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RESEARCH ARTICLE

Role of Ferroptosis in the Progression of COVID-19 and the Development of Long COVID

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Abstract: ***Objective*:** This study aimed to examine the role of ferroptosis on the pathogenesis and progression of COVID-19.

***Materials and Methods*:** A total of 127 patients who were hospitalized for COVID-19 were categorized into two groups according to the intensity of oxygen therapy (high-flow or low-flow). Clinical characteristics, laboratory parameters, plasma markers, and peripheral blood mononuclear cell (PBMC) markers were measured at baseline and one or two weeks after treatment. Telephone follow-up was performed 3 months after discharge to assess long COVID.

***Results*:** Patients receiving high-flow oxygen therapy had greater levels of neutrophils, D-dimer, C reactive protein, procalcitonin, plasma protein levels of tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), IL-17, acyl-CoA synthetase long-chain family member 4 (ACSL4), and PBMC mRNA level of *TNF-α* but had lower levels of lymphocytes and plasma glutathione peroxidase 4 (GPX4). There were negative correlations of plasma GPX4 and cystine/glutamate transporter-11 (SLC7A11) with TNF-α, IL-6, and IL-17 and positive correlations of ACSL4 with inflammatory markers in plasma and PBMCs. The plasma levels of TNF-α, IL-6, IL-17, and ACSL4 were significantly lower after treatment than at baseline, but there were higher post-treatment levels of lymphocytes, GPX4, and SLC7A11. Patients with long COVID had a lower baseline level of plasma SLC7A11.

***Conclusion*:** Ferroptosis is activated during the progression of COVID-19, and a low baseline level of a ferroptosis marker (SLC7A11) may indicate an increased risk for long COVID-19. Ferroptosis has potential as a clinical indicator of long COVID and as a therapeutic target.

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1. INTRODUCTION

The first reported human infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was in Wuhan in December 2019, and this was soon followed by the coronavirus disease 2019 (COVID-19) global pandemic [1]. Molecular studies conducted from December 2019 to November 2020 suggested that the SARS-CoV-2 genome acquired about 2 mutations per month [2, 3]. As of 2 October 2022, SARS-CoV-2 was responsible for more than 615 million infections and 6.5 million deaths worldwide, and this has had significant effects on societies and especially healthcare systems throughout the world [1, 4]. SARS-CoV-2 continues to undergo mutational and antigenic changes, and the accumulation of these changes in the genome has altered the fitness of this virus. There are now five SARS-CoV-2 variants of concern, namely Al-

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pha, Beta, Gamma, Delta, and Omicron. These different variants have specific mutations and amino acid changes in the spike protein and other proteins that impact the disease severity and clinical presentation of patients [5]. For example, the Omicron variant appears less likely to cause severe disease than other variants but is more transmissible and is less affected by vaccination [6-8]. In contrast to other variants, which can readily become established in the lungs, Omicron tends to stay in the upper respiratory tract (nose, throat, and bronchi) [9, 10]. However, Omicron infections can nonetheless lead to severe disease, pneumonia, respiratory failure, and even death [11].

Patients with COVID-19 typically present with many complications, as well as physiological alterations and changes in various biochemical and clinical markers. For example, laboratory findings demonstrated the presence of elevated levels of proinflammatory $CD4^+$T and $CD8^+$T cells, excessive production of cytokines ('cytokine storm'), hypercoagulability, and dysregulation of iron homeostasis (especially iron overload) during the pathogenesis of COVID-19 [12]. Multiple studies have shown that hyper-inflammatory responses play a central role in the progression of severe COVID-19 [13, 14], including the activation of neutrophils and macrophages, and the overproduction of proinflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) [15-17]. Moreover, free iron released into the circulation in patients with COVID-19 can cause oxidative damage to the lungs and other organs. Iron overload may also result in inflammation and immune dysfunction [18-20]. The study of Chakurkar *et al.* reported several markers of iron metabolism (serum iron, ferritin, and serum catalytic iron) were associated with adverse outcomes in COVID-19 and suggested that iron chelation therapy could be considered for COVID-19 [21]. SARS-CoV-2 infection can lead to depletion of glutathione (GSH), inactivation of glutathione peroxidase 4 (GPX4), dysregulation of iron metabolism, and increased peroxidation of poly-unsaturated fatty acids (PUFAs), all of which are signs of oxidative stress [12]. These findings suggest that SARS-CoV-2 may trigger ferroptosis (iron-dependent programmed cell death) in multiple organs, a pathological response that may lead to multiorgan damage [22, 23]. This led us to hypothesize that molecular interaction of the systems responsible for cellular iron regulation and the systems responsible for inflammatory processes may contribute to the pathogenesis of COVID-19.

Long COVID, also referred to as post-COVID-19 syndrome, is characterized by symptoms of chest pain or tightness, cough, fatigue or breathlessness, ageusia and anosmia, and headache [24-28]. After infection with SARS-CoV-2, up to 30% of individuals may develop long COVID [29-32]. These various symptoms can last for many months after the initial symptoms of COVID-19. Patients with long COVID may experience systemic inflammatory and neuroinflammatory responses, endothelial damage, and reactivation of latent pathogens [33, 34]. Patients with severe acute COVID-19 typically develop hyperactivation of cytokines and increased inflammation, and these may also contribute to long COVID in some patients [34]. Several researches suggest that ferroptosis is associated with neuropsychiatric symptoms and cognitive decline in individuals with SARS-CoV-2 infection [35-37].

Moreover, Jankauskas *et al.* demonstrated that ferroptosis markers, such as GPX4 and SLC7A11, in the serum of non-survivors were significantly dysregulated in endothelial cells. This dysregulation may be linked to the development of cardiovascular and cerebrovascular disorders [38, 39]. Elucidation of the molecular mechanism of long COVID will likely aid in the development of new therapeutic strategies, and this motivated us to examine the role of ferroptosis in the pathogenesis of COVID-19and long COVID.

At the end of 2022, after China recognized COVID-19 as a Class B infectious disease, there was a resurgence of infections, most of which were by the Omicron BA.5 strain. We examined data from 127 patients who were hospitalized at a single institution in China and received low-flow oxygen therapy (LFOT) or high-flow nasal cannula oxygen therapy (HFNC) and examined the role of ferroptosis in the progression of COVID-19 and the development of long COVID. Our general purpose was to identify novel markers and possible therapeutic targets for COVID-19 and long COVID, and to identify COVID-19 patients who have the greatest risk of progression to long COVID.

2. MATERIALS AND METHODS

2.1. Study Design and Participants

This study reviewed the records of 127 consecutive adult patients who were hospitalized and received oxygen therapy for COVID-19 at the First Affiliated Hospital of Bengbu Medical College (Anhui Province, China) from December 1, 2022 to January 1, 2023. The diagnosis was based on a positive result from a real-time reverse transcriptase-polymerase chain reaction (RT-

PCR) test or an antigen test. All patients were treated according to the Novel Coronavirus Pneumonia Diagnosis and Treatment Protocol (version 10). Patients who were not admitted for COVID-19 or who were hospitalized for less than 48 hours were excluded. The study protocol was approved by the local Ethics Committee, and the study procedures followed the tenets of the Declaration of Helsinki and Good Clinical Practice guidelines.

