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ORIGINAL RESEARCH

# Prognostic Value of C-Reactive Protein, Procalcitonin, and Interferon-Alpha in COVID-19 and Their Association with PANoptosis: A Cohort Study in Guangxi, China

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**Objective:** Numerous biomarkers of COVID-19 severity have been studied recently, while their relevance in the post-COVID-19 era requires further validation. This study sought to identify reliable biomarkers within a single cohort through a comprehensive comparative analysis and to investigate the underlying molecular mechanisms.

**Material and Methods:** In this prospective, observational cohort study, we comprehensively assessed 41 markers in 97 COVID-19 pneumonia patients and 53 healthy volunteers. Receiver operating characteristics and Kaplan–Meier curves were plotted to determine the predictive value of effective biomarkers. Subsequently, flow cytometry, immunofluorescence, and Western blot analysis were conducted to evaluate the inflammatory cell death induced by the identified biomarkers in peripheral blood cells.

**Results:** Based on the screening, plasma IL-6, IL-8, IL-10, IFN-α, PCT, and CRP at hospital admission were highly associated with disease severity ($P < 0.001$). A pairwise combination of CRP, PCT, and IFN-α could induce PANoptosis in neutrophils, lymphocytes, and platelets.

**Conclusion:** The value of IL-6, IL-8, IL-10, IFN-α, PCT, and CRP in predicting the severity of COVID-19 was further verified in our study, and superior to novel biomarkers. Our data confirmed for the first time that CRP, PCT, and/or IFN-α triggers PANoptosis in lymphocytes, neutrophils, and platelets, which may prompt severe progression in COVID-19 patients.

**Keywords:** COVID-19, C-reactive protein, procalcitonin, interferon alpha, PANoptosis

## Introduction

The coronavirus disease 2019 (COVID-19) pandemic, instigated by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has precipitated a worldwide public health crisis over the past 4 years. Vaccines and treatments have effectively reduced the impact of the virus, but its rapid mutation and high transmission still pose a threat to people.[1] The report from The Chinese Center for Disease Control and Prevention (CDC) indicates that sporadic cases persist, and individuals with underlying conditions continue to be susceptible to severe illness.[2] This underscores the critical necessity for ongoing research



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and surveillance to guide the development of long-term control strategies. Plenty of biomarkers linked to the severity of COVID-19 and adverse outcomes have been emerging during a period of intense viral pathogenicity.[3] In the post-COVID-19 era, as COVID-19 transitions into a seasonal illness and coexists with influenza A (IAV), it remains uncertain whether previously established parameters continue to be applicable. Thus, we selected several biomarkers which have demonstrated superior performance and potential predictive value in recent studies for a comprehensively horizontal comparisons within one cohort, which aid in identifying reliable biomarkers, understanding potential molecular mechanisms, and discovering personalized treatment strategies.

Depending on their clinical application, the biomarkers correlated with COVID-19 disease severity can be divided into three categories: routinely tested clinical laboratory markers, non-routinely tested anti-viral markers, and no clinic-applied novel biomarkers. Routinely tested clinical laboratory markers mainly included the following kinds: inflammatory and immunological parameters, such as C-reactive protein (CRP), procalcitonin (PCT); hematological parameters, including lymphocyte count (LYC), neutrophil-to-lymphocyte ratio (NLR), and red blood cell distribution width (RDW); and organ damage indicators, such as Creatine Kinase-MB (CK-MB), aspartate aminotransferase (AST), and alanine aminotransferase (ALT).[3] Apart from the above, it has been observed that crucial anti-viral markers interferons (IFNs) were not routinely monitored in COVID-19 patients in most hospitals in China. IFNs are critical immunological parameters that mediate the early innate immune response to viral infections. Dysregulated IFN responses have been observed with the infection of coronaviruses, including suppressed levels of IFNs and I–IFN excessive induction.[4,5] We hope our study could increase the understanding of IFNs effects on COVID-19. Additionally, a few novel predictive biomarkers of COVID-19 have been identified, such as calprotectin (S100A8/A9), 8-hydroxydeoxyguanosine (8-OHdG), TNF (tumor necrosis factor)-related apoptosis-inducing ligand (TRAIL). S100A8/A9, which is synthesized and secreted locally at the inflammatory site, presents a more promising biomarker for COVID-19 compared to traditional inflammatory biomarkers such as C-reactive protein and calcitonin.[6] Numerous studies have indicated that the disruption of redox homeostasis contributes to the pathophysiology, progression, and outcomes of COVID-19.[7,8] 8-OHdG is a common biomarker for oxidative DNA damage,[9] but its predictive value for COVID-19 severity is controversial.[10] Severe COVID-19 exhibited higher levels of 8-OHdG in a Bieganski cohort of 72 patients,[11] whereas no statistically significant differences were observed in another Brazilian cohort.[12] Despite extensive research efforts, few of these novel biomarkers have been routinely detected by clinicians in the management of COVID-19. Therefore, we hope to evaluate comprehensively the predictive value of biomarkers associated with COVID-19 and discover a more appropriate clinical biomarker for early disease classification, which will hopefully guide future treatments for COVID-19.

Certain biomarkers demonstrate both prognostic value and specific bioactivities, which are potentially effective therapeutic targets. Interleukin-6 (IL-6) is produced in response to infections or tissue injuries, and its dysregulated excessive and persistent synthesis can lead to inflammatory disorders.[13] In response to inflammatory conditions, IL-6 pathway inhibitors (such as siltuximab and tocilizumab) have demonstrated effectiveness in patients with severe or critical COVID-19.[14] IFN is a bioactive factor with immunoregulatory properties, which could modulate immune cell differentiation, proliferation, and cytokine production.[15] To our knowledge, a hallmark of critical COVID-19 is the reduction of IFN-I. IFN therapy has been proposed for COVID-19 treatment, but the clinical outcomes have been disappointing. This may be attributed to IFN-induced inflammatory responses, the occurrence of cytokine storms, and the process of PANoptosis.[16] Moreover, PCT and CRP were often used as biomarkers for bacterial infection and inflammation,[17] and their bioactivities are gradually being recognized.[18–20] Laura Brabenec et al identified that PCT specifically targets the endothelium and induces hyperpermeability by disrupting the stability of endothelial adhesion junctions.[19] Native pentameric CRP (pCRP) and modified/monomeric CRP (mCRP) exhibit potent pro-inflammatory activities, including prolonging neutrophil apoptosis and inducing the release of neutrophil extracellular traps (NETs).[21] Given the pathogenicity of markers, there are reasons to speculate that the adverse outcomes of COVID-19 are potentially caused by the overproduction of biomarkers. PANoptosis has been confirmed as a pathological mechanism of cytokine storm and could be correlated with adverse outcomes.[22] It represents a distinct and physiologically relevant inflammatory programmed cell death (PCD), exhibiting hallmark traits of pyroptosis, apoptosis, and necroptosis.[23] PANoptosis can be triggered by inflammatory cytokines, such as TNF-α plus IFN-γ,[24,25] and this cell death process, in turn, leads to an amplification of cytokine release, culminating in a life-threatening cytokine storm, ultimately resulting in systemic inflammation, multi-organ

failure, and lethality.[26–29] Whether the potentially active markers described above can promote adverse outcomes by inducing PANoptosis needs further investigation.

In this study, we enrolled both COVID-19 patients and healthy individuals and conducted a comprehensive analysis to explore accurate and reliable biomarkers for predicting prognosis in the post-COVID era. Furthermore, our observations regarding the pathogenic impact of these biomarkers on peripheral blood cells may enhance the understanding of the molecular mechanisms involved in the increased mortality risk associated with COVID-19, thereby facilitating the development of personalized therapeutic strategies.

