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Inflammatory markers, iron-related parameters and anemia of inflammation dynamics in acute infections: Insights from comparison of a COVID-19 pneumonia and a sepsis cohort

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ABSTRACT

BACKGROUND: Iron is essential for nearly all organisms, including microbes, as a functional component of many proteins and enzymes involved in vital biochemical functions. In vertebrates, iron metabolism is mostly regulated by the hepatic hormone hepcidin, which plays a role in the so-called “nutritional immunity”, the battle for nutrient metals at the host–pathogen interface. During acute infection, hepcidin production is mainly induced by interleukin (IL)-6, leading to iron sequestration in macrophages and, in turn, iron-restricted erythropoiesis. Both hypoferremia and inflammatory cytokines suppress erythropoiesis, possibly leading to anemia of inflammation (AI). However, host response to invading pathogens is complex, mainly studied in vitro or in animal models, and emerging evidence suggests different patterns of iron homeostasis alteration depending on infection etiology.

AIMS: This study aimed to: (1) compare the evolution of inflammatory markers, iron metabolism, and AI in two different cohorts of patients hospitalized for severe acute infections; investigate (2) predictors of in-hospital mortality; (3) mechanisms of hepcidin regulation in vivo; (4) possible predictors of microbial etiology.

MATERIALS AND METHODS: A total of 447 patients were enrolled within 48 hours from admission to a medical ward of Verona University Hospital and followed up during hospitalization; 391 were affected by COVID-19 pneumonia and 56 by sepsis. Clinical, biochemical, and iron-related parameters were obtained at defined time points. Cytokine assays were assessed for the COVID-19 pneumonia cohort. Immunological analysis of circulating mononuclear cells was performed for the sepsis cohort, which was followed up after 3 months from discharge.

RESULTS: Although the COVID-19 pneumonia patients were older (71 [61-80] vs 65 [53-76] years, p=0.001) and more frequently comorbid (85.2 vs 59.6%, p=0.004) than septic patients, the two cohorts did not differ

significantly for disease severity and mortality rate. Baseline anemia was far more frequent in the sepsis compared to the COVID-19 pneumonia cohort (78.6 vs 31.2%, $p<0.001$), and all sepsis nonsurvivors were anemic. At 3-month follow-up, anemia prevalence was still relevant (38.1%) despite iron biomarkers generally returning to normal values. The baseline biochemical and iron-metabolism profile differed significantly among the two cohorts, with lower C-reactive protein (CRP) and white blood cell (WBC) count and higher ferritin and hepcidin levels observed for COVID-19 pneumonia patients.

In COVID-19 pneumonia patients, female sex, increased age and IL-8, and a decrease of PaO_2/FiO_2 (P/F) ratio and transferrin upon admission significantly predicted mortality. Hemoglobin, CRP, Neutrophil to Lymphocyte ratio, creatinine, erythroferrone, soluble transferrin receptor, IL-1Ra, IL-6, and tumor necrosis factor-α, but not P/F ratio, were significantly associated with basal hepcidin values.

In the sepsis cohort, basal hepcidin >160 ng/mL was associated significantly with 30-day mortality (log-rank 4.154, $p=0.042$). Circulating myeloid precursors were detected across all time points. Their counts were higher in nonsurvivors compared to survivors, in whom a partial, although not full, reduction was observed at 3-month follow-up.

Baseline anemia, high CRP and WBC count, and low hepcidin values were significantly associated with a higher risk of sepsis and a lower risk of COVID-19 pneumonia in binary logistic regression.

CONCLUSIONS: This study of well-characterized patients highlights a profound alteration of iron homeostasis during acute COVID-19 pneumonia and sepsis, showing important differences possibly reflecting different inflammatory patterns. In-hospital mortality was associated with different factors in the two cohorts. In COVID-19 pneumonia, inflammation and erythropoiesis, rather than the hypoxic drive, seem to upregulate hepcidin at baseline. Specific hematological biomarkers have been identified as possible predictors of infection etiology.

ABBREVIATIONS

ALT: alanin aminotransferase

AST: aspartate aminotransferase

CK: creatine phosphokinase

CRP: C-reactive protein

eGFR: estimated glomerular filtration rate

ERFE: erythroferrone

FiO_2: inspiratory oxygen fraction

Hb: hemoglobin

ICU: intensive care unit

IFN: interferon

IL: interleukin

LD: lactate dehydrogenase

MCV: mean corpuscular volume

N/L ratio: ratio of neutrophil to lymphocyte count

PaO_2: arterial partial oxygen tension

P/F: ratio of arterial partial oxygen tension to inspiratory oxygen fraction

RDW: red blood cell distribution width

SOFA: sequential organ failure assessment

sTfR: soluble transferrin receptor

sTfR index: ratio of soluble transferrin receptor to log (10) ferritin

TNF: tumor necrosis factor

TSAT: transferrin saturation

WBC: white blood cells

WHO: World Health Organization

* p-value <0.05

** p-value <0.01

*** p-value <0.001

**** p-value < 0.0001

VARIABLES' NORMAL REFERENCE RANGES

The following reference values are provided by the local laboratory:

Variable	Normal reference range
Albumin	35 - 50 g/L
ALT	10 - 50 U/L
AST	10 - 50 U/L
CK	40 - 300 U/L
Creatinine	0.59 - 1.29 mg/dL
CRP	0 - 5 mg/L
eGFR	60 - 999 mL/min/1.73 m^2
Ferritin	30 - 300 µg/L
Fibrinogen	2.00 - 4.00 g/L
Hb	135 - 160 g/L
Iron	55 - 161 µg/dL
LD	135 - 225 U/L
Lymphocytes	1.20 - 4.00 10^9/L
MCV	80.0 - 99.0 fL
Neutrophils	1.80 - 8.00 10^9/L
Platelets	150 - 400 10^9/L
RDW	11.5 - 15.0 %
Transferrin	2.00 - 3.60 g/L
TSAT	16.0 - 40.0 %
WBC	4.30 - 10.00 10^9/L

For iron-related proteins, refer to the Materials and Methods section.

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1. INTRODUCTION

1.1 Systemic and cellular iron homeostasis

Iron is an essential trace element for nearly all living organisms, with rare exceptions such as Borrelia burgdorferi [1] and certain Lactobacilli [2]. In vertebrates, several vital biochemical functions, like oxygen transport, oxygen sensing, energy production, host defense, and DNA replication and repair, rely on iron [3]. Physiological minor iron losses occur daily through enterocytes and skin cell shedding, while higher amounts of iron are lost during menstruation. Moreover, increased iron consumption characterizes childhood and pregnancy, especially during the second and third trimesters [4]. These unavoidable iron losses consist of about 1-2 mg per day and are counterbalanced by an equivalent amount of iron absorbed by the duodenal enterocytes. Furthermore, iron from senescent erythrocytes is efficiently recycled through the reticuloendothelial system and is mainly used by the bone marrow for erythropoiesis, accounting for approximately 20-25 mg per day [5]. On the other hand, iron's biochemical properties of readily accepting and donating electrons confer to this element immense reactivity, which in turn can cause cellular damage and death, also known as ferroptosis [6]. The total body iron content in an adult male is approximately 3–4 g. However, the plasma iron pool is significantly smaller, accounting for approximately 3-4 mg, and is represented by transferrin-bound Fe^{3+}, which allows the safe transport of iron. In healthy individuals, transferrin saturation (TSAT) ranges from 20 to 45%. The cellular uptake of circulating transferrin-bound iron is mediated by transferrin receptor protein 1 (TFR1), expressed by all cell types, through endocytosis of the transferrin-Fe^{2+}-TFR1 complex. Duodenal enterocytes uptake non-transferrin-bound iron from the intestinal lumen through the symporter divalent metal transporter 1 (DMT1). An alternative mechanism for cellular iron uptake is endocytosis of ferritin-bound iron present in exosomes [6]. Moreover, macrophages catch haem iron through erythrophagocytosis, or upon hemolysis by internalization of hemoglobin-haptoglobin and haem-haemopexin complexes [6]. Once inside the cells, iron is used for many biological processes or can be safely stored

in ferritin, a large protein formed of 24 chains, made of both heavy (H) and light (L) subunits, which is able to store up to 4500 iron atoms [5].

1.1.1 Systemic iron homeostasis

Systemic iron homeostasis must be finely tuned and is orchestrated by hepcidin, a disulfide-rich 25 amino acid peptide produced by hepatocytes [7]. Hepcidin controls iron export to the plasma by inducing ubiquitination and lysosomal degradation of the iron-exporter ferroportin in the so-called hepcidin-ferroportin axis. This latter protein is expressed mainly in enterocytes, macrophages, and hepatocytes and releases iron from the cytoplasm into the extracellular space, where it is converted from Fe^{2+} to Fe^{3+} by membrane-associated ferroxidases (such as hephaestin or ceruloplasmin) and bound to transferrin to be safely transported through the bloodstream [6]. In erythrocytes, in which ubiquitination machinery is lacking, hepcidin blocks iron efflux by directly occluding the ferroportin channel [8]. Through the described mechanisms, hepcidin inhibits dietary iron uptake from duodenal enterocytes and iron release from macrophages and hepatocytes. Transcription of the *HAMP* gene that encodes for hepcidin is stimulated by several stimuli. During inflammation, it is upregulated by IL-6 within a few hours, causing iron redistribution and sequestration in macrophages [9]. This mechanism is mediated by the JAK-STAT3 signalling pathway [6]. Among cytokines, type I interferon (IFN) stimulates hepcidin transcription, tumor necrosis factor (TNF)-α has been associated with the suppression of hepcidin mRNA levels in hepatoma cells, while IL-1α did not show any significant effect [10, 11]. IL-22, a cytokine of the IL-10 family produced during responses against extracellular pathogens, can upregulate hepcidin transcription in an IL-6 independent way, possibly involving the bone morphogenetic protein (BMP)/SMAD pathway [10]. This pathway is normally activated by high TSAT and high liver iron levels and is repressed by erythroferrone (ERFE), whose transcription by erythroblasts is, in turn, increased by erythropoietin (EPO) [6]. Therefore, erythropoiesis expansion downregulates hepcidin. Recently, another hepatic mechanism inhibiting hepcidin transcription has been identified, that is the hepatokine fibrinogen-

like 1 (FGL1) induction in the liver in response to hypoxia during the recovery from anemia [12].

1.1.2 Cellular iron homeostasis

At a cellular level, four key regulatory pathways of iron metabolism have been described [6]:

1. *The iron regulatory protein (IRP) – Iron response element (IRE) network*

IRP 1 and 2 proteins regulate the post-transcriptional expression of key genes involved in iron metabolism (i.e. *FTL* and *FTH1* encoding for ferritin light and heavy chain, respectively, *SLC40A1* encoding for ferroportin, *SLC11A2* encoding for DMT1) by binding to IRE sequences of RNA. IRP-IRE binding blocks the translation of ferritin and ferroportin, thus reducing iron storage and export, and upregulates DMT1, thus enhancing iron uptake, with the effect of increasing cellular iron availability. Among the iron-related genes regulated by IRP1 and IRP2 are those encoding for HIF2α and ALAS2, which mediate IRP-dependent regulation of EPO and haem synthesis, respectively, both important for iron utilization in erythrocytes. This pathway is indirectly regulated by iron and oxygen level sensing.

2. *The prolyl hydroxylase domain (PHD) – hypoxia-inducible factor (HIF) axis*

Under normal conditions, PHD enzymes hydroxylate 2 proline residues in HIF1 and HIF2, causing their ubiquitination and degradation. For its activity, PHD requires oxygen and iron. Under hypoxia and iron deficiency (ID), HIF is then stabilized, translocates into the nucleus, and promotes the transcription of target genes, such as *EPO* and *Fgl1*. In kidney interstitial cells, HIF2 activates EPO synthesis, which in turn increases ERFE transcription in erythroblasts. In hypoxic hepatocytes, HIF upregulates *Fgl1* expression [12]. Both these mechanisms downregulate hepcidin.

3. *The nuclear receptor co-activator (NCOA4) – mediated ferritinophagy system*

NCOA4 is an autophagy receptor and, in iron-repleted cells, is targeted for proteasomal degradation. Under iron-deficiency conditions, NCOA4 is stabilized due to reduced HERC2-mediated proteasomal degradation. This allows NCOA4 self-oligomerization and its interaction with FTH1. The ferritin-NCOA4 complex is then processed by autophagolysosomes for degradation to increase the cellular labile iron pool and, finally, iron availability. Under hypoxia, *NCOA4* transcription is promoted by HIF2 [6].

4. The nuclear factor erythroid 2-related factor 2 (NRF2) regulatory hub

Oxidative stress activates the transcription factor NRF2, which induces the expression of approximately 250 antioxidant response element (ARE)-containing genes variably involved in the protection from free radical damage. This transcriptional factor regulates several genes involved both in heme biosynthesis (*ABCB6* and *FECH*) and catabolism (*HMOX-1*, *BLVRB*, and *AMBP*) [6]. In erythroid cells, A1M, the protein encoded by *AMBP*, prevents intracellular oxidation by heme- and hemoglobin-induced reactive oxygen species [13]. Moreover, NRF2 also regulates the expression of ferroportin and BMP6.

1.2 Iron metabolism during infections

During infections, the host and the invading pathogens compete for iron, given its essential role for nearly all organisms. Vertebrate strategies that either prevent access to trace elements or exploit their toxicity against invading pathogens (in the case of iron through reactive oxygen species and nitric oxide) are known as “nutritional immunity” [14, 15]. Many pathogens have evolved mechanisms to evade or subvert iron-targeted nutritional immunity, like bacterial iron-chelating compounds named siderophores, and this is corroborated by the large amount of the genome dedicated to iron-acquiring mechanisms [3, 11]. As discussed before, cytokines produced during infections also exert control on iron metabolism, mainly influencing hepcidin production, which stands at the crossroad between iron homeostasis and host response to infections. The structure of hepcidin is similar to defensins, peptides with antimicrobial activity [16], although its direct antimicrobial activity in *in vivo* physiological conditions seems unlikely

[3]. Inflammation-mediated hepcidin upregulation via IL-6 drives the retention of iron in macrophages and its reduced absorption through a posttranslational control of ferroportin, which is accompanied by a decrease in serum iron and TSAT (the so-called hypoferremia of inflammation) [2]. Very recently, a hepcidin-independent pathway has been identified that directly downregulates ferroportin gene transcription after inflammatory stimuli downstream of NF-κB signaling [17].

More broadly, innate immune response to pathogens upregulates hepcidin transcription through SMAD (i.e., SMAD2, SMAD3, and SMAD1/5/8) and STAT3 signaling [18-20]. The adaptive immunity involving the proliferation of B and T lymphocytes is iron-dependent [21, 22]; the activation of these cells is associated with expression of the iron-acquiring transferrin receptor (CD71) [11], and up-regulation of hepcidin has been reported after lymphocyte activation [23]. Inflammatory cytokines can also promote the synthesis of other iron-binding and iron-transporting molecules, such as lactoferrin, siderocalin, haptoglobin, and haemopexin, owing to iron sequestration. On the contrary, TFR1 expression is reduced in macrophages by IFNγ during intracellular infections [3]. Recently, a missense mutation in the *TFRC* gene encoding for TFR1 was discovered as the cause of a combined immunodeficiency with functional impairment of both T and B lymphocytes [24].

The host's iron status can modulate the risk of some infections, such as susceptibility to intracellular microorganisms [11], and the response to vaccination [22, 25]. Patients with iron overload secondary to hereditary hemochromatosis are at higher risk of severe infections caused by the siderophilic bacteria Vibrio vulnificus and Yersinia enterocolitica, and studies in hepcidin-deficient mice have shown a higher susceptibility to siderophilic bacteria and a lower growth of macrophage-resident bacteria [3]. ID is frequent in populations exposed to malaria, could have a protective role in limiting Plasmodium falciparum replication, and prophylactic iron supplementation was associated with worse outcomes in high malaria transmission settings, imposing early interruption of a large randomized, placebo-controlled clinical trial in preschool children [26]. Similarly, iron replacement was associated with a higher incidence of infections (malaria,

brucellosis, and tuberculosis) compared to placebo in iron-deficient Somali nomads [27]. Intriguingly, iron metabolism alterations might, to some extent, differ depending on infection etiology [3, 28, 29].

