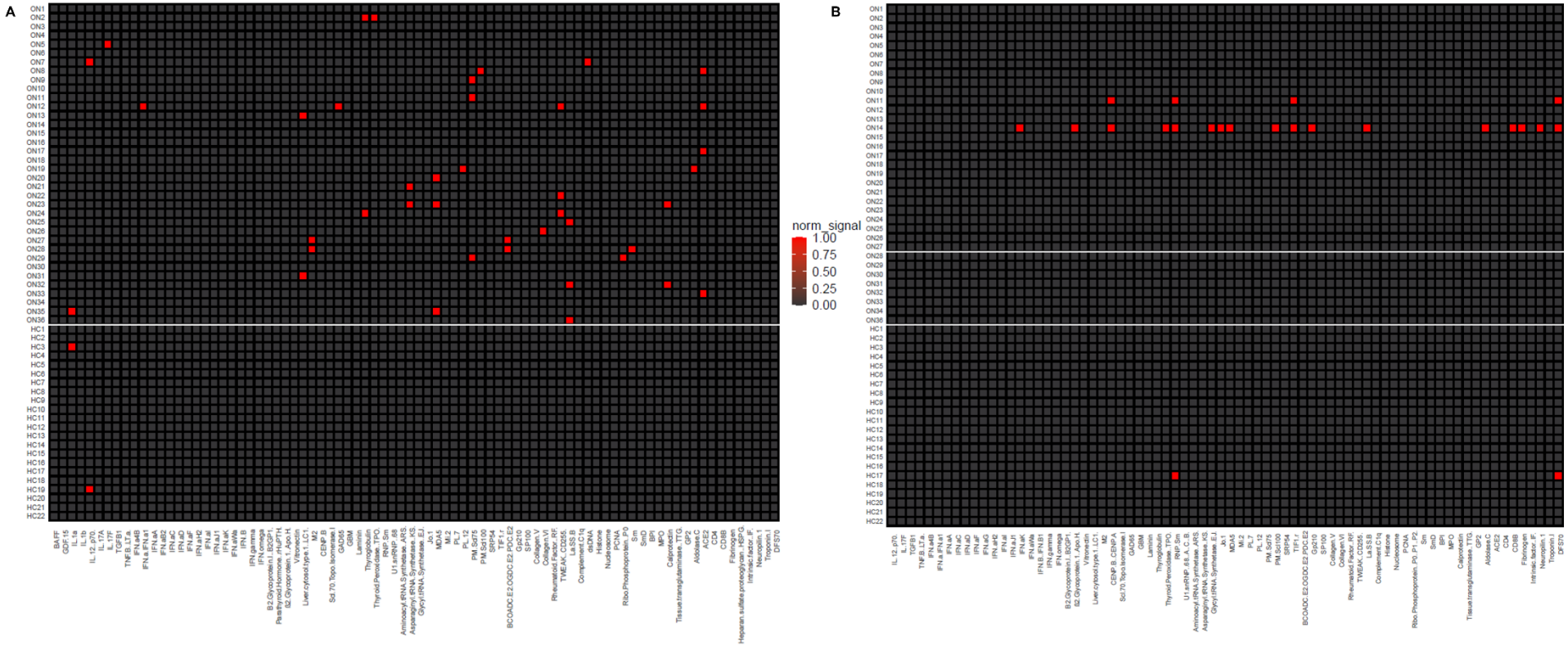


Figure E1. Microarray Autoantibody Profiling

Serum IgG (A) and IgM (B) antibody reactivities against 102 autoantigens were assessed. Positivity was calculated for each autoantigen based on the median + 3SD in the age-sex matched health control group, denoted by HC

