

Viral dynamics and immune correlates of COVID-19 disease severity

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Summary: PCR cycle threshold value ≤ 30 significantly predicts viral culture isolation, and increases with illness duration. Earlier seroconversion with higher peak IgM/IgG levels occurred in severe disease, as were higher levels of inflammatory markers including IL-6 as a key interacting cytokine.

Abstract

Background: Key knowledge gaps remain in the understanding of viral dynamics and immune response of SARS-CoV-2 infection.

Methods: We evaluated these characteristics and established their association with clinical severity in a prospective observational cohort study of 100 patients with PCR-confirmed SARS-CoV-2 infection (mean age 46 years, 56% male, 38% with comorbidities). Respiratory samples (n=74) were collected for viral culture, serum samples for measurement of IgM/IgG levels (n=30), and plasma samples for levels of inflammatory cytokines and chemokines (n=81). Disease severity was correlated with results from viral culture, serologic testing, and immune markers.

Results: 57 (57%) patients developed viral pneumonia, of whom 20 (20%) required supplemental oxygen including 12 (12%) invasive mechanical ventilation. Viral culture from respiratory samples was positive for 19 of 74 patients (26%). No virus was isolated when the PCR cycle threshold (Ct) value was >30 or >14 days after symptom onset. Seroconversion occurred at a median of 12.5 days (IQR 9-18) for IgM and 15.0 days (IQR 12-20) for IgG; 54/62 patients (87.1%) sampled at day 14 or later seroconverted. Severe infections were associated with earlier seroconversion and higher peak IgM and IgG levels. Levels of IP-10, HGF, IL-6, MCP-1, MIP-1 α , IL-12p70, IL-18, VEGF-A, PDGF-BB and IL-1RA significantly correlated with disease severity.

Conclusion: We found virus viability was associated with lower PCR Ct value in early illness. A stronger antibody response was associated with disease severity. The overactive proinflammatory immune signatures offers targets for host-directed immunotherapy which should be evaluated in randomised controlled trials.

Key words: COVID-19, cytokines, serology, viral culture, immunology

Background

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused great harm to health and the global economy [1, 2].

Understanding of SARS-CoV-2 pathogenesis has advanced at an unprecedented speed, but key gaps remain and preliminary findings require validation.

Studies of COVID-19 patients have described the inflammatory milieu in severe infections, with raised neutrophils, suppressed lymphocytes and elevated inflammatory mediators [3, 4]. However, most studies are limited to comparing severe against non-severe infections and lack serial data [5, 6]. A clearer definition of disease pathogenesis will support the development of risk stratification tools and therapeutics targeting critical pathways in the inflammatory cascade.

Early seroconversion has been reported with IgG to the receptor binding domain (RBD) detected at day seven [7-10]. However, evidence of correlation between antibody titres and disease severity is conflicting [8, 10-12]. There remains a need for more detailed assessment of antibody kinetics to help determine the impact of antibody-dependent enhancement in COVID-19 pathogenesis, as well as guide convalescent plasma harvesting and use of serological assays for diagnosis [13].

SARS-CoV-2 can be detected from the nasopharynx for a median of 2-3 weeks following onset of symptoms [14]. Several studies have reported that in immunocompetent individuals, virus is typically only cultured from respiratory samples during the first week of illness when viral loads are highest [8, 15, 16]. This suggests transmission risk declines in the second week. This finding requires confirmation in larger cohorts as it has important implications for infection control and isolation protocols [17].

In this multi-pronged study, we describe the serologic evolution, inflammatory response and pattern of viral shedding and viability in patients with virologically confirmed COVID-19 in Singapore, and analyse the contributions these make to severe infections.

Methods

Patient Recruitment

All individuals confirmed to have COVID-19 by SARS-CoV-2 real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and admitted to any of seven public hospitals in Singapore were eligible for inclusion in this study. RT-PCR was performed on respiratory samples as previously described [18] (details in Supplementary Appendix).

Data and Specimen Collection

Clinical information was extracted from the medical record using a standardized data collection form adapted from the International Severe Acute Respiratory and Emerging Infection Consortium (ISARIC) case record form [19]. Serial blood and respiratory samples were collected during hospitalisation and follow-up post-discharge (days 1, 3, 7, 14, 21 and 28 after enrolment). A stool and urine sample was also collected for SARS-CoV-2 PCR and culture on Day 1.

Clinical Management

At the time of the study, all patients with COVID-19 in Singapore were admitted to airborne infection isolation rooms regardless of disease severity. Supportive therapy including supplemental oxygen and symptomatic treatment were administered as required. Patients with moderate to severe hypoxia were transferred to ICU for further management and invasive mechanical ventilation as required.

Patients were discharged from hospital only after resolution of symptoms and when two consecutive nasopharyngeal swabs >24 hours apart were negative for SARS-CoV-2 by RT-PCR. Follow-up visits were arranged on day 28 of enrolment.

Virus Isolation

Material from nasopharyngeal swabs was collected in universal transport media and used to inoculate Vero-E6 cells (ATCC®CRL-1586TM) for virus isolation in an Animal Biological Safety Level 3 laboratory. Urine and stool samples were collected and transported fresh for virus culture. Stools were filtered before inoculation. Cells were cultured at 37°C for seven days or less if cytopathic effect (CPE) was observed, and three blind passages were performed. CPE consisted of rounded cells and extensive cell death, usually by day four post-inoculation. Positive isolation was confirmed by the observation of CPE and virus-specific PCR. Total RNA was extracted from all samples using E.Z.N.A. Total RNA Kit I (Omega Bio-Tek Inc.) according to the manufacturer's instructions and samples were analyzed by RT-PCR for the detection of SARS-CoV-2 as previously described [20].

Capture ELISA to detect IgM and IgG

Serum collected during the acute and convalescent phases of infection were tested for SARS-CoV-2 receptor binding domain specific IgM and IgG using capture ELISA (details in Supplementary Appendix).

