Plasma RIPK3 And HMGB1 Predict Severe COVID-19 Progression In ICU Patients: A Single-Center Cohort Study

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Abstract

Background: Severe progression of coronavirus disease 2019 (COVID-19) causes respiratory failure and critical illness. Recently, these pathologies have been associated with necroptosis, a receptor-interacting serine/threonine-protein kinase 3 (RIPK3) dependent regulated form of inflammatory cell death. Investigations of indicator necroptosis proteins like RIPK3, mixed lineage kinase domain-like pseudokinase (MLKL), receptor-interacting serine/threonine-protein kinases 1 (RIPK1), and high-mobility group box 1 (HMGB1) in clinical COVID-19 manifestations are lacking.

Methods: A prospective prolonged cohort study including 46 intensive care unit (ICU) patients classified with moderate and severe COVID-19 was conducted with daily measured plasma levels of indicator necroptosis proteins like RIPK3, MLKL, RIPK1, and HMGB1 by enzyme-linked immunosorbent assay (ELISA). On this basis, a multiple logistic (regression) classification for the prediction of severe COVID-19 progression was performed.

Results: We found significantly elevated RIPK3, MLKL, HMGB1, and RIPK1 levels in COVID-19 patients admitted to the ICU compared to healthy controls throughout the ongoing disease, indicating necroptotic processes. Above all, with combined measurements of RIPK3 and HMGB1 plasma levels, we were able to time-independently predict COVID-19 severity with 84% accuracy, 90% sensitivity, and 76% specificity.

Conclusion: We suggest that HMGB1 and RIPK3 are potential biomarkers to identify high-risk COVID-19 patients and developed a classifier for COVID-19 severity.

Introduction

Coronavirus disease 2019 (COVID-19) is the most challenging pandemic in recent human history. In November 2021, the World Health Organization reported 249.743.428 cases of COVID-19 with 5.047.652 deaths globally [1]. It is crucial for the disease outcome and appropriate treatment to develop a method to determine the exact point of COVID-19 exacerbation. Patients suffering from critical COVID-19 often present with respiratory failure as well as features of sepsis, such as coagulopathy, lymphopenia, and high plasma levels of pro-inflammatory cytokines [2].

In various comparable non-COVID-19-related inflammatory diseases, it is already established that the receptor-interacting serine/threonine-protein kinase 1 and 3 (RIPK1 and RIPK3), as well as the mixed lineage kinase domain-like pseudokinase (MLKL), are associated with disease progression as important regulators of necroptotic cell death [3, 4]. For example, the examination of lung tissue sections in H7N9 virus infection, in which acute respiratory distress syndrome (ARDS) was the main cause of death, showed significantly higher RIPK1, RIPK3, phospho-RIPK3, MLKL, and phospho-MLKL protein levels [5]. These data suggest that severe H7N9 infection is associated with necroptosis of the lung epithelium which contributes to ARDS. This hypothesis is supported by results that showed significantly increased RIPK3 levels not only in the plasma of ARDS patients but also in bronchoalveolar lavage fluid [6]. Furthermore, elevated RIPK3 levels in the plasma of patients with severe sepsis or septic shock also indicate that the RIPK3 signaling pathway is activated under septic conditions [7]. Necroptosis, also referred to as RIPK3-dependent necrosis, is executed by phosphorylated and activated RIPK1 and RIPK3, which form a complex known as the necrosome [8–10]. Subsequently, the effector molecule MLKL is phosphorylated, enabling it to oligomerize and migrate to the cell membrane, leading to the release of damage-associated molecular patterns (DAMPs), cell rupture, and lytic cell death [11]. This promotes cytokine production and an excessive immune response [12]. High-mobility group box 1 (HMGB1), considered as one of the most relevant DAMPs released by necroptotic cells, usually binds to DNA as well as chromatin and exerts its function in chromatin modification and DNA repair [13–18]. When released during inflammatory cell death, HMGB1 triggers immunological processes, inducing recruitment of immune cells and expression as well as the release of pro-inflammatory cytokines (interleukin 6 (IL-6); IL-1 β ; tumor necrosis factor- α (TNF- α)), as similarly described in COVID-19 [13, 19–21]. Extracellular HMGB1 is furthermore capable of forming complexes with cytokines amplifying hyperinflammation [22, 23]. Moreover, high serum HMGB1 levels in non-COVID-19 patients were linked to fatal ARDS [24]. Besides, reactive oxygen species (ROS) production is associated with necroptosis, and mitochondrial ROS (mtROS) production also plays a crucial role in peripheral lymphocytes in severe disease conditions [25–27].

Against this background, we decided to conduct a close monitoring of plasma levels of the necroptosis-related proteins RIPK3, MLKL, RIPK1, and the DAMP HMGB1 in COVID-19 patients throughout intensive care unit (ICU) stay. The current single-center cohort study aims to investigate the prognostic potential of RIPK3, MLKL, HMGB1, and RIPK1 in COVID-19 progression as feasible biomarkers. Using long-term measurement data, we were able to build a classifier that predicts COVID-19 exacerbation independently of time. In addition, we analyzed cell death and mtROS in peripheral leukocytes of ICU COVID-19 patients in single measurements, to verify if these parameters differ in COVID-19 patients as shown before in severe disease conditions, e.g. sepsis patients [27].

