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SARS-CoV-2 neutralizing antibodies decline over one year and patients with severe COVID-19 pneumonia display a unique cytokine profile ☆

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ABSTRACT

Objectives: As coronavirus disease 2019 (COVID-19) rages on worldwide, there is an urgent need to characterize immune correlates of protection from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and to identify immune determinants of COVID-19 severity.
Methods: This study examined the longitudinal profiles of neutralizing antibody (NAb) titers in hospitalized COVID-19 patients clinically diagnosed with mild symptoms, pneumonia, or severe pneumonia, up to 12 months after illness onset, using live-virus neutralization. Multiplex, correlation, and network analyses were used to characterize serum-derived inflammatory cytokine profiles in all severity groups.
Results: Peak NAb titers correlated with disease severity, and NAb titers declined over the course of 12 months regardless of severity. Multiplex analyses revealed that IP-10, IL-6, IL-7, and VEGF-α were significantly elevated in severe pneumonia cases compared to those with mild symptoms and pneumonia cases. Correlation and network analyses further suggested that cytokine network formation was distinct in different COVID-19 severity groups.
Conclusions: The study findings inform on the long-term kinetics of naturally acquired serological immunity against SARS-CoV-2 and highlight the importance of identifying key cytokine networks for potential therapeutic immunomodulation.

1. Introduction

The highly transmissible and pathogenic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), has so far infected about 237 million people worldwide, leading to more than 4.8 million deaths within a period of 22 months. In Thailand, approximately 1.7 million cases and over 17 000 deaths have been confirmed at the time of writing (WHO COVID-19 Dashboard). The ongoing COVID-19 pandemic has taken a significant toll on global public health and economies, calling for a deeper understanding of immune correlates of protection against SARS-CoV-2 that may be vital for the implementation of mitigation strategies and development of treatments and vaccines.

The clinical spectrum of COVID-19 includes asymptomatic infection, a mild to moderate self-limiting disease, and a severe critical illness resulting primarily from pulmonary inflammation and diffuse alveolar damage, which can lead to acute respiratory

distress syndrome (ARDS) requiring invasive mechanical ventilation (Gandhi et al., 2020; Guan et al., 2020; Huang et al., 2020). The mechanisms underlying the severe clinical manifestations of COVID-19 are still unclear, although epidemiological studies suggest that older age, male sex, a high body mass index, comorbidities, and race (particularly Black, Asian, and Minority Ethnic (BAME) individuals) are associated with elevated disease severity and/or an increased risk of in-hospital death from COVID-19 (Li et al., 2020; Tian et al., 2020).

Neutralizing antibodies (NAbs) are an important immune correlate of protection against SARS-CoV-2. Studies in mouse and non-human primate models suggest that the passive transfer of NAbs against SARS-CoV-2 can help mitigate the disease (Cross et al., 2021; Zost et al., 2020), while clinical trials have shown partial success of early treatment with high-titer convalescent plasma (Chen et al., 2020; Liu and Aberg 2021). Importantly, NAb titers have been shown to correlate with protection against SARS-CoV-2 reinfection (Addetia et al., 2020; Khoury et al., 2021; Lumley et al., 2021). Thus, an understanding of the long-term kinetics and durability of anti-SARS-CoV-2 NAbs would help inform on the host's natural response to infection. Several studies have shown that robust NAb titers persist over at least 5–8 months, although data on the longevity of these protective antibodies beyond 8 months are still scarce, and the majority of these studies did not use live-virus neutralization assays (Dan et al., 2021; Gaebler et al., 2021; Wajnberg et al., 2020).

The role of specific cytokine networks in COVID-19 severity is still unclear. Studies suggest either no correlation (He et al., 2020) or even an inverse correlation (Argyropoulos et al., 2020) between SARS-CoV-2 viral load and COVID-19 severity, suggesting that clinical deterioration may be immune-mediated, independent of viral replication in at least certain subsets of patients. Patients with severe COVID-19 may experience cytokine-release syndrome (CRS), also colloquially referred to as a 'cytokine storm', systemic hyperinflammation that can lead to ARDS, secondary hemophagocytic lymphohistiocytosis (sHLH) (Mehta et al., 2020), pulmonary edema, multiple organ failure, and death (de la Rica et al., 2020). The use of adjunctive cytokine-targeted therapy, including the US Food and Drug Administration (FDA)-approved monoclonal antibody interleukin 6 (IL-6) receptor antagonist tocilizumab, has been reported to confer clinical improvement in COVID-19 patients (Rubin et al., 2021), suggesting that an intervention in specific inflammatory networks may help attenuate the disease.

This study was performed to investigate the antibody and cytokine profiles of COVID-19 patients clinically diagnosed with mild symptoms, pneumonia, and severe pneumonia. A longitudinal study was conducted of SARS-CoV-2-directed NAbs in these patients up to 12 months post-infection, using *in vitro* neutralization of live virus, which is the 'gold standard' method for NAb assessment that is often circumvented due to its time-consuming nature and the need for a biosafety level 3 (BSL-3) facility (Gundlapalli et al., 2020; Kaufer et al., 2020). A snapshot of inflammatory cytokine profiles in these groups is also provided. Patients with pneumonia and severe pneumonia were profiled separately in acknowledgment of the gamut of COVID-19 clinical manifestations, as there is still a paucity of information on the distinct immune-mediated pathologies underlying various classifications of SARS-CoV-2-induced pneumonia.

