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ORIGINAL ARTICLE

Autoimmunity and Clinical Immunology

Autoantibodies in COVID-19 correlate with antiviral humoral responses and distinct immune signatures

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Abstract

Background: Several autoimmune features occur during coronavirus disease 2019 (COVID-19), with possible implications for disease course, immunity, and autoimmune pathology. In this study, we longitudinally screened for clinically relevant systemic autoantibodies to assess their prevalence, temporal trajectory, and association with immunity, comorbidities, and severity of COVID-19.

Methods: We performed highly sensitive indirect immunofluorescence assays to detect antinuclear antibodies (ANA) and antineutrophil cytoplasmic antibodies (ANCA), along with serum proteomics and virome-wide serological profiling in a multicentric cohort of 175 COVID-19 patients followed up to 1 year after infection, eleven vaccinated individuals, and 41 unexposed controls.

Results: Compared with healthy controls, similar prevalence and patterns of ANA were present in patients during acute COVID-19 and recovery. However, the paired analysis revealed a subgroup of patients with transient presence of certain ANA patterns during acute COVID-19. Furthermore, patients with severe COVID-19 exhibited a high prevalence of ANCA during acute disease. These autoantibodies were quantitatively associated with higher SARS-CoV-2-specific antibody titers in COVID-19 patients and in vaccinated individuals, thus linking autoantibody production to increased antigen-specific humoral responses. Notably, the qualitative breadth of antibodies cross-reactive with other coronaviruses was comparable in ANA-positive and ANA-negative individuals during acute COVID-19. In autoantibody-positive patients, multiparametric characterization demonstrated an inflammatory signature during acute COVID-19 and alterations of the B-cell compartment after recovery.

Conclusion: Highly sensitive indirect immunofluorescence assays revealed transient autoantibody production during acute SARS-CoV-2 infection, while the presence of

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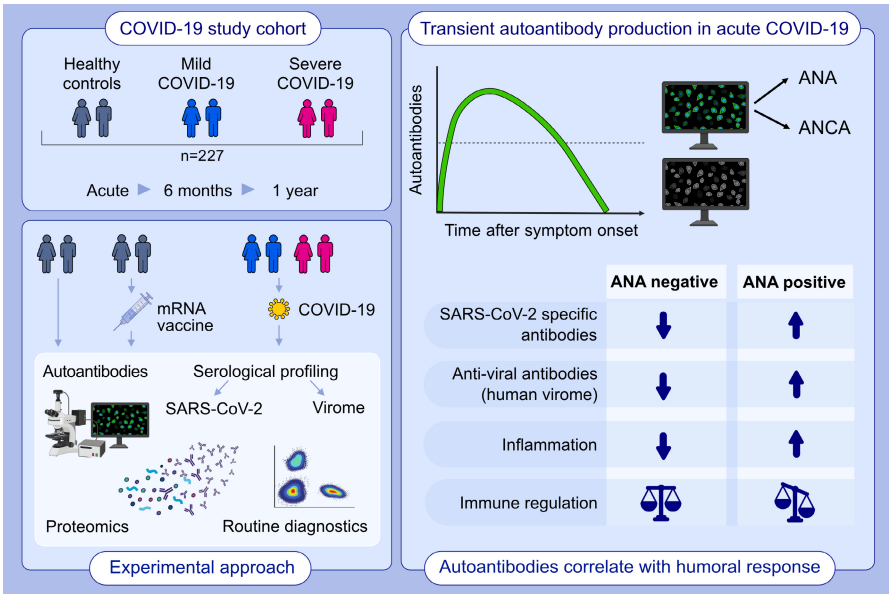
autoantibodies in COVID-19 patients correlated with increased antiviral humoral immune responses and inflammatory immune signatures.

KEYWORDS
antinuclear antibodies, autoantibodies, COVID-19, SARS-CoV-2, VirScan



GRAPHICAL ABSTRACT
In a multicentric cohort of 175 COVID-19 patients, 11 vaccinated individuals, and 41 unexposed controls, we measured ANA and ANCA, along with serum proteomics and virome-wide serological profiling. Paired analysis revealed the transient presence of ANA patterns and ANCA during acute COVID-19. The presence of autoantibodies correlated with increased virus-specific humoral immune responses and a proinflammatory immune signature.

1 | INTRODUCTION

Acute coronavirus disease 2019 (COVID-19) causes a large clinical spectrum, ranging from a mild condition in the majority of cases to fatal disease in 1–2% of subjects.[1–3] Several features of acute COVID-19 resemble clinical manifestations of systemic inflammatory and autoimmune diseases, such as fatigue, myalgia, hyperinflammation, thrombosis, and skin rashes.[3,4] Furthermore, COVID-19 may trigger the onset of autoimmune pathology, as reported for Guillain-Barré syndrome, antiphospholipid syndrome, vasculitis, and multisystem inflammatory syndrome in children.[5–9] *Vice versa*, autoimmune phenomena have been connected to the pathogenesis of severe COVID-19. Pre-existing autoantibodies targeting the type I interferon pathway have been found in about 10% of COVID-19 cases with critical disease.[10–12]

Other acute or chronic viral infections have been associated with autoimmune responses, which have been proposed to arise by molecular mimicry, epitope spreading, or bystander activation.[13] Various autoantibodies have been described in association with COVID-19, including antinuclear antibodies (ANA),[14–20] antineutrophil cytoplasmic antibodies (ANCA),[15,16,21] antiphospholipid antibodies,[5,8,14,17,19,22] and antibodies targeting different extracellular antigens.[11,16] While the presence of different autoantibodies has been associated with severe COVID-19 and worse outcome,[11,15,17–19] it remains unclear to what extent autoantibodies are triggered by acute infection, even though transient autoreactivity and new development of autoantibodies have been suggested in a subgroup of COVID-19 patients.[16,20] Furthermore, several aspects of autoantibodies in COVID-19, including their interplay with virus-specific humoral responses and their durability after acute infection, need further elucidation. In this study, we comprehensively characterized autoantibodies by using highly sensitive indirect immunofluorescence (IIF) assays in a multicentric prospective cohort of 227 individuals.

2 | RESULTS

2.1 | Presence of systemic autoantibodies during acute COVID-19 and recovery

We performed a comprehensive immunological characterization of 175 individuals with confirmed COVID-19 up to 1 year after infection (Figure 1A and Table 1, Table S1), including autoantibody screening by IIF, serum proteomics, and serological profiling. 41 individuals with

