Dysregulated innate and adaptive immune responses discriminate disease severity in COVID-19

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Brief summary: Increasing COVID-19 disease severity is characterized by higher circulating levels of inflammatory markers, differential expression of HGF (higher) and SCF (lower), a hyperinflammatory innate immune system and a severely dysfunctional adaptive immune system.

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Abstract

The clinical spectrum of COVID-19 varies and the differences in host response characterizing this variation have not been fully elucidated. COVID-19 disease severity correlates with an excessive pro-inflammatory immune response and profound lymphopenia. Inflammatory responses according to disease severity were explored by plasma cytokine measurements and proteomics analysis in 147 COVID-19 patients. Furthermore, peripheral blood mononuclear cell cytokine production assays and whole blood flow cytometry were performed. Results confirm a hyperinflammatory innate immune state, while highlighting hepatocyte growth factor and stem cell factor as potential biomarkers for disease severity. Clustering analysis reveals no specific inflammatory endotypes in COVID-19 patients. Functional assays reveal abrogated adaptive cytokine production (interferon-gamma, interleukin-17 and interleukin-22) and prominent T cell exhaustion in critically ill patients, whereas innate immune responses were intact or hyperresponsive. Collectively, this extensive analysis provides a comprehensive insight into the pathobiology of severe to critical COVID-19 and highlight potential biomarkers of disease severity.

Key words

COVID-19; Disease severity; Biomarkers; Cytokines; Proteomics; Innate immunity; Adaptive immunity; Flow cytometry; Exhaustion markers

Background

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has spread rapidly across the world and was officially declared a pandemic in March 2020 [1]. The clinical spectrum of coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2, varies from asymptomatic disease or mild respiratory symptoms, to severe pneumonia, respiratory failure and death [2]. So far, more than eighty million people have been infected, leading to more than 1,800,000 deaths worldwide [3]. Although much has been learned about the pathogenesis of COVID-19 in a very short time, the complex dysregulation of the immune system involved in progression of this disease still remains incompletely understood.

The most severe complication of COVID-19 is respiratory failure due to acute respiratory distress syndrome (ARDS), requiring ventilatory support in the intensive care unit (ICU). Inflammation plays a central role in the pathogenesis of ARDS [4]. Evidence suggests that an exuberant innate immune response induced by SARS-CoV-2 characterizes more severe disease, as illustrated by higher concentrations of circulating pro-inflammatory cytokines in critically ill (ICU) COVID-19 patients as compared to non-ICU patients [2, 5-7]. Furthermore, severely ill patients display a compromised adaptive immune system, characterized by T cell exhaustion and profound lymphopenia [5, 8-10]. These findings point towards dysregulation of both innate and adaptive immunity and the degree of perturbance might be associated with disease severity, potentially leading to the development of clinically useful biomarkers. Therefore, in this study, we integrated plasma cytokine measurements and proteomics to explore the inflammatory response in hospitalized patients with severe (non-ICU) and critical (ICU) COVID-19. Furthermore, ex vivo functional evaluation of innate and adaptive immune responses in COVID-19 patients and healthy controls was performed to provide a comprehensive understanding of the host response in COVID-19.

Methods

Study design and patients

All patients aged ≥ 18 years with polymerase chain reaction (PCR)-confirmed or clinically presumed COVID-19 admitted to medical wards and ICU in the Radboudumc between 05-03-2020 and 21-04-2020 were eligible for enrolment. Presumed infection was defined based on clinical signs and symptoms, specific CT findings and clinical expert consensus [11]. Disease severity was defined according to the patient's need for intensive care at the time of plasma sampling (critical in ICU versus severe in non-ICU patients). Since 37/38 (97.4%) of the ICU patients received invasive mechanical ventilation (one patient received ventilatory support by Optiflow), ward of hospitalization was considered a good and pragmatic representation of disease severity in our study performed during the high-intensity healthcare situation of the first wave of the pandemic in the Netherlands. For ex vivo peripheral blood mononuclear cell (PBMC) stimulation experiments and flow cytometry, sex-matched healthy controls were recruited for comparison. Demographic characteristics of healthy controls are provided in Supplementary Table 1. A graphical overview of the study design is provided in Supplementary Fig 1.

Ethical approval

All applicable study protocols were approved by the local ethics board before initiation of the study. All patients admitted to hospital (or their representatives) either provided verbal informed consent for (non-ICU wards) or did not object to (ICU) participation before enrollment. This study was performed in accordance with the latest version of the

declaration of Helsinki, the International Conference on Harmonisation Good Clinical Practice (ICH GCP) guidelines and local regulations.

Sample processing and data collection

Plasma was obtained from ethylenediaminetetraacetic acid (EDTA) blood by centrifugation and stored at either -20 °C for later enzyme-linked immunosorbent assay (ELISA) or at -80 °C for later proteomics analysis.

Clinical data were obtained from patients' medical files, and processed in encoded form in electronic case report forms (Castor electronic data capture, Amsterdam, the Netherlands).

Cytokine and chemokine ELISAs

IL-6 and TNF-α plasma concentration were measured using commercially available ELISA (Quantikine ELISA kits, R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Cytokine concentrations in supernatants of ex vivo PBMC experiments were assessed by commercially available ELISA (DuoSet ELISA kits, R&D Systems, Inc., Minneapolis, MN, USA, for TNF-α, IL-1β, IL-6, IL-1Ra, IL-17 and IL-22 and Sanquin Reagents, Amsterdam, The Netherlands, for IFN-γ). For all cytokines, measured values below the lower limit of detection (LLoD; provided in the Supplementary Methods) are represented by this lowest detection value.

Proteomics analysis

Circulating plasma protein expression was assessed using the commercially available multiplex proximity extension assay from Olink Proteomics AB (Uppsala, Sweden) [12]. Proteins from three different panels were measured (Inflammation, Cardiometabolic and Cardiovascular II), resulting in data on 269 different proteins in total.

To improve the chance of true positive discoveries, we validated the findings by assessing immune biomarkers in two cohorts: a discovery cohort for identification of proteins differentially expressed in ICU patients as compared to non-ICU patients, and a validation cohort to validate the findings from the discovery cohort.

Measurements were performed on two batches on separate occasions. The first batch included plasma samples donated between 18 and 25-03-2020, the second batch included plasma samples donated between 23-03-2020 and 23-04-2020. Because the second batch encompassed samples from a larger number of patients, patients whose samples were measured at this time were retrospectively assigned to the discovery cohort, whereas those whose samples were measured in the (smaller) first batch were retrospectively assigned to the validation cohort.

Proteins are expressed on a log2-scale as normalised protein expression (NPX) values, and were normalised using bridging samples to correct for batch variation. A more detailed description of the proteomics analysis is provided in the Supplementary Methods.

PBMC isolation and ex vivo stimulation

A detailed description of PBMC isolation and ex vivo stimulation experiments is provided in the Supplementary Methods. In short, PBMCs were isolated from EDTA blood by Ficoll-Paque PLUS differential density gradient centrifugation using SepMate (Stemcell Technologies Inc., Vancouver, BC, Canada) isolation tubes. Cells were washed with PBS, resuspended in supplemented RPMI 1640 Dutch modified culture medium (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA), and counted using a Sysmex XN-450 automated differential hematology analyser (Sysmex Corporation, Kobe, Japan). Isolated PBMCs were added to 96-well round-bottom plate wells (Greiner Bio-One International GmbH, Kremsmünster, Austria) and incubated with RPMI, LPS (serotype 055:B5, Sigma-Aldrich, St. Louis, MO, USA) or heat-killed *Candida albicans* yeast cells (strain UC820) for 24 hours at 37 °C and 5% CO_2 to assess TNF-α, IL-1β, IL-6 and IL-1Ra production. For 7 days' stimulation experiments (to assess IFN-γ, IL-17 and IL-22 production), wells were supplemented with 10% pooled human serum. After incubation, supernatants were collected and stored at -20 °C before ELISA measurements were performed.

