NK Cell Mitochondrial Membrane Potential-Associated Model Predicts Outcomes in Critically Ill Patients with COVID-19

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Purpose: This study investigated potential predictive models associated with natural killer (NK) cell mitochondrial membrane potential (MMP or ΔΨm) in predicting death among critically ill patients with COVID-19.

Patients and Methods: We included 97 patients with COVID-19 of different severities attending Peking Union Medical College Hospital from December 2022 to January 2023. Patients were divided into three groups according to oxygen and mechanical ventilation use during specimen collection and were followed for survival and death at 3 months. The lymphocyte subpopulation MMP was detected via flow cytometry. We constructed a joint diagnostic model by integrating identified key indicators and generating receiver operating curves (ROCs) and evaluated its predictive performance for mortality risk in critically ill patients.

Results: The NK-cell MMP median fluorescence intensity (MFI) was significantly lower in critically ill patients who died from COVID-19 (p<0.0001) and significantly and positively correlated with D-dimer content in critically ill patients (r=0.56, p=0.0023). The random forest model suggested that fibrinogen levels and NK-cell MMP MFI were the most important indicators. Integrating the above predictive models for the ROC yielded an area under the curve of 0.94.

Conclusion: This study revealed the potential of combining NK-cell MMP with key clinical indicators (D-dimer and fibrinogen levels) to predict death among critically ill patients with COVID-19, which may help in early risk stratification of critically ill patients and improve patient care and clinical outcomes.

Keywords: COVID-19, mortality prediction, NK cell, mitochondrial membrane potential, fibrinogen, D-dimer

Introduction

The coronavirus disease 2019 (COVID-19) has caused a global epidemic. Even though the pathogenicity of current strains has decreased compared to the wild-type virus, individuals with underlying health issues and the elderly may still face increased risk of mortality.[1–3] China experienced a widespread outbreak of Omicron BA.5.2 and BF.7 variants starting from December 2022.[4] Recent studies estimate that the surge in SARS-CoV-2 infections from December 2022 to February 2023 resulted in over one million deaths in China.[5] This highlights the importance of assessing disease severity and risk of death in hospitalized critically ill patients.

Mitochondrial membrane potential (MMP) is crucial in maintaining mitochondrial health. Detecting the MMP via fluorescent probes can quantify the mitochondrial damage,[6–8] which has started to be utilized for diagnosing and treating conditions like sepsis, schizophrenia, and HIV.[9–11]Recent studies suggest a possible association between the altered

MMP of lymphocyte subsets and COVID-19 progression. On one hand, dysregulation of the proportion and function of lymphocyte subsets, including T, B, and NK cells, occurs in COVID-19 patients.[12] On the other hand, there are abnormalities in lymphocyte energy metabolism and dysregulation of MMP in COVID-19 patients.[13,14] It is suggested that mitochondrial dysfunction of T cells, B cells, and NK cells is involved in the pathogenesis of COVID-19.[15–19] Research has also found that circulating NK cell mitochondrial mass is the best discriminative indicator of severe and moderate disease,[20] suggesting a role for lymphocyte MMP in disease progression in severe patients. However, changes in lymphocyte subset mitochondrial function in critically ill and deceased patients remain unclear.

In addition to changes in lymphocyte subsets and MMP, COVID-19 patients also experience multi-organ damage and metabolic disorders. For instance, critically ill patients may suffer from coagulation abnormalities[21] and cardiac dysfunction, while significant changes occur in infection indicators and inflammatory cytokine levels.[22,23] However, the relationship between these indicators and changes in lymphocyte MMP in critically ill patients, whether deceased or survived, remains unclear and has not been reported.

Given the potential significance of the MMP and its association with clinical indicators, we focused on investigating the NK cell MMP role as a predictive biomarker for the mortality risk in critically ill patients. By integrating the NK cell MMP with other relevant indicators, we developed a diagnostic model to rapidly identify patients with higher mortality risk. Incorporating these findings into clinical practice can help improve patient care and outcomes in the ongoing fight against COVID-19.

Materials and Methods

Study Population and Clinical Characteristics

This study included a cohort of patients who sought medical care for COVID-19 at Peking Union Medical College Hospital (Beijing, China) through outpatient or inpatient services from December 2022 to February 2023. Inclusion criteria comprised: (1) newly hospitalized patients over 18 years old; (2) confirmed SARS-CoV-2 infection via nucleic acid testing of nasopharyngeal swabs; (3) no prior antiviral treatment for COVID-19; (4) complete clinical history and examination information; and (5) absence of autoimmune diseases or tumors.

97 patients who met the inclusion criteria were categorized into three groups based on their oxygen supplementation and mechanical ventilation requirements at the sample collection time. The mild disease group consisted of 23 patients who did not require oxygen supplementation or mechanical ventilation. The severe disease group included 31 patients who required oxygen supplementation but did not require mechanical ventilation. The critically ill group comprised 43 patients who required mechanical ventilation.

After specimen collection, the patients were followed up for three months to record their survival outcomes. Among the 43 critically ill patients, 16 survived, whereas 27 died within three months. All the patients in the mild and severe disease groups survived. Basic patient information, including general characteristics and laboratory findings, was collected, and summarized in Table 1.

This study was approved by the Ethics Committee of Peking Union Medical College Hospital (K3600) and complies with the Declaration of Helsinki. All participating patients provided informed consent.

Flow Cytometry and Mitochondrial Membrane Potential Detection

Fresh peripheral blood samples collected in ethylene diamine tetra acetic acid K2 (EDTA-K2) tubes were used for flow cytometry analysis.

