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Persistently Elevated Plasma Concentrations of RIPK3, MLKL, HMGB1, and RIPK1 in Patients with COVID-19 in the Intensive Care Unit

To the Editor:

Coronavirus disease (COVID-19) is one of the most challenging pandemics in recent human history. Patients suffering from critical COVID-19 often develop respiratory failure and display clinical features of sepsis, including coagulopathy, lymphopenia, and high plasma concentrations of proinflammatory cytokines (1). In comparable non–COVID-19–related inflammatory diseases, like pneumonia, sepsis, and acute respiratory distress syndrome, it has been shown that important regulators of necroptotic cell death, such as RIPK1 and RIPK3 (receptor-interacting serine/threonine-protein kinase 1 and 3) and MLKL (mixed lineage kinase domain-like pseudokinase), are associated with severe disease progression (2–4). Furthermore, HMGB1 (high-mobility group box 1) is considered as one of the most relevant DAMPs (damage-associated molecular patterns) released by necroptotic cells (5, 6). When released during inflammatory cell death, HMGB1 triggers immunological processes, inducing recruitment of immune cells, as well as expression and secretion of proinflammatory cytokines (IL-6, IL-1β, TNF-α), which also has been observed in patients with COVID-19 (5, 7–9).

To elucidate an association of the necroptosis-related proteins RIPK3, MLKL, HMGB1, and RIPK1 and critical COVID-19 progression, we examined for the first time in daily assessed measurements their plasma concentrations in patients with COVID-19 in the intensive care unit (ICU).

Some of the results of these studies have been previously reported in the form of a preprint (Research Square, November 19, 2021; https://www.researchsquare.com/article/rs-1064345/v1).

We conducted a prospective single-center cohort study of 46 patients with COVID-19 (≥18 yr) admitted to the ICU of the University Hospital Frankfurt am Main, Germany, between June 2020 and January 2021. ICU admission occurred on Day 7 (interquartile range, 4–11) after symptom onset. During ICU stay, 28 patients showed a moderate and 18 patients a severe COVID-19 progression defined by the requirement for mechanical ventilation. The study was performed in accordance with the Declaration of Helsinki. Approval from the local ethics committee was obtained before the study was conducted (references #20-643, #20-982), and a waiver regarding the requirement of written informed consent from patients with COVID-19 was authorized. All participants of the control group provided written informed consent.

Patients with severe COVID-19 were older ($P = 0.033$), showed extended ICU stay ($P < 0.001$), and had increased mortality rate ($P < 0.001$) compared with patients with moderate COVID-19. Of all investigated comorbidities, we found a significantly increased rate of arterial hypertension in patients with severe compared with moderate COVID-19 ($P = 0.016$) (Table 1).

Patients' laboratory values during ICU stay, reflecting immunologic and inflammatory abnormalities, are displayed in Table 1.

Control samples were drawn once from 15 healthy donors (≥18 yr) to compare healthy physiological conditions to COVID-19. Patient blood samples were obtained daily from admission until ICU discharge or death. Plasma RIPK3, MLKL, HMGB1, and RIPK1 concentrations were determined by ELISAs. A detailed methods section is provided in the data supplement.

The following plasma concentrations were determined as mean control concentrations: RIPK3: 0.61 ± 0.3 ng/ml; MLKL: 0.36 ± 0.23 ng/ml; HMGB1: 140.7 ± 54.94 ng/ml; and RIPK1: 8.79 ± 8.01 ng/ml. For patients with moderate or severe COVID-19 in the ICU, the mean plasma concentrations with SDs of RIPK3, MLKL, HMGB1, and RIPK1 were calculated. It should be noted that the weighting of individual patients varies according to the measurement period. In patients with moderate COVID-19, we observed persistently significantly higher concentrations of RIPK3 (4.12 ± 3.21 ng/ml), MLKL

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Author Contributions: U.H. designed research. K.R. performed experiments. K.R. and H.N. collected data. K.R., S.R.T., and U.H. performed the analyses. K.R., S.R.T., S.C., and U.H. wrote the manuscript with input from H.N., E.H.A., A.v.K., and K.Z. All authors critically revised and approved the manuscript.