Flow cytometry

Whole blood cell counts were obtained using a Coulter Ac-T Diff cell counter (Beckman Coulter, Inc., Brea, CA, USA) which was calibrated daily. 1 ~ 1.5 mL of whole blood was incubated in lysis buffer to lyse erythrocytes. Remaining leukocytes were washed twice with PBS and resuspended in PBS + 0.2% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) to achieve a final concentration of 5 x 10^6/mL. 200 µL of cell suspension was

transferred for cell surface staining for CD45, CD8, CD4 and CD279. More detailed information on the fluorochrome conjugate monoclonal antibodies used are provided in the Supplementary Methods. All reagents were titrated and tested before they were used in the current study. Stained cells were measured on a 10-color Navios flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) equipped with three solid-state lasers (488 nm, 638 nm, and 405 nm). HLA-DR expression on monocytes was detected using Anti-HLA-DR/Anti-Monocyte Quantibrite assay (BD Biosciences, San Jose, CA, USA). This assay is described in more detail in the Supplementary Methods. Flow cytometry data were analysed using Kaluza Analysis Software version 2.1 (Beckman Coulter, Inc., Brea, CA, USA).

Statistical analysis

For continuous variables, groups were compared using Mann-Whitney *U* test or Kruskal-Wallis test with Dunn's multiple comparison test, assuming non-Gaussian distribution of variables. Nominal variables were compared using the Chi-square or Fisher's exact test, as appropriate. A *P*-value < 0.05 was considered statistically significant. Receiver-operating characteristic (ROC) analyses for the performance of biomarkers in distinguishing disease severity were performed by designating values from non-ICU patients as control values and those from ICU patients as patient values. Differential expression (DE) analysis of Olink proteins between ICU and non-ICU groups was performed using the R package limma, applying a linear model with age and sex as covariates. Limma uses an empirical Bayes method to moderate the standard errors of the estimated log fold changes [13]. The Benjamini-Hochberg procedure was applied to correct for multiple testing, and an FDR < 0.05 was considered statistically significant. Statistical analyses were performed using

GraphPad Prism 5 or 8 for Windows (GraphPad Software, Inc., San Diego, CA, USA) or R/Bioconductor (https://www.R-project.org/).

Results

Patient cohort and characteristics

147 Hospitalized COVID-19 patients donated plasma, 38 of whom were admitted to ICU and 109 to non-ICU clinical wards. Patient characteristics at hospital admission are provided in Table 1. Demographic characteristics did not differ between the ICU and non-ICU groups at the time of hospitalization. However, in ICU patients, time to plasma sampling was significantly longer, co-morbid pulmonary and auto-immune diseases were significantly less frequent and C-reactive protein (CRP) levels and computed tomography (CT) severity scores at admission were significantly higher.

Inflammatory markers in plasma

At the time of plasma sampling, ICU patients displayed a significantly higher concentration of CRP, D-dimer and ferritin versus non-ICU patients (CRP: ICU: median 248 mg/L versus non-ICU: 82 mg/L; D-dimer: ICU: median 2665 ng/mL versus non-ICU: median 1250 ng/mL; ferritin: ICU: median 1608 µg/L versus non-ICU: median 915 µg/L; Fig 1A-B and Supplementary Fig 2) . Furthermore, plasma levels of IL-6 were significantly higher in ICU versus non-ICU patients (median concentrations of 182.0 pg/mL and 40.0 pg/mL, respectively; Fig 1C). Although TNF-α plasma levels were relatively low in both groups, they were also significantly higher in ICU patients (ICU: median 18.5 pg/mL versus non-ICU: 16.0 pg/mL; Fig 1D), although differences were small.

In-depth proteomics analysis

For proteomics analysis, the discovery cohort consisted of 101 (83 non-ICU and 18 ICU) and the validation cohort of 46 patients (26 non-ICU and 20 ICU; Supplementary Fig 1). Patient characteristics between the two cohorts were generally well matched (Supplementary Table 2). However, the discovery cohort contained relatively fewer ICU patients than the validation cohort (17.8% versus 43.5%, respectively, $P = 0.002$).

After quality control, normalisation of all assessed proteins for all samples and correction for age and sex as covariates, 40 proteins were found to be significantly higher in ICU patients versus non-ICU patients, whereas 24 were significantly lower (false discovery rate [FDR] < 0.05; Fig 2A). In the validation cohort, this was the case for 19 and 30 proteins, respectively. Overlap analysis and correcting *P*-values for multiple testing revealed 27 proteins overlapping between the two cohorts that were significantly differentially expressed in ICU versus non-ICU patients (Fig 2B). Among these, hepatocyte growth factor (HGF; log2 fold change [logFC] 1.39, adjusted *P*-value = 1.19×10^{-6}), chemokine (C-C motif) ligand 20 (CCL20; logFC 1.41, adjusted *P*-value = 5.48×10^{-5}) and IL-6 (logFC 1.44, adjusted *P*-value = 1.61×10^{-3}) were upregulated most strongly and most significantly in ICU patients as compared to non-ICU patients. In contrast, stem cell factor (SCF; logFC -1.43, adjusted *P*-value = 3.14×10^{-7}), delta and notch-like epidermal growth factor-related receptor (DNER; logFC -0.49, adjusted *P*-value 3.38×10^{-6}), vascular endothelial growth factor D (VEGFD; logFC -0.80; adjusted *P*-value 4.0610×10^{-6}) and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL; logFC -0.63, adjusted *P*-value 1.61×10^{-4}) were most significantly downregulated in critically ill versus less severely ill COVID-19 patients.

Receiver-operating characteristic analyses

ROC analyses for discriminating ICU from non-ICU patients were performed on DE of HGF and SCF and compared to CRP, D-dimer, ferritin, and plasma concentrations of IL-6, as determined by ELISA. Comparison of the area under the curve (AUC) for CRP (0.8724), D-dimer (0.8206), ferritin (0.6684), IL-6 (0.8797) and DE of HGF (0.8696) and SCF (0.8385) revealed that the latter three demonstrated strong potential in discriminating disease severity, with AUC values similar to those for CRP and D-dimer and higher than that of ferritin (Supplementary Fig 3).

Clustering analysis of differential protein expression

To identify any potential inflammatory endotypes in COVID-19, unsupervised hierarchical clustering analysis was performed on those proteins that were significantly differentially expressed between ICU and non-ICU patients in the discovery cohort. This revealed no significantly different protein signatures between patients: no specific inflammatory endotypes were identified (Fig 3).

Cytokine production capacity and flow cytometry

Immune cells' cytokine production and markers of activation and exhaustion were assessed in patients with COVID-19 and compared to healthy controls (HCs). In general, immune cells of COVID-19 patients did not demonstrate significant differences in the production capacity of TNF-α, IL-1β, IL-6 and IL-1 receptor antagonist (IL-1Ra) as compared to healthy controls upon stimulation with lipopolysaccharide (LPS) or *C. albicans* (Fig 4A-D). However, ICU patients demonstrated a significantly lower TNF-α response upon stimulation with *C. albicans* as compared to HCs (HC: median 2198.0 pg/mL, IQR 1427.0-3002.0 pg/mL, versus ICU: median 430.9 pg/mL, IQR 174.3-1140.0 pg/mL, versus non-ICU: median 441.2 pg/mL,

IQR 319.9-1955 pg/mL; HC versus ICU $P = 0.0231$; Fig 4A). No significant differences were observed between ICU and non-ICU patients.