Multiplex Microbead-Based Immunoassay

Plasma samples were collected during acute and convalescent phases and treated with a solvent/detergent based on Triton™ X-100 (1%) for virus inactivation [21]. Immune mediator levels were measured using Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex™ Panel 1 (ThermoFisher Scientific) (details in Supplementary Appendix). Cytokine levels were also measured in 23 healthy donor plasma as baseline controls.

Statistics

Patients were categorised into three groups for comparison: no pneumonia on chest radiographs (CXR) throughout admission; pneumonia on CXR without hypoxia; pneumonia with hypoxia (desaturation to $\leq 94\%$) needing supplemental oxygen. Day one was defined as the first day of symptom onset.

Fold change (FC) of antibody titres compared with negative controls were calculated. Kruskal-Wallis test followed by Dunn's multiple comparison test was used to determine significance of antibody levels.

Unpaired t-test was applied to ascertain significant difference in the immune mediator levels between the COVID-19 patients and healthy controls at different time points post illness onset. One-way ANOVA analysis with post-hoc t-test corrected using the method of Bonferroni was used to discern the differences in immune mediator levels between the various disease severity groups. One-way ANOVA results were corrected for multiple testing using the method of Benjamini and Hochberg.

TM4-MeV Suite (version 10.2) was used to compute hierarchical clustering and heat map on the immune mediators (details in Supplementary Appendix). Biological processes and immune pathways were predicted and illustrated using the Search Tool for the Retrieval of Interacting Genes/Proteins database (STRING) (version 11.0; available at: <https://string-db.org>). All interactions were derived from high-throughput lab experiments and previous knowledge in curated databases at a confidence threshold of 0.8.

Students T-test or Mann-Whitney U test was used as appropriate for continuous variables and Fisher's exact test for categorical variables. P-values < 0.05 were considered statistically significant. Plots were generated using GraphPad Prism (version 7), MedCalc Statistical Software (version 19.2.1) or R (version 3.3.1).

Ethical approval

Written informed consent was obtained from all participants. The study protocol at all sites was approved by the National Healthcare Group Domain Specific Review Board, Study Reference 2012/00917; additional study protocol at Singapore General Hospital approved by the SingHealth Centralised Institutional Review Board, Study Reference 2018/3045.

Results

Clinical presentation, treatment and outcome

130 COVID-19 patients were diagnosed in Singapore from the first known case on 22 January 2020 to 6 March 2020 (Figure 1). Among the 100 (77%) enrolled in this study, viral pneumonia was diagnosed by CXR in 57, of whom 20 required supplemental oxygen for hypoxia, and 12 required invasive mechanical ventilation (Table 1).

Following resolution of infection and viral shedding, 97 patients have been discharged and after 90 days of follow up, no re-infection or recrudescence has been detected. Three patients have died. Deaths occurred 27 days or more after hospital admission, and while all were assessed by the managing clinician as related to COVID-19 they followed viral clearance and prolonged invasive mechanical ventilation.

Viral shedding and cultures

SARS-CoV-2 was detectable from nasopharyngeal swabs by PCR up to 48 days after symptom onset. Mean duration of viral shedding by PCR was 16.7 days (95% CI 15.2-18.3). Cessation of viral shedding by PCR occurred in 4% by day 7, 30% by day 14, 78% by day 21 and 91% by day 28. There were no differences in the duration of viral shedding stratified by disease severity (Supplementary Figures 1 and 2).

Viral culture was attempted from 152 respiratory samples where SARS-CoV-2 was detected by PCR. These samples were collected from 74 patients and SARS-CoV-2 was isolated from 21 samples from 19 (26%) patients (Supplementary Table 1). No virus was isolated from samples where the Ct value was >30, or when the sample was collected >14 days after symptoms onset (Figure 2, Supplementary Figures 2 and 3). There was no correlation between virus isolation by culture and infection severity, patient demographics or symptomatology (data not shown). Viral culture was attempted from the stools of 34 patients and urine of 24 patients during acute infection; in all virus was not isolated. Seven (21%) patients had virus detectable by PCR in stool.

IgM and IgG titres correlate with disease severity

Serial serum samples for ELISA were available for 30 patients. Anti-SARS-CoV-2 IgM was first detectable in 17.9% in the first week of illness, 39.3% in the second week, 35.7% in the third week and 7.1% after the third week, and correspondingly for IgG, 7.7%, 26.9%, 50.0% and 15.4%. The median time to seroconversion was 12.5 days (IQR 9-18) for IgM and 15.0 days (IQR 12-20) for IgG. The total seroconversion rate from 62 patients sampled on day 14 or later was 87.1%. Time to IgG seroconversion was significantly shorter for patients with pneumonia and hypoxia compared to less severe infections (median 12.5 vs 18 days, $p=0.013$, Mann-Whitney). There was also significant correlation between disease severity and peak IgM and IgG levels (Figure 3). However, IgM and IgG antibody levels were not associated with duration of viral shedding (Figure 3), baseline viral Ct values from respiratory samples, age or comorbidities (Supplementary Figure 5).

Proinflammatory signature of severe COVID-19

A total of 321 plasma samples were available from 81 patients. Levels of IL-2, IL-18, IFN- γ , TNF- α , MCP-1, HGF, BDNF, LIF, PLGF-1 and bNGF were significantly elevated compared with healthy controls for their first plasma samples taken soon after admission (Figure 4A). Proinflammatory cytokines and chemokines (IL-2, IL-18, IFN- γ , and MCP-1) were elevated at the early phase of

infection and were reduced to healthy basal range at around 15 days post-illness onset. In contrast, the elevated levels of lung injury associated growth factors (HGF, BDNF, LIF, PLGF-1 and bNGF) persisted throughout the infection course (Figure 4A). Stratification of COVID-19 patients with plasma sample nearest to onset of pneumonia and hypoxia revealed the pro-inflammatory cytokines IP-10, HGF, IL-6, MCP-1, MIP-1 α , IL-12p70 and IL-18, growth factors VEGF-A and PDGF-BB, and anti-inflammatory cytokine IL-1RA were associated with severity; levels were significantly elevated between patients with pneumonia without hypoxia and patients with hypoxia compared with patients without pneumonia except MCP1 which was only significantly higher in patients with hypoxia (Figure 4B and 4C; Supplementary Tables 2 and 3).

