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T Lymphocyte Mitochondrial Markers as Independent Risk Factors for Poor Prognosis of COVID-19

Mengying Yang*, Qianqian Li*, Mengxin Huang, Xiaoman Liu, Baogui Wang

Department of Infectious Disease, Fuyang People's Hospital, Fuyang, Anhui, People's Republic of China

*These authors contributed equally to this work

Correspondence: Baogui Wang, Department of Infectious Disease, Fuyang People's Hospital, No. 501 Sanqing Road, Yingzhou District, Fuyang City, Anhui Province, 236000, People's Republic of China, Tel +86-13805582057, Email wangbaogui99@163.com

Background: Severe Acute Respiratory Syndrome Virus 2 (SARS-CoV-2) primarily targets mitochondria. However, the description of mitochondrial signaling in immune cells remains limited in COVID-19. This study aimed to elucidate the pivotal roles played by immune cells and mitochondria in the pathogenesis of COVID-19 and the resulting clinical outcomes.

Methods: We obtained epidemiological characteristics, laboratory parameters and T cell mitochondrial damage indicators in 296 COVID-19 patients. And we further evaluated the predictive value of novel T lymphocyte mitochondrial markers and conventional immune inflammatory markers as clinical outcomes in COVID-19 patients. Finally, Binary logistic regression analysis was conducted to identify the independent risk factors associated with the prognosis of patients with COVID-19.

Results: The severe group exhibited lower counts of Mito+CD3+, Mito+CD4+, and Mito+CD8+ cells compared to the non-severe group. Significantly higher positive rates of CD3+, CD3+CD4+, and CD3+CD8+T cell mitochondrial damage were observed in the severe group compared to the non-severe group. The CD3+CD8+T cells MMP-low% had the highest AUC value of 0.864 (95% CI =0.794–0.934) to evaluate COVID-19 outcome. Binary logistic regression analysis showed that CD3+T cells MMP-low%, CD3+CD4 +T cells MMP-low% and CD3+CD8+T cells MMP-low% were independent risk factors for adverse outcomes in COVID-19 patients.

Conclusion: Our research suggests that a substantial proportion of COVID-19 patients exhibited mitochondrial impairment with T-lymphocyte. T cells mitochondrial markers can serve as predictive factors and independent risk factors for predicting adverse outcomes in COVID-19 patients.

Keywords: COVID-19, SARS-Co V-2, mitochondrial damage, T cell subpopulations, prognosis

Introduction

Novel coronavirus disease 2019 (COVID-19), caused by Severe Acute Respiratory Syndrome Virus 2 (SARS-CoV-2), is a novel and highly infectious respiratory disease associated with rapid transmission and severe health complications.[1] Severe cases of COVID-19 frequently exhibit a phenomenon referred to as a "cytokine storm" and heightened inflammation due to significant immune dysfunction. Monitoring the immune responses of patients with COVID-19 is essential for accurately predicting disease severity and clinical outcomes. T-cell responses can be detected in nearly all individuals infected with SARS-CoV-2.[2–4] Severe COVID-19 patients have consistently presented with lymphopenia or lower T-cell counts, serving as a potential predictor of disease severity.[5] Severe cases are marked by a significant decline in CD4+ and CD8+ T lymphocytes, and the deficiency of SARS-CoV-2-specific CD4+ T cells is closely associated with the disease progression.[6] Those requiring intensive care support exhibit deficiencies in CD4+ and CD8+ T lymphocytes, humoral immune deficiencies, and a pro-inflammatory immune phenotype.[7] However, the precise molecular mechanisms underlying T-cell depletion and impaired immune responses remain incompletely understood in COVID-19.

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The interplay of viral infection, mitochondria, and the immune response constitutes a complex regulatory network where mitochondrial signaling profoundly influences the survival, activity, and functional regulation of immune cells. The host's immune cells and their mitochondrial system are likely pivotal in viral infection and the ultimate disease outcome. Mitochondrial and mitochondrial damage-associated molecular patterns (mtDAMPs), such as mitochondrial DNA (mtDNA), cardiolipin, n-formyl peptides, and cytochrome c, have been recognized for their essential roles in orchestrating the host's immune response against various viral infections.[8] Recent studies have elucidated the critical role of mitochondria in SARS-CoV-2 infection. SARS-CoV-2 is known to target host mitochondria,[9] representing a central factor in the pathogenesis of COVID-19. The viral proteins and RNA are localized in the mitochondria of host cells, where they hijack the mitochondrial structures of immune cells for their proliferation, consequently impairing mitochondrial function.[10,11] Given the significant role of mitochondria in SARS-CoV-2 infection, they have emerged as a potential target for therapeutic interventions in COVID-19. However, there is still a lack of sufficient clinical research to evaluate the efficacy of immune cell mitochondrial function in immune surveillance of COVID-19 patients, as well as its role in predicting disease severity and clinical outcomes. Our study analyzed the T lymphocyte subpopulation in COVID-19 patients using flow cytometry, combined with mitochondrial damage detection, to confirm the critical role of immune cells and their mitochondria in the pathogenesis and prognosis of COVID-19 patients with different severities, aiming to elucidate the important role of mitochondrial damage in the cross-talk between SARS-CoV-2 infection and host immunity.

