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Inflammation and iron metabolism dysregulation as hallmarks of COVID-19 severity

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Background: Coronavirus Disease 2019 (COVID-19) can cause severe clinical outcomes by triggering an excessive immune response and systemic inflammation. As disease severity increases, levels of key inflammatory markers rise accordingly. Inflammation is also associated with alterations in iron metabolism, contributing to immune dysfunction. In this study, we investigated the relationship between inflammation, iron metabolism, and COVID-19 severity to identify potential biomarkers and improve understanding of disease mechanisms. We also perform an exploratory analysis to investigate whether these features associated also with long COVID-19 severity.

Materials and methods: We conducted a retrospective, multicentre, case-control-cross-sectional observational study including 144 COVID-19 hospitalized patients and 139 COVID-19-negative controls (individuals referred for vaccination), enrolled at two hospitals in the Lombardy region, Italy. Patients were divided into four groups according to disease severity. Laboratory parameters included serum markers of inflammation (IL-6, S100A8/A9, and C-reactive protein) and iron metabolism (iron, ferritin, hepcidin, total transferrin, and transferrin saturation). Finally, a publicly available peripheral blood mononuclear cell (PBMC) transcriptomic dataset was analyzed to evaluate whether inflammation and iron dysmetabolism associated with long COVID-19 severity.

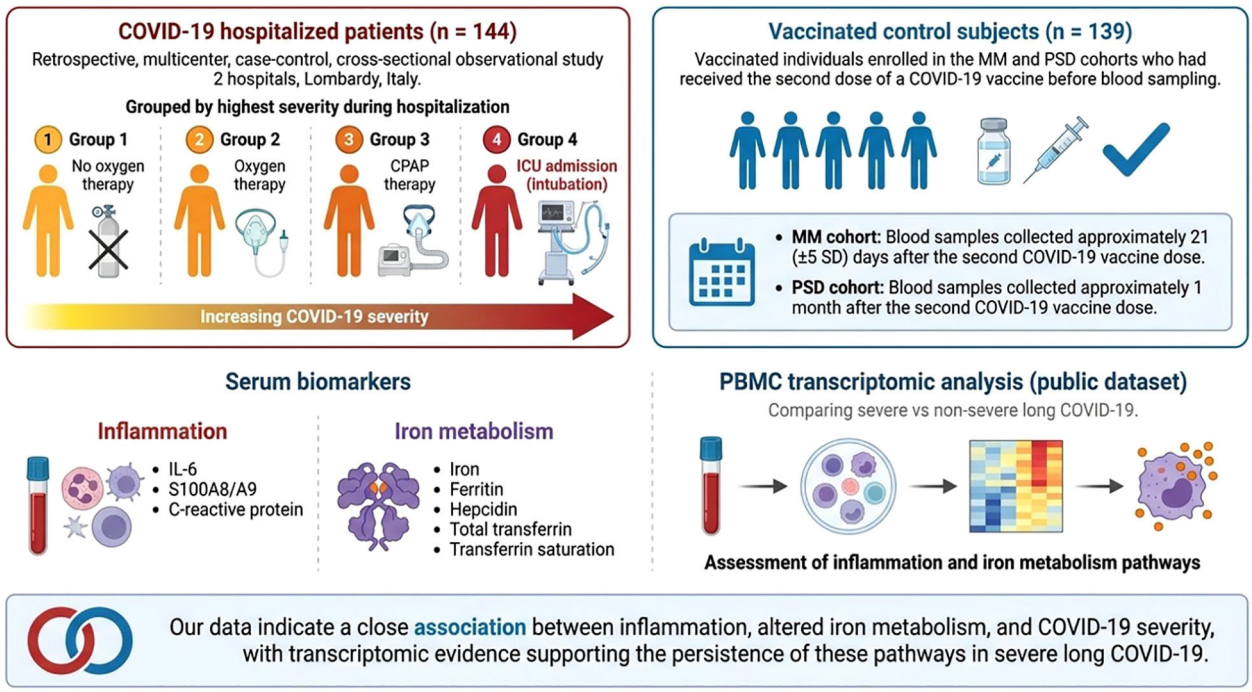
Results: COVID-19 patients exhibited a marked increase in inflammatory markers (IL-6, S100A8/A9, C-reactive protein), as well as ferritin and hepcidin, while serum iron, transferrin levels, and saturated transferrin levels were lower compared to controls. Moreover, increasing COVID-19 severity was associated with higher ferritin, inflammatory markers, white blood cell and neutrophil counts, whereas total transferrin progressively decreased with disease severity. PBMC alterations in

iron metabolism and inflammation were observed in patients with severe long COVID-19.

Conclusions: Our data indicate a close association between inflammation, altered iron metabolism, and COVID-19 severity, with transcriptomic evidence supporting the persistence of these pathways in severe long COVID-19.

KEYWORDS

COVID-19, hepcidin, inflammation, iron metabolism, S100A8/A9



GRAPHICAL ABSTRACT

1 Introduction

Since it first began spreading globally in late 2019, the novel coronavirus SARS-CoV-2, the causative agent of Coronavirus Disease 2019 (COVID-19), has given rise to numerous sub-variants, causing breakthrough infections spreading worldwide (1–3). In May 2023, the World Health Organization recognized COVID-19 as a potential long-term endemic condition and emphasized the need for sustained vigilance (4). In this context, high-risk individuals remain particularly susceptible to severe and potentially life-threatening outcomes (5). Furthermore, the incidence of long COVID-19 positively correlates with the severity of the SARS-CoV-2 viral infection's acute phase (6–8). Thus, understanding the biological mechanisms through which SARS-CoV-2 infection progresses to severe disease remains a critical and timely area of investigation (9, 10).

SARS-CoV-2 infection is characterized by a systemic inflammatory response that can lead to a wide spectrum of clinical manifestations, ranging from asymptomatic or mild illness to severe pneumonia, acute respiratory distress syndrome, cardiovascular complications, multi-organ failure, and death (11–14). The activation of the innate immune response triggers an excessive release of inflammatory mediators (15). Among these, interleukin-6 (IL-6) and the damage-associated molecular pattern molecule S100A8/A9 are consistently elevated in patients with moderate to severe COVID-19, and their persistent elevation has been associated with chronic inflammation, immune dysregulation, and tissue damage in diverse pathological contexts (16–18).