Further network analyses showed association between the aforementioned immune mediators and clinical parameters (pneumonia, hypoxia and ICU admission). The intertwined relationship of the cytokines is shown in Figure 5A. In addition, Ingenuity Pathway Analysis (IPA) revealed canonical pathways associated with these immune mediators and severity, with the top ten canonical pathways involved in inflammatory diseases and cell signalling (Figure 5B). The top canonical pathway highlights the common immune mediators between influenza and SARS-CoV-2 infection, including MCP-1, IL-1RA, IP-10 and IL-6 (Figure 5C). In addition to IPA, STRING prediction of protein-protein interactions identified IL-6 as a direct interacting partner with other severity-associated immune mediators (Figure 5D).

Longitudinal comparison of these immune mediators associated with severity in 12 ICU patients was performed to explore their role as prognostic markers for severe COVID-19 (Supplementary Figure 6). Four of these twelve patients (CT009, CT032, CT037 and CT057) recovered and were discharged at the time of study. Interestingly, HGF and VEGF-A were distinctly separated into two levels, with the four discharged patients having lower HGF and VEGF-A levels, and HGF approaching healthy baseline levels during convalescence, which further indicates that high levels of these cytokines were associated with poor prognosis in ICU patients. Similarly, these patients had lower levels of MCP-1

and IL-6 compared with other ICU patients despite having a less distinct separation. Longitudinal comparison of other immune mediators, including IP-10, IL-18, PDGF-BB and IL-1RA, revealed decreasing levels with days post-ICU admission and approaching healthy baseline levels during the latter period of post-ICU admission (Supplementary Figure 6).

Discussion

This study of 100 patients in the first few months of the COVID-19 pandemic in Singapore provides a detailed overview of clinical presentation, progress and outcomes. Case detection and contact tracing in Singapore is rigorous with the main gap possibly in missing asymptomatic cases [22]. From the cohort, 43% of patients never developed pneumonia, 37% developed pneumonia without hypoxia and 20% pneumonia with hypoxia. We found no relationship between illness severity and duration of viral shedding or PCR Ct values. The central role of the immune response to SARS-CoV-2 in COVID-19 was evident from the strong correlation between disease severity and levels of IgG/IgM and inflammatory immune mediators in our cohort.

Viral shedding of SARS-CoV-2 from the respiratory tract has been observed to persist for several weeks and potentially up to months [18, 23-25]. Whether the virus remains infectious throughout this prolonged viral shedding is an important question to resolve. Smaller studies have found that viable virus was readily isolated from immunocompetent individuals during the first week of illness, but were unable to successfully isolate viruses in culture from day 8 onwards despite detectable viral load by PCR [8, 25, 26]. However, a case report from Taiwan showed it was possible to culture the virus up till day 18 [27], while positive cultures up to Day 20 has been reported from a study of patients with severe infection [15]. We found that successful virus culture was associated with PCR Ct value ≤ 30 . Virus was isolated up to Day 14 post-symptom onset, though the majority were cultured at day 10 or earlier. Using PCR Ct value to guide decision making on de-isolation may be an

alternative to using day of illness and provide an additional level of reassurance. However, this requires further validation.

We observed seroconversion of IgM at a median of 12.5 days and IgG at 15.0 days. Similar to a Hong Kong study of 16 patients, there were no significant differences of IgM and IgG titres by age or comorbidities [10]. We found IgM and IgG titres correlated with disease severity, similarly found in two Chinese studies of 173 [7] and 285 patients respectively [28].

We found a lower seroconversion rate of 87.1% in 62 patients from days 14 or later. A Chinese study of 173 patients reported that 94.3% of patients were IgM positive by days 15-39 and 79.8% IgG positive by days 15-39 [7]. A Hong Kong study reported IgM positivity of 94% after day 14 and IgG positivity 100% after day 14 in a smaller number of 16 patients [10]. The largest study from China of 285 patients reported IgM positivity of 94% days 20-22 and IgG positivity of 100% by days 17-19 [28]. Different sample sizes and antibody assays may account for differing seroconversion rates, which merits further investigation in a large cohort over a longer period.

Serological testing is vital for determining the prevalence of infection in sero-surveys and as part of epidemiological investigations to understand transmission of clusters [29]. The timing of seroconversion may guide the timing of plasmapheresis for convalescent plasma [30, 31]. The role of antibodies in long-term immunity after infection needs further investigation.

Consistent with SARS and Middle East respiratory syndrome (MERS), SARS-CoV-2 infection triggers a cytokine storm in a subset of patients with markedly increased levels of pro-inflammatory cytokines, chemokines and growth factors [32, 33]. Notably, higher levels of IL-6, MCP-1, IP-10, IL-18, IL-1RA, PDGF-BB, HGF, VEGF-A, IL-12p70 and MIP-1 α were associated with severe disease in our study. This robust induction of pro-inflammatory mediators indicates that innate immune cell responses and anti-viral T-cell responses are responsible for SARS-CoV-2 pathogenesis in COVID-19 patients [34]. In addition, elevation of growth factors, including HGF [35], PLGF-1 [36] and LIF [37], illuminates the

repair mechanisms following acute lung injury during SARS-CoV-2 infection. Previous studies showed that ICU patients had more significant cytokine activation compared with non-ICU patients [1]. Our longitudinal cytokine profiling in ICU patients further evaluated prognostic values of specific cytokines. We observed better prognosis in critically ill patients with lower levels of acute lung injury associated growth factors, HGF and VEGF-A at the time of admission to ICU. Our data suggest that the differences in degree of lung injury could reflect recovery rate of patients in ICU. HGF and VEGF-A may serve as early indicators of poor prognosis and may provide guidance to make pre-emptive clinical decisions in critically ill patients.