A total of 100 μL of well-mixed anticoagulated peripheral blood samples were added to corresponding flow tubes containing flow antibodies. The tubes were gently mixed and incubated in the dark for 15 min. Then, 2 mL of lysing solution was added, and the tubes were mixed again and incubated in the dark for an additional 15 min. The remaining lysed samples were discarded after centrifugation at 300 g for 5 min at 25 °C. The panel of flow antibodies included PE-CD3, PE-CD56, FITC-CD8, FITC-CD19, and PE-CY7-CD4 (UBBIO, Zhejiang, China). The average fluorescence intensity of the same fluorophore differs among different monoclonal antibodies, which is used to distinguish between antibodies in the same channel.[24] This flow cytometry antibody panel utilizing two markers within the same fluorescence

Table I General Characteristics and Laboratory Findings of Patients with Mild, Severe, and Critical COVID-19

Characteristics	Mild (n = 23)	Severe (n = 31)	Critical (n = 43)	p-value	Summary
Sex				0.0449	*
Male	12	21	35		
Female	11	9	8		
Age (years)	62.87 ± 15.86	71.87 ± 15.83	70.65 ± 15.62	0.0886	ns
PT	11.98 ± 1.01	12.68 ± 1.60	13.46 ± 2.38	0.0430	*
APTT	26.95 ± 3.46	28.89 ± 5.178	34.91 ± 8.11	<0.0001	****
TT	16.75 ± 1.56	23.46 ± 15.22	36.37 ± 41.45	0.0705	ns
D-Dimer	1.38 ± 1.58	4.67 ± 6.82	6.501 ± 7.15	0.0325	*
Fbg	4.36 ± 1.62	3.16 ± 1.68	3.03 ± 1.37	0.0232	*
INR	0.98 ± 0.09	1.04 ± 0.14	1.11 ± 0.21	0.0398	*
NT-proBNP	635.70 ± 709.50	2805.00 ± 6489.00	4078.00 ± 6160.00	0.1021	ns
hscTnI	22.44 ± 63.37	1050.00 ± 3950.00	235.80 ± 462.00	0.2270	ns
CK	52.00 ± 44.01	116.50 ± 156.60	835.50 ± 2983.00	0.2664	ns
Myo	55.00 ± 26.14	234.60 ± 324.60	2980.00 ± 9575.00	0.2490	ns
CRP	11.17 ± 16.27	37.94 ± 72.86	45.26 ± 51.99	0.0539	ns
PCT	0.12 ± 0.08	4.46 ± 18.65	0.87 ± 1.67	0.0085	**
IL6	25.94 ± 34.94	105.10 ± 204.90	365.60 ± 400.50	<0.0001	****
IL8	29.50 ± 17.82	93.85 ± 131.20	130.90 ± 132.50	<0.0001	****
IL10	5.00 ± 0.00	6.29 ± 3.12	12.34 ± 10.98	<0.0001	****
TNF-α	14.08 ± 5.95	15.79 ± 7.19	24.32 ± 23.63	<0.0001	****

Notes: Ordinary one-way ANOVA was used for statistical analysis; data are shown as mean ± standard deviation. *p < 0.05, **p < 0.01, ****p < 0.0001.
Abbreviations: APTT, activated partial thromboplastin time; CK, creatine kinase; CRP, C-reactive protein; Fbg, fibrinogen; hscTnI, high-sensitivity cardiac troponin I; IL, Interleukin; MFI, mean fluorescence intensity; MMP, mitochondrial membrane potential; myo, myoglobin; NK, natural killer; NT-proBNP, N-terminal pro-brain natriuretic peptide; PCT, procalcitonin; PT, prothrombin time; ROC, receiver operating characteristic; TNF, tumor necrosis factor. ns, not significant.

channel (Figure S1a) has been demonstrated to exhibit high concordance with the classic fluorescence antibody detection panel (Figure S1b) when assessing T cell (Figure S1c and S1d), NK cell (Figure S1e and S1f), and B cell (Figure S1g and S1h) subsets. The washed cells were resuspended in 200 μL of PBS and transferred to an 8-well centrifuge tube containing MitoDye (UBBIO, Zhejiang, China). The samples were mixed and incubated in a constant temperature incubator at 37 °C for 30 min. Then, the samples were analyzed using a flow cytometer (Cytek Biosciences, CA, USA), and MitoDye was detected in the same channel as APC. The gating strategy for detecting mitochondrial membrane potential (MMP) in lymphocyte subsets using MMP-MITO probes is depicted in Figure S2a–S2e.

Detection of Clinical Indicators

Prothrombin time (PT), Fbg level, activated partial thromboplastin time (APTT), thrombin time (TT), and D-dimer level were determined by Sysmex CS-5100 Automated Coagulation Analyzer (Sysmex, Kobe, Japan). High-sensitivity troponin I (hs-TnI), N-terminal pro-brain natriuretic peptide (NT-pro BNP), creatine kinase (CK), and myoglobin (Myo) levels were determined via a Siemens Atellica IM 1600 (Siemens Healthcare, Tarrytown, NY, USA). Serum levels of procalcitonin (PCT) were determined via the VIDAS PCT assay (bioMérieux, Marcy L'Etoile, France), and C-reactive protein (CRP) levels were tested via QuikRead go (Orion Diagnostica Oy, Espoo, Finland). Cytokine measurements of interleukin (IL)-6, IL-8, IL-10, and tumor necrosis factor-alpha (TNF-α) were performed using the IMMULITE 1000 Automated Immunoanalyzer (Siemens Healthcare).