2. Methods

All experiments involving live SARS-CoV-2 were performed in a certified BSL-3 facility in the Department of Microbiology, Faculty of Science, Mahidol University. The experimental protocols were approved by Mahidol University, and all methods were performed following standard protocols approved by the institutional review committee.

2.1. Patients and clinical specimen collection

A total of 75 COVID-19 patients hospitalized from March 2020 to May 2020 were enrolled in this study. Sequential serum samples were collected through the Chakri Naruebodindra Medical Institute (CNMI), Faculty of Medicine Ramathibodi Hospital, Mahidol University, Samut Prakan, Thailand between March 2020 and March 2021. Patients were confirmed to be infected with SARS-CoV-2 by RT-PCR on nasopharyngeal and throat swab specimens through amplification of SARS-CoV-2 ORF1AB and N target gene fragments (Sansure Biotech Inc., Changsha, PR China). Sera were stored at $-80°C$ until use.

2.2. Clinical definitions

Pneumonia was defined as clinical symptoms of respiratory tract infection together with abnormal lung imaging compatible with pneumonia. Patients with pneumonia were classified as severe pneumonia based on the following criteria: respiratory rate >30 breaths/min, severe respiratory distress, or an oxygen saturation ≤93% on room air (World Health Organization, "Clinical management of severe acute respiratory infection when novel coronavirus (nCoV) infection is suspected: interim guidance", January 28, 2020; https://www.who.int/publications-detail/clinical-management-of-severe-acute-respiratory-infection-when-novel-coronavirus-(ncov)-infection-is-suspected). Patients who had no symptoms, uncomplicated (mild) symptoms, or non-severe pneumonia were described as having mild to moderate disease.

2.3. Immunoglobulin detection

IgG and IgM antibodies directed against SARS-CoV-2 spike (S) and nucleocapsid (N) were detected using the fully automated MAGLUMI analyzers (Snibe, Shenzhen, China) according to the manufacturer's protocols. SARS-CoV-2 S1-targeted IgG and IgA were detected by ELISA (Euroimmun, Medizinische Labordiagnostika AG). The optical density (OD) was detected at 450 nm. A ratio of each sample reading to the calibrator was calculated for each sample (OD ratio).

2.4. Live-virus microneutralization assay

SARS-CoV-2 virus (SARS-CoV-2/01/human/Jan2020/Thailand) isolated from a confirmed COVID-19 patient at Bamrasnaradura Infectious Diseases Institute, Nonthaburi, Thailand, was used for the in vitro experiments. Sera were heat inactivated at 56°C for 30 minutes then two-fold serially diluted starting from 1:10. Equal volumes of SARS-CoV-2 were spiked into the serial dilutions at an infectious dose of 100 $TCID_{50}$ (50% tissue culture infectious dose) and incubated for 1 hour at 37°C. Vero E6 cells (ATCC USA) were pre-seeded in Dulbecco's modified Eagle medium (DMEM) supplemented with 2% fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml of streptomycin. One hundred microliters of the virus–serum mixtures at different dilutions were added to 1×10^4 pre-seeded Vero E6 cell monolayers in duplicate on a 96-well microtiter plate, then incubated for 2 days at 37°C and 5% CO_2. The last two columns contained the virus control, cell control, and virus back-titration. Medium was discarded and cells were fixed and permeabilized with ice-cold 1:1 methanol/acetone fixative for 20 minutes at 4°C. Cells were washed three times with $1 \times$ phosphate buffered saline (PBS) containing 0.05% Tween 20 and then blocked with a blocking buffer consisting of 2% bovine serum albumin (BSA) and 0.1% Tween 20 in $1 \times$ PBS

for 1 hour. After washing three more times with wash buffer, SARS-CoV/SARS-CoV-2 nucleocapsid mAb (Sino Biological; Cat. No. 40143-R001) diluted 1:5000 in 1 × PBS containing 0.5% BSA and 0.1% Tween 20 was added to each well and incubated for 2 hours at 37°C. Detection antibody was removed by washing the plate three more times, then 1:2000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit polyclonal antibody (Dako, Denmark A/S; Cat. No. P0448) was added and the plate incubated at 37°C for 1 hour. Plates were washed three more times, then 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added (KPL; Cat. No. 5120-0075) for 10 minutes. The reaction was stopped with 1 N HCl. Absorbance was measured at 450 and 620 nm (reference wavelength) with an ELISA plate reader (Tecan Sunrise).

The average ODs at 450 and 620 nm were determined for the virus and cell control wells, and the neutralizing endpoint was determined by 50% specific signal calculation. The virus neutralizing endpoint titer of each serum was expressed as the reciprocal of the highest serum dilution with an OD value less than X (World Health Organization, 2011), which was calculated as follows:

$$X = [(\text{average } A450 - A620 \text{ of } 100 \times TCID_{50} \text{ virus control wells}) \\ - (\text{average } A450 - A620 \text{ of cell control wells})]/2 \\ + (\text{average } A450 - A620 \text{ of cell control wells})$$

Sera that tested negative at 1:10 dilution were assigned a titer of <10. Sera were considered positive if the NAb titer was ≥20. Live SARS-CoV-2 viruses at passage 3 or 4 and Vero E6 cells at 20 maximum passages were employed.