In contrast, after 7 days of incubation with *C. albicans*, patients' PBMCs displayed a severely defective production of the T helper lymphocyte-derived cytokines interferon gamma (IFN-γ; median concentrations in HC, non-ICU and ICU were 133.6 pg/mL, 37.2 pg/mL, and 19.5 pg/mL, respectively), IL-17 (median concentrations in HC, non-ICU and ICU: 693.4 pg/mL, 532.1 pg/mL, and 80.2 pg/mL, respectively) and IL-22 (median concentrations in HC, non-ICU and ICU 2604.0 pg/mL, 627.9 pg/mL, and 156.0 pg/mL, respectively; Fig 5A-C), with trends towards lower cytokine production in the more severely ill (ICU) patients. Of note, the percentage of lymphocytes within the PBMC fraction differed significantly among groups (median of 79.5% in HC, 51.2% in non-ICU, and 35.6% in ICU) and the percentage of neutrophils in this fraction was significantly higher in the ICU group versus HC group (median of 27.2% and 0.8%, respectively; Fig 5D). Furthermore, the percentage of monocytes was significantly different between HC and non-ICU patients, but not between HC and ICU patients (median of 18.4% in HC, 39.8% in non-ICU and 30.7% in ICU).

Flow cytometry of whole blood samples from a subset of patients and HCs demonstrated significantly lower human leukocyte antigen (HLA)-DR expression on $CD14^+$ monocytes in ICU patients as compared to non-ICU patients and HCs (median mean fluorescent intensity [MFI] area values of 15794, 30825 and 33039, respectively; Fig 6A). Although most patients demonstrated values in the lower normal range or just below, none displayed values comparable to those seen in patients suffering from bacterial sepsis [14]. Cluster of differentiation (CD) 279 (also known as programmed cell death protein 1, PD-1) expression on $CD4^+$ T cells, ranging from naive to effector memory cells re-expressing CD45RA

(TEMRA), however, was significantly upregulated in both non-ICU and ICU patients, as compared to HCs (Fig 6B-C), indicative of lymphocyte exhaustion (percentage of TEMRA $CD4^+$ cells positive for CD279: HC: median 1.9%, non-ICU: median 14.7%; ICU: median 19.1%).

Discussion

Our study provides several important findings. Our comprehensive approach demonstrates that critically ill COVID-19 patients are characterized by higher plasma concentrations of CRP, D-dimer, ferritin, IL-6 and TNF-α compared to less severely ill patients. In parallel, in-depth analysis of differential protein expression highlights several potential biomarkers of disease severity. Of these, HGF and SCF can differentiate between critical and severe illness with approximately equal discriminatory performance as CRP, D-dimer and circulating IL-6, and better performance than ferritin. Next, clustering analysis of differential protein expression demonstrates that patients do not form clusters based on specific inflammatory endotypes. Furthermore, patients' innate immune cells show equal or even higher pro-inflammatory cytokine production after ex vivo stimulation, whereas adaptive cytokine production is significantly decreased in a seemingly severity dependent manner. Moreover, patients' $CD4^+$ T cells display increased expression of PD-1, a marker of apoptosis and T cell exhaustion. On the other hand, HLA-DR expression on monocytes is significantly lower than in healthy controls. Collectively, all these findings point towards a general concept of a homogeneous inflammatory state in patients with COVID-19, combined with compromised T cell immune responses.

The observed relationship between the degree of elevation of pro-inflammatory markers and disease severity is in line with previous studies and has been recognized early in the COVID-19 pandemic [2, 5-7]. One may argue that differences in severity might be attributed to longer

disease duration, as time from hospital admission to blood sampling differed between these two groups (4 days in ICU versus 2 days in non-ICU patients). However, while this may have been a potential confounder theoretically, our population shows remarkable consistency in disease severity over time: only 3/109 non-ICU patients made a transition to the ICU during study follow-up, indicating a deterioration in disease severity. In-depth proteomics analysis revealed a multitude of potential markers of disease severity confirming that IL-6 is strongly upregulated in critically ill patients. Among these, prominently upregulated proteins include HGF and CCL20. Other studies have also shown increased HGF plasma concentrations in COVID-19 related to disease severity [15, 16] and have demonstrated upregulated expression of HGF induced by pro-inflammatory cytokines [17] and in viral infections such as hepatitis B [18]. CCL20 is a chemokine with a strong chemotactic effect on lymphocytes [19] and is also upregulated by pro-inflammatory cytokines. Therefore, its upregulation in critical COVID-19 might serve as a compensatory mechanism for lymphopenia, since multiple studies demonstrate a correlation between disease severity and degree of lymphopenia [2, 8]. In contrast, CCL20 upregulation might cause lymphocyte abundance in the tissues [20], leading to lymphocyte depletion in the peripheral blood.

In our proteomics analysis, SCF was most strongly downregulated. This ligand of the c-Kit receptor is a crucial factor in maintaining hematopoietic stem cells and lymphoid progenitor cells [21, 22]. Furthermore, a positive correlation between SCF and specific COVID-19 neutralizing antibody titers has recently been demonstrated [23]. One could speculate that reduced SCF expression might contribute to the observed lymphopenia in COVID-19 and lower antibody titers.

Additional ex vivo functional analysis confirmed an enhanced innate cytokine response in COVID-19 patients (with the exception of the TNF-α response to *C. albicans*), and monocytic HLA-DR expression was only mildly decreased as opposed to the severe immune suppression seen in bacterial sepsis [14]. However, adaptive cytokine production was severely abrogated with apparent correlations with disease severity. This is in line with a recent study demonstrating impaired IFN-γ production in lymphocytes after stimulation with anti-CD3/anti-CD28 [24]. Adaptive immune system dysfunction was further supported by findings of increased expression of PD-1 on $CD4^+$ T lymphocytes, consistent with previous data [10, 25]. In conclusion, our findings point towards a disease state characterized by a hyperinflammatory innate immune system and a defective adaptive immune system due to profound lymphopenia, exhausted T cells and decreased functionality. These findings are supported by a very recent study on the systems biology of severe versus mild COVID-19 patients, which has also shown a combination of increased systemic inflammation, low HLA-DR expression on monocytes and a defective interferon pathway [26].

Our study has several limitations. First, due to the explorative nature of our study, direct conclusions on causality between immunological profiles and disease severity cannot be inferred. However, we identified several potential biomarkers of severity, warranting further investigation, especially regarding their possible pathophysiological role in disease course and severity. Second, plasma sampling was not performed on predefined timepoints as a consequence of this study's pragmatic design. Differences in hospitalisation duration at the time of sampling might have influenced our results. Additionally, no correction for other potential confounders, such as comorbidity and medication use, was performed due to this approach. Third, we classified disease severity according to admission to ICU versus non-ICU wards, which might differ from classifications employed by other studies. Fourth, in the

proteomics analysis, patient assignment to the discovery or validation cohort was not completely random, but instead ultimately based on the date of plasma donation. Although introduction of bias due to this approach cannot be excluded, the fact that no significant changes in diagnostic work-up or therapeutic management of COVID-19 occurred between the sample collection periods of either cohort appears to minimise this risk. Last, in our ex vivo stimulation experiments, cellular composition of the PBMC fraction differed between the groups. Contamination with low-density granulocytes after Ficoll density centrifugation has been described before in sepsis [27]. To what degree cytokine production has been influenced by these differences is unclear. As the experiments were performed with PBMCs isolated from EDTA blood, and depletion of intracellular calcium has been suggested to impact cytokine production capacity, an impact on the overall strength of cytokine production capacity cannot be fully excluded, although potent cytokine release has been observed in this study.

In conclusion, our integrated and extensive approach demonstrates essential differences in innate and adaptive immune responses between severely and critically ill COVID-19 patients, presenting potential biomarkers of disease severity and elucidating its pathobiology. It further highlights a severely dysfunctional adaptive immune response, in the presence of a hyperinflammatory innate immune system. Further investigations of the crosstalk between innate and adaptive immunity in COVID-19 and their relationship with disease severity are highly warranted.

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Conflicts of interest

L.P.G.D. reports grants from ZonMw and EU H2020 during the conduct of the study, membership of the SCCM/ESICM/SSC COVID-19 guideline committee, membership of the ESICM COVID-19 taskforce and is the chair of the Dutch intensivists (NVIC) taskforce infectious threats. E.J.G-B reports grants from Swedish Orphan BioVitrum, XBiotech, UCB, Novartis, ImmunoSep Horizon 2020 and RISKinCOVID, grants and personal fees from BioMerieux, ThermoFisher BRAHMS, InflaRx GmbH and Abbott Ch and personal fees from Angelini Italy, outside the submitted work.