2.2. Treatments and Comorbidities

The following treatments used during hospitalization were recorded: azvudine, dexamethasone and other systemic corticosteroids, antibiotics (cephalosporins, quinolones, and others), anticoagulant therapy, intravenous immunoglobulin (IVIG), LFOT (flow ≤ 6 L/min), and HFNC (flow > 6 L/min). The presence of the following chronic comorbidities was recorded: hypertension, diabetes, cardiovascular diseases, cerebrovascular diseases, chronic respiratory diseases (chronic obstructive pulmonary disease, asthma, other lung conditions), and other diseases.

2.3. Routine Laboratory Parameters

White blood cells (WBCs), neutrophils (NEUTs), and lymphocytes (LYMs) were determined using an automated hematology analyzer (Beckman Coulter LH750; California, USA). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CRE), blood urea nitrogen (BUN), and C-reactive protein (CRP) were measured enzymatically using an automated analyzer (Olympus AU2700; Olympus, Tokyo, Japan), according to the manufacturer's instructions. D-dimer and fibrinogen (FIB) were measured using an automated coagulometer (Sysmex CA-1500; Sysmex Corporation, Kobe, Japan). Procalcitonin (PCT) was measured using an immunoquantitative analyzer (QMT8000; Wuhan Easy Diagnosis Biomedicine CO., Ltd, Wuhan, China).

2.4. Measurement of Inflammation Markers and Ferroptosis Markers in Plasma

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes from all 127 patients at admission and from 41 of these patients at 7 days or 14 days after treatment onset. Plasma levels of TNF-α (ZC-35733), IFN-γ (ZC-32271), IL-6 (ZC-32446), interleukin-17 (IL-17, ZC-32412), glutathione peroxidase 4 (GPX4, ZC-54591), acyl-CoA synthetase long-chain family member 4 (ACSL4, ZC-55786), and solute carrier family 7 member 11 (SLC7A11, ZC-56248) were determined using enzyme-linked immunosorbent assay (ELISA) kits from Shanghai Zcibio Technology Co. (Shanghai, China), according to the manufacturer's instructions.

2.5. Quantitative RT-PCR Analysis of Peripheral Blood Mononuclear Cells

A peripheral blood sample (7 mL) was taken from each subject at admission, and peripheral blood mononuclear cells (PBMCs) were separated using Ficoll Hypaque Density Gradient centrifugation (LTS1077-1; Tianjin Haoyang Biological Manufacture Co., Ltd; Tianjin; China). Total RNA was isolated using the TRIzol reagent (Invitrogen, California, USA). Then, complementary DNA (cDNA) was synthesized using a high-capacity cDNA reverse transcription kit (Takara, Hokkaido, Japan), and qRT-PCR was performed in an ABI 7500 real-time PCR system (Applied Biosystems, California, USA) using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus; Takara, Japan). Target genes were identified using the National Center of Biotechnology Information (NCBI). Then, Primer Premier version 5 was used to design the primers, which were evaluated by Oligo software and Primer-BLAST in NCBI (Table **S1**). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control, the $2^{-\Delta\Delta Ct}$ method was used to quantify expression, and each experiment was performed three times.

Table 1. Characteristics of COVID-19 patients who received low-flow oxygen therapy and high-flow oxygen therapy.*

Characteristic	**Total (n = 127)**	**LFOT (n = 99)**	**HFNC (n = 28)**	***P-value***
Age, years	70.76 ± 1.36	71.26 ± 1.42	68.96 ± 3.62	0.758
Sex	-	-	-	0.816
Male	75 (59.1%)	59 (59.6%)	16 (57.1%)	-
Female	52 (40.9%)	40 (40.4%)	12 (42.9.%)	-
Comorbidities	110 (86.6%)	87 (79.1%)	23 (20.9%)	-
Hypertension	61 (48.0%)	50 (57.5%)	11 (47.8%)	0.294
Diabetes	29 (26.4%)	25 (28.7%)	4 (17.4%)	0.222

(Table 1) contd....

Characteristic	**Total (n = 127)**	**LFOT (n = 99)**	**HFNC (n = 28)**	***P-value***
Cardiovascular disease	23 (20.9%)	17 (19.5%)	6 (26.1%)	0.606
Cerebrovascular disease	13 (11.8%)	10 (11.5%)	3 (13.0%)	0.999
Chronic respiratory disease	14 (12.7%)	12 (13.8%)	2 (8.7%)	0.688
Other	52 (47.2%)	41 (47.1%)	11 (47.8%)	0.840
Treatment during hospitalization		-	-	-
Antiviral	26 (20.5%)	19 (19.2%)	7 (25%)	0.501
Corticosteroid	81 (63.8%)	61 (61.6%)	20 (71.4%)	0.340
Anticoagulant	56 (44.1%)	40 (40.4%)	16 (57.1%)	0.115
Intravenous immunoglobulin	29 (22.8%)	19 (19.2%)	10 (35.7%)	0.066
Antibiotic	116 (91.3%)	89 (89.9%)	27 (96.4%)	0.481
BMI (kg/m^2)	23.88 ± 3.67	24.27 ± 0.38	22.52 ± 0.54	0.057
WBCs ($10^9/L$)	7.76 ± 4.15	7.54 ± 0.43	8.54 ± 0.77	0.076
NEUTs ($10^9/L$)	6.04 ± 0.37	5.71 ± 0.41	7.19 ± 0.79	0.014
LYMs ($10^9/L$)	1.10 ± 0.06	1.16 ± 0.06	0.89 ± 0.11	0.021
D-dimer (mg/L)	2.26 ± 4.06	1.43 ± 0.20	4.45 ± 1.46	0.048
FIB (g/L)	4.57 ± 0.17	4.57 ± 0.20	4.57 ± 0.35	0.722
ALT (U/L)	27.45 ± 25.80	26.20 ± 2.02	31.56 ± 7.92	0.284
AST (U/L)	31.01 ± 21.03	30.47 ± 2.12	32.81 ± 4.64	0.915
CRP (mg/L)	50.11 ± 63.14	38.68 ± 5.27	86.85 ± 16.04	0.005
PCT (ng/mL)	0.54 ± 2.162	0.14 ± 0.03	1.31 ± 0.88	0.001
CRE (μmol/L)	108.39 ± 11.70	91.90 ± 7.75	161.52 ± 41.59	0.209
BUN (mmol/L)	7.27 ± 0.68	6.17 ± 0.55	10.81 ± 2.15	0.009
Plasma markers (protein)	-	-	-	-
TNF-α (pg/mL)	73.23 ± 1.24	70.95 ± 1.38	81.29 ± 2.26	0.001
IFN-γ (pg/mL)	450.65 ± 7.71	457.82 ± 8.70	425.31± 16.04	0.092
IL-6 (pg/mL)	37.61 ± 0.60	36.82 ± 0.70	40.40 ± 0.97	0.014
IL-17 (pg/mL)	250.21 ± 4.82	244.50 ± 5.69	270.41 ± 7.62	0.032
GPX4 (ng/mL)	118.45 ± 3.44	123.17 ± 4.00	101.74 ± 5.60	0.017
ACSL4 (pg/mL)	1569.48 ± 29.77	1522.71 ± 35.242	1734.87 ± 39.28	0.003
SLC7A11 (ng/mL)	3.82 ± 0.183	3.95 ± 0.23	3.37± 0.21	0.513
PBMC markers (mRNA)**	53	38	15	-
TNF-α	1.59 ± 0.16	1.13 ± 0.10	2.74 ± 0.37	<0.001
IFN-γ	2.00 ± 0.38	1.65 ± 0.36	2.89 ± 0.75	0.358
GPX4	2.02 ± 0.18	1.81 ± 0.16	2.58 ± 0.46	0.286
ACSL4	1.11 ± 0.10	0.99± 0.11	1.42 ± 0.22	0.099
SLC7A11	0.81 ± 0.10	0.90 ± 0.13	0.58 ± 0.08	0.380