Materials And Methods

COVID-19 cohort

This is a prospective single-center cohort study of 46 COVID-19 patients (≥ 18 -years) who were admitted to the ICU of the University Hospital Frankfurt am Main, Germany, between June 2020 and January 2021. During ICU stay, blood samples were obtained daily at 8 a.m. from admission until ICU discharge. Inflammatory parameters including C-reactive protein (CRP), IL-6, procalcitonin (PCT), lactate dehydrogenase (LDH), and peripheral leukocyte count were obtained daily at 4 a.m. and measured by the hospital's central laboratory and compared to the hospital's central

laboratory's threshold levels (CRP: 0.5 mg/dl, IL-6: 7 pg/ml, PCT: 0.5 ng/ml, LDH: 248 U/l, peripheral leukocyte count: 10.41 /nl). Control samples were drawn from 15 non-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-infected healthy donors (≥ 18 -years) to compare healthy physiological conditions to COVID-19. The study was conducted in compliance with good clinical practice and current guidelines. Intubation was considered in patients with COVID-19 and severe hypoxemia ($\text{PaO}_2/\text{FiO}_2 < 150$ mmHg) and respiratory rates > 30 /min. A $\text{PaO}_2/\text{FiO}_2$ of < 100 mmHg in two consecutive measurements was an indication to perform mechanical ventilation, according to the German guideline [28]. Based on this, it was feasible to distinguish between patients with severe and moderate COVID-19 according to their requirement for intubation throughout their ICU stay. Patients were transferred to a normal ward if their oxygen requirement was < 6 l/min and their $\text{SpO}_2 > 90\%$. Patients who were not mechanically ventilated due to patient will ($n=3$), despite indication, were also assigned to the group of patients with severe COVID-19. The time of symptom onset was specified by the patient.

Plasma preparation and measurement

Whole blood samples were drawn into citrate tubes (SARSTEDT S Monovetten, Nümbrecht, Germany, Citrat 3,13%). Samples were centrifuged for 10 minutes at 2000 g and plasma was stored at -80°C until further processing.

Enzyme-linked immunosorbent assays (ELISAs) (RIPK3 – CSB-EL019737-HU Cusabio, Wuhan, China; RIPK1 – MBS 9137722; MLKL – MBS 9137385, MyBioSource, San Diego, USA; RIPK1 – HEE640Hu, Cloud-Clone Corp., Katy, USA; MLKL – OKEH03401, Aviva Systems Biology, San Diego, USA; HMGB1 – NBP2-62766, Novus Biologicals, Toronto, Canada) were used to determine the plasma concentration of RIPK3, RIPK1, MLKL, and HMGB1.

Statistical analysis

Statistical analyses were carried out with GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, CA, USA) and R v4.0.3 (R Foundation for Statistical Computing, Vienna, Austria) [29]. Descriptive variables were calculated using means with standard deviation (SD); medians and interquartile ranges (IQRs, P25%-P75%), as well as counts and percentages. For continuous variables, two-tailed Student's t- or Mann-Whitney U tests were performed. ANOVAs with Tukey's post hoc test for multiple comparisons or Kruskal-Wallis tests with Dunn's post hoc test for multiple comparisons were used to examine more than two groups. Adjusted p-values from post hoc tests were indicated (p_{adj}). For categorical data, Fisher's exact test was performed. Principal Component Analysis (PCA) was performed to investigate the relevance of the measurement parameters and their correlations. A p-value < 0.05 was considered statistically significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Multiple logistic (regression) classifier

A multiple logistic regression analysis was performed including 28 patients with severe COVID-19, defined by the requirement for mechanical ventilation at the time of the respective blood collection, and 18 patients with moderate COVID-19. The COVID-19 cohort was censored (0=ICU discharge and 1=death). The day of the censoring event was labeled as day E and chosen as a reference point. Data from the days E to E-3 were used for training and testing the model. The remaining data up to E-7 were used as complementary validation data. The labeled data were split randomly into a training (70%) and a test set (30%). Each set contained binary class information about the patients' severity status (moderate=0 and severe=1) as well as the quantitative measurements of plasma RIPK3, MLKL, HMGB1, and RIPK1 levels.

Different models were trained and evaluated, including single or combined variables. All models were calculated using a generalized linear model (GLM) of the binomial family to find a classifier (caret package) [30]. A 10-fold cross-validation was performed to exclude a subsample bias and prevent the model from being overfitted. Each model was calculated as a regular logistic regression to compare the relative goodness-of-fit with Akaike's information criterion (AIC). The ideal predictors for the classifier were selected by evaluation of the classification performance indicators, e.g., predictors with the highest accuracy and the lowest AIC after cross-validation were chosen. The final classifier was built with training data that showed mean accuracies $> 90\%$ on days E to E-3. This model was tested with the separated validation set to determine the overall predictive quality of the classifier, e.g., with general performance parameters (Accuracy, Sensitivity, and Specificity). The odds and Odds Ratio (OR) of the predictor variables were determined from the coefficients of the final regression model. Finally, multiple logistic regression models were performed using measurements of RIPK3, HMGB1, CRP, IL-6, PCT, LDH, and peripheral leukocyte count including a training and a test set.

Results

Demographic characteristics and laboratory parameters

Between June 2020 and January 2021, we considered 46 COVID-19 patients admitted to the ICU, of whom 28 patients showed a moderate and 18 patients a severe COVID-19 progression during ICU stay. Overall, ICU admission occurred on day seven (4-11) after symptom onset. Patients with severe COVID-19 were older ($p=0.033$), showed extended ICU stay ($p<0.001$), and increased mortality rate ($p<0.001$) compared to patients with moderate COVID-19 (Table 1). The median survival time after ICU admission in patients with severe COVID-19 was 17 (15-35) days. Of all investigated comorbidities, we found a significantly increased rate of arterial hypertension in patients with severe compared to moderate COVID-19 ($p=0.016$) (Table 1).

We also examined median levels of the patients' laboratory parameters during ICU stay, reflecting immunologic and inflammatory abnormalities (Table 1). Median levels of CRP ($p<0.001$), IL-6 ($p<0.001$), PCT ($p<0.001$), LDH ($p=0.033$), and peripheral leukocyte count ($p<0.001$) were significantly higher in patients with severe compared to moderate COVID-19. Furthermore, CRP (95.7%), IL-6 (87%), and LDH (87%) median levels in these patients were elevated compared to the hospital's central laboratory's threshold. Moreover, elevated peripheral leukocyte counts (55.6% versus 14.3%; $p=0.007$) and PCT levels (61.1% versus 10.7%; $p=0.001$) were significantly more common in patients with severe compared to moderate COVID-19.