A central role for IL-6 in lung injury has been postulated with higher levels associated with mortality in two separate studies and severe immune-mediated injury in lung tissues of patients who died from COVID-19 [38, 39]. Similarly, the IL-1 pathway has been highlighted to contribute towards SARS-CoV-2 pathogenesis [40]. Importantly, our study highlighted the upregulation of IL-1 pathway mediators IL-18, MCP-1, and VEGF-A in critically-ill patients, providing further evidence that dysregulation in the IL-1 pathway could contribute to the hyperinflammatory state, especially in fatal cases.

STRING analysis revealed potential protein-to-protein interactions in severe COVID-19 infection, in which IL-6 is the direct interacting partner of other cytokines associated with disease severity. Thus, several approved IL-6 receptor antagonists could be repurposed to treat severe SARS-CoV-2 infection. Given the elevated level of VEGF-A, JAK inhibitors should also be investigated as a potential therapeutic option [41, 42]. However, the serial data presented here indicates that the therapeutic window for intervention is narrow. Immune modulators will need to be active before the inflammatory cascade causing acute lung injury develops.

Our study has several limitations. Active case finding through contact tracing is likely to have identified the majority of symptomatic infections in Singapore over this time period. However,

atypical or subclinical infections may have been missed. No infections were detected in children (<18 years) or residents of long term care facilities. Individuals were enrolled as soon as possible following admission, but while biological samples were collected serially, they were not all acquired at the same timepoint after symptom onset. Additionally, some laboratory data were incomplete, and clinical data such as date of symptom onset is subject to recall bias. We also did not investigate the effect of different SARS-CoV-2 lineages or mutations on study outcomes [43]. Finally, samples were processed consistently but numerous factors determine the success of viral cultures, and correlation between culturability and infectiousness is unclear.

In conclusion, we found virus viability was associated with lower PCR Ct value in early illness. This merits further investigation in terms of infectivity and infection control. SAR-CoV-2 IgM and IgG did not appear until days 15-21 of illness with implications on the role of rapid diagnostic antibody assay and the timing of plasmapheresis for convalescent plasma. A stronger antibody response was associated with disease severity suggesting a role in immune pathogenesis. Finally, the overactive proinflammatory immune signatures offers targets for host-directed immunotherapy which should be evaluated in randomised controlled trials.

Author Contributions

BEY, Y-SL, and DCL designed the study protocol. BEY, SWXO, PYC, SK, LYAC, SP, SWT, LS, PP, YD, PT, and JGL collected the data. LFPN, DEA, WNC, TMM, S-WF, Y-HC, CWT, BL, OR, LC, TB, RTPL, LR, and L-FW conducted the laboratory investigations including serologic, immunologic, and viral isolation. BEY and LWA Conducted the data analysis. BEY, SWXO, LFPN, WNC, and DCL drafted the manuscript. Y-SL, LR, L-FW, and DCL provided overall supervision. All authors read and approved the final manuscript.

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Dr Young had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Disclaimer

The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

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Declarations of Interest

BY reports personal fees from Roche and Sanofi, outside the submitted work. All other no interest declared.

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Table 1: Demographics, presenting symptoms, parameters and laboratory investigations stratified by disease severity.

	All	No pneumonia (Group A)	Pneumonia but no hypoxia (Group B)	p-value (A vs B)	Pneumonia and hypoxia (Group C)	p-value (A vs C)
	n=100	n=43	n=37		n=20	
Demographics						
Age, years	44 (35 to 56)	37 (31 to 50)	44 (35 to 55)	0.080	62 (49 to 68)	< 0.0001
Sex, male (%)	56 (56%)	23 (53%)	19 (51%)	1.0000	6 (70%)	0.28
Ethnicity (Chinese)	83 (83%)	33 (77%)	31 (84%)	0.58	19 (95%)	0.15
Co-morbidity, any	38 (38%)	12 (28%)	14 (38%)	0.47	12 (60%)	0.025
Diabetes	10 (10%)	2 (5%)	3 (8%)	0.66	5 (15%)	0.033
Hypertension	19 (19%)	2 (5%)	8 (22%)	0.039	9 (45%)	0.0003
Charlson's score	0 (0-0)	0 (0-0)	0 (0-0)	0.25	0 (0-1)	0.0067
Presenting Symptoms						
Duration of symptoms before admission	6 (2 to 9)	5 (2 to 9)	6 (4 to 9)	0.24	7 (2.4 to 8)	0.65
Fever	76 (76%)	26 (60%)	31 (84%)	0.027	19 (95%)	0.0059
Cough	70 (70%)	29 (67%)	25 (68%)	1.000	16 (80%)	0.38
Dyspnoea	17 (17%)	3 (7%)	6 (16%)	0.76	8 (40%)	0.022
Sore throat and/or rhinorrhoea	47 (47%)	29 (67%)	17 (46%)	0.070	6 (30%)	0.0071
Diarrhoea	19 (19%)	9 (21%)	6 (16%)	0.78	4 (20%)	1.000
Baseline parameters						
Temperature, °C	37.6 (37.0 to 38.3)	37.4 (36.9 to 38.0)	37.8 (37.0 to 38.2)	0.26	38.5 (37.7 to 39.0)	0.0045
Respiratory rate, per minute	18 (17 to 19)	18 (17 to 18)	18 (17 to 18)	0.31	20 (19 to 20)	0.0002
Heart rate, beats per minute	89 (77 to 99)	85 (75 to 98)	86 (76 to 105)	0.53	95 (86 to 104)	0.017
Systolic blood pressure, mmHg	132 (120 to 144)	131 (121 to 139)	132 (121 to 146)	0.76	131 (112 to 144)	0.74
Pulse oximeter oxygen saturation (%)	98 (96 to 99)	98 (98 to 100)	98 (97 to 99)	0.20	96 (95 to 97)	< 0.0001
Baseline laboratory investigations						
Haemoglobin, g/dL	13.9 (12.9 to 15.1)	13.9 (13.1 to 15.2)	14.4 (13.3 to 15.3)	0.33	13.1 (12.3 to 14.2)	0.033
Platelet, x10 ⁹ /L	193 (151 to 259)	213 (183 to 263)	185 (144 to 259)	0.14	157 (134 to 198)	0.0017
Lymphocyte, x10 ⁹ /L	1.1 (0.8 to 1.5)	1.4 (0.9 to 1.9)	1.1 (0.8 to 1.5)	0.070	0.5 (0.8 to 1.1)	0.0001
Neutrophil, x10 ⁹ /L	3.0 (2.3 to 4.1)	2.6 (2.3 to 4.1)	3.1 (2.2 to 3.7)	0.97	3.2 (2.4 to 5.1)	0.32
CRP, mg/L (n=81)	11.4 (3.3 to 47.4)	2.8 (1.1 to 9.4)	11.9 (7.1 to 22.1)	<0.0001	87.9 (64.6 to 153.1)	< 0.0001
LDH, U/L (n=90)	443 (365 to 595)	368 (342 to 416)	463 (389 to 585)	0.0024	632 (525 to 871)	< 0.0001
Creatinine, µmol/L (n=97)	67 (54 to 83)	65 (53 to 79)	69 (52 to 81)	0.78	76 (65 to 92)	0.038
ALT, U/L (n=85)	34 (28 to 40)	23 (15 to 32)	27 (15 to 44)	0.27	36 (30 to 56)	0.0014
PCR Ct value at diagnosis	28.2 (24.3 to 33.3)	28.0 (21.3 to 34.5)	29.3 (25.4 to 33.9)	0.35	27.0 (24.8 to 31.2)	0.94