Note: *Values are given as mean ± SD or n (%).
**mRNA levels are relative to *GAPDH*.
Abbreviations: LFOT, low-flow oxygen therapy; HFNC, high-flow nasal cannula oxygen therapy; BMI, body mass index; WBCs, white blood cells; NEUTs, neutrophils; LYMs, lymphocytes; FIB, fibrinogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; PCT, procalcitonin; CRE, creatinine; BUN, blood urea nitrogen; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma; IL-17, interleukin-17; IL-6, interleukin-6; GPX4, glutathione peroxidase 4; ACSL4, acyl-CoA synthetase long-chain family member 4; SLC7A11, solute carrier family 7 member 11.

2.6. Assessment of Long COVID

Follow-up telephone assessments were conducted with each participant 3 months after hospital discharge; if the participant was unavailable, the follow-up was conducted with a proxy. When telephone calls from three different days were unanswered, the patient was classified as "loss of contact". These telephone calls were used to ask a series of questions about the presence of symptoms, including low-grade

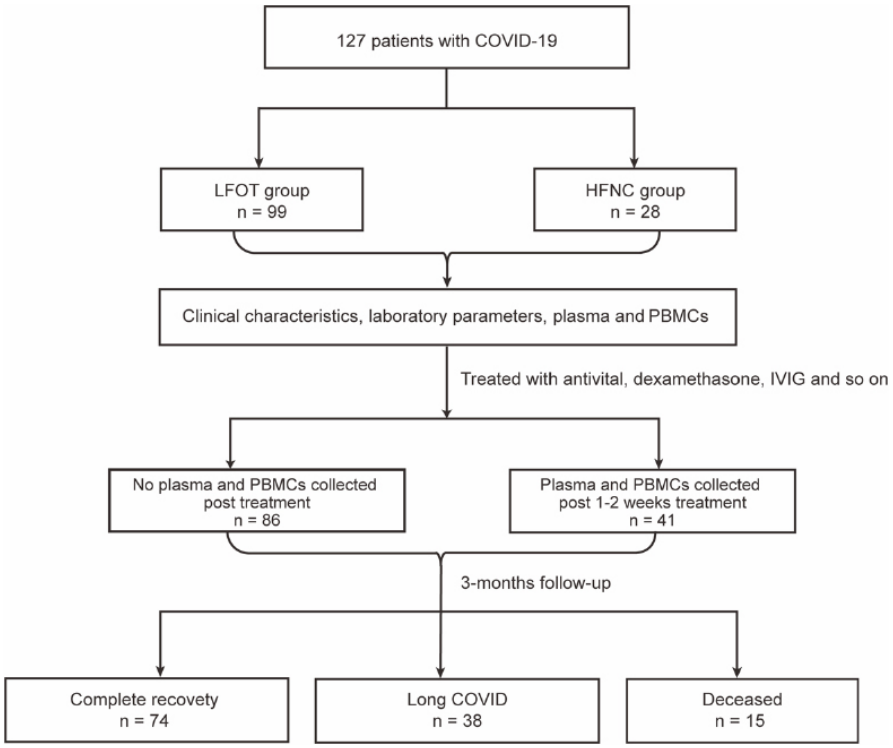

Fig. (1). Disposition of patients who were hospitalized for COVID-19 from December 1, 2022 to January 1, 2023. **Abbreviations**: LFOT, low-flow oxygen therapy; HFNC, high-flow nasal cannula oxygen therapy; IVIG, human immunoglobulin of intravenous injection; PBMC, peripheral blood mononuclear cell.

fever, dyspnea, palpitation, cough and sputum production, weakness, sleep difficulties, pain, chest distress, depression/anxiety, and memory loss. Long COVID was defined as new or ongoing symptoms three months after the onset of acute COVID-19.

2.7. Statistical Analysis

Statistical analysis was performed using SPSS version 25.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 9 (GraphPad Software, California, USA). Continuous variables were presented as means ± standard deviations (SDs), and the independent samples *t*-test was used to determine the significance of differences between groups. Frequency data were expressed as numbers with percentages, and the Chi-squared test or Fisher's exact test was used to determine the significance of differences between groups. A one-way analysis of variance (ANOVA) was used to compare values that were recorded from three groups. Spearman's rank correlation was used to assess the association between markers of ferroptosis and inflammatory factors. All reported *P*-values are two-sided, and a *P*-value below 0.05 was considered significant.

3. RESULTS

3.1. Demographic, Clinical, and Laboratory Characteristics of Patients

We examined 127 adult patients who were hospitalized at our institution with COVID-19 and recorded their characteristics at baseline (Fig. **1** and Table **1**). Fifteen of these patients (11.8%) died after discharge, mainly due to exacerbations of underlying pulmonary, heart, or kidney disease. The median patient age was 71-years-old, and there were 75 males (59.1%) and 52 females (40.9%). Ninety-nine patients (78.0%) required oxygen support from LFOT, and the other 28 patients (22.0%) required HFNC. These two groups had no significant differences in demographic characteristics, comorbidities, or treatments received during hospitalization (all $P > 0.05$). Overall, the most common comorbidity was hypertension (61 patients, 48.0%), followed by diabetes (29 patients, 26.4%), and cardiovascular disease (23 patients, 20.9%). Most patients received treatment with a corticosteroid (63.8%) and an antibiotic (91.3%), and 56 patients (44.1%) received an anticoagulant.