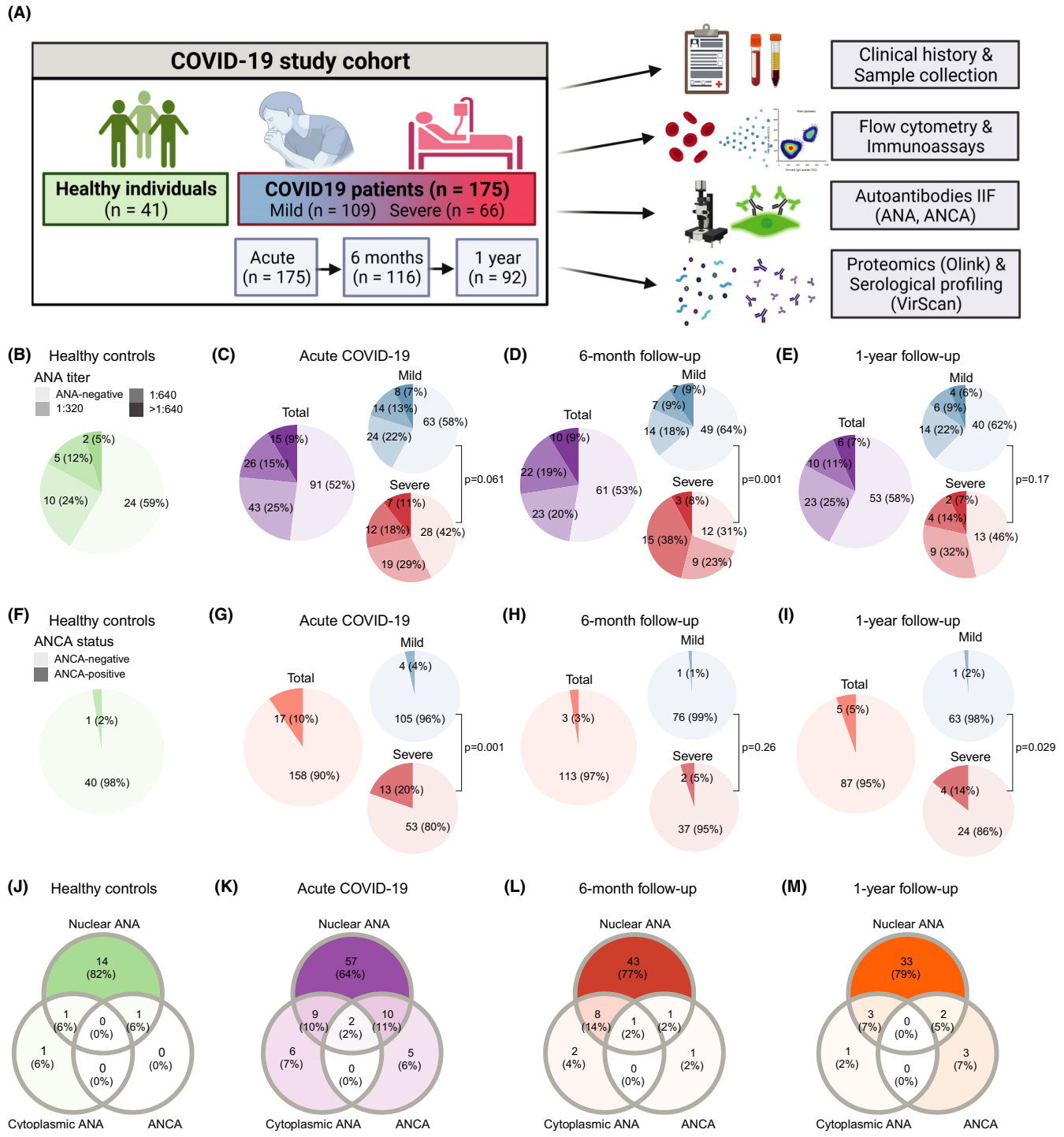


FIGURE 1 Prevalence of autoantibodies in healthy controls and COVID-19 patients during acute disease and follow-up. (A) Study overview. (B–I) Prevalence of ANA titers (B–E) and ANCA (F–I) in healthy controls ($n = 41$) and COVID-19 patients during acute disease ($n = 175$), 6 months ($n = 116$) and 1 year ($n = 92$) after symptom onset. (J–M) Venn diagrams depicting co-occurrence of nuclear ANA, cytoplasmic ANA, and ANCA in healthy individuals (J; $n = 17$), acute COVID-19 patients (K; $n = 89$), and COVID-19 patients 6 months (L; $n = 56$) or 1 year (M; $n = 42$) after SARS-CoV-2 infection that presented with at least one type of autoantibody. *p*-values indicate comparison of ANA (B–E) and ANCA (F–I) prevalence between mild and severe COVID-19 patients using the Fisher's exact test

negative history and serology for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection were included as controls (Figure 1A and Table 1). Furthermore, eleven unexposed individuals were sampled before and after vaccination with BNT162b2 (Table S2).

Using a highly sensitive IIF screening assay, we detected titers of 1:320 and above in 17 of 41 (41.4%) healthy individuals (Figure 1B), which followed an insignificant trend toward higher ANA prevalence in older participants (Figure S1A). The prevalence of ANA

TABLE 1 COVID-19 study cohort characteristics

	Healthy controls	COVID-19 Acute disease		COVID-19 6-month follow-up	
Disease severity		**Mild**	**Severe**	**Mild**	**Severe**
n (%)	41	109 (62.3%)	66 (37.7%)	77 (66.3%)	39 (33.7%)
Patient characteristics					
Age	32 (28–52)	34 (28–52)	68 (57–78)	36 (29–53)	64 (58–74)
Days after symptom onset		10 (7–16)	14 (9–27)	194 (185–205)	211 (194–224)
Sex (female)	24 (58.5%)	54 (49.5%)	27 (40.9%)	40 (51.3%)	14 (35.9%)
Vaccinated	0	0	0	9 (11.7%)	3 (7.7%)
Hospitalized	–	20 (18.3%)	66 (100%)	12 (15.6%)	39 (100%)
Laboratory parameters					
Lymphocyte count (G/L)	1.80 (1.48–2.33)	1.81 (1.18–2.23) ns	0.76 (0.56–1.10)****	1.96 (1.67–2.37) ns	1.77 (1.48–2.34) ns
CRP (mg/L)	0.6 (0.4–1.6)	1.3 (0.6–5.3)**	59.2 (32.2–119.0)****	0.6 (0.6–1.3) ns	1.7 (2.2–5.1)**
TNF-α (ng/L)	8.1 (6.4–10.0)	9.8 (7.6–12)**	16.4 (13.0–20.6)****	9 (6.8–10.9) ns	11.8 (9.3–15.0)****
IL-6 (ng/L)	0.5 (0–1.1)	1.3 (0.1–4.9)***	19.5 (7.4–57.0)****	0.9 (0–2.1)*	1.6 (0.3–5.3)**
S1-specific IgA (OD ratio)	0.33 (0.25–0.46)	1.77 (0.73–4.81)****	7.24 (2.52–10.2)****	2.52 (1.54–4.96)****	5.09 (3.12–7.75)****
S1-specific IgG (OD ratio)	0.20 (0.17–0.25)	0.61 (0.27–2.19)****	5.12 (0.32–9.33)****	2.67 (1.29–5.69)****	6.91 (5.18–8.49)****
Comorbidities					
Hypertension (*n*)	5 (12.2%)	12 (11.0%) ns	38 (57.8%)***	6 (7.8%) ns	21 (53.8%)**
Diabetes (*n*)	2 (4.9%)	6 (5.5%) ns	19 (28.8%)*	4 (5.2%) ns	12 (30.7%)**
Heart disease (*n*)	1 (2.4%)	6 (5.5%) ns	24 (36.6%)***	2 (2.6%) ns	15 (38.5%)***
Lung disease (*n*)	5 (12.2%)	10 (9.2%) ns	12 (18.8%)*	5 (6.5%) ns	11 (28.2%) ns
Malignancy (*n*)	1 (2.4%)	3 (2.8%) ns	8 (12.1%)**	3 (3.9%) ns	5 (12.8%) ns
Kidney disease (*n*)	0 (0%)	8 (7.3%) ns	17 (25.8%)***	3 (3.9%) ns	10 (25.6%)**
Autoimmune disease (*n*)	3 (7.3%)	6 (5.5%) ns	7 (10.6%) ns	6 (7.8%) ns	6 (15.4%) ns

Note: Medians and interquartile ranges (in parentheses) are specified for continuous variables, with *p*-values obtained by the Mann–Whitney *U* test, compared with healthy individuals. Numbers of individuals (*n*) and percentages of the corresponding subgroup (in parentheses) are shown for categorical variables, with *p*-values calculated by the Fisher's exact test, in comparison with healthy individuals.

Abbreviations: ns, nonsignificant; OD, optical density.

*$p < .05$.; **$p < .01$.; ***$p < .001$.; ****$p < .0001$.

positivity in healthy individuals was similar to that in COVID-19 patients during acute disease (48.0%, odds ratio (OR) = 1.30, $p = .49$) and 6 months (47.4%, OR = 1.27, $p = .59$), and 1 year after recovery (42.3%, OR = 1.04, $p = 1$) (Figure 1C–E). Most of the observed ANA titers were just above the positivity threshold of 1:320. Interestingly, we observed a trend of higher ANA prevalence in individuals with severe COVID-19 compared with mild disease during acute infection (OR 1.85, $p = .061$), which was significantly higher 6 months after recovery (OR = 3.81, $p = .0015$) (Figure 1C,D).

Similarly, we used an IIF assay to detect ANCA. ANCA prevalence was similar in mild COVID-19 patients during acute disease (3.6%) compared with healthy individuals (2.4%) (Figure 1F,G). Conversely, we observed a significantly higher ANCA prevalence in severe acute COVID-19 patients (19.7%), both compared with healthy subjects ($p = .016$) and mild COVID-19 cases ($p = .00096$) (Figure 1F,G), which returned to ranges seen in healthy individuals after 6 months (5.1%, $p = .61$) and 1 year (14.3%, $p = .15$) (Figure 1H,I).

In several patients, nuclear ANA, cytoplasmic ANA, or ANCA were detected concurrently, particularly during acute COVID-19 (Figure 1J–M). Moreover, ANCA showed a tendency to be more frequent in ANA-positive (14.3%) compared with ANA-negative (5.5%) individuals during acute COVID-19 ($p = .06$) (Figure 1K).

2.2 | Characteristics of ANA and ANCA patterns in acute COVID-19

To gain a qualitative appreciation, we classified ANA patterns according to the international consensus on ANA pattern anticell (AC) nomenclature.[23] ANA patterns were very similar in healthy controls and COVID-19 patients at all three sampling time points, and in some participants, different patterns were detected concurrently (Figure 2A–D, Figure S2A–D). The most common nuclear patterns were fine-granular nuclear (AC-4 or AC-4 like) and nucleolar (AC-8, AC-9, and AC-10), whereas the most common cytoplasmic patterns were speckled (AC-19 and AC-20) (Figure 2E–G).