Construction of a Random Forest Model and Diagnostic Models

A random forest model was built using Sklearn (version 1.0.2). Due to a small overall sample size, 50% of the samples were randomly selected as the training set, while the remaining 50% were used as the test set. The area under the curve

(AUC) of the final model and the feature importance of each metric were calculated. The above analysis was performed in *Python* (version 3.9.13).

Diagnostic models were created using R (version 4.2.2). The pROC package (version 1.18.0) in R was utilized for the multi-indicator combined diagnosis. After exporting the predicted values, receiver operating characteristic (ROC) curves were generated using GraphPad Prism *9*.

Statistical Analysis

The flow cytometry data were analyzed using NovoExpress software (version 1.4.1). The quantitative data were presented as the mean ± standard deviation, Unpaired t-tests or Mann–Whitney *U*-test was used in comparison between the two groups. Data was analyzed by one-way analysis of variance test among 3 or more groups. Categorical variables were using Fisher's exact test or chi square ($\chi2$) test. Pearson correlation analysis was conducted using the PerformanceAnalytics (version 2.0.4) package in *R* (version 4.2.2) for pairwise correlation analysis and visualization. Significant differences were defined as P < 0.05. Data comparisons and statistical graphs were generated using GraphPad Prism 9.

Results

MMP^{low} NK Cell Proportion is Significantly Reduced in Severe and Critically Ill Patients with COVID-19

Patients in the cohort were categorized into three groups (mildly, severely, and critically ill) based on oxygen supplementation or mechanical ventilation requirements. The counts and percentage of peripheral blood lymphocytes were significantly decreased in the severely and critically ill groups, with a greater decrease observed in the critically ill group than in the severely ill group (Figure S3a and S3b). Further flow cytometry analysis subdivided the lymphocytes into CD3^{+}T, CD3^{-}CD19^{+}B, and CD3^{-}CD56^{+}NK cell subsets. Peripheral blood NK cells exhibited a significant downward trend in severely and critically ill patients, whereas the proportion of B cells significantly increased (Figure 1A). The proportion of T cells in critically ill patients showed an upward trend but did not reach statistical significance (Figure 1A). Subsequently, a combined algorithm using both the flow cytometry measurements of the subset proportions and the absolute cell counts obtained from routine blood tests were applied to calculate the absolute values of the peripheral blood T, B, and NK cells. The absolute counts of NK and T cells were significantly decreased in severely and critically ill patients, whereas no significant difference was observed in the absolute count of B cells (Figure 1B).

Based on differences in the proportions and absolute counts of the lymphocyte subsets, we further investigated the MMP in the peripheral blood cell subsets of mildly, severely, and critically ill patients with COVID-19 using MMP probes. We found that the NK cells were the only subset showing a significant decrease in the MMP^{low} proportion in both severely and critically ill patients compared to mildly ill patients (Figure 1C). In contrast, the MMP^{low} subset of the B cells only exhibited a significant increase in severely ill patients compared with that in mildly ill patients (Figure 1C). There were no significant differences observed in the MMP MFI of T, B, and NK cell between the severely and critically ill patients with COVID-19 (Figure S2c–S2e).