# Materials and Methods

## Study Design and Workflow

A schematic overview of the experimental design and analytical workflow is provided in Figure 1. Briefly, the study enrolled 97 COVID-19 patients and 53 healthy individuals. COVID-19 patients admitted to the hospital were categorized into severe and non-severe groups. Within 24 hours of admission, vital signs and laboratory test results (including CRP levels, PCT levels, routine blood tests, and other inspection indicators) were recorded. Peripheral blood samples were separated to obtain plasma, and additional markers were analyzed to identify biomarkers associated with the severity of COVID-19. Whole blood samples were collected from healthy individuals, from which lymphocytes, neutrophils, and platelets were isolated. The identified biomarkers were subsequently employed to stimulate human peripheral blood cells in vitro, either individually or in combination. The impact of these biomarkers on PANoptosis/NETs was evaluated using flow cytometry, immunofluorescence, and Western blot.

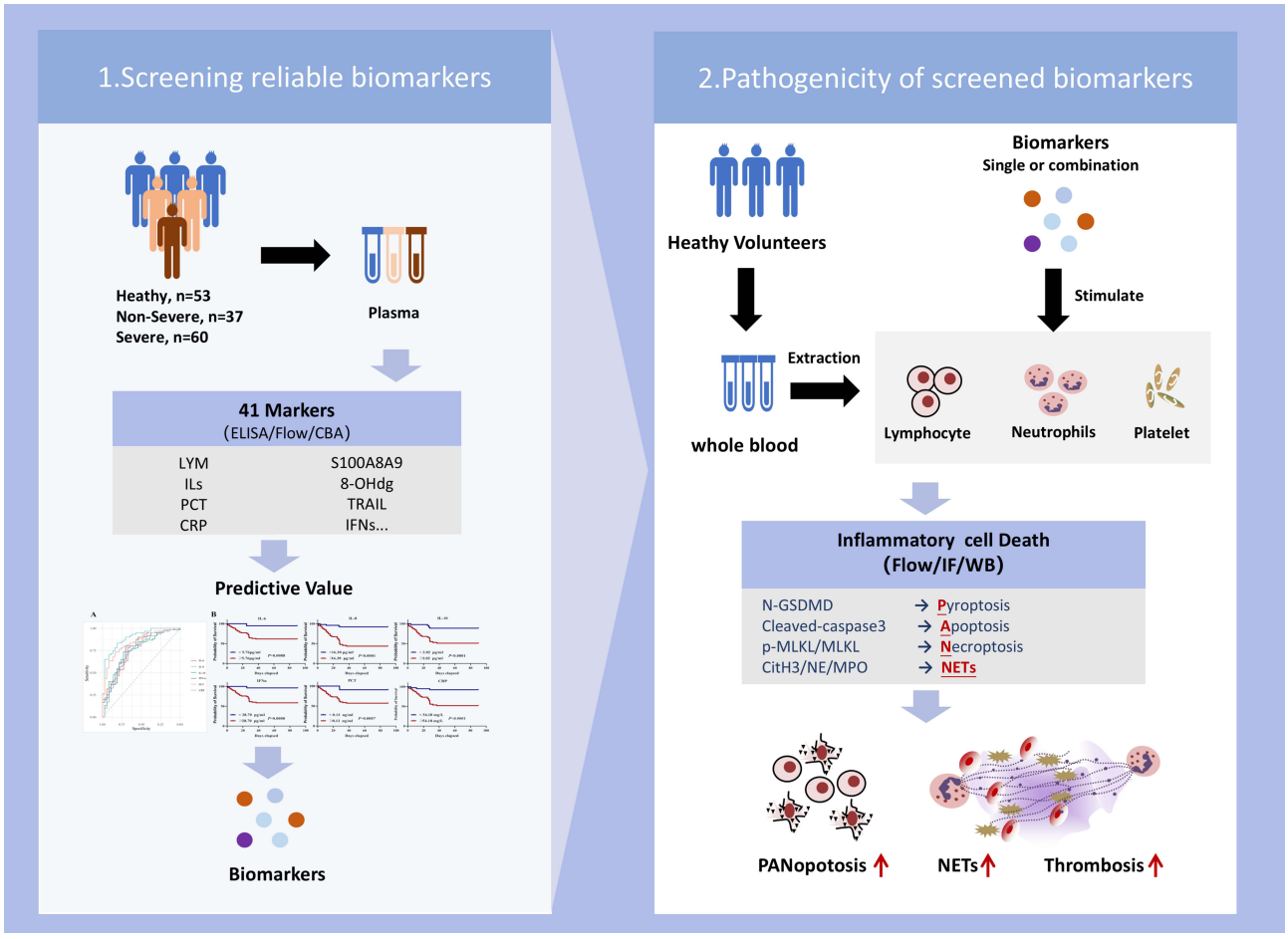


**Figure 1** Schematic overview of the study design and workflow.


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# Study Populations

## Healthy Control Subjects

Healthy volunteers between 22 and 57 years of age were recruited from the Health Management Center at Liuzhou People's Hospital. Inclusion criteria were healthy male or non-pregnant, non-breastfeeding female participants, aged 18–80 years and BMI of 19–26 kg/m$^2$. Individuals were excluded if they met any of the following conditions: (1) A history of chronic or severe diseases affecting major organ systems (eg, cardiovascular, hepatic, renal, respiratory, hematologic, endocrine, immunological, neurological and gastrointestinal) within the past 3 years; (2) Presence of acute respiratory symptoms (eg, fever, cough) or use of antibiotics/antiviral medications within 2 weeks; (3) Receipt of any vaccination within four weeks.

## COVID-19 Subjects

COVID-19 patients were enrolled from the intensive care unit (ICU), emergency department, respiratory and critical care unit, department of infectious diseases, and traditional Chinese medicine department at Liuzhou People's Hospital. The study period extends from January 9, 2023, to January 27, 2024, and includes patients with laboratory-confirmed COVID-19 diagnoses.

The diagnosis of COVID-19 was conducted in accordance with the "Diagnosis and treatment protocol for COVID-19 patients (Tentative 10th Version)" in China.[30] Ninety-seven COVID-19 patients were aged between 24 and 91 years. Fifty-three healthy people were aged between 22 and 57 years.

The selection of subjects for this study was according to the following inclusion criteria: (1) Patients confirmed with COVID-19 through positive SARS-CoV-2 tests; (2) Age ≥ 18 years old; and (3) Patients or their agents sign informed consent after being fully informed. The exclusion criteria were pregnant and lactating women. Patients were categorized into non-severe and severe groups based on the COVID-19 classification specified in their discharge diagnosis: the non-severe group, comprising individuals with mild and moderate conditions, and the severe group, consisting of those with severe and critical conditions. A flowchart of the enrolled patients was shown in Figure 2.

Patients were monitored for 90-day mortality through electronic medical records and phone follow-ups.