Prolonged measurements of plasma RIPK3, MLKL, HMGB1, and RIPK1 in COVID-19 intensive care patients

During ICU stay, we measured RIPK3, MLKL, HMGB1, and RIPK1 levels daily in COVID-19 patients from admission until discharge or death (Fig. 1). In patients with moderate as well as severe COVID-19, we found significantly higher levels of RIPK3 (Fig. 1a), MLKL (Fig. 1b), HMGB1 (Fig. 1c), and RIPK1 (Fig. 1d) compared to healthy controls (the median length of ICU stay in patients with moderate COVID-19 was 6 (4-8) days and with severe COVID-19 was 16 (13.5-21.8) days).

Additionally, we examined these measurements with symptom onset as a baseline (Fig. S1).

The PCA, including RIPK3, MLKL, HMGB1, and RIPK1, revealed a positive association between HMGB1 and MLKL (Fig. 2a,c,d). Combined measurements of RIPK3, MLKL, HMGB1, and RIPK1 segregated COVID-19 patients from healthy controls, whereas an overlap between patients with severe COVID-19 and patients with moderate COVID-19 remained in a two-dimensional PCA scatterplot (Fig. 2b). Therefore, we next examined RIPK3, MLKL, HMGB1, and RIPK1 plasma levels individually and in various combinations to predict disease severity more accurately.

Prediction of severe COVID-19 progression with a multiple logistic (regression) classifier: model selection

With randomly selected training data from the day of the censoring event E (ICU discharge or death) to E-3 (three days before the censoring event), we evaluated models with different predictor combinations in a time-independent manner (Table 2). Although a model consisting of HMGB1, RIPK3, and RIPK1 and a model consisting of solely RIPK3 achieved better accuracies with 78%, their fits and thus model qualities (Akaike's information criterion (AIC)) were worse, resulting in a model with HMGB1 and RIPK3 with a marginally superior fit ($AIC=65.91$), pointing to a simpler and, therefore, more applicable model. While this was an improvement towards the other models, a slightly lower classification accuracy $Acc_{HMGB1+RIPK3}=77\%$ was achieved. However, this model required only two measurements, opposed to three.

To further evaluate on which days plasma RIPK3, MLKL, HMGB1, and RIPK1 levels distinguished best between severe and moderate COVID-19 progression, we looked at individual days and markers backward from the censoring event (E). On days E-3 until the event, HMGB1 (Fig. 3a-d, third column) and on days E-3 and E-1, RIPK3 (Fig. 3b,d, first column) plasma levels were significantly elevated in patients with severe compared to those with moderate COVID-19. In contrast, RIPK1 and MLKL levels did not differ significantly between severe and moderate COVID-19 (Fig. 3a-d, second and fourth column) and were therefore not included in our further analysis. Consequently, plasma RIPK3 and HMGB1 were selected for further evaluation.

Evaluation of the discriminatory ability of combined RIPK3 and HMGB1 plasma levels for building the COVID-19 severity classifier

To organize data for training and testing the selected model, combined RIPK3 and HMGB1 plasma levels are viewed backward from the censoring event (E). Table 3 shows the classification performance of predicting COVID-19 progression using the training and test data. The days starting from day E to E-3 were analyzed separately. In the training data, E, E-1, and E-3 achieved accuracies of 100% ($CI_{95\%,E}[78;100]$), ($CI_{95\%,E-1}[79;100]$), and ($CI_{95\%,E-3}[79;100]$), as well as high sensitivity and specificity (100%), indicating that the combined measurement of HMGB1 and RIPK3 levels on these days discriminated well between patients with moderate and severe COVID-19. On day E-2, the accuracy was lower (82%) ($CI_{95\%,E-2}[57;96]$), mainly due to a loss in specificity. In the test data, the event day was classified at 76% ($CI_{95\%,E,test}[61;87]$) and day E-1 was classified lowest at 72% accuracy ($CI_{95\%,E-1,test}[57;84]$), while day E-2 reached 78% ($CI_{95\%,E-2,test}[64;89]$). On day E-3, the test data were best classified with an accuracy of 83% ($CI_{95\%,E-3,test}[69;92]$).

The most stable and optimal results in discriminating between patients with moderate and severe COVID-19 were found to be days E-1 and E-3, as indicated by significantly higher RIPK3 and HMGB1 plasma levels (Fig. 3b,d, first and third column) as well as by the performance of the training and test data using combined RIPK3 and HMGB1 measurements from these days (Table 3). Therefore, HMGB1 and RIPK3 plasma levels from these days were used in building the final classifier.

Prediction of severe COVID-19 progression with combined RIPK3 and HMGB1 measurements

Table 4 shows the performance of the final classifier with the training, test, and validation data. The overall accuracies of discriminating between a moderate and severe COVID-19 progression were high ($>83\%$). The fraction of false positives and false negatives was low, resulting in specificity and sensitivity levels $>74\%$. The test set reached 83% accuracy as well as 89% sensitivity and 74% specificity (Fig. 4b), which was exceeded by the validation set using data from up to day E-7 (excluding E-1 and E-3) with 84% accuracy, 90% sensitivity, and 76% specificity (Fig. 4c). This was particularly accurate up to 6 days before the censoring event (Fig. 4d).

Also, RIPK3 plasma levels of patients with moderate COVID-19 approached the healthy control levels before ICU discharge (Fig. 4e). Notably, HMGB1 plasma levels indicated significant differences between patients with moderate and severe COVID-19 at a very early stage (E-8) (Fig. 4f). Therefore, the combination of circulating levels of RIPK3 and HMGB1 can be used to time-independently classify COVID-19 patients admitted to the ICU into potential disease severity states (Fig. 4a-c).

The odds of changing COVID-19 severity based on RIPK3 and HMGB1 levels

To further estimate these findings, a logistic regression model was calculated with the full data over the entire observation period of plasma RIPK3 and HMGB1 levels as independent variables and disease progression of COVID-19 as the dependent variable. With this model, the odds of changing the disease severity state were estimated (Table 5).

For every unit change in RIPK3, the log-odds of disease severity change from moderate to severe were increased by 0.305 (Odds Ratio (OR)=1.36 (CI_{95%}[1.22; 1.52])). Similarly, every unit change in HMGB1 increased the log-odds by 0.006 (OR=1.006 (CI_{95%}[1.004; 1.007])).