2.5. Cytokine measurement

Viruses in serum samples were inactivated with 10% Triton X-100 for 1 hour at room temperature. The concentrations of 25 cytokines, chemokines, and growth factors were measured in duplicate using the Milliplex Human Cytokine/Chemokine/Growth Factor Panel A (HCYTA-60K; Merck Millipore, Burlington, MA, USA) following the manufacturer's instructions for the following biomarkers: FGF-2, G-CSF, GM-CSF, IFNα2, IFN-γ, IL-1β, IL-1RA, IL-2, IL-3, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 p70, IL-17A, IL-22, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-AA, PDGF-BB, TNF-α, and VEGF-A. The plate was read on a Luminex MAGPIX (Luminex Multiplexing Instrument, Merck Millipore) with a minimum of 50 beads collected per analyte per well. Belysa software was used to analyze the data.

2.6. Statistical analysis

Statistical analyses were done using GraphPad Prism version 9.0.0 (GraphPad Software Inc., La Jolla, CA, USA), IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA), and R version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria). The statistical tests used for each comparison are indicated in the figure legends. Heatmap was performed using the R package ComplexHeatmap. Missing values were imputed with the lower limit of detection (LLOD). Data were log-transformed, scaled, and subjected to k-means clustering. The optimal number of clusters was determined using the elbow method and silhouette coefficients. Dendrograms were drawn with the Pearson correlation as a distance metric and linkage was determined by the Ward.D method. Columns were annotated with patient characteristics. Correlation matrix analysis was performed using the R package corrplot. Correlation was determined using Spearman's test and data were subjected to hierarchical clustering. Only input pairs with $P \leq 0.05$ are displayed, with a confidence interval of 95% (95% CI). Network analysis of serum-derived inflammatory mediators was performed using Graphia 2.2 (Freeman et al., 2020). The k-nearest neighbors

algorithm (k-NN) classification method, Markov clustering (MCL), and edge reduction with $k = 3$ were applied. Edges represent interactions with Spearman's rank correlation value of >0.2, set to ensure the inclusion of all cytokines.

3. Results

3.1. Patient characteristics

A total of 75 patients with RT-PCR-confirmed COVID-19, hospitalized at the CNMI, Faculty of Medicine Ramathibodi Hospital, were included in the study. Patients were stratified into three clinical groups based on disease severity: mild symptoms, pneumonia, and severe pneumonia. Patient characteristics are presented in Table 1. The median age of the cohort was 39.8 years (interquartile range (IQR) 31.8–49.9 years); 50.7% were female and 49.3% were male. The median age of patients with severe COVID-19 pneumonia was 54.8 years (IQR 50.3–64.0 years), while patients with pneumonia and mild symptoms had median ages of 39.3 (IQR 32.7–48.4) and 34.8 (IQR 27.0–39.9) years, respectively. The median number of days after illness onset at the time of study enrollment was 12 (IQR 7–12.5) for mild cases, 10 (IQR 7–14) for pneumonia cases, and 14 (IQR 9.5–17.5) for severe pneumonia cases. Age was significantly higher in severe pneumonia cases compared to mild symptoms cases and pneumonia cases (**Supplementary Material** Figure S1).

In this cohort, patients who had severe pneumonia were mostly male (80%), in contrast to those with pneumonia (45.9% male) and with mild symptoms (34.8% male). Within the cohort, 20% of the patients had comorbidities, and severe pneumonia cases were more likely to present with comorbidities (60%) compared to pneumonia cases (16.2%) and mild symptoms cases (0%). The most common comorbidities in this cohort were dyslipidemia (8.0%) and diabetes mellitus (10.7%). The geometric mean peak NAb titer was 3279.1 (95% CI 2108.8–5098.7) in severe pneumonia cases, 581.3 (95% CI 332.8–1015.3) in pneumonia cases, and 197.6 (95% CI 89.7–435.1) in mild symptoms cases.

3.2. SARS-CoV-2 neutralizing antibody titers correlate with IgG and IgA titers and wane over the course of 1 year regardless of disease severity

The spike (S) and nucleocapsid (N) proteins are key targets for vaccine design (Ahmed et al., 2020). This study compared the levels of S- and N-specific IgG and IgM, as well as subunit 1 (S1)-IgG and IgA in the three severity groups. Severe pneumonia cases had the highest seropositivity rates across all Ig targets, followed by pneumonia and mild symptoms cases. Levels of all Ig targets were significantly higher in severe pneumonia cases compared to mild symptoms cases. S and N-IgG was also significantly higher in severe cases compared to pneumonia cases (Figure 1A–D). Associations of Ig and NAb titers across clinical disease severity levels were then defined. S and N-IgG, S1-IgG, and S1-IgA showed strong positive correlations with NAbs in all severity groups, while S and N-IgM titers were positively correlated with NAbs in pneumonia and severe pneumonia cases, but not significantly so in mild cases (Figure 1E–H).