Antineutrophil cytoplasmic antibodies patterns observed during acute COVID-19 and follow-up were mostly cytoplasmic (Figure 2H).

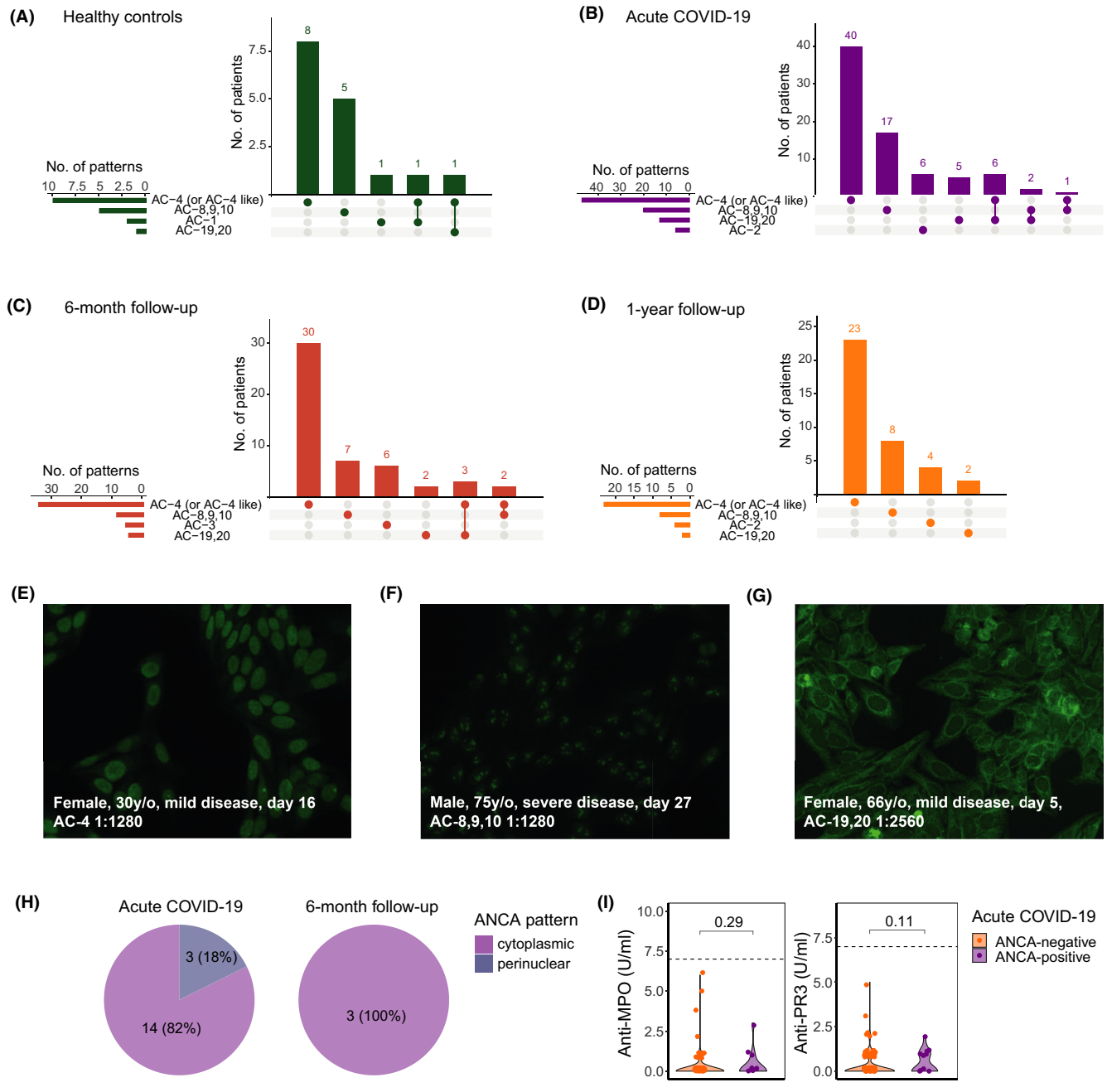


FIGURE 2 IIF pattern of autoantibodies in acute and recovered COVID-19. (A–D) Intersection plots showing counts of the four most prevalent ANA patterns (horizontal bars) and counts of pattern combinations (vertical bars) as indicated by the dot matrix, for healthy controls (A), and COVID-19 patients during acute disease (B), 6 months (C), and 1 year after symptom onset (D). (E–G) Example IIF pictures showing the most common nuclear, including fine-granular (E) and nucleolar (F), and cytoplasmic, including speckled (G), ANA patterns observed in the study cohort. All images were recorded at a dilution of 1:320. y/o—years old. (H) IIF ANCA patterns observed in ANCA-positive COVID-19 patients during acute disease ($n = 17$) and 6 months after recovery ($n = 3$). (I) Anti-MPO and anti-PR3 antibodies during acute COVID-19 ($n = 175$) in ANCA-positive and ANCA-negative individuals. Dashed lines indicate diagnostic cut-off values

However, cytoplasmic patterns were atypical, and accordingly, none of the ANCA-positive patients showed positivity for either antimyeloperoxidase (MPO) or antiproteinase 3 (PR3) antibodies (Figure 2I), suggesting other antigen specificities than commonly found in ANCA-associated vasculitis.[24,25]

2.3 | Temporal trajectory of autoantibodies in individual COVID-19 patients

To appreciate changes in ANA and ANCA on an individual level, we performed the paired analysis of all followed-up COVID-19 patients

($n = 129$). For mild and severe COVID-19 patients combined, we observed similar proportions of patients with isolated ANA positivity during acute disease (14.7%) and follow-up (12.4%). However, a trend toward a higher proportion of new ANA development at follow-up visit was evident in patients with severe COVID-19 (20.5%) compared to patients with mild COVID-19 (8.2%) (OR = 2.84, $p = .054$) (Figure 3A,B). To also account for subtle changes in IIF patterns, we conducted a blinded, paired analysis of IIF images to identify patterns that were transiently present either during acute disease or follow-up. Strikingly, we found transient patterns in 11 of 62 (17.7%) ANA-positive COVID-19 patients during acute disease, with speckled cytoplasmic (AC-19 and 20), nucleolar (AC-8, 9, 10), and mitotic being the most frequent patterns (Figure 3C,D). In stark contrast, only three of 59 (5.1%) ANA-positive individuals presented with a pattern during follow-up that was not present during acute disease, thus demonstrating that transient ANA patterns were significantly more prevalent during acute COVID-19 ($p = .045$) (Figure 3C).

For ANCA, we observed that of ten patients that tested positive during acute COVID-19, eight were negative during follow-up, whereas only two remained positive. Furthermore, only one