Model comparison for the prediction of severe COVID-19 progression using multiple inflammatory variables

To compare the predictive power of established inflammatory markers as well as RIPK3 and HMGB1, we additionally performed a multiple logistic regression model including measurements of RIPK3, HMGB1, CRP, IL-6, PCT, LDH, and peripheral leukocyte count. Interestingly, in the training set, the combination of measured RIPK3, HMGB1, and PCT levels reached the highest accuracy (93%). In order to be able to represent the plot in two dimensions, with one variable on the x-axis and one on the y-axis, we chose a model with two measurements from our potential biomarkers. In fact, the combination of RIPK3 and HMGB1 levels discriminated best between moderate and severe COVID-19 progression with an accuracy of 86% in the training set (Table 6). In the test set, both models performed similarly, with an accuracy of 83.7% (Table 7). Therefore, plasma RIPK3 and HMGB1 are the most suitable candidates for predicting COVID-19 severity.

Discussion

In this study, plasma RIPK3, MLKL, HMGB1, and RIPK1 levels of COVID-19 patients are obtained in daily-assessed measurements throughout the whole ICU stay. Based on these data, we developed for the first time a classifier built on RIPK3 and HMGB1 as potential biomarkers to discriminate between moderate and severe COVID-19 progression after ICU admission with an accuracy of 84%. Several independent lines of evidence support this conclusion.

First, COVID-19 intensive care patients showed continuously significantly higher plasma RIPK3 levels than healthy controls throughout their ICU stay, strongly indicating ongoing RIPK3-dependent necroptosis. In addition to previous investigations that considered RIPK3 levels at single time points [31, 32], we revealed in our prolonged study that patients with severe COVID-19 possess higher RIPK3 plasma levels in a time-dependent manner. Also, patients with moderate COVID-19 showed decreasing RIPK3 levels, corresponding to their recovery.

Second, we observed significant long-term elevations of HMGB1 in COVID-19 intensive care patients compared to healthy controls. Elevations of HMGB1 levels were associated with the requirement for mechanical ventilation and fatal outcome, as also demonstrated in our supplemental data and previous studies [33, 34]. Notably, we also revealed significant elevations of plasma HMGB1 corresponding to severe COVID-19 progression in a disease-dependent time course. Chen *et al.* observed an association between exogenous human HMGB1 and stimulated angiotensin-converting enzyme 2 (ACE2) expression as an entry receptor for SARS-CoV-2 in cultured human lung epithelial cells, indicating a feedback loop that possibly worsens patients' outcomes [33]. RIPK3 and extracellular HMGB1 also contribute to endothelial dysfunction and loss of barrier integrity, considered to be involved in COVID-19 pathology [6, 35–37]. Since high extracellular levels of HMGB1 are particularly harmful, our results provide evidence for HMGB1 as a potential drug target in COVID-19, as has been successfully demonstrated for IL-6 signaling [38].

Third, we found that plasma MLKL and RIPK1 levels were tendentially higher in COVID-19 ICU patients compared to healthy controls, indicating an involvement of necroptosis in COVID-19 pathology. Accordingly, upregulation of phosphorylated MLKL was detected in lung tissue of SARS-CoV-2-infected mice and post mortem human lungs. *In vitro*, MLKL and RIPK3 contributed to cell death induction, as well as cytokine and DAMP release in SARS-CoV-2-infected cells, reinforcing our findings in COVID-19 patients in the ICU [39]. Moreover, phosphorylated and thus activated RIPK1 was detected in pharyngeal epithelial cells of COVID-19 patients, and since respiratory tissues appeared to be a prominent sink for RIPK1 in COVID-19, its interaction with SARS-CoV-2 components is hypothesized [40, 41]. However, MLKL and RIPK1 did not contribute significantly to COVID-19 severity and were therefore not included in the final classifier.

In addition to our prolonged study investigating kinetic variations, our single measurements of RIPK3, MLKL, HMGB1, and RIPK1 supported our hypothesis that necroptosis plays a role in COVID-19, as described in our supplemental data. In this cohort, we also observed a loss of viable peripheral leukocytes in every examined cell subpopulation according to disease severity, particularly in patients receiving extracorporeal membrane oxygenation (ECMO), however, given that there were only 6 patients with this treatment, these results should be interpreted carefully.

To our knowledge, we are the first to perform mtROS measurements using flow cytometry in whole blood samples of COVID-19 patients; therefore, there is still a lack of comparative studies. Other studies were carried out on cell cultures treated with plasma from COVID-19 patients or with single

viral components (open reading frame 3a (ORF-3a) or the SARS-CoV-2 spike protein), as well as SARS-CoV-2-infected monocytes *in vitro* and respiratory samples (sputum/ Bronchoalveolar lavage (BAL)) of COVID-19 patients [42–46]. Nevertheless, it is important to mention these studies, but comparisons should be interpreted with caution. We show in our supplemental data that peripheral leukocytes of our COVID-19 cohort with single measurements had significantly lower levels of mtROS compared to healthy controls.

In COVID-19 patients, cytokines like TNF- α and IL-6 are mainly released by non-circulating cells, indicating pathological processes in infected or damaged tissue [32, 47]. We therefore assume that RIPK3, MLKL, HMGB1, and RIPK1 are released, for instance, by virus-infected lung tissue cells, damaged vascular endothelial cells, or activated immune cells recruited to the site of infection [5, 6, 39, 48–50].

As patients were admitted to the ICU at different disease stages, data from the first day after ICU admission would provide limited information. Therefore, we took data from time points when the disease progression was already clear to build our model using plasma RIPK3 and HMGB1 levels and thereby reduced the variability that resulted from admission to the ICU at different COVID-19 stages. In everyday clinical practice, it is often not possible to predict whether a patient is close to or long before death or ICU discharge. The classifier model avoids this problem with RIPK3 and HMGB1 as promising biomarkers in COVID-19.

This study has several limitations. The timing of mechanical ventilation is a subjective outcome. However, differentiation of severity is possible because, once patients have the indication for intubation, spontaneous breathing and non-invasive ventilation are no longer sufficient and a definite state of disease progression has been reached. Since we intended to examine COVID-19 patients over a prolonged period, we decided to consider the requirement for intubation as a distinction between a moderate and severe COVID-19 progression for the study design. We cannot completely exclude an additional impact of the intubation status on plasma levels of RIPK3, MLKL, HMGB1, and RIPK1.