Longitudinal profiling of NAb titers against live SARS-CoV-2 in sera from all three groups was then performed. NAb titers were arbitrarily stratified into low, medium, and high levels based on criteria for COVID-19 convalescent plasma (CCP) donation (Wendel et al. 2021) and it was observed that a majority of the patients' NAb titers dropped to medium and low titers within 1 year regardless of disease severity (Figure 1I). Patients with severe pneumonia produced significantly higher peak NAb titers (geometric mean 3079.4, 95% CI 1808.4–5244.9) compared to those with

Table 1
Clinical characteristics of enrolled COVID-19 patients based on disease severity (mild symptoms, pneumonia, and severe pneumonia). Median age, sex, geometric mean peak neutralizing antibody titers, median days after illness onset at the time of study enrollment, and comorbidities are listed for each group.

Characteristic	All patients(N = 75)	Mild symptoms(n = 23)	Pneumonia(n = 37)	Severe pneumonia(n = 15)
Age (years), median (IQR)	39.8 (31.8–49.9)	34.8 (27.0–39.9)	39.3 (32.7–48.4)	54.8 (50.3–64.0)
Sex, *n* (%)				
Female	38 (50.7)	15 (65.2)	20 (54.1)	3 (20.0)
Male	37 (49.3)	8 (34.8)	17 (45.9)	12 (80.0)
Geometric mean peak neutralizing antibody titer (95% CI)	576.5 (372.2–893.0)	197.6 (89.7–435.1)	581.3 (332.8–1015.3)	3279.1 (2108.8–5098.7)
Number of days after illness onset at time of study enrollment (first available time-point for all groups), median (IQR)	12 (7–14.25)	12 (7–12.5)	10 (7–14)	14 (9.5–17.5)
Any comorbidity, *n* (%)	15 (20.0)	0	6 (16.2)	9 (60.0)
Hypertension, *n* (%)	5 (6.7)	0	2 (5.4)	3 (20.0)
Coronary artery disease, *n* (%)	3 (4.0)	0	0	3 (20.0)
Dyslipidemia, *n* (%)	6 (8.0)	0	1 (2.7)	5 (33.3)
Diabetes mellitus, *n* (%)	8 (10.7)	0	3 (8.1)	5 (33.3)
Chronic kidney disease, *n* (%)	2 (2.7)	0	1 (2.7)	1 (6.7)
Cancer, *n* (%)[a]	2 (2.7)	0	1 (2.7)	1 (6.7)
HIV, *n* (%)	1 (1.3)	0	1 (2.7)	0
COPD, *n* (%)	1 (1.3)	0	0	1 (6.7)
No chronic comorbidities, *n* (%)	60 (80.0)	23 (100.0)	31 (83.8)	6 (40.0)

CI, confidence interval; COPD, chronic obstructive pulmonary disease; IQR, interquartile range.

[a] Includes any type of cancer.

pneumonia (364.8, 95% CI 184.0–723.3) or mild symptoms (62.9, 95% CI 29.7–132.9) within the first month after illness onset. All 3 groups exhibited a decline in NAb titer between 2 and 12 months after illness onset regardless of disease severity. The biggest drop in mean peak NAb titer occurred between 6 and 12 months for all three groups; severe pneumonia cases experienced an approximate 10-fold decrease, pneumonia 5-fold, and mild symptoms cases experienced a 6-fold decline between 6 and 12 months. At the 12-month mark, NAb titers no longer differed significantly between severity groups, and all 3 groups retained lower NAb titers than were produced during the first month post-illness onset, although only those with severe pneumonia experienced a NAb titer decline that was statistically significant in this regard. Compared to the peak geometric mean titers for each group, after 12 months mild symptoms and pneumonia cases retained approximately 20% of antibody concentrations (geometric mean titers of 40 and 116.2, respectively), while those with severe pneumonia retained about 5% (geometric mean titer of 160) (Figure 1J).

3.3. COVID-19 patients with severe pneumonia display distinct inflammatory cytokine profiles

Next, 25 key cytokines associated with inflammation and cytokine storms (Fajgenbaum and June, 2020; Ragab et al., 2020) were assessed in serum from a sub-cohort of 48 patients with mild symptoms, pneumonia, and severe pneumonia using a multiplex assay. The results showed that IP-10, IL-6, IL-7, and VEGF-α concentrations were significantly elevated in severe pneumonia cases compared to mild symptoms and pneumonia cases. Meanwhile, MCP-1, IL-1RA, and IL-8 concentrations were also significantly higher in severe pneumonia cases compared to mild symptoms cases, and TNF-α was significantly upregulated in severe pneumonia cases compared to pneumonia cases. MIP-1α was higher in healthy controls compared to mild and pneumonia cases (Figure 2A). GM-CSF, IL-2, IL-3, IL-10, IL-12 p70, and IL-22 had mostly low or undetectable expression in the majority of patients regardless of disease severity. Levels of other markers including FGF-2, GCSF, IFN-α2, IFN-γ, IL-1β, IL-9, IL-10, IL-17A, MIP-1β, PDGF-AA, and PDGF-BB did not differ significantly between the groups (**Supplementary Material** Figure S2).