Values are median (IQR) or number (%). Groups were compared using Mann-Whitney U test or Fisher's exact test. ALT: Alanine Aminotransferase; Ct: Cycle threshold; CRP: C-reactive protein; IQR: inter-quartile range; LDH: Lactate Dehydrogenase

Figures

Figure 1. Flow diagram of study recruitment and follow up.

130 patients diagnosed in Singapore as of 6 March 2020. *1 excluded as diagnosis was retrospective via serology rather than PCR. The 100 were enrolled from the following hospitals in Singapore: National Centre for Infectious Diseases (77); National University Hospital (8); Singapore General Hospital (8); Ng Teng Fong General Hospital (3); Changi General Hospital (2); Alexandra Hospital (1); Khoo Teck Puat Hospital (1).

Figure 2: Scatter plot of viral culture results by day from symptom onset and PCR Ct value.

Analysis includes 152 samples from 74 patients where virus was detectable by SARS-CoV-2 PCR. Virus was cultured from 21 (14%) samples from 19 patients.

Figure 3: IgG and IgM readings stratified by (A) duration of viral shedding, (B) disease severity and (C) time to first positive antibody level.

Fold of change (Fc) is calculated by dividing optical density (OD) reading of a test sample by the average OD reading of negative controls. Samples with $Fc > 3$ are considered positive. As sampling time and numbers were not uniform for all patients, we plotted the highest IgM/IgG from a single serum sample for each patient when multiple samples were available.

Figure 4. Immune signatures of COVID-19 patients reveal cytokines associated with disease severity.

Plasma fractions were isolated from the blood of COVID-19 patients ($n = 81$) at different time-points. Time-points closest to event onset, including mild (no pneumonia), pneumonia without hypoxia, or pneumonia with hypoxia, were chosen to identify cytokines that were associated with disease severity. Concentrations of 45 immune mediators were quantified using a 45-plex microbead-based immunoassay. **(A)** Longitudinal profile of detectable cytokines in COVID-19 patients during the acute phase of disease. Statistical analyses were performed using unpaired t-test against the healthy baseline ($*P < 0.05$; $**P < 0.01$, $***P < 0.001$). Cytokine level for healthy controls ($n = 23$) is indicated by the dotted line. **(B)** Heatmap of severity-associated cytokine levels in patients with different disease outcome (healthy controls, $n = 23$; no pneumonia, $n = 34$; pneumonia without hypoxia, $n = 28$; pneumonia with hypoxia, $n = 19$). Each color represents the relative concentration of a particular analyte. Blue and red indicates low and high concentration, respectively. **(C)** Profiles of significant immune mediators are illustrated as scatter plots. One-way ANOVAs were conducted on the logarithmically transformed concentration with post hoc T tests corrected using the method of Bonferroni. ANOVA results were corrected for multiple testing using the method of Benjamini and Hochberg ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$). Cytokine level for healthy controls ($n = 23$) is indicated by the dotted line. Patient samples that are not detectable are assigned the value of logarithm transformation of Limit of Quantification (LOQ).

Figure 5. Network analysis of immune mediators associated with disease severity in COVID-19 patients.

(A) Interactomic analysis by Ingenuity Pathway Analysis (IPA) Software. The network is displayed graphically as nodes and edges. Each node represents either severity-associated cytokine or disease condition. Thickness of edges represent the magnitude of biological relationship between connected nodes. Edges are drawn thicker when the association between parameters is stronger. **(B)** IPA analysis of the eight significant immune mediators associated with disease severity in COVID-19 patients. The chart represents the top ten significantly associated canonical pathways with the immune mediators. **(C)** Venn diagrams show common immune mediators between influenza virus infection and SARS-CoV-2 virus infection. **(D)** Interactive relationships between the immune mediators were determined by STRING analysis, with a confidence threshold of 0.8. IL-6, interleukin 6; IL-1RA, interleukin 1 receptor antagonist; MCP-1, monocyte chemoattractant protein-1; HGF, hepatocyte growth factor; VEGF-A, vascular endothelial growth factor A; PDGF-BB, two beta subunits of platelet-derived growth factor; IP-10, IFN- γ inducible protein, IL-8, interleukin 8; NRP-2, neuropilin 2.