## Plasma Samples

Plasma was isolated for analysis after collecting blood from 97 COVID-19 patients and 53 healthy individuals. Blood samples were collected within 24 hours after admission and transferred immediately to Liuzhou People's Hospital

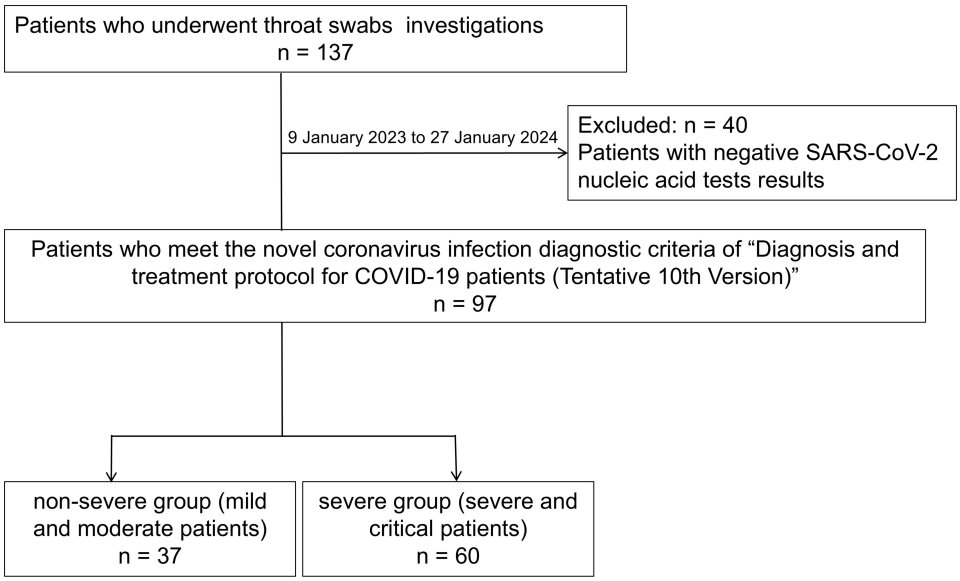


**Figure 2** Flowchart of enrolled patients.

Research Center of Medical Sciences for testing biomarkers. All blood samples were centrifuged at 3000 rpm for 10 minutes at 4°C, and the supernatant was gathered and stored at −80°C.

### Marker Levels Detection

Interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-10 (IL-10) levels were detected by flow cytometry-based Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (IL-6 Flex Set, 558276; IL-8 Flex Set, 558277; IL-10 Flex Set, 558274; BD Biosciences). As described by the manufacturer, the detection range of the assay was 10–2500 pg/mL (The theoretical limit of detection is 0.13 pg/mL).

According to the instructions of the manufacturer, ELISA kits were used to measure plasma Interleukin-1β (IL-1β) (KE00195, Proteintech), IFN-α (E-EL-H6125-96T, Elabscience), IFN-γ (KE00146, Proteintech), IFN-β (E-EL-H0085-96T, Elabscience), TNF-α (70-EK182HS-96, Elabscience), 8-OHdG (E-EL-0028-96T, Elabscience), A disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4) (CSB-E11848h, CUSABIO), S100A8/A9 (439707, BioLegend), TRAIL (CSB-E04750h, CUSABIO).

## Peripheral Blood Cells Isolation and Cultivation

Blood was taken from healthy individuals. As described by the manufacturer's protocol, human neutrophils (NEUs) were separated from whole blood by discontinuous density-gradient centrifugation on Percoll (GE Healthcare, Uppsala, Sweden). Peripheral blood lymphocytes (LYMs) were separated by using human peripheral blood lymphocyte isolation solution (P8610, Solarbio, China). Moreover, Platelets (PLTs) were isolated from human peripheral blood as follows. Peripheral blood was drawn into an acid-citrate-dextrose (ACD) buffer vacutainer, centrifuged at 180×g for 10 minutes to isolate PRP, then centrifuged at 1500×g for 10 minutes to obtain platelets. Then, Isolated cells (NEUs, LYMs, and PLTs) were cultured in RPMI 1640 medium (with 10% FBS and 1% Penicillin-Streptomycin). PLTs culture required supplementation with 0.1 M HEPES.

## Cells Treatment

Cultured human NEUs, LYMs, and PLTs were stimulated with 5 μg/mL C-Reactive Protein from human fluids (C4063, Sigma-Aldrich), 0.65 μg/mL Recombinant Human Procalcitonin protein (ab92843, Abcam), 100 ng/mL IL-6 (HY-P7044, MCE), 100 ng/mL IL-8 (HY-P722044, MCE), 50 ng/mL IL-10 (HY-P7030, MCE) and/or 160 ng/mL IFN-α (HY-P7022, MCE) for 8 h (for PLTs and NEUs) or 24 h (for LYMs) at 37°C and 5% $CO_2$.

## Flow Cytometry Analysis

Cell death was assessed using propidium iodide (PI) staining and flow cytometry analysis. The procedure was described as follows: cells were resuspended in 100 μL of Phosphate Buffered Saline (PBS) containing 2 μg/mL of PI, then incubated for 30 minutes at room temperature and analyzed using a BD LSRFortessa flow cytometer. Data were analyzed using FlowJo version 10 software. Experiments were repeated three times, each experiment was in triplicate.

## Immunofluorescence Assay

A standard protocol for immunofluorescent staining was followed. Briefly, cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature, washed twice with 1×PBS for 10 minutes on a rocker, and permeabilized with 0.25% Triton X-100 at room temperature for 10 minutes. After washing, 5% bovine serum albumin was used to block the cells at room temperature for 1 hour and treated with antibodies against phosphorylated mixed lineage kinase domain like pseudokinase (p-MLKL) (1:400, NOVUSBIO, 954724), antibodies against Gasdermin D (GSDMD) (1:100, Invitrogen, PA5-119680) and antibodies against Cleaved-Caspase-3 (1:100, CST, 9664S), at 4°C overnight. Followed by three washes with 1×PBS, the cells were incubated with 488 Tyramide (YT0070L, UElandy), 555 Tyramide (YT0071L, UElandy) and 640 Tyramide (YT0073L, UElandy) at RT for 1 h. For the last 10 minutes, neutrophil and lymphocyte nuclei were stained with DAPI, while platelets were marked with CD41a. In the end, the slices were washed with PBS and treated with anti-fluorescence quenching agents. Images were acquired using laser confocal microscopy and the immunofluorescence intensity was assessed using the Leica Application suite X (LAX) software. Experiments were repeated three times, each experiment was in triplicate.

## Western-Blot

Protein from NEUs, LYMs, and PLTs was collected 8 or 24 hours after stimulation and assessed with a BCA protein assay kit (Beyotime). Proteins were separated on 12% SurePAGE precast gel and then transferred to PVDF membranes, which were blocked in 5% nonfat milk for 1 hour at room temperature before overnight incubation with primary antibodies at 4°C including GAPDH (1:1000, Proteintech, 60004–1), Cleaved-Caspase-3 (1:1000, CST, 9664S), GSDMD (1:1000, Invitrogen, PA5-119680), N-GSDMD (1:1000, Abcam, ab215203), MLKL (1:1000, Proteintech, 21066-1-AP) and p-MLKL (1:1000, NOVUSBIO, 954724). Subsequently, membranes were incubated with secondary antibody [Goat Anti-Rabbit and Anti-Mouse (1:4000; cat. nos. RGAR001 and SA00001-1, respectively; both Proteintech)] for 2 h at room temperature. Protein bands were detected utilizing an Enhanced Chemiluminescence (ECL) kit (Thermo Fisher Scientific, USA) and visualized under a gel imaging system (Bio-Rad, USA). The band intensities were subsequently analyzed in a semi-quantitatively manner using Quantity One software. Experiments were repeated three times; each experiment was in triplicate.

## Data and Statistics

Statistical analyses were conducted utilizing SPSS version 26.0 and GraphPad Prism version 9.0. Categorical data were compared using the chi-squared or Fisher's exact test, and numerical data were analyzed between two groups using the *t*-test. Repeated measures one-way ANOVA was used to analyze comparisons among multiple groups. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$ and were considered statistically significant. Optimal biomarker cut-off values for disease severity were defined by a receiver operating characteristic (ROC) curve. Survival probabilities were calculated using the Kaplan–Meier method and compared with the Log rank test.

# Results

## Patient Characteristics

A total of 97 patients with COVID-19 were included in this study. As shown in Table 1, 68 patients were male (70.10%) and 29 patients were female (29.90%), with an average age of (69.80 ± 9.59) years. There were 60 (61.86%) and 37 (38.14%) patients in severe and non-severe groups, respectively. The 90-day all-cause mortality rate was 35.05%, with 33 (55.00%) deaths in the severe group and 1 (2.70%) death in the non-severe group. Patients with cardiovascular and cerebrovascular diseases (51.35% vs. 73.33%) or cancer (20% vs. 5.41%) would be more susceptible to developing severe COVID-19. Severe group patients tend to experience longer hospital stays (days: 8.66 ± 4.49 vs. 23.27 ± 14.49), which may result in higher hospitalization expenses. Between the groups, there was no significant variation in vaccination history, age and smoking ($P > 0.05$).