Methods

Patients' Involvement

This retrospective study involved 296 hospitalized patients diagnosed with COVID-19 at Fuyang People's Hospital from December 2022 to March 2023. The cohort comprised 190 non-severe cases, including 113 mild and 77 moderate cases, and 106 severe cases, including 80 severe and 26 critical cases. The diagnosis of active COVID-19 was confirmed by SARS-CoV-2 nucleic acid testing of nasal and pharyngeal throat swab specimens using real-time RT-PCR assay, and the severity of COVID-19 was classified according to National Health Commission of China Diagnosis and Treatment Protocol for SARS-CoV-2 Infection (Trial Version 10). The diagnostic and classification criteria for this study adhered to the guidelines outlined in the Novel Coronavirus Infection Diagnosis and Treatment Protocol Trial Version 10, as promulgated by the Chinese National Health Commission (General Office of the National Health Commission and Comprehensive Department of the National Administration of Traditional Chinese Medicine Diagnosis and Treatment Plan for Novel Coronavirus Infection [EB/OL][2023–03-14]). Cases with substantial missing clinical data were excluded from the analysis. The study was reviewed and approved by Medical Ethics Committee of Fuyang People's Hospital (Ethical Approval Reference Number [2021] No. 27). Due to the fact that this study is only a retrospective study and only collects some clinical data and indicator data retrospectively, without intervening in the patient's treatment process, in view of the above reasons, after review and approval by the Medical Ethics Committee of Fuyang People's Hospital, the patient's informed consent was exempted.

Data Collection

Data for this study were extracted from the hospital's electronic medical record system by two attending physicians. Baseline epidemiological data, including age, gender, and comorbidities, were recorded. All blood samples from patients were collected within 24 h of admission, and conventional testing indicators like blood routine, C-reactive protein, liver and kidney function, coagulation function and myocardial enzyme spectrum as well as T lymphocyte mitochondrial damage project were measured. A comprehensive panel of laboratory indicators was collected and analyzed, including counts of white blood cells, neutrophils, lymphocytes, and platelets, levels of C-reactive protein (CRP), glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, creatine kinase, lactate dehydrogenase, creatine kinase myocardial band (CK-MB) isoenzyme, D-dimers, and Peripheral blood-derived inflammatory immune indicators, including Neutrophils/Lymphocytes Ratio (NLR), Platelets /Lymphocytes Ratio (PLR), Platelets*Neutrophils/Lymphocytes (systemic immune inflammation index, SII), Neutrophils/[White blood cells-neutrophils] (dNLR), Neutrophils*monocytes/lymphocytes

(SIRI) and Neutrophils* monocytes*platelets/lymphocytes (PIV). Flow cytometry was utilized to assess peripheral blood T lymphocyte subsets and mitochondrial damage indicators, encompassing absolute counts and percentages of CD3+, CD3+CD4+, and CD3+CD8+T cells, along with TC mitochondrial damage index, TH mitochondrial damage index, TS mitochondrial damage index, CD45 lymphocyte percentage, and CD4/CD8 ratio, as well as T lymphocytes mitochondrial membrane potential low percentage (MMP-low%). The clinical outcomes of 296 inpatients were statistically analyzed.

Flow Cytometry Detection of Peripheral Blood T Lymphocytes and Mitochondrial Membrane Potential Low Percentage (MMP-Low%)

Peripheral blood samples were collected in EDTA anticoagulant tubes, and the following procedural steps were performed: (1) The mitochondrial staining reagent was extracted from the 96-well plate stored at −20°C and shielded from light by wrapping it in aluminum foil. The 96-well reagent plate, once restored to room temperature, was placed in a centrifuge at 250g for 1 minute; (2) A volume of 20 μL from the "pre-mixed reagent" was dispensed into the corresponding well of the 96-well plate; (3) 100 μL of anticoagulated human peripheral blood sample, previously inverted at least seven times, was placed into the 96-well plate. The plate was positioned on a 96-well plate mixer in a light-protected environment. The mixer was set to medium speed, and the mixture was incubated at room temperature for 15 minutes in darkness; (4) 400 μL of hemolysin (Nuclear Histo-Cyto Lysis Solution, 10×) working solution (preparation method: three parts of NH Lysis Solution 10× were combined with seven parts of purified water; the solution was mixed at room temperature, and placed aside) was added to a 96-well tube, which was subsequently placed on a 96-well plate mixer. The mixer was adjusted to medium speed and incubated at room temperature for 15 minutes. Consequently, the sample was prepared for machine-based detection. The principle of mitochondrial function detection in this study relies on a small molecular ionic probe that permeates the inner mitochondrial membrane through cellular osmosis. On entering the inner mitochondrial membrane, the probe's methylene chloride component facilitates the active methylene and sulfur groups to attach to the polypeptide proteins in the inner membrane, culminating in a stable complex structure and exhibiting corresponding fluorescence intensity. Following staining with the dye, cells can be fixed without causing the mitochondrial probes to fade. Using CytekTM NL-CLC full spectrum flow cytometry to detect mitochondrial membrane potential of peripheral blood T lymphocytes (MMP), The detection reagent is CD8+19 FITC/CD3+56 PE/CD45 PC5 5/CD4 PC7/MitoDye detection kit [Pan Peptide Biotechnology (Zhejiang) Co., Ltd.] Company], in which mitochondrial mass is expressed as MMP, mitochondrial membrane potential low percentage (MMP-low%) refers to the percentage of cells with low mitochondrial membrane potential in the total number of such cells, The higher the value, the more likely it is that the body may be in a state of immune suppression and immune exhaustion.