MFI of NK Cell MMP is Decreased in Critically Ill Patients Who Did Not Survive

After assessing the MMP^{low} subset and the NK cell MMP MFI among the hospitalized patients, we followed up on their survival outcomes over three months. We observed that mortality only occurred in the critically ill patient group. Consequently, we analyzed the relevant indicators of the NK cell MMP in critically ill patients who either survived or did not survive. No significant differences were observed in the proportions and absolute counts of the NK cell subsets (Figure 2A and Table 2). However, there was a substantial upward trend in the MMP^{low} subset and a significant decrease in the NK cell MMP MFI (Figure 2B, 2C and Table 2).
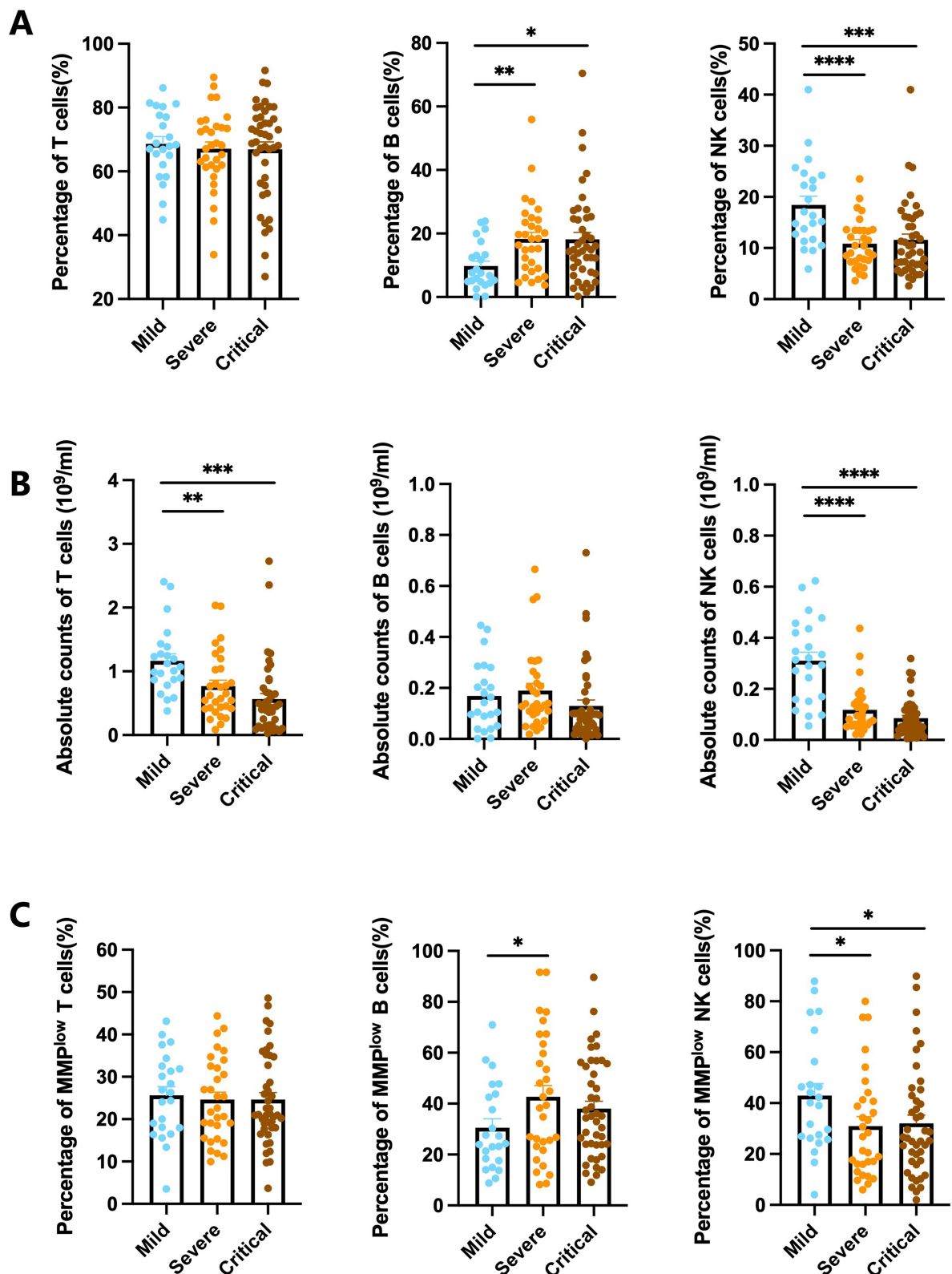
Figure 1 Changes in lymphocyte subset proportions and corresponding MMP in patients with mild, severe, and critical COVID-19. (**A**) Proportional of T, B, and NK subsets among lymphocyte; (**B**) absolute count of T, B, and NK subsets; (**C**) proportional of MMP^{low} cells in s T, B, and NK cells. Unpaired t-tests or Mann–Whitney *U*-test was used for statistical analysis among two groups: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
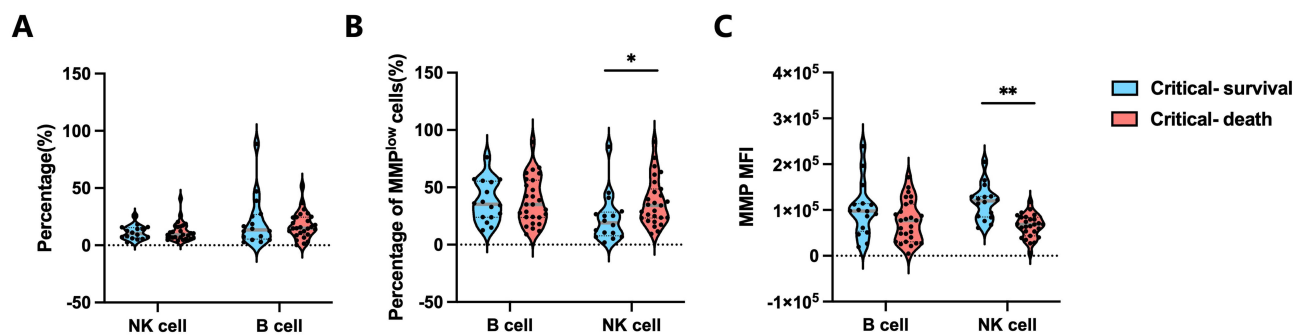
Figure 2 Differences in proportions and mitochondrial membrane potential of B and NK cell subsets between deceased and surviving critically ill patients. (**A**) Proportional of B and NK cell subsets among lymphocytes between deceased and surviving critically ill patients. (**B**) Proportional of of MMP^{low} B and NK cells in deceased and surviving critically ill patients. (**C**) MMP MFI within B and NK cell subsets in deceased and surviving critically ill patients. Unpaired t-tests or Mann–Whitney *U*-test were used for statistical analysis. Data shown as mean ± standard error mean, ns: not significant, *p < 0.05, **p < 0.01.

Correlation Between NK Cell MMP MFI and Clinical Indicators in Patients Who Died

Next, we analyzed the differences in clinical indicators between surviving and dead patients (Table 3). The coagulation indicator Fbg level showed a significant decrease in the number of patients who died, while the average content of D-dimer also exhibited a decreasing trend with a p-value of 0.06 (Figure 3A). The myocardial injury marker NT-proBNP content significantly increased in patients who did not survive, and the mean values of CK, hscTnI, and myo contents were also elevated in the group of patients who did not survive with no significant differences (Figure 3B). Similar

Table 2 General Characteristics and Laboratory Findings of Patients with Critical COVID-19 Who Survived or Died

