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# Cytokines and their relationship to the severity and prognosis of Coronavirus Disease 2019 (COVID-19)

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**Cytokines and their relationship to the severity and prognosis of Coronavirus Disease 2019 (COVID-19)**

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**Abstract**

**Objective:** To delineate the characteristics and clinical significance of plasma inflammatory cytokines altered in COVID-19.

**Design:** Retrospective, single-center cohort study.

**Setting:** Tongji Hospital in Wuhan, china.

**Participants:** Among a cohort of 308 patients with a diagnosis of COVID-19, 138 patients died, while 170 patients recovered and were discharged from the hospital. Date were collected until 27 February 2020.

**Primary and secondary outcome measures:** Clinical characteristics and laboratory findings were obtained from electronic medical records with data collection forms.

**Result:** The percentage of patients having elevated IL-2R, IL-6, IL-8, IL-10, and TNF-α increased with the severity of disease (P <0.0001 for all). The values of IL-2R (P <0.0001), IL-6 (P <0.0001), IL-8 (P = 0.0001), IL-10 (P <0.0001), and TNF-α (P <0.0001) were also 2 to 20-fold higher in dead patients compared to that of the recovered ones. Also, the IL-6 and IL-10 increased in both the progressive patient groups: moderate (P = 0.0026) and the severe (P <0.0001). In multivariable analysis, patients with a higher level of IL-2R (OR 1.001, 95%CI 1.000-1.002, P=0.031) and IL-6 (OR 1.013, 95%CI 1.003-1.024, P=0.015) at admission were associated with increasing odds of in-hospital death, independent of other covariates, including the severity of disease and the lymphocyte count. Additionally, IL-2R (P = 0.002), IL-6 (P<0.001), and IL-10 (P<0.001) were negatively correlated with the total cholesterol levels.

**Conclusion:** Increased pro-inflammatory and anti-inflammatory cytokines, including IL-2R, IL-6, IL-8, TNF-α, and IL-10, showed an obvious association both the severity and in-hospital mortality of COVID-19. Thus, our study indicates that cytokines are valuable in predicting the severity

of COVID-19 and helps in distinguishing critically ill patients from the less affected ones.

**Strengths and limitations of this study**

1. We systematically studied cytokine profiles along with their relationship to the severity and the prognosis of COVID-19 disease.
2. Meanwhile, objective factors of the patients related to their basic level of cytokines were explored.
3. Earlier identification of a severe or a critical case can help in providing clinical intervention, on time.
4. Our study has some limitations. Firstly, this study is a retrospective analysis based on initial inflammatory cytokine levels on admission.
5. Secondly, since the date regarding lymphocyte subsets is not available.

**Introduction**

The outbreak of coronavirus disease, 2019 (COVID-19) caused by Severe Acute Respiratory Syndrome-coronavirus-2 (SARS-CoV-2), is erupting worldwide. [1 2] The World Health Organization (WHO) declared COVID-19 as a pandemic.[3] Although most cases were mild to moderate, increasing COVID-19 cases lead to a significant number of patients developing severe symptoms and death. Currently, a lot of detailed clinical information on COVID-19 is known, but the characteristics of inflammatory cytokines associated with COVID-19 patients remain largely unclear. Severe illness, of almost any etiology, is accompanied by a generalized host inflammatory response. This host immune response process is referred to as Systemic Inflammatory Response Syndrome. If this process is not controlled or is dysfunctional, it will lead to cytokine storm syndrome [4]. Cytokine storm is one of the possible mechanisms underlying rapid disease progression [5]. Recent studies have reported a relationship between the

serum inflammatory cytokine levels and the severity or prognosis of COVID-19 patients. However, there are some inconsistent conclusions. For example, several studies reported interleukin (IL)-6 as a potential biomarker to predict COVID-19 progression or monitor disease severity [6-8]. However, Song et al. found no significant differences in the levels of IL-6 and tumor necrosis factor-α (TNF-α) between severe and non-severe COVID-19 patients [9]. Most of the previous studies only focused on the role of IL-6 in COVID-19. The characteristics and role of other cytokines in COVID-19, especially the anti-inflammatory cytokines, IL-10 and IL-8 were paid little attention. IL-10 can be a parameter in predicting the clinical outcome of patients with severe community-acquired pneumonia and is also the most important anti-inflammatory cytokine in the human immune response [10].

Thus, the authors of this research have systematically studied cytokine profiles along with their relationship to the severity and the prognosis of COVID-19 disease. Meanwhile, objective factors of the patients related to their basic level of cytokines were explored. Earlier identification of a severe or a critical case can help in providing clinical intervention, on time.

## Methods

### Study design and participants

This was a retrospective single-center study, which enrolled 308 patients from January 10, 2020, to February 13, 2020, at Tongji Hospital, Huazhong University of Science and Technology (Wuhan, China). As of February 27, 2020, 138 patients died, while 170 patients recovered and were discharged from the hospital. Data were collected at the time of admission. Diagnosis and clinical classification of COVID-19 were made according to the clinical guidelines (version 5 trial) developed by the National Health Committee of the People's Republic of China

(http://www.nhc.gov.cn/). The clinical classification is as follows: (1) moderate type- including fever, respiratory tract symptoms, and imaging that shows pneumonia. (2) severe type- meeting any of the following criteria: a) respiratory distress, respiratory rate ≥ 30 beats/min; b) in resting state, meaning oxygen saturation ≤ 93%; c) arterial blood oxygen partial pressure/oxygen concentration ≤ 300 mmHg (1 mmHg = 0.133 kPa). (3) critical type- involving one of the following conditions: a) respiratory failure requiring mechanical ventilation; b) presence of shock; c) combined organ failure requiring admission in ICU. For Tongji Hospital is a designated hospital for critically ill patients, the mortality is relatively high in this cohort. Exacerbation from moderate to severe or severe to critical was defined as progression. The primary outcome of the study was in-hospital mortality. The study excluded patients, having secondary vasculitis and uremia that needed maintenance hemodialysis along with the ones who died within 48 h of admission. This study was approved by the Tongji Hospital Ethics Committee. Before enrollment, written informed consent was obtained from patients involved in the study, while data was collected retrospectively.

**Cytokine measurement**

In order to explore the influence of COVID-19 on the secretion of cytokines, Chemiluminescence Immunoassay (CLIA) was performed. Cytokines including IL-1β, IL-2R, IL-6, IL-8 (also known as CXCL8), IL-10, and TNF-α were assessed using the serum samples drawn from the patients, shortly after hospitalization. CLIA was performed by a fully automated analyzer (Immulite 1000, DiaSorin Liaison, Italy or Cobas e602, Roche Diagnostics, Germany) for all the patients according to the manufacturer's instructions. IL-1β kit (#LKL11), IL-2R kit (#LKIP1), IL-8 kit (#LK8P1), IL-10 kit (#LKXP1) and TNF-α kit (#LKNF1) were purchased from DiaSorin (Vercelli, Italy). IL-6 kit (#05109442190) was purchased from Roche Diagnostics, Germany. Patients were divided into elevated and normal groups according to the instructions.

**Statistical analysis**

Continuous variables were expressed as a median with IQR, and categorical variables were described as frequency rates and percentages. We used the Mann-Whitney U test or Kruskal-Wallis test, whichever was appropriate, to compare the differences between the groups. Proportions for categorical variables were compared using the chi-square test, Fisher's exact test, or Yates' continuity corrected chi-square test. Univariate and multivariate logistic regression models (Forward: LR) were applied to screen the risk of death. The Kaplan–Meier method was used to assess the cumulative rate of mortality based on the normal range of cytokines and was compared with the log-rank test. Receiver operating characteristic curve (ROC) analysis was performed to assess the accuracy of inflammatory cytokine levels in predicting death. The tests were two-sided, and a P value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS (version 23.0).

**Patient and public involvement**

This was a retrospective case series study, and no patients were involved in the study design or in setting the research questions or the outcome measures directly. No patients were asked to advise on interpretation or writing up of results.

**Result**

**The Characteristics in COVID-19 patient cohort**

The demographic and clinical characteristics of 308 COVID-19 patients are summarized in Table 1. At the time of admission, 91 (30%) patients were classified as moderate type, 133 (43%) patients as severe, and 84 (27%) patients fulfilled the criteria of critical type. As per the results, 138 (45%) patients died, and 170 (55%) were discharged from the hospital. Moderate patients and survived patients were more likely to be female and younger. The ratio of comorbidity was significantly higher in critical (P <0.0001) and the dead group (P <0.0001). In the laboratory findings, the

levels of lymphocytes, platelets, and the total cholesterol were lower in critical and the dead group, whereas neutrophil count, D-dimer, alanine aminotransferase, blood urea nitrogen, creatinine, procalcitonin, lactic dehydrogenase, C-reactive protein, IL-2R, IL-6, IL-8, IL-10, and TNF-α were significantly higher (P <0.0001 for all). Also, critical (P <0.0001) and the dead (P <0.0001) group were more likely to receive non-invasive ventilation or invasive ventilation. The median time from disease onset to the outcome was 22 (IQR 15.8–30.0) days for non-survivors and 29 (IQR 24.0–34.0) days for the survivors.

Table 1. Demographics and clinical characteristics of COVID-19 patients.

| Variables | Condition at admission | | | P value | Outcome | | P value |
|---|---|---|---|---|---|---|---|
| | Moderate (n=91) | Severe (n=133) | Critical (n=84) | | Survivor (n=170) | Non-survivor (n=138) | |
| Age, years | 55.0 (39.0-67.0) | 66.0 (55.5-73.0) | 70.0 (63.3-78.8) | <0.0001 | 57.0 (43.0-68.0) | 70.0 (62.8-78.0) | <0.0001 |
| Female, n (%) | 51/91 (56%) | 67/133 (50%) | 24/84 (29%) | 0.0003 | 95/170 (56%) | 47/138 (34%) | 0.0001 |
| Comorbidity, n (%) | 35/89 (39%) | 82/132 (62%) | 61/83 (74%) | <0.0001 | 72/167 (43%) | 106/137 (77%) | <0.0001 |
| Hypertension, n (%) | 18/89 (20%) | 51/132 (39%) | 41/83 (49%) | <0.0001 | 43/167 (26%) | 67/137 (49%) | <0.0001 |
| Diabetes, n (%) | 14/89 (16%) | 22/132 (17) | 20/83 (24%) | 0.1621 | 24/167 (14%) | 32/137 (23%) | 0.0443 |
| Cardiovascular disease, n (%) | 5/89 (6%) | 21/132 (16%) | 15/83 (18%) | 0.0158 | 13/167 (8%) | 28/137 (20%) | 0.0013 |
| Cerebrovascular disease, n (%) | 2/89 (2%) | 3/132 (2%) | 7/83 (8%) | 0.0402 | 4/167 (2%) | 8/137 (6%) | 0.2155 |
| Pulmonary disease, n (%) | 6/89 (7%) | 11/132 (8%) | 9/83 (11%) | 0.3380 | 10/167 (6%) | 16/137 (12%) | 0.0775 |
| Chronic kidney disease, n (%) | 0/89 (0%) | 3/132 (2%) | 4/83 (5%) | 0.0353 | 1/167 (1%) | 6/137 (4%) | 0.0715 |
| Signs and symptoms | | | | | | | |
| Fever, n (%) | 76/90 (84%) | 119/133 (90%) | 77/84 (92%) | 0.1318 | 145/169 (86%) | 127/138 (92%) | 0.0875 |
| Cough, n (%) | 54/90 (60%) | 101/132 (77%) | 71/84 (85%) | 0.0002 | 114/169 (68%) | 112/137 (82%) | 0.0047 |
| Laboratory parameters | | | | | | | |