We are aware that measurements of 46 patients must be considered carefully, but regarding the number of blood samples received daily over a longer period of time, the study size is unusually extensive, in particular, compared to other single-center studies.

Moreover, to further explore the disease mechanisms indicated by this study, we suggest additional investigations on necroptosis markers, such as studies on other COVID-19 progressions and stages which we could not take into account e.g., non-hospitalized patients, or patients with post-COVID-19 syndrome. Finally, our data needs to be confirmed in further longitudinal clinical studies with independent cohorts of COVID-19 patients before implementation in clinical algorithms can be considered.

Conclusion

Our classifier with RIPK3 and HMGB1 as promising biomarkers in COVID-19 could help to timely identify future patients who require more intensive monitoring and benefit from maximized immunomodulatory therapy after ICU admission [38, 51]. This model is simple and more accurate than models that, in addition to RIPK3 and HMGB1 plasma levels, considered inflammatory markers such as CRP, IL-6, PCT, LDH, and peripheral leukocyte count.

Abbreviations

Acc	accuracy
ARDS	acute respiratory distress syndrome
AIC	Akaike’s Information criterion
ACE2	angiotensin-converting enzyme 2
BAL	Bronchoalveolar lavage
COPD	chronic obstructive pulmonary disease
COVID-19	coronavirus disease 2019
CRP	C-reactive protein
DAMPs	damage-associated molecular patterns
ECMO	Extracorporeal membrane oxygenation
ELISA	enzyme-linked immunosorbent assay
GLM	generalized linear model
HMGB1	high-mobility group box 1

ICU	intensive care unit
IL	interleukin
LDH	lactate dehydrogenase
MLKL	mixed lineage kinase domain-like pseudokinase
mtROS	mitochondrial ROS
ORF-3a	open reading frame 3a
OR	Odds Ratio
PCA	Principal Component Analysis
PCT	procalcitonin
RIPK3	receptor-interacting serine/threonine-protein kinase 3
RIPK1	receptor-interacting serine/threonine-protein kinases 1
ROS	reactive oxygen species
β	regression coefficient
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SD	standard deviation
SE	standard error
TNF- α	tumor necrosis factor- α

Declarations

Ethics approval and consent to participate

The study was performed in accordance with the Declaration of Helsinki. Approval from the local ethics committee was obtained before the study was conducted (reference #20-643, #20-982) and a waiver regarding the requirement of written informed consent from COVID-19 patients was authorized. All participants of the control group provided written informed consent.

Consent for publication

All authors critically revised and approved the manuscript.

Data and materials availability

All data are available in the main text or the supplementary materials.

Author contributions

Contribution: U.H. designed research; K.R. performed experiments; K.R. and H.N. collected data; K.R., U.H., and S.T. performed the analyses; K.R., U.H., S.T., and S.C. wrote the manuscript with input from H.N., E.A., A.v.K., and K.Z.

Competing interests

KZ: The Department of Anaesthesiology, Intensive Care Medicine & Pain Therapy of the University Hospital Frankfurt received support from B. Braun Melsungen, CSL Behring, Fresenius Kabi, and Vifor Pharma for the implementation of Frankfurt's Patient Blood Management program and KZ received honoraria for scientific lectures and ad board meeting within the last 3 years from CSL Behring, GE Healthcare, Edwards, Haemonetics, implatcast GmbH, med Update GmbH, Pharmacosmos and Vifor Pharma. The remaining authors declare no potential conflicts of interest.

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Tables

Table 1. Patient demographics of the COVID-19 cohort.

	Overall	Moderate	Severe	p
Total ^a	46 (100%)	28 (60.9%)	18 (39.1%)	
Gender, female ^a	12 (26.1%)	8 (28.6%)	4 (22.2%)	0.739
Age ^b , yr	66.5 (49-78)	56 (47.8-72)	73.5 (65.3-81)	0.033
Bodyweight ^b , kg	89 (78.2-109)	91.5 (77.3-111.3)	85 (79.1-98.8)	0.596
ICU stay ^b , d	8 (4.3-16)	6 (4-8)	16 (13.5-21.8)	<0.001
Outcome, death ^a	14 (30.4%)	0 (0%)	14 (77.8%)	<0.001
Comorbidity				
Arterial hypertension ^a	25 (54.3%)	11 (39.3%)	14 (77.8%)	0.016
Diabetes mellitus ^a	13 (28.3%)	5 (17.9%)	8 (44.4%)	0.092
Adipositas ^a	15 (32.6%)	10 (35.7%)	5 (27.8%)	0.749
COPD ^a	6 (13%)	2 (7.1%)	4 (22.2%)	0.191
Asthma bronchiale ^a	3 (6.5%)	2 (7.1%)	1 (5.6%)	1.000
Laboratory parameters				

CRP ^b , mg/dl	7.6 (3.9-14.2)	5.4 (3.4-8.8)	13.72 (8.9-18.7)	0.001
>0.5 mg/dl ^a	44 (95.7%)	26 (92.9%)	18 (100%)	0.513
Leukocyte count ^b , /nl	8.5 (6.4-11.7)	6.7 (5.8-8.8)	11.4 (9-13.3)	<0.001
>10.41 /nl ^a	14 (30.4%)	4 (14.3%)	10 (55.6%)	0.007
IL-6 ^b , pg/ml	35.9 (15-111.6)	21.2 (8.5-37.8)	124 (80.2-207.8)	<0.001
>7 pg/ml ^a	40 (87%)	22 (78.6%)	18 (100%)	0.068
PCT ^b , ng/ml	0.2 (0.1-0.7)	0.1 (0.1-0.2)	0.7 (0.4-2.2)	<0.001
>0.5 ng/ml ^a	14 (30.4%)	3 (10.7%)	11 (61.1%)	<0.001
LDH ^b , U/l	365.5 (315.9-441.8)	352.5 (291.1-399.5)	429.5 (355.1-494)	0.033
>248 U/l ^a	40 (87%)	22 (78.6%)	18 (100%)	0.068

Data are presented as ^an (%) for categorical variables or ^bmedian (interquartile range) for continuous variables. Patients' laboratory parameters are reported as the respective median of the parameter levels obtained during ICU stay. p-values comparing patients with moderate and severe COVID-19 were calculated with Mann-Whitney U test or Fisher's exact test. Additionally, patients' median laboratory parameter levels were compared to the hospital's central laboratory's threshold levels (CRP: 0.5 mg/dl, IL-6: 7 pg/ml, PCT: 0.5 ng/ml, LDH: 248 U/l, peripheral leukocyte count: 10.41 /nl). Respective quantities in the pathological range were determined and then compared among patients with severe and moderate COVID-19 by Fisher's exact test.