1.2.1 Iron metabolism in viral infections

Many viruses can encode proteins to evade the host immune system. For example, some can use TFR1 as a receptor to detect and invade highly metabolically active cells, while others can encode proteins that interfere with major histocompatibility complex (MHC) class I proteins [21]. Of note, HFE is a non-classical MHC class I protein sharing structural homology with classical MHC class I molecules; although it does not participate in antigen presentation, it is involved in iron metabolism by binding to transferrin receptors, regulating iron transport in macrophages, and influencing hepcidin synthesis. HFE can be targeted for proteasomal degradation by a human cytomegalovirus protein, US2, promoting cellular iron loading [21]. It has also been demonstrated that host nutritional status, including iron, drives viruses' genome changes and represents a driving force for emerging viral variants [30]. As hepcidin is an acute-phase protein produced by hepatocytes, it is not surprising that viral infections that damage the liver can be accompanied by changes in iron homeostasis. Moreover, hepatic iron loading can exacerbate chronic viral diseases. Interestingly, in contrast to most bacterial and viral infections, the viraemic phase of both hepatitis B (HBV) and C virus (HCV) infection is not characterized by hepcidin induction [3]. In the case of HCV, the infection per se is associated with iron overload, in which hepcidin suppression is likely to play a key role, either mediated by the virus or due to the host's genetic predisposition. In any case, excess iron is associated with a worse prognosis of HCV-related disease [21]. As regards human immunodeficiency virus (HIV), it severely impairs the immune system directly destroying CD4+ T-helper cells and impairing macrophages and dendritic cells' immune function. Both *in vitro* and *in vivo* evidence indicate that excess iron may promote HIV replication and secondary pathogens infections. At the same time, anemia often accompanies HIV infection and is associated with poor prognosis. Thus, the unbalance of iron homeostasis in both directions seems to be detrimental in

the context of HIV infection [21]. Considering influenza virus infection, evidence in mice showed that persistent hypoferremia suppressed adaptive immune response and was associated with lower viral clearance and more severe lung disease [22].

1.2.2. COVID-19: a brief overview focusing on iron metabolism

In early 2020, a new virus, named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), was identified as the etiological agent of an outbreak of highly deadly atypical pneumonia cases first emerged in the Chinese city of Wuhan [31]. Since then, the SARS-CoV-2-related disease, known as Coronavirus Disease 19 (COVID-19), has been responsible for a rapidly spreading pandemic responsible for more than 777 million reported cases and more than 7 million deaths worldwide (COVID-19 circulation | WHO COVID-19 dashboard). The spike glycoprotein of SARS-CoV-2 binds to the human angiotensin-converting enzyme-2 (ACE-2) receptor on the epithelial cell surface, highly expressed in epithelial cells of the nasal cavity. The host transmembrane protease serine 2 (TMPRSS2) facilitates the entry of the virus into the host cells [32]. SARS-CoV-2 infection is characterized by a large spectrum of clinical manifestations, ranging from the absence of symptoms to severe pulmonary and extrapulmonary multiorgan damage and death [33, 34]. Older age, male sex, and comorbidities (such as cancer, chronic liver or kidney disease, cardiovascular disease, chronic obstructive pulmonary disease, obesity, and diabetes) have been associated with worse outcomes in COVID-19 [35].

The COVID-19 pandemic has boosted an enormous expansion of research, also involving iron metabolism biomarker alterations following acute infection, with some heterogeneity of results given the wide range of disease severities included in the studies [16].

Ferritin, an acute phase reactant, is systematically elevated in severe COVID-19 cases, and hyperferritinemia has been associated with more severe disease and a higher risk of mortality in several studies and meta-analyses [36-38]. Therefore, high ferritin has been proposed as a marker of hyperinflammation and a predictor of worsening and mortality in COVID-19 hospitalized patients [16].

Low serum iron and low TSAT have also been consistently reported in severe COVID-19 cases [39-44]. In a study comparing outpatients and inpatients, hypoferremia was associated with the risk of hospitalization and the degree of respiratory failure of hospitalized patients, with a better area under the curve (AUC) compared to CRP in distinguishing in- and outpatients [40]. Another study, including 30 COVID-19 patients admitted to the Intensive Care Unit (ICU), found that hypoferremia was lower in more severe patients and was even lower than in other critically ill, including septic, patients [39]. However, other studies did not observe significant differences in iron levels upon admission between severity groups [43, 44]. Interestingly, at disease onset, iron levels poorly [40, 41, 43] or did not correlate with hepcidin [42, 45] but did correlate with CRP and IL-6 (negatively) [41-43] and with lymphocyte counts and P/F ratio (positively) [39]. Serum iron and TSAT were significantly lower in ICU compared to non-ICU COVID-19 patients after 7 days from hospitalization [43] and remained significantly lower in patients who required assisted ventilation at day 181–270 post-COVID-19 onset [46].

Increased serum hepcidin has also been reported in COVID-19 patients and associated with more severe disease [38, 41, 44, 46] and worse clinical outcomes [45], but other studies reported inconsistent results and found no association of hepcidin with disease severity [40, 43]. As stated by some authors, COVID-19 could represent a useful *in vivo* model for different pathways of hepcidin upregulation during infection and inflammation [16], and the coexistence of hypoxia in severe COVID-19 pneumonia patients gives the opportunity to study the effect of opposite drivers of iron metabolism. Surprisingly, hepcidin did not differentiate patients according to the severity of inflammation or hypoxia, and no correlation was found between hepcidin and P/F ratio [43]. Conversely, in another study including only non-anemic COVID-19 patients, hepcidin was significantly higher in hypoxic compared to non-hypoxic subjects [44].

Very few data are available regarding EPO and ERFE in COVID-19. In one study on non-anemic patients, baseline EPO was higher in hypoxic patients and increased together with disease severity [44]. Conversely, in another study reporting a high prevalence of anemia, EPO levels were lower in ICU

compared to non-ICU patients but did not differ when grouping for P/F ratio levels [43]. A third study with a long follow-up observed a delayed increase of EPO during hospitalization in more severe patients, in whom a progressive reduction of Hemoglobin (Hb) was also encountered [46]. ERFE showed no significant differences across disease presentation groups but was significantly higher in anemic compared to non-anemic patients [41]. Finally, two small studies reported lower ERFE levels in SARS-CoV-2 compared to non-SARS-CoV-2 infected patients [29, 47].

The heterogeneity of results of the reported studies highlights the entwined interplay between different regulators of iron metabolism and erythropoiesis in complex clinical conditions such as COVID-19 disease.

1.2.3 Iron metabolism in bacterial infections

Interactions between host and bacteria have been studied both in mouse models and, to a smaller extent, in humans. Bacteria have developed many different mechanisms to obtain iron from the host. More than 500 bacterial siderophores, which are small, high-affinity iron-chelating compounds aimed at scavenging iron, have been described. In the case of Yersinia pestis, genetic studies have suggested that this bacterium made a niche transition from enteric to systemic pathogen thanks to the enhanced ability to acquire iron [11]. Some extracellular Gram-negative bacteria, like Neisseria meningitidis, Neisseria gonorrhoeae, and Haemophilus influenzae, have developed specific mechanisms to obtain iron from the host iron-containing proteins (such as transferrin, lactoferrin, Hb, and ferritin) [3]. Moreover, most bacterial infections rapidly increase hepcidin production. The systemic hepcidin-ferroportin axis activation during inflammation leads to iron sequestration in macrophages and could promote the growth of intracellular microorganisms such as Salmonella, Mycobacteria, and Legionella [2]. At the same time, however, local production of IFNγ and nitric oxide stimulate transcription of the gene encoding ferroportin limiting iron loading of macrophage phagosomes [3]. The removal of ferroportin from the phagosome membrane in response to different pathogens has been recently demonstrated in human cells, too, as part of nutritional immunity [48].

1.2.4 Sepsis: a brief overview focusing on iron metabolism

Sepsis is defined as a life-threatening organ dysfunction due to a dysregulated host response to infection. It is recognized as a global health priority, due to its high mortality burden and associated long-term physical, psychological, and cognitive morbidity [49]. In 2017 48.9 million cases and 11 million sepsis-related deaths were estimated worldwide, accounting for almost 20% of all global deaths [50]. Bacterial infections are the most common cause of sepsis, although viruses and fungi may also occur especially in immunocompromised subjects. The most common site of infection associated with sepsis in hospitalized patients is the respiratory tract, followed by abdominal, bloodstream, intravascular line, and urinary tract infections [51, 52]. Staphylococcus aureus, Escherichia coli, Klebsiella spp., Pseudomonas aeruginosa, Enterococci, Streptococci, and coagulase-negative Staphylococci are the most frequently isolated pathogens [52, 53]. Early interventions, in particular initiation of effective antimicrobial therapy and appropriate fluid resuscitation, are a cornerstone of the so-called “golden hour” in sepsis treatment and are associated with better survival in septic shock [54]. The Sequential Organ Failure Assessment (SOFA) score is widely used to assess the severity of organ dysfunction, and a higher SOFA score predicts an increased risk of mortality [55]. Laboratory tests such as WBC and platelets count, CRP, procalcitonin, bilirubin and lactate are of great importance in clinical evaluation, particularly to suspect organ dysfunction and to calculate SOFA score. Unfortunately, neither SOFA score nor a single laboratory marker can capture early-stage infection before the onset of organ dysfunction, they cannot be used as a screening tool for diagnosing sepsis and are not useful in distinguishing infection etiology [56].

Because of severe acute inflammation, iron metabolism is altered during sepsis, and some iron-related parameters have been proposed as outcome predictors in ICU patients.

Ferritin was found typically elevated during ICU stay, septic patients showed the highest ferritin values compared to other causes of organ dysfunction, and hyperferritinemia significantly decreased with sepsis resolution [57, 58]. Moreover, ferritin has been reported to be significantly higher in

nonsurvivors compared to survivors ICU septic patients [59] and was associated with 28-day mortality [60].

On the contrary, serum iron [60] and transferrin levels were decreased in ICU patients, with the lowest values among septic patients [58]. Transferrin was higher while iron was lower in survivors [59]. Moreover, the decrease of transferrin and the rise of iron [61] and TSAT [58] were independent predictors of mortality in ICU patients.

The increase of hepcidin in human sepsis was first demonstrated in a cohort of patients admitted to the emergency department. Hepcidin was positively correlated with IL-6 levels and associated with Hb decline during hospitalization, while no correlation between hepcidin levels and Hb levels at admission was observed [62]. An early increase in serum hepcidin was reported in severe sepsis [60, 63], and hepcidin was significantly higher among septic patients in the ICU compared to critically ill noninfected patients [58]. Interestingly, hepcidin on day 1 of ICU admission was an independent predictor of 28-day mortality in septic patients with a similar AUC to the SOFA score and showed the highest specificity with a cut-off value of 142.6 ng/mL [60]. In the context of bloodstream infections and less severe sepsis cases, iron and hepcidin, but also CRP, WBC, and IL-6, failed to distinguish septic from non-septic infected patients, and the only iron-related parameter that showed a significant difference was siderocalin [64].

In critically ill patients, inflammation, hypoxia, and anemia often coexist and are expected to act as opposite drivers of hepcidin regulation. The effect of hypoxia on iron metabolism was first studied in humans in healthy volunteers during experimental endotoxemia, showing a reduced upregulation of hepcidin in hypoxemic compared to normoxemic subjects [65].

One study reported that the soluble transferrin receptor (sTfR) index, a marker that may be useful in the detection of co-existing ID in patients with chronic inflammation [66], was lower in ICU septic patients compared to healthy controls, was significantly lower in sepsis nonsurvivors compared to survivors, and predicted 28-day mortality. Moreover, it was negatively correlated with hepcidin [60]. Similarly, a lower sTfR index has been reported in sepsis children compared to healthy controls [67]. Despite the

potential usefulness of sTfR index in clinical practice and these promising preliminary results, its role in sepsis has been largely neglected so far.

In one study, EPO was found to be increased in ICU septic patients compared to healthy controls and significantly lower in nonsurvivors compared to survivors [60], but others have reached the opposite conclusion [68, 69] and the correlation between EPO levels and sepsis prognosis is controversial. Finally, a study evaluated ERFE and EPO levels in hypoxic and normoxic patients exposed to endotoxin to induce acute inflammation, reporting higher levels of both markers in the hypoxia group [65].

In conclusion, iron metabolism is profoundly affected by several pathological mechanisms and opposite drivers during sepsis, such as inflammation, hypoxia, ID, anemia, and erythropoiesis disruption.

1.2.5 Iron-related parameters in infections from different etiologies

In the last years, a few authors have reported some differences in iron metabolism alterations depending on infection etiology [28, 29, 47, 70]. Oppen et al. compared typical, atypical bacterial, and viral community-acquired pneumonia (CAP) before the COVID-19 outbreak, finding that higher levels of hepcidin and ferritin at hospital admission predicted atypical bacterial vs viral etiology, even after adjustment for CRP and procalcitonin values [28]. Hegelund and colleagues compared three cohorts of patients diagnosed with either SARS-CoV-2, influenza virus, or bacterial CAP. Upon 48 hours of admission, significantly higher ferritin and hepcidin were observed in the SARS-CoV-2 cohort compared to the other two cohorts, while the bacterial cohort had higher CRP levels compared to the SARS-CoV-2 group. Serum iron levels were comparable across the three groups, while ERFE was higher in the bacterial compared to the SARS-CoV-2 cohort, although Hb was lower [29]. Iron metabolism markers were compared by Delaye et al. in two small cohorts of hospitalized patients with fever, respiratory, or gastrointestinal symptoms tested SARS-CoV-2 positive or negative [47]. Within the 7 days of diagnosis, hepcidin was significantly higher, and ERFE was significantly lower in the SARS-CoV-2 positive group, while no difference was observed for ferritin, iron, transferrin,

TSAT, and sTfR levels. Finally, Hortova et al. reported elevated hepcidin and ferritin in ICU patients admitted for septic shock, severe COVID-19, or after orthopedic surgery. They observed higher hepcidin levels among COVID-19 survivors compared to nonsurvivors but did not mention specific differences in iron-related parameters between the septic shock and the COVID-19 cohorts [70].

1.3 Elements of immunology of COVID-19 and sepsis

During infections, pathogen-derived molecules called pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPS) released by damaged and infected host cells can bind to pattern recognition receptors (PRRs) of the host, which activate innate immune cells and trigger acute inflammatory response. Activation of PRRs results in stimulation of key transcription factors, such as NF-κβ, activator protein-1, and interferon regulatory factor, which in turn promote the production of cytokines and other inflammatory mediators [56]. Pro-inflammatory cytokines, in turn, induce acute phase proteins production by hepatocytes. The cytokine profile produced in response to bacterial and fungal infections includes IL-6, TNF-α, and IL-1β, together with IL-8, mainly produced in response to bacteria, and IFNγ and IL-17 to fungi. Viral infections stimulate type 1 IFN release [56]. However, COVID-19 has revealed a distinct immunological pathway, as the IL-1 - IL-6 axis is likely to represent one of the most relevant signaling pathways of this infection [32]. The majority of severe COVID-19 cases are characterized by a unique pattern of immune dysfunction, driven by excessive release of IL-6, whose main features are monocyte hyperactivation, causing over-production of pro-inflammatory cytokines, and profound lymphopenia and lymphocyte impairment [71], which is in contrast to bacterial sepsis [32]. Beyond IL-6, several studies have measured other cytokines in COVID-19 patients, reporting increased levels of the pro-inflammatory mediators such as TNF-α, IL-1β, IL-8, IFNγ, but also IL-10, which is commonly considered an anti-inflammatory cytokine, even though its pro-inflammatory role has been demonstrated in autoimmune diseases and cancer [72-74]. In COVID-19, IL-10 levels were

significantly higher in ICU compared to non-ICU patients and were strongly positively correlated to IL-6 and CRP levels [72].