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Meetings where the information has previously been presented

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Figure legends

Fig 1. Critically ill COVID-19 patients demonstrate higher levels of inflammatory markers than severely ill patients.

(A) CRP concentrations are significantly higher in COVID-19 patients admitted to the ICU (median 248 mg/L, interquartile range [IQR] 149-306 mg/L) as compared to those admitted to the ward (median 82 mg/L, IQR 47-123 mg/L, $P < 0.0001$) at the time of plasma sampling (non-ICU: n = 108; ICU: n = 37). This pattern is also observed for (B) D-dimer (ICU: median 2665 ng/mL, IQR 1780-5978 ng/mL versus non-ICU: median 1250 ng/mL, IQR 785-1810 ng/mL, $P < 0.0001$; non-ICU: n = 93; ICU: n = 36) concentrations, all of which were determined in the course of routine clinical care. (C) Measurements of circulating IL-6 in patient plasma by ELISA reveal significantly higher concentration in COVID-19 patients admitted to the ICU than in patients admitted to the ward (median 182.0 pg/mL, IQR 90.25-408.0 pg/mL versus 40.0 pg/mL, IQR 23.0-83.3 pg/mL, respectively, $P < 0.0001$; non-ICU: n= 102; ICU: n = 33). (D) Measurements of circulating TNF-α levels in plasma demonstrate significantly higher concentrations in ICU patients as compared to non-ICU patients (ICU: median 18.5 pg/mL, IQR 16.0-26.8 pg/mL versus non-ICU: median 16.0 pg/mL, 16.0-19.3 pg/mL, $P = 0.0082$; non-ICU: n = 98; ICU: n = 30).

CRP, C-reactive protein. IL-6, interleukin-6. TNF-α, tumour necrosis factor alpha. ICU, intensive care unit. ELISA, enzyme-linked immunosorbent assay. Data are shown as median with interquartile range (IQR). Non-ICU and ICU groups were compared using Mann-Whitney *U* test. $^{**}P < 0.01$. $^{***}P < 0.0001$

Fig 2. Proximity extension assay demonstrates differential protein expression in plasma according to COVID-19 disease severity. (A) Volcano plot of differential expression of proteins in the discovery cohort (n = 101), with age and sex included as covariates. Results from all three employed protein panels are displayed (Inflammation, Cardiometabolic and Cardiovascular II panel) as log2 fold change of expression in ICU patients compared to non-ICU patients, plotted against adjusted *P*-values. Proteins significantly differentially expressed in both the discovery and validation cohort are displayed in bold. (B) After overlap analysis of differential protein expression in both the discovery and validation cohorts, 27 proteins were significantly up- or downregulated in ICU patients, as compared to non-ICU patients with adjusted *P*-values < 0.05 after correction for multiple testing. Most significantly upregulated proteins are HGF, CCL20 and IL-6; most significantly downregulated proteins are SCF, DNER, VEGFD and TRAIL.

ICU, intensive care unit. HGF, hepatocyte growth factor. CCL20, chemokine (C-C motif) ligand 20. IL-6, interleukin-6. SCF, stem cell factor. DNER, delta and notch-like epidermal growth factor-related receptor. VEGFD, vascular endothelial growth factor D. TRAIL, tumour necrosis factor-related apoptosis-inducing ligand.

Fig 3. Heatmap of significantly differentially expressed proteins demonstrate no specific inflammatory endotypes.

Unsupervised hierarchical clustering of significantly differentially expressed proteins after multiple testing in the discovery cohort does not identify specific patient endotypes.

ICU, intensive care unit.

Fig 4. Innate cytokine production in COVID-19

Production of the innate cytokines TNF-α (A), IL-1β (B), IL-6 (C) and IL-1RA (D) was not significantly different between healthy controls and COVID-19 patients admitted to non-ICU wards or ICU after stimulation with LPS or heat-killed *C. albicans* yeast cells, with the exception of TNF-α production in response to *C. albicans* stimulation (A). TNF-α: LPS: HC: median 187.5 pg/mL, IQR 76.1-245.4 pg/mL; non-ICU: median 260.1 pg/mL, IQR 175.7-753.4 pg/mL; ICU: median 199.2 pg/mL, IQR 98.0-349.8 pg/mL; *C. albicans*: HC: median 2198 pg/mL, IQR 1427-3002 pg/mL; non-ICU: 441.2 pg/mL, IQR 319.9-1955 pg/mL; ICU: median: 430.9 pg/mL, IQR 174.3-1140 pg/mL; HC versus ICU $P = 0.0231$; IL-1β: LPS: HC: median 2169 pg/mL, IQR 1809-3391 pg/mL; non-ICU: median 2456 pg/mL, IQR 1143-3246 pg/mL; ICU: median 2315 pg/mL, IQR 1144-5413 pg/mL; *C. albicans*: HC: median 3021 pg/mL, IQR 2374-4959 pg/mL; non-ICU: median 1953 pg/mL, IQR 1461-2136 pg/mL; ICU: median 1964 pg/mL, IQR 1148-3120 pg/mL; IL-6: LPS: HC: median 20001 pg/mL, IQR 5152-23811 pg/mL; non-ICU: median 16865 pg/mL, IQR 6218-29887 pg/mL; ICU: median 17260 pg/mL, IQR 8334-27553 pg/mL; *C. albicans*: HC: median 4338 pg/mL, IQR 3387-7193 pg/mL; non-ICU: median 8252 pg/mL, IQR 5232-13557 pg/mL; ICU: median 6310 pg/mL, IQR 5070-9856 pg/mL; IL-RA: LPS: HC: median 12749 pg/mL, IQR 10089-15141 pg/mL; non-ICU: median 14655 pg/mL, IQR 11697-33901 pg/mL; ICU: median 13349 pg/mL, IQR 10953-17000 pg/mL; *C. albicans*: HC: median 11023 pg/mL, IQR 7976-14168 pg/mL; non-ICU: median 11341 pg/mL, IQR 7499-19824 pg/mL; ICU: median 9862 pg/mL, IQR 6345-12465 pg/mL (HC: n = 8; non-ICU: n = 8; ICU: n = 9; for IL-RA: RPMI: non-ICU: n = 5; ICU: n = 6).

TNF-α, tumour necrosis factor alpha. IL, interleukin. HC, healthy controls. ICU, intensive care unit. RPMI, RPMI 1640 Dutch modified culture medium. LPS, lipopolysaccharide. *C. albicans*,

heat-killed *Candida albicans* yeast cells. Data are shown as median with interquartile range (IQR). All groups were compared using Kruskal-Wallis test with Dunn's post test comparing all pairs of columns. *$P < 0.05$