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|---|---|---|---|---|---|---|---|
| Neutrophil count, $\times 10^9$/L | 3.1 (2.5-4.1) | 4.0 (2.7-5.7) | 10.1 (7.2-14.7) | <0.0001 | 3.3 (2.4-4.3) | 8.1 (4.9-12.5) | <0.0001 |
| Lymphocyte count, $\times 10^9$/L | 1.2 (0.9-1.6) | 0.9 (0.7-1.3) | 0.5 (0.4-0.8) | <0.0001 | 1.1 (0.8-1.5) | 0.6 (0.4-0.8) | <0.0001 |
| Platelet count, $\times 10^9$/L | 213.0 (160.0-309.0) | 208.5 (149.0-261.3) | 156.0 (94.5-222.0) | <0.0001 | 220.0 (170.8-291.3) | 151.0 (107.0-222.5) | <0.0001 |
| D-Dimer, ug/mL | 0.4 (0.3-0.8) | 1.0 (0.5-2.6) | 18.1 (2.6-21.0) | <0.0001 | 0.5 (0.3-1.0) | 7.9 (1.3-21.0) | <0.0001 |
| ALT, U/L | 18.0 (13.0-24.0) | 23.0 (16.0-39.0) | 33.5 (21.0-55.8) | <0.0001 | 20.0 (13.8-32.5) | 28.0 (18.0-46.5) | <0.0001 |
| BUN, mmol/L | 3.7 (3.1-4.7) | 5.1 (3.7-6.7) | 9.6 (7.0-16.1) | <0.0001 | 3.9 (3.1-5.2) | 8.8 (5.6-12.9) | <0.0001 |
| Creatinine, umol/L | 66.0 (57.0-81.0) | 72.0 (56.0-93.0) | 87.5 (70.5-120.0) | <0.0001 | 65.5 (57.0-82.0) | 86.5 (66.8-120.0) | <0.0001 |
| Total cholesterol, mmol/L | 3.8 (3.2-4.5) | 3.5 (3.1-3.9) | 3.2 (2.8-3.8) | <0.0001 | 3.6 (3.2-4.3) | 3.3 (2.8-3.9) | <0.0001 |
| Procalcitonin, ng/ml | 0.05 (0.04-0.09) | 0.09 (0.05-0.23) | 0.49 (0.17-1.49) | <0.0001 | 0.05 (0.04-0.09) | 0.31 (0.14-1.09) | <0.0001 |
| Lactic dehydrogenase, U/L | 229.0 (190.0-283.0) | 315.0 (219.5-431.8) | 637.0 (490.5-872.5) | <0.0001 | 243.0 (194.8-309.8) | 524.5 (366.0-721.0) | <0.0001 |
| C-reactive protein, mg/L | 7.7 (2.5-24.3) | 49.3 (8.9-93.3) | 112.3 (71.4-187.3) | <0.0001 | 10.7 (2.4-36.7) | 100.5 (62.4-161.2) | <0.0001 |
| Interleukin 1β ≥5pg/mL | 13/91 (14%) | 17/133 (13%) | 11/84 (13%) | 0.8120 | 23/170 (14%) | 18/138 (13%) | 0.9006 |
| Interleukin-2 receptor, U/mL | 475.5 (375.8-630.8) | 799.0 (538.5-1097.0) | 1259.5 (942.3-1825.0) | <0.0001 | 553.0 (402.0-802.0) | 1137.5 (822.0-1584.3) | <0.0001 |
| ≥710 U/L | 19/91 (21%) | 74/133 (56%) | 77/84 (92%) | <0.0001 | 51/170 (30%) | 113/138 (82%) | <0.0001 |
| Interleukin-6, pg/mL | 5.6 (2.7-15.3) | 24.3 (6.7-61.7) | 64.8 (29.42-153.1) | <0.0001 | 7.9 (2.7-22.8) | 59.7 (23.6-137.4) | <0.0001 |
| ≥7 pg/mL | 41/91 (45%) | 100/133 (75%) | 77/83 (92%) | <0.0001 | 90/170 (53%) | 132/137 (96%) | <0.0001 |
| Interleukin-8, pg/mL | 15.4 (7.7-29.4) | 19.5 (12-35.5) | 30.8 (21.0-71.8) | <0.0001 | 16.3 (9.4-28.7) | 26.6 (16.4-60.40) | <0.0001 |
| ≥62 pg/mL | 10/91 (11%) | 13/133 (10%) | 77/84 (92%) | <0.0001 | 14/170 (8%) | 33/138 (24%) | 0.0001 |
| Interleukin-10, pg/mL | 5.0 (5.0-5.1) | 5.9 (5.0-10.80) | 10.9 (6.4-18.7) | <0.0001 | 5.0 (5.0-6.7) | 10.1 (5.4-16.4) | <0.0001 |
| ≥9.1 pg/mL | 10/91 (11%) | 46/133 (35%) | 76/83 (92%) | <0.0001 | 31/170 (18%) | 76/137 (56%) | <0.0001 |
| TNF-α, pg/mL | 7.7 (6.0-9.5) | 8.6 (6.9-11.9) | 11.2 (7.4-18.8) | <0.0001 | 7.8 (6.1-9.7) | 10.9 (7.7-15.9) | <0.0001 |
| ≥8.1 pg/mL | 43/91 (47%) | 80/133 (60%) | 77/84 (92%) | <0.0001 | 82/170 (48%) | 101/138 (73%) | <0.0001 |
| Treatment | | | | | | | |
| Mechanical ventilation, n (%) | 4/91 (4%) | 46/133 (35%) | 79/84 (94%) | <0.0001 | 7/170 (4%) | 122/138 (88%) | <0.0001 |
| Antibiotics treatment, n (%) | 85/91 (93%) | 128/133 (96%) | 84-0/84 (100%) | 0.0191 | 160/170 (94%) | 137/138 (99%) | 0.0343 |

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|---|---|---|---|---|---|---|---|
| Antiviral treatment, n (%) | 90/90 (100%) | 124/126 (98%) | 62/79 (79%) | <0.0001 | 167/167 (100%) | 109/128 (85%) | <0.0001 |
| Corticosteroids, n (%) | 48/90 (53%) | 86/133 (65%) | 74/84 (88%) | <0.0001 | 84/169 (50%) | 124/138 (90%) | <0.0001 |
| Immunoglobin, n (%) | 31/91 (34%) | 77-56/133 (58%) | 47-37/84 (56%) | 0.0031 | 69/170 (41%) | 86/138 (62%) | 0.0001 |
| Duration of complaint, days | 8.0 (4.8-13.0) | 10.0 (7.0-13.5) | 11.0 (7.0-15.0) | 0.0007 | 8.0 (6.0-13.0) | 10.0 (7.0-15.0) | 0.0027 |
| Hospitalization, days | 16.0 (12.0-23.0) | 19.0 (12.0-24.0) | 8.0 (4.0-13.0) | <0.0001 | 19.5 (14.0-24.0) | 10.0 (5.0-17.0) | <0.0001 |
| Duration of disease, days | 26.0 (20.0-31.0) | 29.0 (24.0-35.0) | 19.0 (15.0-29.0) | <0.0001 | 29.0 (24.0-34.0) | 22.0 (15.8-30.0) | <0.0001 |
| Progression | 8/45 (18%) | 52/129 (40%) | - | - | - | - | - |

Data are median (IQR), mean (SD) or n (%). P values were calculated by Mann-Whitney U test, Kruskal-Wallis test, chi-square test, Fisher's exact test or Yates' continuity corrected chi-square test, as appropriate. ALT, Alanine aminotransferase; BUN, blood urea nitrogen; Duration of complaint, time from onset of symptom to hospital admission; Hospitalization, time from hospital admission to outcome; Duration of disease, time from onset of symptom to outcome.



**Plasma cytokine alteration in COVID-19**

The baseline for cytokine concentrations was determined from blood obtained at admission time. The levels and the abnormal ratio of cytokines, including IL-2R, IL-6, IL-8, IL-10, and TNF-α, gradually increased as the disease progressed or resulted in poor prognosis (P <0.0001 for all). No significant difference was observed in IL-1β (P = 0.8120) within each of the groups. Likewise, the values of IL-2R (P <0.0001), IL-6 (P <0.0001), IL-8 (P = 0.0001), IL-10 (P <0.0001) and TNF-α (P <0.0001) were also 2 to 20-fold higher in dead patients compared to that of the recovered ones (Table 1). Additionally, several cytokines, including pro-inflammatory and anti-inflammatory, were elevated at the baseline in patients whose conditions were progressive compared to the patients whose conditions were stable (Figure 1). Specifically, the pro-inflammatory IL-2R (P <0.0001) and TNF-α (P <0.0001) were significantly higher in the progressive group compared with the stable group in the severe type of patients. IL-6 was increased in the progressive group, both in moderate (P = 0.0026) and in severe (P <0.0001) type of patients. Although numerically high,

the differences in IL-8 levels between the two groups did not reach statistical significance. Similarly, the progressive group had higher levels of the anti-inflammatory cytokine, IL-10 in both the types of severity (P = 0.0008 for moderate type, P = 0.0011 for severe type). These results suggest that the progression of COVID-19 was associated with the initial levels of plasma cytokine.

**Correlation between the baseline cytokine levels and the physiological variables measured on admission**

The relationship between plasma cytokines and various physiological variables were assessed and presented in Table 2. Except for IL-1β, other plasma cytokines were positively correlated with age, albumin, creatinine, random blood glucose, D-dimer, lactic dehydrogenase, and CRP (P<0.001 for all). Also, IL-2R (P = 0.002), IL-6 (P<0.001), and IL-10 (P<0.001) were negatively correlated with total cholesterol. There was no significant relationship between the cytokine levels and levels of C3 and C4.

Table 2. Correlation among baseline inflammatory biomarkers and physiologic variables measure on the day of admission.

| | IL-1β | IL -2R | IL -6 | IL -8 | IL-10 | TNF-α |
|---|---|---|---|---|---|---|
| Age | -0.002 | 0.426 | 0.356 | 0.293 | 0.261 | 0.361 |
| | 0.967 | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| Time from onset of symptom | -0.061 | 0.222 | -0.011 | 0.038 | -0.069 | -0.055 |
| to hospital admission | 0.289 | <0.001* | 0.843 | 0.504 | 0.226 | 0.336 |
| Albumin | 0.177 | -0.527 | -0.467 | -0.319 | -0.346 | -0.290 |
| | 0.003* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| ALT | -0.047 | 0.276 | 0.250 | 0.198 | 0.167 | 0.248 |
| | 0.415 | <0.001* | <0.001* | 0.001* | 0.003* | <0.001* |
| Creatinine | -0.037 | 0.332 | 0.355 | 0.226 | 0.284 | 0.439 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 0.512 | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| Uric acid | 0.033 | 0.170 | 0.155 | 0.128 | 0.052 | 0.331 |
| | 0.578 | 0.004* | 0.054 | 0.031* | 0.387 | <0.001* |
| Total cholesterol | -0.021 | -0.177 | -0.332 | -0.080 | -0.276 | -0.103 |
| | 0.709 | 0.002* | <0.001* | 0.159 | <0.001* | 0.071 |
| Random blood glucose | 0.004 | 0.303 | 0.276 | 0.272 | 0.336 | 0.270 |
| | 0.950 | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| D-Dimer | -0.026 | 0.614 | 0.509 | 0.328 | 0.378 | 0.367 |
| | 0.651 | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| Lactic dehydrogenase | -0.065 | 0.598 | 0.562 | 0.339 | 0.472 | 0.391 |
| | 0.255 | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| CRP | 0.008 | 0.618 | 0.776 | 0.345 | 0.528 | 0.493 |
| | 0.894 | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| C3 | -0.226 | -0.235 | -0.026 | -0.066 | 0.093 | -0.262 |
| | 0.52 | 0.137 | 0.882 | 0.652 | 0.576 | 0.075 |
| C4 | 0.62 | 0.001 | -0.056 | -0.156 | 0.070 | -0.21 |
| | 0.053 | 0.991 | 0.711 | 0.295 | 0.673 | 0.163 |

Values present are Spearman correlation coefficients (upper) and P values (lower). IL, interleukin; TNF-α, tumor necrosis factor-α; ALT, alanine aminotransferase; CRP, C-reactive protein; C3, complement 3; C4, complement 4. *Denotes statistically significant correlations.

**Association of plasma cytokines with the in-hospital deaths**

Kaplan-Meier analysis indicated a significantly higher mortality rate in patients with abnormal plasma cytokine values, including elevated IL-2R, IL-6, IL-8, IL-10, and TNF-α (P<0.0001 for all) (Figure 2). Univariate logistic regression analysis revealed that IL-2R, IL-6, IL-8, IL-10, and

TNF-α were related to a poor outcome (Table 3). After adjusting for age, gender, comorbidities, the severity of the disease, and lymphocyte count, the levels of IL-2R and IL-6 were associated with in-hospital mortality (Figure 3). Further, the plasma cytokines were analyzed by ROC analysis to evaluate their ability to predict the in-hospital death rates (Figure 4). The area under the curve was 0.82 (95%CI: 0.78–0.87) for IL-2R, 0.85 (95%CI: 0.81–0.89) for IL-6, 0.69 (95%CI: 0.64–0.75) for IL-8, 0.75 (95%CI: 0.69–0.81) for IL-10, 0.71 (95%CI: 0.65–0.77) for TNF-α (Table 4).

Table 3. Logistic regression to analysis independent factors for predicting mortality of COVID-19 patients.

| Cytokines | P value | OR | 95% CI |
|---|---|---|---|
| IL-1β, pg/mL* | 0.242 | 1.028 | 0.982-1.077 |
| IL -2R, U/mL* | <0.001 | 1.003 | 1.002-1.004 |
| IL -6, pg/mL* | <0.001 | 1.031 | 1.022-1.040 |
| IL -8, pg/mL* | <0.001 | 1.010 | 1.004-1.015 |
| IL-10, pg/mL* | <0.001 | 1.066 | 1.032-1.101 |
| TNF-α, pg/mL* | <0.001 | 1.205 | 1.131-1.285 |

IL, interleukin; TNF, tumor necrosis factor; OR, odds ratio; CI, confidence interval; *Per 1unit increase.