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Figure 1

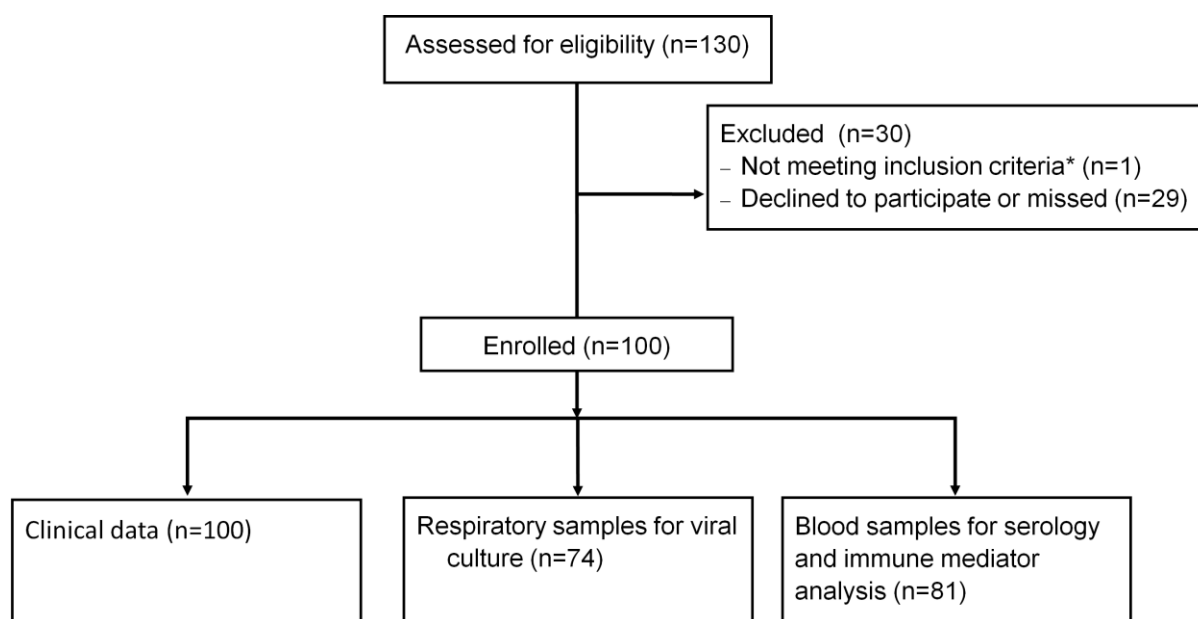


Figure 2

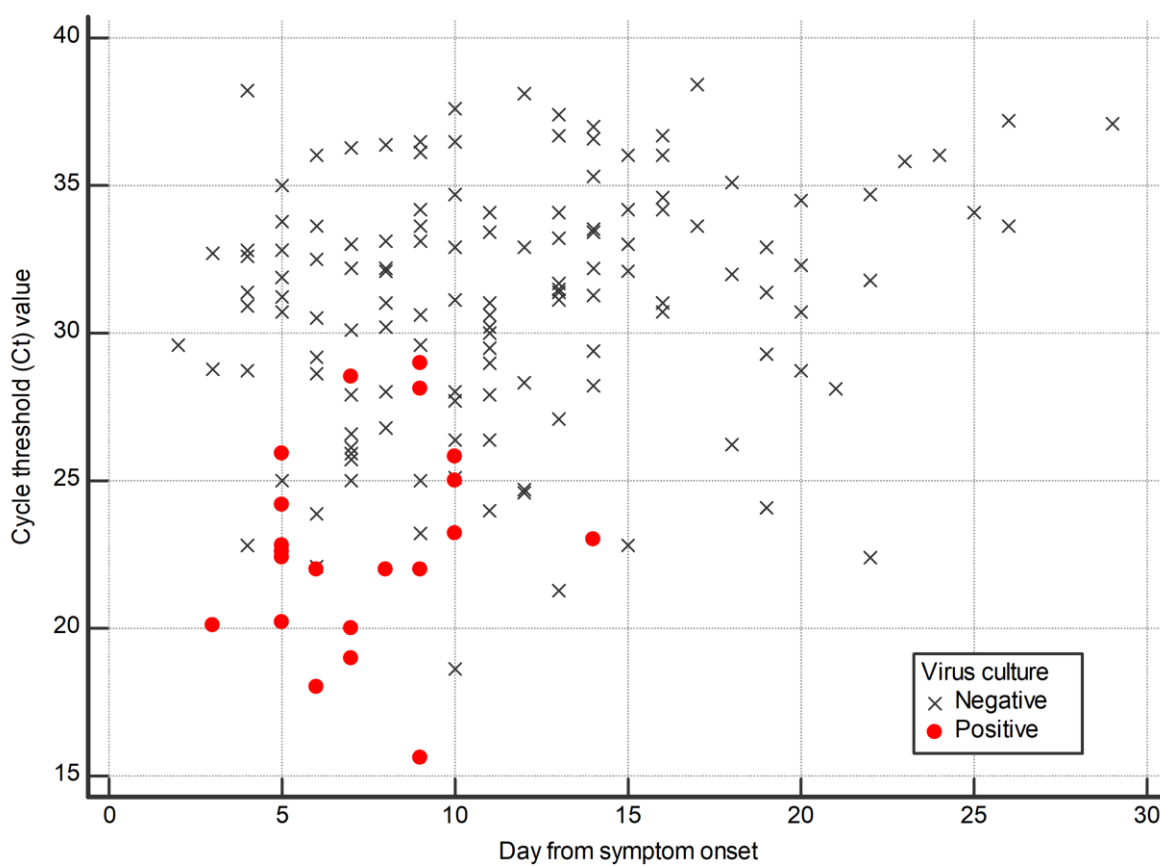