Fig 5. Adaptive cytokine production in COVID-19

Production of the lymphocyte-derived cytokines IFN-γ (A), IL-17 (B) and IL-22 (C) in response to *C. albicans* was severely abrogated in COVID-19 patients as compared to healthy controls. Furthermore, a trend of less cytokine production with increasing disease severity was observed for IFN- γ (A) and IL-22 (C). IFN-γ: HC: median 133.6 pg/mL, IQR 36.6-254.5 pg/mL; non-ICU: median 37.2 pg/mL, IQR 19.5-112.2 pg/mL; ICU: median 19.5 pg/mL, IQR 19.5-19.5 pg/mL; HC versus ICU $P = 0.0007$; IL-17: HC: median 693.4 pg/mL, IQR 423.5-1062.0 pg/mL; non-ICU: median 532.1 pg/mL, IQR 78.0-1763.0 pg/mL; ICU: median 80.2 pg/mL, IQR 78.0-165.2 pg/mL; HC versus ICU $P = 0.0074$; IL-22: HC: median 2604.0 pg/mL, IQR 1372.0-4229.0 pg/mL; non-ICU: median 627.9 pg/mL, IQR 160.9-2490.0 pg/mL; ICU: median 156.0 pg/mL, IQR 156.0-203.8 pg/mL; HC versus ICU $P = 0.0006$ (HC: n = 8; non-ICU: n = 8; ICU: n = 10). (D) Cellular composition of the PBMC fraction after differential density gradient centrifugation differed between healthy controls, non-ICU and ICU COVID-19 patients with regard to the percentage of neutrophils, lymphocytes, monocytes and eosinophils. Percentage of neutrophils within the PBMC fraction: HC: median 0.8%, IQR 0.6-1.8%; non-ICU: median 6.1%, IQR 4.6-17.1%; ICU: median 27.2%, IQR 15.2-36.9%; HC versus ICU $P < 0.0001$. Percentage of lymphocytes within the PBMC fraction: HC: median 79.5%, IQR 71.8-80.8%; non-ICU: median 51.2%, IQR 32.9-70.5%; ICU: median 35.6%, IQR 22.5-44.0%; HC versus non-ICU $P = 0.0355$ and HC versus ICU $P = 0.0002$; Percentage of monocytes within the PBMC fraction: HC: median 18.4%, IQR 17.8-22.1%; non-ICU: median 39.8%, IQR 22.9-55.4%; ICU: median 30.7%,

IQR 24.2-40.4%; HC versus non-ICU $P = 0.0109$). Percentage of eosinophils within the PBMC fraction: HC: median 0.0%, IQR 0.0-0.0%; non-ICU: median 0.0%, IQR 0.0-0.0%; ICU: median 0.1%, IQR 0.0-0.7%; non-ICU versus ICU $P = 0.0353$. Percentage of basophils within the PBMC fraction: HC: median 0.8%, IQR 0.6-1.0%; non-ICU: median 0.5%, IQR 0.3-1.0%; ICU: median: 0.7%, IQR 0.5-1.0%.

IFN-γ, interferon gamma. IL, interleukin. PBMC, peripheral blood mononuclear cell. HC, healthy controls. ICU, intensive care unit. RPMI, RPMI 1640 Dutch modified culture medium. *C. albicans*, heat-killed *Candida albicans* yeast cells. Neutro, neutrophilic granulocytes. Lympho, lymphocytes. Mono, monocytes. Eo, eosinophilic granulocytes. Baso, basophilic granulocytes. Data are shown as median with interquartile range (IQR). All groups were compared using Kruskal-Wallis test with Dunn's post test comparing all pairs of columns. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$

Fig 6. Monocyte HLA-DR expression and $CD4^+$ lymphocyte CD279 expression in COVID-19

(A) $CD14^+$ Monocytes obtained from ICU COVID-19 patients displayed significantly lower expression of HLA-DR by flow cytometry than healthy controls and non-ICU COVID-19 patients (mean fluorescent intensity [MFI] area in HC: median 33039, IQR 31287-40813; non-ICU: median 30825, IQR 23903-42977; ICU: median 15794, IQR 14740-19928; HC versus ICU $P = 0.0085$ and non-ICU versus ICU $P = 0.0191$ (HC: n = 5; non-ICU: n = 11; ICU: n = 7). (B-C) CD279 (PD-1) is expressed on a significantly higher percentage of $CD4^+$ lymphocytes from COVID-19 patients admitted to non-ICU wards or ICU than in healthy controls, as shown for naive (B) and terminally differentiated (C) $CD4^+$ lymphocytes (percentage of naive $CD4^+$ cells positive for CD279: HC: median 0.2%, IQR 0.1-0.3%; non-ICU: median 2.5%, IQR 0.9-5.0%; ICU: median: 0.8%, IQR 0.7-2.2%; HC versus non-ICU $P = 0.0023$ and HC versus ICU $P =$

0.0348; percentage of TEMRA $CD4^+$ cells positive for CD279: HC: median 1.9%, IQR 1.0-4.1%; non-ICU: median 14.7%, IQR 8.3-18.8%; ICU: median 19.1%, IQR 8.6-29.4%; HC versus non-ICU $P = 0.0059$ and HC versus ICU $P = 0.0021$; HC: n = 10; non-ICU: n = 11; ICU: n = 7).

HLA-DR, human leukocyte antigen - DR isotype. CD, cluster of differentiation. TEMRA, T effector memory cell re-expressing CD45RA. HC, healthy controls. ICU, intensive care unit. MFI, mean fluorescent intensity.

Data are shown as median with interquartile range (IQR). All groups were compared using Kruskal-Wallis test with Dunn's post test comparing all pairs of columns. *$P < 0.05$. **$P < 0.01$

Table 1. Patient characteristics at hospital admission

	Total (n = 147)	Non-ICU[a] (n = 109)	ICU[a] (n = 38)	*P*-value (Non-ICU vs. ICU)
Age (years)	66 (54 - 73)	66 (52 - 73)	67 (57 - 73)	0.945
Male sex (n, %)	99 (67)	71 (65)	28 (74)	0.333
BMI (kg/m^2)	26.9 (23.8 - 29.3)	26.5 (23.7 - 29.3)	27.6 (25.0 - 29.9)	0.342
BMI > 30 kg/m^2 (n, %)	30 (21.1)	22 (21.2)	8 (21.1)	0.9896
Time from first COVID-19 symptoms to hospital admission (days)	7 (5 - 10)	7 (5 - 10)	5 (6 - 10)	0.770
Time from hospital admission to plasma sampling (days)	3 (2 - 4)	2 (2 - 3)	4 (3 - 6)	< 0.001
PCR proven COVID-19 (n, %)	138 (94)	103 (94)	35 (92)	0.6956
Co-morbidities				
Haematological malignancy	13 (8.8)	10 (9.2)	3 (7.9)	1.000
Solid organ malignancy	31 (21.1)	23 (21.1)	8 (21.1)	0.995

SCT	6 (4.1)	5 (4.6)	1 (2.6)	1.000
SOT	7 (4.8)	7 (6.4)	0 (0)	0.191
Pulmonary disease (including COPD)	35 (23.8)	31 (28.4)	4 (10.5)	0.028
Cardiovascular disease (including hypertension)	84 (57.1)	64 (58.7)	20 (52.6)	0.514
Hypertension	57 (38.8)	41 (37.6)	16 (42.1)	0.625
Diabetes mellitus	31 (21.1)	21 (19.3)	10 (26.3)	0.359
CKD, requiring RRT	1 (0.7)	1 (0.9)	0 (0)	1.000
CKD, no RRT	9 (6.1)	9 (8.3)	0 (0)	0.112
Auto-immune disease, including IBD	23 (15.6)	22 (20.2)	1 (2.6)	0.009
HIV/AIDS	1 (0.7)	1 (0.9)	0 (0)	1.000
Liver disease	6 (4.1)	6 (5.5)	0 (0)	0.339
Other	130 (88.4)	101 (92.7)	29 (76.3)	0.007
WBC (x 10^9/L)	7.1 (5.2 - 9.3)	6.9 (4.5 - 9.2)	7.5 (6.0 - 10.3)	0.180
Neutrophils (x 10^9/L)	5.6 (3.5 - 7.7)	5.6 (3.3 - 8.0)	5.8 (4.4 - 7.4)	0.741
Lymphocytes (x 10^9/L)	0.8 (0.5 - 1.1)	0.7 (0.5 - 1.1)	0.8 (0.4 - 1.2)	0.815

Monocytes (x 10^9/L)	0.4 (0.3 - 0.7)	0.4 (0.3 - 0.8)	0.4 (0.2 - 0.5)	0.242
CRP (mg/L)	82 (44 - 151)	72 (40 - 115)	139 (87 - 225)	< 0.001
Ferritin (µg/L)	795 (377 - 1468)	785 (378 - 1385)	1025 (227 - 2157)	0.414
D-dimer (ng/mL)	870 (533 - 1733)	890 (525 - 1875)	650 (370 - 1995)	0.649
CO-RADS	5 (5 - 6)	5 (5 - 6)	5 (5 - 6)	0.144
CT severity score	12 (9 - 15)	12 (9 - 15)	17 (13 - 19)	0.002

Data are presented as median with interquartile range (IQR) or n (%).