Table 4. Analysis of receiver-operating characteristics curve for predicting in-hospital mortality in COVID-19 patients.

| Variables | P value | AUC (95%CI) | Sensitivity | Specificity | Cut-off value | PPV | NPV |
|---|---|---|---|---|---|---|---|
| Interleukin-2 receptor | <0.001 | 0.82 (0.78-0.87) | 0.80 | 0.73 | 755.50 | 0.72 | 0.55 |
| Interleukin-6 | <0.001 | 0.85 (0.81-0.89) | 0.77 | 0.75 | 22.80 | 0.71 | 0.80 |
| Interleukin-8 | <0.001 | 0.69 (0.64-0.75) | 0.66 | 0.62 | 20.50 | 0.58 | 0.55 |
| Interleukin-10 | <0.001 | 0.75 (0.69-0.81) | 0.80 | 0.66 | 5.15 | 0.65 | 0.55 |
| TNF-α | <0.001 | 0.71 (0.65-0.77) | 0.56 | 0.79 | 10.05 | 0.68 | 0.55 |

TNF-**α**, tumor necrosis factor-**α**; AUC, area under curves; PPV = positive predictive value; NPV = negative predictive value

## Discussion

COVID-19 has rapidly spread throughout the world and is labeled as a pandemic by WHO. Both clinical features and the serum markers associated with the severity of COVID-19 patients have been reported[11-14]. However, insufficient knowledge about the specific alterations in the cytokine levels could be the immune mechanism underlying rapid disease progression. In the present study, we systematically analyzed immunological characteristics, particularly cytokine profiles and their relationship with the severity, mortality, and prognosis of COVID-19.

Consistent with the previous report, we noted lymphocytopenia and increased inflammatory cytokine concentration in the majority of the severe and critical cases, which was markedly worse when compared to the moderate cases. This indicates that an impaired immune system and a cytokine storm might be associated with COVID-19 severity. Further, the plasma concentration of cytokines in the progressive group was higher than the stable group in severe type of cases. Although, the differences in IL-2R, IL-8, and TNF-α between the two groups did not reach statistical significance in moderate type cases, which might be partly due to the limited sample size; a recent study has shown that elevated cytokines were dynamically correlated with the disease severity[5 15 16]. Additionally, SARS-CoV and MERS-CoV infections were also characterized by elevated levels of inflammatory cytokines with severe lung injury[17-20].

Cytokines are proteins, glycoproteins, or signaling peptides with potent various biological functions at picomolar concentration[21]. IL-2R, IL-6, IL-8, and TNF-α were major pro-inflammatory factors required to initiate a series of effective immune cascade events to an infection or a tissue injury site. The anti-inflammatory IL-10 was found to inhibit the monocyte inflammatory response directly and negatively regulate the cascade of pro-inflammatory cytokines, which induced monocyte hyporesponsiveness in sepsis and multiple organ dysfunction[22]. It is reported that coronavirus infection, its rapid replication, as well as the delayed INF-I signaling, activates inflammatory monocyte-macrophage, resulting in an

increased cytokine concentration, vascular leakage, and pathogenic T cell response[23]. Besides, lymphocytopenia observed in severe and critical patients may impair T cells, which dampens the overactive innate immune response and further aggravate the inflammatory response[24]. In line with the previous study, we suggest that excessive cytokine secretion may be associated with the progressive group. Nevertheless, the underlying cellular source and the mechanism involving cytokine accumulation remains to be determined.

Meanwhile, at the time of admission, serum levels of both pro-inflammatory and anti-inflammatory cytokines, including IL-2R, IL-6, IL-8, TNF-α and IL-10, were significantly higher in the case of non-survivors compared to that of the survivors. Previous studies have shown that pro-inflammatory cytokines predict mortality in patients with sepsis, acute respiratory distress syndrome, and the severe infection seen after burnt injuries[20 25]. Also, elevated cytokines are a concern as an independent risk factor of a poor outcome in acute renal failure[26]. In line with the previous research, univariate logistic regression analysis suggested that IL-2R, IL-6, IL-8, IL-10, and TNF-α were related to in-hospital mortality incidents in the present cohort. The association between IL-2R, IL-6, and in-hospital mortality was maintained after adjusting for demographics and other cofounders. A similar conclusion of elevated cytokines related to in-hospital mortality has been found in SARS-CoV or MERS-CoV infection[18-20]. In contrast, both concentration and proportion of IL-1β were not increased in most of the patients, with only 13% of non-survivors showing elevated levels of IL-1β. While the secondary inflammatory cytokine IL-6, considered more distal than IL-1 in the inflammatory cascade, was a significant predictor of survival and had a higher AUC value. The specific immune cascade response and the cellular origin of cytokines in COVID-19 warrants further exploration.

In conclusion, both pro-inflammatory and anti-inflammatory cytokines alteration, including IL-2R, IL-6, IL-8, TNF-α, and IL-10, shows an obvious association with the severity and in-hospital mortality of COVID-19.

**Competing interest:** The authors declare that they have no conflict of interest.

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**Contributors:** QQL, AYC, XCZ, LH and HFL did the data collection, QQL and AYC wrote the first draft of the manuscript. AYC, YRW and QQL analyzed the data, provided edits of the first draft of the manuscript. TW and FH participated in the revision of the manuscript and approved the final version.

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**References**

1. Wang LS, Wang YR, Ye DW, et al. A review of the 2019 Novel Coronavirus (COVID-19) based on current evidence. *International journal of antimicrobial agents* 2020:105948. doi: 10.1016/j.ijantimicag.2020.105948 [published Online First: 2020/03/24]

2. Sohrabi C, Alsafi Z, O'Neill N, et al. World Health Organization declares global emergency: A review of the 2019 novel coronavirus (COVID-19). *International journal of surgery (London, England)* 2020;76:71-76. doi: 10.1016/j.ijsu.2020.02.034 [published Online First: 2020/03/01]

3. WHO Director-General's opening remarks at the media briefing on COVID-19-11 March 2020

4. Chousterman BG, Swirski FK, Weber GF. Cytokine storm and sepsis disease pathogenesis. *Seminars in immunopathology* 2017;39(5):517-28. doi: 10.1007/s00281-017-0639-8 [published Online First: 2017/05/31]

5. Mehta P, McAuley DF, Brown M, et al. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet* 2020;395(10229):1033-34. doi: 10.1016/S0140-6736(20)30628-0

6. Gong J, Dong H, Xia SQ, et al. Correlation Analysis Between Disease Severity and Inflammation-related Parameters in Patients with COVID-19 Pneumonia. *medRxiv* 2020:2020.02.25.20025643. doi: 10.1101/2020.02.25.20025643

7. Ulhaq ZS, Soraya GV. [Not Available]. *Medecine et maladies infectieuses* 2020 doi: 10.1016/j.medmal.2020.04.002 [published Online First: 2020/04/08]

8. Liu T, Zhang J, Yang Y, et al. The potential role of IL-6 in monitoring severe case of coronavirus disease 2019. *medRxiv* 2020:2020.03.01.20029769. doi: 10.1101/2020.03.01.20029769

9. Song C-Y, Xu J, He J-Q, et al. COVID-19 early warning score: a multi-parameter screening tool to identify highly suspected patients. *medRxiv* 2020:2020.03.05.20031906. doi: 10.1101/2020.03.05.20031906

10. Wu CL, Chan MC, Chang GC, et al. Etiology and cytokine expression in patients requiring mechanical ventilation due to severe community-acquired

pneumonia. *Journal of the Formosan Medical Association = Taiwan yi zhi* 2006;105(1):49-55. doi: 10.1016/s0929-6646(09)60108-x [published Online First: 2006/01/28]

11. Wu C, Chen X, Cai Y, et al. Risk Factors Associated With Acute Respiratory Distress Syndrome and Death in Patients With Coronavirus Disease 2019 Pneumonia in Wuhan, China. *JAMA Intern Med* 2020 doi: 10.1001/jamainternmed.2020.0994
12. Young BE, Ong SWX, Kalimuddin S, et al. Epidemiologic Features and Clinical Course of Patients Infected With SARS-CoV-2 in Singapore. *Jama* 2020 doi: 10.1001/jama.2020.3204
13. Zhang JJ, Dong X, Cao YY, et al. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy* 2020 doi: 10.1111/all.14238
14. Zhou F, Yu T, Du R, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *The Lancet* 2020 doi: 10.1016/s0140-6736(20)30566-3
15. Chen X, Zhao B, Qu Y, et al. Detectable serum SARS-CoV-2 viral load (RNAaemia) is closely correlated with drastically elevated interleukin 6 (IL-6) level in critically ill COVID-19 patients. *Clin Infect Dis* 2020 doi: 10.1093/cid/ciaa449
16. Chen G, Wu D, Guo W, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. *J Clin Invest* 2020 doi: 10.1172/JCI137244

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17. Jiang Y, Xu J, Zhou C, et al. Characterization of Cytokine/Chemokine Profiles of Severe Acute Respiratory Syndrome. *American Journal of Respiratory and Critical Care Medicine* 2005;171(8):850-57. doi: 10.1164/rccm.200407-857OC

18. Zhou J, Chu H, Li C, et al. Active replication of Middle East respiratory syndrome coronavirus and aberrant induction of inflammatory cytokines and chemokines in human macrophages: implications for pathogenesis. *J Infect Dis* 2014;209(9):1331-42. doi: 10.1093/infdis/jit504

19. Chien JY, Hsueh PR, Cheng WC, et al. Temporal changes in cytokine/chemokine profiles and pulmonary involvement in severe acute respiratory syndrome. *Respirology* 2006;11(6):715-22. doi: 10.1111/j.1440-1843.2006.00942.x

20. Kong SL, Chui P, Lim B, et al. Elucidating the molecular physiopathology of acute respiratory distress syndrome in severe acute respiratory syndrome patients. *Virus Res* 2009;145(2):260-9. doi: 10.1016/j.virusres.2009.07.014

21. Bienvenu J, Monneret G, Fabien N, et al. The clinical usefulness of the measurement of cytokines. *Clin Chem Lab Med* 2000;38(4):267-85. doi: 10.1515/CCLM.2000.040

22. Sfeir T, Saha DC, Astiz M, et al. Role of interleukin-10 in monocyte hyporesponsiveness associated with septic shock. *Crit Care Med* 2001;29(1):129-33. doi: 10.1097/00003246-200101000-00026

23. Channappanavar R, Fehr AR, Vijay R, et al. Dysregulated Type I Interferon and Inflammatory Monocyte-Macrophage Responses Cause Lethal Pneumonia

in SARS-CoV-Infected Mice. *Cell Host Microbe* 2016;19(2):181-93. doi: 10.1016/j.chom.2016.01.007

24. Kim KD, Zhao J, Auh S, et al. Adaptive immune cells temper initial innate responses. *Nat Med* 2007;13(10):1248-52. doi: 10.1038/nm1633

25. Kraft R, Herndon DN, Finnerty CC, et al. Predictive Value of IL-8 for Sepsis and Severe Infections After Burn Injury: A Clinical Study. *Shock* 2015;43(3):222-7. doi: 10.1097/SHK.0000000000000294

26. Simmons EM, Himmelfarb J, Sezer MT, et al. Plasma cytokine levels predict mortality in patients with acute renal failure. *Kidney Int* 2004;65(4):1357-65. doi: 10.1111/j.1523-1755.2004.00512.x

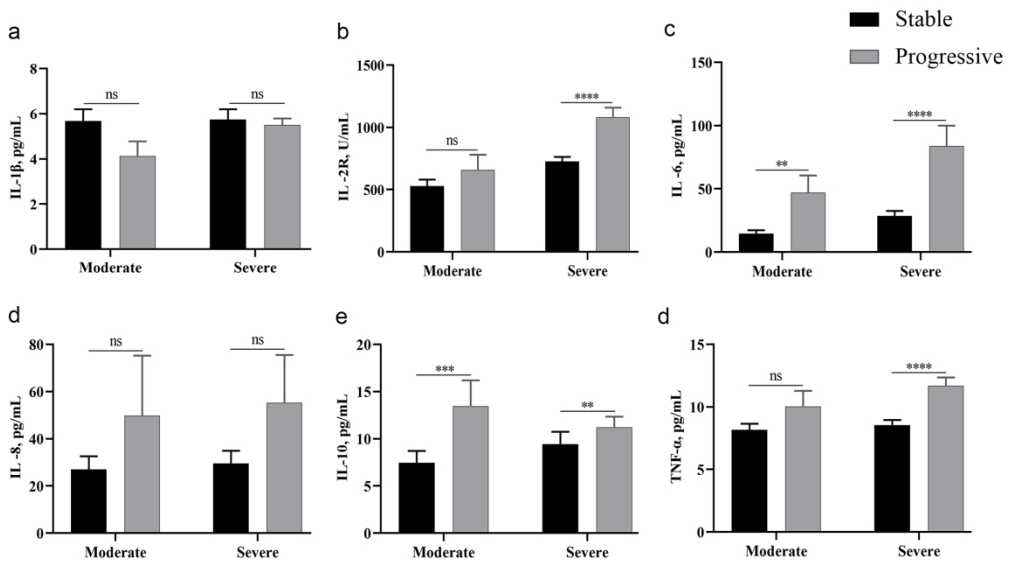
**Figure legend**

Figure 1. Cytokine values for COVID-19 patients on admission versus progressive group and stable group in both the moderate and severe types. The error bars represent mean ±SEM. IL, interleukin; TNF, tumor necrosis factor; P values were calculated by the Mann-Whitney U test, * P<0.05, **P<0.01, ***P<0.001, ***P<0.0001.

Figure 2. Cumulative incidence of in-hospital mortality in patients with coronavirus disease 2019 subgrouped by cytokines. (a) Interleukin 1β, (b) Interleukin 2R, (c) Interleukin 6, (d) Interleukin 8, (e) Interleukin 10, and (f) TNF-α.