Characteristics	Total Critical Patients (n = 43)		p-value	Summary
	Surviving (n = 16)	Dead (n = 27)		
Sex (%)			0.4433	ns
Male	12	23		
Female	4	4		
Age (years)	61.56 ± 19.35	76.04 ± 9.87	0.0023	**
PT	13.16 ± 1.91	13.64 ± 2.64	0.5259	ns
APTT	32.79 ± 6.26	36.17 ± 8.90	0.1902	ns
TT	38.30 ± 40.68	35.23 ± 42.63	0.8174	ns
D-Dimer	9.103 ± 10.36	4.96 ± 3.75	0.0656	ns
Fbg	3.96 ± 1.55	2.48 ± 0.89	0.0003	***
INR	1.08 ± 0.17	1.12 ± 0.24	0.5459	ns
NT-proBNP	1314.00 ± 1369.00	5614.00 ± 7210.00	0.0283	*
hscTnI	59.40 ± 50.01	333.80 ± 554.40	0.0643	ns
CK	413.70 ± 955.20	1089.00 ± 3708.00	0.4957	ns
Myo	486.50 ± 715.80	4434.00 ± 11,882.00	0.2250	ns
CRP	37.62 ± 35.67	49.79 ± 59.79	0.4648	ns
PCT	0.62 ± 0.61	1.02 ± 2.05	0.4754	ns
IL6	361.50 ± 422.20	368.00 ± 396.60	0.9620	ns
IL8	86.93 ± 56.03	157.60 ± 157.70	0.1168	ns
IL10	11.37 ± 13.89	12.92 ± 9.08	0.6831	ns
TNF-α	18.55 ± 6.88	28.16 ± 29.66	0.2441	ns

Notes: Unpaired two-tailed Student's *t*-tests were used for statistical analysis; data shown as mean ± standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001.
Abbreviations: APTT, activated partial thromboplastin time; CK, creatine kinase; CRP, C-reactive protein; Fbg, fibrinogen; hscTnI, high-sensitivity cardiac troponin I; IL, interleukin; MFI, mean fluorescence intensity; MMP, mitochondrial membrane potential; myo, myoglobin; NK, natural killer; NT-proBNP, N-terminal pro-brain natriuretic peptide; PCT, procalcitonin; PT, prothrombin time; ROC, receiver operating characteristic; TNF, tumor necrosis factor. ns, not significant.

Table 3 Feature Importance and Importance Rank in Random Forest Model

Importance Rank	Feature	Feature Importance	Importance Rank	Feature	Feature Importance
1	Fbg	0.1639	14	PLT	0.0275
2	NK MMP MFI	0.1534	15	hscTnI	0.0261
3	NT-pro BNP	0.0915	16	WBC	0.0232
4	TNF-α	0.0494	17	PCT	0.0228
5	IL-10	0.0465	18	EOS#	0.0198
6	NEUT#	0.0464	19	IL-6	0.0193
7	INR	0.0377	20	CK	0.0175
8	TT	0.0361	21	CRP	0.0171
9	D-Dimer	0.0346	22	PT	0.0117
10	MMP^{low} NK	0.0322	23	LYABS	0.0111
11	IL-8	0.0320	24	LY%	0.0111
12	MONO#	0.0289	25	Myo	0.0089
13	APTT	0.0279	26	BASO#	0.0033

Abbreviations: WBC, white blood cell count; LY%, lymphocyte percentage; LYABS, absolute lymphocyte count; NEUT#, neutrophil count; MONO#, monocyte count; BASO#, basophil count; EOS#, eosinophil count. APTT, activated partial thromboplastin time; Fbg, fibrinogen; hscTnI, high-sensitivity cardiac troponin I; IL, interleukin; MFI, median fluorescence intensity; MMP, mitochondrial membrane potential; MMPlow, subset of NK cells with low MMP; myo, myoglobin; NK, natural killer; NT-proBNP, N-terminal pro-brain natriuretic peptide; PCT, procalcitonin; PT, prothrombin time; TNF, tumor necrosis factor; INR, international normalized ratio.

patterns were observed for the infection markers CRP and PCT, as well as cytokines IL-6, IL-8, IL-10, and TNF, with increased average levels but no significant differences (Figure 3C and 3D).

We further analyzed the correlation between the NK cell MMP MFI and the clinical indicators. Correlation between NK cell MMP MFI and age, gender was not found in patients who died (Figure S4). In the overall cohort of total critically ill patients, the NK cell MMP MFI showed a weak positive correlation with D-dimer content (r = 0.39, p = 0.01) and a weak negative correlation with NT-proBNP content (r = −0.33, p = 0.04) (Figure S5a–S5c and Table S1). Among the patients who did not survive, a stronger correlation was detected between the NK cell MMP MFI and D-dimer content, with an R-value of 0.5617 and a p-value of 0.0023 (Figure 4A, 4B and Table S2) while no correlation was found between NK cell MMP MFI and cytokines or myocardial injury markers (Figure S5d and S5e).

NK Cell MMP and Clinical Indicators in Predicting Mortality: Insights from Random Forest Model

We randomly selected 50% of the critically ill patients as the training set to construct a random forest model, which yielded a diagnostic model with an AUC of 0.81. Within this random forest model, the NK cell MMP MFI was the second most important predictor, with a feature importance ranking of 0.1534. The Fbg content was the most important feature, with a feature importance ranking of 0.1639. Other features included in the random forest model and their importance are listed in Table 3.