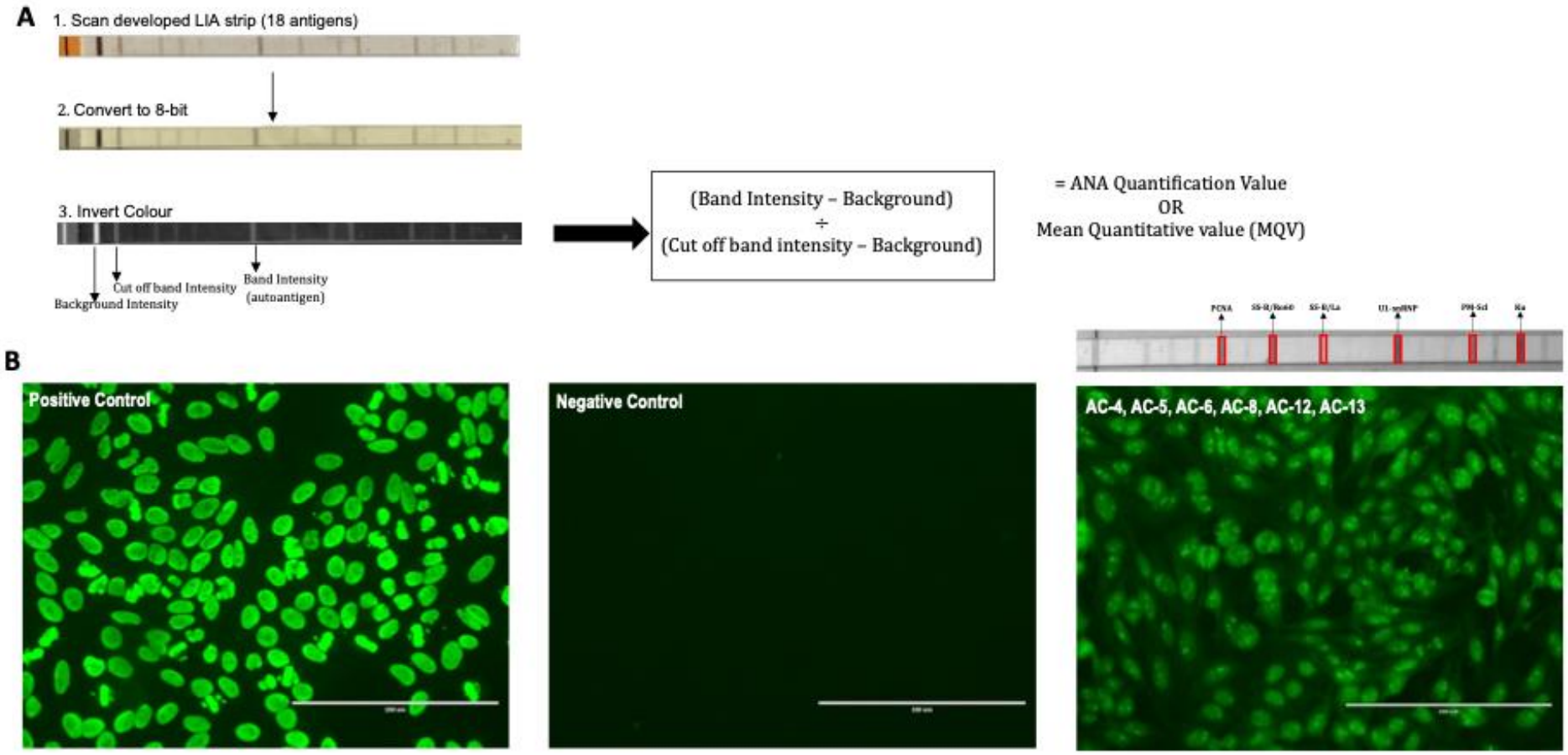


Figure E2. Validation of ANA/ENA Quantification Using Rapid Assessment Strips

(A) A scan of the rapid line immunoassay was converted into 8-bit and inverted to visualize band intensity. The fluorescence of each band was normalized to the background and cut-off band intensity to obtain the mean quantitative value (MQV). (B) HEp-2 cells were stained with sera of post-COVID patients and evaluated for anti-cell staining patterns as outlined by the International Consensus on Antinuclear Antibody (ANA) Patterns (ICAP). The patterns were then compared to positive ANA reactivities on the line immunoassay to confirm reliability and consistency of results.