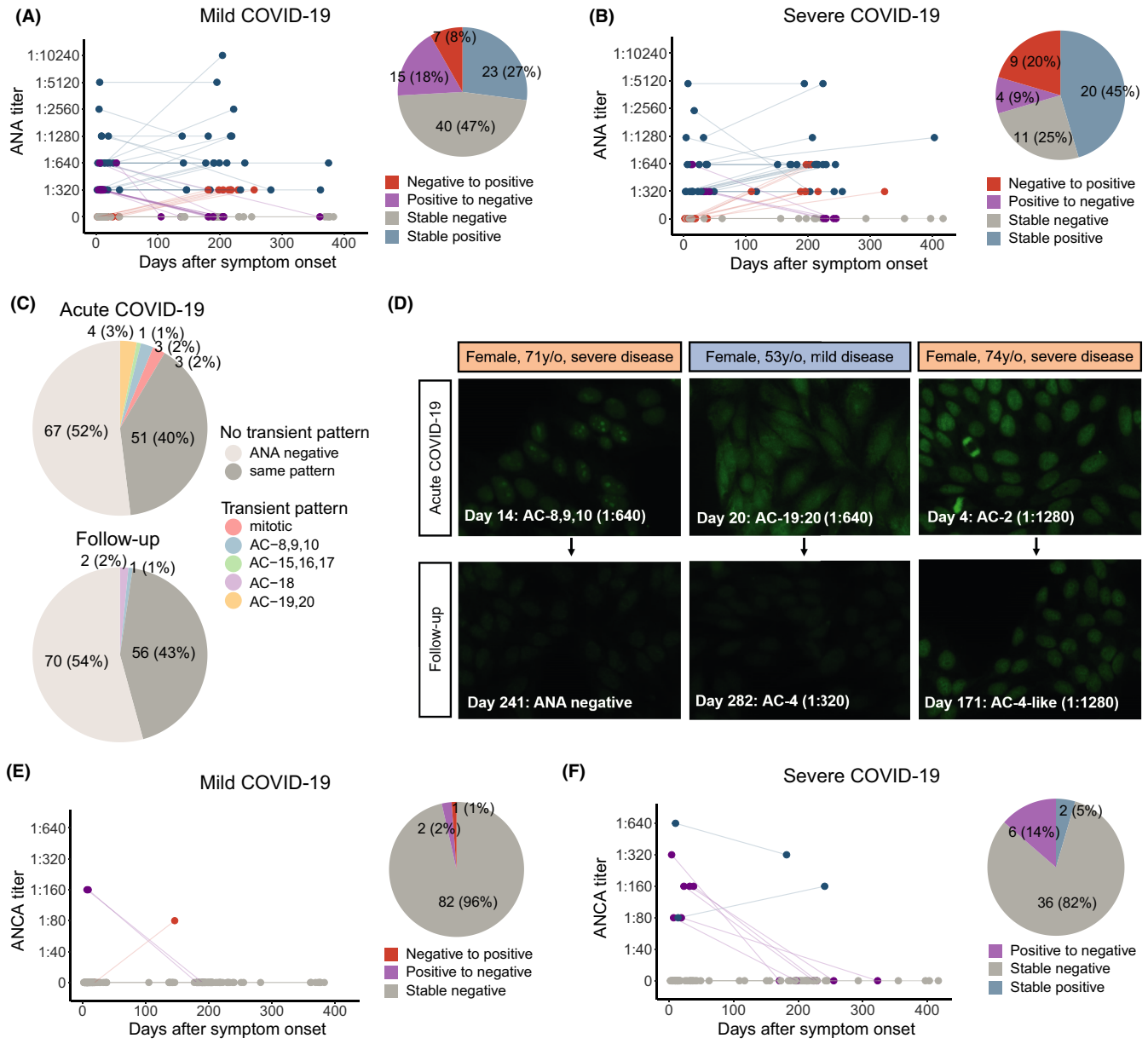


FIGURE 3 Paired longitudinal comparison indicates transient induction of autoantibodies in acute COVID-19. (A–B) Temporal trajectory of ANA titers in mild (A, $n = 85$) and severe (B, $n = 44$) COVID-19 patients, showing the first available follow-up sample, i.e., at 6 months ($n = 116$) or 1 year ($n = 13$) after symptom onset. Colors indicate development of ANA status from acute disease to follow-up. (C) Results from blinded, paired IIF picture analysis ($n = 129$). Patterns that were uniquely observed at one timepoint are colored. (D) Exemplary IIF pictures of three patients exhibiting transient ANA patterns during acute COVID-19, with a transient nucleolar (left), cytoplasmic (middle) or mitotic (right) pattern. All pictures were recorded at a dilution of 1:320. y/o, years old. (E–F) Temporal trajectory of ANCA titers in mild (E, $n = 85$) and severe (F, $n = 44$) COVID-19 patients. Colors indicate development of ANCA status from acute disease to follow-up

patient newly exhibited positive ANCA at follow-up (Figure 3E,F). Collectively, we found that a subgroup of individuals shows ANA and atypical ANCA production during the acute phase of COVID-19, which usually subsides during follow-up.

2.4 | Virus-specific responses in autoantibody-positive and SARS-CoV-2-vaccinated subjects

To elucidate the influence of autoantibody production during acute infection, we investigated the correlation of autoantibodies with specific humoral immune responses to SARS-CoV-2. We longitudinally assessed SARS-CoV-2 spike 1 (S1)-specific immunoglobulin A (IgA) and IgG titers and found that the presence of ANA was associated with higher concentrations of S1-specific antibodies in COVID-19 patients during acute disease, which extended to 6 months after recovery (Figure 4A). Conversely, 1 year after recovery, we did not observe any differences (Figure S3). The presence of ANA correlated significantly with S1-specific IgG levels even after accounting for age, disease severity, and sampling time point in a multiple linear regression model, which was not the case of S1-specific IgA (Figure 4B, Table S3). Similarly, we found higher S1-specific IgA and a trend toward higher IgG titers in patients that tested positive for ANCA during acute disease (Figure 4C).

To elucidate whether autoantibodies were associated with an increased humoral immune response only after natural SARS-CoV-2 infection or also after other antigen-specific immune responses, we measured ANA and S1-specific antibodies in 11 individuals before and after COVID-19 vaccination with BNT162b2 (Figure 4D–F, Table S2). Although a tendency of an increased ANA prevalence following the first vaccine shot was apparent, no significant difference was observed between sampling timepoints (Figure 4D). We observed higher S1-specific IgA in ANA-positive individuals when combining data from 2 and 4 weeks after the first vaccine shot, whereas no difference was observed for IgG (Figure 4E,F). In summary, these findings suggest the presence of autoantibodies is associated with increased S1-specific humoral responses following acute COVID-19 up to 6 months after recovery and following SARS-CoV-2 vaccination.

2.5 | Human virome-wide serological profiling in acute COVID-19

Next, we sought to investigate qualitative aspects of antibody responses during acute COVID-19 with respect to previous antiviral humoral responses in ANA-positive and ANA-negative individuals. Based on the phage immunoprecipitation sequencing (PhIP-seq) technology (VirScan),[26] we performed human virome-wide serological profiling in 97 acute COVID-19 patients and 18 healthy controls. We assessed the results of antibodies directed to 112 different viruses (Table S4), with data for a total of 87,890 epitopes, consisting of 56-amino acid (AA)-long, overlapping peptides. The library comprised all six human coronaviruses (HCoV) described before the COVID-19 pandemic, including HCoV-HKU1, HCoV-NL63, HCoV-229E, betacoronavirus 1 (BCoV1, including HCoV-OC43), severe acute respiratory syndrome-related coronavirus (SARS-CoV), and Middle East respiratory syndrome-related coronavirus (MERS-CoV).

A multivariate analysis using the summed epitope hits per viral species revealed distinct differences in COVID-19 patients compared with healthy controls, which were particularly pronounced more than 1 week after symptom onset (Figure 5A). Between-group comparisons of COVID-19 patients and healthy controls revealed a significant difference ($p < .005$) in summed epitope hits for eight viral species (Figure 5B). Of these, four enterovirus species were more abundant in healthy controls. Conversely, antibodies targeting cytomegalovirus (CMV) and Pegivirus A, and those directed to SARS-CoV and MERS-CoV were significantly more abundant in COVID-19 patients, whereas antibodies targeting the four common coronaviruses HCoV-HKU1, HCoV-NL63, HCoV-229E, and BCoV1 showed a parallel but insignificant trend ($p > .005$) (Figure 5B). Antibodies directed to all coronavirus species correlated positively with time from symptom onset (Figure 5C), thus indicating the production of cross-reactive antibodies during acute COVID-19.

To further study antibodies targeting CoVs in acute COVID-19, we evaluated serological profiles on a singular epitope level. We found a significantly higher ($p < .05$) proportion of COVID-19 patients tested positive for a total of 18 CoV epitopes compared with healthy controls, of which 16 were in the spike and two in the nucleoprotein (Figure 5D, Figure S4A). Since healthy individuals tested negative for these but positive for only one epitope (Figure 5D,E), we hypothesized these 18 CoV epitopes enriched in COVID-19 patients were targeted by antibodies newly produced during acute COVID-19 and cross-reactive with shared epitopes of other CoVs. Pairwise protein alignment of these epitopes with corresponding SARS-CoV-2 proteins allowed identification of regions of SARS-CoV-2 spike and nucleoprotein targeted by cross-reactive antibodies, comprising two segments (AA positions 777–886 and 1105–1195) of spike S2 domain and one segment of nucleoprotein (AA 140–252), which have been previously identified in COVID-19 patients.[27] Patients with severe COVID-19 tested positive for significantly more cross-reactive antibodies than mild disease patients (Figure 5E). However, no significant difference was observed in ANA-positive compared with ANA-negative patients (Figure 5F), although the proportion of ANA-positive patients that tested positive was slightly higher for most cross-reactive epitopes (Figure 5G).