Establishment of Diagnostic Model Containing NK Cell MMP and Clinical Indicators

We further investigated the independent and combined predictive effects of the NK cell MMP MFI and the laboratory markers identified in the above analysis. Through ROC analysis, we found that the AUC of the NK cell MMP MFI was 0.87 (95% CI: 0.7587 to 0.9820), that of the D-dimer content was 0.60 (95% CI: 0.4182 to 0.7785), and that of the Fbg content was 0.81 (95% CI: 0.6405 to 0.9706) (Figure 5A–5C). The combined prediction model involving the NK cell MMP MFI, Fbg content, and D-dimer content achieved the best prediction Results, reaching an AUC of 0.94 (95% CI: 0.8627 to 1.000) (Figure 5D).
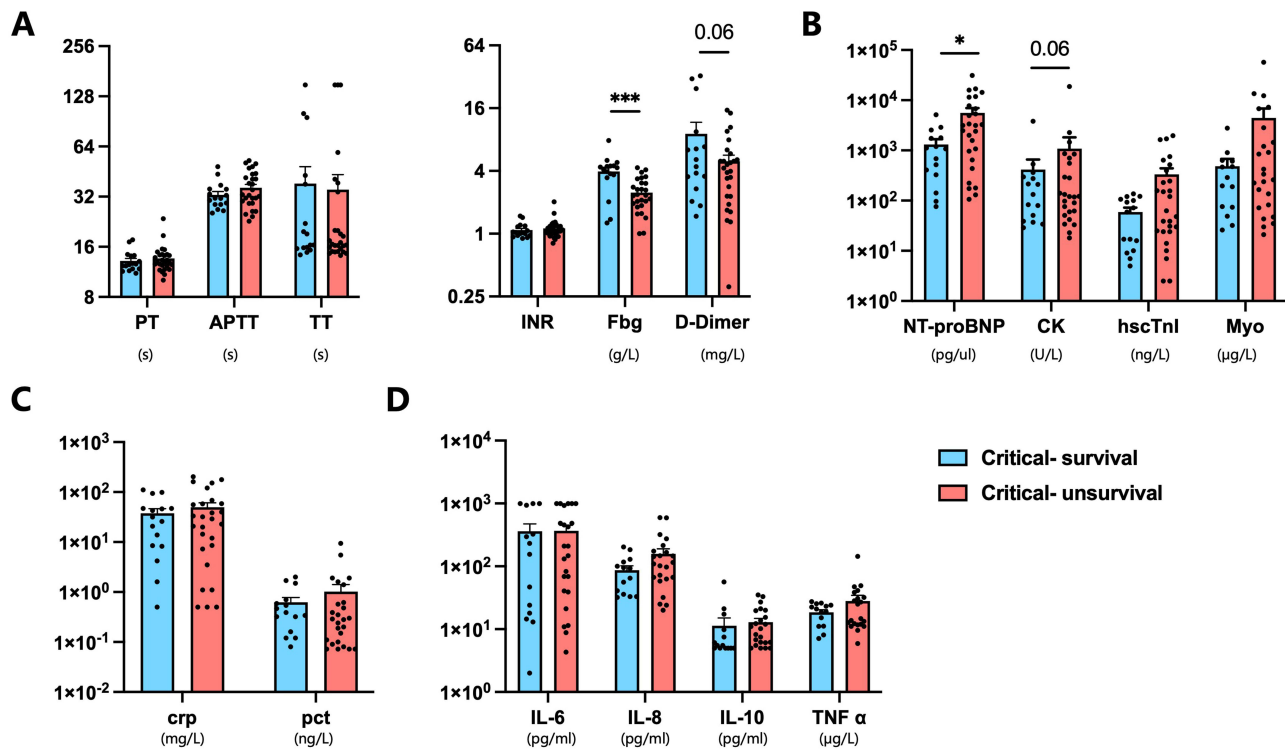
Figure 3 Changes in clinical parameters in deceased and surviving patients with critical COVID-19. (**A**) Levels of coagulation markers PT, APTT, TT, INR, Fbg, and D-dimer in deceased and surviving critically ill patients. (**B**) Levels of myocardial injury markers NT-proBNP, CK, hscTnI, and Myo in deceased and surviving critically ill patients. (**C**) Levels of infection-related markers CRP and PCT and coagulation markers PT, APTT, TT, INR, Fbg, and D-dimer between patient groups. (**D**) Differences in levels of cytokines IL-6, IL-8, IL-10, and TNF-α between patient groups. Unpaired t-tests or Mann–Whitney *U*-test were used for statistical analysis. Data shown as mean ± standard error mean, ns: not significant, *p < 0.05.
Abbreviations: APTT, activated partial thromboplastin time, CK, creatine kinase; CRP, C-reactive protein; Fbg, fibrinogen; hscTnI, high-sensitivity cardiac troponin I; IL, interleukin; MFI, median fluorescence intensity; MMP, mitochondrial membrane potential; myo, myoglobin, NT-proBNP; N-terminal pro-brain natriuretic peptide; PCT, procalcitonin; PT, prothrombin time; TT, thrombin time; TNF, tumor necrosis factor.
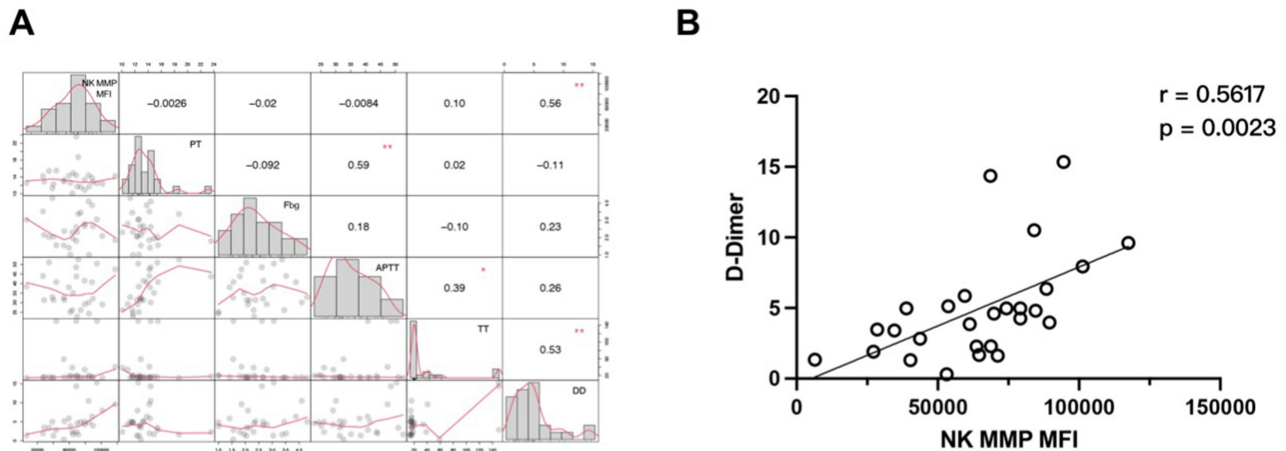