Several strategies have been tested to classify and predict COVID-19 severity (19, 20). Accumulating evidence indicates that the SARS-CoV-2-induced systemic inflammation at the basis of COVID-19's most severe outcomes is linked to alterations in iron

Abbreviations: BF, brain fog; COVID-19, Coronavirus Disease 2019; CPAP, continuous positive airway pressure; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; GEO_Gene Expression Omnibus; hsCRP, high sensitivity C Reactive Protein test; ICU, intensive care unit; IL-6, interleukin-6; MM, MultiMedica; PBMCs_peripheral blood mononuclear cells; PSD, Policlinico San Donato; RBC, red blood cell; TSAT, transferrin saturation; UMAP, Uniform Manifold Approximation and Projection; WBC, white blood cell.

metabolism. Specifically, COVID-19 patients often exhibit significantly reduced serum iron, transferrin saturation (TSAT) and transferrin levels, accompanied by markedly elevated ferritin, and hepcidin. These changes reflect functional iron deficiency, where iron is present within the body but unavailable for physiological use (21).

Iron sequestration is mediated by hepcidin, a central regulator of iron homeostasis. Hepcidin is a liver-derived peptide that responds to inflammatory stimuli by inhibiting cellular iron efflux. Upon binding to ferroportin on cellular surfaces, hepcidin induces its internalization, thereby blocking both intestinal iron absorption and the release of iron from macrophages. The net result is a decline in serum iron, despite preserved total body iron stores (22).

In the context of COVID-19, this adaptive response, aimed at limiting iron availability to invading pathogens, can become maladaptive. Iron is essential for numerous cellular processes, including DNA synthesis, mitochondrial respiration, and immune cell proliferation and function (23). In particular, T lymphocytes and natural killer cells are highly dependent on iron (24). Consequently, iron sequestration may impair the host's ability to mount an effective immune response, potentially contributing to viral persistence and worsening disease severity.

Decreased iron availability leads to reduced hemoglobin synthesis and may contribute to the anemia observed in patients with COVID-19. Elevated ferritin further reflects the combined effects of inflammation and iron dysregulation (25).

Despite these observations, the relationship between inflammation, iron metabolism and COVID-19 severity remains incompletely understood. Additionally, how these alterations influence basic hematological parameters requires further investigation.

In this study, we aimed to characterize the inflammatory, iron metabolism, and hematological profiles in COVID-19 patients to identify biomarkers associated with disease severity and shedding light on the pathophysiological mechanisms contributing to the complications of COVID-19 and long COVID-19.

2 Materials and methods

2.1 Subject recruitment

This retrospective, multicentre, case-control-cross-sectional observational study included participants recruited between March and May 2021 at IRCCS MultiMedica (MM) and IRCCS Policlinico San Donato (PSD), Milan, Italy. Demographic and clinical data were collected for each enrolled subject, and SARS-CoV-2 infection was assessed by reverse transcription-quantitative polymerase chain reaction. SARS-CoV-2-positive patients were enrolled during the first and second COVID-19 waves for the PSD cohort and during the third COVID-19 wave for the MM cohort. SARS-CoV-2-positive patients were divided into four groups based on COVID-19 severity, classified according to the highest severity experienced during hospitalization (1): no oxygen therapy, (2) oxygen therapy, (3) treatment with continuous positive airway pressure (CPAP), and (4) admission to the intensive care unit (ICU) with intubation.

COVID-19-negative control group consisted of individuals enrolled in the MM and PSD cohorts who had received the second dose of a COVID-19 vaccine before blood sampling. In the PSD cohort, control subjects were healthcare workers who received the BNT162b2 mRNA vaccine (26). Previous SARS-CoV-2 infection was excluded by anti-nucleocapsid IgG testing. In the MM cohort, control subjects were individuals enrolled during the Italian national COVID-19 vaccination program who self-reported no history of COVID-19 infection. Participants received authorized anti-SARS-CoV-2 vaccines available at the time of enrollment, including the BNT162b2 mRNA vaccine (Pfizer-BioNTech), mRNA-1273 vaccine (Moderna), and ChAdOx1 nCoV-19 vaccine (AstraZeneca).

2.2 Ethics approval and consent to participate

The experimental protocols for control groups (MM: 479.2021, PSD: 48/2021/spall/PU/403-2021), and COVID-19 patients (MM: 453.2020, PSD: 75/INT/2020), received approval from the respective Hospital Institutional Ethics Committees. The study adhered to the principles of the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all participants prior to enrolment.

2.3 Serum sample collection

Serum samples were obtained from COVID-19 patients and control subjects. For control subjects, blood samples were collected approximately 21 days (± 5) after the second COVID-19 vaccine dose in the MM cohort and approximately 1 month after the second vaccine dose in the PSD cohort. Blood samples from the PSD cohort were collected, processed, and stored at the BioCor Biobank according to the same standardized operating procedures adopted at MM, ensuring harmonized pre-analytical handling across centers. Briefly, the serum was collected by allowing the blood to clot for 30–45 minutes at room temperature (18-22 °C), followed by centrifugation at 2700×g for 15 minutes. All samples underwent one freeze-thaw cycle before analysis.

2.4 Laboratory tests

A panel of consolidated blood markers was assessed, including white (WBC) ($x10^3$/uL) and red (RBC) blood cell count ($x10^6$/uL), lymphocytes ($x10^3$/uL), monocytes ($x10^3$/uL), neutrophils ($x10^3$/uL), creatinine (mg/dL), using routine automated laboratory analyzers. Serum concentrations of iron (µg/dL), TSAT (%), ferritin (ng/mL), total transferrin (mg/dL), C-reactive protein (CRP) (mg/dL), and hepcidin (ng/mL) were determined. Iron, ferritin, transferrin, and CRP were measured using an automatic analyzer. CRP was detected using a high-sensitivity test (hsCRP). Hepcidin levels were quantified using the high-sensitivity human Hepcidin 25 enzyme-linked immunosorbent assay (ELISA) kit (cod. EIA5782, DRG) using Dynex Technology. Quality controls for both high and low cytokine concentrations were included in each plate, and all values fell within the ranges specified in the kit certificates of analysis.

2.5 Measurement of serum levels of inflammation markers

Serum concentrations of inflammation markers were measured using high-sensitivity ELISA kits for human IL-6 (BMS213-2HS, Invitrogen) and S100A8/A9 (MRP8/14, BUHLMANN), following the manufacturer's instructions.