ICU, intensive care unit. BMI, body mass index. SCT, stem cell transplantation. SOT, solid organ transplantation. COPD, chronic obstructive pulmonary disease. CKD, chronic kidney disease. RRT, renal replacement therapy. HIV, human immunodeficiency virus. AIDS, acquired immunodeficiency syndrome. WBC, white blood cell count. CO-RADS, Dutch COVID-19 reporting and data system. CRP, C-reactive protein. CT, computed tomography.

[a] Classification based on the location of the patient at the time of plasma sampling.

Figure 1

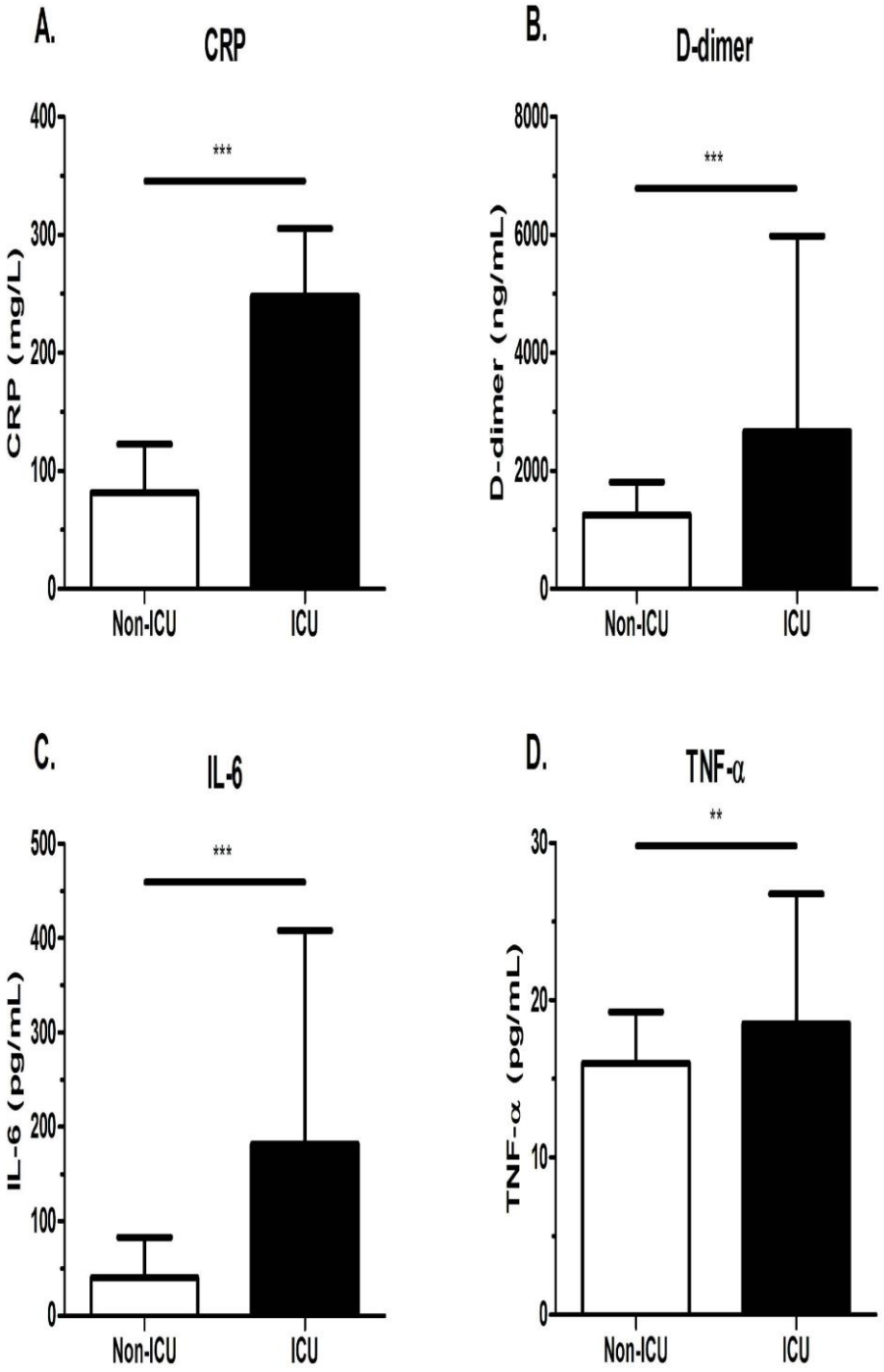
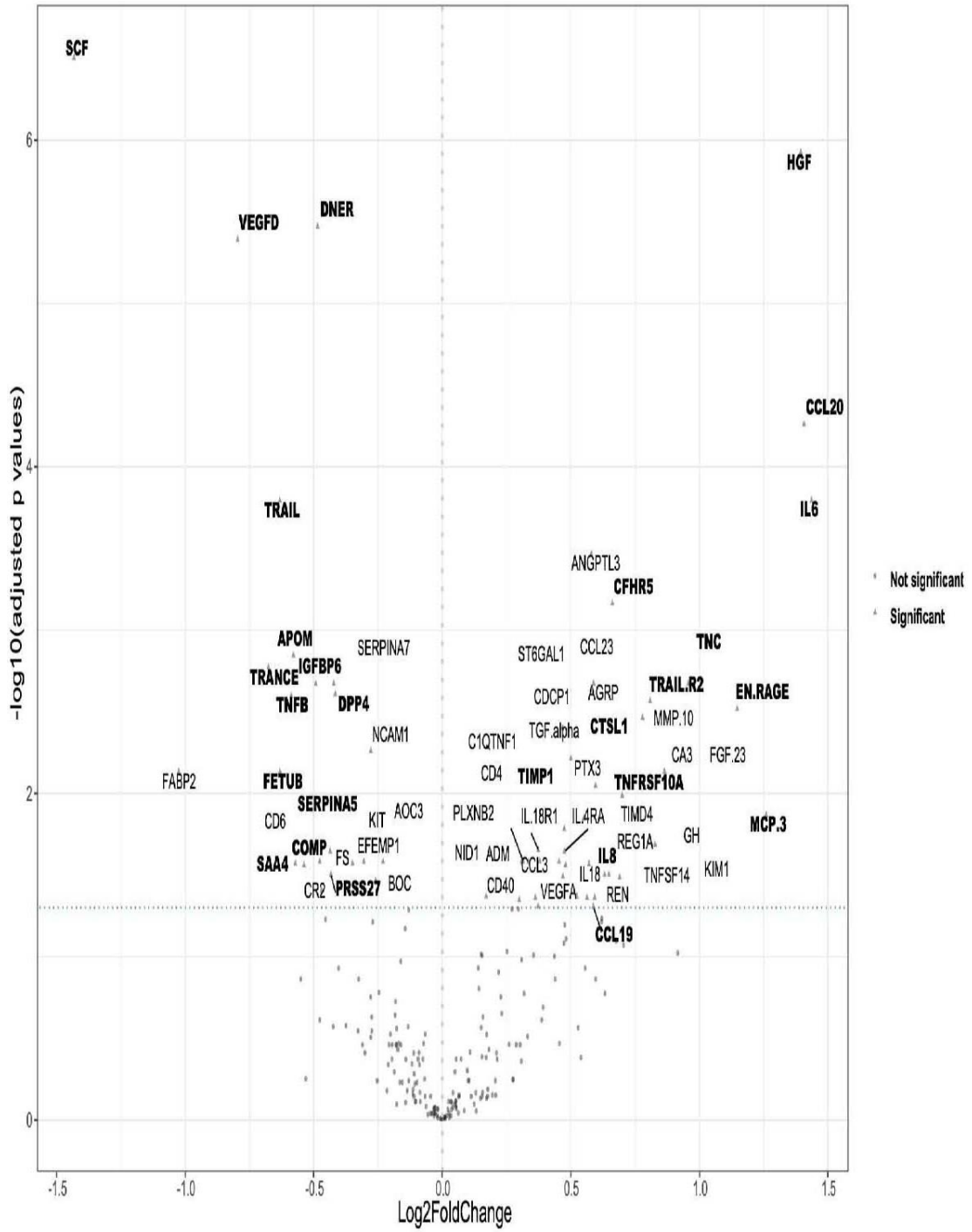