COPD, chronic obstructive pulmonary disease

Table 2. GLM model performances of the training data for prediction of COVID-19 severity.

Model	Accuracy (%)	AIC
HMGB1 + RIPK3	0.77	65.91
HMGB1 + RIPK3 + RIPK1	0.78	67.88
HMGB1 + RIPK3 + RIPK1 + MLKL	0.75	69.23
HMGB1	0.75	72.17
HMGB1 + MLKL	0.74	73.91
HMGB1 + RIPK1	0.76	74.03
RIPK3	0.78	91.99
MLKL + RIPK3	0.77	93.11
MLKL	0.60	105.61
MLKL + RIPK1	0.61	106.45
RIPK1	0.57	107.52

Classification accuracies (%) and AICs values of the training data from day E to E-3

Table 3. Classification of the severity status on multiple days on and before the censoring event.

The classification performance of predicting COVID-19 severity using the training and test data consisting of combined RIPK3 and HMGB1 plasma levels on day E to E-3.

CI, confidence interval; Acc, accuracy

Table 4. Classifier evaluation of the training, test, and validation data.

	day	n0	n1	Accuracy	Acc2.5% CI	Acc97.5% CI	Sensitivity	Specificity
Training data	E	12	3	1.00	0.78	1.00	1.00	1.00
	E-1	10	6	1.00	0.79	1.00	1.00	1.00
	E-2	9	8	0.82	0.57	0.96	0.89	0.75
	E-3	11	5	1.00	0.79	1.00	1.00	1.00
Test data	E	27	19	0.76	0.61	0.87	0.93	0.53
	E-1	27	19	0.72	0.57	0.84	0.67	0.79
	E-2	27	19	0.78	0.64	0.89	0.82	0.74
	E-3	27	19	0.83	0.69	0.92	1.00	0.58

Training data		reference		Test data		reference		Validation data		reference	
		moderate	severe			moderate	severe			moderate	severe
predicted	moderate	21	0	predicted	moderate	24	5	predicted	moderate	57	11
	severe	0	11		severe	3	14		severe	6	35
Accuracy			1.00	Accuracy			0.83	Accuracy			0.84
Sensitivity			1.00	Sensitivity			0.89	Sensitivity			0.90
Specificity			1.00	Specificity			0.74	Specificity			0.76

Confusion matrices of the classification performances on training, test, and validation data with corresponding accuracy, sensitivity, and specificity

Table 5. Logistic regression parameters of fitting the severity state with RIPK3 and HMGB1 plasma levels.

	β	SE	Z value	2.5% CI	97.5% CI	p-value	significant	RIPK3 and HMGB1 plasma levels of the total measurements are included in a logistic regression to calculate the odds of disease-related severity change. The model parameters of the fit are presented (**p<0.001). β , regression coefficient; SE, standard error
(Intercept)	-2.970	0.413	-7.191	-3.816	-2.193	>0.001	***	
RIPK3	0.305	0.055	5.530	0.201	0.418	>0.001	***	
HMGB1	0.006	0.001	6.017	0.004	0.007	>0.001	***	

Table 6. Training set for COVID-19 severity classification using multiple inflammatory variables.

Data is split into severe and moderate as described in our main method section

Acc, accuracy

Table 7. Test set for COVID-19 severity classification using multiple inflammatory variables.