## Early Prediction of Severe COVID-19 by Plasma Markers

In our cohort, 41 parameters were assessed for their potential in predicting severe COVID-19 progression, among which 28 indicators were derived from local hospital laboratories (see Table 2) were evaluated. Our results found that severe group had significantly higher WBC counts (11.58 ± 4.82 vs. 7.57 ± 2.35×$10^9$/L, $P < 0.001$), NEUR (86.19 ± 7.27 vs. 76.38 ± 10.65%, $P = 0.001$), CRP (96.17 ± 50.43 vs. 52.72 ± 40.52 mg/L, $P < 0.001$), and BUN (10.75 ± 5.83 vs. 5.76 ± 2.32 mmol/L, $P < 0.001$) than non-severe patients. However, lymphocyte counts (0.72 ± 0.44 vs. 0.98 ± 0.42×$10^9$/L, $P = 0.044$), Ca (1.98 ± 0.17 vs. 2.09 ± 0.08, $P = 0.002$), and Albumin (29.13 ± 4.59 vs. 36.79 ± 4.72 g/L, $P<0.001$) were significantly lower than non-severe patients. In addition, compared with non-severe cases, no significant decrease in platelet counts were found in severe cases. However, coagulation markers, PT (15.32 ± 2.92 vs. 13.74 ± 1.14, $P < 0.001$), APTT (44.16 ± 9.08 vs. 39.58 ± 5.92, $P = 0.008$), and TT (18.43 ± 2.36 vs. 17.34 ± 1.00, $P = 0.003$) were all significantly prolonged in severe patients, while the prothrombin time activity percentage (PTA) was markedly decreased (80.64 ± 18.84 vs. 95.70 ± 15.50, $P < 0.001$). Results indicated that with the progression of the disease, severe patients had signs of coagulopathy.

In addition, the following 13 biomarkers were determined in the plasma of COVID-19 patients and healthy volunteers: IL-1β, IL-6, IL-8, IL-10, PCT, 8-OHdG, TNF-α, IFN-α, IFN-β, IFN-γ, S100A8/A9, TRAIL, and ADAMTS4. TNF-α, IFN-γ, IL-1β, and ADAMTS4 were excluded from the analysis due to their undetectable levels

**Table 1** Demographic and Epidemiological Baseline Data

| Clinical Classification | | | | |
|---|---|---|---|---|
| | **Total (N=97)** | **Non-Severe (N=37)** | **Severe (N=60)** | ***P*-value** |
| **Gender** | | | | 0.024 |
| Male | 68(70.10%) | 21(56.76%) | 47(78.33%) | |
| Female | 29(29.90%) | 16(43.24%) | 13(21.67%) | |
| **Age** | 69.80±9.59 | 68.92±10.58 | 70.35±8.95 | 0.592 |
| **Vaccination history** | | | | 0.386 |
| 1 dose | 3(3.09%) | 2(5.41%) | 1(1.67%) | |
| 2 doses | 9(9.28%) | 2(5.41%) | 7(11.67%) | |
| 3 doses | 30(30.93%) | 12(32.43%) | 18(30%) | |
| 4 doses | 2(2.06%) | 1(2.70%) | 1(1.67%) | |
| Unvaccinated | 22(22.68%) | 5(13.51%) | 17(28.33%) | |
| No record | 31(31.96%) | 15(40.54%) | 16(26.67%) | |
| **Hospital stays** | 17.70±12.33 | 8.66±4.49 | 23.27±14.49 | <0.001 |
| **Death** | 34(35.05%) | 1(2.70%) | 33(55.00%) | <0.001 |
| **Comorbidities** | | | | |
| Cardiovascular and cerebrovascular diseases | 63(64.95%) | 19(51.35%) | 44(73.33%) | 0.028 |
| Chronic lung disease | 23(23.71%) | 6(16.22%) | 17(28.33%) | 0.173 |
| Diabetes | 23(23.71) | 11(29.73%) | 12(20.00%) | 0.274 |
| Chronic liver disease | 9(9.28%) | 3(8.11%) | 6(10.00%) | 0.755 |
| Chronic kidney disease | 16(16.49%) | 5(13.51%) | 11(18.33%) | 0.534 |
| Cancer | 14(14.43%) | 2(5.41%) | 12(20%) | 0.047 |
| Maintenance dialysis patients | 4(4.12%) | 0(0.00%) | 4(6.67%) | 0.141 |
| Immunosuppression | 2(2.06%) | 1(2.70%) | 1(1.67%) | 0.727 |
| Obesity | 1(1.03%) | 0(0.00%) | 1(1.67%) | 0.619 |
| **Smoking** | | | | 0.081 |
| Yes | 16(16.49%) | 3(8.11%) | 13(21.67%) | |
| No | 81(83.51%) | 34(91.89%) | 47(78.33%) | |

**Table 2** Laboratory Parameters Features of COVID-19 Patients

| Clinical Classification | | | |
|---|---|---|---|
| | **Non-Severe (N=37)** | **Severe (N=60)** | ***P*-value** |
| **Body temperature(°C)** | 36.86±0.51 | 37.05±0.62 | 0.270 |
| **Heart rate** | 87.19±9.30 | 95.07±13.70 | 0.010 |
| **SBP** | 126.64±13.59 | 125.4±14.59 | 0.750 |
| **DBP** | 71.54±9.69 | 68.85±11.70 | 0.344 |
| **RR** | 19.84±1.54 | 20.87±5.48 | 0.335 |
| **WBC (×$10^9$/L)** | 7.57±2.35 | 11.58±4.82 | <0.001 |
| **LY(%)** | 15.38±8.24 | 7.44±4.51 | <0.001 |
| **LYM (×$10^9$/L)** | 0.98±0.42 | 0.72±0.44 | 0.044 |
| **NEUR(%)** | 76.38±10.65 | 86.19±7.27 | 0.001 |
| **PLT (×$10^9$/L)** | 226.97±70.21 | 187.25±79.91 | 0.085 |
| **PT** | 13.74±1.14 | 15.32±2.92 | <0.001 |
| **PTA** | 95.70±15.50 | 80.64±18.84 | <0.001 |
| **APTT** | 39.58±5.92 | 44.16±9.08 | 0.008 |
| **TT** | 17.34±1.00 | 18.43±2.36 | 0.003 |
| **FIB** | 5.39±1.64 | 5.92±1.68 | 0.136 |
| **HGB (g/L)** | 112.84±15.97 | 107.65±25.82 | 0.551 |

(*Continued*)


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**Table 2** (Continued).

| | Clinical Classification | | |
|---|---|---|---|
| | **Non-Severe (N=37)** | **Severe (N=60)** | ***P*-value** |
| **HCT (%)** | 33.47±4.55 | 31.42±6.95 | 0.316 |
| **Tbil (umol/L)** | 10.30±3.76 | 11.99±6.95 | 0.478 |
| **Albumin (g/L)** | 36.79±4.72 | 29.13±4.59 | <0.001 |
| **ALT (U/L)** | 27.39±14.12 | 35.48±23.46 | 0.336 |
| **AST (U/L)** | 30.22±12.74 | 73.77±78.39 | 0.278 |
| **K** | 3.65±0.38 | 3.87±0.44 | 0.079 |
| **Na** | 138.35±3.15 | 139.62±4.75 | 0.322 |
| **Cl** | 102.80±3.47 | 103.09±6.32 | 0.849 |
| **Ca** | 2.09±0.08 | 1.98±0.17 | 0.002 |
| **CRE (umol/L)** | 128.79±92.52 | 157.95±115.53 | 0.568 |
| **BUN (mmol/L)** | 5.76±2.32 | 10.75±5.83 | <0.001 |
| **CRP (mg/L)** | 52.72±40.52 | 96.17±50.43 | 0.001 |