To explore potential correlations of ANA with humoral responses against other viruses, we compared seroreactivity against all tested viral epitopes and ANA positivity. Several epitopes were detected more frequently ($p < .005$) in ANA-positive, but not in ANA-negative, individuals (Figure 5H). Three of the identified peptides were located on Epstein-Barr virus (EBV) nuclear antigen 2 (EBNA-2), to which ANA-positive participants showed more epitope hits, independent of age (Figure S4B). When combining data of all available epitopes, we found significantly more hits in ANA-positive compared with ANA-negative participants, which was most pronounced at a younger age (Figure 5I). Thus, human

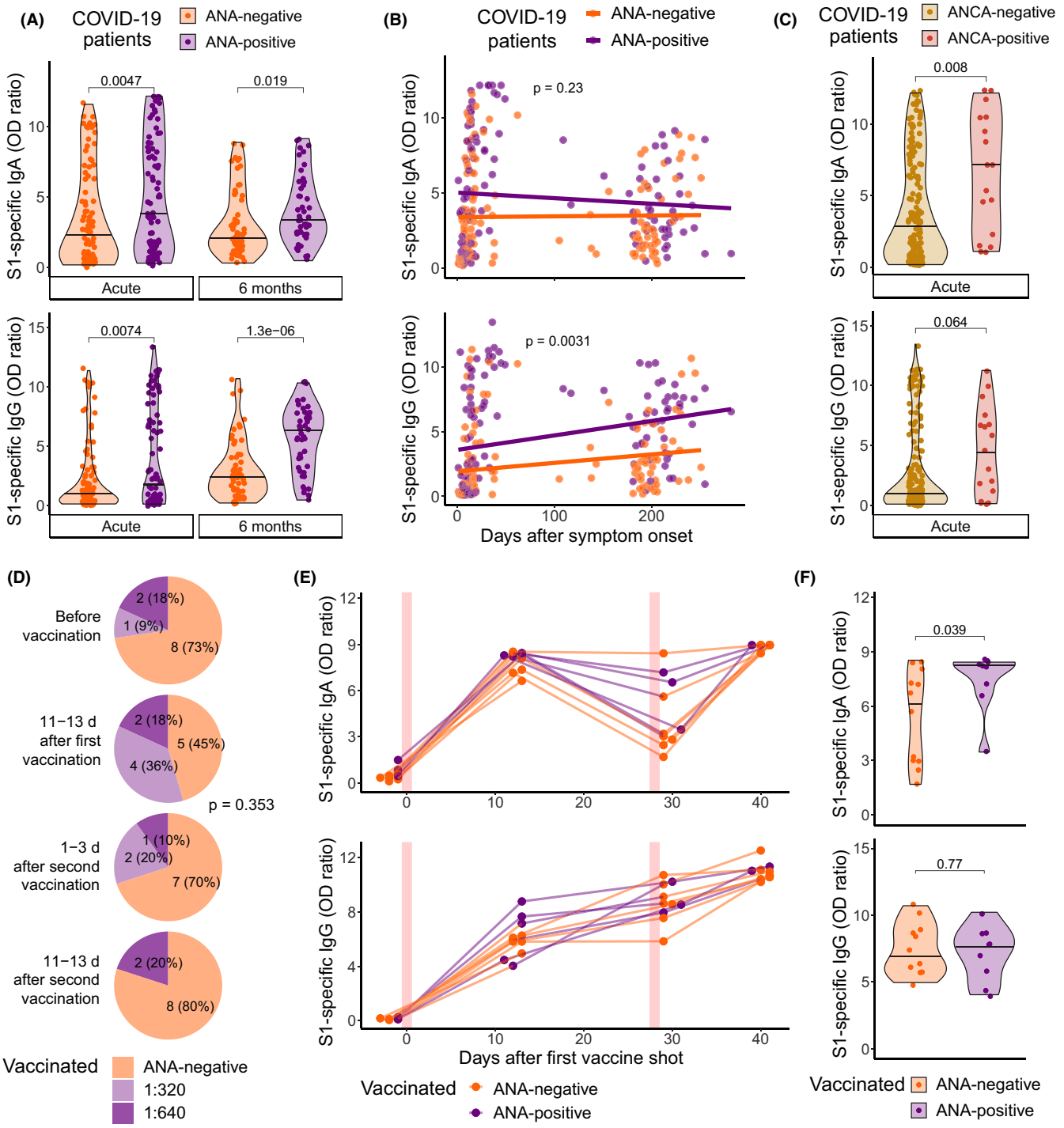


FIGURE 4 Presence of autoantibodies is associated with an increased virus-specific humoral response after SARS-CoV-2 infection and vaccination. (A–B) S1-specific IgA and IgG in ANA-positive and ANA-negative COVID-19 patients during acute disease ($n = 175$) and 6 months after recovery ($n = 104$). (B) p-values indicate significance of the correlation of ANA positivity as an independent parameter in a multiple linear regression model accounting for age, disease severity and sampling time point (Table S3). (C) S1-specific IgA and IgG in ANCA-positive and ANCA-negative COVID-19 patients during acute disease ($n = 175$). (D) ANA prevalence and titers in previously unexposed individuals ($n = 11$) before and after vaccination with BNT162b2 at indicated time points. The p-value was calculated using the chi-squared test of independence. (E) S1-specific IgA and IgG before and after COVID-19 vaccination ($n = 11$). Red vertical lines indicate the time points of first and second vaccination with BNT162b2. (F) S1-specific IgA and IgG in ANA-positive and ANA-negative participants following COVID-19 vaccination with BNT162b2, combining data from 10–13 days after the first ($n = 11$) and 1–3 days after the second ($n = 10$) vaccination

virome-wide serological profiling in acute COVID-19 revealed antibodies cross-reactive to other coronaviruses, whereas ANA-positive participants produced antibodies to more viral epitopes on a virome-wide level.

2.6 | Association of autoantibodies with inflammatory signature during acute COVID-19

Several studies have associated autoantibodies with severe COVID-19.[11,15,17–19] Thus, we sought to further characterize ANA-positive and ANA-negative COVID-19 patients during acute disease by proteomics comprising 86 inflammatory markers, cytokine measurements, flow cytometry, clinical history, and routine diagnostic analyses. The proportion of participants with a known autoimmune disease was low in our cohort and indifferent in individuals without or with ANA or ANCA (Table 2), and none of the study participants developed a symptomatic autoimmune disease following SARS-CoV-2 infection. We found a higher prevalence of comorbidities in autoantibody-positive patients, including hypertension and heart disease, but no significant sex difference (Table 2).

A multivariate analysis with 130 variables, including demographic parameters, routine diagnostic measurements, and inflammation markers obtained by proteomics (Table S5), allowed for a nearly complete separation of severe COVID-19 patients from healthy individuals, with mild COVID-19 patients exhibiting intermediate characteristics (Figure 6A). Several markers contributing to severe COVID-19 were significantly higher ($p < .05$) in ANA-positive than ANA-negative COVID-19 patients, revealing an inflammatory signature associated with severe disease in ANA-positive individuals (Figure 6B). Importantly, ANA-positive COVID-19 patients were older and experienced longer hospitalization (Figure 6C), and many inflammation markers, including C-reactive protein (CRP) and interleukin (IL)-6, were elevated compared with ANA-negative patients (Figure 6D). Furthermore, ANA positivity was associated with T-cell activation as suggested by higher soluble IL-2 receptor alpha (sIL-2Rα) serum concentrations and increased proportions of activated $CD38^+$ $HLA\text{-}DR^+$ $CD4^+$ and $CD8^+$ T cells (Figure 6E). Similar trends toward an inflammatory signature were also observed in ANCA-positive individuals during acute COVID-19, although these results were limited due to the lower prevalence of ANCA (Figure S5A,B).

Finally, we assessed the characteristics of ANA-positive and ANA-negative COVID-19 patients 6 months after acute disease to identify alterations in the absence of acute inflammation. A multivariate analysis of 43 parameters, including patient characteristics and routine diagnostic measurements, revealed differences comparing ANA-positive and ANA-negative individuals, with several inflammation markers, including IL-6, tumor necrosis factor–alpha (TNF-α), and sIL-2Rα, being significantly higher in ANA-positive participants (Figure 6F,G, Table S6). Interestingly, we also observed differences in Ig subclasses, with significantly higher IgG1 and significantly lower IgM in ANA-positive individuals (Figure 6H). Furthermore, marked changes in B-cell subsets were apparent, with higher frequencies of IgD^+ $CD27^-$ naïve B cells and lower frequencies of IgD^+ $CD27^+$ nonswitched and IgD^- $CD27^+$ switched memory B cells in ANA-positive individuals (Figure 6I). Altogether, in autoantibody-positive COVID-19 patients, we found an inflammatory signature during acute disease resembling alterations found in severe disease and changes in inflammation markers, Ig subclasses, and B cells 6 months after recovery.

3 | DISCUSSION

Although autoantibodies targeting nuclear, cytoplasmic, and soluble autoantigens following viral infections have been well described,[28,29] their significance has remained ill-defined. In this study, we used highly sensitive assays to detect ANA and ANCA, representing systemic autoantibodies, in patients up to 1 year after infection with SARS-CoV-2. Firstly, we found transient ANA and ANCA in a subgroup of participants during acute COVID-19, highlighting the importance of careful interpretation of diagnostic autoantibody assays during acute viral infections and of re-testing autoantibody-positive individuals several months after recovery from acute viral infections. Autoantibody production could result from the activation of autoreactive B and T cells recognizing viral epitopes by means of molecular mimicry.[30,31] Alternatively, antigen-independent 'bystander' activation of autoreactive B and T cells by cytokines and other inflammatory mediators could drive autoantibody production.[32,33] Particularly in severe COVID-19, which is associated with early neutrophilia and pronounced neutrophil extracellular trap (NET) formation,[34,35] NETs expose shielded intracellular self-antigens,[25,36,37] thus causing the production of ANCA and ANA. Interestingly, a high prevalence of IgA ANCA has been reported in acute COVID-19 patients showing chilblain-like lesions.[21] In our study, all ANCA-positive subjects tested negative for anti-MPO and anti-PR3 antibodies and showed no indication of ANCA-associated vasculitis. Thus, it remains elusive whether these ANCA have pathogenic potential.