Statistical Analysis

Statistical analysis was conducted using SPSS 26.0 software. All measurement data underwent a normality test, applying the Shapiro–Wilk method before analysis. Data adhering to a normal distribution were expressed as $\bar{x} \pm s$. The independent samples *t*-test was employed to compare different groups. Data not conforming to a normal distribution were presented as median and quartiles (M [P25, P75]); relevant inter-group comparisons were performed using the Mann–Whitney *U*-test. Enumeration data were represented as frequencies and constituent ratios. Groups were compared through the chi-square test or Fisher's exact probability method. The area under the curve (AUC) of the receiver operating characteristic curve (ROC) was calculated to assess the performance in the prediction of fatal outcomes. Univariate and multivariate logistic regression analysis was employed to explore the independent risk factors associated with the prognosis of those diagnosed with COVID-19. All variables were modeled separately to avoid collinearity. Statistical significance was set at $p < 0.05$.

Results

Demographic Characteristics

Table 1 illustrates the demographic characteristics of the severe and non-severe groups. The severe group exhibited an older median age compared to the non-severe group (76.00 vs 70.50) ($p<0.001$). The proportion of male in the severe

Table 1 Demographics Characteristics

Demographics Characteristics	Total Number of Cases (n=296)	Non-severe Group (n=190)	Severe Group (n=106)	Statistical Value	P-value
Age (years)	72.00(63.25,80.00)	70.50(59.00,78.00)	76.00(69.00,84.00)	−4.286	<0.001
Gender n (%)					
Male	174 (58.78%)	102 (53.68%)	72 (67.92%)	5.695	0.017
Comorbidities n (%)					
Hypertension	123 (41.55%)	71 (37.37%)	52 (49.06%)	3.827	0.050
Diabetes	68 (22.97%)	34 (17.89%)	34 (32.08%)	7.732	0.005
Cardiovascular disease	68 (22.97%)	30 (15.79%)	38 (35.85%)	15.472	<0.001
Chronic pulmonary disease	95 (32.09%)	48 (25.26%)	47 (44.34%)	11.361	0.001
Cerebrovascular disease	25 (8.45%)	16 (8.42%)	9 (8.49%)	0.000	0.984

group is higher than that in the non-severe group (67.92% vs 53.68%) ($p<0.05$). In the severe group, the proportions of hypertension, chronic pulmonary disease, diabetes, cardiovascular disease, and cerebrovascular disease were more significant than those in the non-severe group. Specifically, the proportions of diabetes, chronic pulmonary disease, and cardiovascular disease exhibited substantial differences between the two groups ($p=0.005$, $p<0.001$, $p=0.001$, respectively).

Laboratory Indices and Clinical Outcomes

As shown in Table 2, the severe group exhibited higher levels of white blood cells and neutrophils than the non-severe group. Conversely, the severe group showed significantly lower counts of lymphocytes and platelets ($p<0.001$, $p<0.001$, $p<0.001$, $p<0.001$, respectively). Furthermore, glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, creatine kinase, lactate dehydrogenase, CK-MB, D-dimer, and CRP levels were substantially elevated in the severe group

Table 2 Laboratory Indices and Clinical Outcome

Laboratory Indices	Normal Range	Total Number of Cases (n=296)	Non-severe Group (n=190)	Severe Group (n=106)	Statistical Value	P-value
White blood cells	(3.5–9.5)*10^9/L	6.71(4.75,9.58)	5.88(4.54,8.32)	8.32(5.57,11.93)	−4.646	<0.001
Neutrophils	(1.8–6.3)*10^9/L	5.04(3.12,7.54)	3.84(2.87,6.11)	6.80(3.97,10.46)	−5.746	<0.001
Lymphocytes	(1.1–3.2)*10^9/L	1.06(0.63,1.56)	1.24(0.81,1.69)	0.64(0.46,1.12)	6.636	<0.001
Platelets	(125–350)*10^9/L	185.50(131.00,245.00)	196.00 (149.75,249.25)	144.50 (93.75,223.50)	3.676	<0.001
C-reactive protein	(0.0–5.0)mg/L	28.05(6.98,81.70)	15.93(4.18,47.98)	63.97 (26.66,140.56)	−6.774	<0.001
Glutamic-pyruvic transaminase	(9.0–50)U/L	20.55(12.90,36.08)	19.30(12.60,31.55)	25.40(13.47,48.35)	−2.362	0.018
Glutamic oxaloacetic transaminase	(15–40)U/L	22.45(16.65,37.45)	20.95(15.28,29.50)	32.05(20.98,52.93)	−5.511	<0.001

(*Continued*)

Table 2 (Continued).

Laboratory Indices	Normal Range	Total Number of Cases (n=296)	Non-severe Group (n=190)	Severe Group (n=106)	Statistical Value	P-value
Creatine kinase	(50–310) U/L	49.35(33.25,106.38)	46.95(32.67,78.70)	66.20 (33.50,184.35)	−2.979	0.003
Lactate dehydrogenase	(120–250)U/L	232.70(181.50,316.25)	207.35 (171.50,252.38)	332.45 (237.42,451.85)	−8.583	<0.001
Creatine kinase isoenzyme MB	(0.8–5.0)ng/mL	2.41(1.65,3.62)	2.12(1.47,3.07)	3.25(1.96,5.36)	−5.305	<0.001
D-dimer	(0.0–0.5)mg/L	0.76(0.35,2.09)	0.54(0.31,1.14)	1.72(0.80,4.12)	−7.229	<0.001
Peripheral blood-derived inflammatory immune indicators						
NLR		4.54(2.56,8.88)	4.21(2.40,8.66)	5.54(2.72,10.09)	−1.738	0.082
PLR		179.31(99.36,337.84)	157.81 (79.04,285.14)	188.99 (110.46,364.51)	2.08	0.037
SII		808.42 (408.52,1704.05)	789.02 (365.26,1713.44)	832.33 (440.64,1709.67)	0.512	0.609
dNLR		2.81(1.64,5.01)	2.61(1.53,4.66)	3.06(1.78,6.43)	−2.042	0.041
SIRI		1.96(0.88,4.31)	1.54(0.79,3.00)	3.68(1.49,6.66)	−5.634	< 0.001
PIV		317.46(140.77, 786.32)	277.32(136.75, 560.84)	444.59(154.02, 1282.81)	−2.916	0.004
Clinical outcomes					59.838	<0.001
Discharge after improvement		266 (89.86%)	190 (100.00%)	76 (71.70%)		
Died		30 (10.14%)	0	30 (28.30%)		