**Abbreviations**: SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; RR, Respiratory Rate; WBC, White Blood Cell Count; LY(%), Lymphocyte Percentage; LYM, Absolute Lymphocyte Count; NEUR, Neutrophil Percentage; PLT, Platelet Count; PT, Prothrombin Time; PTA, Prothrombin Time Activity; APTT, Activated Partial Thromboplastin Time; TT, Thrombin Time; FIB, Fibrinogen; HGB, Hemoglobin; HCT, Haematocrit; Tbil, Total Bilirubin; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; CRE, Creatinine; BUN, Blood Urea Nitrogen; CRP, C-reactive protein.

in the plasma samples of the initial 35 patients in our cohort. By analyzing the remaining 9 parameters, we found that 8 parameters other than 8-OHdG exhibit significant diagnostic efficacy. However, only IL-6, IL-8, IL-10, PCT, and IFN-α showed significant predictive value for disease severity (see Figure 3). All together, these results indicated that IL-6,

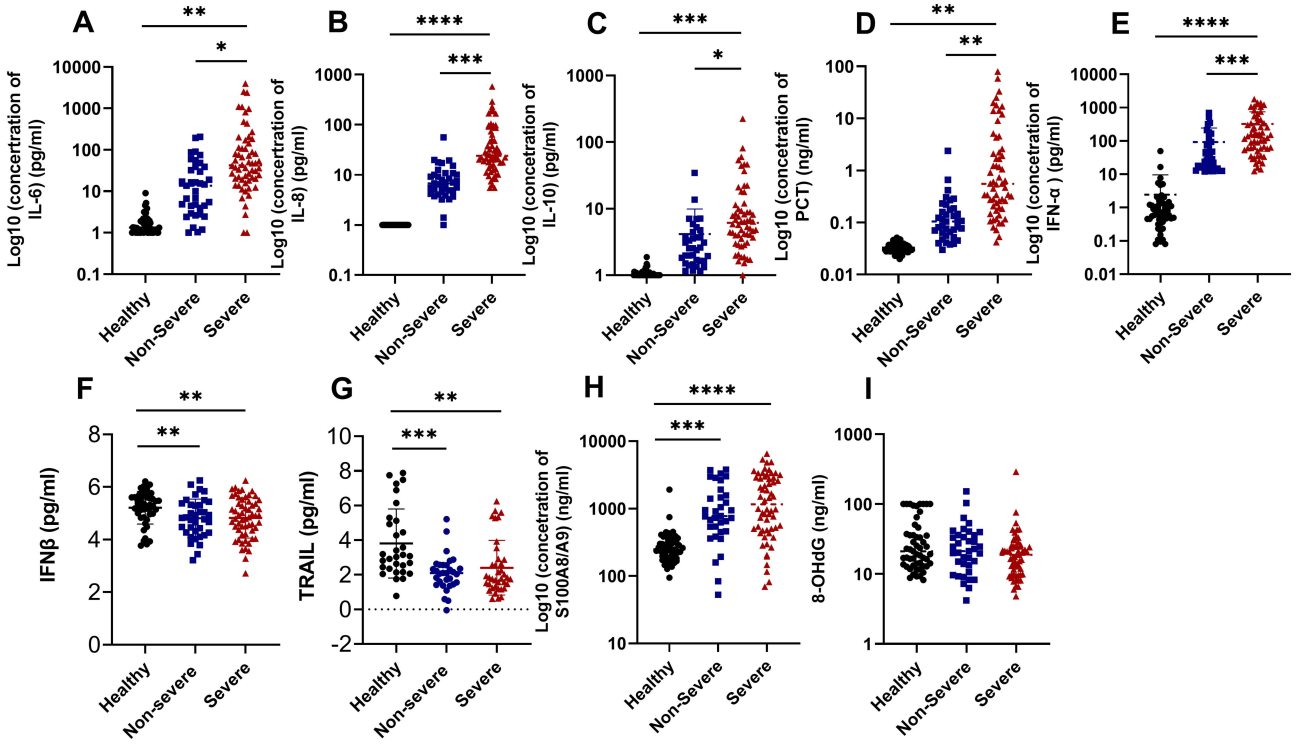


**Figure 3** The laboratory parameters in healthy controls and at hospital admission of patients with COVID-19 pneumonia. The plasma concentrations of IL-6 (**A**), IL-8 (**B**), IL-10 (**C**), PCT (**D**), IFN-α (**E**), IFN-β (**F**), S100A8/A9 (**G**), TRAIL (**H**), 8-OHdG (**I**), were analyzed in healthy controls (n = 53) and patients with non-severe (n = 37), and severe (n = 60) COVID-19. All data were represented as means±SD. The results of different groups were compared by employing ANOVA followed by the Dunnett post hoc test. **P*< 0.05, ***P* < 0.01, ****P*< 0.001, *****P*< 0.0001.

**Table 3** ROC Analysis of Various Indicators for COVID-19 Severity

| Variables | AUC | 95% Confidence Interval | *P*-value | Cut-off | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| **IL-6** | 0.753 | 0.653–0.854 | <0.001 | 18.085 | 0.783 | 0.667 |
| **IL-8** | 0.894 | 0.830–0.958 | <0.001 | 9.980 | 0.833 | 0.806 |
| **IL-10** | 0.746 | 0.643–0.850 | <0.001 | 2.855 | 0.733 | 0.722 |
| **IFN-α** | 0.761 | 0.660–0.863 | <0.001 | 48.719 | 0.800 | 0.694 |
| **PCT** | 0.841 | 0.762–0.920 | <0.001 | 0.235 | 0.733 | 0.833 |
| **CRP** | 0.736 | 0.630–0.842 | <0.001 | 54.180 | 0.750 | 0.667 |

IL-8, IL-10, IFN-α, PCT, and CRP may be biomarkers of COVID-19 disease severity, and may cause changes in the number and function of neutrophils, lymphocytes, and platelets.

## The Predictive Capacity of Plasma IL-6, IL-8, IL-10, IFN-α, PCT and CRP for Severity and Death

To assess the prognostic utility of IL-6, IL-8, IL-10, IFN-α, PCT, and CRP between the non-severe and severe COVID-19 groups, an ROC curve was generated, and the AUC was determined. Accordingly, the AUC values of IL-6, IL-8, IL-10, IFN-α, PCT and CRP were as follows: 0.753 (95% CI, 0.653-0.854), 0.894 (95% CI, 0.830-0.958), 0.746 (95% CI, 0.643-0.850), 0.761 (95% CI, 0.660-0.863) 0.841 (95% CI 0.762-0.920) and 0.736 (95% CI, 0.630-0.842), respectively (see Table 3). The optimal cut-off values of these biomarkers for distinguishing between non-severe and severe groups, along with their corresponding sensitivity and specificity values were shown in Table 3. The study population was divided into a low-level group and a high-level group based on the optimal cut-off value. The survival curve of the 90-day survival rate was drawn through the Kaplan–Meier method. Kaplan–Meier curves revealed that IL-6 ≥ 18.085 pg/mL, IL-8 ≥ 9.980 pg/mL, IL-10 ≥ 2.855 pg/mL, IFN-α ≥ 48.719 pg/mL, PCT ≥ 0.235 ng/mL or CRP ≥ 54.180 mg/L had more unfavorable outcome (see Figure 4).