As regards sepsis, both pro- and anti-inflammatory responses are activated early, with a concomitant increase of IL-6, IL-8, TNF-α but also IL-10 levels in septic patients [75]. After the acute inflammatory phase aimed to control pathogen invasion, the anti-inflammatory response becomes predominant to restore homeostasis. Sometimes the pro-inflammatory host response to the invading pathogen can be excessive and generate a “cytokine storm” with deleterious clinical effects and poor prognosis. On the contrary, early excessive immunosuppression can lead to death due to failure to control pathogens [76]. In sepsis survivors, the long-term persistence of never-reached immune homeostasis has been linked to a high rate of comorbidities and mortality [77]. A recent area of research focuses on emergency myelopoiesis in response to infection, which has been associated with chronic critical illness in sepsis survivors [75]. Hematopoietic stem and progenitor cells express several receptors for PAMPs and are activated by cytokines such as type I and II interferons, IL-1, and TNF so that ongoing infection and inflammation influence the hematopoietic system and drive the bone marrow output of cell types according to the immunological needs of the organism [78]. Indeed, neutrophils and monocytes are promptly recruited to sites of localized tissue damage by inflammatory cytokines. Immature myeloid cells migrating into the blood during infections could become functionally active myeloid-derived suppressor cells with potent immunosuppressing functions acting both on innate and adaptive immune responses [75]. Imprinting of maladaptive myelopoiesis during the initial phases of sepsis could persist after infection resolution and drive chronic disruption of immune homeostasis, causing persistent hyperinflammation and immunosuppression [78]. Pathogen-specific myelopoietic responses have been described in bacterial, fungal, and viral infections. Moreover, emergency myelopoiesis dysregulation has been described in severe COVID-19, with features of type I IFN responses and increased concentrations of IL-6 and IL-1β, which play a central role in myeloid activation [78]. Epigenetic alterations of hematopoietic stem and progenitor

cells in individuals recovering from severe COVID-19 have been reported and may account, at least in part, for the post-COVID-19 symptoms [79]. Recently, inflammatory stress erythropoiesis and iron dysregulation, characterized by monocyte iron loading and increased iron demand in proliferating lymphocytes, have also been described in patients with post-acute sequelae of COVID-19 [46].

In conclusion, together with microbiological cultures, which are still considered the “golden standard” for identifying pathogens and are a cornerstone of appropriate antimicrobial therapy, classical and emerging host-derived inflammatory biomarkers could represent a complementary diagnostic tool to earlier distinguish the main types of infection etiologies, including bacterial and viral sepsis [56]. Given that the alterations in iron-related parameters are partially distinct in infections, including SARS-CoV-2 and bacterial infections, iron metabolism markers may add useful information in differentiating etiology, but evidence in humans is still sparse.

1.4 Anemia of inflammation

Anemia can be considered an important off-target effect of iron metabolism alterations during acute or chronic immune activation [2], called anemia of inflammation (AI) or anemia of chronic disease [80]. AI is the second most prevalent form after iron deficiency anemia (IDA). The most frequent diseases associated with AI are acute and chronic infections, cancer, autoimmune disorders, chronic kidney disease, and congestive heart failure [81]. Defined by the World Health Organization (WHO) as Hb levels < 130 g/L for men and < 120 g/L for women [82], anemia is a common feature of critically ill patients, with an estimated prevalence ranging from one to two out of three patients at ICU admission and reaching 95% within the following 3 days [83, 84]. Of note, approximately 37% of ICU patients receive red blood cell (RBC) transfusions, which are associated with higher mortality rates [84]. Other treatment regimens, such as exogenous EPO [85] or iron administration [86], have failed to improve outcomes.

The pathophysiology of AI is characterized by (1) inflammation-driven hepcidin upregulation leading to iron dysregulation and iron-restricted

erythropoiesis, (2) direct suppression of erythropoiesis by inflammatory cytokines such as IL-1, TNF, and IFNγ, which cause impaired hypoxia-mediated EPO stimulation, and (3) decreased erythrocyte survival [81].

Several mechanisms contribute to inflammatory suppression of erythropoiesis, such as reduced EPO production, impaired EPO–EPO receptor signalling, and hematopoietic stem cell dysfunction. Erythroid cell proliferation and differentiation are impaired by the blunted EPO effect and by iron restriction caused by hepcidin and cytokines. Moreover, various inflammatory mediators directly target erythroid cells and induce apoptosis. The decreased red blood cell survival is probably a minor factor in chronic conditions but is likely to play an important role in acute infections, severe sepsis, or other critical illnesses characterized by a high level of cytokine activation when anemia is detected early (after hours or a few days) and therefore cannot be attributed to impaired erythropoiesis [81]. Decreased erythrocyte lifespan is commonly ascribed to hemolysis and massive erythrophagocytosis by activated macrophages.

As regards COVID-19, a certain degree of heterogeneous alterations in red blood cell morphology was found in more than half of hospitalized patients and was associated with mortality [87]. Furthermore, elevated RDW, which may reflect erythropoietic stress, has been associated with poor prognosis [88]. Although anemia is not a predominant characteristic of COVID-19 and lower basal Hb has inconsistently been associated with more severe cases [39, 41, 43, 44, 89], a decrease of Hb levels has been observed in moderate to severe patients up to 3 months post-onset [46]. Moreover, prolonged iron dysregulation and maldistribution were found in patients with post-acute sequelae of COVID-19 months after infection [46].

During sepsis, anemia may not be found at hospital presentation, but it develops frequently and early [90], probably enhanced also by large intravenous fluid administration, repeated phlebotomies, and blood losses. Persistent elevation of inflammatory cytokines, such as IL-6, IL-8, and TNF-α, was associated with functional iron-restricted erythropoiesis without evidence of systemic ID in critically ill septic patients [91]. As different causes of anemia may coexist, and both anemia and RBC transfusions are associated with a significantly increased risk for adverse events in septic

patients, the identification of the exact etiology could provide more appropriate treatment and better outcomes to patients with AI. However, standard iron biomarkers have failed to predict ID in septic patients so far [92]. Importantly, low hepcidin has been proposed as a diagnostic tool for detecting ID in critically ill patients and as an independent predictor of 1-year mortality [93]. sTfR index has also shown accuracy in diagnosing IDA in the context of AI [66]. Hence, exploring the ability of new diagnostic tools to identify the cause of anemia during acute infections is of great clinical importance.

2. AIMS OF THE STUDY

The present work aimed to:

1. Describe and compare inflammatory patterns, iron metabolism, and anemia of inflammation at baseline and during disease evolution in two cohorts of well-characterized patients hospitalized for acute severe infections of different etiologies (COVID-19 pneumonia and sepsis) and followed up across a three to five-time points span
2. Elucidate the possible role of early (within 48 hours of hospital admission) parameters, including iron biomarkers, in the prediction of COVID-19 pneumonia and sepsis outcomes
3. Explore the interrelation of different mechanisms of hepcidin regulation *in vivo*.

Finally, given the emerging evidence on different inflammatory patterns possibly reflecting a specific response of the host to different pathogens, we explored the ability of well-recognized acute-phase biomarkers and iron-related parameters to predict microbial etiology.

3. MATERIALS AND METHODS

3.1 Study population

This is a retrospective analysis based on data collected prospectively. In this study, two cohorts of patients were recruited.

The first cohort, termed the *COVID-19 pneumonia cohort*, enrolled consecutive patients aged 18 years or older diagnosed with SARS-CoV-2 pneumonia and admitted either to the low and medium-intensity Internal Medicine wards or to the Infectious Disease ward of the Verona University Hospital between April 2020 and May 2021. This registry was part of the scientific project named “Conoscerlo per Sconfiggerlo, Alleanza contro COVID-19 (ENACT)” of the University of Verona funded by Fondazione Cariverona. The project was approved by the local Ethical Committee (No. 2646CESC).
Patients were recruited in the study according to the following criteria:

- Inclusion criteria:
  - SARS-CoV-2 RNA detection by reverse-transcription real-time polymerase chain reaction (PCR) in a nasopharyngeal swab sample at hospital admission
  - Clinical and radiological features of COVID-19 pneumonia
  - Blood sample collection within 48 hours from the hospital admission.

- Exclusion criteria:
  - Patients admitted directly to the ICU.

Baseline (T0) demographic, clinical, biochemical, and arterial blood gas analysis data, together with radiological features, were collected within 48 hours of hospitalization. Patients’ in-hospital follow-up included clinical assessment, pharmacological treatment, and sampling for biochemical monitoring of COVID-19 evolution, which was repeated at +/- 7 days (T1)

and +/- 14 days (T2) from hospitalization if the patient was still hospitalized. At the same time points, blood samples exceeding after routine analysis were collected for research purposes and stored at -80°C by the local facility Laboratorio Universitario di Ricerca Medica (LURM). Information about in-hospital evolution was retrospectively collected from the medical records.

The second cohort, named the *sepsis cohort*, recruited consecutive patients admitted to the same wards with a diagnosis of sepsis between July 2023 and February 2025. This is an interim analysis of a broader and still ongoing prospective validation study conducted by the Immunology, Internal Medicine, and Infectious Disease Units of the Azienda Ospedaliera Universitaria Integrata of Verona. This validation study is a part of the clinical research project “Persistent, aberrant myelopoiesis as etiological factor for chronic illness and metastatic disease” performed by the Istituto Oncologico Veneto and funded by the Piano Nazionale di Ripresa e Resilienza (PNRR-MAD-2022-12375871).
For patient recruitment, the following inclusion and exclusion criteria were applied:

- Inclusion criteria:
  - Patients aged 18-79 years
  - Patients newly diagnosed with sepsis according to the international definition of sepsis (The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3))
  - Blood sample collection within 48 hours from the hospital admission.

- Exclusion criteria:
  - Patients aged less than 17 or 80 or more years
  - Patients with SARS-CoV-2 infection
  - Patients with septic shock at the time of sepsis diagnosis and admitted directly to the ICU

- Patients having a do-not-intubate indication due to severe pre-existing clinical conditions and short life expectancy before the acute infection.

Baseline (T0) demographic, clinical, biochemical, and arterial blood gas analysis data, together with cultures for microbiological purposes, were collected at diagnosis and possibly before starting antimicrobial treatment. Patients' in-hospital follow-up included clinical assessment, pharmacological treatment, and sampling for biochemical monitoring of sepsis evolution, which was repeated at +/- 5 days (T1), +/- 10 days (T2) from hospitalization, and at discharge (T3). Blood sampling was repeated 3 months after discharge for patients discharged alive (T4). At the same time points, blood samples for research purposes (3 EDTA plasma tubes of 7 mL) were collected and stored at -80°C by the local Immunology Unit. Information about in-hospital evolution was collected retrospectively by consulting the medical records.

Both studies are conducted in accordance with the Note for Guidance on Good Clinical Practice (CPMP/ICH-135/95) and the Helsinki Declaration (revision 2013). Patients received the standard-of-care treatment for their condition according to current international guidelines. All patients provided informed consent, which was orally acquired for the COVID-19 cohort in accordance with the Ethical Committee that waived the requirement of written consent due to the SARS-CoV-2 pandemic.

3.2 Serum assays

3.2.1 Iron, ferritin and transferrin assays

Plasma samples were used to determine iron metabolism assays. In both cohorts, serum iron, serum ferritin, and serum transferrin were assessed by the local laboratory on the day of collection on heparin-plasma or subsequently on stored samples, when necessary, except for serum iron of the sepsis cohort patients that could not be measured afterward on EDTA plasma due to iron chelation. The following reference values for iron

parameters are provided by the local laboratory: iron 55 - 161 µg/dL, ferritin 30 - 300 µg/L, transferrin 2.00 - 3.60 g/L, TSAT 16.0 - 40.0 %.

3.2.2 Hepcidin assay

The Intrinsic Hepcidin IDx™ ELISA kit (Intrinsic Lifesciences, The BioIron Company™, USA) has been used for hepcidin measurement. It is a monoclonal antibody-based assay that binds with high affinity to the N-terminus of hepcidin-25, which is required for its bioactivity and binding to ferroportin. This antibody also binds low-abundance, N-terminus isomers of hepcidin-25 with lower affinities. The kit is a competitive binding assay between hepcidin-25 in the test specimen and a biologically active biotinylated human hepcidin-25 tracer for a constant number of high-affinity anti-hepcidin-25 N-terminal-specific monoclonal antibody binding sites. Concentration values are obtained by interpolation from the standard calibration curve by GraphPad Prism software (Boston, MA, USA) and adjusted for the dilution factor. Concentrations are expressed in ng/mL. The hepcidin normal range is 6.2 - 82.6 ng/mL. The method's sensitivity is 2.5 ng/mL. As reported by the manufacturer, a strong correlation was observed between serum and EDTA plasma hepcidin measurements.

3.2.3 Erythroferrone assay

ERFE analysis was performed using the Human Erythroferrone IETM ELISA kit (Intrinsic Lifesciences, The BioIron Company™, USA), which is a double monoclonal antibody sandwich ELISA assay. Absorbance values were used for data interpolation and reduction by GraphPad Prism software (Boston, MA, USA). Concentrations were expressed in ng/mL. The limit of detection of human erythroferrone ELISA kit is 0.1 ng/mL. The median ERFE concentration obtained with the same methodology in healthy individuals was reported in a recent publication as 0.51 ng/mL (IQR: 0.12–1.25), with men having significantly higher levels than women (0.67 vs 0.32 ng/mL) [94].

3.2.4 sTfR assay

sTfR was measured using the DRG sTfR ELISA kit (DRG Instruments GmbH, Germany) and performed according to manufacturers' instructions. This ELISA assay is a sandwich ELISA, where the microtiter wells are coated with a monoclonal (mouse) antibody directed towards a unique antigenic site of the sTfR molecule. Absorbance values were used for data interpolation and reduction by GraphPad Prism software (Boston, MA, USA). Concentrations were expressed in ng/mL. The mean normal expected value reported by the manufacturer for men and women is 1430 ng/mL (range min-max: 480-3090 ng/mL), observed in a group of 176 healthy individuals. Values have been then transformed from ng/mL to mg/L.

3.2.5 Cytokines analysis

For the COVID-19 pneumonia cohort only, a panel of inflammatory cytokines was assessed at three time points (T0, T1, and T2). The panel included the following cytokines: IFNγ, IL-1Ra, IL-1β, IL-6, IL-8, IL-10, IL-22, and TNF-α. Their quantification was performed with an innovative automated kit for multiple immunoassays called Ella (Ella™ Assay, Bio-techne, USA). This system is based on microfluidic technology and uses "custom-made" cartridges. Each sample diluted 1:2 is loaded on two different Simple Plex Cartridges, with 32 wells each, one including IL-1β, IL-6, IL-8, and TNF-α and the other IFNγ, IL-1Ra, IL-10, and IL-22. In this immunoassay, the sample is run through a microfluidic channel that specifically binds the protein of interest. The channel is then washed to remove nonspecific bindings and then flows through a detection reagent. Measurements are performed in triplicate for each sample. An internal calibration curve allows for the absolute quantitation of the analyte of interest. Dedicated software allows for measurements and calibration curve visualization.

3.2.6 Immunological cells analysis

The aforementioned PNRR study aims to assess epigenetic, transcriptional, and metabolic changes in the myeloid compartment during the initial phases of sepsis to understand how the negative training of myelopoiesis can lead

myeloid cells to acquire features that could be the basis for the persistence of unsolved inflammation/immunosuppression. Thus, for the sepsis cohort only, immunological analysis was performed. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Paque density gradient centrifugation and used to identify major cell types. Single-cell analysis will also be performed to elucidate the functional role of altered monocytes, but these results are not available so far.

1. Flow cytometry analysis of CD14+ARG1+ Cells
    - Cell staining

PBMCs were washed with FACS buffer (PBS containing 2% FBS and 0.05% sodium azide) and stained with a cocktail of surface antibodies. The following fluorochrome-conjugated monoclonal antibodies were used: CD3-FITC, CD19-FITC, CD56-FITC (used as a lineage-defining 'cocktail' or Lin), HLA-DR-PerCP-Cy5.5, CD14-APC-H7, CD16-PE-Cy7, CD66b-BV605, and CD34-PE. Following surface staining, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Permeabilized cells were then stained intracellularly with Arginase 1 (ARG1)-Alexa Fluor 647 antibody.