Abbreviations: NLR, Neutrophils/Lymphocytes Ratio; PLR, Platelets /Lymphocytes Ratio; SII, Platelets*Neutrophils/Lymphocytes (systemic immune inflammation index, SII); dNLR, Neutrophils/[White blood cells-neutrophils]; SIRI, Neutrophils*monocytes/lymphocytes; PIV, Neutrophils* monocytes*platelets/lymphocytes.

compared to the non-severe group ($p<0.05$, $p<0.001$, $p<0.05$, $p<0.05$, $p<0.001$, $p<0.001$ and $p<0.001$, respectively). For peripheral blood derived inflammatory immune indicators, the severe group had higher levels of NLR, PLR, SII, dNLR, SIRI and PIV than the non-severe group; PLR, dNLR, SIRI and PIV displayed significant differences between the two groups ($p<0.05$, $p<0.05$, $p<0.001$, $p<0.01$, respectively). The overall number of discharged patients after improvement was 266 (89.86%), and the number of deaths was 30 (10.14%) in both groups. 100% of patients improved and discharged in the non severe group. 76 (71.70%) patients were discharged and 30 (28.30%) died in the severe group.

Peripheral Blood T Lymphocytes and Mitochondrial Membrane Potential Low Percentage (MMP-Low%)

Flow cytometry was employed to assess T-lymphocytes and mitochondrial damage indicators as well as mitochondrial membrane potential low percentage (MMP-low%). Figure 1 illustrate typical flow cytometry graphs displaying T-cell subset counts and mitochondrial damage assessment in COVID-19 patients. As demonstrated in Table 3 and Figure 2 (According to the requirements, Figure 2 has been changed to a scatter plot), among the 296 patients, the median absolute counts of Mito +CD3+ T cells (CD3+T cells with normal mitochondrial function), Mito+CD3+CD4+ T cells (CD3+CD4+T cells with normal mitochondrial function), and Mito+CD3+CD8+ T cells (CD3+CD8+T cells with normal mitochondrial function) fell below the lower limit of the normal range. The severe group exhibited significantly lower counts of Mito+CD3+ T cells, Mito

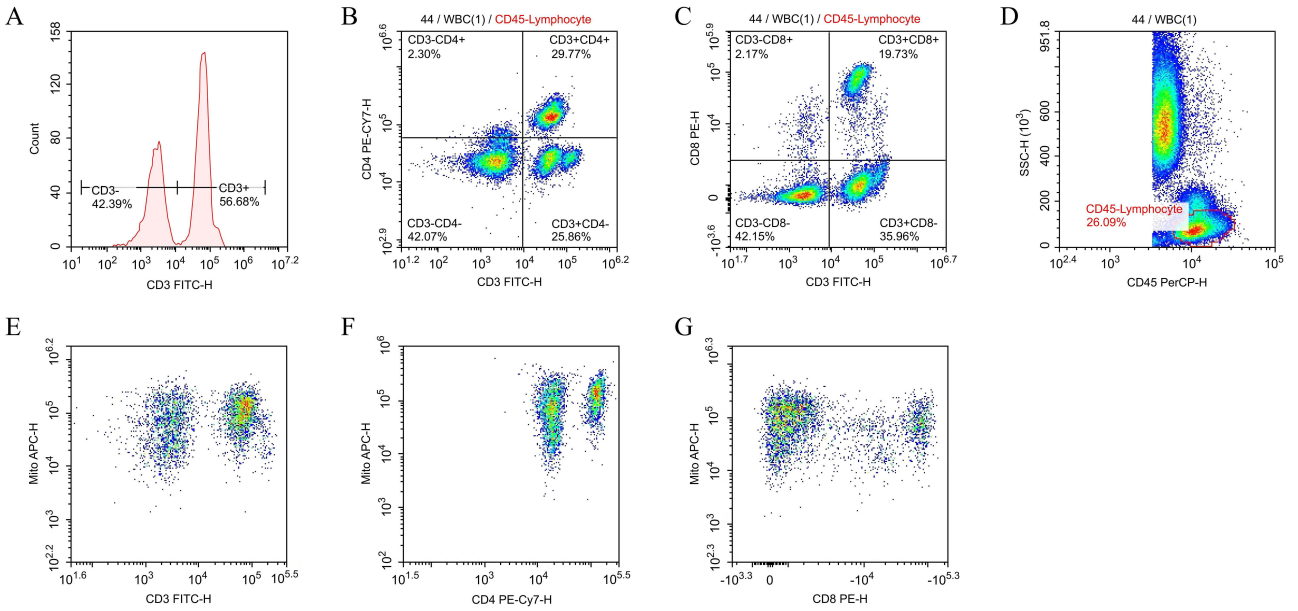

Figure 1 (**A-G**) A typical example of flow cytometry images for T lymphocyte count and mitochondrial damage detection of COVID-19 patient; (**A-D**) represent the CD3+T cell population, CD3+CD4+T cell subpopulation, CD3+CD8+T cell subpopulation, and CD45 lymphocyte percentage, respectively; (**E-G**) represent the mitochondrial damage index of CD3+T cells, CD3+CD4+T cells, and CD3+CD8+T cells, respectively.