## Co-Stimulation of CRP, PCT, or IFN-α Induced PANoptosis in Peripheral Blood Cells

Five screened pro-inflammatory biomarkers were used to stimulate LYMs, NEUs, and PLTs, respectively. The results showed that the combination of 5 biomarkers (defined as Cocktail) led to significant death of peripheral blood cells, but the biomarker stimulation alone did not induce significant cell death (see Figure 5A–C). Then we measured cell death in LYMs, NEUs, and PLTs treated with various combinations of biomarkers. Interestingly, whether in LYMs, NEUs, and PLTs, the pairings of CRP,

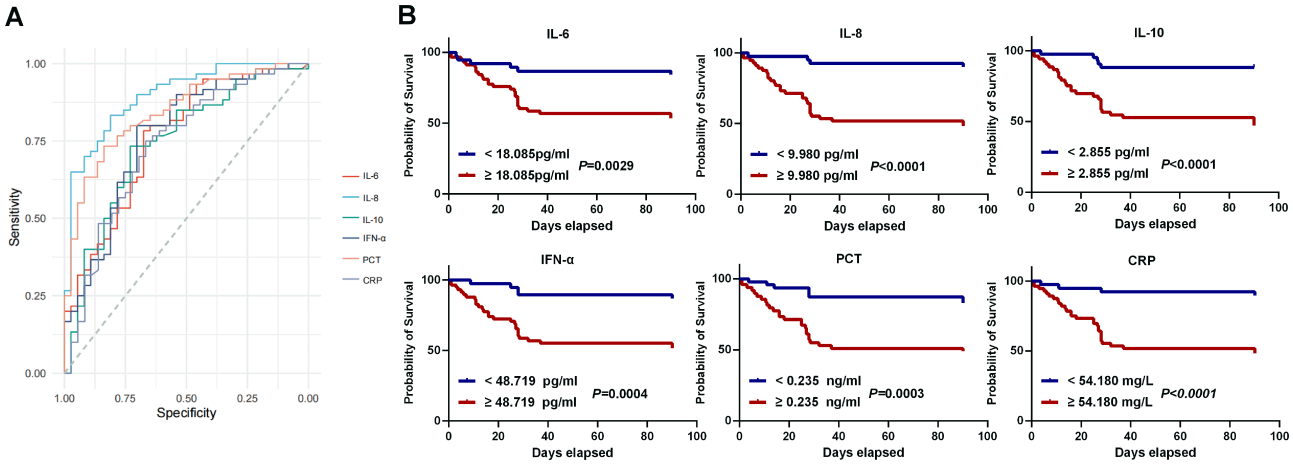


**Figure 4** The predictive capacity of plasma IL-6, IL-8, IL-10, IFN-α, PCT and CRP for severity and death (**A**): Receiver operating characteristics (ROC) curves of IL-6, IL-8, IL-10, IFN-α, PCT and CRP for prediction of severity among COVID-19 patients. (**B**): Kaplan–Meier curves of overall survival (OS) for different IL-6, IL-8, IL-10, IFN-α, PCT and CRP levels in COVID-19 patients.


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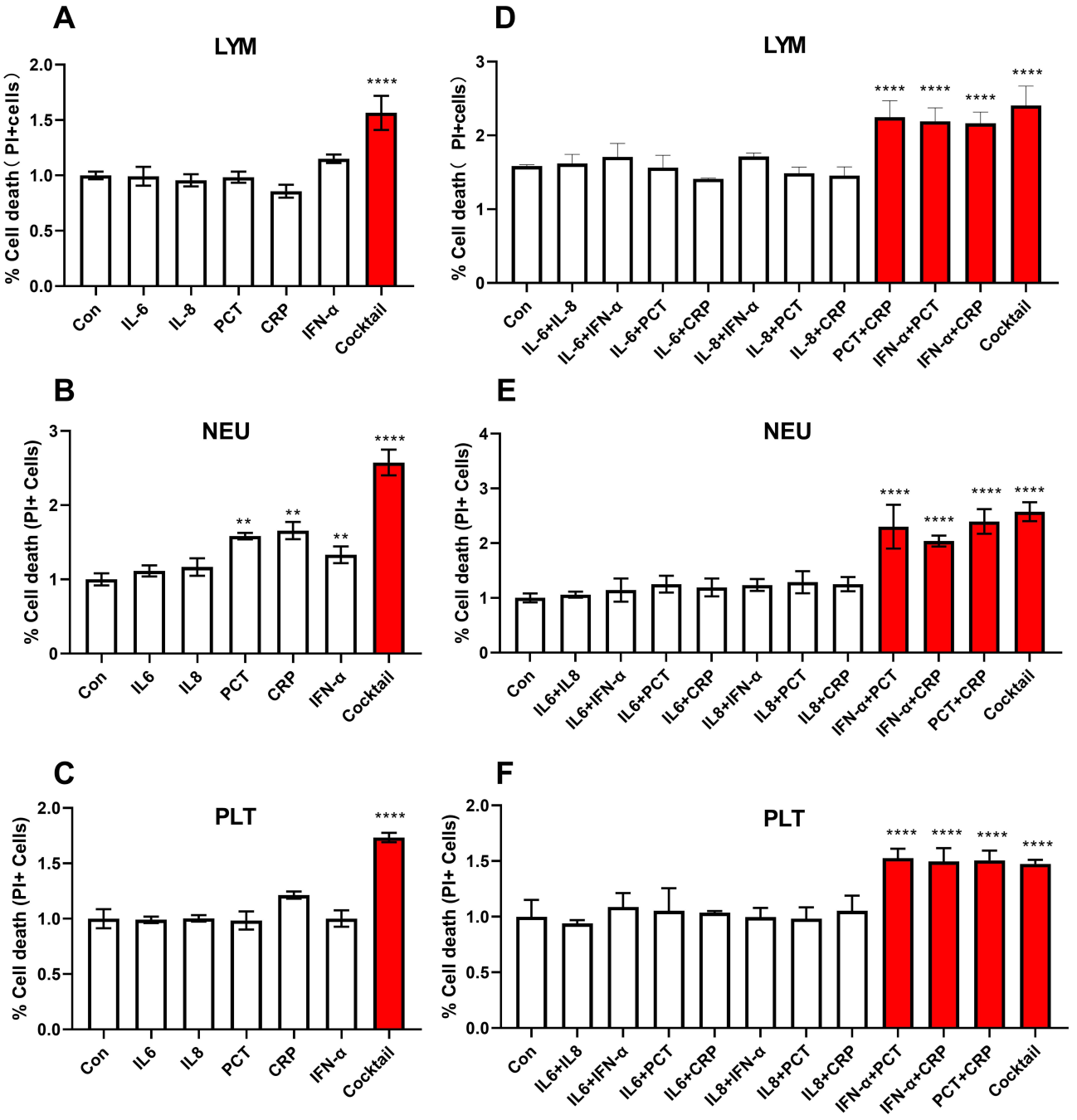


**Figure 5** Co-stimulation of CRP, PCT or IFN-α induced cell death in peripheral blood cells (**A**–**C**) Percentage of cell death in LYMs and PLTs after 8 h of treatment, and in NEUs after 24 h of treatment, as assessed by flow cytometry with PI staining. "Cocktail" contained all 5 pro-inflammatory biomarkers (IL-6, IL-8, IFN-α, PCT and CRP). (**D**–**F**) The percentage of cell death in LYMs and PLTs after 8 h of treatment, and in NEUs after 24 h of treatment with the indicated combinations. Data from three independent experiments (n=3 per group) were compared using ANOVA with Dunnett post hoc test for group comparisons. $^{**}P < 0.01$, $^{****}P < 0.0001$.