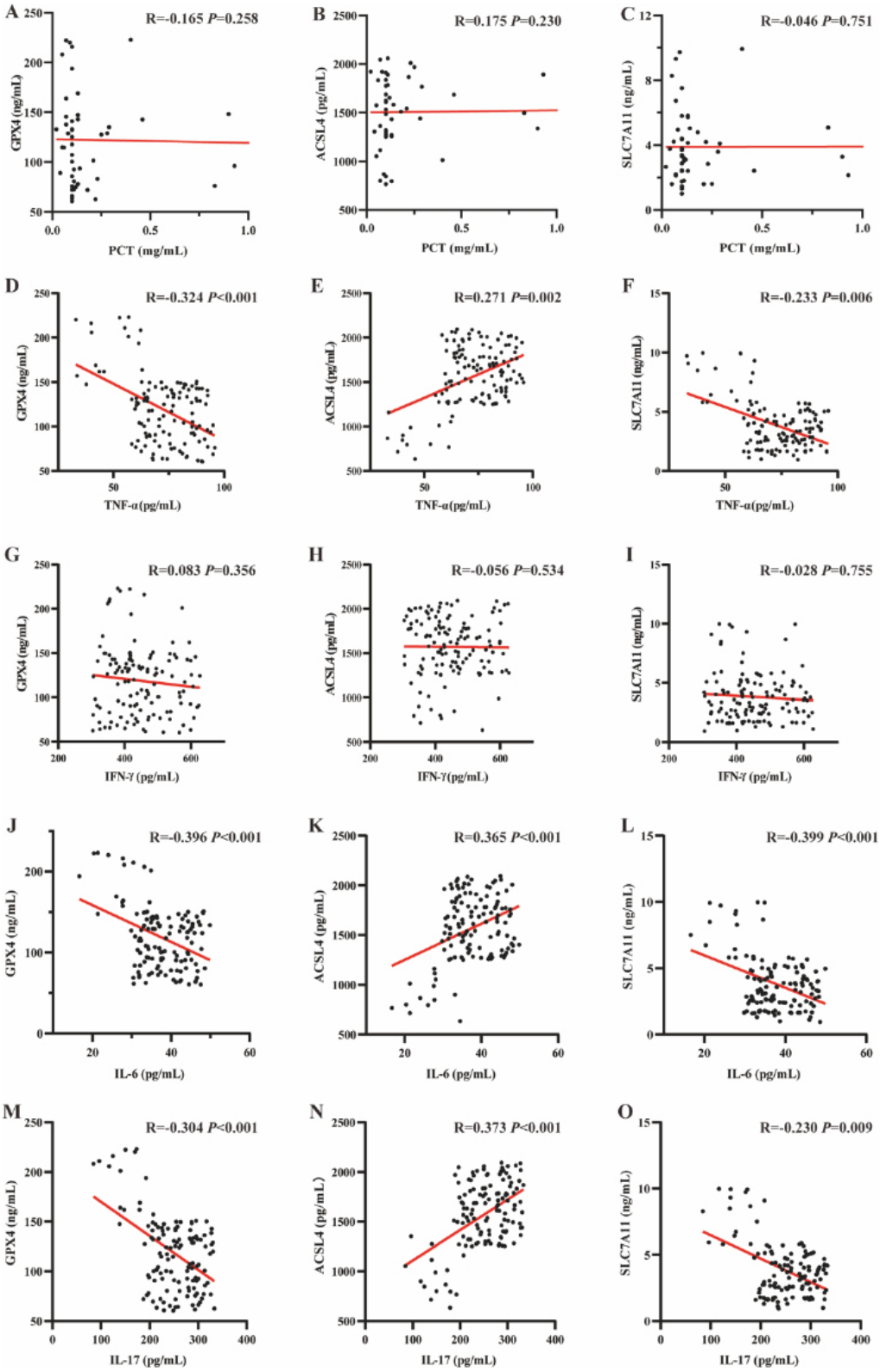

Fig. (2). Correlations in the baseline plasma levels of ferroptosis marker proteins (vertical axes) and inflammation marker proteins (horizontal axes). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

The LFOT and HFNC groups had significant differences in many laboratory variables. In particular, the HFNC group had significantly higher levels of NEUTs, D-dimer, CRP, PCT, and BUN but a significantly lower level of LYMs (all $P < 0.05$). The HFNC group also had significantly higher plasma levels of four markers (TNF-α, IL-17, IL-6, and ACSL4) but a significantly lower level of plasma GPX4 (all $P < 0.05$). qRT-PCR analysis of PBMCs indicated that the HFNC group had a significantly higher level of *TNF-α* mRNA ($P < 0.05$).

3.2. Relationship of Ferroptosis Markers and Inflammatory Markers at Admission

We examined the possible role of ferroptosis in the progression of COVID-19 by using ELISA measurements at admission to determine the correlations of plasma levels of GPX4 (inhibitor of ferroptosis), ACSL4 (promoter of ferroptosis), and SLC7A11 (inhibitor of ferroptosis) with plasma levels of PCT, TNF-α, IFN-γ, IL-6, and IL-17 (markers of inflammation; Figs. **2A-O**). The results showed that PCT had no significant correlations with GPX4, ACSL4, or SLC7A11 (all $P > 0.05$). TNF-α had negative correlations with GPX4 and SCLC7A11 and a positive correlation with ACSL4 (all $P < 0.01$). INF-γ had no significant correlations with GPX4, ACSL4, or SLC7A11 (all $P > 0.05$). IL-6 had negative correlations with GPX4 and SLC7A11 and a positive correlation with ACSL4 (all $P < 0.01$). IL-17 had negative correlations with GPX4 and SCLC7A11 and a positive correlation with ACSL4 (all $P < 0.01$).

We also used qRT-PCR analysis of PBMCs to measure correlations in the expression of *TNF-α* and *IFN-γ* with *GPX4*, *ACSL4*, and *SLC7A11* (Figs. **3A-F**). *TNF-α* had no significant correlation with *GPX4* ($P > 0.05$), a significant positive correlation with *ACSL4* ($P < 0.01$), and a significant negative correlation with *SLC7A11* ($P < 0.05$). *IFN-γ* had no significant correlation with *GPX4* or *SLC7A11* (both $P > 0.05$), but had a significant positive correlation with *ACSL4* ($P < 0.01$).

For further analysis, we stratified the entire patient population into three groups according to the levels of four plasma markers of inflammation (TNF-α, IFN-γ, IL-6, and IL-17) and two PBMC markers of inflammation (*TNF-α* and *IFN-γ*), as shown in Table **2**. We then analyzed the relationships of three plasma markers of ferroptosis with each of the four plasma markers of inflammation (Figs. **4A-L**). When stratified by TNF-α, the GPX4 and SLC7A11 levels were higher in group 1 than in the other groups, but the ACSL4 level was lower in group 1 than in the other groups. When stratified by IFN-γ, there were no significant differences in the levels of GPX4, ACSL4, and SLC7A11. When stratified by IL-6, group 1 had significantly higher levels of GPX4 or SCL7A11 than the other groups. When stratified by IL-17, group 1 had a significantly higher level of GPX4 than the other groups and a lower level of ACSL4 than group 3.