2.6 Bioinformatic analysis of publicly available transcriptomic data from GEO in long COVID-19 PBMCs

Publicly available transcriptomic data were retrieved from the Gene Expression Omnibus (GEO) under accession number GSE251849. The dataset includes peripheral blood mononuclear cells (PBMCs) from long COVID-19 patients with brain fog (BF) (n = 5) and without BF (n = 6). Differential gene expression analysis was performed using GEO2R. Statistical significance was assessed using the Benjamini-Hochberg false discovery rate, with a threshold of adjusted p-value < 0.05 and no log2 fold-change cutoff. The HALLMARK_HEME_METABOLISM and the HALLMARK_INFLAMMATORY_RESPONSE gene sets were obtained from the Molecular Signatures Database (27) [1] both comprised 200 genes, of which 170 and 156, respectively, were detected in the dataset. Over-representation analysis was performed using a two-tailed Fisher's exact test to assess the enrichment of the gene sets among the differentially expressed genes of the dataset. The exact probability was calculated using MedCalc Software Ltd. Fisher exact probability calculator (Version 23.5.1; accessed April 15, 2026).

2.7 Statistical analysis

Categorical variables were presented as frequencies and percentages and compared between groups by Chi-Square test or Fisher's exact test, as appropriate. Continuous variables were summarized with median and quartiles, and compared between COVID-19 positive and negative subjects by non-parametric Wilcoxon test[1]. For comparisons between the four COVID-19 severity levels, the non-parametric Kruskall Wallis test was used. Wilcoxon test with *post-hoc* Bonferroni adjustment was performed for multiple comparisons analysis. To evaluate the association between variables of interest, we performed Spearman correlation and linear regression analysis. Given the large number of linear regression and correlation analyses conducted, multiple-comparison correction was performed using the Benjamini-Hochberg false discovery rate (FDR) procedure. Statistical significance was defined as an FDR-adjusted q-value < 0.05. Furthermore, to assess the association between inflammatory markers or iron metabolism parameters and COVID-19 infection, logistic regression models adjusted by age and sex were performed, and Odds Ratios with 95% Confidence Intervals were calculated. Furthermore, to account for baseline differences between groups, propensity score matching was performed to obtain age- and sex-matched cohorts. Differences in inflammatory and iron metabolism parameters were subsequently re-evaluated between the matched COVID-19 (n = 60) and non-COVID-19 (n = 60) groups. Statistical analyses were implemented by SAS Software 9.4 and p-values <0.05 were considered statistically significant, with the exception of the COVID-19 severity levels analysis, where the p-value significance level was set to 0.0125.

[1] Available at: https://www.gsea-msigdb.org

3 Results

3.1 Cohort description and COVID-19 severity classification

Patient cohort consisted of 144 patients hospitalized for COVID-19 [median age (years) (quartiles): 66.5 (59.5-76.0), 64.6% males] (Table 1) and 139 subjects negative for COVID-19 (controls), (median age: 55.0 (55.0-61.6), 30.9% males). Among COVID-19-positive patients, considering the highest severity experienced during hospitalization, 10 (6.9%) did not require oxygen therapy (group 1), 46 (31.9%) required oxygen therapy (group 2), 35 (24.3%) required treatment with CPAP (group 3), 53 (36.8%) required admission to the ICU (group 4), and 49 (34.0%) patients died in the hospital.

3.2 Circulating inflammatory cytokines are enhanced in COVID-19 patients

To assess whether COVID-19 is associated with alterations in inflammation and iron metabolism, we measured inflammatory markers in COVID-19 patients compared to COVID-19 negative

TABLE 1 COVID-19 patients' characteristics.

Characteristics	Values
N total enrolled patients	144
Not-survived at hospital discharge, n (%)	49 (34.0)
O_2 therapy not required n (%)	10 (6.9)
O_2 therapy required n (%)	46 (31.9)
CPAP required in ward n (%)	35 (24.3)
Intubation required in ICU n (%)	53 (36.8)
Age: Median(quartiles)	*66.5 (59.5-76)
Males, n (%)	93 (64.6)
WBC ($x10^3$/uL), Median(quartiles)	8.0 (5.3-10.3)
RBC ($x10^6$/uL), Median(quartiles)	4.3 (3.7-4.7)
Neutrophils ($x10^3$/uL), Median(quartiles)	6.7 (4.1-9.6)
Lymphocytes ($x10^3$/uL), Median(quartiles)	0.9 (0.6-1.3)
Monocytes ($x10^3$/uL), Median(quartiles)	0.5 (0.3-0.8)
Creatinine (mg/dL), Median(quartiles)	0.8 (0.6-1.1)
Cardiovascular diseases	31 (21.7)
Diabetes	32 (22.4)

CPAP, continuous positive airway pressure ventilation system. ICU, intensive care unit; CRP, C-reactive protein; WBC, white blood cells; RBC, red blood cells. *Age of the patients from the Policlinico San Donato cohort was provided as decade ranges for privacy reasons. Therefore, all patients aged in a given decade were arbitrarily assigned the middle value of the given decade.

controls. In both groups, blood tests were analysed to compare the expression level of inflammatory markers IL-6, S100A8/A9, and CRP between the two conditions (Figures 1a–c). IL-6, S100A8/A9, and CRP were found to be increased in COVID-19 patients compared to the control group ($p \leq 0.0001$ for all comparisons).

3.3 COVID-19 patients show altered iron metabolism

Next, we measured parameters related to iron metabolism in the same study cohorts. Compared to controls, COVID-19 significantly affected all the variables assessed (Figures 1d–h). Indeed, serum iron ($p < 0.0001$), total transferrin ($p < 0.0001$) and TSAT ($p < 0.05$) were significantly reduced, while ferritin ($p < 0.0001$) and hepcidin ($p < 0.0001$) were significantly higher in serum from COVID-19 patients compared to COVID-19 negative controls. To account for potential confounding effects arising from differences in baseline characteristics between COVID-19-positive and COVID-19-negative subjects, an age- and sex-matched subgroup of 60 subjects per group was selected and the analyses were repeated. Re-evaluation of the parameters confirmed the original findings, with all variables remaining highly significant ($p < 0.0001$), except for TSAT, which no longer showed a significant difference (data not shown).