PCT, or IFN-α (defined as IFN-α + CRP, IFN-α + PCT, and CRP + PCT) significantly led to the occurrence of cell death (equivalent to Cocktail, see Figure 5D–F). Therefore, we speculate that the changes in the quantity and function of peripheral blood cells are mainly caused by CRP, PCT, and IFN-α. To identify if this death was PANoptosis, which is highly linked to inflammation. We detected the expression of the apoptotic marker cleaved-caspase3, pyroptosis marker GSDMD, and necroptosis markers p-MLKL/MLKL by immunofluorescence and western-blot. As illustrated in Figure 6, results demonstrated that the pairings combination of CRP, PCT, or IFN-α synergistically induced cleavage of caspase-3, GSDMD and phosphorylation of MLKL (Figure 6A–C). Furthermore, LYMs, NEUs, and PLTs exhibited variable degrees of PANoptosis in

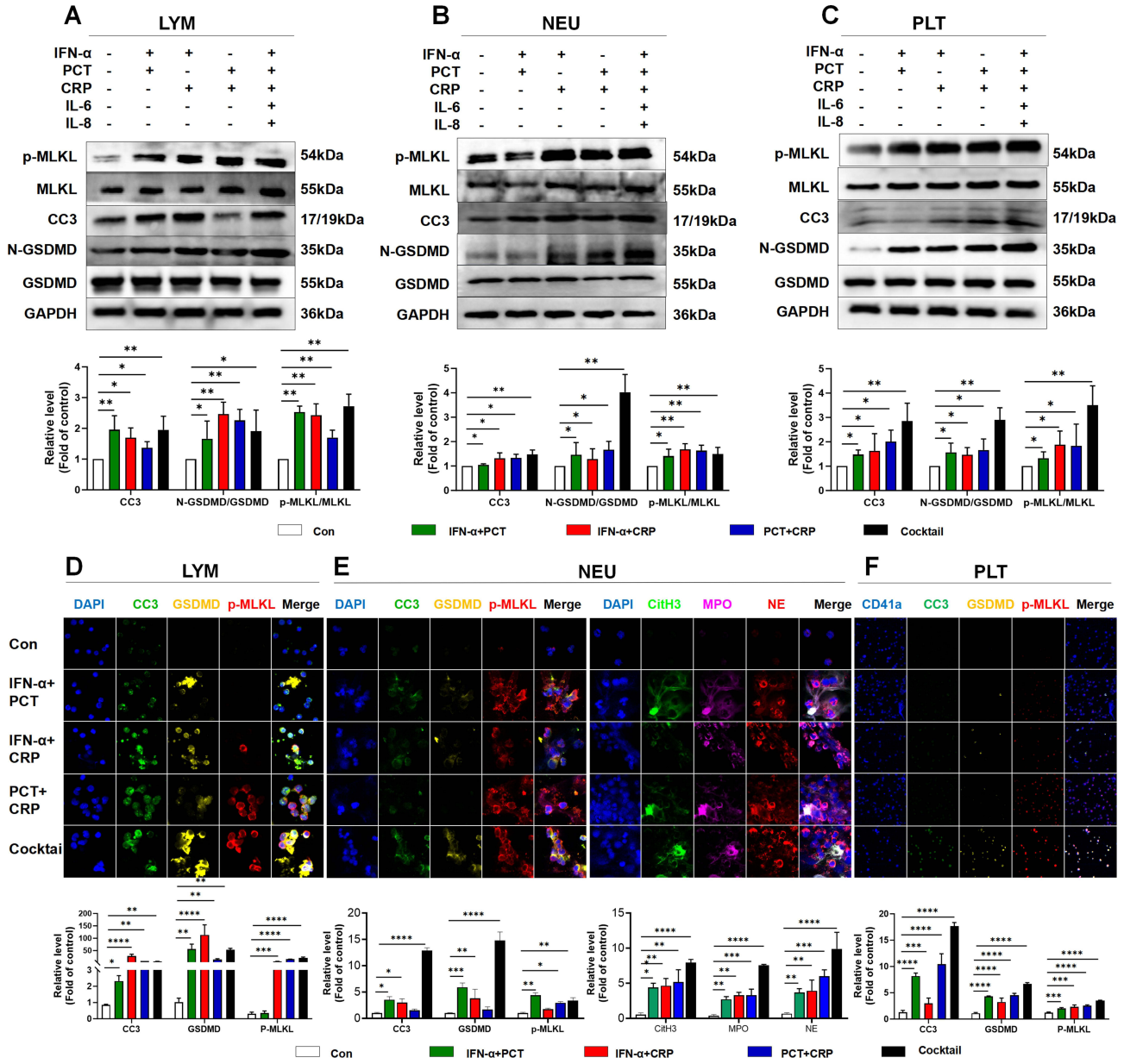


**Figure 6** Co-stimulation of CRP, PCT, or IFN-α induced PANoptosis in peripheral blood cells (**A**–**C**): Western blot analysis of PANoptosis markers, including the apoptotic marker cleaved- caspase-3 (CC3), the pyroptosis marker N-GSDMD/GSDMD, and the necroptosis markers p-MLKL/MLKL, was performed in co-treatment with CRP, PCT, or IFN-α in LYMs (**A**) and PLTs (**C**) after 8 h of co-treatment, and in NEUs (**B**) after 24 h of co-treatment. (**D**–**F**): Immunofluorescence analysis of PANoptosis markers (cleaved-caspase3, GSDMD, and p-MLKL) and NETs biomarkers (CitH3, MPO, and HE) in co-treatment with CRP, PCT, or IFN-α in LYMs (**D**) and PLTs (**F**) after 8 h of co-treatment, and in NEUs (**E**) after 24 h of co-treatment. Magnification ×200, Scale bar = 20 μm. All data were represented as means±SD. Data from three independent experiments (n=3 per group) were compared using ANOVA with Dunnett post hoc test for group comparisons. *$P$ < 0.05, **$P$ < 0.01, ***$P$< 0.001, ****$P$< 0.0001.

immunofluorescence (see Figure 6D–F). In addition, NEUs have also been shown to release NETs ($CitH3^+MPO^+NE^+$) after co-stimulation of CRP, PCT, or IFN-α (see Figure 6E). Collectively, these data suggested that co-stimulation of CRP, PCT, or IFN-α sensitizes the peripheral blood cells to undergo PANoptosis.

## Discussion

In this study, we found that (1) By assessing 41 markers, it is found that IL-6, IL-8, IL-10, IFN-α, PCT, and CRP have better predictive value for COVID-19 severity, which superior to novel biomarkers; (2) Peripheral S100A8/A9, TRAIL, and IFN-β have potential as markers for SARS-CoV-2 infection, but they are not reliable indicators of COVID-19


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severity; (3) Severe group had significantly increased neutrophil, decreased lymphocyte counts and coagulopathy; (4) The pairings of CRP, PCT, or IFN-α led to PANoptosis in NEUs, LYMs, and PLTs.