Figure 3. Association of inflammatory cytokines with in-hospital mortality in COVID-19 patients. Odds ratios (ORs) of each variable were obtained using multivariate logistic regression models after adjustment for age, gender, comorbidities, the severity of the disease, and lymphocyte count. The severity was staged based on the guidelines for diagnosis and treatment of COVID-19 (trial fifth edition) published by the Chinese National Health Commission on February 4, 2020. 95% CI, 95% confidence interval.

Figure 4. Receiver operating characteristic curve of Interleukin 2R, Interleukin 6, Interleukin 8, Interleukin 10, and TNF-α for in-hospital death.

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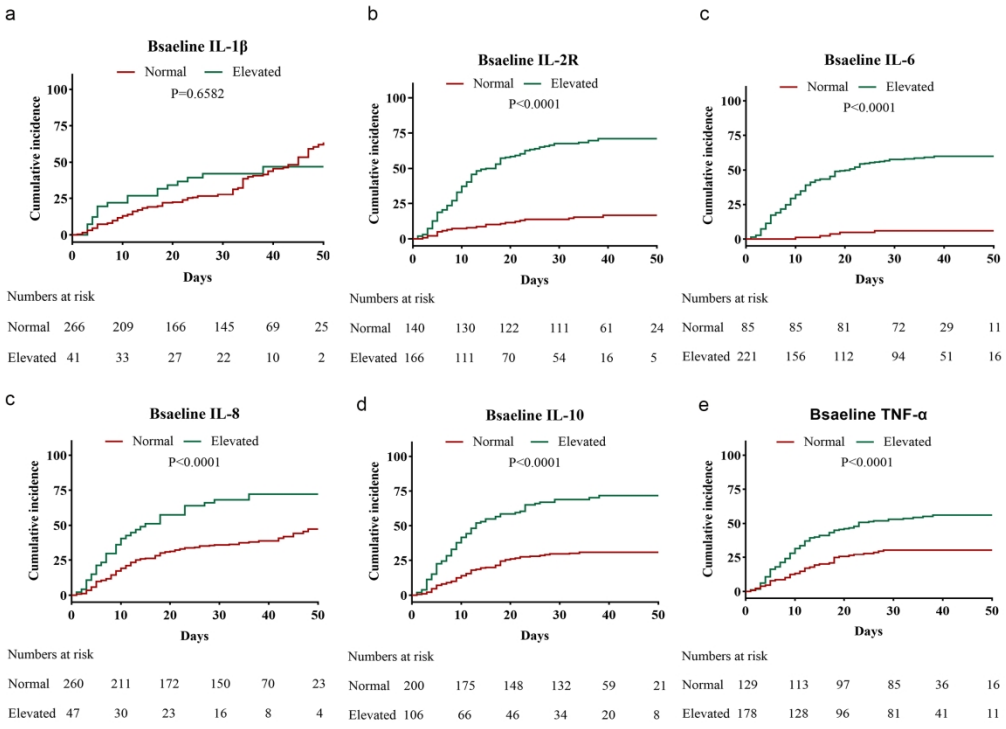


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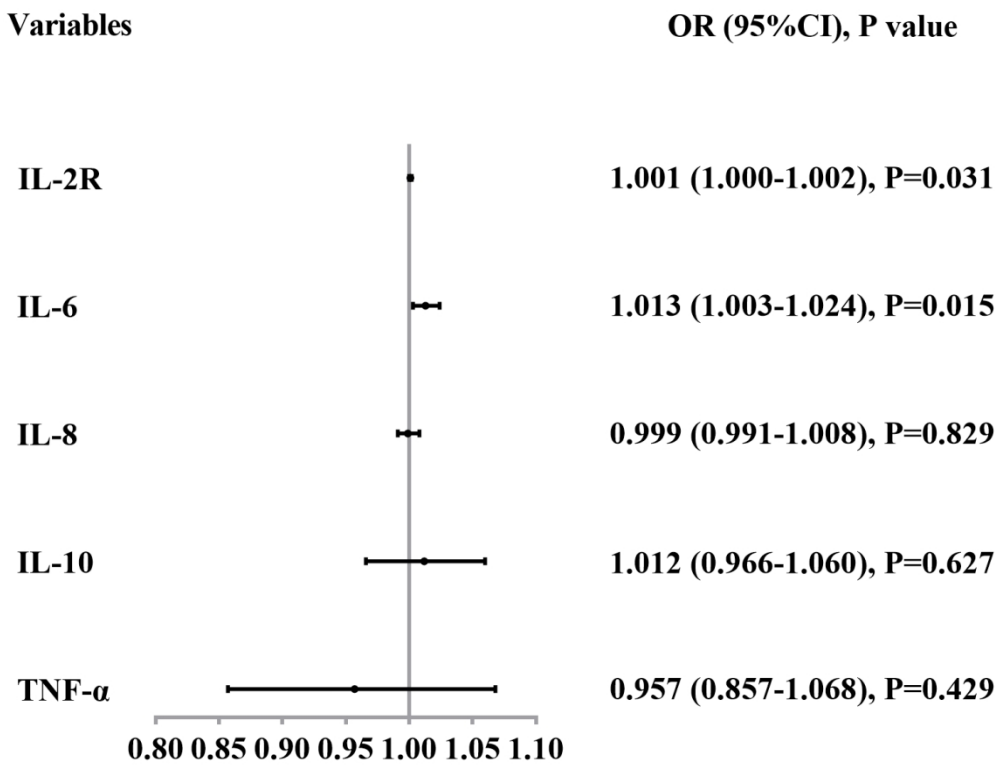


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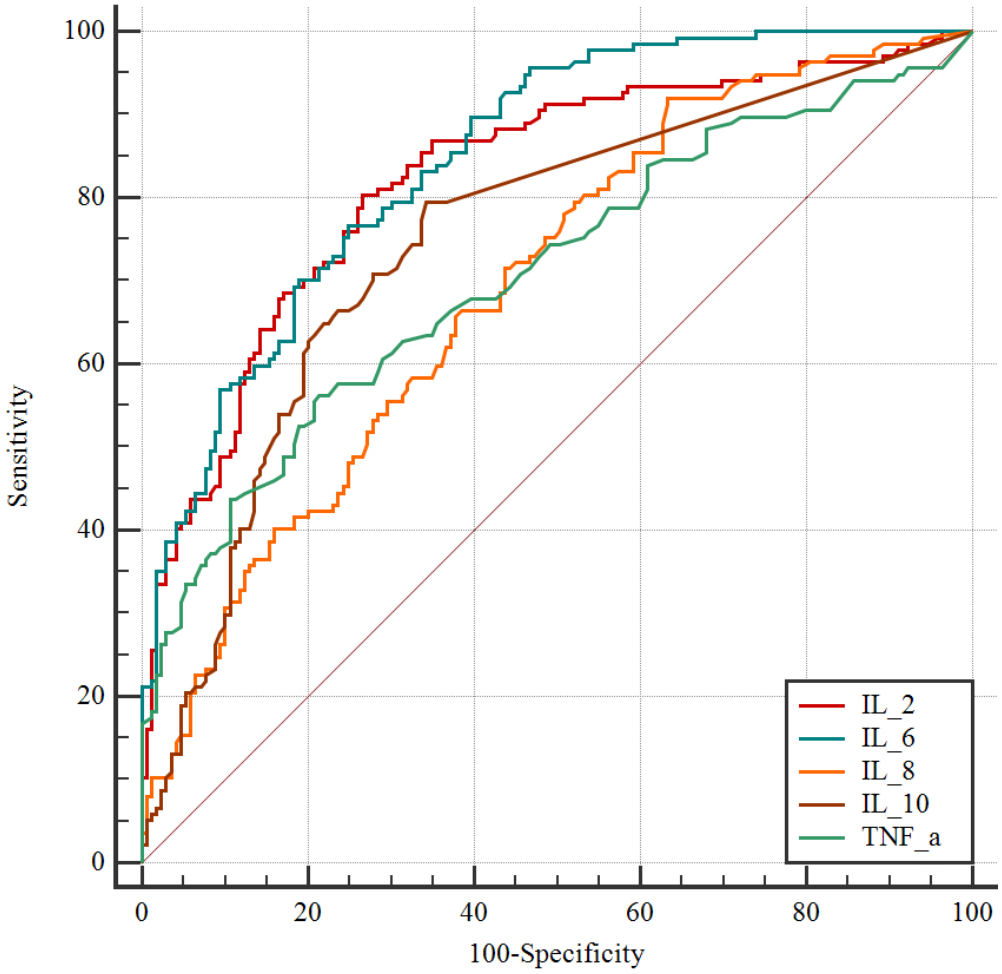


Figure 4. Receiver operating characteristic curve of Interleukin 2R, Interleukin 6, Interleukin 8, Interleukin 10, and TNF-α for in-hospital death.

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STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

| | Item No | Page No | Recommendation |
|---|---|---|---|
| **Title and abstract** | 1 | 1-2 | (*a*) Indicate the study's design with a commonly used term in the title or the abstract |
| | | | (*b*) Provide in the abstract an informative and balanced summary of what was done and what was found |
| | | | **Introduction** |
| Background/rationale | 2 | 3 | Explain the scientific background and rationale for the investigation being reported |
| Objectives | 3 | 4 | State specific objectives, including any prespecified hypotheses |
| | | | **Methods** |
| Study design | 4 | 4 | Present key elements of study design early in the paper |
| Setting | 5 | 4-5 | Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection |
| Participants | 6 | 4 | (*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up |
| | | | (*b*) For matched studies, give matching criteria and number of exposed and unexposed |
| Variables | 7 | 4-5 | Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable |
| Data sources/ measurement | 8* | 5 | For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group |
| Bias | 9 | 5 | Describe any efforts to address potential sources of bias |
| Study size | 10 | 4 | Explain how the study size was arrived at |
| Quantitative variables | 11 | 5 | Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why |
| Statistical methods | 12 | 6 | (*a*) Describe all statistical methods, including those used to control for confounding |
| | | | (*b*) Describe any methods used to examine subgroups and interactions |
| | | | (*c*) Explain how missing data were addressed |
| | | | (*d*) If applicable, explain how loss to follow-up was addressed |
| | | | (*e*) Describe any sensitivity analyses |
| | | | **Results** |
| Participants | 13* | 6 | (a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed |
| | | | (b) Give reasons for non-participation at each stage |
| | | | (c) Consider use of a flow diagram |
| Descriptive data | 14* | 6 | (a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders |
| | | | (b) Indicate number of participants with missing data for each variable of interest |
| | | | (c) Summarise follow-up time (eg, average and total amount) |
| Outcome data | 15* | 7 | Report numbers of outcome events or summary measures over time |
| Main results | 16 | 11 | (*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear |

| | | | |
|---|---|---|---|
| | | | which confounders were adjusted for and why they were included |
| | | | (*b*) Report category boundaries when continuous variables were categorized |
| | | | (*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period |
| Other analyses | 17 | 9-10 | Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses |
| | | | **Discussion** |
| Key results | 18 | 12 | Summarise key results with reference to study objectives |
| Limitations | 19 | 14 | Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias |
| Interpretation | 20 | 13 | Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence |
| Generalisability | 21 | 13-14 | Discuss the generalisability (external validity) of the study results |
| | | | **Other information** |
| Funding | 22 | 15 | Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based |

*Give information separately for exposed and unexposed groups.

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# Cytokines and their relationship to the severity and prognosis of Coronavirus Disease 2019 (COVID-19): a retrospective cohort study.

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# Cytokines and their relationship to the severity and prognosis of Coronavirus Disease 2019 (COVID-19): a retrospective cohort study.

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Keywords: SARS-CoV-2; COVID-19; cytokines; pneumonia

Word count: 3077

**Abstract**

**Objective:** To delineate the characteristics and clinical significance of plasma inflammatory cytokines altered in COVID-19.

**Design:** Retrospective, single-center cohort study.

**Setting:** Tongji Hospital in Wuhan, china.

**Participants:** Among a cohort of 308 patients with the diagnosis of COVID-19, 138 patients died, while 170 patients recovered and were discharged from the hospital. The data were collected until February 27 2020.

**Primary and secondary outcome measures:** Clinical characteristics and laboratory findings were obtained from electronic medical records with data collection forms.

**Result:** The percentage of patients having elevated IL-2R, IL-6, IL-8, IL-10, and TNF increased with the severity of disease (P <0.0001 for all). The values of IL-2R (P <0.0001), IL-6 (P <0.0001), IL-8 (P = 0.0001), IL-10 (P <0.0001), and TNF (P <0.0001) were also 2 to 20-fold higher in dead patients compared to that of the recovered ones. Also, the IL-6 and IL-10 increased in both the progressive patient groups: moderate (P = 0.0026) and the severe (P <0.0001). In multivariate analysis, patients with a higher level of IL-2R (OR 1.001, 95%CI 1.000–1.002, P = 0.031) and IL-6 (OR 1.013, 95%CI 1.003–1.024, P = 0.015) at admission were associated with increasing odds of in-hospital death, independent of other covariates, including the severity of disease and the lymphocyte count.