- Flow cytometry and gating strategy

A four-laser flow cytometer (e.g., BD LSR Fortessa) was used for data acquisition. Lymphocytes, monocytes, and granulocytes were initially gated based on forward and side scatter properties. A lineage-negative (Lin^-) population was identified by excluding cells expressing CD3, CD19, and CD56. Within the monocyte gate (based on FSC/SSC and positive for CD14 and CD16), the frequency of CD14+ARG1+ cells was determined. The gating strategy involved: (1) gating on live cells, (2) identifying the monocyte population (FSC-A vs. SSC-A), (3) selecting CD14 and CD16 markers to define classical, intermediate, and non-classical monocytes, and (4) analysing the percentage of CD14+ARG1+ cells within the monocyte population. On the CD14+ARG1+ fraction of cells, the expression levels of CD34 as a marker of immature cells was additionally evaluated.

2. Isolation of CD14+ cells and immunosuppression assay
    - CD14+ cell isolation

CD14+ cells isolated from PBMCs collected at T0 and 3 months (T4) using anti-CD14 magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the isolated CD14+ cells was confirmed by flow cytometry and was consistently greater than 95%.

- T cell activation and co-culture

T cells were isolated from healthy donors using an untouched T cell isolation kit. T cells were activated with plate-bound anti-CD3 and soluble anti-CD28 antibodies at a concentration of 1 µg/mL each. Activated T cells were labelled with CFSE (carboxyfluorescein succinimidyl ester) to track proliferation. The labelled, activated T cells were then co-cultured with the isolated CD14+ cells from sepsis patients at T0 and T4 at effector:target ratios of 1:3. After 96 hours, T cell proliferation was assessed by flow cytometry by measuring the dilution of the CFSE signal. T cell proliferation in the presence of patient-derived CD14+ cells was compared to T cell proliferation in the absence of these cells.

3.3 Statistical Analysis

Categorical variables are presented as frequencies and percentages. Continuous variables are presented as mean ± standard deviation (SD) or median [interquartile range] if the normality of distribution was not confirmed by the Shapiro-Wilk test. Categorical data were compared using the Chi-Squared test, while the T-student test or the Mann-Whitney U test were used for continuous data, as appropriate. Comparison between repeated measurements of the same variables at different time points was assessed by the Wilcoxon matched pairs signed rank test or the Friedman test, as appropriate.

Binary logistic regression analysis was performed to identify possible predictors of in-hospital mortality and infection etiology among baseline parameters. In the COVID-19 pneumonia cohort, linear regression analysis

was performed to explore the main drivers of hepcidin upregulation at infection onset. Due to the small number of cases in the sepsis cohort, multivariable analysis was not feasible in this group.

Thirty-day survival was analyzed using the Kaplan-Meier method, and differences between groups were assessed by the Log-rank test.

For immunological analyses, comparisons between groups were made using a one-way ANOVA with post-hoc tests.

The analysis was performed on complete cases.

A two-sided $p < 0.05$ was considered statistically significant. Statistical analyses were performed using IBM SPSS 28.0 software (SPSS Inc., Chicago, Illinois, USA) and GraphPad Prism 10.0.2 (GraphPad Software, Boston, MA, USA).

4. RESULTS

4.1 COVID-19 pneumonia cohort

4.1.1 Cohort characteristics upon admission

A total of 391 patients diagnosed with COVID-19 pneumonia were recruited, with a median age of 71 years, two-thirds of whom were males (Table 1). The burden of comorbidities was high, with a prevalence of 85.2% and a median number of 2 [1-3]. Hypertension was the most common chronic disease, followed by diabetes, chronic heart diseases, chronic respiratory diseases, and neoplasia. The median time from symptoms onset and admission was 7 [4-10] days, while the length of hospitalization was 11 [6-20] days and did not significantly differ between survivors and patients deceased during hospital stay. Survivors were younger and more frequently had a mute previous medical history or a lower comorbidity burden compared to the deceased. A higher proportion of females died during hospitalization. Compared to men, women were significantly elderly (median age 74 vs 68 years, p-value 0.002) and more frequently comorbid (94.0 vs 80.6%, p-value <0.001). According to the WHO definition, anemia was detected upon admission in 31.2% of subjects, and one out of two of the deceased patients was anemic.

As regards the biochemical profile upon admission, the cohort was characterized by increased levels of inflammatory markers such as CRP, fibrinogen, and D-dimer, and low lymphocyte counts and P/F ratio, the latter consistent with pneumonia-related respiratory failure. Compared to survivors, deceased patients had significantly higher CRP, neutrophil counts, Neutrophil to Lymphocyte (N/L) ratio, creatinine, and D-dimer, and lower Hb, lymphocyte counts, albumin, ALT, and P/F ratio.

The overall in-hospital mortality rate of the COVID-19 pneumonia cohort was 18.7% (n=73). Of survivors, 104 patients (32.6%) were admitted to the ICU, of whom 38 (36.5%) received mechanical ventilation.

Table 1. Baseline demographic and clinical characteristics, and biochemical parameters of the COVID-19 pneumonia cohort according to in-hospital mortality.

	COVID-19 pneumonia cohort (n=391)	Survived (n=318)	Deceased (n=73)	p-value
Demographic and clinical characteristics				
Age, years	71 [61-80]	67 [58-76]	83 [77-87]	**<0.001**
Males, n (%)	258 (66.0)	218 (68.6)	40 (54.8)	**0.025**
Comorbidities (any), n (%)	333 (85.2)	262 (82.4)	71 (97.3)	**0.001**
Hypertension	217 (55.5)	171 (53.8)	46 (63.0)	0.152
Atrial fibrillation	42 (10.7)	23 (7.2)	19 (26.0)	**<0.001**
Chronic Ischaemic Heart Disease	41 (10.5)	28 (8.8)	13 (17.8)	**0.040**
Chronic Heart Failure	24 (6.1)	11 (3.5)	13 (17.8)	**<0.001**
Chronic Respiratory Disease	57 (14.6)	44 (13.8)	13 (17.8)	0.494
Chronic Kidney Disease	26 (6.6)	14 (4.4)	12 (16.4)	**<0.001**
Chronic Liver Disease	16 (4.1)	14 (4.4)	2 (2.7)	0.750
Diabetes	78 (19.9)	64 (20.1)	14 (19.2)	0.984
Active solid neoplasia	19 (4.9)	14 (4.4)	5 (6.8)	0.565
Active hematologic neoplasia	19 (4.9)	12 (3.8)	7 (9.6)	0.079
Anemia	122 (31.2)	85 (26.7)	37 (50.7)	**<0.001**
Biochemical parameters				
C-reactive protein, mg/L	73 [43-129]	70 [40-123]	104 [61-157]	**0.002**
Hb, g/L	134.0 [122.3-143.0]	135.0 [125.0-144.0]	125.0 [116.5-140.0]	**<0.001**
MCV, fL	90 [86.8-93.7]	89.2 [86.6-93.0]	91.9 [88.4-95.5]	**<0.001**
RDW, %	13.2 [12.6-14.4]	13.1 [12.5-14.1]	14.4 [13.1-16.0]	**<0.001**
WBC, 10^9/L	7.11 [5.03-10.22]	6.94 [5.06-9.94]	8.55 [4.83-13.34]	0.074
Neutrophils, 10^9/L	5.74 [3.79-8.78]	5.56 [3.82-8.40]	7.02 [3.51-11.65]	**0.023**
Lymphocytes, 10^9/L	0.74 [0.53-1.06]	0.79 [0.57-1.08]	0.59 [0.39-0.88]	**<0.001**
N/L, ratio	7.34 [4.06-13.01]	6.97 [4.02-11.06]	12.63 [4.22-20.43]	**<0.001**
Platelets, 10^9/L	207 [159-268]	211 [162-273]	192 [146-254]	0.143
Creatinine, mg/dL	0.85 [0.71-1.04]	0.84 [0.70-0.99]	1.01 [0.75-1.60]	**<0.001**
eGFR, mL/min	84 [64-96]	87 [69-99]	61 [33-84]	**<0.001**
Albumin, g/L	35.8 [32.6-38.7]	36.6 [33.4-39.2]	33.1 [29.4-36.9]	**<0.001**
AST, U/L	37 [28-52]	36 [27-50]	40 [30-57]	0.117
ALT, U/L	31 [21-46]	32 [22-49]	26 [18-43]	**0.028**
Fibrinogen, g/L	5.78 [4.73-6.92]	5.78 [4.80-6.84]	5.73 [4.42-7.45]	0.705
D-dimer, g/L	1060 [703-1767]	1020 [635-1565]	1625 [970-3309]	**<0.001**
LD, U/L	328 [268-406]	323 [264-398]	351 [276-428]	0.141
CK, U/L	112 [56-236]	104 [56-218]	145 [60-255]	0.114
P/F, ratio	238 [133-290]	257 [152-295]	145 [102-241]	**<0.001**

4.1.2 Iron metabolism and cytokine profile upon admission

Baseline iron-related proteins and cytokine assays are reported in Table 2. Consistent with the hyperinflammatory state, high levels of ferritin and hepcidin, together with hypoferremia, were observed. Basal hepcidin was

not associated with 30-day mortality in the Kaplan-Meier survival analysis. Regarding cytokines, increased levels of IFNγ, IL-1Ra, IL-6, IL-10, and TNF-α were detected. Deceased patients had significantly lower transferrin, IL-1β, and higher ERFE, IL-6, IL-8, IL-10, and TNF-α levels compared to survivors.

Table 2. Baseline iron metabolism and cytokine assays of the COVID-19 pneumonia cohort.

	Entire cohort (n=391)	**Survived (n=318)**	**Deceased (n=73)**	**p-value**
Iron markers				
Iron, μg/dL	32 [23-52]	32 [24-53]	34 [23-46]	0.703
Ferritin, μg/L	809 [437-1294]	811 [442-1250]	778 [396-1537]	0.612
Transferrin, g/L	1.65 [1.40-1.94]	1.68 [1.42-1.96]	1.54 [1.23-1.91]	**0.010**
TSAT, %	14 [10-24]	14 [10-24]	16 [11-26]	0.167
Hepcidin, ng/mL	201.01 [130.74-265.28]	201.07 [134.63-262.68]	198.02 [95.67-286.39]	0.949
ERFE, ng/mL	0.41 [0.14-1.11]	0.37 [0.14-0.92]	0.76 [0.21-1.61]	**<0.001**
sTfR, mg/L	1.74 [1.42-2.22]	1.70 [1.42-2.10]	1.95 [1.34-2.79]	0.099
sTfR index	0.62 [0.47-0.84]	0.61 [0.47-0.79]	0.65 [0.45-0.98]	0.214
Cytokines, pg/mL				
IFNγ	3.59 [1.07-11.90]	3.70 [1.21-14.05]	2.80 [0.81-8.49]	0.138
IL-1Ra	1188 [744-2347]	1137 [695-2219]	1626 [782-2739]	0.055
IL-1β	0.27 [0.15-0.52]	0.29 [0.17-0.54]	0.21 [0.09-0.39]	**0.015**
IL-6	21.9 [9.4-49.6]	18.8 [8.2-46.0]	39.3 [12.8-72.4]	**<0.001**
IL-8	7.8 [4.9-11.7]	7.0 [4.8-10.6]	10.4 [8.1-18.9]	**<0.001**
IL-10	13.2 [7.5-24.6]	12.7 [7.0-22.6]	17.1 [9.6-35.2]	**0.009**
IL-22	17.0 [10.3-26.8]	16.6 [10.0-25.4]	20.1 [12.3-36.4]	0.082
TNF-α	13.2 [10.5-17.4]	12.9 [10.2-16.6]	15.8 [12.0-25.7]	**<0.001**

4.1.3 Variable trends across different time points

As stated in the materials and methods section, blood samples were recorded at pre-defined time points during hospitalization (T0-T2). Table 3 reports the trends of main biochemical parameters, iron metabolism markers, and cytokines. Regarding common biochemical parameters, a progressive decline of Hb, CRP, and N/L ratio and a rise in WBC count were observed during hospitalization. The statistical significance of these trends was confirmed by Friedman's tests for repeated measures (Figure 1). Both CRP decrease, and WBC increase were evident already at T1, while median values tended to stabilize between T1 and T2. Median Hb progressively declined, with an increasing prevalence of anemia (46.7% at T1 and 79.3% at T2).

Table 3. Trends of biochemical parameters, iron metabolism and cytokine assays of the COVID-19 pneumonia cohort across study's time points.

	T0 (n=391)	T1 (n=211)	T2 (n=93)
Biochemical parameters			
C-reactive protein, mg/L	73 [43-129]	26 [10-74]	41 [12-114]
Hb, g/L	134.0 [122.3-143.0]	128.0 [113.0-142.0]	114.0 [102.3-125.0]
MCV, fL	90.0 [86.8-93.7]	91.3 [88.0-94.3]	92.4 [89.6-95.0]
RDW, %	13.2 [12.6-14.4]	13.2 [12.5-14.5]	13.6 [13.0-14.9]
WBC, 10^9/L	7.11 [5.03-10.22]	10.30 [8.39-13.05]	11.02 [8.12-14.68]
Neutrophils, 10^9/L	5.74 [3.79-8.78]	8.36 [6.29-10.48]	8.25 [5.29-11.16]
Lymphocytes, 10^9/L	0.74 [0.53-1.06]	1.07 [0.64-1.53]	1.26 [0.78-1.90]
N/L, ratio	7.34 [4.06-13.01]	7.79 [4.31-14.28]	5.84 [3.69-12.30]
Platelets, 10^9/L	207 [159-268]	272 [204-347]	204 [150-282]
Creatinine, mg/dL	0.85 [0.71-1.04]	0.79 [0.66-0.98]	0.84 [0.64-1.02]
P/F, ratio	238 [133-290]	171 [119-238]	216 [132-278]
Iron markers			
Iron, µg/dL	32 [23-52]	77 [51-113]	62 [38-87]
Ferritin, µg/L	809 [437-1294]	1045 [665-1731]	1041 [550-1624]
Transferrin, g/L	1.65 [1.40-1.94]	1.45 [1.23-1.81]	1.41 [1.14-1.79]
TSAT, %	14 [10-24]	39 [26-53]	33 [19-45]
Hepcidin, ng/mL	201.01 [130.74-265.28]	156.88 [100.60-228.66]	151.01 [81.44-216.36]
ERFE, ng/mL	0.41 [0.14-1.11]	0.14 [0.14-0.53]	0.38 [0.14-1.17]
sTfR, mg/L	1.74 [1.42-2.22]	1.36 [1.05-1.83]	1.11 [0.81-1.42]
sTfR index	0.62 [0.47-0.84]	0.43 [0.29-0.60]	0.34 [0.23-0.46]
Cytokines, pg/mL			
IFNγ	3.59 [1.07-11.90]	1.02 [0.52-2.57]	1.14 [0.50-2.45]
IL-1Ra	1188 [744-2347]	1312 [888-2491]	1720 [1003-3276]
IL-1β	0.27 [0.15-0.52]	0.44 [0.24-0.89]	0.52 [0.26-1.01]
IL-6	21.9 [9.4-49.6]	17.3 [6.5-48.7]	19.3 [9.87-46.1]
IL-8	7.8 [4.9-11.7]	8.2 [5.9-12.5]	8.4 [6.2-13.3]
IL-10	13.2 [7.5-24.6]	6.5 [3.9-13.3]	6.0 [4.1-10.0]
IL-22	17.0 [10.3-26.8]	16.7 [9.8-34.7]	17.5 [7.6-35.7]
TNF-α	13.2 [10.5-17.4]	15.6 [11.9-20.4]	17.0 [12.9-23.6]

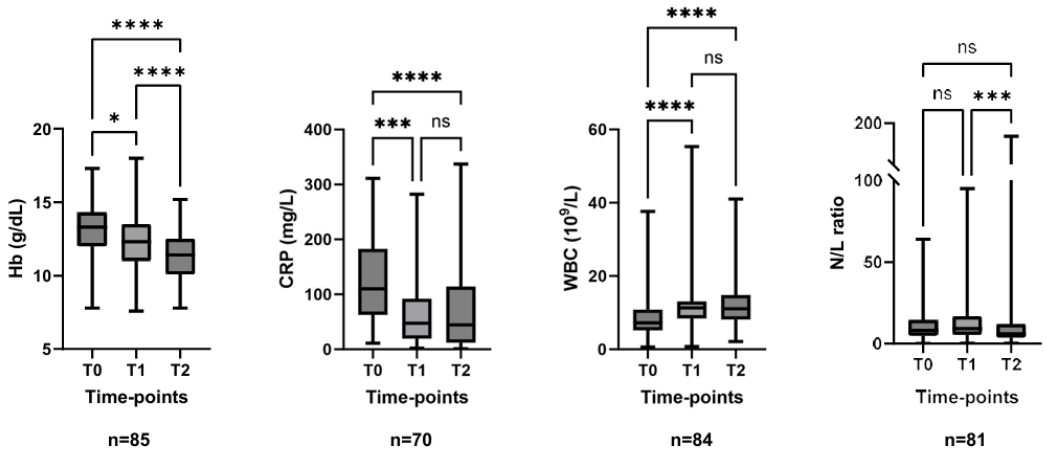

Figure 1. Friedman test for repeated measures of Hb, CRP, WBC, and N/L ratio across the study's time points, COVID-19 pneumonia cohort. Box and

whisker plots (* p-value <0.05, *** p-value <0.001, **** p-value < 0.0001, ns=non-significant, n=number of patients analysed).