The relationship between cytokine concentrations and patient characteristics were then assessed through a clustered heatmap. Data were annotated with the patients' disease severity (labeled 'Severity'), NAb antibody titer at the time of sampling ('Titer'), number of days from disease onset at the time of sampling ('Onset'), age, and sex. The analysis resulted in two patient clusters and three cytokine clusters. Cytokine cluster 1 consisted of PDGF-AA and PDGF-BB, cluster 2 comprised GCSF, MCP-1, IL-8, IL-6, IP-10, IL-1RA, IL-10, MIP1β, IL-7, and VEGF-α, while cluster 3 included GM-CSF, IL-3, IL-2, IL-12 p70, IL-22, IFN-γ, MIP1α, TNF-α, IL-1β, FGF-2, IL-17A, IFN-α2, and IL-9. Patient cluster A (*n* = 42), exhibited mixed cytokine profiles, while cluster B (*n* = 6) comprised 100% of severe pneumonia cases; these patients generally displayed upregulation of cytokine cluster 2. Severe pneumonia cases in cluster A had a median age of 54.8 years and 62.5% were male, while those in cluster B had a median age of 54.7 years and 100% were male (Figure 2B).

Although patients with severe pneumonia were significantly associated with older age compared to the other groups (**Supplementary Material** Figure S1), and the majority of the patients with severe pneumonia were male (12/15), there was no statistically significant correlation between target cytokine levels and patient age or sex within each severity group. Cytokine levels and days after illness onset were also not significantly correlated, with the exception of VEGF-α, which displayed a positive correlation (data not shown).

3.4. Cytokines form tight-knit inflammatory networks associated with COVID-19 severity

To determine the strength of association between target cytokines in the context of clinical disease severity, separate hierarchical correlation matrix analyses were performed for each group. All groups displayed differential patterns of pairwise positive and negative correlations (Figure 3A–C).

Next, the relationship between pairs of target cytokines in different severity groups was visualized using network analyses. The three groups were found to have distinct cytokine network topologies characterized by differential groups of closely associated inflammatory mediators (Figure 3D–F). Notably, there was a significant positively correlated group comprising IL-6, IL-1RA, MIP-
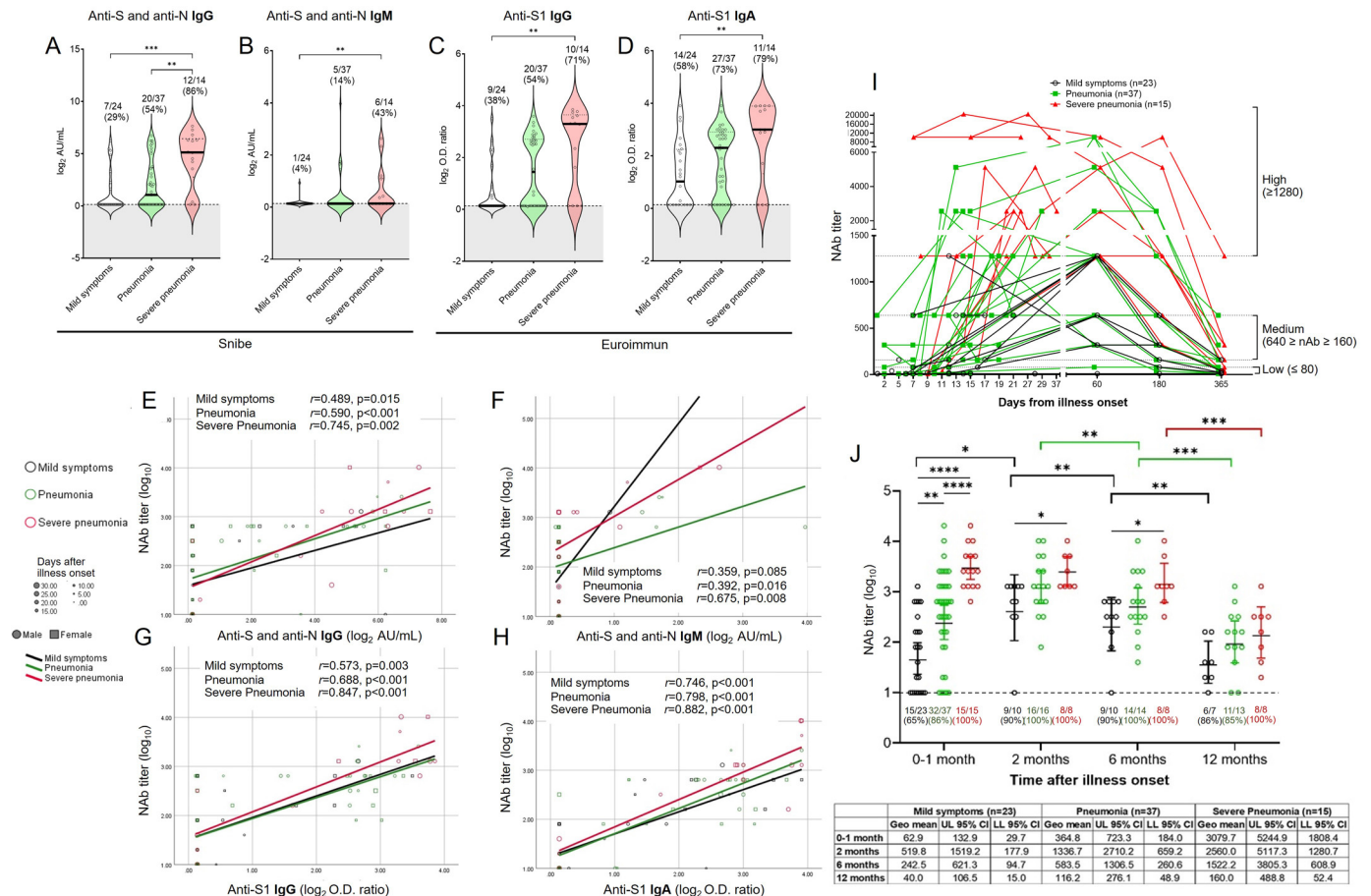
Figure 1. COVID-19 patient antibody profiles based on disease severity. (A) S- and N-specific IgG, (B) S- and N-specific IgM, (C) S1-specific IgG, and (D) S1-specific IgA titers at the time of study enrollment. The fractions and percentages of patients who were seropositive for each Ig subclass are presented. Correlations between neutralizing antibody (NAb) titer and (E) S- and N-specific IgG, (F) S- and N-specific IgM, (G) S1-specific IgG, and (H) S1-specific IgA titers at the time of study enrollment. The correlation coefficient (r) by Spearman rank correlation analysis and *P*-values are shown for each disease severity subgroup. Colors represent the disease severity group, dot sizes represent the number of days after illness onset, and shapes represent sex. (I) Longitudinal profiling of NAb titers in hospitalized COVID-19 patients with mild symptoms (black), pneumonia (green), and severe pneumonia (red) over 12 months. Days 60, 180, and 365 are approximate dates counting from the reported day of illness onset. (J) NAb titers segregated into time intervals (0–1 month, 2 months, 6 months, and 12 months) after illness onset. The highest titer for each subject within each interval is presented. Mixed-effects models with the Geisser–Greenhouse correction and Tukey's multiple comparisons test were used to calculate statistical significance, with individual variances computed for each comparison. Horizontal bars represent geometric means and error bars denote 95% confidence intervals (CI). Dotted horizontal line represents the NAb lower limit of detection (10). The fractions and percentages of patients retaining NAb positivity (positivity cutoff at ≥20) within each group at different time-points are displayed above the *x*-axis. Row statistics including the geometric mean and the upper and lower bounds of the 95% CI for each time interval are tabulated below the graph. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