To identify reliable biomarkers in post-COVID-19 era, we comprehensively assessed 41 markers within a single cohort. With the present study, we found that routinely tested clinical laboratory markers IL-6, IL-8, IL-10, PCT, CRP, and anti-viral markers IFN-α were superior to novel biomarkers for predicting the severity and adverse outcomes in COVID-19 patients. This was validated in concordance with the literature,[3,31] highlighting the importance of inflammation-associated markers in predicting COVID-19 severity. Activated immune cells uncontrollably released proinflammatory mediators, as called a "cytokine storm", which could contribute to an abnormal systemic inflammatory response and severe pathological features observed in COVID-19.[32] Cytokines, especially interleukins (ILs), TNF-α, and IFN-γ can be employed as indicators to assess the severity of COVID-19.[31] Unexpectedly, TNF-α and IFN-γ were excluded from the analysis due to their undetectable levels in the plasma samples of the initial 35 patients in our cohort. It indicated that not all severe patients exhibited high expression of TNF/IFN-γ. Similarly, some studies have determined that COVID-19 patients display a high heterogeneity in TNF/IFN-γ levels, which may contribute to uncontrolled inflammation.[25,33] Depending on this, the lack of regularity in their levels may hinder accurate assessment of disease severity. However, the clinical significance of TNF/IFN-γ should not be underestimated. Clinicians could stratify patients based on the heterogeneity of TNF/IFN-γ, potentially facilitating the development of personalized therapeutic strategies. Apart from the above, novel inflammatory markers S100A8/A9 and TRAIL are not well validated in our cohort. They have the potential to be used as diagnostic markers for SARS-CoV-2 infection, but not as indicators of the severity of COVID-19. However, S100A8/A9 has been confirmed as a potential biomarker for COVID-19 severity in some researches.[34,35] Additionally, the role of TRAIL as severity marker for COVID-19 has been debated, the literature reported that Low TRAIL concentrations are associated with severe disease.[36] In contrast, we and others have shown that TRAIL is not a good predictor of the severity of COVID-19.[37] Such inconsistency may be attributed to the high baseline severity of this cohort, as the biomarker in question was nonspecific and consequently did not correlate with progression to severity. Next, we will perform a more comprehensive validation in a significantly expanded patient cohort. Notably, among the screened six markers identified in our cohort, IFN-I is the key cytokine with an important function in antiviral responses. A noticeable phenotype was identified in severe patients, consisting of no IFN-β and low IFN-α production and activity during the first days of infection, which was correlated with a sustained viral load and an intensified inflammatory reaction.[38] Differently, our study showed that IFN-α was markedly elevated and IFN-β slightly decreased. This inconsistency may be attributed to the fact that majority of patients included in our cohort were those admitted to the ICU, with the IFN response having missed the period of delayed or down-regulated interferon responses in the early phase. Despite this, it could still largely reflect adverse outcomes in COVID-19, regardless of elevation or reduction. Regrettably, IFN-I is not routinely evaluated as a clinical biomarker by clinicians in our country. So, we highly recommend that IFN-I should be widely incorporated into clinical assessments, especially in cases of viral infection, to enhance its utility in clinical settings.

We next evaluated the impact of screened biomarkers on peripheral blood cells, we found for the first time that pairwise combination with CRP, PCT, or IFN-α can induce PANoptosis in peripheral blood cells. PANoptosis, an inflammatory cell death that simultaneously involves pyroptosis (P), apoptosis (A), and necroptosis (N),[39] is one of the pathogenesis of SARS-CoV-2 infection. It may play a role in the development of cytokine storms, tissue damage, and acute respiratory distress syndrome.[40]

In addition to COVID-19, elevated levels of CRP and PCT are acknowledged as biomarkers indicative of disease severity in influenza virus and respiratory syncytial virus (RSV) infections, and are generally correlated with a poor prognosis.[41–43] Furthermore, an imbalance in the IFN response is a sign of severe influenza infection.[44,45] Despite differences in the initial viral triggers, the subsequent convergence towards a hyperinflammatory state and cell death may constitute a common "final pathway" that results in organ damage in severe pneumonia across various etiologies.[46] Consequently, we propose the hypothesis that PANoptosis, as induced by CRP, PCT, or IFN-α, may not be exclusive to SARS-CoV-2 but could potentially signify a universal marker of dysregulated host immunity in cases of severe viral pneumonia. This compelling hypothesis forms the basis for our immediate future research direction: to validate this biomarker cocktail and mechanistic model in cohorts of patients with severe influenza and RSV infections.

Peripheral blood cells have been shown as effector cells of cell death.[24,25,47] Severe COVID-19 cases exhibited leukocytosis, neutrophilia, lymphopenia, and thrombocytopenia.[48,49] Similar findings were noted within our research,

except that the manifestations of thrombocytopenia were not readily apparent instead of coagulation. Determining whether this effect is related to PANoptosis, induced by CRP, PCT, and/or IFN-α is the main focus of this study. Lymphopenia and immunosuppression are hallmarks of COVID-19.[50,51] Postmortem analyses of spleens and lymph nodes from COVID-19 patients have revealed an absence of germinal centers,[52] potentially attributable to lymphocyte cell death induced by CRP, PCT, and IFN-α. Neutrophils, another important innate immune cell subset, their activation are a significant clinical feature in COVID-19.[53] In our study, severe patients exhibit a significant increase in circulating neutrophils, the pairwise combination of CRP, PCT, or IFN-α induced PANoptosis and NETosis. NETosis, a type of programmed cell death in neutrophils, is now more commonly identified as a mediator of pathophysiological disorders subsequent to SARS-CoV-2 infection, which thus confers severe organ damage.[54,55] Whether increased neutrophil numbers are associated with PANoptosis and NETosis is still not fully clarified. To the best of our knowledge, the bone marrow has substantial reserves of neutrophils. In response to infection or stress, cytokines are released, facilitating the increased mobilization of immature neutrophils from the bone marrow.[56] And, severe cases of COVID-19 have been linked to elevated levels of immature neutrophils that are $CD33^{+}CD10^{-}$ or $CD10^{low}CD101-$[57,58]. Thus, we postulated that enhanced neutrophils production by bone marrow hematopoietic progenitors possibly associated with the aid of neutrophil death induced by CRP, PCT, or IFN-α. Besides, severe patients had signs of thrombocytopenia and coagulopathy in our research, in line with other reports.[49,59–62] The massive release of inflammatory mediators activates the coagulation system, which leads to coagulation disturbance and thrombocytopenia.[63] PANoptosis could amplify cytokine release, culminating in a life-threatening cytokine storm,[26–29] which may result in thrombocytopenia and coagulopathy.

Taken together, a comprehensive understanding of the dynamic interactions between COVID-19, markers, and PANoptosis is crucial for the development of targeted antiviral therapies to combat COVID-19 and mitigate potential complications.

## Study Limitations

Our study has some limitations which must be considered. Firstly, this research is a prospective study conducted at a single center with a limited number of participants, which limits the generalizability of our findings to other medical institutions in other environments; further sample size expansion and multi-center validation are needed. Secondly, as this analysis relied on baseline biomarker measurements to predict outcomes, it does not capture the potential impact of evolving treatments administered during hospitalization on the disease course. Furthermore, while this study included healthy controls to establish assay baselines, a key limitation is the lack of a disease control group (eg, patients with influenza pneumonia). Future studies directly comparing biomarker profiles between COVID-19 and other etiologies of severe respiratory infection are warranted to determine the specificity of the identified biomarker panel for COVID-19 progression. Lastly, although we adjusted for major comorbidities, we did not collect detailed data on socioeconomic status or lifestyle factors, which may act as residual confounders, future studies incorporating these social determinants of health and serial biomarker measurements will provide a more comprehensive understanding of the predictors of COVID-19 severity.

## Conclusion

The value of IL-6, IL-8, IL-10, IFN-α, PCT, and CRP in predicting the severity of COVID-19 was further verified in our study, and superior to novel biomarkers. The identified biomarker panel could help clinicians early identify patients at high risk of progression to severe COVID-19. Furthermore, we confirmed for the first time that CRP, PCT, and/or IFN-α triggers PANoptosis in lymphocytes, neutrophils, and platelets, which may prompt severe progression in COVID-19 patients. It is important to highlight that immune dysregulation in COVID-19 patients exhibits heterogeneity and is dynamic, requiring personalized treatment strategies.

## Data Sharing Statement

Data sets used and/or analyzed during the current study period are available from the corresponding author, Ying Wu, upon reasonable request.

## Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki. The cohort protocol was approved by the Medical Ethics Committee of Liuzhou People's Hospital under the reference number KY2023-002-01. Our research was registered on Chinese medical research registration information system (www.medicalresearch.org.cn) under the number MR-45-23-010004. All enrolled patients and healthy donors provided written informed consent.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

All authors declare no competing interests in this work.

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