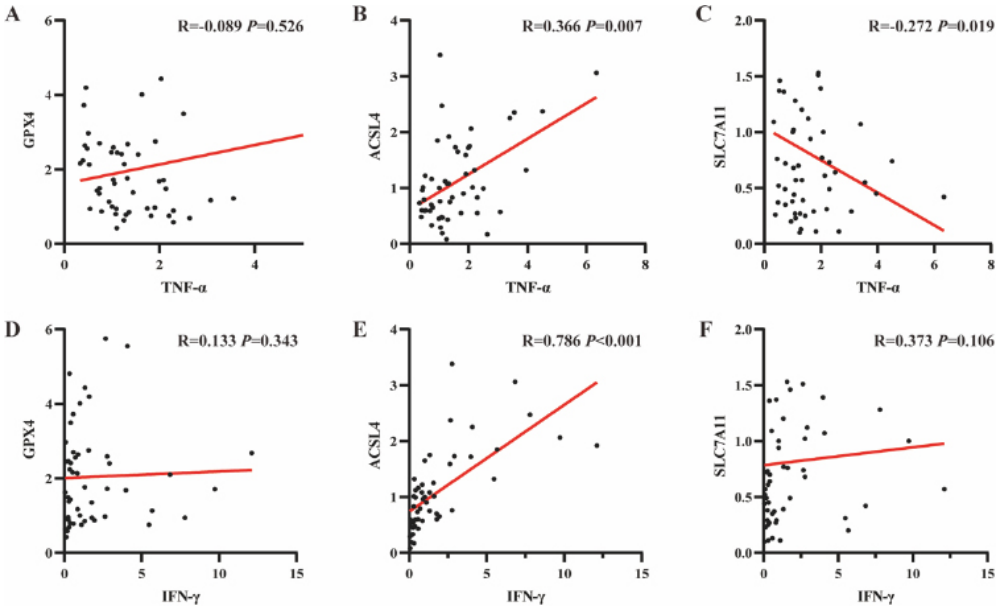

Fig. (3). Correlations in the baseline PBMC levels of ferroptosis marker mRNAs (vertical axes) and inflammation marker mRNAs (horizontal axes). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

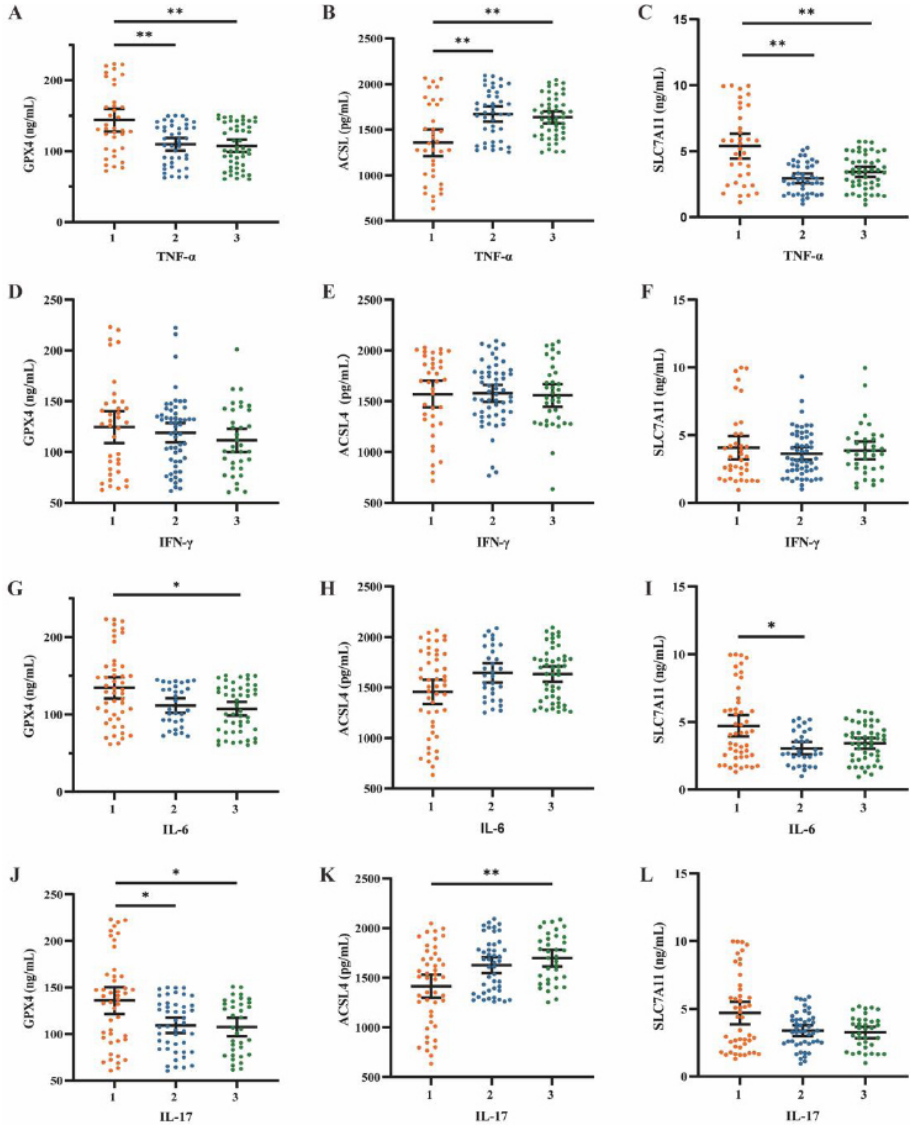

Fig. (4). Relationship of the baseline plasma levels of ferroptosis marker proteins (vertical axes) with different inflammation marker proteins (horizontal axes). *$P < 0.05$ and **$P < 0.01$ for between-group comparisons. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Criteria used to stratify plasma markers and PBMC markers of inflammation.

Markers	Group 1	Group 2	Group 3
Plasma (protein)	-	-	-
TNF-α (pg/mL)	<65	65 to 80	>80
IFN-γ (pg/mL)	<400	400 to 500	>500
IL-6 (pg/mL)	<35	35 to 40	>40
IL-17 (pg/mL)	<230	230 to 290	>290
PBMC (mRNA)**	-	-	-
TNF-α	<1	1 to 1.5	>1.5
IFN-γ	<0.5	0.5 to 1.5	>1.5

Note: **mRNA levels are relative to *GAPDH*.

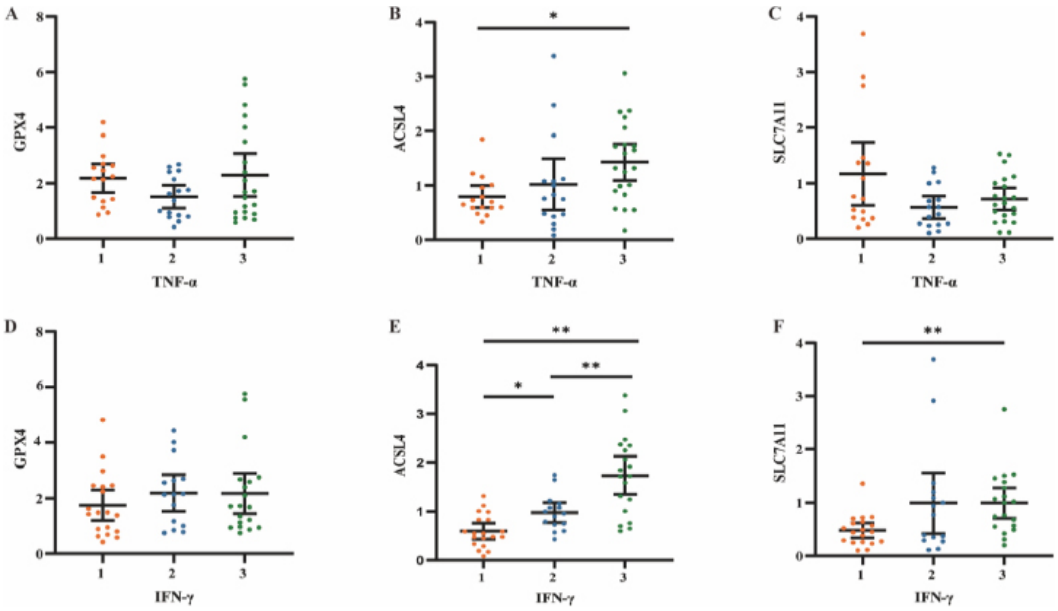

Fig. (5). Relationship of the baseline PBMC levels of ferroptosis marker mRNAs (vertical axes) with different inflammation marker mRNAs (horizontal axes). **P* < 0.05 and ***P* < 0.01 for between-group comparisons. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

We performed a similar analysis for the two PBMC mRNAs (Figs. **5A-F**). When stratified by *TNF-α*, there were no differences in the level of *GPX4*, but group 1 had a lower level of *ACSL4* than group 3. When stratified by *IFN-γ*, the three groups had no differences in the level of *GPX4.* However, the levels of *ACSL4* were higher in group 2 than in group 1 and higher in group 3 than in the two other groups; the level of *SCL7A11* was higher in group 3 than in group 1.