**Conclusion:** Increased pro-inflammatory and anti-inflammatory cytokines, including IL-2R, IL-6, IL-8, TNF, and IL-10, showed an obvious association with both the severity and in-hospital mortality of COVID-19. Thus, our study indicates that cytokines are valuable in predicting the severity of COVID-19 and helps in distinguishing critically ill patients from the less affected ones.

**Strengths and limitations of this study**

1. This study systematically investigated the cytokine profiles among patients with COVID-19 disease.
2. The major finding is that the levels of cytokines are valuable in predicting the severity and prognosis of the COVID-19.
3. The quality of the study is reduced by the lack of observing the relationship between dynamic cytokine levels and the progression of COVID-19.
4. This study is a retrospective single-center cohort analysis based on initial inflammatory cytokine levels on admission.
5. The data regarding lymphocyte subsets is not available.

## Introduction

The outbreak of coronavirus disease, 2019 (COVID-19) caused by Severe Acute Respiratory Syndrome-coronavirus-2 (SARS-CoV-2), is erupting worldwide. [1] [2] The World Health Organization (WHO) declared COVID-19 as a pandemic.[3] Although most cases were mild to moderate, increasing COVID-19 cases led to a significant number of patients developing severe symptoms and death. Currently, a lot of detailed clinical information on COVID-19 is known, but the characteristics of inflammatory cytokines associated with COVID-19 patients remain largely unclear.

Severe illness, of almost any etiology, is accompanied by a generalized host inflammatory response. This host immune response process is referred to as Systemic Inflammatory Response Syndrome. If this process is not controlled or is dysfunctional, it will lead to cytokine storm syndrome [4]. Cytokine storm is one of the possible mechanisms underlying rapid disease progression [5]. Recent studies have reported a relationship between the serum inflammatory cytokine levels and the severity or prognosis of COVID-19 patients. However, there are some inconsistent conclusions. For example, several studies reported interleukin (IL)-6 as a potential biomarker to predict COVID-19 progression or monitor disease severity [6-8]. However, Song et al. found no significant differences in the levels of IL-6 and tumor necrosis factor (TNF) between severe and non-severe COVID-19 patients [9]. Most of the previous studies only focused on the role of IL-6 in COVID-19. The characteristics and role of other cytokines in COVID-19, especially the anti-inflammatory cytokine IL-10, have been paid little attention. IL-10 can be a parameter in predicting the clinical outcome of patients with severe community-acquired pneumonia and is also the most important anti-inflammatory cytokine in the human immune response [10].

Thus, the authors of this research have systematically studied cytokine profiles along with their relationship to the severity and the prognosis of

COVID-19 disease. Meanwhile, objective factors of the patients related to their basic level of cytokines were explored. Earlier identification of a severe or a critical case can help in providing clinical intervention on time.

## Methods

### Study design and participants

This was a retrospective single-center study, which enrolled 308 patients from January 10, 2020, to February 13, 2020, at Tongji Hospital, Huazhong University of Science and Technology (Wuhan, China). As of February 27, 2020, 138 patients died, while 170 patients recovered and were discharged from the hospital. Data were collected at the time of admission. Diagnosis and clinical classification of COVID-19 were made according to the clinical guidelines (version 5 trial) developed by the National Health Committee of the People's Republic of China (http://www.nhc.gov.cn/). The clinical classification is as follows: (1) moderate type- including fever, respiratory tract symptoms, and imaging that shows pneumonia. (2) severe type- meeting any of the following criteria: a) respiratory distress, respiratory rate ≥ 30 beats/min; b) in resting state, meaning oxygen saturation ≤ 93%; c) arterial blood oxygen partial pressure/oxygen concentration ≤ 300 mmHg (1 mmHg = 0.133 kPa). (3) critical type- involving one of the following conditions: a) respiratory failure requiring mechanical ventilation; b) presence of shock; c) combined organ failure requiring admission in ICU. Tongji Hospital is a designated hospital for critically ill patients. In this cohort, we observed relatively high mortality. Exacerbation from moderate to severe or severe to critical was defined as progression. The primary outcome of the study was in-hospital mortality. The study excluded patients, having secondary vasculitis and uremia that needed maintenance on hemodialysis along with the ones who died within 48 h of admission. This study was approved by the Tongji Hospital Ethics Committee. Before enrollment,

written informed consent was obtained from patients involved in the study, while data were collected retrospectively.

**Cytokine measurement**

In order to explore the influence of COVID-19 on the secretion of cytokines, Chemiluminescence Immunoassay (CLIA) was performed. Cytokines, including IL-1β, IL-2R, IL-6, IL-8 (also known as CXCL8), IL-10, and TNF were assessed using the serum samples drawn from the patients shortly after hospitalization. CLIA was performed by a fully automated analyzer (Immulite 1000, DiaSorin Liaison, Italy or Cobas e602, Roche Diagnostics, Germany) for all the patients according to the manufacturer's instructions. IL-1β kit (#LKL11), IL-2R kit (#LKIP1), IL-8 kit (#LK8P1), IL-10 kit (#LKXP1) and TNF kit (#LKNF1) were purchased from DiaSorin (Vercelli, Italy). IL-6 kit (#05109442190) was purchased from Roche Diagnostics, Germany. The patients were divided into elevated and normal groups according to the instructions.

**Statistical analysis**

Continuous variables were expressed as a median with IQR, and categorical variables were described as frequency rates and percentages. We used the Mann-Whitney U test or Kruskal-Wallis test, whichever was appropriate, to compare the differences between the groups. Proportions for categorical variables were compared using the chi-square test, Fisher's exact test, or Yates' continuity corrected chi-square test. Univariate and multivariate logistic regression models (Forward: LR) were applied to screen the risk of death. The Kaplan–Meier method was used to assess the cumulative rate of mortality based on the normal range of cytokines and was compared with the log-rank test. Receiver operating characteristic curve (ROC) analysis was performed to assess the accuracy of inflammatory cytokine levels in predicting death. The tests were two-sided, and a P value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS (version 23.0).

## Patient and public involvement

This was a retrospective single-center cohort study, and no patients were involved in the study design or in setting the research questions or the outcome measures directly. No patients were asked for advice on interpretation or writing up of results.

## Result

### The Characteristics in COVID-19 patient cohort

The demographic and clinical characteristics of 308 COVID-19 patients are summarized in Table 1. At the time of admission, 91 (30%) patients were classified as moderate type, 133 (43%) patients as severe, and 84 (27%) patients fulfilled the criteria of critical type. As per the results, 138 (45%) patients died, and 170 (55%) were discharged from the hospital. Moderately ill and surviving patients were more likely to be female and younger. The ratio of comorbidity was significantly higher in critical (P <0.0001) and the dead group (P <0.0001). In the laboratory findings, the levels of lymphocytes, platelets, and the total cholesterol were lower in the critical and the dead group, whereas neutrophil count, D-dimer, alanine aminotransferase, blood urea nitrogen, creatinine, procalcitonin, lactic dehydrogenase, C-reactive protein, IL-2R, IL-6, IL-8, IL-10, and TNF were significantly higher (P <0.0001 for all). Also, critical (P <0.0001) and the dead (P <0.0001) groups were more likely to receive non-invasive ventilation or invasive ventilation. The median time from disease onset to the outcome was 22 (IQR 15.8–30.0) days for non-survivors and 29 (IQR 24.0–34.0) days for the survivors.

Table 1. Demographics and clinical characteristics of COVID-19 patients.

| | Condition at admission | | | Outcome | |
|---|---|---|---|---|---|

| Variables | Moderate (n = 91) | Severe (n = 133) | Critical (n = 84) | P value | Survivor (n = 170) | Non-survivor (n = 138) | P value |
|---|---|---|---|---|---|---|---|
| Age, years | 55.0 (39.0–67.0) | 66.0 (55.5–73.0) | 70.0 (63.3–78.8) | <0.0001 | 57.0 (43.0–68.0) | 70.0 (62.8–78.0) | <0.0001 |
| Female, n (%) | 51/91 (56%) | 67/133 (50%) | 24/84 (29%) | 0.0003 | 95/170 (56%) | 47/138 (34%) | 0.0001 |
| Comorbidity, n (%) | 35/89 (39%) | 82/132 (62%) | 61/83 (74%) | <0.0001 | 72/167 (43%) | 106/137 (77%) | <0.0001 |
| Hypertension, n (%) | 18/89 (20%) | 51/132 (39%) | 41/83 (49%) | <0.0001 | 43/167 (26%) | 67/137 (49%) | <0.0001 |
| Diabetes, n (%) | 14/89 (16%) | 22/132 (17) | 20/83 (24%) | 0.1621 | 24/167 (14%) | 32/137 (23%) | 0.0443 |
| Cardiovascular disease, n (%) | 5/89 (6%) | 21/132 (16%) | 15/83 (18%) | 0.0158 | 13/167 (8%) | 28/137 (20%) | 0.0013 |
| Cerebrovascular disease, n (%) | 2/89 (2%) | 3/132 (2%) | 7/83 (8%) | 0.0402 | 4/167 (2%) | 8/137 (6%) | 0.2155 |
| Pulmonary disease, n (%) | 6/89 (7%) | 11/132 (8%) | 9/83 (11%) | 0.3380 | 10/167 (6%) | 16/137 (12%) | 0.0775 |
| Chronic kidney disease, n (%) | 0/89 (0%) | 3/132 (2%) | 4/83 (5%) | 0.0353 | 1/167 (1%) | 6/137 (4%) | 0.0715 |
| Signs and symptoms | | | | | | | |
| Fever, n (%) | 76/90 (84%) | 119/133 (90%) | 77/84 (92%) | 0.1318 | 145/169 (86%) | 127/138 (92%) | 0.0875 |
| Cough, n (%) | 54/90 (60%) | 101/132 (77%) | 71/84 (85%) | 0.0002 | 114/169 (68%) | 112/137 (82%) | 0.0047 |
| Laboratory parameters | | | | | | | |
| Neutrophil count, $\times 10^9$/L | 3.1 (2.5–4.1) | 4.0 (2.7–5.7) | 10.1 (7.2–14.7) | <0.0001 | 3.3 (2.4–4.3) | 8.1 (4.9–12.5) | <0.0001 |
| Lymphocyte count, $\times 10^9$/L | 1.2 (0.9–1.6) | 0.9 (0.7–1.3) | 0.5 (0.4–0.8) | <0.0001 | 1.1 (0.8–1.5) | 0.6 (0.4–0.8) | <0.0001 |
| Platelet count, $\times 10^9$/L | 213.0 (160.0–309.0) | 208.5 (149.0–261.3) | 156.0 (94.5–222.0) | <0.0001 | 220.0 (170.8–291.3) | 151.0 (107.0–222.5) | <0.0001 |
| D-Dimer, ug/mL | 0.4 (0.3–0.8) | 1.0 (0.5–2.6) | 18.1 (2.6–21.0) | <0.0001 | 0.5 (0.3–1.0) | 7.9 (1.3–21.0) | <0.0001 |
| ALT, U/L | 18.0 (13.0–24.0) | 23.0 (16.0–39.0) | 33.5 (21.0–55.8) | <0.0001 | 20.0 (13.8–32.5) | 28.0 (18.0–46.5) | <0.0001 |
| BUN, mmol/L | 3.7 (3.1–4.7) | 5.1 (3.7–6.7) | 9.6 (7.0–16.1) | <0.0001 | 3.9 (3.1–5.2) | 8.8 (5.6–12.9) | <0.0001 |
| Creatinine, umol/L | 66.0 (57.0–81.0) | 72.0 (56.0–93.0) | 87.5 (70.5–120.0) | <0.0001 | 65.5 (57.0–82.0) | 86.5 (66.8–120.0) | <0.0001 |
| Total cholesterol, mmol/L | 3.8 (3.2–4.5) | 3.5 (3.1–3.9) | 3.2 (2.8–3.8) | <0.0001 | 3.6 (3.2–4.3) | 3.3 (2.8–3.9) | <0.0001 |
| Procalcitonin, ng/mL | 0.05 (0.04–0.09) | 0.09 (0.05–0.23) | 0.49 (0.17–1.49) | <0.0001 | 0.05 (0.04–0.09) | 0.31 (0.14–1.09) | <0.0001 |
| Lactic dehydrogenase, U/L | 229.0 (190.0–283.0) | 315.0 (219.5–431.8) | 637.0 (490.5–872.5) | <0.0001 | 243.0 (194.8–309.8) | 524.5 (366.0–721.0) | <0.0001 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C-reactive protein, mg/L | 7.7 (2.5–24.3) | 49.3 (8.9–93.3) | 112.3 (71.4–187.3) | <0.0001 | 10.7 (2.4–36.7) | 100.5 (62.4–161.2) | <0.0001 |
| Interleukin 1β ≥5pg/mL | 13/91 (14%) | 17/133 (13%) | 11/84 (13%) | 0.8120 | 23/170 (14%) | 18/138 (13%) | 0.9006 |
| Interleukin-2 receptor, U/mL | 475.5 (375.8–630.8) | 799.0 (538.5–1097.0) | 1259.5 (942.3–1825.0) | <0.0001 | 553.0 (402.0–802.0) | 1137.5 (822.0–1584.3) | <0.0001 |
| ≥710 U/L | 19/91 (21%) | 74/133 (56%) | 77/84 (92%) | <0.0001 | 51/170 (30%) | 113/138 (82%) | <0.0001 |
| Interleukin-6, pg/mL | 5.6 (2.7–15.3) | 24.3 (6.7–61.7) | 64.8 (29.42–153.1) | <0.0001 | 7.9 (2.7–22.8) | 59.7 (23.6–137.4) | <0.0001 |
| ≥7 pg/mL | 41/91 (45%) | 100/133 (75%) | 77/83 (92%) | <0.0001 | 90/170 (53%) | 132/137 (96%) | <0.0001 |
| Interleukin-8, pg/mL | 15.4 (7.7–29.4) | 19.5 (12–35.5) | 30.8 (21.0–71.8) | <0.0001 | 16.3 (9.4–28.7) | 26.6 (16.4–60.40) | <0.0001 |
| ≥62 pg/mL | 10/91 (11%) | 13/133 (10%) | 77/84 (92%) | <0.0001 | 14/170 (8%) | 33/138 (24%) | 0.0001 |
| Interleukin-10, pg/mL | 5.0 (5.0–5.1) | 5.9 (5.0–10.80) | 10.9 (6.4–18.7) | <0.0001 | 5.0 (5.0–6.7) | 10.1 (5.4–16.4) | <0.0001 |
| ≥9.1 pg/mL | 10/91 (11%) | 46/133 (35%) | 76/83 (92%) | <0.0001 | 31/170 (18%) | 76/137 (56%) | <0.0001 |
| TNF, pg/mL | 7.7 (6.0–9.5) | 8.6 (6.9–11.9) | 11.2 (7.4–18.8) | <0.0001 | 7.8 (6.1–9.7) | 10.9 (7.7–15.9) | <0.0001 |
| ≥8.1 pg/mL | 43/91 (47%) | 80/133 (60%) | 77/84 (92%) | <0.0001 | 82/170 (48%) | 101/138 (73%) | <0.0001 |
| Treatment | | | | | | | |
| Mechanical ventilation, n (%) | 4/91 (4%) | 46/133 (35%) | 79/84 (94%) | <0.0001 | 7/170 (4%) | 122/138 (88%) | <0.0001 |
| Antibiotics treatment, n (%) | 85/91 (93%) | 128/133 (96%) | 84–0/84 (100%) | 0.0191 | 160/170 (94%) | 137/138 (99%) | 0.0343 |
| Antiviral treatment, n (%) | 90/90 (100%) | 124/126 (98%) | 62/79 (79%) | <0.0001 | 167/167 (100%) | 109/128 (85%) | <0.0001 |
| Corticosteroids, n (%) | 48/90 (53%) | 86/133 (65%) | 74/84 (88%) | <0.0001 | 84/169 (50%) | 124/138 (90%) | <0.0001 |
| Immunoglobin, n (%) | 31/91 (34%) | 77–56/133 (58%) | 47–37/84 (56%) | 0.0031 | 69/170 (41%) | 86/138 (62%) | 0.0001 |
| Duration of complaint, days | 8.0 (4.8–13.0) | 10.0 (7.0–13.5) | 11.0 (7.0–15.0) | 0.0007 | 8.0 (6.0–13.0) | 10.0 (7.0–15.0) | 0.0027 |
| Hospitalization, days | 16.0 (12.0–23.0) | 19.0 (12.0–24.0) | 8.0 (4.0–13.0) | <0.0001 | 19.5 (14.0–24.0) | 10.0 (5.0–17.0) | <0.0001 |
| Duration of disease, days | 26.0 (20.0–31.0) | 29.0 (24.0–35.0) | 19.0 (15.0–29.0) | <0.0001 | 29.0 (24.0–34.0) | 22.0 (15.8–30.0) | <0.0001 |
| Progression | 8/45 (18%) | 52/129 (40%) | - | - | - | - | - |