Figure 2a



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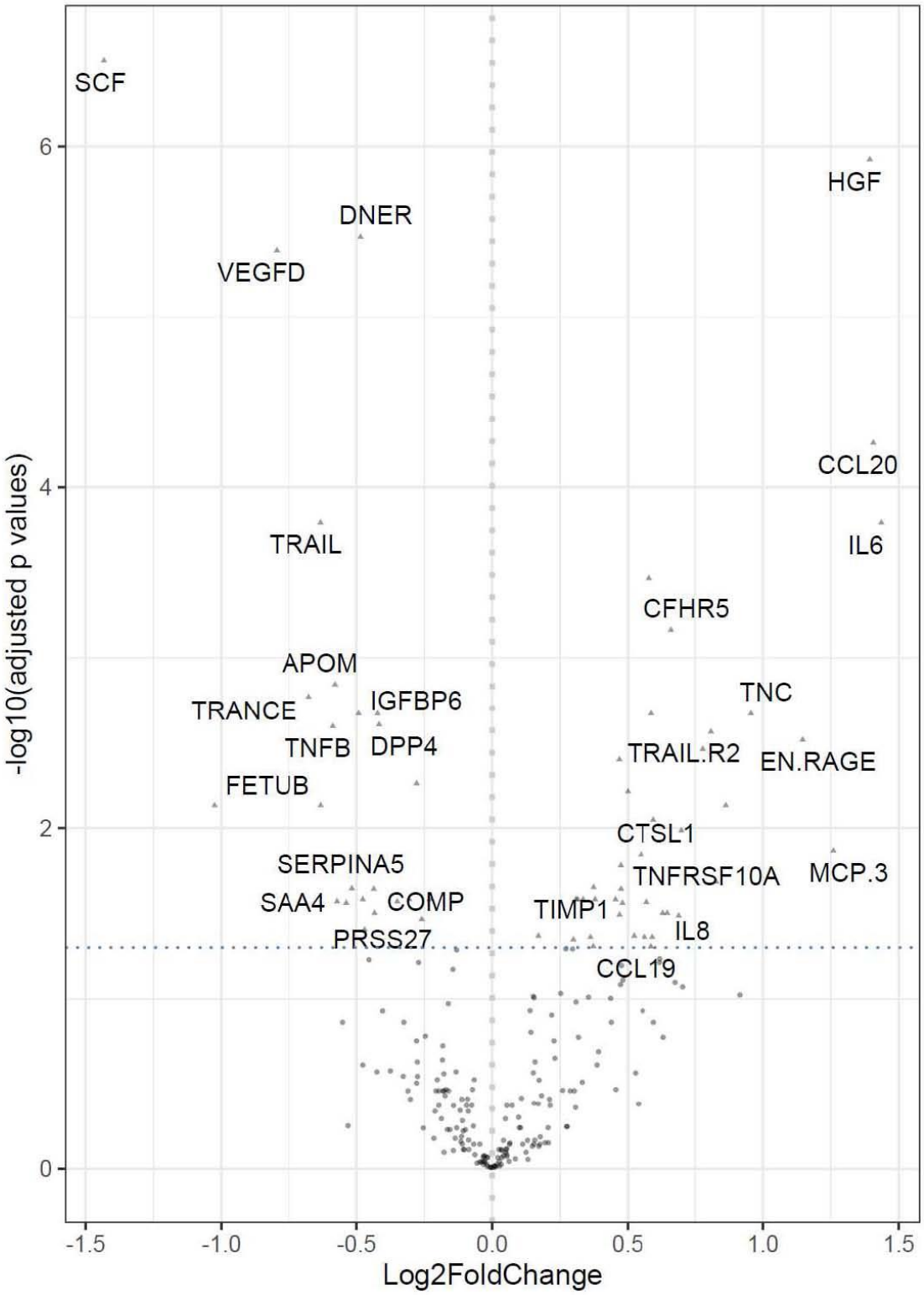