Secondly, we found distinct features in autoantibody-positive COVID-19 patients during acute disease and recovery. Autoantibodies were associated with prolonged hospitalization and inflammation markers during acute disease, supporting recent findings.[11,15,17–19,38,39] Whereas autoantibodies targeting type I interferons have been linked to severe COVID-19,[10–12,16] severe COVID-19 could decrease self-tolerance by tissue damage and inflammation, altogether leading to the generation of autoantibodies. However, confounding factors should be considered, such as age and comorbidities, affecting the prevalence of autoantibodies[40] and the risk of severe COVID-19.[1] Following these considerations, we found changes in ANA-positive individuals even 6 months after acute COVID-19, indicating ongoing low-grade inflammation. Furthermore, we observed alterations of the B-cell compartment, including increased naïve and decreased memory B cells, previously associated with presymptomatic and early-stage autoimmune diseases.[41–43] Differences in total Ig concentrations have been found in autoimmune diseases[44] and patients suffering from postacute COVID-19 syndrome (PACS).[45] Thus, we have previously identified

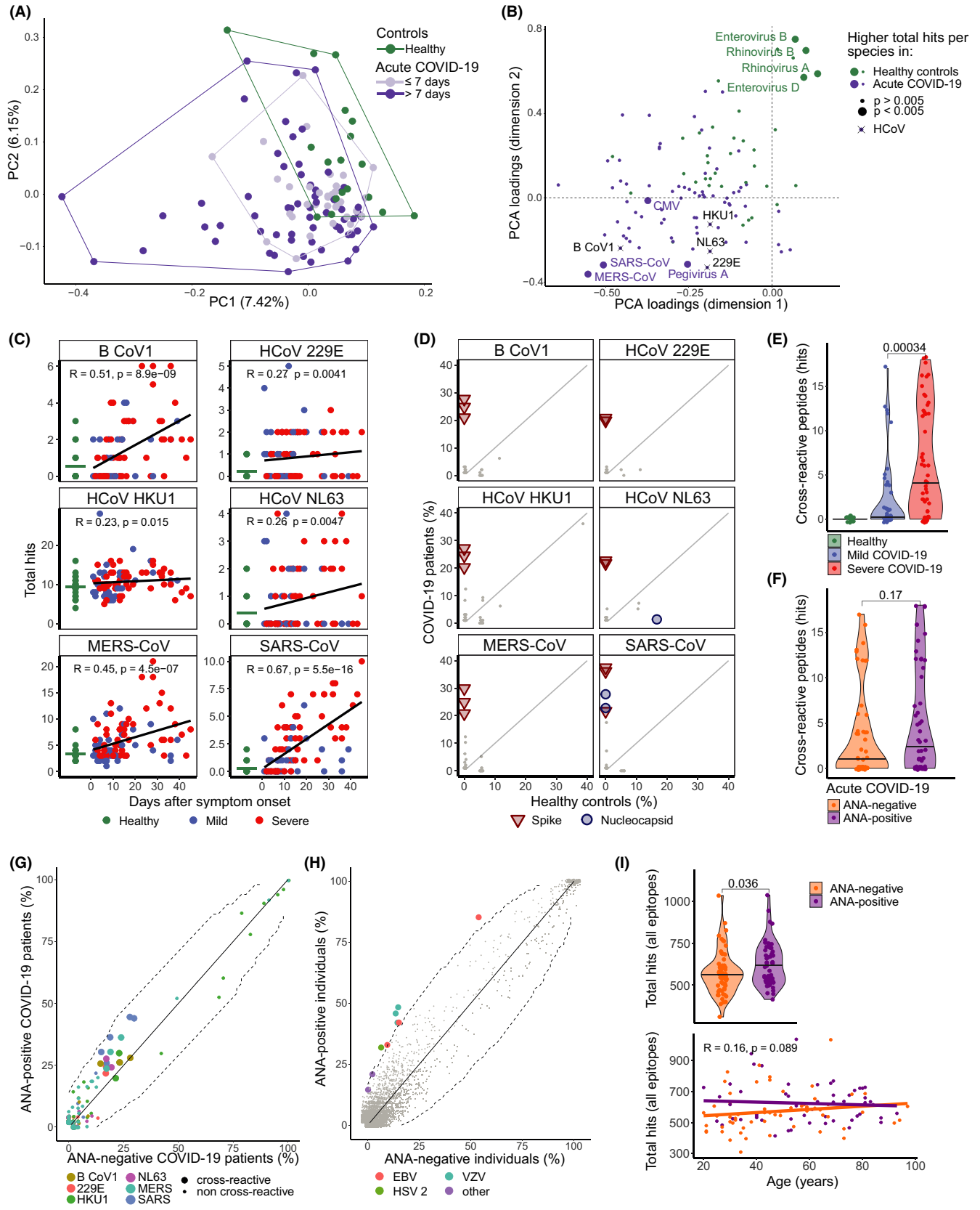


FIGURE 5 Legend on next page

FIGURE 5 Comprehensive serological profiling (VirScan) in ANA-positive and ANA-negative individuals during acute COVID-19. (A) Principal component analysis (PCA) of 112 viral species, including data of 18 healthy individuals and 96 acute COVID-19 patients, grouped by time point of sample collection after symptom onset. Each dot represents an individual participant. (B) Loadings of PCA depicted in (A), with each viral species shown as individual dots (Table S4). Colors indicate participant groups with higher mean epitope hits per species. Viral species with significant difference ($p < .005$) between COVID-19 patients and healthy controls are shown as large colored dots. Black crosses indicate insignificant differences of coronaviruses ($p > .005$). (C) Temporal association of summed epitope hits of six coronaviruses after symptom onset, shown for acute COVID-19 patients ($n = 97$) and healthy controls ($n = 18$). Horizontal green bars represent means of healthy controls. (D) Percentage of healthy controls and COVID-19 patients with positive results for epitopes of six coronavirus species. Significantly enriched epitopes ($p < .05$) of spike and nucleocapsid are indicated accordingly. (E–F) Summed hits for cross-reactive epitopes, comparing healthy controls and patients with mild and severe COVID-19 (E) or COVID-19 patients with or without ANA (F). (G) Percentage of ANA-positive and ANA-negative COVID-19 patients with positive results for cross-reactive and non-cross-reactive epitopes of six coronavirus species. Dashed lines mark significance threshold at $p < .05$. (H) Percentage of ANA-positive and ANA-negative study participants ($n = 115$) with positive results, shown for all available epitopes. Significantly enriched epitopes ($p < .005$) are colored. EBV—Epstein–Barr virus; HSV-2—herpes simplex virus 2; VZV—varizella-zoster virus; other—other viruses comprising Aichivirus A and Mamastrovirus 1. (I) Summed epitope hits per individual including all available epitopes, comparing ANA-negative and ANA-positive participants (top; $n = 115$) and as a function of age (bottom)

an Ig signature in PACS, including low total IgM, and found clinical risk factors, including increased age and severe disease course.[45] Although a direct link to autoantibody development in PACS has not been reported, a misdirected immune response may underly both manifestations. Notably, none of the participants newly developed a symptomatic autoimmune disease during the study period, but larger studies of longer duration are needed to confirm these data.

Thirdly, we observed higher S1-specific antibody titers in autoantibody-positive COVID-19 patients, indicating a more robust, functional humoral immune response, since S1-specific antibodies have been associated with virus-neutralizing capacity.[46–48] Similar to our results, recent reports found increased antiviral humoral responses in autoantibody-positive individuals during acute COVID-19, although the interrelation remained unclear.[16,19,22] Interestingly, following COVID-19 mRNA vaccination in systemic lupus erythematosus (SLE) patients, higher concentrations of SARS-CoV-2-specific IgG positively correlated with anti-dsDNA antibodies,[39] which is in line with our data of increased S1-specific IgA production in ANA-positive individuals following vaccination. These findings indicate an inherent capacity of ANA-positive individuals to mount more robust antibody responses upon antigen challenge. Further investigations are needed to elucidate the differences in antigen-specific IgA and IgG production after mRNA vaccination. Human virome-wide serological profiling revealed the production of cross-reactive antibodies to other coronaviruses during acute COVID-19, particularly in severe disease, consistent with broader humoral immune responses in severe COVID-19.[27] Whereas antibodies targeted similar cross-reactive coronavirus epitopes in ANA-positive and ANA-negative COVID-19 patients, more antibodies targeted EBV antigen EBNA-2 in ANA-positive individuals. Higher humoral responses against EBV have been described in ANA-positive individuals, irrespective of autoimmune disease.[49,50] Also, EBV has been associated with the development of ANA and SLE.[51] Furthermore, severe acute COVID-19 is characterized by extrafollicular B-cell activation,[20,38,52] which is found in autoimmune disease and associated with activation of autoreactive B cells. This increased response could allow for rapid formation of virus-specific antibody-secreting cells,[20] potentially explaining why individuals with autoantibodies exhibit higher humoral responses during acute COVID-19. Whether autoantibody-positive subjects also show increased SARS-CoV-2-specific long-lived T-cell responses[53] remains to be investigated.