Considering iron-related parameters, we observed an early and marked increase of both iron and TSAT, together with a mild further increase of ferritinemia, which, however, remained highly elevated across all time points. Hepcidin and sTfR index decreased gradually, especially during the second half of the observation period, while no significant variation of transferrin, ERFE, and sTfR was detected (Figure 2).

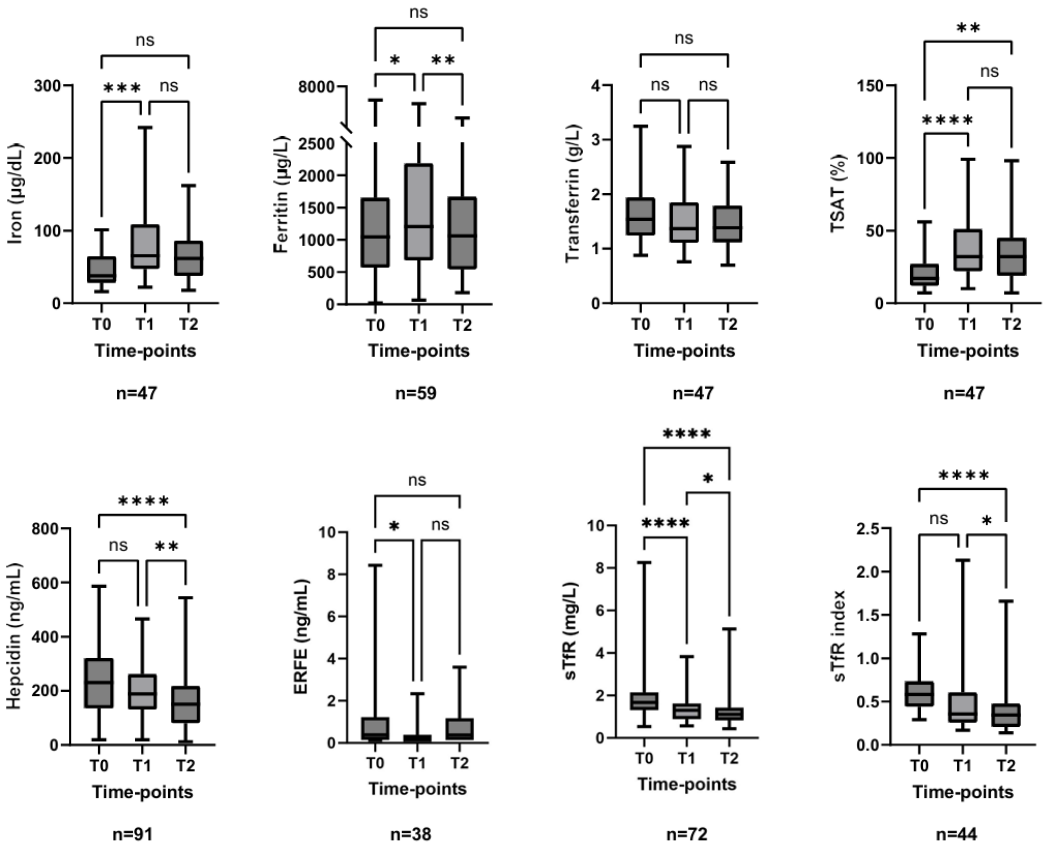

Figure 2. Friedman test for repeated measures of iron metabolism markers across the study's time points, COVID-19 pneumonia cohort. Box and whisker plots (* p-value <0.05, ** p-value <0.01, *** p-value <0.001, **** p-value < 0.0001, ns=non-significant, n=number of patients analysed).

Cytokines' global trends between T0 and T2 are reported in Figure 3. A general reduction of IFNγ and IL-10 levels and a rise of IL-1Ra, IL-1β, and TNF-α were observed, while IL-6, IL-8, and IL-22 showed an essentially stable trend.

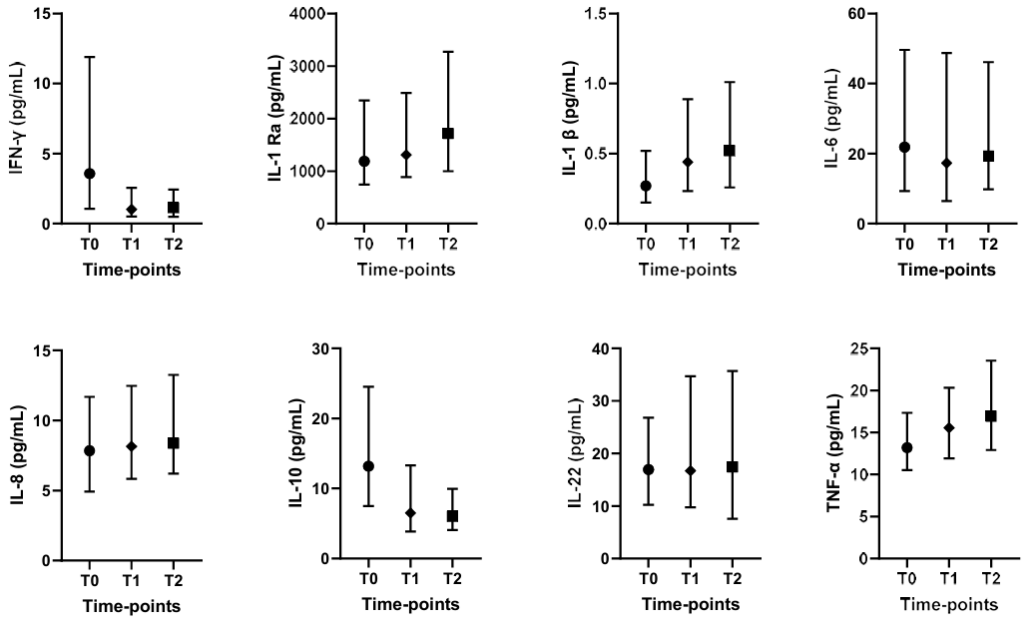

Figure 3. Cytokines' trends across the study's time points, COVID-19 pneumonia cohort. Median (symbols) with interquartile range (bars).

Considering pairwise comparisons, there was an early and highly significant decrease of IFNγ and IL-10 and a less significant increase of IL-1Ra and IL-1β, especially at 2 weeks from admission. The trends of the other cytokines included in the study were not significant (Figure 4).

Comparing variables' trends among survivors and deceased patients, we observed a significantly different evolution for several parameters. Nonsurvivors showed a significant increase in CRP (p=0.004), WBC (p=0.027), and neutrophils (p=0.003) between T1 and T2. N/L ratio (p<0.001) and IL-6 (p=0.012) tended to systematically increase in the deceased, while the same parameters progressively decreased in survivors. Transferrin was almost stable in survivors while it decreased in the deceased (p=0.007). Finally, P/F (p<0.001) and IL-8 (p=0.020) showed a divergent evolution during hospitalization in the two groups (Figure 5). Trends for other serum parameters did not significantly differ according to the outcome.

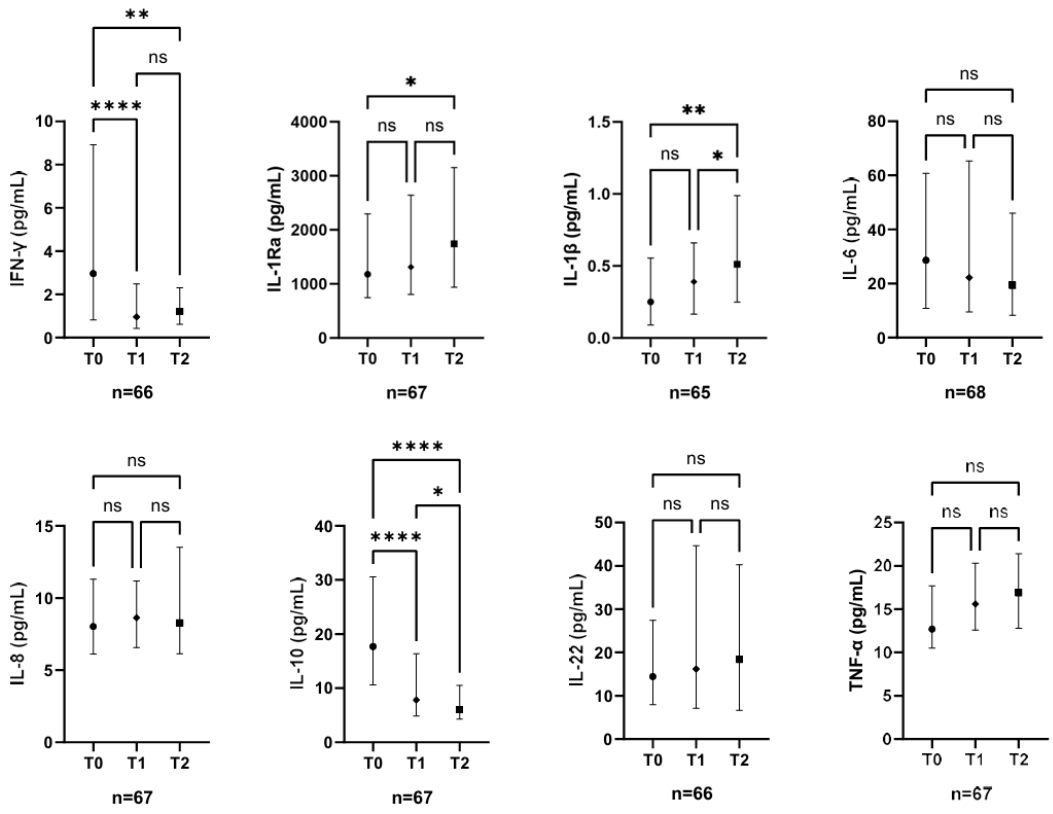

Figure 4. Friedman test for repeated measures of cytokines across the study's time points, COVID-19 pneumonia cohort. Box and whisker plots (* p-value <0.05, ** p-value <0.01, **** p-value < 0.0001, ns=non-significant, n=number of patients analysed).

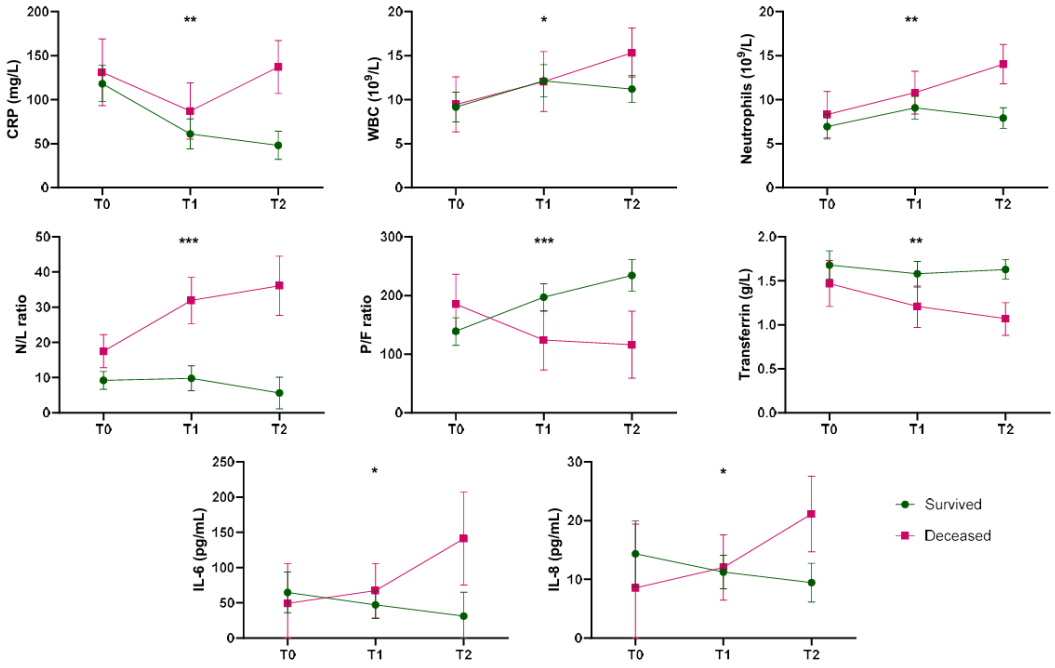

Figure 5. Friedman test for repeated measures across the study's time points, grouping for mortality outcome. Means (dots) ± SD (bars) are reported. Only significant tests are shown (* p-value <0.05, ** p-value <0.01, *** p-value <0.001, exact p-values are reported in the text).

4.1.4 Multivariable analysis

A binary logistic regression analysis was performed to identify possible predictors of in-hospital mortality. Although significantly different between survivors and nonsurvivors at the univariable level, albumin and D-dimer were excluded due to many missing values. Female sex, increase of age and IL-8, and decrease of P/F ratio and transferrin significantly predicted mortality at the multivariable level (Table 4).

Table 4. Binary logistic regression for prediction of in-hospital mortality.

	B	S. E.	p-value	Exp (B)
Age	**0.049**	**0.013**	**<0.001**	**1.050**
Gender (M)	**-1.059**	**0.423**	**0.012**	**0.347**
Anemia (yes)	0.529	0.425	0.213	1.697
CRP	-0.006	0.003	0.072	0.994
N/L ratio	0.049	0.025	0.050	1.051
Creatinine	-0.023	0.225	0.919	0.977
ALT	-0.008	0.008	0.289	0.992
P/F ratio	**-0.008**	**0.002**	**0.001**	**0.992**
Transferrin	**-1.619**	**0.443**	**<0.001**	**0.198**
ERFE	0.027	0.088	0.760	1.027
IL-1β	-0.725	0.514	0.158	0.485
IL-6	0.000	0.003	0.981	1.000
IL-8	**0.020**	**0.009**	**0.025**	**1.020**
IL-10	-0.006	0.011	0.593	0.994
TNF-α	0.006	0.033	0.856	1.006

A multivariable analysis was carried out to explore the upregulation of hepcidin at the onset of COVID-19 pneumonia. The following variables, expected to be variably involved in hepcidin regulation mechanisms, were included in the analysis: Hb, CRP, N/L ratio, P/F (as a proxy for hypoxia), creatinine, iron, ERFE, sTfR, IL-1Ra, IL-6, IL-22, and TNF-α (Table 5). Hb, CRP, N/L ratio, creatinine, ERFE, sTfR, IL-1Ra, IL-6, and TNF-α were significantly associated with basal hepcidin values. Interestingly, the P/F ratio was not found to be significantly associated with hepcidin values. These findings suggest that inflammation and erythropoiesis, rather than the hypoxic drive, seem to upregulate hepcidin at baseline in patients hospitalized for COVID-19 pneumonia. Given the positive coefficient of Hb and the negative coefficients of ERFE and sTfR, hepcidin regulation might be influenced by pre-existing erythron homeostasis. When the same

multivariable model was restricted to nonanemic subjects, CRP, sTfR, IL-1Ra, IL-6, and TNF-α remained significantly associated with hepcidin. On the other hand, considering only anemic patients, Hb, CRP, N/L ratio, creatinine, ERFE, IL-1Ra, and IL-6 were significantly associated with hepcidin levels.