1β, TNF-α, MCP-1, GCSF, and IP-10 in severe pneumonia cases (Figure 3F), some of which were significantly upregulated in severe cases compared to mild and pneumonia cases (Figure 2A).

4. Discussion

The kinetics and duration of NAb titers in response to viral infection are not always accurately predictable from the early phases of infection (Sallusto et al., 2010), although many valuable studies have extrapolated the trajectory of SARS-CoV-2 NAb production through applications of machine learning algorithms (Legros et al., 2021; Wheatley et al., 2021; Chia et al. 2021). Moreover, most longitudinal studies have not used live SARS-CoV-2 clinical isolates in neutralization tests, which require BSL-3 certified laboratories, and have often opted for safer alternatives such as pseudotyped SARS-CoV-2-based neutralization assays instead (Dan et al., 2021; Gaebler et al., 2021; Nie et al., 2020). In the present study, temporal changes in NAb titers were monitored in hospitalized COVID-19 patients with varying disease severity over a period of up to 1 year after illness onset using live-virus neutralization.

The decline in NAb titers over time, regardless of COVID-19 severity, may be due to transient plasmablast expansion, which show signs of decay less than 10 days after the onset of COVID-19 symptoms (Laing et al., 2020). NAb decline may also stem from a biphasic shift between antibodies produced by short-lived plasma cells during the acute phase to those subsequently generated by the 10–20% that differentiate into long-lived memory plasma cells (Turner et al., 2021). It is still unclear why antibody levels correlate with COVID-19 severity; high viral loads may result in higher disease severity in some patients (Fajnzylber et al., 2020), which in turn may result in a robust production of antibodies in response to extended antigen exposure. Unfortunately, cycle threshold (Ct) values, which semi-quantitatively assess the SARS-CoV-2 viral load, were not recorded for the present cohort, but correlations between viral load and cytokine levels would be an important issue to address in the future. Alternatively, theories of antibody-dependent enhancement (ADE) in COVID-19 are emerging, but so far no definitive role for ADE in human coronaviruses has been established (Lee et al., 2020). Nevertheless, a recent study suggests that NAbs may expand coronavirus cell tropism by binding to Fc receptor-expressing immune cells and guiding viral entry
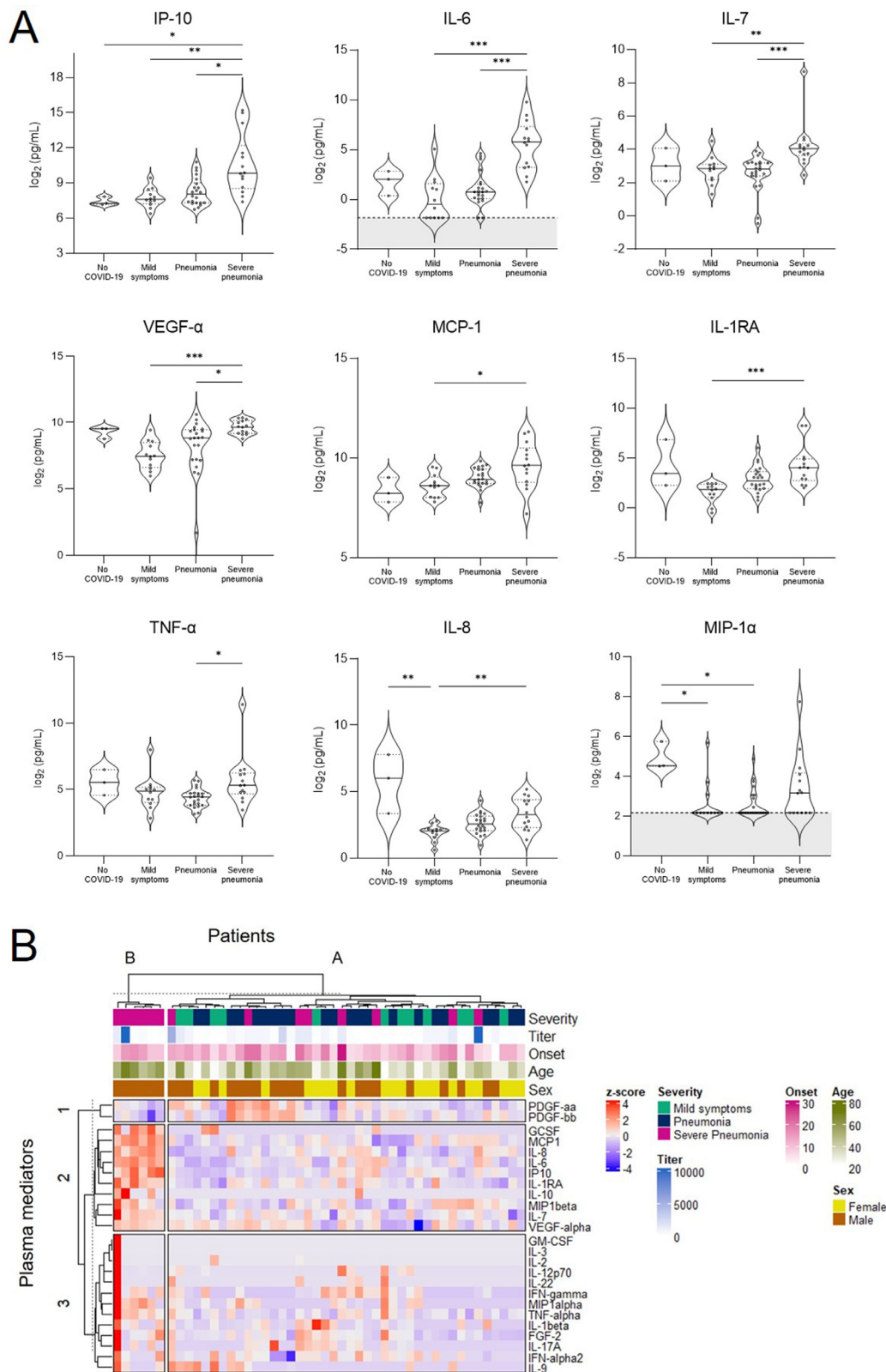