Limitations of this study include the use of highly sensitive IIF assays that yielded a high prevalence of positive results in healthy subjects and COVID-19 patients. Most of the measured ANA titers were at or just above the threshold level, which usually would be considered of irrelevant clinical significance. Moreover, we did not assess the specificity of autoantibodies, but recent studies have shown reactivity to a wide spectrum of autoantigens.[11,16]

Altogether, our study shows autoantibodies in COVID-19 appear to be transient and correlate with increased antiviral humoral immune responses and a distinct immune signature. As questions arise regarding long-term consequences of COVID-19, including the risk of immune dysregulation and autoimmune disease, understanding the mechanisms involved in balancing self-tolerance, and protective immune responses become crucial to recognize and manage patients at risk for developing autoimmune diseases.

4 | METHODS

4.1 | Human subjects and patient characteristics

Following written informed consent, adult individuals were recruited for medical history and blood sampling between April 2020 and May 2021. The study was approved by the Cantonal Ethics Committee of Zurich (BASEC #2016-01440). The cohort comprised mild and severe COVID-19 patients, healthy controls, and vaccinated individuals.

COVID-19 patients (Table 1, Table S1): 175 patients with reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)-confirmed SARS-CoV-2 infection were included during acute COVID-19 at four hospitals in the Canton of Zurich, Switzerland. COVID-19 was classified for maximum disease severity according to the World Health Organization (WHO) classification criteria into mild disease – including asymptomatic ($n = 4$), mild illness ($n = 93$),

TABLE 2 Characteristics of ANA- or ANCA-positive and ANCA-negative COVID-19 patients at acute disease or 6 months after infection

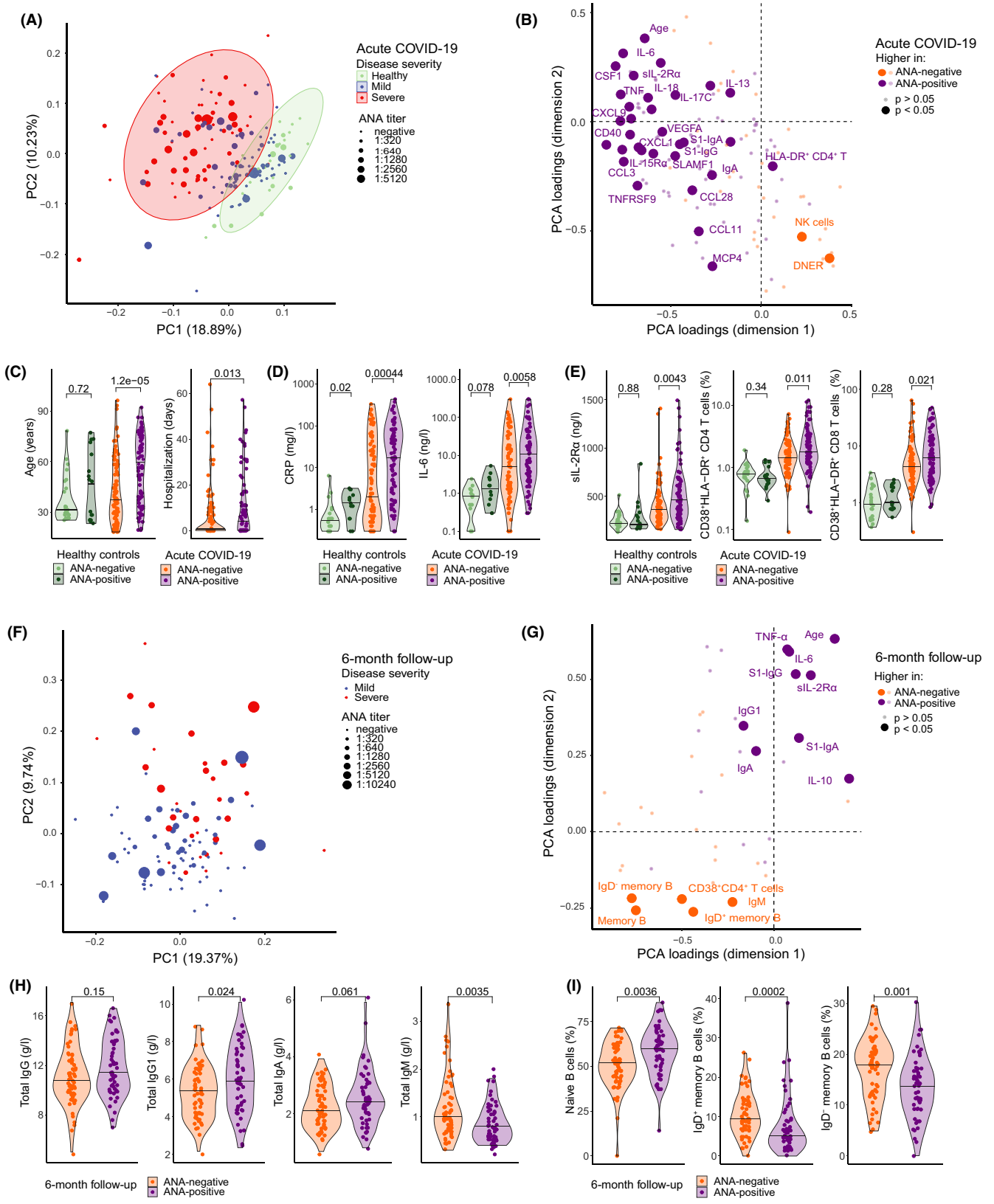
	COVID-19 Acute disease			COVID-19 6-month follow-up		
ANA	**ANA-negative**	**ANA-positive**	***p*-value**	**ANA-negative**	**ANA-positive**	***p*-value**
Patient characteristics						
n (%)	91 (52.0%)	84 (48.0%)	–	61 (52.6%)	55 (47.4%)	–
Age	38 (30–58)	60.5 (38–73)	***	33(29–47)	61 (45–69)	****
Days after symptom onset	11 (7–16)	12 (7–19)	ns	195 (186–206)	204 (183–218)	ns
Sex (female)	43 (47.3%)	38 (45.2%)	OR 0.92, ns	29 (47.5%)	25 (45.5%)	OR 0.95, ns
Comorbidities						
Hypertension	18 (19.7%)	32 (38.1%)	OR 2.48, *	8 (12.1%)	19 (34.5%)	OR 3.39, **
Diabetes mellitus	12 (13.2%)	13 (15.5%)	OR 1.20, ns	6 (9.8%)	10 (18.2%)	OR 1.99, ns
Heart disease	9 (9.9%)	21 (25.0%)	OR 3.02, **	1 (1.6%)	16 (29.1%)	OR 23.68, ***
Lung disease	15 (16.5%)	7 (8.3%)	OR 0.46, ns	9 (14.8%)	7 (12.7%)	OR 0.87, ns
Kidney disease	11 (12.1%)	14 (16.7%)	OR 1.45, ns	2 (3.3%)	11 (20.0%)	OR 7.14, **
Malignancy	4 (4.4%)	8 (9.5%)	OR 2.28, ns	4 (6.6%)	5 (9.1%)	OR 1.40, ns
Autoimmune disease	8 (8.7%)	5 (5.9%)	OR 0.66, ns	5 (8.2%)	7 (12.7%)	OR 1.62, ns
ANCA	**ANCA-negative**	**ANCA-positive**	***p*-value**	**ANCA-negative**	**ANCA-positive**	***p*-value**
Patient characteristics						
n (%)	158 (90.3%)	17 (9.7%)	–	113 (97.4%)	3 (2.6%)	–
Age	44 (32–65)	71 (57–80)	***	43 (31–64)	69 (64–70)	–
Days after symptom onset	11 (7–16)	11 (9–19)	ns	199 (187–216)	182 (164–212)	–
Sex (female)	74 (46.8%)	7 (41.2%)	OR 0.79, ns	52 (46.0%)	1 (33.3%)	–
Comorbidities						
Hypertension	41 (25.9%)	9 (52.4%)	OR 3.19*	27 (23.9%)	0 (0%)	–
Diabetes mellitus	21 (13.2%)	4 (23.5%)	OR 2.00, ns	15 (13.3%)	1 (33.3%)	–
Heart disease	24 (15.2%)	6 (35.2%)	OR 3.02, ns	16 (14.2%)	1 (33.3%)	–
Lung disease	21 (13.3%)	1 (5.9%)	OR 0.41, ns	15 (13.3%)	1 (33.3%)	–
Kidney disease	22 (13.9%)	3 (17.6%)	OR 1.32, ns	12 (10.6%)	1 (33.3%)	–
Malignancy	10 (5.9%)	2 (11.8%)	OR 1.96, ns	8 (7.1%)	1 (33.3%)	–
Autoimmune disease	12 (7.6%)	1 (5.9%)	OR 0.76, ns	12 (10.6%)	1 (33.3%)	–

Note: For continuous variables, medians and interquartile ranges (in parentheses) are specified, with *p*-values obtained by the Mann–Whitney *U* test comparing individuals with and without autoantibodies. For categorical variables, numbers of individuals (*n*) and percentages of the corresponding subgroup (in parentheses) and odds ratios (OR) with *p*-values indicating significance in the Fisher's exact test are shown.