Variables	Accuracy (%)	Acc2.5% CI	Acc97.5% CI	p-value	Sensitivity	Specificity
HMGB1 + RIPK3 + PCT	0.931	0.862	0.972	0	0.966	0.884
HMGB1 + RIPK3 + IL-6 + PCT	0.931	0.862	0.972	0	0.966	0.884
HMGB1 + RIPK3 + CRP + IL-6 + PCT	0.931	0.862	0.972	0	0.966	0.884
HMGB1 + RIPK3 + PCT + LDH	0.931	0.862	0.972	0	0.966	0.884
HMGB1 + RIPK3 + IL-6 + PCT + LDH	0.931	0.862	0.972	0	0.966	0.884
HMGB1 + RIPK3 + CRP + IL-6 + PCT + LDH	0.931	0.862	0.972	0	0.966	0.884
HMGB1 + RIPK3 + IL-6 + PCT + Leukocyte count	0.931	0.862	0.972	0	0.966	0.884
HMGB1 + RIPK3 + CRP + IL-6 + PCT + Leukocyte count	0.931	0.862	0.972	0	0.966	0.884
HMGB1 + RIPK3 + IL-6 + PCT + LDH + Leukocyte count	0.931	0.862	0.972	0	0.983	0.86
HMGB1 + RIPK3 + CRP + PCT	0.921	0.85	0.965	0	0.948	0.884
HMGB1 + RIPK3 + PCT + Leukocyte count	0.921	0.85	0.965	0	0.948	0.884
HMGB1 + RIPK3 + CRP + PCT + Leukocyte count	0.921	0.85	0.965	0	0.948	0.884
HMGB1 + RIPK3 + PCT + LDH + Leukocyte count	0.921	0.85	0.965	0	0.966	0.86
HMGB1 + RIPK3 + CRP + PCT + LDH + Leukocyte count	0.921	0.85	0.965	0	0.966	0.86
HMGB1 + RIPK3 + CRP + IL-6 + PCT + LDH + Leukocyte count	0.921	0.85	0.965	0	0.966	0.86
HMGB1 + RIPK3 + CRP + PCT + LDH	0.911	0.838	0.958	0	0.948	0.86
HMGB1 + RIPK3 + CRP + IL-6 + LDH	0.891	0.813	0.944	0	0.931	0.837
RIPK3 + IL-6 + PCT + Leukocyte count	0.891	0.813	0.944	0	0.966	0.791
RIPK3 + CRP + IL-6 + PCT + Leukocyte count	0.891	0.813	0.944	0	0.966	0.791
HMGB1 + RIPK3 + CRP + IL-6 + LDH + Leukocyte count	0.891	0.813	0.944	0	0.931	0.837
HMGB1 + RIPK3 + CRP + IL-6	0.881	0.802	0.937	0	0.931	0.814
HMGB1 + RIPK3 + CRP + LDH	0.881	0.802	0.937	0	0.931	0.814
HMGB1 + RIPK3 + CRP + IL-6 + Leukocyte count	0.881	0.802	0.937	0	0.931	0.814
RIPK3 + PCT + Leukocyte count	0.881	0.802	0.937	0	0.966	0.767
RIPK3 + CRP + PCT + Leukocyte count	0.881	0.802	0.937	0	0.966	0.767
HMGB1 + CRP + IL-6 + PCT + Leukocyte count	0.881	0.802	0.937	0	0.948	0.791
HMGB1 + RIPK3 + LDH + Leukocyte count	0.881	0.802	0.937	0	0.931	0.814
HMGB1 + RIPK3 + CRP + LDH + Leukocyte count	0.881	0.802	0.937	0	0.931	0.814
RIPK3 + IL-6 + PCT + LDH + Leukocyte count	0.881	0.802	0.937	0	0.966	0.767
RIPK3 + CRP + PCT + LDH	0.871	0.79	0.93	0	0.966	0.744
HMGB1 + CRP + IL-6 + PCT + LDH	0.871	0.79	0.93	0	0.948	0.767
HMGB1 + RIPK3 + CRP + Leukocyte count	0.871	0.79	0.93	0	0.931	0.791
HMGB1 + IL-6 + PCT + Leukocyte count	0.871	0.79	0.93	0	0.948	0.767
HMGB1 + IL-6 + PCT + LDH + Leukocyte count	0.871	0.79	0.93	0	0.948	0.767
HMGB1 + CRP + IL-6 + PCT + LDH + Leukocyte count	0.871	0.79	0.93	0	0.948	0.767
RIPK3 + CRP + IL-6 + PCT + LDH + Leukocyte count	0.871	0.79	0.93	0	0.966	0.744
HMGB1 + RIPK3	0.861	0.778	0.922	0	0.897	0.814
HMGB1 + RIPK3 + CRP	0.861	0.778	0.922	0	0.914	0.791
HMGB1 + RIPK3 + IL-6	0.861	0.778	0.922	0	0.897	0.814

Variables	Accuracy (%)	Acc2.5% CI	Acc97.5% CI	p-value	Sensitivity	Specificity
HMGB1 + CRP + LDH	0.93	0.809	0.985	0	1	0.812
HMGB1 + CRP + IL-6 + LDH	0.93	0.809	0.985	0	1	0.812
HMGB1 + CRP	0.884	0.749	0.961	0	0.963	0.75
HMGB1 + IL-6	0.884	0.749	0.961	0	1	0.688
HMGB1 + CRP + IL-6	0.884	0.749	0.961	0	0.963	0.75
HMGB1 + PCT	0.884	0.749	0.961	0	1	0.688
HMGB1 + CRP + PCT	0.884	0.749	0.961	0	1	0.688
HMGB1 + IL-6 + PCT	0.884	0.749	0.961	0	1	0.688
HMGB1 + CRP + IL-6 + PCT	0.884	0.749	0.961	0	1	0.688
HMGB1 + IL-6 + LDH	0.884	0.749	0.961	0	1	0.688
HMGB1 + PCT + LDH	0.884	0.749	0.961	0	1	0.688
HMGB1 + CRP + PCT + LDH	0.884	0.749	0.961	0	1	0.688
HMGB1 + IL-6 + PCT + LDH	0.884	0.749	0.961	0	1	0.688
HMGB1 + CRP + IL-6 + PCT + LDH	0.884	0.749	0.961	0	1	0.688
HMGB1 + CRP + Leukocyte count	0.884	0.749	0.961	0	0.963	0.75
HMGB1 + IL-6 + Leukocyte count	0.884	0.749	0.961	0	1	0.688
HMGB1 + CRP + IL-6 + Leukocyte count	0.884	0.749	0.961	0	0.963	0.75
HMGB1 + CRP + PCT + Leukocyte count	0.884	0.749	0.961	0	1	0.688
HMGB1 + CRP + IL-6 + PCT + Leukocyte count	0.884	0.749	0.961	0	1	0.688
HMGB1 + CRP + LDH + Leukocyte count	0.884	0.749	0.961	0	0.963	0.75
HMGB1 + IL-6 + LDH + Leukocyte count	0.884	0.749	0.961	0	1	0.688
RIPK3 + IL-6 + LDH + Leukocyte count	0.884	0.749	0.961	0	0.926	0.812
HMGB1 + CRP + IL-6 + LDH + Leukocyte count	0.884	0.749	0.961	0	0.963	0.75
HMGB1 + CRP + PCT + LDH + Leukocyte count	0.884	0.749	0.961	0	1	0.688
HMGB1 + CRP + IL-6 + PCT + LDH + Leukocyte count	0.884	0.749	0.961	0	1	0.688
HMGB1 + RIPK3 + CRP	0.86	0.721	0.947	0.001	0.889	0.812
HMGB1 + RIPK3 + CRP + IL-6	0.86	0.721	0.947	0.001	0.926	0.75
RIPK3 + PCT	0.86	0.721	0.947	0.001	0.926	0.75
RIPK3 + CRP + PCT	0.86	0.721	0.947	0.001	0.926	0.75
HMGB1 + RIPK3 + CRP + LDH	0.86	0.721	0.947	0.001	0.889	0.812
RIPK3 + PCT + LDH	0.86	0.721	0.947	0.001	0.926	0.75
RIPK3 + CRP + PCT + LDH	0.86	0.721	0.947	0.001	0.926	0.75
HMGB1 + RIPK3 + Leukocyte count	0.86	0.721	0.947	0.001	0.926	0.75
RIPK3 + CRP + Leukocyte count	0.86	0.721	0.947	0.001	0.889	0.812
HMGB1 + RIPK3 + CRP + Leukocyte count	0.86	0.721	0.947	0.001	0.889	0.812
RIPK3 + CRP + IL-6 + Leukocyte count	0.86	0.721	0.947	0.001	0.889	0.812
HMGB1 + PCT + Leukocyte count	0.86	0.721	0.947	0.001	1	0.625
RIPK3 + CRP + PCT + Leukocyte count	0.86	0.721	0.947	0.001	0.926	0.75
HMGB1 + RIPK3 + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.926	0.75
RIPK3 + CRP + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.889	0.812