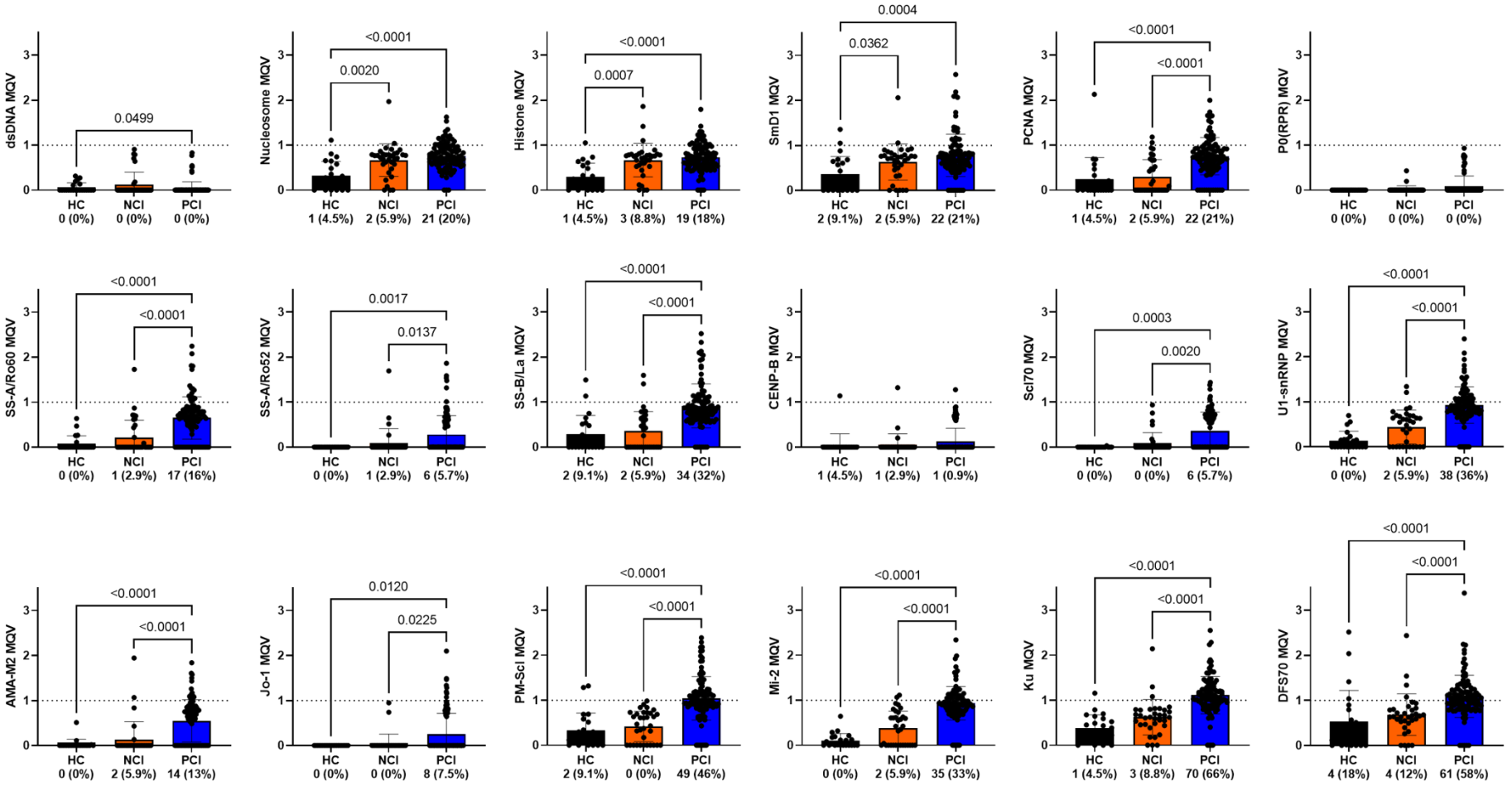


Figure E3. Differential Antinuclear Antibody Signatures at 3 Months Post-Recovery

Statistical analysis was performed with Kruskal-Wallis test with Dunn's multiple comparisons to assess difference between healthy controls (n=22), non-COVID infection controls (n=34), and post-COVID patients (n=106) at 3 months post-infection.