3.4 Iron metabolism parameters associate with inflammatory markers and blood count in COVID-19 patients

Next, we explored how each parameter describing iron metabolism was associated with inflammatory cytokines and blood count values in COVID-19 positive subjects through linear regression analyses of collected data (Table 2). Results were reported as FDR-adjusted q-values. Serum iron increase was associated with a decrease of CRP ($q < 0.01$), and IL-6 ($q < 0.01$). Increasing levels of serum ferritin were associated with a decrease in lymphocyte count (for a 10-unit increase of ferritin, $q < 0.05$). Increased hepcidin was associated with an increase of CRP ($q < 0.01$) and IL-6 ($q < 0.01$) while a decrease of lymphocyte count ($q < 0.01$). Increasing levels of total transferrin were associated with a decrease of CRP (for 10-unit increase of transferrin, $q < 0.05$), but increase of lymphocytes (for 10-unit increase of transferrin, $q < 0.01$). Furthermore, the increase of saturation of circulating transferrin was found to be associated with decrease of CRP ($q < 0.05$). Moreover, performing correlation analysis, in addition to the associations already identified by linear regression, we also observed negative correlations between transferrin and neutrophils ($q < 0.01$) and TSAT and IL6 ($q < 0.05$), as well as positive correlations between ferritin and S100A8/A9 and CRP ($q < 0.05$ and $q < 0.05$) and between monocytes and TSAT and iron ($q < 0.05$ and $q < 0.05$).

3.5 Iron metabolism and the inflammatory profile inform on the risk of being affected by COVID-19

To evaluate the association between studied parameter and COVID-19, we performed logistic regression adjusted by age and sex. The results showed a positive association between an increase in inflammatory cytokine concentration , including IL-6 and S100A8/A9, and CRP levels, and COVID-19 positivity (Table 3). Moreover, higher levels of ferritin and hepcidin were associated with the presence of COVID-19. On the other hand, lower levels of iron and transferrin were associated with COVID-19.

3.6 COVID-19 severity associates with an increase in serum interleukin-6 and alarmin S100A8/A9

In order to inquire into the association between inflammation and COVID-19 severity, patients affected by COVID-19 were sorted by severity into four groups: 1) no oxygen therapy; 2) oxygen therapy required; 3) treatment with CPAP, and 4) admission to the ICU with intubation. IL-6, S100A8/A9, and CRP data from groups 2, 3, and 4 were then compared with group 1 (Figure 2). It was observed that sera from patients in group 4 were significantly enriched in IL-6 compared to group 1 ($p < 0.0125$). Interestingly, S100A8/A9 was enriched in sera from patients of groups 3 and 4 compared to group 1 ($p < 0.0125$).

3.7 COVID-19 severity associates with an increase in serum ferritin and a decrease in serum transferrin

We then studied iron metabolism across COVID-19 severity levels. Hematological levels of iron, ferritin, transferrin, TSAT, and hepcidin were compared between the four groups. It was found that, compared to group 1, serum from patients in group 4 contained significantly higher levels of ferritin ($p < 0.0125$) and lower levels of total transferrin ($p < 0.0125$). No statistically significant differences were observed between the groups for TSAT, iron, and hepcidin (Figure 3).

3.8 COVID-19 severity affects blood count parameters

Iron metabolism and inflammation are strongly linked to the number, type, and functionality of circulating blood cells. Hence, to shed further light on the mechanisms involved in the interplay between COVID-19, iron metabolism, and inflammation, data from the blood count of COVID-19 positive patients were collected. As shown in Table 4, creatinine, RBC count, lymphocytes, or monocytes number were not differentially distributed across COVID-19 severity levels ($p > 0.05$). However, a positive trend was observed in WBC ($p < 0.01$) and neutrophil count ($p < 0.05$) across COVID-19 severity levels. Interestingly, neutrophil displayed a slight, gradual increase in their concentration from severity group 1 to 3, a trend that does not apply to group 4.

3.9 Long COVID-19 severity associates with dysregulation of heme metabolism and immune response genes in PBMCs

To investigate whether the role of iron metabolism in COVID-19 severity is maintained in long COVID-19 severity, we performed a bioinformatics exploratory analysis on publicly available datasets.