Data are median (IQR), mean (SD) or n (%). P values were calculated by Mann-Whitney U test, Kruskal-Wallis test, chi-square test, Fisher's exact test or Yates' continuity corrected chi-square test, as appropriate. ALT, Alanine aminotransferase; BUN, blood urea nitrogen; Duration of Complaint, time from onset

of symptom to hospital admission; Hospitalization, time from hospital admission to outcome; Duration of disease, time from onset of symptom to outcome.

**Plasma cytokine alteration in COVID-19**

The baseline for cytokine concentrations was determined from blood obtained at admission time. The levels and the abnormal ratio of cytokines, including IL-2R, IL-6, IL-8, IL-10, and TNF, gradually increased as the disease progressed or resulted in poor prognosis ($P < 0.0001$ for all). No significant difference was observed in IL-1β ($P = 0.8120$) within each of the groups. Likewise, the values of IL-2R ($P < 0.0001$), IL-6 ($P < 0.0001$), IL-8 ($P = 0.0001$), IL-10 ($P < 0.0001$), and TNF ($P < 0.0001$) were also 2 to 20-fold higher in dead patients compared to that of the recovered ones (Table 1). Additionally, several cytokines, including pro-inflammatory and anti-inflammatory, were elevated at the baseline in patients whose conditions were progressive compared to the patients whose conditions were stable (Figure 1). Specifically, the pro-inflammatory IL-2R ($P < 0.0001$) and TNF ($P < 0.0001$) were significantly higher in the progressive group compared with the stable group in the severe type of patients. IL-6 was increased in the progressive group, both in moderate ($P = 0.0026$) and in severe ($P < 0.0001$) type of patients. Although numerically high, the differences in IL-8 levels between the two groups did not reach statistical significance. Similarly, the progressive group had higher levels of the anti-inflammatory cytokine, IL-10, in both the types of severity ($P = 0.0008$ for moderate type, $P = 0.0011$ for severe type). These results suggest that the progression of COVID-19 was associated with the initial levels of plasma cytokine.

**Correlation between the baseline cytokine levels and the physiological variables measured on admission**

The relationship between plasma cytokines and various physiological variables were assessed and presented in Table 2. Except for IL-1β, other

plasma cytokines were positively correlated with age, albumin, creatinine, random blood glucose, D-dimer, lactic dehydrogenase, and CRP (P<0.001 for all). Also, IL-2R (P = 0.002), IL-6 (P<0.001), and IL-10 (P<0.001) were negatively correlated with total cholesterol. There was no significant relationship between the cytokine levels and levels of C3 and C4.

Table 2. Correlation among baseline inflammatory biomarkers and physiologic variables measured on the day of admission.

| | IL-1β | IL –2R | IL –6 | IL –8 | IL-10 | TNF |
|---|---|---|---|---|---|---|
| Age | -0.002 | 0.426 | 0.356 | 0.293 | 0.261 | 0.361 |
| | 0.967 | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| Time from onset of symptom | -0.061 | 0.222 | -0.011 | 0.038 | -0.069 | -0.055 |
| to hospital admission | 0.289 | <0.001* | 0.843 | 0.504 | 0.226 | 0.336 |
| Albumin | 0.177 | -0.527 | -0.467 | -0.319 | -0.346 | -0.290 |
| | 0.003* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| ALT | -0.047 | 0.276 | 0.250 | 0.198 | 0.167 | 0.248 |
| | 0.415 | <0.001* | <0.001* | 0.001* | 0.003* | <0.001* |
| Creatinine | -0.037 | 0.332 | 0.355 | 0.226 | 0.284 | 0.439 |
| | 0.512 | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| Uric acid | 0.033 | 0.170 | 0.155 | 0.128 | 0.052 | 0.331 |
| | 0.578 | 0.004* | 0.054 | 0.031* | 0.387 | <0.001* |
| Total cholesterol | -0.021 | -0.177 | -0.332 | -0.080 | -0.276 | -0.103 |
| | 0.709 | 0.002* | <0.001* | 0.159 | <0.001* | 0.071 |
| Random blood glucose | 0.004 | 0.303 | 0.276 | 0.272 | 0.336 | 0.270 |
| | 0.950 | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| D-Dimer | -0.026 | 0.614 | 0.509 | 0.328 | 0.378 | 0.367 |
| | 0.651 | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |

| | | | | | | |
|---|---|---|---|---|---|---|
| Lactic dehydrogenase | -0.065 | 0.598 | 0.562 | 0.339 | 0.472 | 0.391 |
| | 0.255 | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| CRP | 0.008 | 0.618 | 0.776 | 0.345 | 0.528 | 0.493 |
| | 0.894 | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| C3 | -0.226 | -0.235 | -0.026 | -0.066 | 0.093 | -0.262 |
| | 0.52 | 0.137 | 0.882 | 0.652 | 0.576 | 0.075 |
| C4 | 0.62 | 0.001 | -0.056 | -0.156 | 0.070 | -0.21 |
| | 0.053 | 0.991 | 0.711 | 0.295 | 0.673 | 0.163 |

Values represent Spearman's correlation coefficients (upper) and P values (lower). IL, interleukin; TNF, tumor necrosis factor; ALT, alanine aminotransferase; CRP, C-reactive protein; C3, complement 3; C4, complement 4. *Denotes statistically significant correlations.

**Association of plasma cytokines with the in-hospital deaths**

Kaplan-Meier analysis indicated a significantly higher mortality rate in patients with abnormal plasma cytokine values, including elevated IL-2R, IL-6, IL-8, IL-10, and TNF (P<0.0001 for all) (Figure 2). Univariate logistic regression analysis revealed that IL-2R, IL-6, IL-8, IL-10, and TNF were related to a poor outcome (Table 3). After adjusting for age, gender, comorbidities, the severity of the disease, and lymphocyte count, the levels of IL-2R and IL-6 were associated with in-hospital mortality (Figure 3). Further, the plasma cytokines were analyzed by ROC analysis to evaluate their ability to predict the in-hospital death rates (Figure 4). The area under the curve was 0.82 (95%CI: 0.78–0.87) for IL-2R, 0.85 (95%CI: 0.81–0.89) for IL-6, 0.69 (95%CI: 0.64–0.75) for IL-8, 0.75 (95%CI: 0.69–0.81) for IL-10, 0.71 (95%CI: 0.65–0.77) for TNF (Table 4).

Table 3. Logistic regression to analysis independent factors for predicting mortality of COVID-19 patients.

| Cytokines | P-value | OR | 95% CI |
|---|---|---|---|
| IL-1β, pg/mL* | 0.242 | 1.028 | 0.982–1.077 |

| | | | |
|---|---|---|---|
| IL –2R, U/mL* | <0.001 | 1.003 | 1.002–1.004 |
| IL –6, pg/mL* | <0.001 | 1.031 | 1.022–1.040 |
| IL –8, pg/mL* | <0.001 | 1.010 | 1.004–1.015 |
| IL-10, pg/mL* | <0.001 | 1.066 | 1.032–1.101 |
| TNF, pg/mL* | <0.001 | 1.205 | 1.131–1.285 |

IL, interleukin; TNF, tumor necrosis factor; OR, odds ratio; CI, confidence interval; *Per 1unit increase.

Table 4. Analysis of receiver-operating characteristics curve for predicting in-hospital mortality in COVID-19 patients.

| Variables | P-value | AUC (95%CI) | Sensitivity | Specificity | Cut-off value | PPV | NPV |
|---|---|---|---|---|---|---|---|
| Interleukin-2 receptor | <0.001 | 0.82 (0.78–0.87) | 0.80 | 0.73 | 755.50 | 0.72 | 0.55 |
| Interleukin-6 | <0.001 | 0.85 (0.81–0.89) | 0.77 | 0.75 | 22.80 | 0.71 | 0.80 |
| Interleukin-8 | <0.001 | 0.69 (0.64–0.75) | 0.66 | 0.62 | 20.50 | 0.58 | 0.55 |
| Interleukin-10 | <0.001 | 0.75 (0.69–0.81) | 0.80 | 0.66 | 5.15 | 0.65 | 0.55 |
| TNF | <0.001 | 0.71 (0.65–0.77) | 0.56 | 0.79 | 10.05 | 0.68 | 0.55 |

TNF, tumor necrosis factor; AUC, area under curves; PPV = positive predictive value; NPV = negative predictive value

## Discussion

COVID-19 has rapidly spread throughout the world and is labeled as a pandemic by WHO. Both clinical features and the serum markers associated with the severity of COVID-19 patients have been reported[11-14]. However, we do not know the exact reasons for the specific alterations in cytokine levels; it might be due to the immune response triggering the rapid disease progression. In the present study, we systematically analyzed immunological characteristics, particularly cytokine profiles and their relationship with the severity, mortality, and prognosis of COVID-19.

Consistent with the previous report, we noted lymphocytopenia and increased inflammatory cytokine concentration in the majority of the severe

and critical cases, which was markedly worse when compared to the moderate cases. This indicates that an impaired immune system and a cytokine storm might be associated with COVID-19 severity. Further, the plasma concentration of cytokines in the progressive group was higher than the stable group in severe type of cases. Although, the differences in IL-2R, IL-8, and TNF between the two groups did not reach statistical significance in moderate type cases, which might be partly due to the limited sample size; a recent study has shown that elevated cytokines were dynamically correlated with the disease severity[5 15 16]. Additionally, SARS-CoV and MERS-CoV infections were also characterized by elevated levels of inflammatory cytokines with severe lung injury[17-20].