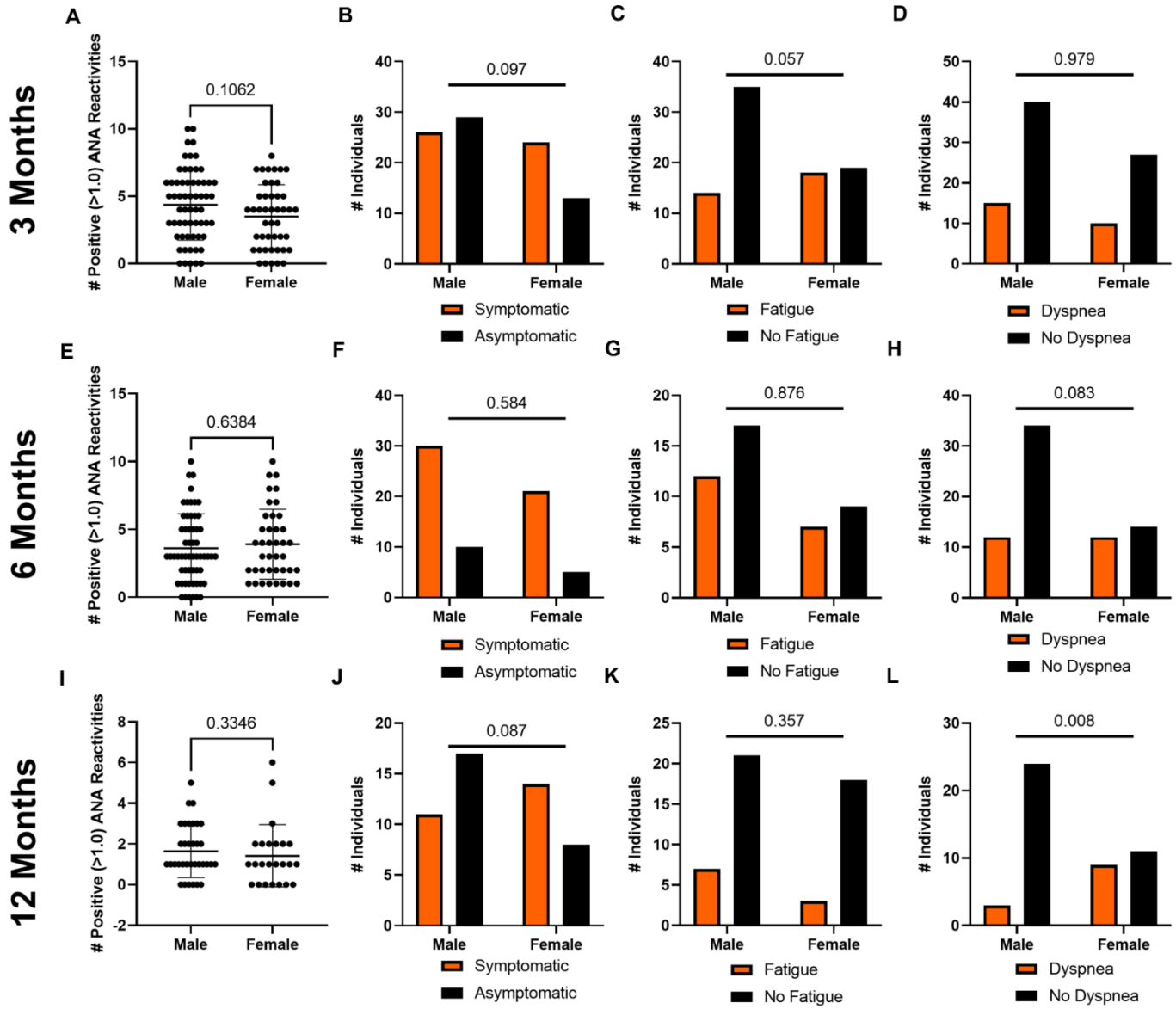


Figure E4. Impact of Sex on PASC Autoimmunity and Symptomaticity

(A) The frequency of positive ANA reactivities in our PCI cohort stratified by sex. Chi-squared analysis was conducted to evaluate the impact of sex on (B) symptomaticity, (C) fatigue, and (D) dyspnea at 3 months (A-D), 6 months (E-H) and 12 months (I-L) Significance was determined as $P<0.05$.