+CD3+CD4+ T cells, and Mito+CD3+CD8+ T cells compared to the non-severe group ($p<0.001$, $p<0.001$ and $p<0.001$, respectively). The percentages of CD45 lymphocytes, CD3+ T cells, and CD3+CD4+ T cells were significantly lower in the severe group compared to the non-severe group ($p<0.001$, $p<0.05$ and $p<0.05$, respectively). There were no considerable differences in CD8 cell percentage and CD4/CD8 ratio between the two groups. In both groups, 178 cases were positive for TC mitochondrial damage index (CD3+T cell mitochondrial damage) (accounting for 60.14%), 149 for TH mitochondrial damage index (CD3+CD4+T cell mitochondrial damage) (accounting for 50.34%), and 190 for TS mitochondrial damage

Table 3 T Lymphocyte and Their Mitochondrial Damage Indicators

T lymphocyte and Mitochondrial Damage Indicators	Normal Range	Total Number of Cases (n=296)	Non-severe Group (n=190)	Severe Group (n=106)	Statistical Value	P-value
Mito+CD3+ T cells	723–2271	640.00 (376.25,973.00)	759.00 (527.00,1151.00)	413.50 (255.50,662.25)	6.973	<0.001
Mito+CD3+CD4+ T cells (Th cells)	396–1309	360.00 (200.75,562.00)	437.50 (280.25,659.25)	233.00 (130.00,356.00)	6.714	<0.001
Mito+CD3+CD8+ T cells (Ts cells)	224–1014	222.00 (133.25,389.00)	277.50 (159.75,433.00)	157.00 (86.80,275.00)	5.503	<0.001
TC mitochondrial damage index (Mito +CD3+ MDI) positive n (%)	Negative	178 (60.14%)	85 (44.74%)	93 (87.74%)	52.477	<0.001
TH mitochondrial damage index (Mito +CD3+CD4 MDI) positive n (%)	Negative	149 (50.34%)	63 (33.16%)	86 (81.13%)	62.642	<0.001
TS mitochondrial damage index (Mito +CD3+CD8 MDI) positive n (%)	Negative	190 (64.19%)	100 (52.63%)	90 (84.91%)	30.832	<0.001

(*Continued*)

Table 3 (Continued).

T lymphocyte and Mitochondrial Damage Indicators	Normal Range	Total Number of Cases (n=296)	Non-severe Group (n=190)	Severe Group (n=106)	Statistical Value	P-value
CD45 lymphocyte (%)	>5	13.72(7.25,23.84)	17.96 (11.00,27.09)	7.26 (5.14,13.60)	8.136	<0.001
Mito+CD3+ T cells (%)	56–84	62.05(51.87,69.62)	64.19 (55.03,70.98)	59.12 (46.77,67.75)	2.795	0.005
Mito+CD3+CD4+ T cells (%)	28–53	34.78±11.57	35.95±11.07	32.68±12.20	2.35	0.019
Mito+CD3+CD8+ T cells (%)	16–42	21.82(15.57,29.04)	21.82 (16.51,28.98)	21.98 (14.62,29.43)	0.641	0.522
Mito+CD4/Mito+CD8 ratio	0.7–2.8	1.66(1.03,2.44)	1.68(1.10,2.49)	1.58(0.96,2.37)	0.934	0.35
CD3+T MMP-low%	25.72–68.5	48.58 (33.16, 60.96)	46.7 (29.39, 58.2)	51.8 (38.04, 68.99)	−3.335	< 0.001
CD3+CD4+T MMP-low%	19.6–53.1	45.42 (32.26, 52.61)	41.33 (24.83, 49.16)	50.92 (39.17, 56.49)	−5.819	< 0.001
CD3+CD8+T MMP-low%	30.1–77.6	47.1 (35.78, 68.51)	40.88 (31.12, 57.69)	56.69 (40.96, 78.89)	−5.774	< 0.001

Abbreviations: TC, CD3+T lymphocyte; TH, CD3+CD4+T lymphocyte; TS, CD3+CD8+T lymphocyte; Mito+CD3+ T cells, CD3+T cells with normal mitochondrial function; Mito+CD3+CD4+ T cells, CD3+CD4+T cells with normal mitochondrial function; Mito+CD3+CD8+ T cells, CD3+CD8+T cells with normal mitochondrial function; MMP-low%, mitochondrial membrane potential low percentage.

index (CD3+CD8+T cell mitochondrial damage) (accounting for 64.19%). It is worth noting that the positive percentages of TC, TH, and TS mitochondrial damage in the severe group were significantly higher than those in the non-severe group ($p<0.001$ for all). Additionally, CD3+T cells MMP-low%, CD3+CD4+T cells MMP-low% and CD3+CD8+T cells MMP-low% in the severe group were significantly higher than those in the non-severe group ($p<0.001$ for all).

T Lymphocyte MMP-Low% Have Excellent Efficacy in Predicting the Outcome of COVID-19

To further evaluate the predictive value of novel T lymphocyte mitochondrial markers (CD3+T cells MMP-low%, CD3+CD4+T cells MMP-low% and CD3+CD8+T cells MMP-low%) and conventional immune inflammatory markers (SIRI, PIV, PLR, dNLR and CRP) as clinical outcomes in COVID-19 patients, the area under the ROC curve (AUC) was calculated. As shown in Table 4, the CD3+CD8+T cells MMP-low% had the highest AUC value of 0.864 (95% CI =0.794–0.934) to evaluate COVID-19 outcome. Additionally, CD3+CD4+T MMP-low% (AUC = 0.826, Sen = 100%, Spe = 71.8%), CD3+T MMP-low% (AUC = 0.795, Sen = 80%, Spe = 88.3%), CRP(AUC = 0.839, Sen = 86.7%, Spe = 75%), and dNLR(AUC = 0.821, Sen = 70%, Spe = 85%) equally had good predictive value.