This letter has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Table 1. Patient Demographics of the COVID-19 Cohort

	Overall	Moderate	Severe	*P* Value
Total	46 (100)	28 (60.9)	18 (39.1)	
Sex, F	12 (26.1)	8 (28.6)	4 (22.2)	0.739
Age, yr	66.5 (49–78)	56 (47.8–72)	73.5 (65.3–81)	0.033
BMI, kg/m^2	29 (26–34.2)	29.2 (25.7–34.4)	28.6 (26.5–32.9)	0.842
ICU stay, d	8 (4.3–16)	6 (4–8)	16 (13.5–21.8)	<0.001
Outcome, death	14 (30.4)	0 (0)	14 (77.8)	<0.001
Comorbidity				
Arterial hypertension	25 (54.3)	11 (39.3)	14 (77.8)	0.016
Diabetes mellitus	13 (28.3)	5 (17.9)	8 (44.4)	0.092
Obesity	20 (43.5)	13 (46.4)	7 (38.9)	0.763
COPD	6 (13)	2 (7.1)	4 (22.2)	0.191
Bronchial asthma	3 (6.5)	2 (7.1)	1 (5.6)	1.000
Laboratory values				
CRP, mg/dl	7.6 (3.9–14.2)	5.4 (3.4–8.8)	13.72 (8.9–18.7)	0.001
Leukocyte count, /nl	8.5 (6.4–11.7)	6.7 (5.8–8.8)	11.4 (9–13.3)	<0.001
IL-6, pg/ml	35.9 (15–111.6)	21.2 (8.5–37.8)	124 (80.2–207.8)	<0.001
PCT, ng/ml	0.2 (0.1–0.7)	0.1 (0.1–0.2)	0.7 (0.4–2.2)	<0.001
LDH, U/L	365.5 (315.9–441.8)	352.5 (291.1–399.5)	429.5 (355.1–494)	0.033

Definition of abbreviations: BMI = body mass index; COPD = chronic obstructive pulmonary disease; COVID-19 = coronavirus disease; CRP = C-reactive protein; LDH = lactate dehydrogenase; PCT = procalcitonin.
Data are presented as *n* (%) for categorical variables or median (interquartile range) for continuous variables. Patients' laboratory values are reported as the respective median of the parameter concentrations obtained during ICU stay. ICU stay was defined as the days from admission to discharge or death. For more information on data collection and analysis, *see* data supplement. *P* values comparing patients with moderate and severe coronavirus disease were calculated with Mann-Whitney *U* test or Fisher exact test. Patients' median laboratory values are presented. Hospital's central laboratory's threshold concentrations are CRP: 0.5 mg/dl; leukocyte count: 10.41/nl; IL-6: 7 pg/ml; PCT: 0.5 ng/ml; and LDH: 248 U/L.

(1.41 ± 0.87 ng/ml), HMGB1 (220.57 ± 130.26 ng/ml), and RIPK1 (47.57 ± 44.24 ng/ml) compared with healthy control subjects throughout the monitoring period (Figures 1A–1D). The median length of ICU stay in patients with moderate COVID-19 was 6 (4–8) days. Patients with severe COVID-19 had a longer ICU stay, with a median of 16 (13.5–21.8) days until discharge or death. Therefore, we found continuously higher RIPK3 concentrations (5.96 ± 3.97 ng/ml), MLKL concentrations (1.66 ± 1.25 ng/ml), and HMGB1 concentrations (337.08 ± 215.28 ng/ml) compared with healthy control subjects over a longer time (Figures 1A–1C). RIPK1 concentrations (Figure 1D) showed a broad range; however, these were well separated from control concentrations, with significantly higher concentrations in patients with severe COVID-19 (63.7 ± 70.22 ng/ml). In addition, we examined these measurements with symptom onset as a baseline (*see* Figure E1 in the data supplement). Notably, on the majority of days after ICU admission, mean RIPK3, MLKL, HMGB1, and RIPK1 plasma concentrations of patients with severe COVID-19 were higher than those of patients with moderate COVID-19, which suggests that these proteins could be used as potential markers for severe COVID-19 progressions (Tables E1–E6 and Figures E2 and E3).