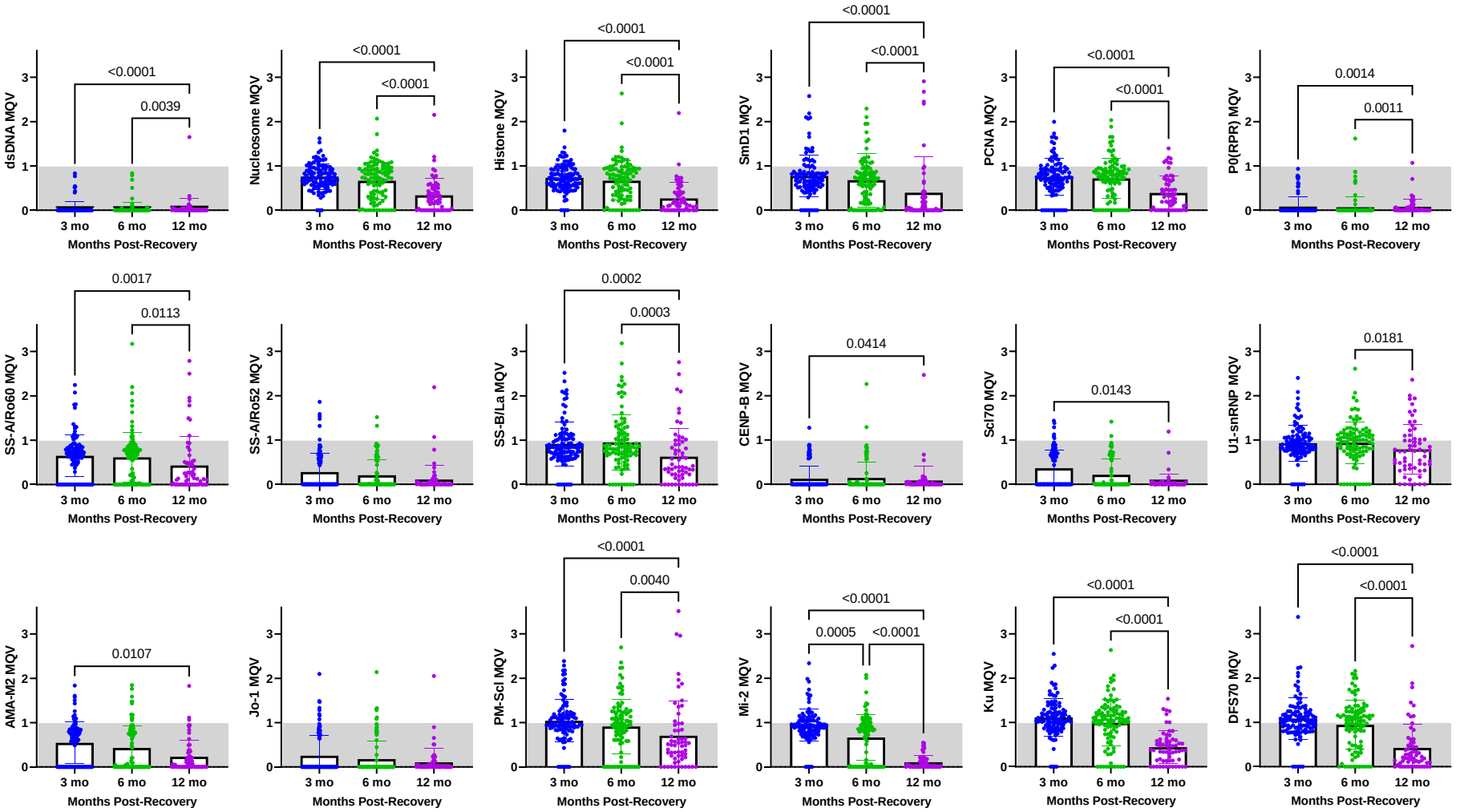


Figure E5. Differential Antinuclear Antibody Signatures at 3, 6, and 12 Months Post-Recovery

Statistical analysis was performed with Kruskal-Wallis test with Dunn's multiple comparisons to assess differences in circulating autoantibodies in patients at 3 months (n=106), 6 months (n=98), and 12 months (n=58) post-recovery.