3.3. Changes in Ferroptosis Markers and Inflammatory Markers from Baseline to Post-treatment

We also analyzed changes in the laboratory test results, ELISA results, and qRT-PCR results for 41 patients from baseline to one or two weeks after onset of treatment (Table **3**). Relative to baseline, there were higher post-treatment levels of LYMs and plasma levels of GPX4 and SLC7A11; however, there were lower post-treatment levels of BUN and plasma levels of TNF-α, IL-6, IL-17, and ACSL4 (all $P < 0.05$).

3.4. Relationship of Inflammation and Ferroptosis Markers at Baseline with Long COVID-19

Fifteen patients died, and the other 112 patients completed the post-COVID-19 telephone survey. Based on analysis of different self-reported symptoms, 38 of these patients (33.9%) had long COVID (Table **4**). The most commonly reported symptoms of long COVID were cough and sputum production (36.8%), dyspnea (31.6%), and weakness (26.3%). We then compared the baseline levels of inflammation and ferroptosis markers in the groups with and without long COVID. The only significant difference was that the long COVID group had a significantly lower baseline level of plasma SLC7A11 ($P = 0.007$).

Table 3. Changes in the levels of routine blood indicators, inflammatory markers, and ferroptosis markers from baseline to post-treatment.*

Characteristic	Baseline (n = 41)	Post-treatment (n = 41)	Change	*P-values*
Age, years	72.46 ± 2.32	-	-	-
Sex	-	-	-	-
Male	29 (70.7%)	-	-	-
Female	12 (29.3%)	-	-	-
WBCs (10^9/L)	7.50 ± 0.56	8.33 ± 0.73	−0.78 ± 0.69	0.350

(Table 3) contd....

Characteristic	Baseline (n = 41)	Post-treatment (n = 41)	Change	*P-values*
NEUTs (10^9/L)	6.07 ± 0.56	6.57 ± 0.75	−0.45 ± 0.66	0.660
LYMs (10^9/L)	0.86 ± 0.07	1.09 ± 0.07	−0.24 ± 0.07	0.002
D-dimer (mg/L)	3.63 ± 1.12	3.33 ± 0.71	0.57 ± 1.03	0.566
FIB (g/L)	4.65 ± 0.25	4.31 ± 0.26	0.42 ± 0.41	0.313
ALT (U/L)	28.92 ± 5.30	33.19 ± 7.20	−5.24 ± 8.33	0.434
AST (U/L)	35.03 ± 3.85	28.94 ± 3.34	5.36 ± 4.94	0.308
CRE (μmol/L)	117.54 ± 25.33	121.65 ± 22.86	−1.42 ± 9.57	0.544
BUN (mmol/L)	7.75 ± 1.26	7.03 ± 1.15	0.88 ± 0.98	0.031
Plasma (protein)	-	-	-	-
TNF-α (pg/mL)	71.78 ± 2.24	58.79 ± 1.88	12.98 ± 2.49	<0.001
IFN-γ (pg/mL)	454.72 ± 13.21	454.44 ± 15.52	0.28 ± 21.82	0.990
IL-6 (pg/mL)	36.87 ± 1.17	30.84 ± 1.08	6.03 ± 1.22	0.001
IL-17 (pg/mL)	243.21 ± 8.69	209.98 ± 7.31	33.23 ± 9.34	0.001
GPX4 (ng/mL)	121.78 ± 6.12	139.54 ± 5.32	−17.76 ± 7.01	0.015
ACSL4 (pg/mL)	1523 ± 60.52	1348.06 ± 35.86	175.24 ± 61.06	0.007
SLC7A11 (ng/mL)	3.52 ± 0.30	4.99 ± 0.21	−1.46 ± 0.25	0.001
PBMC (mRNA)**	-	-	-	-
TNF-α	1.88 ± 0.31	1.59 ± 0.22	0.29 ± 0.36	0.400
IFN-γ	2.51 ± 0.67	1.82 ± 0.45	0.69 ± 0.70	0.512
GPX4	1.87 ± 0.14	2.00 ± 0.20	−0.13 ± 0.19	0.486
ACSL4	1.09 ± 0.13	1.15 ± 0.14	−0.06 ± 0.10	0.554
SLC7A11	0.73 ± 0.12	0.72 ± 0.13	0.01 ± 0.13	0.621

Note: *Values are given as mean ± SD or n (%).
**mRNA levels are relative to *GAPDH*.

Table 4. Baseline levels of inflammatory markers and ferroptosis markers in patients with and without long COVID-19.*

-	Total (n = 112)	Non-long COVID (n = 74)	Long COVID (n = 38)	*P-values*
Age, years	69.23 ± 1.44	69.92 ± 1.86	67.89 ± 2.21	0.281
Sex	-	-	-	0.580
Male	63 (56.3%)	43 (58.1%)	20 (52.6%)	-
Female	49 (43.8%)	31 (41.9%)	18 (47.4%)	-
Symptoms	-	-	-	-
Low-grade fever	-	-	2 (5.26%)	-
Dyspnea	-	-	12 (31.6%)	-
Palpitation	-	-	1 (2.63%)	-
Cough and sputum production		-	14 (36.8%)	-
Weakness	-	-	10 (26.3%)	-
Sleep difficulties	-	-	2 (5.26%)	-
Pain	-	-	5 (13.2%)	-
Chest distress	-	-	3 (7.89%)	-
Depression anxiety	-	-	4 (10.52%)	-
Memory loss	-	-	3 (7.89%)	-
Plasma (protein)	-	-	-	-
TNF-α (pg/mL)	76.58 ± 1.00	77.44 ± 1.30	74.88 ± 1.53	0.217

(Table 4) contd....