The principal component analysis revealed a positive association between HMGB1 and MLKL (Figures 1E, 1G, and 1H). Combined measurements of RIPK3, MLKL, HMGB1, and RIPK1 segregated patients with COVID-19 from healthy control subjects, whereas an overlap between patients with severe COVID-19 and patients with moderate COVID-19 remained in a two-dimensional principal component analysis scatterplot (Figure 1F). In particular, the plasma concentrations of patients with severe COVID-19 showed a high variability in contrast to healthy control subjects and patients with moderate COVID-19. Therefore, we further examined RIPK3, MLKL, HMGB1, and RIPK1 plasma concentrations individually and in various combinations and found that RIPK3 and HMGB1 in particular could indicate severe COVID-19 progression (Tables E1–E6 and Figures E2 and E3).

To the best of our knowledge, we showed for the first time, using daily measurements, constantly elevated plasma concentrations of the necroptosis-related proteins RIPK3, MLKL, HMGB1, and RIPK1 in patients with moderate and severe COVID-19 compared with healthy control subjects throughout their whole ICU stay.

Elevated RIPK3 serum concentrations in patients with COVID-19 at one distinct time point have been described previously (10). However, in the current study, we monitored the entire critical period of COVID-19 and also detected significantly higher plasma concentrations of MLKL, HMGB1, and RIPK1 in addition to RIPK3.

Several studies of these four necroptotic key player proteins in COVID-19 disease models, (e.g., in mice, *in vitro*, or respiratory tissues of patients with COVID-19) support our findings (11–14). Together with our results, this implies a notable link between RIPK3-, MLKL-, HMGB1-, and RIPK1-mediated signaling pathways and critical COVID-19, hinting at excessive inflammatory cell death, which contributes to the clinical manifestation of patients with COVID-19. To further explore the disease mechanisms indicated by this study and possible diagnostic options based on our findings, we suggest additional research on necroptosis markers, such as studies