Table E1. Inflammatory Mediators Associated with Prevalent ANAs and Symptomaticity at 12 Months Post-COVID

Multiple Logistic Regression Analysis: Cytokines vs ANAs – 12 Months						
Cytokine	**Predicted ANA**	**ANA Prevalence at 12 Months**	**Estimate**	**Standard Error**	**Z-Score**	**P-Value**
TNFα	SS-A/Ro60	12%	2.449	1.436	1.71	0.088
TNFα	U1-snRNP	30%	1.306	0.771	1.69	0.091
IL-6	PCNA	9%	4.166	1.824	2.28	0.022
IL-8	DFS70	12%	4.631	2.667	1.74	0.083
IL-8	PM-Scl	21%	-2.527	1.253	-2.02	0.044
CRP	PM-Scl	21%	2.122	1.219	1.74	0.082
CRP	SmD1	11%	4.329	2.605	1.66	0.097
VCAM-1	Ku	11%	3.9456	2.109	1.87	0.061

Multiple Logistic Regression Analysis: Cytokines vs Symptoms						
Cytokine	**Predicted Symptom**	**Timepoint**	**Estimate**	**Standard Error**	**Z-Score**	**P-Value**
D-dimer	Fatigue	3 months	1.010	0.395	2.56	0.011
ICAM-1	Cough		1.136	0.518	2.19	0.028
D-dimer	Dyspnea		0.553	0.245	2.26	0.024
D-dimer	Any Symptom		1.305	0.536	2.44	0.015
TNFα*	Fatigue	12 months	4.645	2.018	2.30	0.021
TNFα*	Any Symptom		2.399	1.105	2.17	0.030

A multiple regression analysis was performed to determine predictive power of cytokines on ANA reactivity at 12 months with significance set as $P<0.1$. All significantly predicted ANAs had a prevalence between 9-30% at 12 months post-COVID. In addition, a multiple regression analysis was conducted to determine if cytokines predict symptomaticity at each timepoint.

Table E2. Impact of Comorbidities on PASC Autoimmunity and Symptomaticity

Patient Comorbidity at 12 Months	**# (%)** N=57	**Avg. #aAbs w/ Comorb.**	**Avg. #aAbs w/o Comorb.**	**P-Value**	**Cough**	**P-Value**	**Fatigue**	**P-Value**	**Dyspnea**	**P-Value**
Cardiovascular	30 (53%)	1.5	1.7	0.8912	8/28	0.3062	5/28	0.7317	4/28	0.0922
Respiratory	11 (19%)	2.5	1.4	0.0916	4/9	0.0928	3/9	0.3636	4/9	0.1995
Gastrointestinal	6 (11%)	2.2	1.5	0.7298	0/6	0.3168	2/6	0.5859	2/6	0.6210
Endocrine	12 (21%)	2	1.5	0.2529	1/11	0.4162	2/11	>0.9999	4/11	0.4267
Renal	8 (14%)	2.1	1.5	0.1162	3/8	0.3506	4/8	**0.0407**	3/8	0.3858
Others	8 (14%)	2.3	1.5	0.2115	1/7	>0.9999	3/7	0.1326	2/7	>0.9999

The frequency of positive ANA reactivities in our post-COVID cohort at 12 months stratified by pre-existing comorbidities. Kruskal-Wallis test was conducted to determine whether comorbidities affected the autoimmune profile in our post-COVID population. Additionally, chi-squared analysis was performed to evaluate the impact of comorbidities on our symptoms at 12 months post-recovery. Significance was determined for all analyses as $P<0.05$.