Abbreviation: ns, nonsignificant.

*$p < .05$.; **$p < .01$.; ***$p < .001$.; ****$p < .0001$.

FIGURE 6 ANA-positive COVID-19 patients exhibit a proinflammatory signature. (A) PCA accounting for 130 parameters (Table S5) including data of healthy controls ($n = 28$) and acute COVID-19 patients ($n = 146$). Participants with missing values were excluded from this analysis. 95% confidence ellipses (t-distributed) are shown for healthy controls and severe COVID-19 patients. (B) Loadings (variable coordinates) of the PCA depicted in (A), with each parameter shown as an individual dot. Colors indicate the group of COVID-19 patients with higher mean for each parameter, and parameters with significant differences ($p < .05$) are represented as large dots, and selected parameters are annotated (Table S5). (C–E) Comparison of ANA-negative and ANA-positive individuals among healthy controls or acute COVID-19 patients. (C) Patient characteristics, including duration of hospitalization ($n = 174$) and age ($n = 216$). (D) Inflammation markers, including CRP ($n = 209$) and IL-6 ($n = 215$). (E) T-cell activation, including sIL-2Rα ($n = 215$), and $CD38^+HLA\text{-}DR^+$ $CD4^+$ ($n = 210$) and $CD8^+$ ($n = 209$) T cells. (F–G) PCA (F) and loadings (G) accounting for 43 parameters (Table S6) including data of COVID-19 patients 6 months after recovery ($n = 107$). Participants with missing values were excluded from this analysis. (H–I) Comparison of ANA-negative and ANA-positive COVID-19 patients 6 months after recovery. (H) Concentration of total Ig subclasses in serum ($n = 116$). (I) Frequency of B-cell subsets, including IgD^+CD27^- naïve, IgD^+CD27^+ nonswitched memory and IgD^-CD27^+ switched memory B cells ($n = 114$)



and mild pneumonia (n = 12)—and severe disease—including severe pneumonia (n = 30) and acute respiratory distress syndrome (n = 36).[54] Follow-up visits for medical history and blood collection were conducted approximately 6 months and 1 year after symptom onset. Unreachable individuals or those declining further participation were lost to follow-up.

Healthy controls (Table 1): 41 participants with negative history of SARS-CoV-2 infection and serology were recruited. Five individuals developed COVID-19 after inclusion and were subsequently allocated to the patient cohort.

Vaccinated individuals (Table S2): 11 individuals with negative history and serology for SARS-CoV-2 infection were sampled once before vaccination, once after the first, and twice after the second mRNA vaccination with BNT162b2 (BioNTech-Pfizer).

4.2 | Autoantibody detection

Antinuclear antibodies was measured by IIF on HEp-2 cells (Euroimmun) with a cut-off dilution of 1:320. ANCA was measured by IIF on neutrophils fixed by ethanol and formalin (Euroimmun) with a cut-off dilution of 1:40. IIF imaging was performed using a diagnostic, computer-aided microscopy system (Euroimmun). ANA patterns were classified according to the international consensus on ANA patterns anticell (AC) nomenclature[23] by blinded trained personnel. For paired analyses of ANA patterns, 129 pairs of IIF pictures at 1:320 dilution were blinded for patient characteristics and sampling time point and examined pairwise by the same observer. Antibodies against myeloperoxidase and proteinase 3 were measured on Phadia™ 250 (ThermoFisher Scientific) or on Bioflash® (Werfen) according to the manufacturer's instructions.

4.3 | Immunoassays

Immunoassays for Ig subsets, anti-SARS-CoV-2 spike S1-specific IgA and IgG, interleukin (IL)-1β, IL-2, IL-5, IL-6, IL-10, IL-12, interferon-γ (IFN-γ), sIL-2Rα, and tumor necrosis factor α (TNF-α), were performed in accredited laboratories at University Hospital Zurich. Serum Ig subsets were quantified on an Optilite® turbidimeter (The Binding Site Group). S1-specific IgA and IgG were measured by enzyme-linked immunosorbent assays (ELISA) (Euroimmun), as established.[46] IL-1β, IL-2, IL-6, IFN-γ, and sIL-2Rα were determined by ELISA (R&D Systems) on Opsys Reader™ (Dynex). IL-5, IL-10, and IL-12 were measured by cytometric bead assays (BD Biosciences) on a Navios cytometer (Beckman Coulter). TNF-α was determined with a kit (R&D Systems) using MagPix® (ThermoFisher Scientific).

4.4 | Flow cytometry

As established,[55] blood samples were processed and analyzed in accredited laboratories at University Hospital Zurich. Blood samples were lysed with VersaLyse, fixed with IOTest3 solution, and stained with antibodies (Beckman Coulter; Table S7). Absolute cell counts were determined using Flow Set Pro Fluorospheres calibration beads on Navios (Beckman Coulter).

4.5 | Serum proteomics

Serum samples were analyzed by a proximity extension assay-based technology 92-plex inflammation panel (Olink®), as established.[33,55,56] Six parameters were excluded because less than 50% of samples showed results above the detection limit.

4.6 | Human virome-wide serological profiling

As established,[7,57] serum samples were inactivated, normalized for total IgG concentration, and incubated as duplicates with a bacteriophage library displaying linear, 56-amino acid long viral epitopes. IgG-phage complexes were captured with magnetic beads, lysed, and quantified by next-generation sequencing. Blank beads samples were used as negative controls. Reads were mapped to the epitope library with Bowtie2, and counts were obtained using SAMtools. A previously described binning strategy was used to identify positivity for epitopes,[58] with a minimum z-score of 3.5 for both sample replicates compared with negative controls. Results for a total of 112 different human viruses were included in the further analysis (Table S4), whereas eukaryotes, prokaryotes, non-human viruses, and human viruses with no variance or a maximal summed epitope hit count below three were excluded.

4.7 | Statistics

Statistical analyses were performed using R (version 4.1.0). Unless otherwise specified, the between-group comparison was performed using two-tailed, nonparametric, unpaired testing (Mann–Whitney *U*) for numeric variables and odds ratios with the Fisher's exact test for categorical variables, with p-values of $<.05$ defined as significant. Missing values were omitted. Principal component analyses (PCA) were performed using stats (4.2.0) and factoextra (1.0.7) with scaled, centered variables, and loadings are shown as variable coordinates. Spearman's rank correlation was used for associations of numeric variables. Pairwise protein alignment for 56-amino acid (AA) long peptides with SARS-CoV-2 spike (Uniprot Entry P0DTC2) and nucleoprotein (P0DTC9) was generated using Biostrings (2.60.2), with BLOSUM62 substitution matrix and gap opening and extension penalty of −11 and −1, respectively. Data visualization was performed using ggplot2 (version 3.3.5), ggfortify (0.4.12), ggVennDiagram (1.1.4), UpSetR (1.4.0), and corrplot (0.90). Horizontal lines in violin plots represent medians. Regression lines represent simple linear regression models.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in relation to this work.

AUTHOR CONTRIBUTIONS

P.T. contributed to patient recruitment and data collection, analysis, and interpretation. C.C. contributed to patient recruitment, data collection, and data interpretation. Y.Z., S.H., and S.A. contributed to patient recruitment and data collection. C.P., Z.T., and P.B. contributed to the data collection. M.E.R. contributed to patient recruitment and clinical management. E.B., A.R., M.S.-H., L.C.H., and J.N. contributed to patient recruitment. E.P.-M. contributed to data analysis. O.B. conceived the project and interpreted the data. P.T. and O.B. wrote the manuscript. All authors edited and approved the final draft of the article.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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