Figure 3

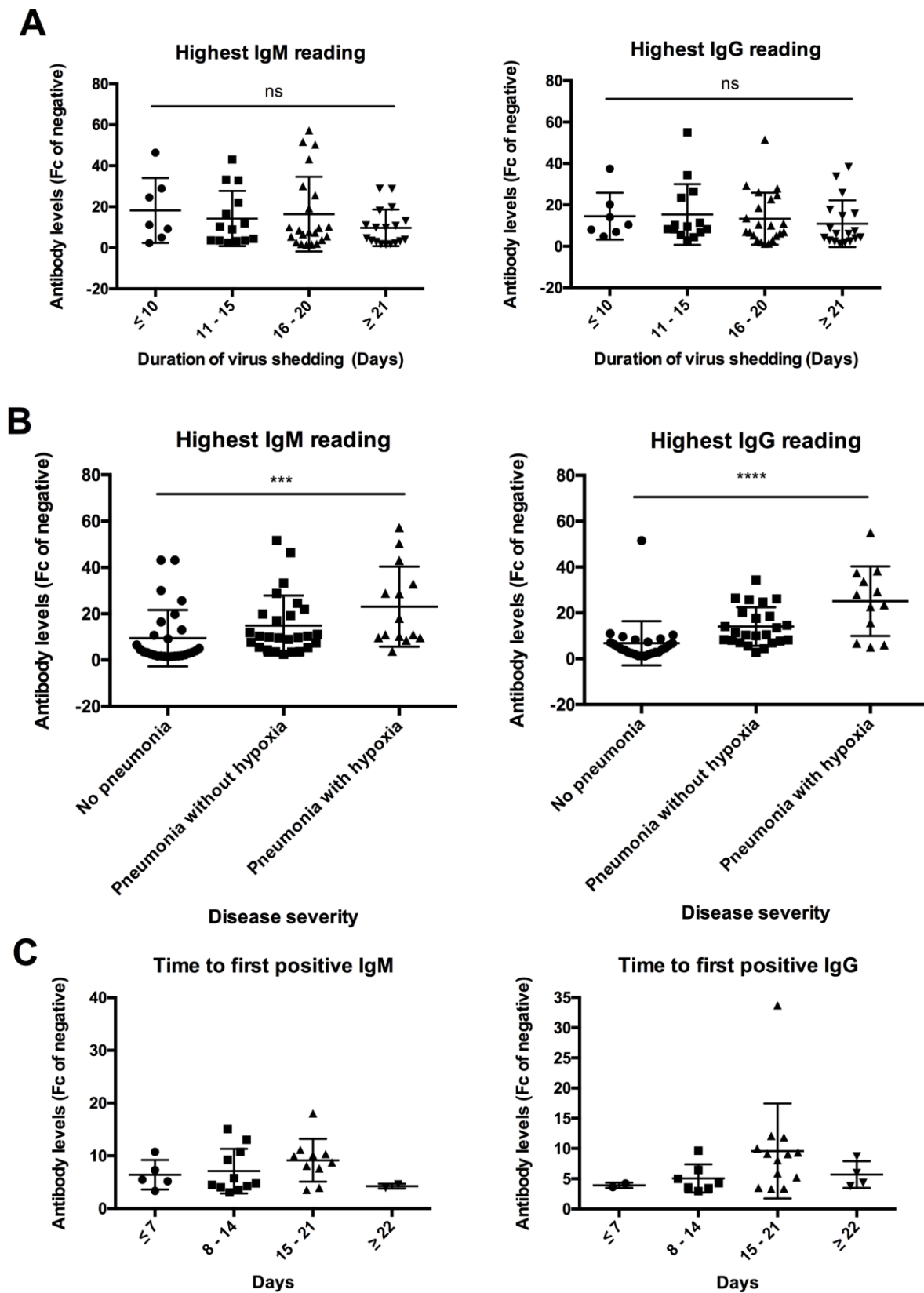


Figure 4

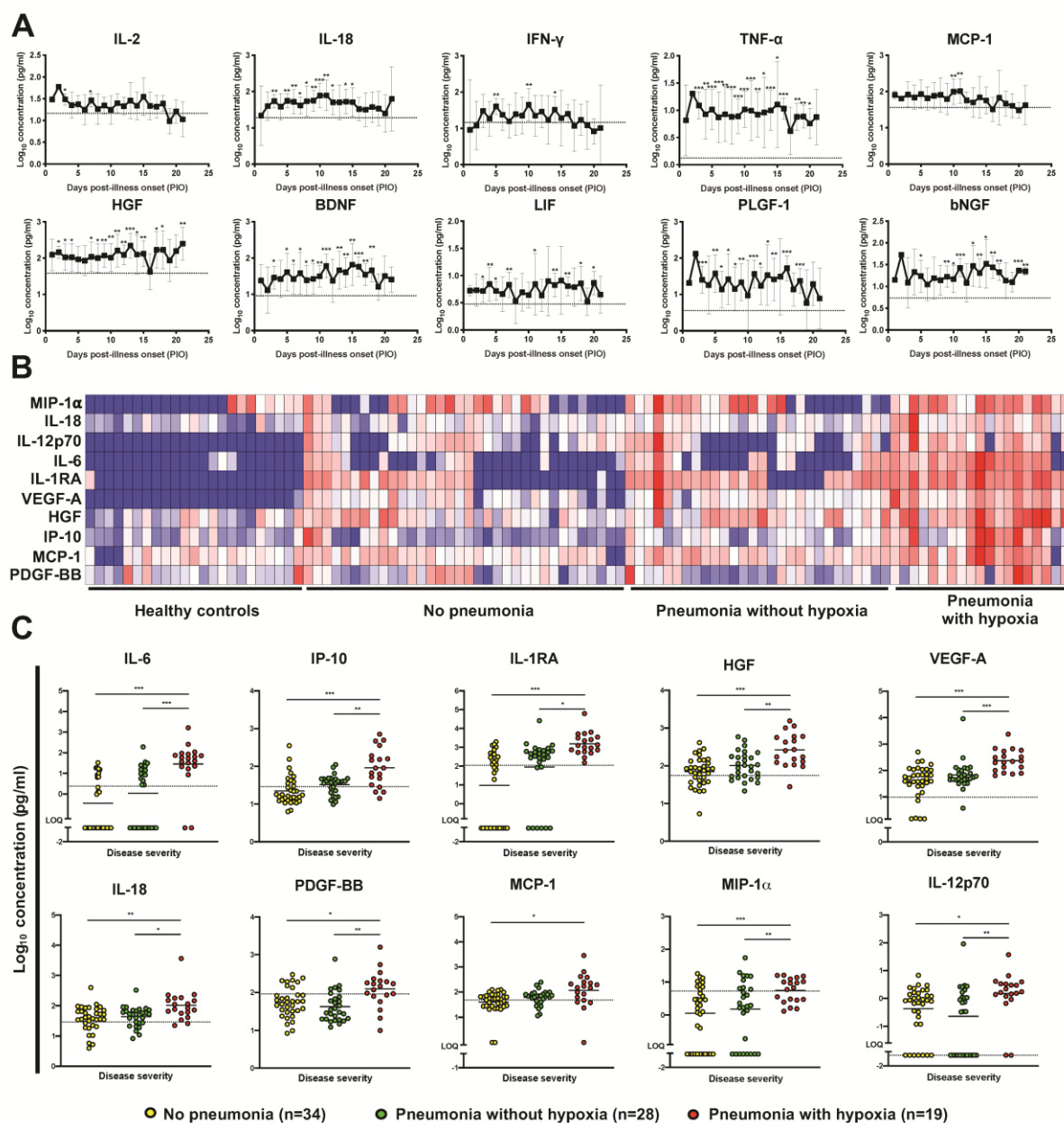