Figure 2. Cytokine expression in COVID-19 cases with mild symptoms, pneumonia, and severe pneumonia. (A) Multiplex analysis of target serum-derived inflammatory cytokines in COVID-19 patients with mild symptoms ($n = 12$), pneumonia ($n = 22$), and severe pneumonia ($n = 14$), and in healthy controls ($n = 3$) at the time of study enrollment. Healthy control blood was collected pre-pandemic. The non-parametric Kruskal–Wallis test with Dunn's multiple comparisons test was used to determine the statistical significance of mediator levels between all three groups at the time of study enrollment. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. Dashed horizontal lines represent the lower limit of detection for each target. Only targets with significantly different levels between groups are shown here; the rest can be found in **Supplementary Material** Figure S2. (B) Clustered heatmap of 25 inflammatory cytokines from hospitalized COVID-19 patients at the time of study enrollment. Values below the lower limit of detection were imputed for each target, and cytokine data were log-transformed, scaled, and both rows and columns were subjected to the k-means clustering algorithm. Positive z-score values are red and negative values are blue. Columns were annotated with patient characteristics including disease severity ('Severity'), NAb titer ('Titer'), number of days from illness onset at the time of study enrollment ('Onset'), age, and sex.
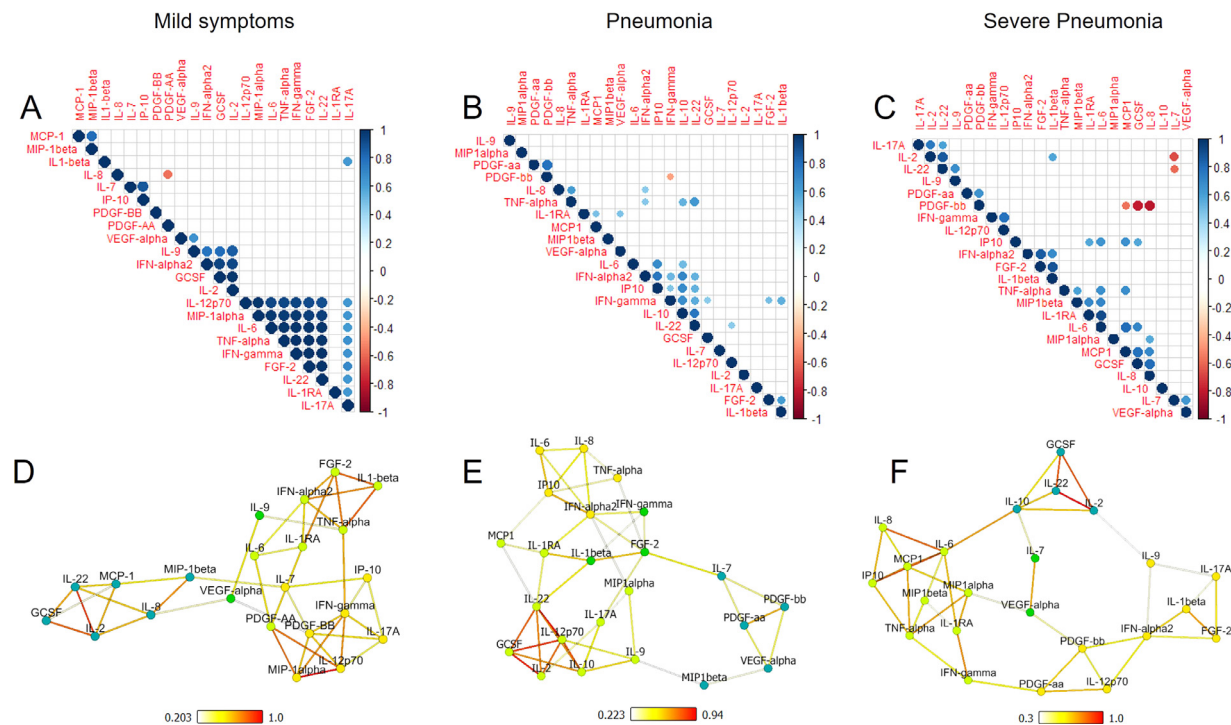
Figure 3. Correlograms and network analyses of serum-derived inflammatory cytokines across COVID-19 disease severity groups. Correlation matrices of cytokine expression in hospitalized COVID-19 patients with (A) mild symptoms ($n = 12$), (B) pneumonia ($n = 22$), and (C) severe pneumonia ($n = 14$) at the time of study enrollment. Only significantly correlated ($P \leq 0.05$) mediator interactions are shown. Positive and negative correlations are shown in blue and red, respectively. The size and color intensity of the dots are proportional to the Spearman correlation coefficients (r_S). Pairwise correlation networks showing positive relationships between cytokines in hospitalized COVID-19 patients with (D) mild symptoms, (E) pneumonia, and (F) severe pneumonia. Nodes represent cytokines/chemokines/growth factors and edges represent positive correlations. Node color represents clusters based on the Markov cluster (MCL) algorithm. Edge color represents the Spearman rank correlation coefficient value between connecting nodes.