Table 5. Linear regression. Dependent variable: hepcidin.

	Unstandardized coefficients		Standardized coefficients		
	B	**S. E.**	**Beta**	**T**	**p-value**
(Constant)	32.066	52.215		0.614	0.540
Hb	**9.795**	**3.110**	**0.157**	**3.150**	**0.002**
CRP	**0.297**	**0.070**	**0.214**	**4.212**	**<0.001**
N/L ratio	**1.537**	**0.618**	**0.128**	**2.488**	**0.013**
P/F ratio	0.023	0.059	0.019	0.396	0.692
Creatinine	**23.486**	**5.433**	**0.216**	**4.323**	**<0.001**
Iron	-0.009	0.234	-0.002	-0.039	0.969
ERFE	**-6.345**	**2.190**	**-0.145**	**-2.898**	**0.004**
sTfR	**-16.985**	**5.638**	**-0.156**	**-3.013**	**0.003**
IL-1Ra	**0.017**	**0.005**	**0.204**	**3.266**	**0.001**
IL-6	**0.305**	**0.090**	**0.199**	**3.387**	**<0.001**
IL-22	-0.009	0.009	-0.048	-1.043	0.298
TNF-α	**-1.745**	**0.728**	**-0.132**	**-2.398**	**0.017**

4.2 Sepsis cohort

4.2.1 Cohort characteristics upon admission

As reported in Table 6, the sepsis cohort is composed of 56 patients aged 65 [53-76] years, mainly males. The rate of comorbidities was approximately 60%, hypertension and cardiovascular diseases were the most prevalent chronic conditions, followed by chronic respiratory diseases and diabetes. Five patients died during hospitalization with a mortality rate of 8.9%. Anemia was common at baseline, affecting around 80% of the cohort, mostly mild to moderate, and all 5 deceased patients were anemic. As expected, markers of inflammation such as CRP, fibrinogen, WBC counts, and N/L ratio were high. Mean albumin and P/F ratio were reduced compared to normal values, possibly reflecting the multiorgan involvement of sepsis. Compared to survivors, deceased patients only differed for higher SOFA score and creatinine levels, and lower lymphocyte counts and P/F ratio.

As regards the causes of sepsis, Figure 6 reports the rates of pathogens isolated from cultures of biological samples, mainly blood or urine, but also others such as pharyngeal swabs or cerebrospinal fluid. The most identified microorganism was Escherichia coli, followed by Streptococcus pneumoniae and Legionella pneumophila. Consistently, lower respiratory tract and urinary tract infections were the most frequent clinical features associated with sepsis. In 35% of cases, mostly affected by lower respiratory tract infections, no pathogens were identified despite appropriate and multiple microbiological studies.

Table 6. Baseline demographic and clinical characteristics, and biochemical parameters of the sepsis cohort according to in-hospital mortality.

	Sepsis cohort (n=56)	Survived (n=51)	Deceased (n=5)	p-value
Demographic and clinical characteristics				
Age, years	65 [53-76]	65 [50-76]	74 [68-76]	0.278
Males, n (%)	34 (60.7)	30 (58.8)	4 (80.0)	0.638
Comorbidities (any), n (%)	39 (59.6)	34 (66.7)	5 (100)	0.309
Hypertension	32 (57.1)	28 (54.9)	4 (80.0)	0.379
Cardiovascular Diseases	20 (35.7)	17 (33.3)	3 (60.0)	0.336
Chronic Respiratory Disease	13 (23.2)	10 (19.6)	3 (60.0)	0.076
Chronic Kidney Disease	11 (19.6)	9 (17.6)	2 (40.0)	0.251
Chronic Liver Disease	5 (8.9)	3 (5.9)	2 (40.0)	0.058
Diabetes	13 (23.2)	11 (21.6)	2 (40.0)	0.580
Neoplasia (active)	3 (5.4)	2 (3.9)	1 (20.0)	0.249
Neoplasia (previous)	8 (14.3)	7 (13.7)	1 (20.0)	0.552
Immunorheumathological Disease	7 (12.5)	6 (12.0)	1 (20.0)	0.508
Anemia	44 (78.6)	39 (76.5)	5 (100)	0.574
SOFA score	2 [0-4]	2 [0-3]	8 [4-9]	**0.005**
Biochemical parameters				
C-reactive protein, mg/L	222 (±109)	220 (±105)	239 (±158)	0.718
Hb, g/L	111.9 (±18.3)	112.8 (±18.4)	102.6 (±16.4)	0.236
MCV, fL	92.3 [88.4-96.4]	92.4 [88.3-96.5]	92.0 [84.9-96.0]	0.747
RDW, %	14.0 [13.1-15.9]	14.0 [13.0-15.7]	16.1 [14.7-21.4]	0.055
WBC, 10^9/L	11.13 [7.14-14.70]	11.04 [7.32-14.87]	13.11 [4.17-13.90]	0.703
Neutrophils, 10^9/L	8.20 [5.19-12.66]	7.91 [5.25-12.33]	11.61 [3.36-12.98]	0.988
Lymphocytes, 10^9/L	0.92 [0.53-1.63]	1.12 [0.59-1.68]	0.44 [0.42-0.47]	**0.006**
N/L, ratio	8.42 [3.97-14.53]	8.27 [3.76-12.40]	15.90 [7.21-31.23]	0.117
Platelets, 10^9/L	237 [143-286]	239 [164-290]	115 [35-250]	0.055
Creatinine, mg/dL	1.02 [0.81-1.69]	0.97 [0.78-1.62]	1.62 [1.45-2.61]	**0.046**
Albumin, g/L	31.3 (±5.7)	31.5 (±5.7)	30.2 (±6.4)	0.673
AST, U/L	28 [22-45]	26 [21-44]	40 [31-50]	0.090
ALT, U/L	23 [18-34]	22 [18-77]	29 [24-41]	0.157
Fibrinogen, g/L	6.89 (±2.09)	7.02 (±2.07)	6.26 (±2.42)	0.519
P/F, ratio	257 (±109)	281 (±98)	113 (±44)	**0.009**

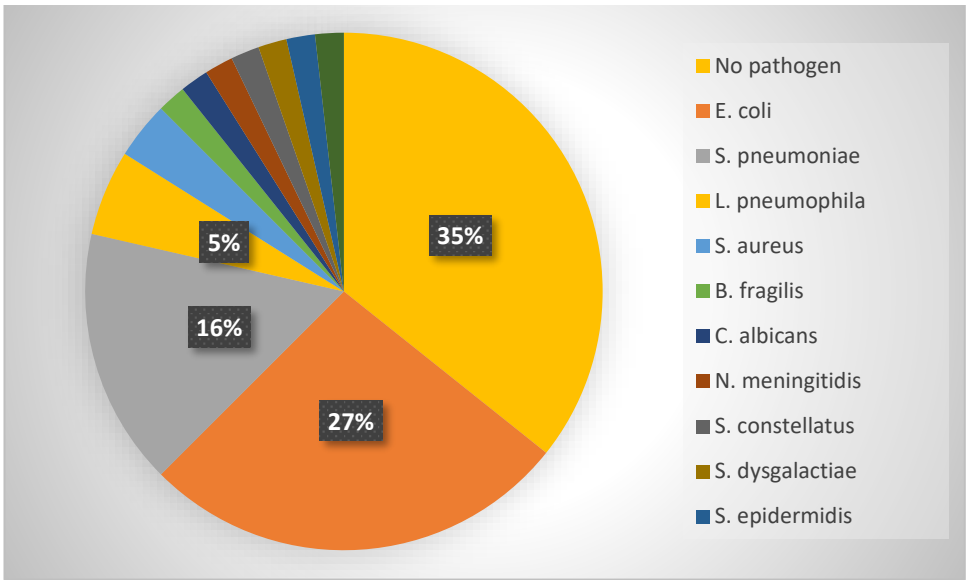

Figure 6. Rates of pathogens isolated from samples' cultures.

4.2.2 Iron metabolism upon admission

As expected, iron metabolism of the entire cohort was profoundly altered with respect to normal values, with high ferritin and hepcidin, and low iron, TSAT, and ERFE (Table 7). Iron-related parameters did not differ significantly among survivors and nonsurvivors, except for transferrin and sTfR index, which were lower in the deceased group.

Table 7. Baseline iron metabolism assays of the sepsis cohort.

	Sepsis cohort (n=56)	**Survived (n=51)**	**Deceased (n=5)**	**p-value**
Iron markers				
Iron, µg/dL	25 [18-71]	25 [18-77]	28 [17-38]	0.668
Ferritin, µg/L	505 [221-735]	485 [219-701]	686 [367-2958]	0.251
Transferrin, g/L	1.61 (±0.38)	1.66 (±0.35)	1.18 (±0.39)	**0.007**
TSAT, %	15 [9-30]	15 [8-30]	19 [13-24]	0.815
Hepcidin, ng/mL	151.35 (±98.33)	135.22 (±102.97)	215.87 (±35.69)	0.147
ERFE, ng/mL	0.61 [0.39-0.91]	0.60 [0.28-0.80]	0.77 [0.46-2.22]	0.272
sTfR, mg/L	1.46 [1.10-1.83]	1.47 [1.16-1.95]	1.10 [0.68-1.48]	0.128
sTfR index	0.48 [0.42-0.74]	0.56 [0.44-0.87]	0.35 [0.24-0.45]	**0.016**

Interestingly, baseline hepcidin greater than 160 ng/dL, which approximated the median cohort value, was significantly associated with 30-day mortality as shown by the Kaplan-Meier function reported in Figure 7.

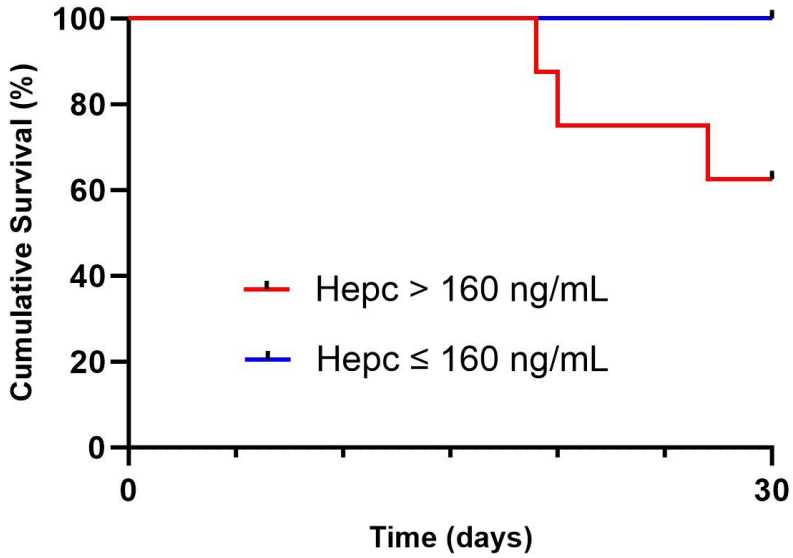

Figure 7. Kaplan-Meier survival curves at 30 days from disease onset according to hepcidin value, sepsis cohort (log-rank 5.185, p=0.023; n=20).

4.2.3 Variable trends across different time points

As described in the Materials and Methods section, blood samples were recorded at pre-defined time points both during hospitalization (T0, T1, T2, T3) and at 3 months after discharge (T4). At the time of the present analysis, 21 out of 35 survivors attended the 3-month follow-up (60%). The other 16 patients discharged alive have not yet reached the 3-month time point. Trends of the main variables were analyzed between admission and discharge (T0-T3) except for hepcidin, ERFE, sTfR, and sTfR index (T0-T4), due to more samples available for these time points to maximize statistical power. Therefore, trends refer only to survivors. Patients who received intravenous iron supplementation during hospitalization were excluded from iron metabolism analysis.

As expected, inflammatory markers such as CRP, WBC count, and N/L ratio markedly decreased during hospitalization and returned around normal values upon discharge. Conversely, median Hb persisted low (Figure 8).

Iron-related proteins' trends are reported in Figure 9 and reflect the progressive resolution of the profound unbalance observed at sepsis onset. Iron and transferrin increased, while ferritin decreased significantly in the time span between sepsis onset and clinical resolution. Compared to hospital admission, after 3 months, hepcidin was significantly lower, and the

sTfR index was significantly higher. Nevertheless, anemia prevalence was still high at T4 (n=8 patients, 38.1%).

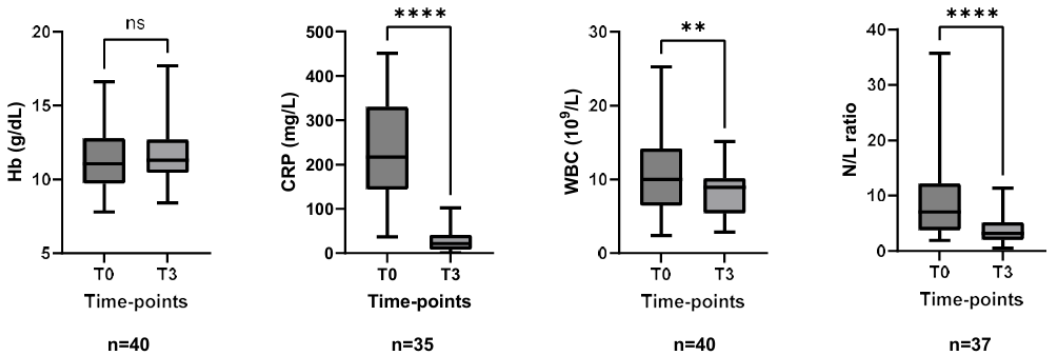

Figure 8. Wilcoxon matched pairs signed rank test of biochemical parameters between admission (T0) and discharge (T3), sepsis cohort. Box and whisker plots (** p-value <0.01, **** p-value < 0.0001, ns=non-significant, n=number of patients analysed).

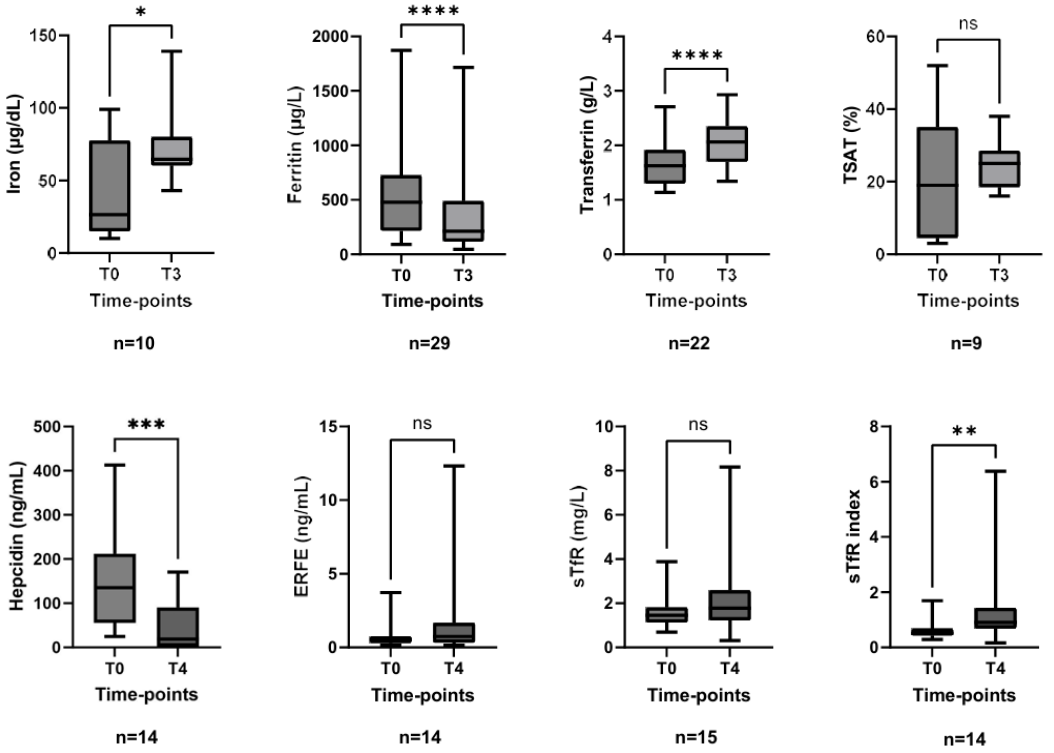

Figure 9. Wilcoxon matched pairs signed rank test of iron metabolism markers between admission (T0) and discharge (T3) or at 3-month follow-up (T4), sepsis cohort. Box and whisker plots (* p-value <0.05, ** p-value <0.01, *** p-value <0.001, **** p-value < 0.0001, ns=non-significant, n=number of patients analysed).