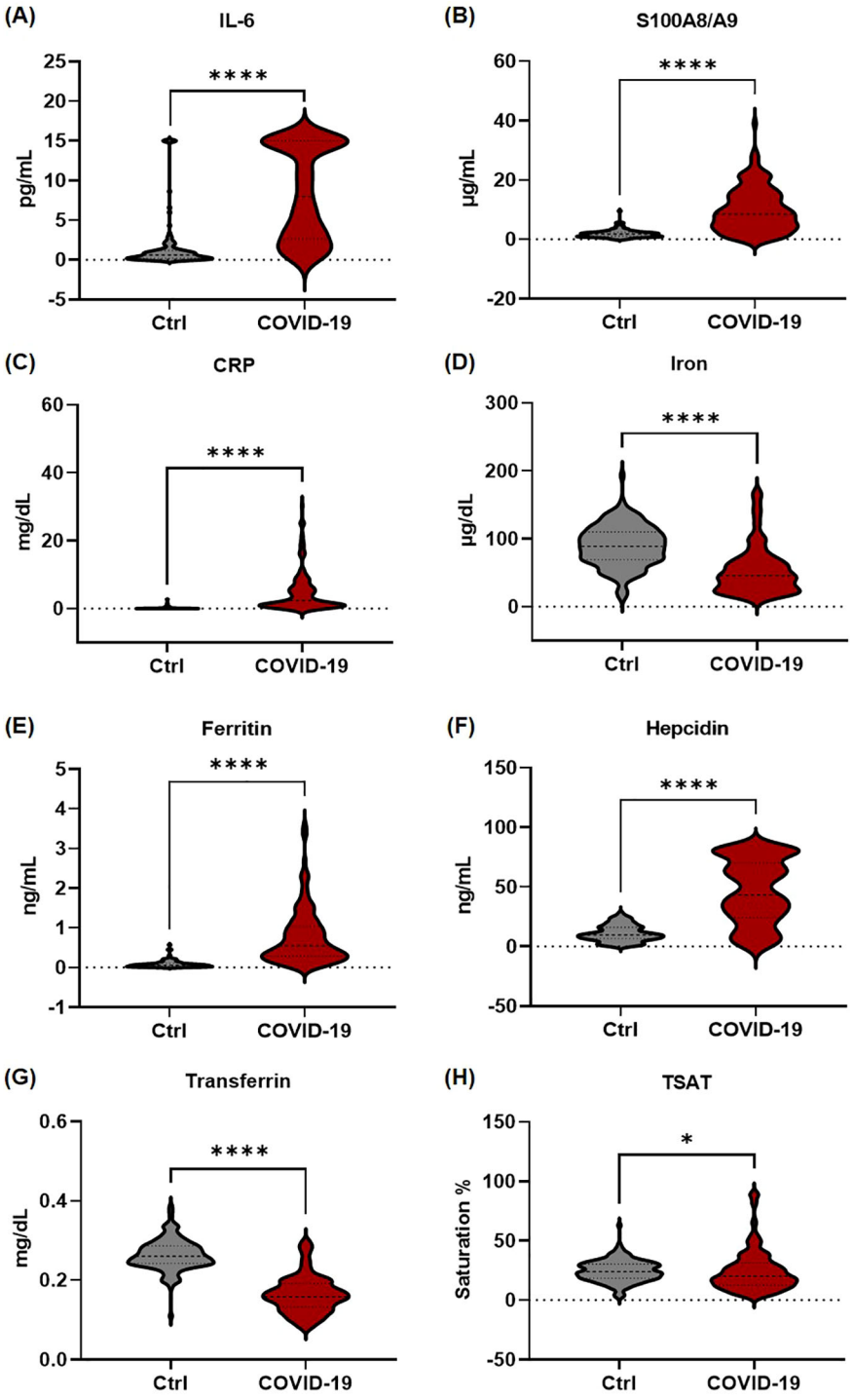


FIGURE 1
Comparison of circulating inflammatory cytokines and iron metabolism parameters between COVID-19 and control patients. Comparison of IL-6 **(A)**, S100A8/A9 **(B)**, CRP **(C)**, iron **(D)**, ferritin **(E)**, hepcidin **(F)**, transferrin **(G)**, and transferrin saturation **(H)** serum levels between control and COVID-19 patients. COVID-19=Coronavirus Disease 2019; CRP, C-reactive protein; IL-6, interleukin-6; TSAT, transferrin saturation; Ctrl, control patients. '*'=$p < 0.05$; '****' =$p < 0.0001$. N = 139 and 144 for control and COVID-19 patients, respectively.

We compared gene expression in PBMCs between long COVID-19 patients with and without BF and evaluated the over-representation of heme metabolism genes and inflammatory response genes, which are closely related to iron metabolism, among the resulting differentially expressed genes. The two patient groups exhibited clear clustering, as illustrated by the Uniform Manifold Approximation and Projection (UMAP) projection (Figure 4a).

A total of 16,134 genes were tested, and 2,071 differentially expressed genes were identified, of which 1,238 were upregulated and 833 downregulated in patients with BF compared to those without (Figure 4b). The HALLMARK_HEME_METABOLISM gene set included 200 genes (the complete gene list is available as Supplementary Data 1), of which 170 were detected in the dataset. Among these, 31 genes were significantly differentially expressed

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TABLE 2 Linear regression analysis of iron metabolism in relation to inflammation and blood count parameters.

Inflammatory, blood count, and biochemical variables	Iron metabolism variables				
	Iron	*Ferritin^*	*Hepcidin*	*Transferrin^*	*TSAT*
CRP	**-0.06****	0.02	**0.07****	**-0.31***	**-0.08***
S100A8/A9	0.02	0.01	0.004	-0.23	0.05
IL6	**-0.05****	0.003	**0.06****	-0.23	-0.07
RBC	0.002	0.001	0.005	0.02	0.001
WBC	0.01	0.001	-0.01	-0.18	0.02
Neutrophils	0.01	0.01	-0.02	-0.29	0.02
Lymphocytes	0.002	**-0.002***	**-0.01****	**0.04****	-0.001
Monocytes	0.004	0	-0.004	-0.02	0.01
Creatinine	-0.001	0	0.002	-0.02	0

Each cell reports the regression coefficient from a univariate linear regression model where the dependent variable (outcome) is the inflammation or blood count/biochemical parameters (in rows) and the independent variable is the iron metabolism parameter (in columns). Significance is reported with Benjamini-Hochberg adjustment (FDR-adjusted q-values). ^Regression coefficients for ferritin and transferrin were reported for 10-unit increase. CRP, C-reactive protein; IL-6, interleukin 6; RBC, red blood cells; WBC, white blood cells; TSAT, transferrin saturation. Statistically significant models are reported in bold, with '*' standing for $q < 0.05$, '**' for $q < 0.01$.

between the two groups, including 24 upregulated and 7 downregulated in patients with BF. Over-representation analysis using two-tailed Fisher's exact test showed a significant enrichment of the HALLMARK_HEME_METABOLISM gene set among differentially expressed genes (exact probability = 0.0381), indicating a non-random over-representation of iron/heme-related genes among differentially expressed transcripts. The same analysis was performed using the HALLMARK_INFLAMMATORY_RESPONSE gene set (the complete gene list is available as Supplementary Data 2); in this case, 36 genes out of the 156 found in the dataset resulted deregulated (11 downregulated and 25 upregulated), highlighting a strong effect of long COVID-19 on the immune response as proved by the over-representation analysis (two-tailed Fisher's exact, exact probability = 0.0004). These results indicate a statistically significant over-representation of HALLMARK_HEME_METABOLISM and HALLMARK_INFLAMMATORY_RESPONSE genes among differentially expressed genes between long COVID-19 patients with and without BF, suggesting that altered inflammatory response and iron/heme-related transcriptional programs of PBMCs may be associated with severity of long COVID-19.

TABLE 3 Age- and sex-adjusted logistic models correlating serum inflammatory and iron metabolism parameters to the diagnosis of COVID-19.

Variable	*Odds Ratio (95%CI)*	*P value*
IL-6	1.31 (1.21 – 1.43)	**<.0001**
S100A8/A9	1.71 (1.44-2.04)	**<.0001**
CRP	50.18 (16.53 – 152.33)	**<.0001**
Iron	0.97 (0.96 – 0.98)	**<.0001**
*Ferritin**	1.13 (1.09 – 1.17)	**<.0001**
Hepcidin	1.13 (1.09 – 1.17)	**<.0001**
*Transferrin**	0.65 (0.58 – 0.72)	**<.0001**
TSAT	1.00 (0.98 – 1.02)	0.9278

Odds ratio values for inflammatory cytokines and iron metabolism parameters in association with SARS-CoV-2 positivity. *Odds ratios for total transferrin and ferritin were calculated for 10-units increase. CI, confidence interval; IL-6, interleukin 6; TSAT, transferrin saturation. N = 139 and 144 for control and COVID-19 patients, respectively. Bold values indicate statistically significant results.