Logistic Regression Analysis of Risk Factors Associated with Prognosis in COVID-19 Patients

Binary logistic regression analysis was conducted to identify the independent risk factors associated with the prognosis of patients with COVID-19. Univariate logistic regression analysis on all variables mentioned in the above article was conducted, and further multivariate logistic regression analysis on variables with statistical differences was performed. All variables in Table 5 were modeled separately to avoid collinearity. All models were controlled for age, sex, hypertension, diabetes, chronic pulmonary disease, SIRI, NLR, C-reactive protein, Glutamic-pyruvic transaminase, Creatine kinase, Lactate dehydrogenase, D-dimer. As shown in Table 5, CD3+T cells MMP-low%, CD3+CD4+T cells

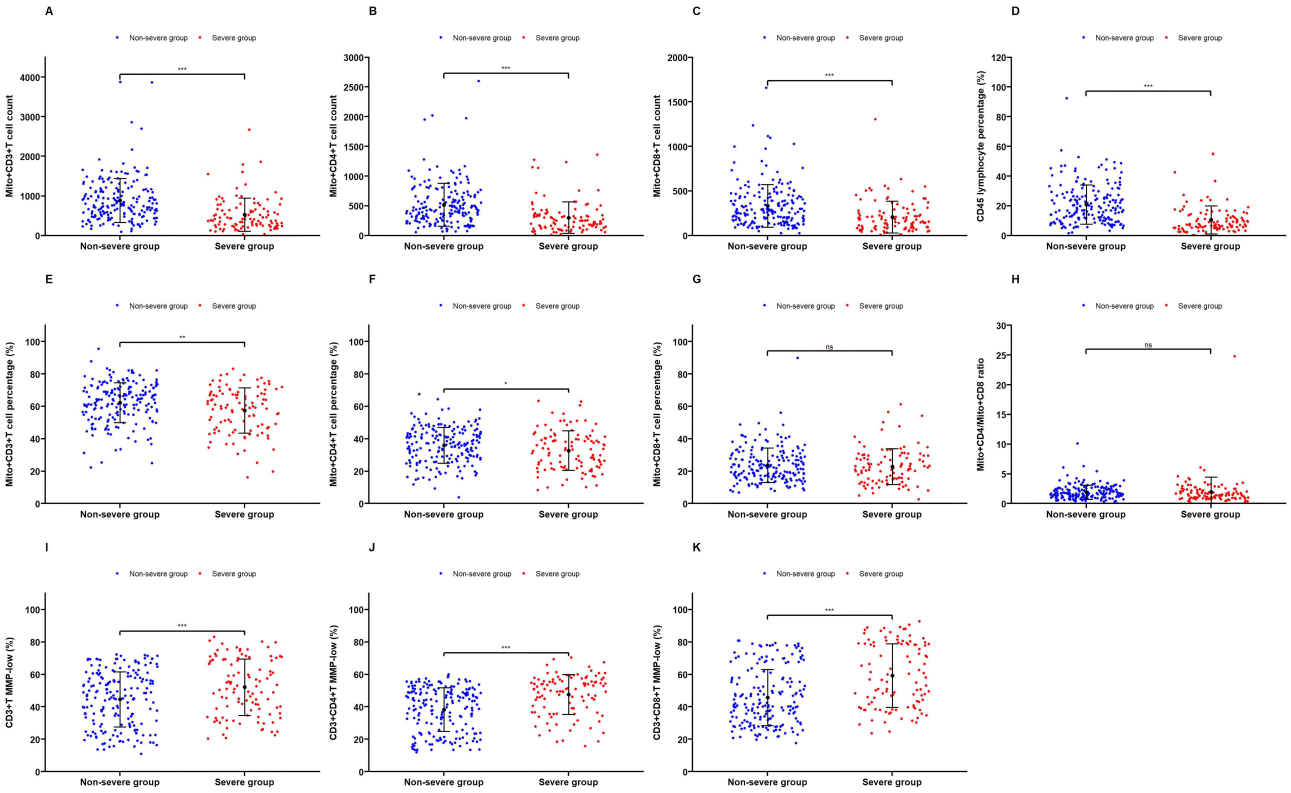

Figure 2 (**A-K**) Peripheral blood T lymphocyte and mitochondrial membrane potential low percentage (MMP-low%) in both groups. (**A-C**) represent the absolute count of Mito+CD3+T cells, Mito+CD3+CD4+T cells, and Mito+CD3+CD8+T cells in both groups, respectively. (**D-H**) D-G represent the percentage of CD45 lymphocytes, Mito +CD3+T cells, Mito+CD3+CD4+T cells and Mito+CD3+CD8+T cells in both groups, respectively. H represents the CD4/CD8 ratio in both groups. (**I-K**) represent CD3 +T cells MMP-low%, CD3+CD4+T cells MMP-low% and CD3+CD8+T cells MMP-low% in both groups, respectively. *P < 0.05, **P < 0.01, ***P < 0.001.

MMP-low% and CD3+CD8+T cells MMP-low% were independent risk factors for adverse outcomes in COVID-19 patients.