Figure 4 Correlation analysis between MMP MFI of NK cells in deceased patients and coagulation-related clinical parameters. (**A**) Overview of pairwise correlation analysis between NK cell MMP MFI and coagulation-related clinical parameters (PT, APTT, Fbg, TT, and D-dimer levels) in deceased critically ill patients. (**B**) Correlation analysis between NK cell MMP MFI and D-dimer levels in deceased critically ill patients. Pearson's correlation analysis was used for analyses.
Abbreviations: APTT, activated partial thromboplastin time; TT, thrombin time; Fbg, fibrinogen; MFI, median fluorescence intensity; MMP, mitochondrial membrane potential; PT, prothrombin time.

Discussion

This study aimed to investigate the changes in the MMP of NK cells among critically ill patients with COVID-19 and evaluate the predictive function of a model constructed using the NK cell MMP MFI and key clinical indicators for
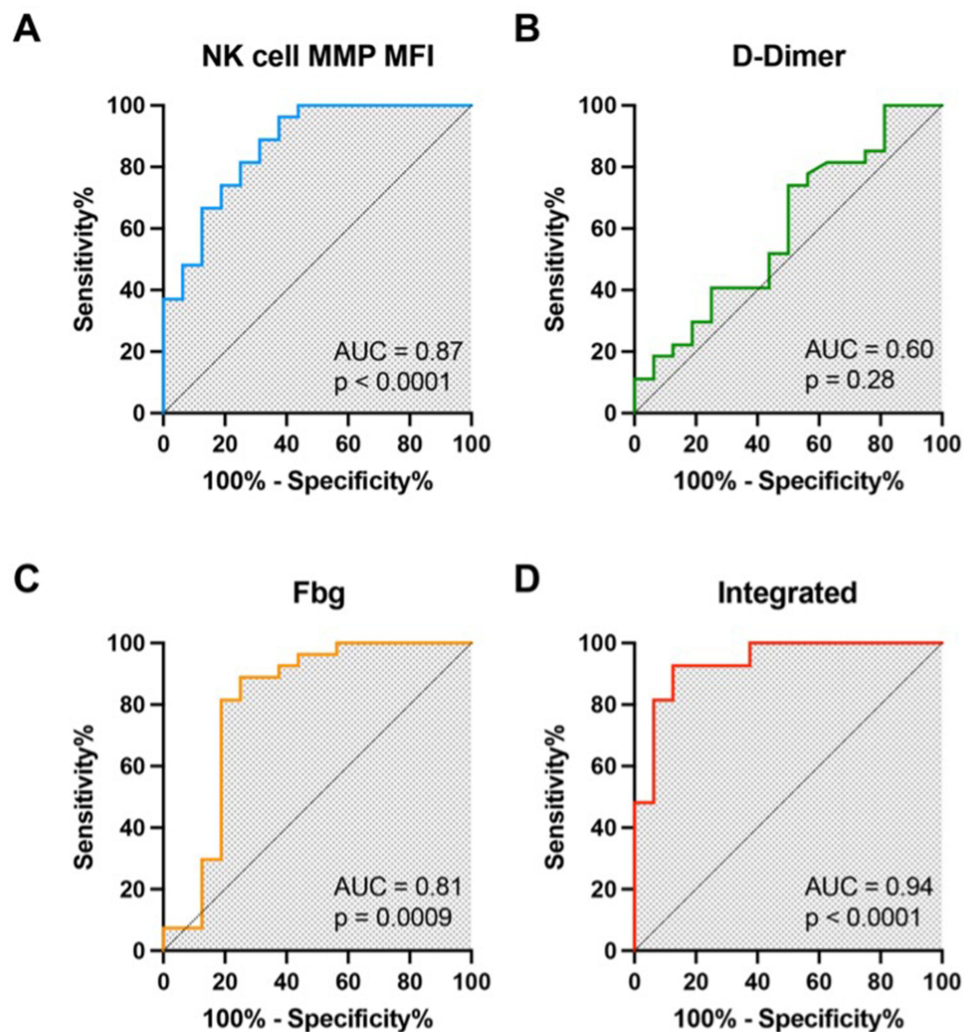
Figure 5 Diagnostic value and combined diagnostic model of NK cell MMP MFI and clinical features. (**A-C**) ROC curve of NK cell MMP MFI (a), D-dimer level (b), and Fbg level (c) to distinguish between death and survival of critically ill patients. (**D**) ROC curve of combined diagnostic model including NK cell MMP MFI, Fbg level, and D-dimer level.
Abbreviations: ROC, receiver operating characteristic; NK, natural killer; MMP, mitochondrial membrane potential; MFI, median fluorescence intensity.

mortality in these patients. We confirmed previous research findings regarding the MMP, suggesting alterations in the MMP in disease states.[25–27] We revealed an abnormal decrease in the NK cell MMP MFI in critically ill patients with COVID-19 who did not survive. We integrated this indicator with the coagulation indicators identified through correlation analysis and a random forest model for mortality diagnosis, providing novel insights for mortality prediction in critically ill patients with COVID-19.