4 Discussion

Results of the present study demonstrate an association between iron metabolism, inflammation, and COVID-19 severity by analyzing a cohort of patients afferent to two hospitals in Milan, Italy. Furthermore, the association between iron metabolism and disease severity has also been highlighted in long COVID-19 patients through bioinformatic analyses of publicly available data.

Our data reinforce and extend previous reports that S100A8/A9, IL-6, and CRP serum concentrations are increased in COVID-19 patients (28–30) in a larger cohort, exploring a wider array of markers. Moreover, we quantifying serum iron, ferritin, transferrin, hepcidin, and transferrin saturation in function of SARS-CoV-2 infection. Our results are in keeping with the description of anemia of inflammation, a condition in which IL-6 upregulates hepcidin in the liver. Hepcidin binds ferroportin in enterocytes and macrophages, reducing serum iron. Consistent with our findings, anemia of inflammation is marked by an uncoupling of ferritin and serum iron levels. Our results are consistent with the observation of an altered profile of cytokines and iron metabolism described in COVID-19 syndrome (29, 31). Odds ratio analysis showed that inflammatory and iron metabolism markers were associated with COVID-19 disease (29, 31). It is important to note that these alterations have also been reported in long COVID-19 syndrome (32).

We show that inflammatory markers increase along with the rise of COVID-19 severity, extending previous observations on less granular severity-based classifications (21, 33–35). Notably, the rise in circulating inflammatory cytokines is linked to iron metabolism modulation through the interaction of IL-6 with hepcidin (36). Consistently, hepcidin is upregulated in COVID-19 patients compared to controls, while iron is significantly downregulated in COVID-19 patients compared to controls. These observations challenge previous results found in a cohort of 50 patients sorted by severity into mild, severe, and critical groups based on oxygen

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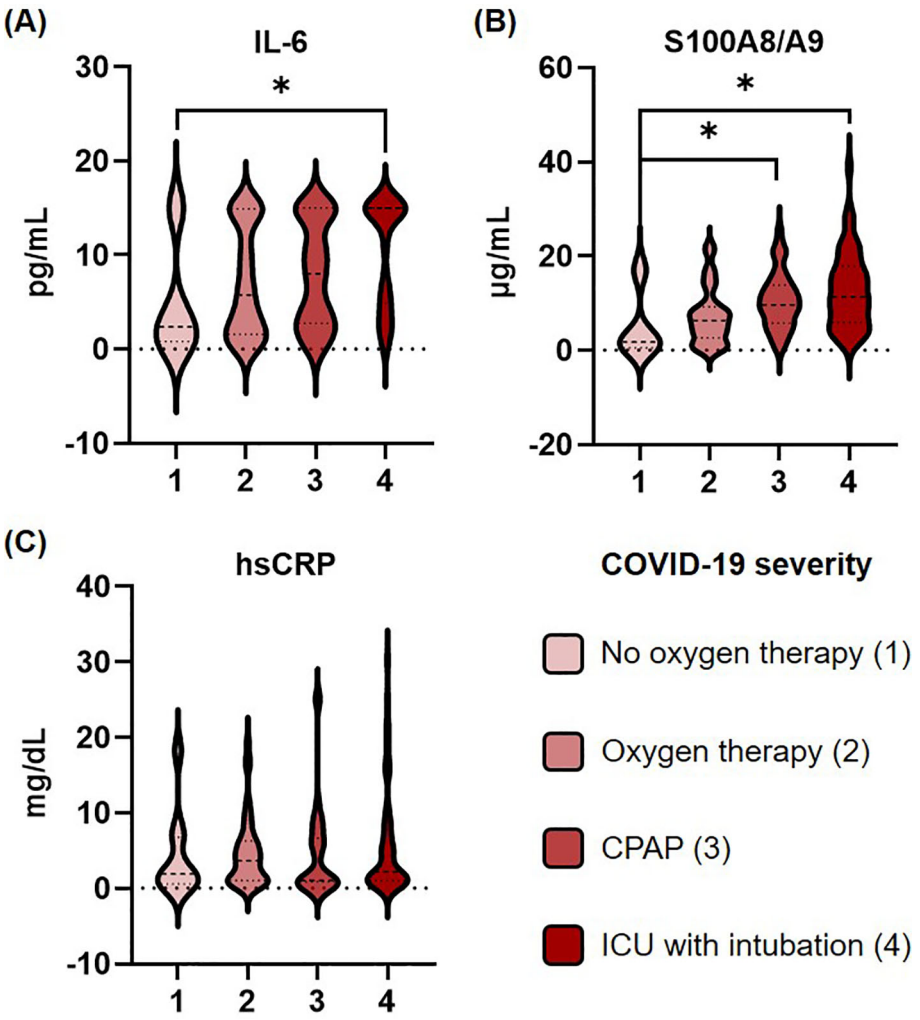

FIGURE 2
Comparison of inflammatory cytokines between different levels of COVID-19 severity. Comparison of IL-6 **(A)**, S100A8/A9 **(B)**, and hsCRP **(C)** serum levels in subjects divided for COVID-19=Coronavirus Disease 2019, -6, hsCRP=high-sensitivity C-reactive protein test, CPAP=continuous positive airway pressure; ICU=intensive care unit. Due to Bonferroni correction, the significance threshold for these comparisons is 0.0125. '*'=$p < 0.0125$ vs COVID-19 severity level 1. N = 10, 46, 35, and 53 for COVID-19 severity level 1, 2, 3, and 4, respectively.

saturation, the use of artificial ventilation, and organ failure (37). In a study, serum iron levels were found to be predictive of COVID-19 severity and, when measured after antiviral treatment, of mortality. The authors also described a trend in lymphocyte count in correlation with COVID-19 severity, which suggests some differences in the pathological landscape compared with our study; possibly, due to the fact that patients were enrolled exclusively during the first wave of the pandemic, while our study includes the first three waves. Other studies confirmed the reduction in serum iron in COVID-19 patients compared to healthy controls with no significant differences between mild, moderate, and severe cases, accompanied by a reduction of transferrin and a pathological increase in ferritin (21, 38). Our data do not show any difference within severity groups, hence adding up to the discussion on the roles of each of the studied elements as markers and predictors of COVID-19 outcomes.