4.2.4 Myeloid precursors

Regarding immunological assays, Figure 10 A shows the percentage of circulating arginase-positive myeloid precursors, a subpopulation of monocytes characterized by $CD14^+ARG1^+$ markers, detected in the peripheral blood of septic patients during the study time span. These cells are usually undetectable in blood samples of healthy donors but have been found in patients' plasma at every time point and were still present in survivors at 3 months after clinical sepsis resolution. Figure 10 B represents the percentage of T cell proliferation after incubation with these arginase-positive myeloid precursors, showing their immunosuppressive effect on T lymphocytes. Deceased patients are shown as red dots: at baseline, the immunosuppressive effect of myeloid precursors is clearly more marked in nonsurvivors, while in survivors at follow-up the effect tends to reduce.

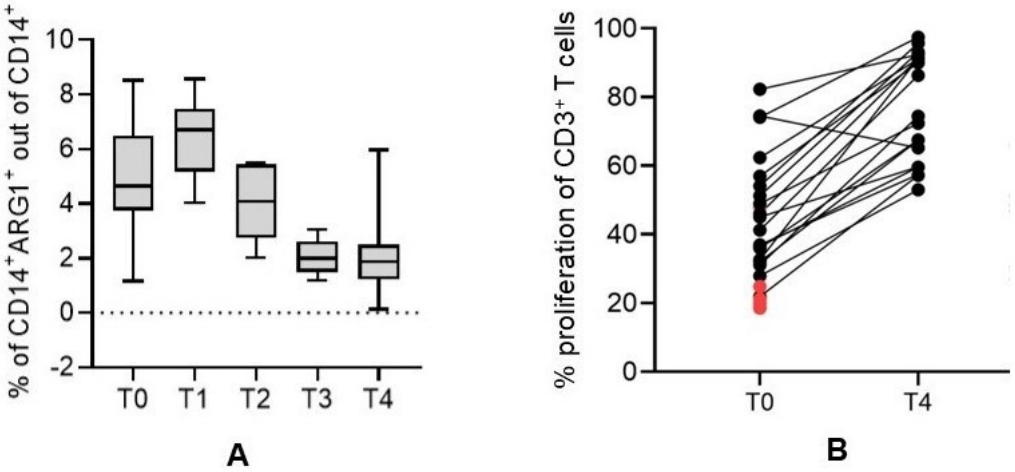

Figure 10. Sepsis cohort. **A:** Percentage of arginase-positive myeloid cells ($CD14^+ARG1^+$) on total circulating monocytes ($CD14^+$) across study time points. **B:** T cell proliferation assays indicating immunosuppression by $CD14^+$ cells.

4.3 Comparison of the COVID-19 pneumonia and the sepsis cohort

4.3.1 Cohorts' characteristics upon admission

Baseline characteristics of the two cohorts are reported and compared in Table 8. Patients of the COVID-19 pneumonia cohort were significantly elderly (71 vs 65 years, p=0.001) and more comorbid (85.2 vs 59.6%, p=0.004) than those of the sepsis cohort. Moreover, they experienced a higher, although not statistically significant, mortality rate (18.7 vs 8.9%, p=0.072). Baseline anemia was instead far more frequent in the sepsis cohort (78.9 vs 31.2%, p<0.001).

The biochemical profile upon admission differed significantly among the two cohorts (Table 8 and Figure 11). Septic patients had a median higher CRP, WBC, neutrophils and lymphocytes count, RDW, creatinine, and fibrinogen compared to COVID-19 pneumonia subjects, which in turn exhibited higher Hb, albumin, ferritin, hepcidin, and sTfR values. Interestingly, the two cohorts did not differ for other parameters such as N/L and P/F ratio, and iron and ERFE levels.

Table 8. Baseline demographic and clinical characteristics, and biochemical parameters of the COVID-19 pneumonia and the sepsis cohort.

	COVID-19 pneumonia cohort (n=391)	Sepsis cohort (n=56)	p-value
Demographic and clinical characteristics			
Age, years	71 [61-80]	65 [53-76]	**0.001**
Males, n (%)	258 (66.0)	34 (60.7)	0.438
Comorbidities (any), n (%)	333 (85.2)	39 (59.6)	**0.004**
Hypertension	217 (55.5)	32 (57.1)	0.817
Cardiovascular Diseases	81 (20.7)	20 (35.7)	**0.012**
Chronic Respiratory Disease	57 (14.6)	13 (23.2)	0.096
Chronic Kidney Disease	26 (6.6)	11 (19.6)	**<0.001**
Chronic Liver Disease	16 (4.1)	5 (8.9)	0.110
Diabetes	78 (19.9)	13 (23.2)	0.570
Active neoplasia	38 (9.7)	3 (5.4)	0.290
Anemia	122 (31.2)	44 (78.6)	**<0.001**
In-hospital mortality	73 (18.7)	5 (8.9)	0.072
Biochemical parameters			
C-reactive protein, mg/L	73 [43-129]	208 [141-310]	**<0.001**
Hb, g/L	134.0 [122.3-143.0]	110.5 [98.0-124.0]	**<0.001**
MCV, fL	90.0 [86.8-93.7]	92.3 [88.4-96.4]	**0.025**
RDW, %	13.2 [12.6-14.4]	14.0 [13.1-15.9]	**<0.001**
WBC, 10^9/L	7.11 [5.03-10.22]	11.13 [7.14-14.70]	**<0.001**
Neutrophils, 10^9/L	5.74 [3.79-8.78]	8.20 [5.19-12.66]	**<0.001**
Lymphocytes, 10^9/L	0.74 [0.53-1.06]	0.92 [0.53-1.63]	**0.019**
N/L, ratio	7.34 [4.06-13.01]	8.42 [3.97-14.53]	0.404
Platelets, 10^9/L	207 [159-268]	237 [143-286]	0.560
Creatinine, mg/dL	0.85 [0.71-1.04]	1.02 [0.81-1.69]	**<0.001**
Albumin, g/L	35.8 [32.6-38.7]	31.0 [29.3-34.7]	**<0.001**
AST, U/L	37 [28-52]	28 [22-45]	**0.001**
ALT, U/L	31 [21-46]	23 [18-34]	**0.004**
Fibrinogen, g/L	5.78 [4.73-6.92]	6.49 [5.09-8.48]	**0.031**
P/F, ratio	238 [133-290]	271 [166-333]	0.115
Iron markers			
Iron, µg/dL	32 [23-52]	25 [18-71]	0.260
Ferritin, µg/L	809 [437-1294]	505 [221-735]	**<0.001**
Transferrin, g/L	1.65 [1.40-1.94]	1.61 [1.30-1.86]	0.223
TSAT, %	14 [10-24]	15 [9-30]	0.994
Hepcidin, ng/mL	201.01 [130.74-265.28]	152.27 [74.45-211.89]	**0.019**
ERFE, ng/mL	0.41 [0.14-1.11]	0.61 [0.39-0.91]	0.096
sTfR, mg/L	1.74 [1.42-2.22]	1.46 [1.10-1.83]	**0.012**
sTfR index	0.62 [0.47-0.84]	0.48 [0.42-0.74]	0.069

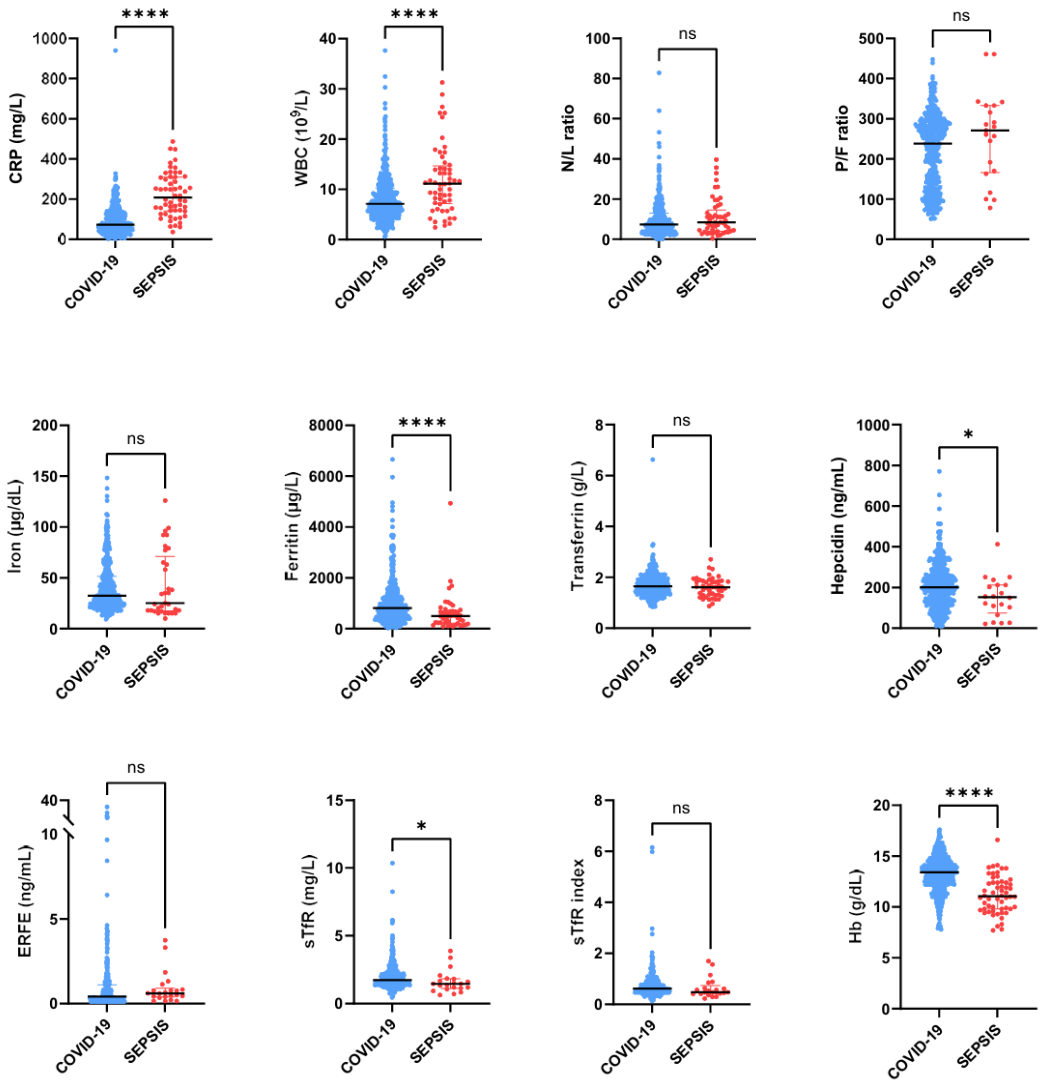

Figure 11. Mann-Whitney tests for comparison of baseline (T0) biochemical parameters and iron metabolism markers of the two cohorts. Scatter dot plot, median (line) with interquartile range (* p-value <0.05, **** p-value < 0.0001, ns=non-significant, n=number of patients analysed).

4.3.2 Variable trends across different time points

The evolution of serum parameters of the COVID-19 pneumonia and the sepsis cohort throughout the study's time points is displayed in Figures 12 and 13. A similar progressive decline in Hb levels between T0 and T2 is observed, as a probable consequence of persisting inflammation, while a clear trend inversion is seen upon sepsis resolution. Interestingly, despite a common decrease in CRP levels, WBC clearly increased over time in the COVID-19 pneumonia cohort but declined in the sepsis cohort.

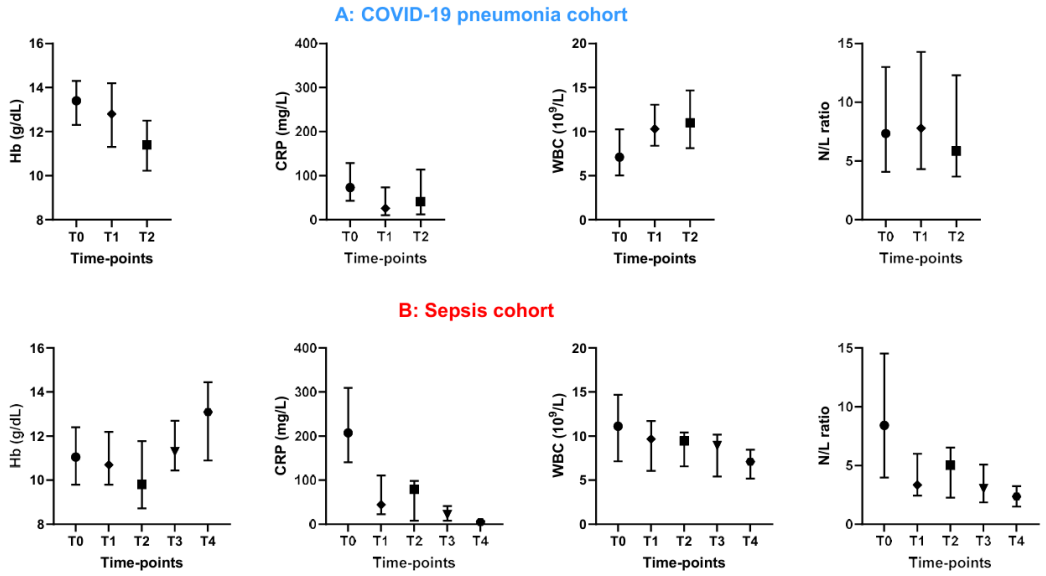

Figure 12. Comparison of biochemical parameters' trends of the two cohorts across studies' time points, as defined in the Materials and Methods section. Median (symbols) with interquartile range (bars). **A:** COVID-19 pneumonia cohort; **B:** Sepsis cohort.

As regards iron-related proteins, sideremia and TSAT showed a similar increasing trend in both groups and stabilized upon infection resolution in the sepsis cohort. Transferrin was quite stable across T0-T2 time points in both cohorts and increased only by sepsis resolution. On the other hand, in addition to significantly higher levels at baseline, ferritin trends seem to differ, with a progressive rise among COVID-19 patients compared to a more stable and, later, declining trend among septic patients. Hepcidin, which was higher at baseline in the COVID-19 pneumonia cohort, declined at T1 in both groups, while at T2 remained stable in the COVID-19 but increased in the sepsis cohort. Also, the sTfR and sTfR index showed a different evolution between cohorts, with a progressive decline among COVID-19 patients and a rise among septic ones. Finally, ERFE trends appeared quite similar, and values rose only upon the resolution of sepsis. Given that blood samples at discharge (T3) and 3 months later (T4) were available for discharged alive septic patients only, comparisons between cohorts were not possible for those time points. Despite this discrepancy, to be thorough, we decided to report data trends for all available time points of both groups.