HMGB1 + RIPK3 + CRP + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.889	0.812
HMGB1 + RIPK3 + IL-6 + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.926	0.75
RIPK3 + CRP + IL-6 + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.889	0.812
HMGB1 + PCT + LDH + Leukocyte count	0.86	0.721	0.947	0.001	1	0.625
RIPK3 + PCT + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.926	0.75
HMGB1 + RIPK3 + PCT + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.926	0.75
RIPK3 + CRP + PCT + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.926	0.75
HMGB1 + RIPK3 + CRP + PCT + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.926	0.75
HMGB1 + IL-6 + PCT + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.963	0.688
RIPK3 + IL-6 + PCT + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.926	0.75
HMGB1 + RIPK3 + IL-6 + PCT + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.926	0.75
RIPK3 + CRP + IL-6 + PCT + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.926	0.75
HMGB1 + RIPK3 + CRP + IL-6 + PCT + LDH + Leukocyte count	0.86	0.721	0.947	0.001	0.926	0.75
HMGB1 + RIPK3	0.837	0.693	0.932	0.002	0.926	0.688
RIPK3 + CRP	0.837	0.693	0.932	0.002	0.889	0.75
HMGB1 + RIPK3 + IL-6	0.837	0.693	0.932	0.002	0.926	0.688
HMGB1 + RIPK3 + PCT	0.837	0.693	0.932	0.002	0.926	0.688

Data is split into severe and moderate

Acc, accuracy

Figures

Fig. 1

Figure 1

Elevated RIPK3, MLKL, HMGB1, and RIPK1 plasma levels in COVID 19 intensive care patients. Mean longitudinal levels of the COVID 19 cohort with 28 patients with moderate (green) and 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 levels are plotted by days after ICU admission. Significant differences between patients with moderate COVID 19 and healthy controls (Ctrl) were found on days 0 to 7 and 11 (RIPK3); 1 to 7 (MLKL and HMGB1); 1, 2, and 4 to 7 (RIPK1). Significant differences between patients with severe COVID 19 and healthy controls were found on days 1 to 16, 18, 21, 22, 24 to 27, and 33 (RIPK3); 1 to 9, 12, 14 to 16, and 19 (MLKL); 0 to 9, 11, 13 to 16, and 27 (HMGB1); 3, 5 to 10, 15, 20, 21, 25, and 28 (RIPK1). Statistical differences between plasma levels of patients with moderate and severe COVID 19 and 15 healthy controls (black dotted line) were assessed using unpaired two sided Student's t test; *p<0.05

Fig. 2

Figure 2

PCA of RIPK3, MLKL, HMGB1, and RIPK1 plasma levels in COVID 19 intensive care patients. (a) PCA variable correlations plot of RIPK3, MLKL, HMGB1, and RIPK1 plasma levels. With two dimensions, 62.5% of the variance is expressed. (b) PCA scatter plot of the three groups (healthy control=Ctrl, blue circle; moderate COVID 19=yellow triangle; severe COVID 19=orange square) with plasma levels of all four markers. (c) Bar plot of variables' (RIPK3, MLKL, HMGB1, and RIPK1) contribution to Dim1 in percentage. The red dashed line indicates the expected average contribution (25%). (d) Bar plot of the same variables' contribution to Dim2 in percentage. Dim, dimension; cos2, squared coordinates (quality of representation)

Fig. 3

Figure 3

Elevated RIPK3 and HMGB1 plasma levels in patients with severe COVID 19 progression. Box plots of RIPK3, MLKL, HMGB1, and RIPK1 plasma levels on (a) the last day on the ICU=E including 15 patients with moderate and 6 patients with severe COVID 19, (b) one day before=E 1 including 16 patients with moderate and 10 patients with severe COVID 19, (c) two days before=E 2 including 14 patients with moderate and 11 patients with severe COVID 19, and (d) three days before=E 3 including 13 patients with moderate and 8 patients with severe COVID 19. Statistical differences between patients with moderate and severe COVID 19 were assessed using an unpaired two sided Student's t test and were regarded significant at $p \leq 0.05$

Fig. 4

Figure 4

Classification evaluation. From the coefficients of the generalized linear model (GLM), a linear discriminator can be constructed and projected into the feature space (black line), constituted by the measured plasma levels of RIPK3 and HMGB1 (moderate=green, severe=red). (a) The training data from the days E 1 and E 3 that were used in building the classifier are shown in a scatterplot, separated by the linear classifier. (b) Projection of the test data into feature space, using the discriminator to evaluate the classification performance. Five red dots are classified as green (moderate) and three green dots are classified as red (severe). Some dots are close to the discriminator with high classification ambiguity. (c) Validation of the classifier using data from up to day E 7 (excluding E 1 and E 3). The fraction of false negatives is larger (11 dots) than the false positives (6 dots). (d) The classifier's mean prediction accuracy was plotted with 95% confidence intervals up to 14 days before the event (excluding E 2 and E 3). Mean RIPK3 (e) and HMGB1 (f) plasma levels of patients with moderate (green) and severe (red) COVID 19 are plotted from day E 14 until the censoring event with 95% confidence intervals. Mean plasma levels of healthy controls (Ctrl) are represented as black dashed lines

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [STROBEchecklistv4combined.docx](#)
- [SupplementNOTrackChanges.docx](#)