Our findings should be interpreted in light of several limitations. First, the characteristics of the control population should be considered when interpreting these results. Control subjects were vaccinated individuals enrolled within healthcare-related cohorts and therefore may not represent a strictly healthy reference population. The vaccination status itself, as well as the healthcare worker setting of the PSD cohort, could potentially influence immune and inflammatory parameters. Moreover, differences in demographic characteristics, including age and sex distribution, between patients and controls may represent a potential source of residual confounding, despite statistical adjustments. Therefore, the control group should be considered a vaccinated reference population rather than an ideal healthy population.

Furthermore, the analysis according to COVID-19 severity is limited by the relatively small number of patients in some severity categories. In particular, the group with the lowest disease severity included only 10 patients, and this subgroup was used as a reference category for comparisons across severity groups. The limited sample size may reduce statistical power and increase uncertainty in intergroup comparisons, potentially affecting the stability of severity-related trends. Therefore, findings based on disease severity should be interpreted with caution and require confirmation in larger cohorts with more balanced representation across severity categories.

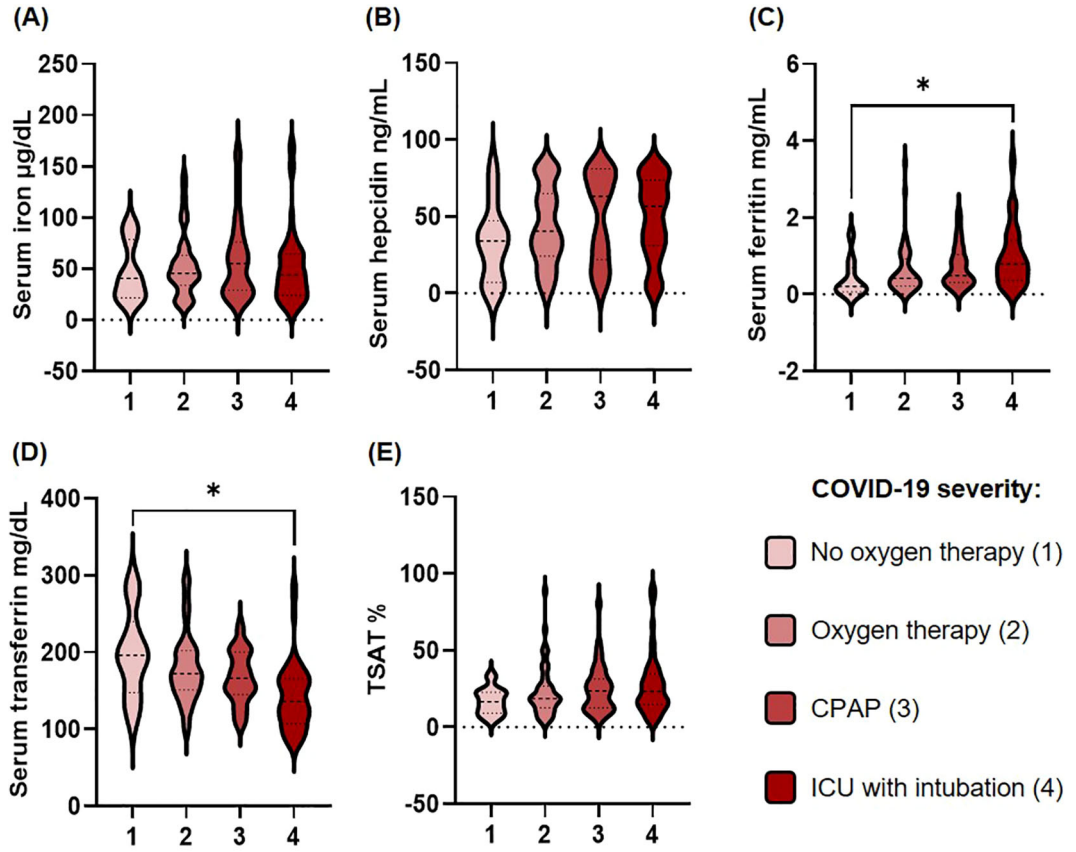

FIGURE 3
Comparison of iron metabolism parameters between different levels of COVID-19 severity. Comparison of iron **(A)**, hepcidin **(B)**, ferritin **(C)**, transferrin **(D)**, and transferrin saturation (TSAT) **(E)** serum levels in subjects divided for COVID-19 severity level. COVID-19=Coronavirus Disease 2019; CPAP=continuous positive airway pressure; ICU=intensive care unit. Due to Bonferroni correction, the significance threshold for these comparisons is 0.0125. '*'=$p < 0.0125$ vs COVID-19 severity level 1. N = 10, 46, 35, and 53 for COVID-19 severity level 1, 2, 3, and 4, respectively.

Finally, the interpretation of severity-related findings should also consider the potential influence of ICU treatments and blood sampling timing, which may affect inflammatory markers, hematological parameters, and iron metabolism. These factors may introduce additional variability in biological measurements and should be taken into account when interpreting associations between disease severity and laboratory alterations.

Iron availability is essential for cellular metabolism and regulates the function and proliferative capacity of leukocytes (39). On the other hand, hepcidin-induced iron overload increases susceptibility to ferroptotic cell death (40). Our data showed no statistical differences for RBC between the different severity groups, similar to what was previously reported (17, 37). However, a consensus is lacking in literature when it comes to lymphocyte and monocyte counts (41–44). It was previously established that the relative amounts of circulating lymphocyte-derived extracellular vesicles are indicative of COVID-19 severity (45), suggesting that similar changes in the number of producing cell types or activity may occur. However, we show no difference in monocyte nor lymphocyte counts in relation to COVID-19 severity.