Cytokines are proteins, glycoproteins, or signaling peptides with potent various biological functions at picomolar concentration[21]. IL-2R, IL-6, IL-8, and TNF were major pro-inflammatory factors required to initiate a series of effective immune cascade events to an infection or a tissue injury site. The anti-inflammatory IL-10 was found to inhibit the monocyte inflammatory response directly and negatively regulate the cascade of pro-inflammatory cytokines, which induced monocyte hyporesponsiveness in sepsis and multiple organ dysfunction[22]. It is reported that coronavirus infection, its rapid replication, as well as the delayed INF-I signaling, activate inflammatory monocyte-macrophage, resulting in an increased cytokine concentration, vascular leakage, and pathogenic T cell response[23]. Besides, lymphocytopenia observed in severe and critical patients may impair T cells, which dampens the overactive innate immune response and further aggravate the inflammatory response[24]. In line with the previous study, we suggest that excessive cytokine secretion may be associated with the progressive group. Nevertheless, the underlying cellular source and the mechanism involving cytokine accumulation remains to be determined.

Meanwhile, at the time of admission, serum levels of both pro-inflammatory and anti-inflammatory cytokines, including IL-2R, IL-6, IL-8, TNF and IL-10, were significantly higher in the case of non-survivors compared to that of the survivors. Previous studies have shown that

pro-inflammatory cytokines predict mortality in patients with sepsis, acute respiratory distress syndrome, and the severe infection seen after burnt injuries[20 25]. Also, elevated cytokines are a concern as an independent risk factor of a poor outcome in acute renal failure[26]. In line with the previous research[27], univariate logistic regression analysis suggested that IL-2R, IL-6, IL-8, IL-10, and TNF were related to in-hospital mortality incidents in the present cohort. The association between IL-2R, IL-6, and in-hospital mortality was maintained after adjusting for demographics and other cofounders. A similar conclusion of elevated cytokines related to in-hospital mortality has been found in SARS-CoV or MERS-CoV infection[18-20]. IL-1 is released after the binding of SARS-CoV-2 to the Toll Like Receptor (TLR), then mediates lung inflammation, fever and fibrosis, and provokes severe respiratory problems[28 29]. However, both concentration and proportion of IL-1β, in contrast, were not increased in most of the patients, with only 13% of non-survivors showing elevated levels of IL-1β. While the secondary inflammatory cytokine IL-6, considered more distal than IL-1 in the inflammatory cascade, was a significant predictor of survival and had a higher AUC value. The specific immune cascade response and the cellular origin of cytokines in COVID-19 deserve further exploration.

At present, many drugs with variable efficacies have been proposed for the treatment of COVID-19 induced cytokine storm[28-31]. The interplay of the main pro-inflammatory IL-6 and TNF contributes to the cytokine storm. Thus, the targeted therapy of IL-6 and TNF should not be neglected in COVID-19 patients. Tocilizumab, a specific monoclonal antibody that blocks IL-6, has been used for severe COVID-19 patients with confirmed elevated levels of IL-6[30]. Previous reports have shown that the use of anti- IL-6 treatment with tocilizumab led to a reduction in fever and lung lesion opacity, and recovered the percentage of lymphocytes in peripheral blood[32]. Chloroquine and hydroxychloroquine can suppress the production of various cytokines, such as IL-1, IL-6, and TNF, via Toll-like receptor signaling and cGAS stimulation of interferon genes[33]. However, the therapeutic benefit of chloroquine in patients with COVID-19 remains controversial[34 35]. Anti- TNF therapy is used in some

COVID-19 patients with autoimmune diseases. A case report showed that treatment with anti-TNF seems to have a protective effect on the evolution of severe types, thereby preventing the damaging effects of cytokine storm[36]. However, anti-TNF has been associated with an increased risk of respiratory complications or death[37]. Overall, immunomodulatory agents with good safety profiles for severe COVID-19 remain limited.

Our study had some limitations. First, this study is a retrospective analysis based on the initial inflammatory cytokines on admission. We did not describe the kinetic change in cytokines profiles in COVID-19 patients. The function of cytokine and the role of cytokine accumulation for pulmonary and other organs remains to be elucidated. Therefore, the relationship between prognosis significance and time-dependent changes in cytokines remains unknown. Second, since data regarding the lymphocyte subsets are not available, further studies are needed to analyze the correlation between the change in lymphocyte subsets and humoral immune response. Third, Tongji Hospital was assigned as a designed hospital for severely or critically ill patients with covid-19, so there was a bias in critical patient selection for prognostic research. Thus, the case fatality ratio in our study cannot reflect the true mortality of COVID-19. Last but not least, this was a retrospective observational single center study. Whether the results of the present study are applicable to the other regions is questionable due to the potential differences in treatment protocol and time for patients to receive treatment.

In conclusion, both pro-inflammatory and anti-inflammatory cytokines alteration, including IL-2R, IL-6, IL-8, TNF, and IL-10, show an obvious association with the severity and in-hospital mortality in COVID-19 patients.

**Competing interest:** The authors declare that they have no conflict of interest.

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**Availability of data and materials:** Please contact the corresponding author for all data requests.

**Contributors:** QQL, AYC, XCZ, LH and HFL did the data collection, QQL and AYC wrote the first draft of the manuscript. AYC, YRW and QQL analyzed the data, provided edits of the first draft of the manuscript. TW and FH participated in the revision of the manuscript and approved the final version.

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**References**

1. Wang LS, Wang YR, Ye DW, et al. A review of the 2019 Novel Coronavirus (COVID-19) based on current evidence. *International journal of antimicrobial agents* 2020:105948. doi: 10.1016/j.ijantimicag.2020.105948 [published Online First: 2020/03/24]

2. Sohrabi C, Alsafi Z, O'Neill N, et al. World Health Organization declares global emergency: A review of the 2019 novel coronavirus (COVID-19).

*International journal of surgery (London, England)* 2020;76:71-76. doi: 10.1016/j.ijsu.2020.02.034 [published Online First: 2020/03/01]

3. COVID WVpco. https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---11-march-2020.

4. Chousterman BG, Swirski FK, Weber GF. Cytokine storm and sepsis disease pathogenesis. *Seminars in immunopathology* 2017;39(5):517-28. doi: 10.1007/s00281-017-0639-8 [published Online First: 2017/05/31]

5. Mehta P, McAuley DF, Brown M, et al. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet* 2020;395(10229):1033-34. doi: 10.1016/S0140-6736(20)30628-0

6. Gong J, Dong H, Xia SQ, et al. Correlation Analysis Between Disease Severity and Inflammation-related Parameters in Patients with COVID-19 Pneumonia. *medRxiv* 2020:2020.02.25.20025643. doi: 10.1101/2020.02.25.20025643

7. Ulhaq ZS, Soraya GV. [Not Available]. *Medecine et maladies infectieuses* 2020 doi: 10.1016/j.medmal.2020.04.002 [published Online First: 2020/04/08]

8. Liu T, Zhang J, Yang Y, et al. The potential role of IL-6 in monitoring severe case of coronavirus disease 2019. *medRxiv* 2020:2020.03.01.20029769. doi: 10.1101/2020.03.01.20029769

9. Song C-Y, Xu J, He J-Q, et al. COVID-19 early warning score: a multi-parameter screening tool to identify highly suspected patients. *medRxiv* 2020:2020.03.05.20031906. doi: 10.1101/2020.03.05.20031906

10. Wu CL, Chan MC, Chang GC, et al. Etiology and cytokine expression in patients requiring mechanical ventilation due to severe community-acquired pneumonia. *Journal of the Formosan Medical Association = Taiwan yi zhi* 2006;105(1):49-55. doi: 10.1016/s0929-6646(09)60108-x [published Online First: 2006/01/28]

11. Wu C, Chen X, Cai Y, et al. Risk Factors Associated With Acute Respiratory Distress Syndrome and Death in Patients With Coronavirus Disease 2019 Pneumonia in Wuhan, China. *JAMA Intern Med* 2020 doi: 10.1001/jamainternmed.2020.0994

12. Young BE, Ong SWX, Kalimuddin S, et al. Epidemiologic Features and Clinical Course of Patients Infected With SARS-CoV-2 in Singapore. *Jama* 2020 doi: 10.1001/jama.2020.3204

13. Zhang JJ, Dong X, Cao YY, et al. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy* 2020 doi: 10.1111/all.14238

14. Zhou F, Yu T, Du R, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *The Lancet* 2020 doi: 10.1016/s0140-6736(20)30566-3

15. Chen X, Zhao B, Qu Y, et al. Detectable serum SARS-CoV-2 viral load (RNAaemia) is closely correlated with drastically elevated interleukin 6 (IL-6) level in critically ill COVID-19 patients. *Clin Infect Dis* 2020 doi: 10.1093/cid/ciaa449

16. Chen G, Wu D, Guo W, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. *J Clin Invest* 2020 doi:

10.1172/JCI137244

17. Jiang Y, Xu J, Zhou C, et al. Characterization of Cytokine/Chemokine Profiles of Severe Acute Respiratory Syndrome. *American Journal of Respiratory and Critical Care Medicine* 2005;171(8):850-57. doi: 10.1164/rccm.200407-857OC

18. Zhou J, Chu H, Li C, et al. Active replication of Middle East respiratory syndrome coronavirus and aberrant induction of inflammatory cytokines and chemokines in human macrophages: implications for pathogenesis. *J Infect Dis* 2014;209(9):1331-42. doi: 10.1093/infdis/jit504

19. Chien JY, Hsueh PR, Cheng WC, et al. Temporal changes in cytokine/chemokine profiles and pulmonary involvement in severe acute respiratory syndrome. *Respirology* 2006;11(6):715-22. doi: 10.1111/j.1440-1843.2006.00942.x

20. Kong SL, Chui P, Lim B, et al. Elucidating the molecular physiopathology of acute respiratory distress syndrome in severe acute respiratory syndrome patients. *Virus Res* 2009;145(2):260-9. doi: 10.1016/j.virusres.2009.07.014

21. Bienvenu J, Monneret G, Fabien N, et al. The clinical usefulness of the measurement of cytokines. *Clin Chem Lab Med* 2000;38(4):267-85. doi: 10.1515/CCLM.2000.040

22. Sfeir T, Saha DC, Astiz M, et al. Role of interleukin-10 in monocyte hyporesponsiveness associated with septic shock. *Crit Care Med* 2001;29(1):129-33. doi: 10.1097/00003246-200101000-00026

23. Channappanavar R, Fehr AR, Vijay R, et al. Dysregulated Type I Interferon and Inflammatory Monocyte-Macrophage Responses Cause Lethal Pneumonia in SARS-CoV-Infected Mice. *Cell Host Microbe* 2016;19(2):181-93. doi: 10.1016/j.chom.2016.01.007

24. Kim KD, Zhao J, Auh S, et al. Adaptive immune cells temper initial innate responses. *Nat Med* 2007;13(10):1248-52. doi: 10.1038/nm1633

25. Kraft R, Herndon DN, Finnerty CC, et al. Predictive Value of IL-8 for Sepsis and Severe Infections After Burn Injury: A Clinical Study. *Shock* 2015;43(3):222-7. doi: 10.1097/SHK.0000000000000294

26. Simmons EM, Himmelfarb J, Sezer MT, et al. Plasma cytokine levels predict mortality in patients with acute renal failure. *Kidney Int* 2004;65(4):1357-65. doi: 10.1111/j.1523-1755.2004.00512.x

27. Cheng A, Hu L, Wang Y, et al. Diagnostic performance of initial blood urea nitrogen combined with D-dimer levels for predicting in-hospital mortality in COVID-19 patients. *Int J Antimicrob Agents* 2020:106110. doi: 10.1016/j.ijantimicag.2020.106110

28. Conti P, Ronconi G, Caraffa A, et al. Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by Coronavirus-19 (COVI-19 or SARS-CoV-2): anti-inflammatory strategies. *J Biol Regul Homeost Agents* 2020;34(2):327-31. doi: 10.23812/CONTI-E

29. Conti P, Gallenga CE, Tete G, et al. How to reduce the likelihood of coronavirus-19 (CoV-19 or SARS-CoV-2) infection and lung inflammation mediated by IL-1. *J Biol Regul Homeost Agents* 2020;34(2):333-38. doi: 10.23812/Editorial-Conti-2

30. Zhao M. Cytokine storm and immunomodulatory therapy in COVID-19: Role of chloroquine and anti-IL-6 monoclonal antibodies. *Int J Antimicrob Agents* 2020;55(6):105982. doi: 10.1016/j.ijantimicag.2020.105982

31. Wang J, Wang BJ, Yang JC, et al. [Research advances in the mechanism of pulmonary fibrosis induced by coronavirus disease 2019 and the corresponding therapeutic measures]. *Zhonghua Shao Shang Za Zhi* 2020;36(8):691-97. doi: 10.3760/cma.j.cn501120-20200307-00132

32. Xu X, Han M, Li T, et al. Effective treatment of severe COVID-19 patients with tocilizumab. *Proc Natl Acad Sci U S A* 2020;117(20):10970-75. doi: 10.1073/pnas.2005615117

33. Schrezenmeier E, Dorner T. Mechanisms of action of hydroxychloroquine and chloroquine: implications for rheumatology. *Nat Rev Rheumatol* 2020;16(3):155-66. doi: 10.1038/s41584-020-0372-x