-	**Total (n = 112)**	**Non-long COVID (n = 74)**	**Long COVID (n = 38)**	***P-values***
IFN-γ (pg/mL)	454.96 ± 8.14	454.68 ± 10.02	455.50 ± 14.17	0.962
IL-6 (pg/mL)	39.06 ± 0.52	38.88 ± 0.66	39.42 ± 0.86	0.568
IL-17 (pg/mL)	263.73 ± 3.82	259.96 ± 4.78	271.09 ± 6.28	0.158
GPX4 (ng/mL)	108.75 ± 2.68	108.97 ± 3.24	108.32 ± 4.81	0.966
ACSL4 (pg/mL)	1652.16 ± 23.88	1638.01 ± 28.37	1679.71 ± 43.79	0.386
SLC7A11 (ng/mL)	3.25 ± 0.12	3.49 ± 0.16	2.76 ± 0.18	0.007
PBMC (mRNA)**	-	-	-	-
TNF-α	1.25 ± 0.11	1.23 ± 0.13	1.28 ± 0.20	0.989
IFN-γ	1.79 ± 0.43	1.25 ± 0.34	2.69 ± 0.96	0.328
GPX4	2.02 ± 0.19	1.92 ± 0.23	2.19 ± 0.33	0.503
ACSL4	1.04 ± 0.11	0.96 ± 0.14	1.17 ± 0.17	0.229
SLC7A11	0.90 ± 0.12	0.86 ± 0.15	0.97 ± 0.22	0.911

Note: *Values are given as mean ± SD or n (%).
**mRNA levels are relative to *GAPDH*.

4. DISCUSSION

The present study is the first to investigate the role of ferroptosis in the progression of COVID-19 and in the development of long COVID in patients who were infected at a time when the Omicron variant of SARS-CoV-2 was predominant. There were four major findings. First, ferroptosis was significantly upregulated in patients who required HFHC rather than LFOT. Second, ferroptosis was positively correlated with overall inflammation, as indicated by the increased levels of multiple inflammatory markers. Third, a comparison of baseline data and data collected after administration of antivirals and corticosteroids indicated there were decreases in inflammation and ferroptosis following treatment. Fourth, 38 of 127 patients (33.9%) suffered from long COVID-19, and their baseline plasma level of SLC7A11 (a ferroptosis inhibitor) was lower than in patients who did not develop long COVID. These findings indicate that ferroptosis was upregulated during the progression of COVID-19 and during long COVID, and that ferroptosis is less active as the symptoms of COVID-19 resolve.

Many previous studies have examined the pathophysiology of severe and critical COVID-19, and there is evidence that hyper-inflammatory responses contribute to this condition [13, 14]. These hyperinflammatory responses are due to increased activation of myeloid cells (neutrophils, monocytes, and macrophages) and increased secretion of proinflammatory cytokines, other cytokines, and chemokines [15, 16, 40]. Our results are consistent with these previous studies. There is also increasing evidence of a relationship between ferroptosis activity with COVID-19 progression. When affected cells release damage-associated molecular patterns and alarmins, ferroptosis induces an immune response that amplifies cell death and promotes a series of inflammation responses [41]. For example, a 2022 study of COVID-19 found that ferroptosis activity was related to the increased release of inflammatory cytokines and exacerbations in dysfunction of the immune system and inflammatory system, conditions that can lead to multi-organ complications [22]. A 2023 study enrolled 120 critical COVID-19 patients upon ICU admission and separated them into subgroups based on biomarker levels, which proved that the subgroup of patients with high catalytic iron and MDA levels was associated with reduced survival [42]. Another study of COVID-19 found that an elevated level of serum ferritin and low levels of serum iron and transferrin within three days of admission to an ICU were present in more than 75% of critically ill patients [43]. In contrast, Riegler *et al.* compared lung tissues with and without SARS-2 infections and reported no differences in apoptosis and ferroptosis [24]. These previous findings led us to investigate the role of ferroptosis in the progression of COVID-19 by measuring the levels of two inhibitors of ferroptosis (GPX4 and SLC7A11) and one promoter of ferroptosis (ACSL4).

We enrolled 127 consecutive COVID-19 patients, 22.0% of whom required HFNC during hospitalization. Compared to the LFOT group, the HNFC group had a higher level of NEUTs, CRP, and PCT (classical markers of inflammation), and a lower level of LYMs. Multiple studies of patients with COVID-19 have confirmed that increased levels of NEUTs and CRP were positively associated with increased production of inflammatory cytokines and more severe symptoms. For example, Lee *et al.* found that COVID-19 patients with high levels of CRP and NEUTs and low levels of LYMs had an increased need for supplementary oxy-

gen support and also had worse outcomes [25]. The depth of lymphopenia has shown a positive correlation with the severity of COVID-19. Unfortunately, there have been only a few reported therapies aimed at increasing LYMs [44]. In another study, it was found that the median concentration of CRP in patients with severe COVID-19 (80.9 mg/L, range: 0.5-275.2) was significantly higher compared to those with mild COVID-19 (39.8 mg/L, range: 0.5-221.2) [45]. Similarly, our HFNC group had higher plasma levels of TNF-α, IL-6, and IL-17 and a higher PBMC level of *NTF-α*.

TNF-α is a member of the tumor necrosis factor superfamily (TNFSF) and a potent proinflammatory cytokine whose plasma concentration correlates with infection by SARS-CoV-2 [46-48]. Many previous studies of patients with COVID-19 showed that increased serum levels of TNF-α and IFN-γ at admission were associated with increased risk of death [38, 40, 46, 48, 49]. The higher level of TNF-α in our HFNC group is likely related to their more severe or critical disease status. Our LFOT and HFNC groups had no significant difference in plasma IFN-γ levels. A postmortem study of COVID-19 patients found an increased level of mononuclear cells in the lungs but no change in the plasma level of IFN-γ [50]. This may be because of a stronger antiviral response to infection in lymphoid organs and infected tissues. In agreement, Petrone *et al.* found that the whole-blood level of IFN-γ in response to SARS-CoV-2 peptides was unrelated to disease severity, symptom onset, or lymphocyte count [51]. In addition to TNF-α, cell death and viral infection can activate inflammasomes, which produce an excess of cytokines (IL1β, IL-6, IL-18) and chemokines as part of the 'cytokine storm' [52-55]. Notably, Wilson *et al.* reported that the levels of IL-6, IL-8, and other inflammatory factors were similar in COVID-19 patients with critical illness and patients with acute respiratory distress syndrome (ARDS) or sepsis [56].