TABLE 4 **Analysis of blood count and biochemical laboratory parameters according to COVID-19 severity level.**

Variable	*COVID-19 severity class (n)*				*p*
	1 (10)	2 (46)	3 (35)	4 (53)	
WBC ($x10^3$/uL)	8.5 (4-9.7)	6.3 (4.4-8.1)	8.3 (5.9-10.3)	9.2 (6.7-12.7)	**0.0048**
RBC ($x10^6$/uL)	4.1 (3.8-4.6)	4.3 (3.7-4.8)	4.6 (3.9-4.7)	4.1 (3.6-4.6)	0.2109
Neutrophils ($x10^3$/uL)	6.2 (3.1-8.4)	4.7 (3.2-7.6)	7.3 (4.9-9.2)	7.6 (5.1-10.9)	**0.0166**
Lymphocytes ($x10^3$/uL)	1.2 (0.6-1.4)	0.8 (0.6-1.3)	1 (0.7-1.2)	0.8 (0.5-1.4)	0.4738
Monocytes ($x10^3$/uL)	0.4 (0.3-0.5)	0.5 (0.3-0.7)	0.5 (0.4-0.8)	0.6 (0.3-0.9)	0.1067
Creatinine (mg/dL)	0.9 (0.7-1.5)	0.8 (0.7-1.2)	0.8 (0.7-1)	0.7 (0.6-1.3)	0.4061

WBC, white blood cells; RBC, red blood cells. Statistically significant *p* values (≤0.05) are highlighted in bold.

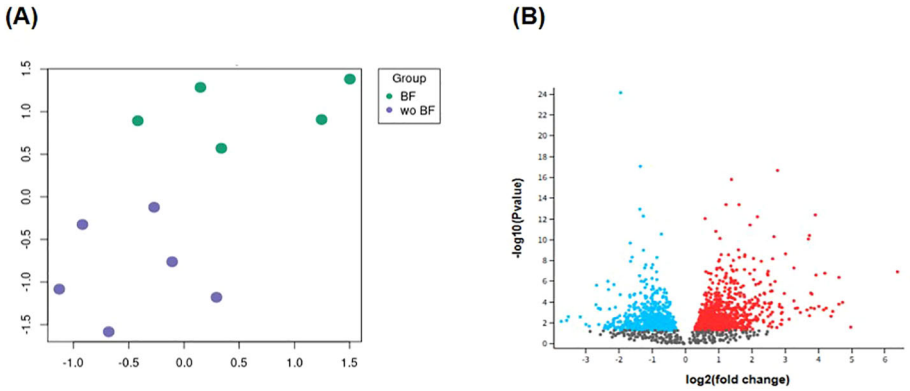

FIGURE 4
Transcriptomic profiling of long COVID-19 patients with or without brain fog. UMAP visualization of PBMC transcriptomic profiles from long COVID-19 patients with BF (N = 5) and without BF (N = 6). Each point represents a sample, colored according to clinical group, showing the distribution and clustering based on global gene expression patterns **(A)**. Volcano plot of differential gene expression between long COVID-19 patients with and without BF. Each point represents a gene, plotted according to log2 fold change and -log10 adjusted p-value (Benjamini-Hochberg false discovery rate). Significantly differentially expressed genes (adjusted p-value < 0.05) are highlighted, with upregulated genes in patients with BF shown in red and downregulated genes in light blue. Data were extracted from the publicly available dataset GSE251849 **(B)**. COVID-19, Coronavirus Disease 2019; UMAP, Uniform Manifold Approximation and Projection; PBMCs, peripheral blood mononuclear cells; BF, brain fog.

Interestingly, however, linear regression analysis showed that lymphocyte count associates negatively with ferritin. These findings suggest that lymphocyte count is linked to alterations in iron metabolism rather than to disease severity per se. In linear regression analysis neutrophil and monocyte count did not associate with any iron metabolism parameter studied. However, neutrophil counts showed a slight, progressive increase from severity group 1 to group 3 suggesting a trend toward increased systemic inflammation with worsening disease severity. However, this trend was not observed in group 4, possibly owing to the more intensive pharmacological treatment administered to critically ill patients. As expected, linear regression analysis revealed a negative association for IL-6 and CRP to iron, transferrin, and its saturation percentage, while showing a positive association to hepcidin. These data support the role of iron metabolism, inflammation, and blood cell counts as COVID-19 severity determinants. More studies are needed to elucidate hepcidin's role in COVID-19 progression, as presented data advocate for it being central.

The bioinformatics analysis conducted on the GSE251849 dataset strengthened the findings of long-lasting inflammation in long COVID-19 patients and highlighted the relevance of iron dysmetabolism association with long COVID-19 severity (46, 47). Several studies have shown that an iron-related signature persisting beyond the acute phase of COVID-19 can identify patients who will later develop long COVID-19 (32, 48, 49), and that this alteration also persists in patients with long COVID-19 compared to healthy subjects or subjects that have completely recovered their functional ability (50, 51). Another study specifically focusing on BF showed that alterations in iron metabolism during hospitalization are associated with the subsequent onset of BF in patients with long COVID-19 (52). Our explorative bioinformatic analysis completes the picture suggesting that alterations in iron/heme-related transcriptional programs in PBMCs may be associated with the severity of long COVID, particularly in patients experiencing BF. Given the limited sample size of the publicly available transcriptomic dataset, these findings should be considered hypothesis-generating and require validation in larger, independent cohorts integrating transcriptomic and circulating biomarkers of iron metabolism.

The relevance of this study lies primarily in demonstrating that a panel of inflammatory and iron metabolism biomarkers comprising IL-6, S100A8/9, ferritin, transferrin, WBC and neutrophil count, can significantly improve the identification of COVID-19 severity, thereby potentially enabling earlier risk stratification and the identification of patients who may benefit from targeted interventions. In addition, our exploratory transcriptomic analysis extends these observations by highlighting immune response and iron/heme-related pathways as promising candidates for future investigation in the pathophysiology of long COVID-19.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Institutional Ethics Committees of IRCCS MultiMedica (Comitato Etico Centrale IRCCS Lombardia; protocols 479.2021

and 453.2020) and San Raffaele Hospital (CET Lombardia; protocols 48/2021/spall/PU/403-2021 and 75/INT/2020), covering both controls and COVID-19 patients.

Author contributions

MM: Data curation, Investigation, Supervision, Writing – original draft, Writing – review & editing. AR: Data curation, Investigation, Supervision, Writing – original draft, Writing – review & editing. MC: Data curation, Investigation, Supervision, Writing – original draft, Writing – review & editing. AliM: Investigation, Writing – review & editing. ET: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. BB: Investigation, Writing – review & editing. LD: Investigation, Writing – review & editing. AleM: Investigation, Writing – review & editing. RC: Investigation, Writing – review & editing. MR: Investigation, Writing – review & editing. FM: Investigation, Writing – review & editing. GA: Conceptualization, Writing – original draft, Writing – review & editing. GS: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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