([Wan et al., 2020](#)), thus the possibility of ADE in COVID-19 exacerbation should not be ruled out.

It has become clear that SARS-CoV-2 infection leads to immune imbalance and dysregulation ([Blanco-Melo et al., 2020](#); [Mathew et al., 2020](#)) and that unbridled cytokine storms are key to COVID-19 immunopathology ([de la Rica et al., 2020](#)). In this study, it was found that several cytokines, including IP-10 (CXCL10), IL-6, IL-7, and VEGF-α, were distinctly upregulated in severe pneumonia cases compared to mild symptoms and pneumonia cases; these may serve as key prognostic markers of COVID-19 severity. It was also found that the formation of cytokine networks was dependent on clinical disease severity. The results of the present study, along with those of multiple studies showing that tocilizumab and corticosteroid use confer varying levels of success ([Neumann et al., 2021; Rosas et al., 2021; Wang et al., 2020](#)), suggest a need for personalized therapies in the context of SARS-CoV-2-driven immunopathogenesis and hyperinflammation.

Several limitations to this study should be pointed out. Firstly, cytokine levels may correlate with severity, but this does not necessarily imply their pathogenic roles. The results also provide a mere snapshot of the cytokine/chemokine/growth factor interactions in the early phase of COVID-19 and do not capture the spatiotemporal dynamics of the expression of these mediators ([Sinha et al., 2020](#)). Longitudinal profiling of pertinent cytokines in larger cohorts of COVID-19 patients would ideally provide a more complete picture of inflammatory networks during distinct stages of disease. Other aspects of the immune response, including T cell- and innate immune cell-mediated responses, should also be investigated further.

The ongoing COVID-19 pandemic has brought the whole world to a standstill. Case numbers have continued to surge in persistent 'waves' across the globe ([Karagiannidis et al., 2021](#); [Kuehn, 2021](#)).

The results of this study highlight the need to maintain protective measures in the face of a potentially transient serological immunity against SARS-CoV-2 and the emergence of unusually divergent viral strains ([Cohen and Burbelo, 2020; Walker et al., 2020; Frampton et al. 2021](#)). There is also a need for further in-depth studies of inflammatory cytokine networks linked to COVID-19 severity, which may lead to new prognostic and/or therapeutic avenues.

Author contributions

VV wrote the manuscript, performed experiments, and analyzed the data. SSri, SM, SK, CSri, CSet, and NI performed experiments and collected data. PP, MK, SH, and SSun supervised the project. AT conceived the original idea and supervised the project.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2021.09.021.

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