Figure 3

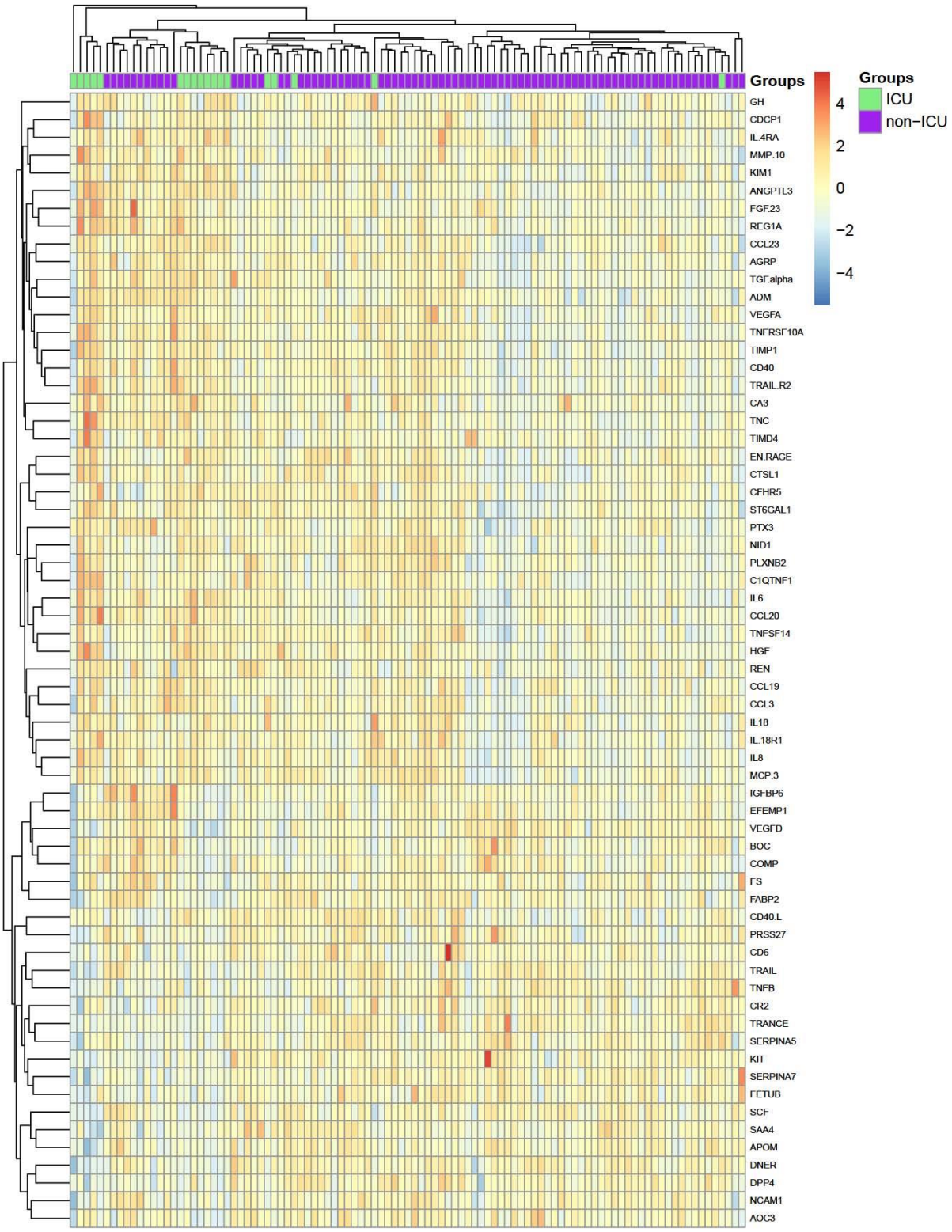

Figure 4

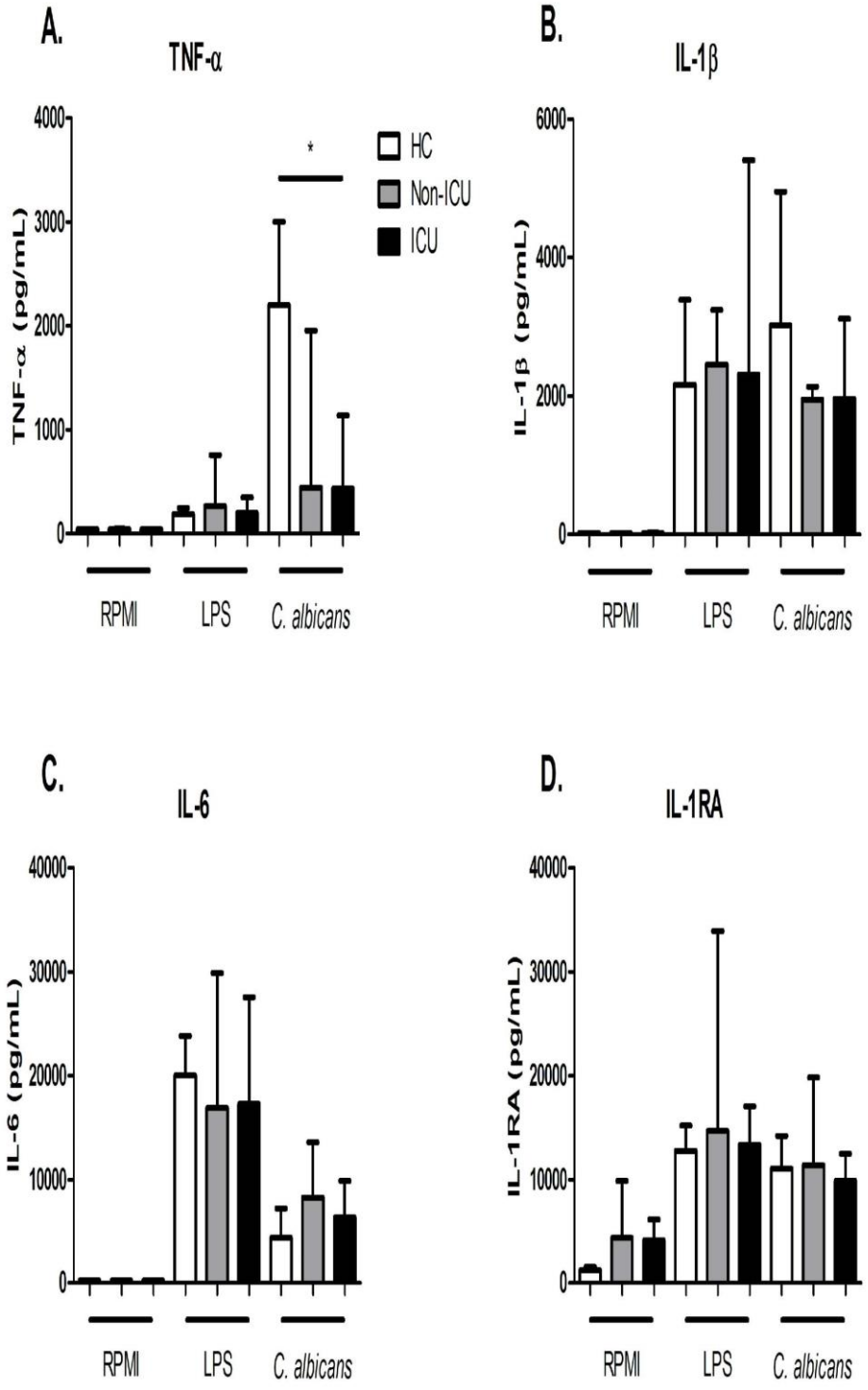

Figure 5

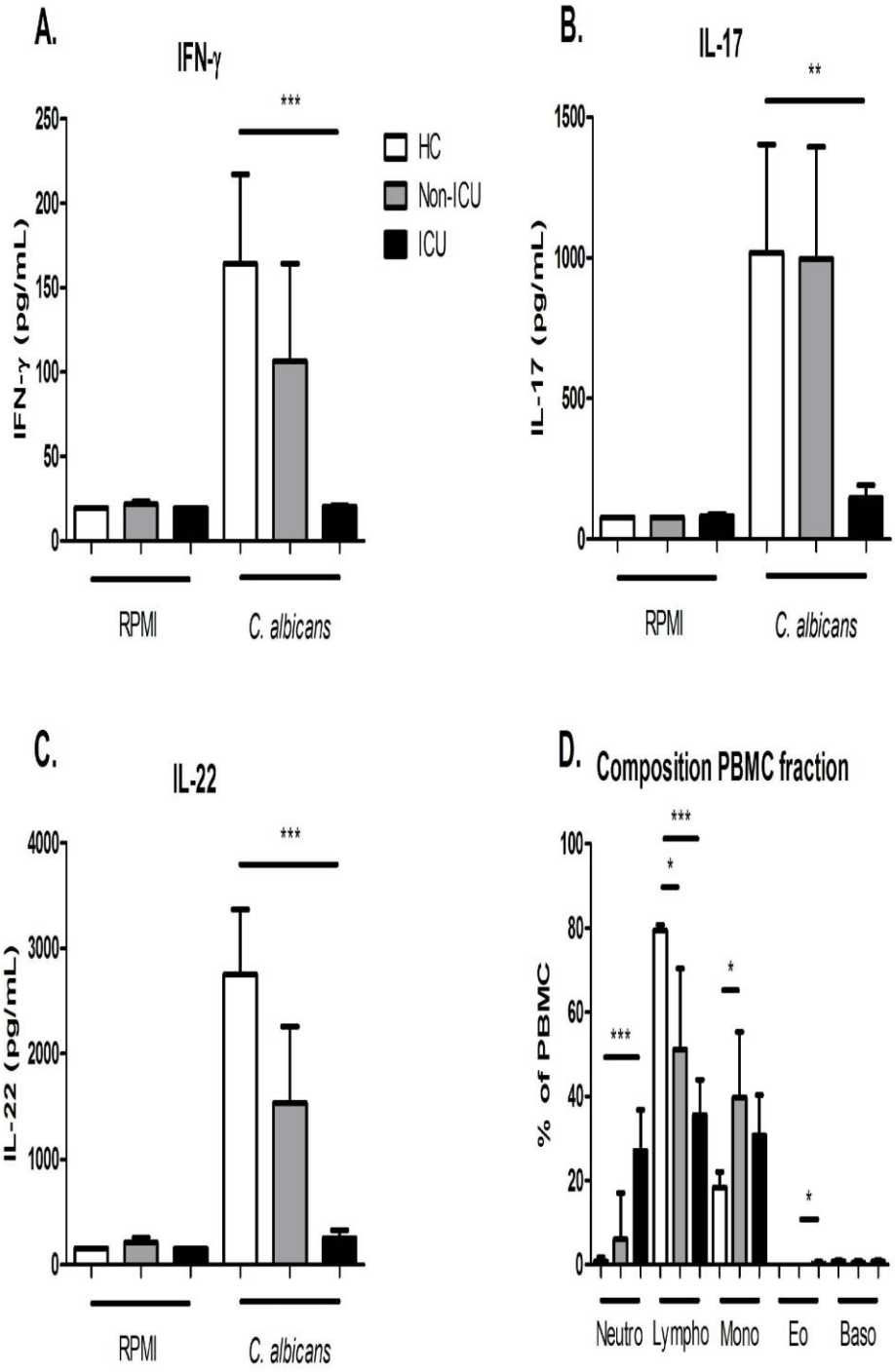

Figure 6