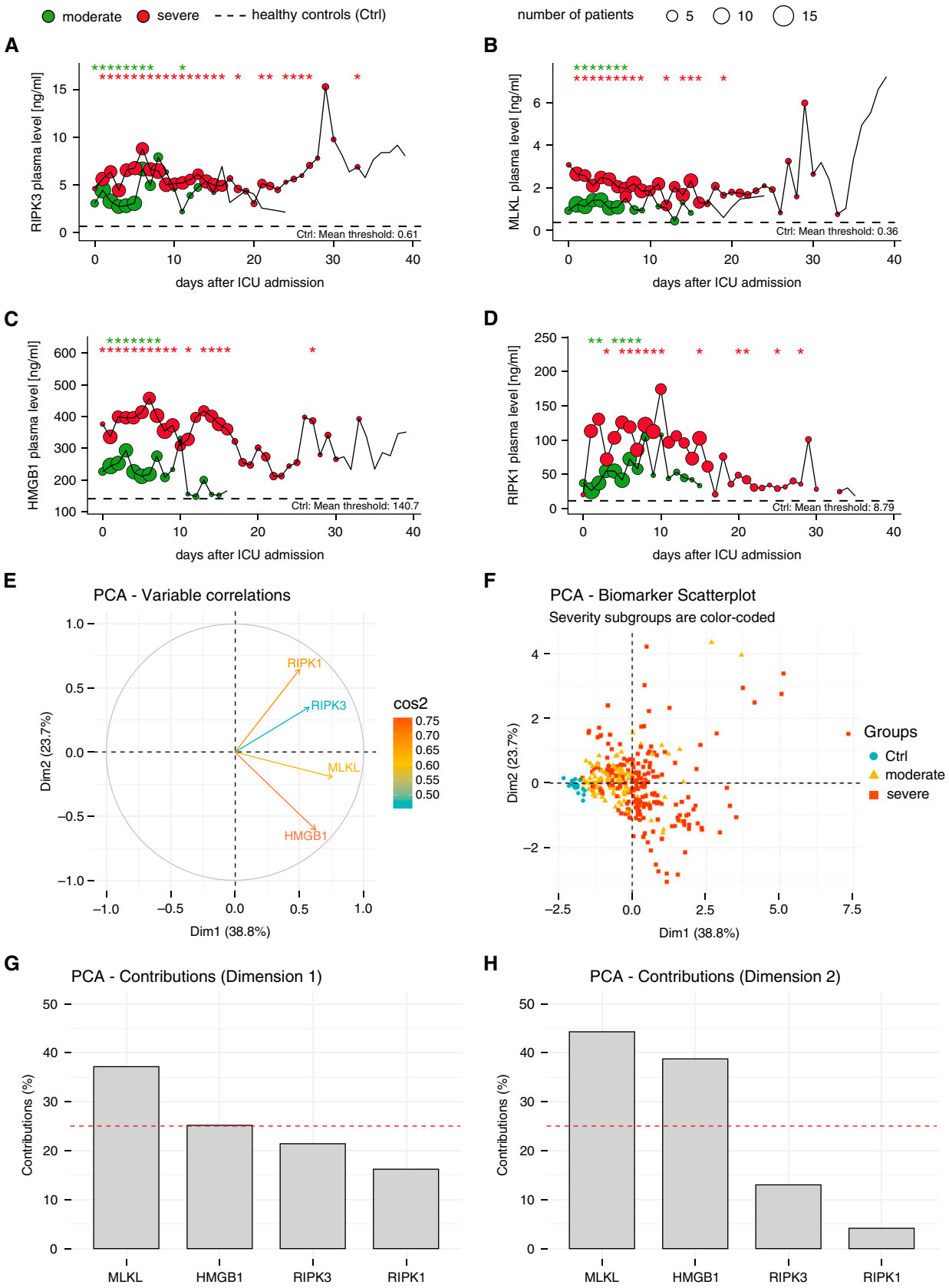

Figure 1. Continuously elevated RIPK3 (receptor-interacting serine/threonine-protein kinase 3), MLKL (mixed lineage kinase domain-like pseudokinase), HMGB1 (high-mobility group box 1), and RIPK1 (receptor-interacting serine/threonine-protein kinase 1) plasma concentrations in patients with coronavirus disease (COVID-19) in intensive care. Mean longitudinal concentrations of 28 patients with moderate (green) and

on nonhospitalized patients, patients in the normal ward, or patients with post–COVID-19 syndrome. However, we cannot completely exclude an impact of intensive care treatment and the intubation status on the measured plasma concentrations. For example, invasive ventilation may lead to increased cell damage (15). Also, a control group of equally ill, ventilated patients without COVID-19 should be considered. ■

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Figure 1. (*Continued*). 18 patients with severe (red) COVID-19 are presented. Bubble size is equivalent to the number of patients. (*A*) RIPK3, (*B*) MLKL, (*C*) HMGB1, and (*D*) RIPK1 plasma concentrations are plotted by days after intensive care unit (ICU) admission. Significant differences between patients with moderate COVID-19 and healthy control subjects (Ctrl) were found on Days 0–7 and 11 (RIPK3); 1–7 (MLKL and HMGB1); and 1, 2, and 4–7 (RIPK1). Significant differences between patients with severe COVID-19 and healthy control subjects were found on Days 1–16, 18, 21, 22, 24–27, and 33 (RIPK3); 1–9, 12, 14–16, and 19 (MLKL); 0–9, 11, 13–16, and 27 (HMGB1); and 3, 5–10, 15, 20, 21, 25, and 28 (RIPK1). Statistical differences between plasma concentrations of patients with moderate and severe COVID-19 and 15 healthy control subjects (black dotted line) were assessed using unpaired two-sided Student's *t* test; $*P < 0.05$. (*E*) Principal component analysis (PCA) variable correlations plot of RIPK3, MLKL, HMGB1, and RIPK1 plasma concentrations. With two dimensions, 62.5% of the variance is expressed. (*F*) PCA scatterplot of the three groups (Ctrl = blue circle; moderate COVID-19 = yellow triangle; severe COVID-19 = orange square) with plasma concentrations of all four markers. (*G*) Bar plot of variables' (RIPK3, MLKL, HMGB1, and RIPK1) contribution to Dimension 1 (Dim1) in percentage. The red dashed line indicates the expected average contribution (25%). (*H*) Bar plot of the same variables' contribution to Dim2 in percentage. cos2 = squared coordinates (quality of representation).