An association exists between decreased NK cell counts and the dysfunction and severity of COVID-19. Mitochondria are associated with various cellular and metabolic functions, including energy production and cellular respiration; therefore, mitochondrial dysfunction is closely associated with altered cellular function.[28,29] Inflammation, increased metabolic changes, and persistent mitochondrial dysfunction occur in patients with severe COVID-19.[30] COVID-19 induces an elevated inflammatory and oxidative state, leading to mitochondrial dysfunction and subsequent cell death.[31]

MMP, an indicator of mitochondrial function, can be detected using novel fluorescent probes via flow cytometry, and changes in MMP can be quantified via fluorescence intensity.[32] Elevated inflammatory and oxidative states of individual T cells predict sepsis well, and combining MMP fluorescence intensity with PCT, an indicator of infection, has better diagnostic efficacy.[9] Recent studies have also found that the mitochondrial function of circulating NK cells can

distinguish between severe and moderate disease.[20] Our study supports and complements these findings; in the critically ill patient group, the MMP of the NK cells in the patients who did not survive significantly decreased.

In previous studies as well as our own research, a higher proportion of males among deceased patients has been observed.[33,34] However, no differences in NK cell MMP MFI between genders were observed in both mild, severe, surviving critically ill, and deceased critically ill cases. This suggests that gender is not a confounding factor affecting the predictive ability of NK cell MMP MFI for mortality risk in critically ill patients. We also observed a negative correlation between NK cell MMP MFI and age in critically ill patients, which is consistent with our finding that critically ill deceased patients tend to be older with lower NK cell MMP MFI. The above findings also complement the existing literature by highlighting the predictive value of the NK cell MMP MFI in identifying critically ill patients at a higher risk of death (Figures 1 and 2).

Critically ill patients with COVID-19 show decreased platelet MMP compared to healthy individuals and non-ICU patients,[35] indicating a possible correlation between the MMP and anticoagulant indicators. We conducted a comprehensive analysis that included hemostasis, myocardial injury, cytokine, and infection markers in critically ill patients with COVID-19. Correlation analysis revealed a significant positive correlation between the NK cell MMP and D-dimer levels, particularly in critically ill patients who did not survive, with a correlation coefficient of 0.56, suggesting a potential association between the NK cell MMP and blood clot formation and dissolution.

Patients with COVID-19 exhibit severe thrombus formation in the lungs, along with mild hypercoagulability, infiltration, and endothelial cell dysfunction in the heart.[36] This is consistent with the findings of our study; in patients with mild, severe, and critical COVID-19, there is a progressive and significant increase in PT, APTT, and D-dimer levels, accompanied by a decrease in Fbg content, suggesting a potentially extensive consumption of clotting factors. However, among the critically ill patients who survived and died, the disparities related to coagulation primarily revolved around a marked reduction in Fbg. This result suggests that Fbg is a better predictor of mortality in critically ill patients than other markers. Our subsequent analyses further validated this observation; in the mortality prediction model constructed using the random forest analysis, the Fbg exhibited the highest degree of feature importance.

In our study, the AUC for the NK cell MMP was 0.87, indicating its moderate predictive ability for the mortality of critically ill patients with COVID-19. The combined diagnostic model incorporating D-dimer, Fbg, and NK cell MMP levels had a better predictive ability with an AUC of 0.94 based on the ROC analysis. Metabolites enriched in mitochondria-related pathways in the peripheral blood can predict death in patients with sepsis.[37] Our findings are consistent with those of this previous study, highlighting the potential role of mitochondrial function in predicting patient outcomes. D-dimer and Fbg levels are also closely associated with mortality in COVID-19,[38,39] supporting their inclusion in our diagnostic model. Including these coagulation markers in conjunction with the NK cell MMP strengthens the predictive power of the model, enabling a more accurate assessment of mortality risk in critically ill patients with COVID-19.

Our study had several limitations. The sample size was relatively small, and the study was conducted in a single center. Therefore, the generalizability of our findings to larger populations and different settings requires further investigation. Despite the limitations, our study provides important insights into the potential utility of the NK cell MMP as a prognostic biomarker in critically ill patients with COVID-19. The non-invasive NK cell MMP MFI assessment using flow cytometry makes it a promising tool for risk stratification and clinical decision-making. The prospective detection of the NK cell MMP MFI, combined with the routine clinical indicators of D-dimer and Fbg levels in a diagnostic model, allows the early identification of patients at a higher mortality risk. This early identification enables timely interventions to improve patient outcomes.

Conclusion

In summary, our research findings indicate a significant decrease in the NK cell MMP MFI, which can be utilized for early mortality prediction in critically ill patients with COVID-19. The diagnostic model combining the NK cell MMP MFI, Fbg level, and D-dimer level demonstrates superior performance compared to the individual diagnostics. Therefore, using these biomarkers to construct a diagnostic model for critical COVID-19 cases can further enhance diagnostic

performance, risk assessment, and development of personalized treatment strategies. Ultimately, this will improve patient care and treatment outcomes in managing COVID-19.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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