Discussion

To date, there is still a lack of research on using immune cell mitochondria as biomarkers for COVID-19 diagnosis or prognosis. In this study, the peripheral blood T-lymphocyte subsets and their mitochondria damage were analyzed in

Table 4 Predictive Value of ROC Curve Evaluation of T Lymphocyte Mitochondrial Markers and Conventional Immune Inflammatory Markers in Predicting the Outcomes of COVID-19

	SIRI	PIV	PLR	dNLR	CRP	CD3+T MMP-low%	CD3+CD4+T MMP-low%	CD3+CD8+T MMP-low%
AUC	0.703	0.589	0.571	0.821	0.839	0.795	0.826	0.864
SE	0.049	0.061	0.066	0.041	0.035	0.039	0.036	0.036
95% CI	0.605–0.8	0.469–0.709	0.441–0.7	0.741–0.901	0.771–0.908	0.718–0.871	0.756–0.896	0.794–0.934
p	<0.001	0.945	0.898	<0.001	<0.001	<0.001	<0.001	<0.001
Youden index	0.315	0.199	0.215	0.55	0.617	0.683	0.718	0.573
Relevant standards	1.828	1950.59	262.422	5.824	60.08	68.965	49.27	77.855

(*Continued*)

Table 4 (Continued).

	SIRI	PIV	PLR	dNLR	CRP	CD3+T MMP-low%	CD3+CD4+T MMP-low%	CD3+CD8+T MMP-low%
Sensibility	0.8	0.267	0.467	0.7	0.867	0.8	1	0.667
Specificity	0.515	0.932	0.748	0.85	0.75	0.883	0.718	0.906

Abbreviations: SIRI, Neutrophils*monocytes/lymphocytes; PIV, Neutrophils* monocytes*platelets/lymphocytes; PLR, Platelets /Lymphocytes Ratio; dNLR, Neutrophils/[White blood cells-neutrophils]; CRP, C-reactive protein; MMP-low%, mitochondrial membrane potential low percentage; AUC, area under the curve; SE: standard error; CI, confidence interval.

Table 5 Logistic Regression Analysis of Risk Factors Associated with Prognosis in COVID-19 Patients

Variable	B	SE	wald	p	OR	CI
CD45 lymphocyte %	−0.089	0.055	2.601	0.107	0.915	0.812–1.007
Mito+CD3+ T cells %	0.005	0.024	0.046	0.83	1.005	0.958–1.056
Mito+CD3+T cells count	0	0.001	0.168	0.682	1	0.998–1.001
Mito+CD3+CD4+T cells count	−0.001	0.001	0.757	0.384	0.999	0.996–1.002
TC mitochondrial damage index	2.433	1.447	2.825	0.093	11.388	1.044–310.276
TH mitochondrial damage index	1.152	0.956	1.452	0.228	3.165	0.568–27.876
TS mitochondrial damage index	1.935	1.163	2.767	0.096	6.927	0.939–98.01
CD3+T MMP-low%	0.086	0.03	8.347	0.004	1.09	1.034–1.164
CD3+CD4+T MMP-low%	0.155	0.055	7.919	0.005	1.168	1.063–1.324
CD3+CD8+T MMP-low%	0.08	0.025	9.879	0.002	1.083	1.035–1.146

Abbreviations: B, regression coefficient; SE, standard error; Wald, Chi square value; OR, odds ratio; CI, confidence interval.

COVID-19 with varying severities. Our findings demonstrate the presence of a substantial number of T-lymphocyte subsets exhibiting mitochondrial damage in both patient groups. Mitochondrial damage is suggested as a potential factor contributing to reduced T-cell counts. Severe COVID-19 patients exhibit lower CD4+ and CD8+T cell counts and more pronounced mitochondrial damage compared to those with the non-severe patients. The CD3+CD8+T cells MMP-low% had the highest AUC value of 0.864 to evaluate COVID-19 outcome. Binary logistic regression analysis showed that CD3+T cells MMP-low%, CD3+CD4+T cells MMP-low% and CD3+CD8+T cells MMP-low% were independent risk factors for adverse outcomes in COVID-19 patients. Our research suggests that T cells mitochondrial markers can serve as predictive factors and independent risk factors for predicting adverse outcomes in COVID-19 patients. This study elaborates on the potential role of immune cells and their mitochondrial function in the progression and clinical outcomes of COVID-19. Assessing the mitochondrial function and related biomarkers of immune cells in response to SARS-CoV-2 infection offers a novel perspective for predicting COVID-19 susceptibility, disease severity, and prognosis. Monitoring the mitochondrial function of immune cells can serve as a noninvasive biomarker for diagnosing and predicting the prognosis of COVID-19.

The host's immune response to viral infection significantly depends on mitochondrial function. Host immune cells and their mitochondria likely play a pivotal role in viral infection, including SARS-CoV-2 infection, and influence disease outcomes. Mitochondria, as one of the most important organelles, play an indispensable role in oxidative phosphorylation, oxidative stress, metabolism of lipids, amino acids, and carbohydrates, and regulation of metabolic and immune reactions within the body.[12,13] Research has demonstrated that the hijacking of host mitochondria by SARS-CoV-2 plays a pivotal role in the pathogenesis of COVID-19. Several protein components of SARS-CoV-2, such as open-reading frames (ORF)-7a and (ORF)-8b, are known to interact with host mitochondrial components, facilitating the proliferation of SARS-CoV-2 within the mitochondrial structure, ultimately leading to impaired mitochondrial

function.[10,11] When taken over by SARS-CoV-2, mitochondria form double-membrane vesicles, resulting in the disruption of mitochondrial membrane integrity, release of mitochondrial DNA into the bloodstream, the activation of immune responses, and ultimately the induction of a cytokine storm and uncontrolled high-inflammatory responses within the body.[9] Clinical studies on mitochondrial DNA in patients with COVID-19 provide support for this perspective. Researchers such as Scozzi, D[14] and Edinger et al[15] confirmed that elevated levels of circulating mtDNA are noninvasive predictive biomarkers for poor prognosis and mortality in those with COVID-19. Plasma mtDNA levels, as indicators of mitochondrial damage and oxidative stress, demonstrate similar AUCs in predicting mortality in COVID-19 compared to traditional indicators such as lactate dehydrogenase, ferritin, and D-dimer that are associated with disease severity. High levels of mtDNA are significantly associated with the severity and adverse outcomes of COVID-19.