We found higher plasma levels of ACSL4 and lower plasma levels of GPX4 and SLC7A11 in the HFNC group than in the LFOT group. ACSL4 promotes ferroptosis in a variety of cell types, including neurons, fibroblasts, vascular cells, and immune cells [57-59]. Infection by the SARS-CoV-2 virus leads to increased production of reactive oxygen species, iron metabolism and transport, and antioxidative defenses. A study of human placental tissues reported that ACSL4 expressions in 23 samples of SARS-COV-2-positive patients were significantly greater than in 6 samples of SARS-CoV-2-negative patients [60]. There is also evidence that ferroptosis and ACSL4 inhibitors can significantly decrease viral replication [60]. Thus, inhibition of ferroptosis has potential as a method for treatment of patients with COVID-19 [61, 62]. Qu *et al.* [63] found that the level of ACSL4 was significantly increased in brain tissues following subarachnoid hemorrhage and inhibiting the expression of ACSL4 and suppressing ferroptosis-reduced inflammation and oxidative stress. In addition, a study of psoriatic lesions showed that the expression of ACSL4 had positive correlations with the expression of TNF-α, IL-6, IL-8, and IL-17α [64]. These results suggest that activation of ACSL4 may exacerbate disease severity by promoting inflammation. Erastin (an inducer of ferroptosis) can enhance the production of TNF-α and IL-6, indicating that ferroptosis promotes inflammatory responses; this response was inhibited when ferrostatin-1 or ACSL4 siRNA was added [65]. Liu *et al.* showed that IFN-γ/TNF-α promoted the expression of ACSL4, TNF-α, IL-6, and IL-8 and that down-regulation of ACSL4 blocked the effects of IFN-γ/TNF-α on activation of ferroptosis [64]. We found that the plasma levels of GPX4 and SLC7A11 were negatively correlated with the levels of TNF-α, IL-6 and IL-17 in COVID-19 patients. A previous study of isolated macrophages and synovial fibroblasts found that TNF-α protected cells from ferroptosis prominently by enhancing the activity of the system x_c^- cysteine/glutamate transporter [66]. A 2023 study reported that 24 h incubation of human endothelial cells with serum from COVID-19 patients who did not survive led to significantly decreased expression of GPX4 [38]. Our results and the results of these other studies demonstrate increased ferroptosis activity during inflammation and suggest that ferroptosis has an important role in the progression of COVID-19.

Our measurements of markers of inflammation and ferroptosis in plasma samples of COVID-19 patients indicated that both of these processes had decreased activity after the treatment. Single-cell RNA-seq analysis of high-risk COVID-19 patients who received corticosteroid treatment revealed a downregulation in 'IFN-γ response' and 'TNF-α signaling *via* NF-κB'in classical/intermediate monocytes [67]. While specific therapies for COVID-19 are currently lacking, researchers have discovered that iron chelators could be effective in its treatment. This is because they can reduce inflammation and prevent the virus from binding to receptors that are essential for host cell entry [68-70].

Between 10% and 25% of COVID-19 patients experience long COVID, which typically manifests as significant limitations of daily activities, increased long-term leave from work, and other sequelae that can cont-

inue for more than one year [28, 71]. The most common symptoms of long COVID in our study population were cough and sputum production (36.8%), dyspnea (31.6%), and weakness (26.3%), which were consistent with present reports [29, 30, 72]. Systemic inflammatory and neuroinflammatory processes, endothelial damage, and reactivation of latent pathogens are possible causes of long COVID [34, 73]. A recent meta-analysis revealed that approximately 30% of patients recovering from SARS-CoV-2 pneumonia displayed persistent fibrotic changes during the initial 12 months post-discharge [74]. These changes are associated with an increased risk of developing chronic cough. Additionally, Gonçalves *et al.* identified frailty as a valuable predictor of persistent cognitive impairment [75]. Several studies showed that a decrease in GSH, inactivation of GPX4, and increased lipid peroxidation occurred in patients who had long COVID and neuropsychiatric sequelae [76, 77]. We found that the baseline plasma SLC7A11 level was lower in patients who developed long COVID ($P < 0.007$); however, our long COVID and non-long COVID groups had no significant differences in the expression of *GPX4*, *ACSL4*, and *SLC7A11* in PBMCs. Our finding that a high baseline level of plasma SLC7A11 was associated with the development of long COVID-19 is suggestive. However, further research is needed to clarify whether ferroptosis plays a role in the pathogenesis of long COVID.

Our study has certain limitations. First, we did not use a healthy control group for comparisons. However, many previous studies have confirmed the presence of inflammation in patients with COVID-19. Moreover, our main purpose was to examine the relationship of ferroptosis with the severity of COVID-19 and with the progression to long COVID. Second, our sample size was relatively small, and all patients were from a single institution. Studies of larger and more diverse populations are therefore required to confirm the reliability of ACSL4 and SLC7A11 as biomarkers for predicting COVID-19 severity and progression to long COVID.

CONCLUSION

Our two major findings are that there is upregulation of ferroptosis during the progression of COVID-19, as indicated by measurements of two ferroptosis inhibitors (SLC7A11 and GPX4) and one ferroptosis promoter (ACSL4), and that ferroptosis activity was positively associated with COVID-19 severity and the level of inflammation. These findings provide an increased understanding of the role of ferroptosis during the pathogenesis of COVID-19 and long COVID, and suggest that future studies should consider targeting of ferroptosis as a novel therapeutic approach.

AUTHORS' CONTRIBUTIONS

WZ conceived and designed the experiments and wrote the paper; SYW, YXH, HKZ, and JCC performed the experiments and analyzed and interpreted the data for this study; SSD, DDL, and ML contributed reagents, materials, analysis tools or data; YG wrote the paper; and CML conceived and designed the experiments.

LIST OF ABBREVIATIONS

COVID-19	=	Corona Virus Disease 2019
PBMC	=	Peripheral Blood Mononuclear Cell
TNF-α	=	Tumor Necrosis Factor-Alpha
IL-6	=	Interleukin 6
IL-17	=	Interleukin 17
ACSL4	=	Acyl-CoA Synthetase Long-chain Family Member 4
mRNA	=	Messenger RNA
GPX4	=	Plasma Glutathione Peroxidase 4
SLC7A11	=	Cystine/glutamate Transporter-11
SARS-CoV-2	=	Severe Acute Respiratory Syndrome Coronavirus 2
GSH	=	Glutathione
PUFAs	=	Poly-unsaturated Fatty Acids
LFOT	=	Low-flow Oxygen Therapy
HFNC	=	High-flow Nasal Cannula Oxygen Therapy
RT-PCR	=	Real-time Reverse Transcriptase-polymerase Chain Reaction
IVIG	=	Intravenous Immunoglobulin
WBCs	=	White Blood Cells
NEUTs	=	Neutrophil
LYMs	=	Lymphocytes
ALT	=	Alanine Aminotransferase
AST	=	Aspartate Aminotransferase
CRE	=	Creatinine
BUN	=	Blood Urea Nitrogen

CRP	= C-reactive Protein
FIB	= Fibrinogen
PCT	= Procalcitonin
EDTA	= Ethylenediaminetetraacetic Acid
IFN-γ	= Interferon Gamma
ELISA	= Enzyme-linked Immunosorbent Assay
cDNA	= Complementary DNA
qRT-PCR	= Quantitative Real-time Reverse Transcriptase-polymerase Chain Reaction
NCBI	= National Center of Biotechnology Information
GAPDH	= Glyceraldehyde-3-phosphate Dehydrogenase
SDs	= Standard Deviations
ANOVA	= A One-way Analysis of Variance
TNFSF	= Tumor Necrosis Factor Superfamily
IL-18	= Interleukin 18
ARDS	= Acute Respiratory Distress Syndrome
siRNA	= Small Interfering RNA

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was approved by the Bengbu Medical College Ethics Committee (approval number: 2022 No. 275).

HUMAN AND ANIMAL RIGHTS

All procedures performed in studies involving human participants were in accordance with the ethical standards of institutional and/or research committee and with the 1975 Declaration of Helsinki, as revised in 2013.

CONSENT FOR PUBLICATION

Informed consent was obtained from all participants.

STANDARDS OF REPORTING

STROBE guidelines were followed.

AVAILABILITY OF DATA AND MATERIALS

Data are available from the corresponding author upon request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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