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Figure 5

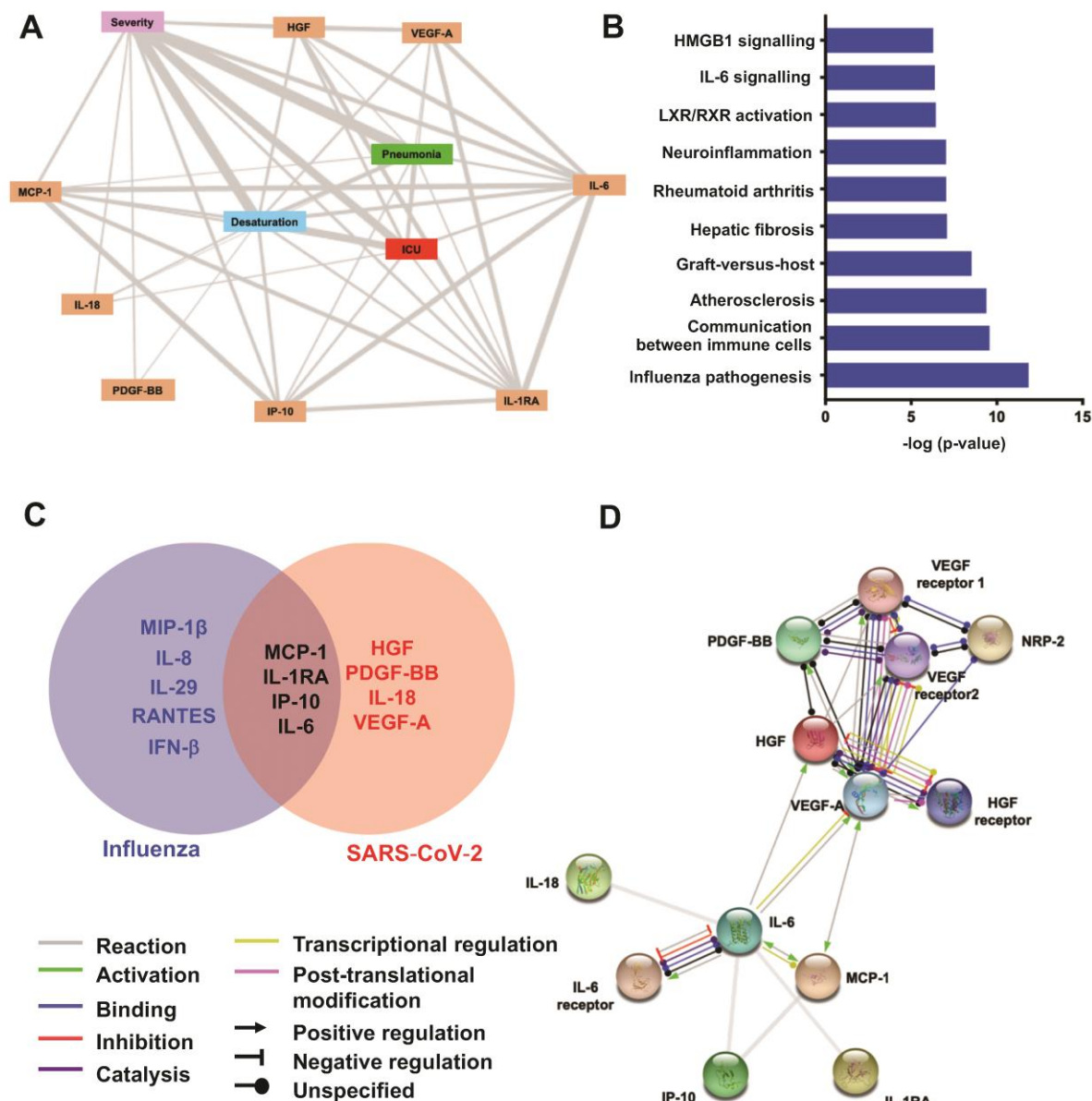


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