Consistent with the results of our study, Yufei et al[16] demonstrated that T-cell dysfunction and apoptosis resulting from mitochondrial damage are significant mechanisms contributing to the reduction of T lymphocytes in those with COVID-19. They characterized the T-cell subsets with mitochondrial dysfunction in patients with COVID-19 using multicolor flow cytometry. T cells exhibiting mitochondrial dysfunction were associated with the loss of CD4+ T cells and hyperactivation of CD8+ T cells. Subsequent in vitro experiments revealed that T cells with mitochondrial dysfunction exhibited a poor response to SARS-CoV-2 peptide stimulation, indicating an impaired antiviral immune response of T cells. Beyond T lymphocytes, other studies have confirmed that the mitochondrial function of natural killer (NK) cells is also significantly impaired in severe SARS-CoV-2 infection.[17] By assessing mitochondrial mass and membrane potential in NK cells, researchers have affirmed that the mitochondrial mass of peripheral blood NK cells can serve as a novel predictive biomarker for severe SARS-CoV-2 infection. Compared to conventional infection biomarkers, the mitochondrial mass of circulating NK cells is the most effective discriminant for assessing the severity of COVID-19. Combining traditional infection biomarkers with novel immune monitoring indicators enhances the prediction of disease severity and clinical outcomes in COVID-19. Our findings reveal a significant presence of T-lymphocyte subsets with mitochondrial damage in individuals with differing degrees of COVID-19, with severely infected patients showing more significant mitochondrial damage in CD4+T cells and CD8+T cells. The mitochondrial damage index of CD3+CD4+T cells independently predicts poor prognosis in patients with COVID-19. Our results underscore the significance of immune cell mitochondrial function in predicting the severity and prognosis of COVID-19, elucidating the critical role of mitochondrial damage at the intersection of SARS-CoV-2 infection and immunity.

SARS-CoV-2 infection can induce mitochondrial damage, and conversely, mitochondrial signaling also participates in regulating the host cell's immune response to viral infection. Further investigations have revealed that mitochondrial energy metabolism might be one of the crucial mechanisms. SARS-CoV-2 manipulates the energy metabolism of host mitochondria, exacerbating COVID-19. The virus seizes control of host mitochondria to influence metabolic pathways, favoring its replication.[18,19] Joseph Guarnieri et al[20] performed an extensive study involving human and rodent samples and demonstrated that SARS-CoV-2 interferes with mitochondrial oxidative phosphorylation(OXPHOS), glycolysis, and other metabolic processes in mammals by suppressing gene expression linked to these pathways, ultimately leading to severe illness and even death in COVID-19. A substantial presence of dysfunctional immune cells marks severe COVID-19. The association between cellular energy metabolism and immune function relies on the flexibility of cellular mitochondria in generating energy from different substrates.[21] Saima Ajaz et al[22] observed that SARS-CoV-2 modulates the mitochondrial metabolism of peripheral blood mononuclear cells(PBMC) in patients with COVID-19. This modulation leads to PBMC mitochondrial dysfunction, altered energy metabolism, and energy deficiency, triggering a compensatory increase in glycolysis. The manipulation of mitochondrial metabolism by SARS-CoV-2 triggers a potent inflammatory response in the body. The study further identified an association between increased plasma fibroblast growth factor-21 levels, and the severity and mortality of COVID-19. Krishnan et al[23] observed the inhibition of mitochondrial OXPHOS coupled with upregulation of glycolysis in CD8+ T cells and monocytes in patients afflicted with severe SARS-CoV-2 infection.

Our study evaluates lymphocytic mitochondrial damage. This approach provides a direct insight into mitochondrial function, frequently called the "biological engine" within immune cells. The fluorescence intensity index we assessed through flow cytometry reflects the quality of T-cell mitochondria and mitochondrial membrane potential. By integrating organelle vitality with immune cell counts, a comprehensive understanding of the body's immune response to SARS-

CoV-2 infection is obtained. Molecular detection technology represents the future of precise assessments, offering promising applications for those with COVID-19. It provides more objective and in-depth immune parameters for clinical purposes, enabling early detection of individuals at risk of developing severe symptoms. This technology further facilitates the better evaluation of disease severity, prognosis, and treatment optimization. The limitations of this study are as follows: (1) The sample size of this study is relatively small, and there may be a need for more clinical studies with larger sample sizes in the future; (2) This study lacks a healthy control group; (3) This study lacks animal models and in vitro cell experiments to further validate our results.

Data Sharing Statement

The data presented in this study are available on request from the corresponding author.

Declarations and Ethics Approval

All procedures performed in the studies involving human participants accorded with the ethical standards of the Medical Ethics Committee of Fuyang People's Hospital, and with the 1964 helsinki Declaration and its later amendments, or other comparable ethical standards.

Author Contributions

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

Disclosure

The authors declare no conflict of interest.

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