34. Fiolet T, Guihur A, Rebeaud M, et al. Effect of hydroxychloroquine with or without azithromycin on the mortality of COVID-19 patients: a systematic review and meta-analysis. *Clin Microbiol Infect* 2020 doi: 10.1016/j.cmi.2020.08.022

35. Castelnuovo AD, Costanzo S, Antinori A, et al. Use of hydroxychloroquine in hospitalised COVID-19 patients is associated with reduced mortality: Findings from the observational multicentre Italian CORIST study. *Eur J Intern Med* 2020 doi: 10.1016/j.ejim.2020.08.019

36. Duret PM, Sebbag E, Mallick A, et al. Recovery from COVID-19 in a patient with spondyloarthritis treated with TNF-alpha inhibitor etanercept. *Ann Rheum*

*Dis* 2020;79(9):1251-52. doi: 10.1136/annrheumdis-2020-217362

37. Brito CA, Paiva JG, Pimentel FN, et al. COVID-19 in patients with rheumatological diseases treated with anti-TNF. *Ann Rheum Dis* 2020 doi: 10.1136/annrheumdis-2020-218171

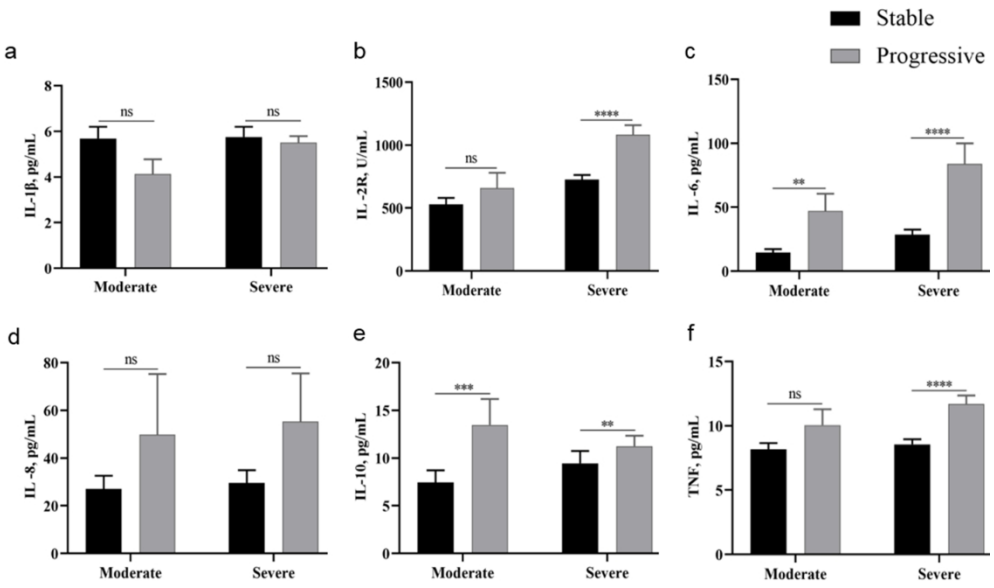
**Figure legend**

Figure 1. Cytokine values for COVID-19 patients on admission versus progressive group and stable group in both the moderate and severe types. The error bars represent mean ±SEM. IL, interleukin; TNF, tumor necrosis factor; P values were calculated by the Mann-Whitney U test, * P<0.05, **P<0.01, ***P<0.001, ***P<0.0001.

Figure 2. Cumulative incidence of in-hospital mortality in patients with coronavirus disease 2019 subgrouped by cytokines. (a) Interleukin 1β, (b) Interleukin 2R, (c) Interleukin 6, (d) Interleukin 8, (e) Interleukin 10, and (f) TNF.

Figure 3. Association of inflammatory cytokines with in-hospital mortality in COVID-19 patients. Odds ratios (ORs) of each variable were obtained using multivariate logistic regression models after adjustment for age, gender, comorbidities, the severity of the disease, and lymphocyte count. The severity was staged based on the guidelines for diagnosis and treatment of COVID-19 (trial fifth edition) published by the Chinese National Health Commission on February 4, 2020. 95% CI, 95% confidence interval.

Figure 4. Receiver operating characteristic curve of Interleukin 2R, Interleukin 6, Interleukin 8, Interleukin 10, and TNF for in-hospital death.

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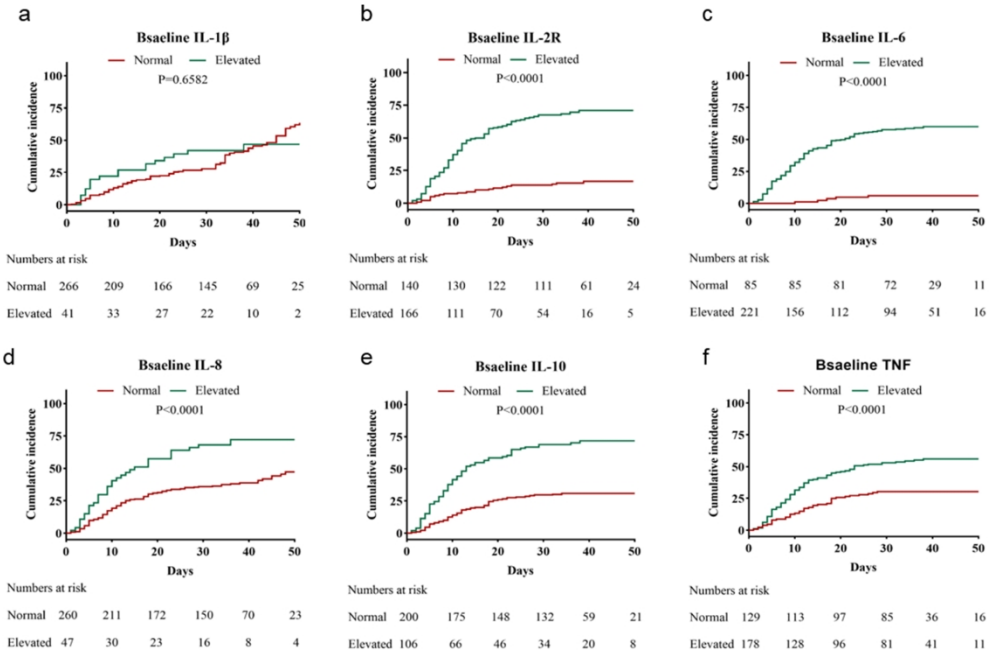


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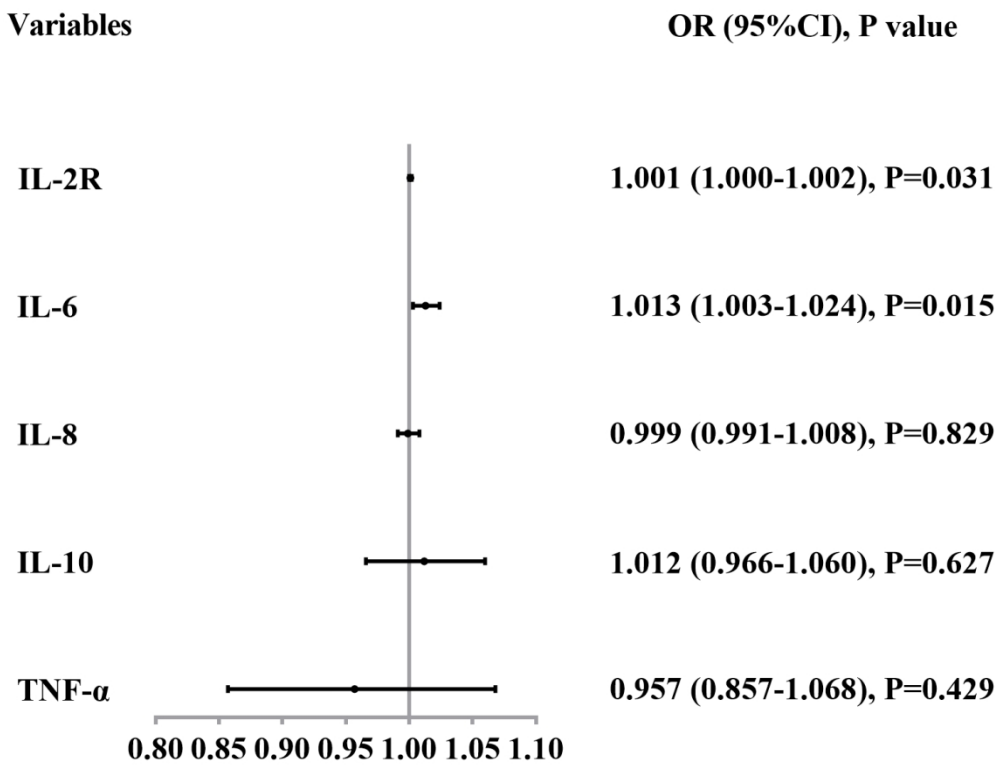


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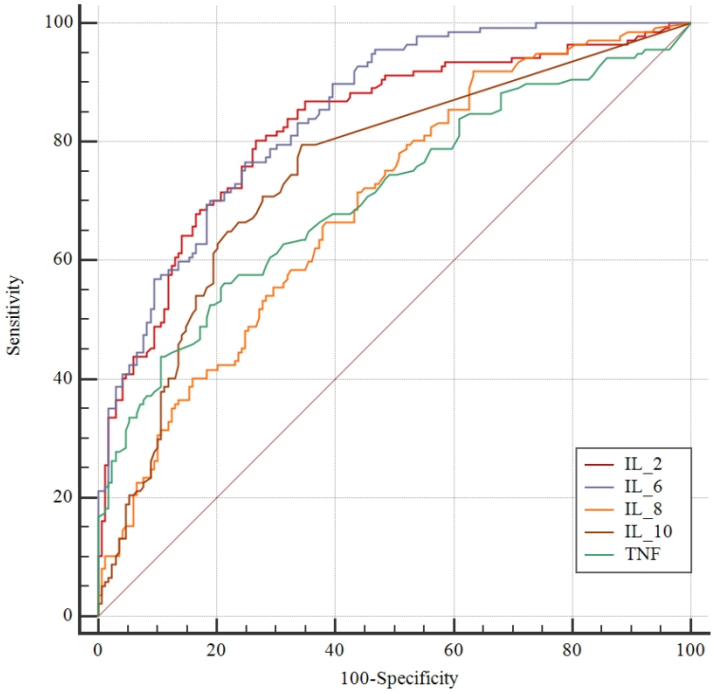


Figure 4. Receiver operating characteristic curve of Interleukin 2R, Interleukin 6, Interleukin 8, Interleukin 10, and TNF for in-hospital death.

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STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

| | Item No | Page No | Recommendation |
|---|---|---|---|
| **Title and abstract** | 1 | 1-2 | (*a*) Indicate the study's design with a commonly used term in the title or the abstract |
| | | | (*b*) Provide in the abstract an informative and balanced summary of what was done and what was found |
| | | | **Introduction** |
| Background/rationale | 2 | 3 | Explain the scientific background and rationale for the investigation being reported |
| Objectives | 3 | 4 | State specific objectives, including any prespecified hypotheses |
| | | | **Methods** |
| Study design | 4 | 4 | Present key elements of study design early in the paper |
| Setting | 5 | 4-5 | Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection |
| Participants | 6 | 4 | (*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up |
| | | | (*b*) For matched studies, give matching criteria and number of exposed and unexposed |
| Variables | 7 | 4-5 | Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable |
| Data sources/ measurement | 8* | 5 | For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group |
| Bias | 9 | 5 | Describe any efforts to address potential sources of bias |
| Study size | 10 | 4 | Explain how the study size was arrived at |
| Quantitative variables | 11 | 5 | Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why |
| Statistical methods | 12 | 6 | (*a*) Describe all statistical methods, including those used to control for confounding |
| | | | (*b*) Describe any methods used to examine subgroups and interactions |
| | | | (*c*) Explain how missing data were addressed |
| | | | (*d*) If applicable, explain how loss to follow-up was addressed |
| | | | (*e*) Describe any sensitivity analyses |
| | | | **Results** |
| Participants | 13* | 6 | (a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed |
| | | | (b) Give reasons for non-participation at each stage |
| | | | (c) Consider use of a flow diagram |
| Descriptive data | 14* | 6 | (a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders |
| | | | (b) Indicate number of participants with missing data for each variable of interest |
| | | | (c) Summarise follow-up time (eg, average and total amount) |
| Outcome data | 15* | 7 | Report numbers of outcome events or summary measures over time |
| Main results | 16 | 11 | (*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear |

| | | | |
|---|---|---|---|
| | | | which confounders were adjusted for and why they were included |
| | | | (*b*) Report category boundaries when continuous variables were categorized |
| | | | (*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period |
| Other analyses | 17 | 9-10 | Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses |
| | | | **Discussion** |
| Key results | 18 | 12 | Summarise key results with reference to study objectives |
| Limitations | 19 | 14 | Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias |
| Interpretation | 20 | 13 | Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence |
| Generalisability | 21 | 13-14 | Discuss the generalisability (external validity) of the study results |
| | | | **Other information** |
| Funding | 22 | 15 | Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based |

*Give information separately for exposed and unexposed groups.

**Note:** An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.