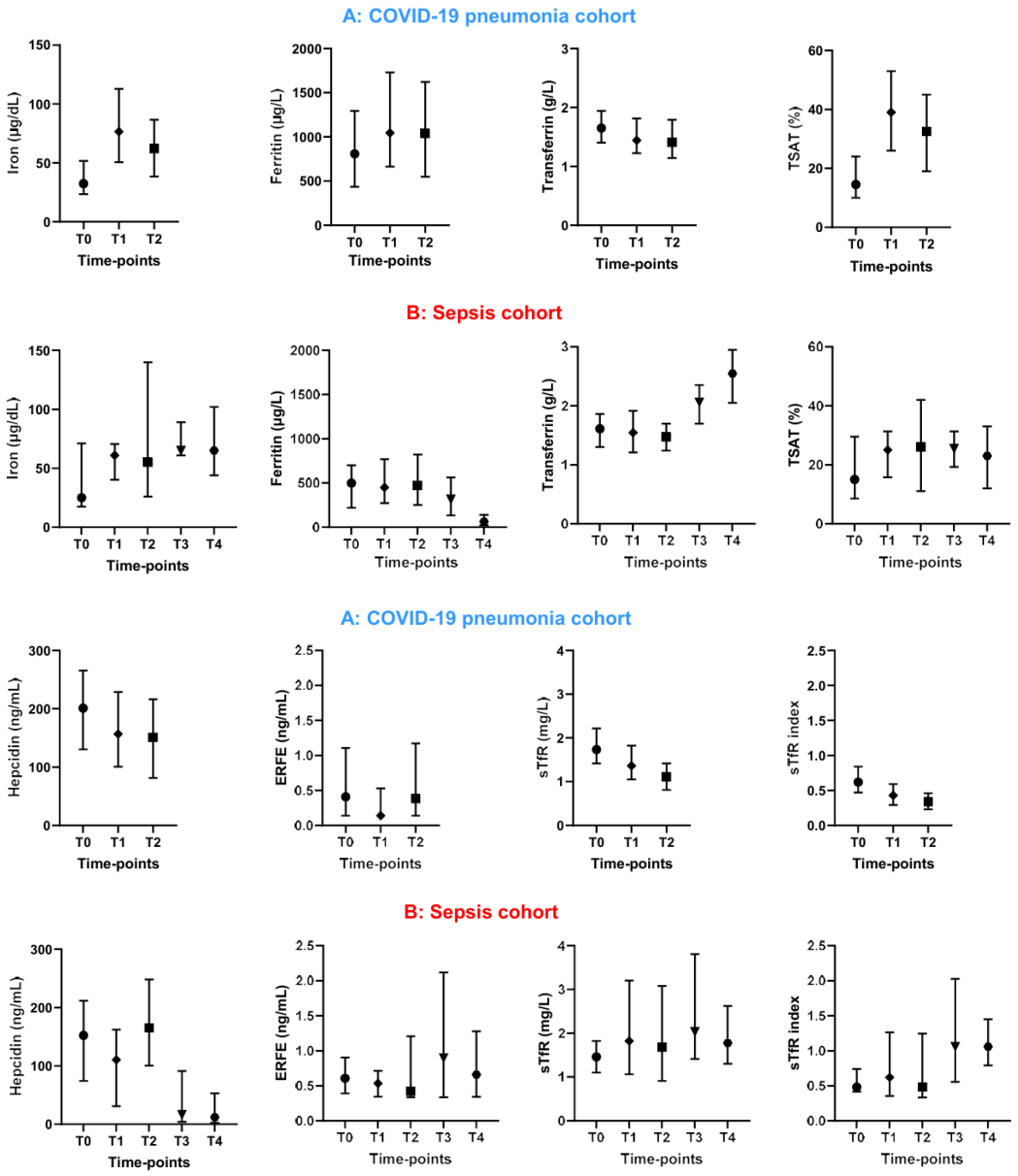

Figure 13. Comparison of iron metabolism markers' trends of the two cohorts across studies' time points, as defined in the Materials and Methods section. Median (symbols) with interquartile range (bars). **A:** COVID-19 pneumonia cohort; **B:** Sepsis cohort.

4.3.3 Multivariable analysis

A binary logistic regression analysis was performed to identify possible predictors of infection etiology (sepsis versus COVID-19 pneumonia). The presence of anemia, high CRP and WBC count, and low hepcidin values at baseline was significantly associated with a higher risk of sepsis even after adjustment for major possible confounders (Table 9).

Table 9. Binary logistic regression for prediction of sepsis versus COVID-19 pneumonia.

	B	S. E.	p-value	Exp (B)
Age	-0.030	0.022	0.180	0.971
Comorbidities (n)	0.139	0.180	0.440	1.149
Anemia (yes)	**1.538**	**0.723**	**0.033**	**4.657**
CRP	**0.011**	**0.003**	**<0.001**	**1.011**
WBC	**0.141**	**0.050**	**0.005**	**1.152**
Creatinine	0.387	0.223	0.096	1.473
Ferritin	0.001	0.000	0.121	1.001
Transferrin	-0.937	0.718	0.192	0.392
Hepcidin	**-0.017**	**0.005**	**<0.001**	**0.983**
ERFE	-0.299	0.323	0.354	0.742
sTfR	-1.483	1.229	0.228	0.227
sTfR index	2.328	2.435	0.339	10.258

Unfortunately, cytokine levels, which could have shed further light on the specific inflammatory process that seems to differentiate the two cohorts, were not available for the sepsis cohort and could not be included in the multivariable analysis.

5. DISCUSSION

To the best of our knowledge, this is the first study reporting a wide panel of clinical, hematological, iron- and inflammation-related parameters collected within 48 hours of hospital admission and following variations at defined time points in two well-characterized cohorts of patients affected by either COVID-19 pneumonia or sepsis.

Our data indicate that iron metabolism is profoundly altered during acute COVID-19 pneumonia and sepsis. Remarkable differences have been observed between the two cohorts, with possible clinical impact.

At hospital admission, marked hyperferritinemia and hypoferremia, together with low transferrin and TSAT and increased hepcidin, were observed in COVID-19 pneumonia patients, as expected from the acute inflammatory response and as reported also in previous studies [16, 37, 38]. Nonsurvivors had significantly lower transferrin and higher ERFE levels compared to survivors, while ferritin, iron, and hepcidin did not differentiate patients' outcomes. Based on our data, we cannot speculate on the source of ERFE (previously known as myonectin), but severe hypoxia could have contributed to skeletal muscle other than erythrocytes production, especially in deceased subjects [95]. In our cohort, baseline transferrin but not anemia significantly predicted in-hospital mortality at the multivariable level, together with age, sex, P/F ratio, and IL-8. These findings are in part in contrast with previous studies, as lower iron and higher hepcidin levels have been reported in nonsurvivors [41, 45]. Moreover, baseline high ferritin [36, 37] and low iron and Hb [37] have been associated with higher mortality risk, even though others found anemia but not iron, ferritin, or transferrin to be associated with in-hospital mortality at multivariable analysis [89]. These discrepancies in results might be due to the heterogeneity of studies. The dynamic of iron metabolism markers during the initial 14 days of hospitalization showed a significant increase of both iron and TSAT in the first week, which reached normal values and then remained stable, together with a mild further increase of ferritin, which, in contrast, remained highly elevated across all time points. Hepcidin and sTfR index decreased more slowly and significantly during the second week, while no significant

variation of transferrin, ERFE, and sTfR values was detected across the study time span. The gradual decline of the sTfR index might represent a marker of progressive erythropoiesis impairment secondary to prolonged inflammation, which in turn could contribute to the decrease of Hb observed in the same time span. Indeed, about one third of COVID-19 pneumonia patients were anemic upon hospital admission, in accordance with a previous report in a similar population [96]. Anemia prevalence was significantly higher in nonsurvivors, and Hb progressively and significantly declined during two weeks of hospital stay, a trend observed also by others especially in more severe COVID-19 cases [43, 46, 96]. Interestingly, transferrin levels were almost stable in survivors, whereas they significantly decreased in patients who died during hospitalization, in accordance with previous reports [96]. Other biomarkers showed a significantly different trend in nonsurvivors compared to survivors (an increase of CRP, IL-6, IL-8, N/L ratio, WBC and neutrophil count, and a decline of P/F ratio), overall suggesting a worsening of severe inflammation, possibly leading to death. Taken together, these observations suggest that in COVID-19 pneumonia patients, a dynamic change of iron disruption occurred during the first two weeks of hospital stay, partially restoring iron homeostasis after an acute hyperinflammatory state, but the increasing prevalence of anemia during the same time span is likely to reflect the detrimental effect of prolonged inflammation on erythropoiesis. Unfortunately, data about bleeding complications and RBC transfusions were not available. Very few data have been published so far regarding the dynamic variation of iron-related parameters in COVID-19 patients, as most studies collected data at hospital admission only. Lanser and collaborators focused on anemia etiology and dynamics and reported data on iron status (but not hepcidin) in the first week of hospitalization for COVID-19, with increasing ferritin and decreasing transferrin, especially in nonsurvivors [96]. A prolonged dysregulation of iron metabolism was also observed by Hanson et al., who reported high hepcidin and low Hb in the first two weeks, and elevated ferritin and reduced iron and TSAT months after COVID-19 in moderate to severe patients [46]. However, sTfR and ERFE were not measured.

To further investigate the mechanisms involved in hepcidin upregulation among COVID-19 pneumonia patients, we performed a multivariable analysis that identified several variables (Hb, CRP, N/L ratio, creatinine, ERFE, sTfR, IL-1Ra, IL-6, and TNF-α) significantly associated with basal hepcidin. Of note, all these parameters refer, directly or indirectly, to either the erythropoietic or the inflammatory drive. On the other hand, the proxy of the hypoxic drive P/F ratio did not predict hepcidin. The hypoxic drive was also inapparent after adjusting for the presence of anemia. Thus, despite the known theoretical role of hypoxia in downregulating hepcidin, our *in vivo* study did not support this mechanism, possibly due to the complexity of the in vivo model discussed here, involving coexisting inflammation, infection, comorbidities, and hypoxia, whose interrelations are difficult to disentangle. Unlike nonanemic patients, in anemic subjects Hb, ERFE, and creatinine contributed significantly to the determination of hepcidin, pointing out a predominant influence of erythropoiesis on hepcidin regulation in the anemia setting. Maira et al. reported similar results, finding comparable hepcidin values and no correlation between hepcidin and P/F ratio irrespective of the severity of COVID-19 [43]. They concluded that the effect of hypoxia prevails over inflammation in more severe COVID-19 patients. However, our multivariable analysis is not in agreement with this suggestion. Gugo and collaborators also found that hepcidin value was not proportional to the level of inflammation in nonanemic hypoxic COVID-19 patients, probably due to a downregulating effect of hypoxia, which was corroborated by higher levels of EPO in the same group [44]. Since we did not measure EPO concentration, we cannot speculate whether the observed ERFE values are adequate and if there are differences due to anemia and severity of hypoxia. Further analysis aimed to compare patients grouped for Hb and P/F ratio levels, possibly including also EPO assay, may improve our understanding of the mechanisms of hepcidin upregulation and the determinants of anemia of inflammation *in vivo*.

The cytokine profile was available for the COVID-19 pneumonia cohort only and reflected a burst of inflammation, especially marked in nonsurvivors, with basal significantly higher levels of IL-6, IL-8, IL-10, and TNF-α compared to survivors, and reduced levels of IL-1β. These observations are

rather in line with the literature, as high levels of IL-6 [97-99] and IL-10 [72, 98] have been associated with worse outcomes, and IL-8 was generally higher in ICU compared to non-ICU patients [98], however higher IL-1β has generally been associated with worse outcomes [97, 100, 101]. As regards cytokines' trends, we observed an early and highly significant decrease of IFNγ and IL-10 and a delayed increase of IL-1Ra and IL-1β during the 2 weeks after COVID-19 diagnosis, suggesting a progressive reduction of the cytokine storm. Few studies have assessed the association of COVID-19 outcomes with a wide cytokine panel, and to date, results are inconclusive [97, 98]. Of note, the variations that emerged from our data could reflect a higher proportion of survivors among followed-up patients and the use of specific drugs, such as Tocilizumab (an anti-IL-6 monoclonal antibody) and Anakinra (a recombinant human IL-1 receptor antagonist), which has been recently demonstrated to reduce IL-1β levels in monocytes of severe COVID-19 patients [101]. Specific treatments have not been analyzed as they were outside the aims of this study.

At baseline, also the sepsis cohort showed increased ferritin and hepcidin and low iron, transferrin, and TSAT, in accordance with the activation of severe inflammation also stated by high CRP, WBC count, N/L ratio, and fibrinogen values. Anemia was highly prevalent, and all deceased patients were anemic. Low transferrin and sTfR index values in the deceased group suggest inflammation rather than ID as the main mechanism underlying anemia. Compared to survivors, deceased patients had higher SOFA score and creatinine levels and lower lymphocyte counts, P/F ratio, transferrin, and sTfR index at baseline. We did not find significant differences in iron or TSAT levels, but the limited number of samples available for baseline iron is likely to represent a bias. These observations suggest that, together with other well-known markers, iron-related parameters might contribute to predicting mortality risk in septic patients, even though we were not able to perform a multivariable analysis due to the small sample size. However, it is of note that hepcidin >160 ng/dL was associated with 30-day mortality. Previous studies found similar results in ICU septic patients, reporting, in particular, a good predictivity of hepcidin [60] and iron [61] for 28-day mortality and an association between mortality and increased iron and

TSAT, and decreased transferrin and sTfR index [59-61]. Overall, these findings are consistent with hypoferremia of inflammation as an innate immune defense against siderophilic pathogens [2].

During follow-up of sepsis survivors, we observed significant dynamic changes in iron-related proteins, with an increase in iron and transferrin and a decrease in ferritin levels upon discharge compared to hospital admission. Moreover, after 3 months, hepcidin was significantly lower, and the sTfR index was significantly higher, but anemia prevalence was still high. These findings likely indicate a complete restoration of iron homeostasis at the resolution of the severe acute inflammation, proven by both clinical improvement and drastic decrease of CRP, WBC count, and N/L ratio. However, the persistence of anemia in almost 40% of patients deserves further research as it could reflect post-septic long-standing impairment of erythropoiesis. Interestingly, we detected circulating myeloid precursors with immunosuppressing functions in the peripheral blood of septic patients across all time points during the hospital stay and, although reduced, even at 3-month follow-up. These cells reflect emergency myelopoiesis in response to infection and might indicate a chronic disruption of immune homeostasis, causing persistent hyperinflammation and immunosuppression, possibly influencing also erythropoiesis [78]. We expect that single-cell analysis will provide further insight into the mechanisms of emergency myelopoiesis and the functional role of altered monocytes in acute and post-acute sepsis phases.

When directly comparing the two cohorts, significant differences in baseline biomarker patterns have been observed. The COVID-19 pneumonia cohort had higher ferritin, hepcidin, and sTfR values compared to the sepsis cohort. In contrast, septic patients had a median higher CRP, WBC, neutrophils and lymphocyte counts, RDW, creatinine, and fibrinogen, and lower Hb and albumin values compared to COVID-19 pneumonia subjects. Anemia was prevalent in both cohorts upon hospital admission, but it was far more frequent in septic patients, in whom a tendency to higher ERFE was also observed that is likely to reflect an erythropoietic stimulus. Interestingly, the P/F ratio was comparable, indicating a similar burden of hypoxia in the two groups. Comparable results have been reported in a previous study on

SARS-CoV-2 and bacterial CAPs, with higher ferritin and hepcidin observed in the SARS-CoV-2 cohort and higher CRP and ERFE and lower Hb in the bacterial cohort [29]. However, this study included a few COVID-19 pneumonia cases and a few severe cases, and data were available only at hospital admission. Interestingly, also Delaye reported higher hepcidin and lower ERFE values in a small study comparing SARS-CoV-2 positive to SARS-CoV-2 negative patients with similar Hb levels [47]. At ICU admission, CRP and creatinine were significantly higher in another septic compared to COVID-19 cohort. However, in this study, iron-related parameters were only compared with healthy donors and with patients undergoing orthopedic surgery [70]. Even though the time points did not fully correspond, the first three (T0-T2) were misaligned for 2 days only, and some observations can be highlighted. WBC increased in the COVID-19 pneumonia and decreased in the sepsis cohort, despite a common decrease of CRP levels. On the other hand, a similar tendency to progressive decline in Hb levels between T0 and T2 is observed, as a probable effect of persisting inflammation on erythropoiesis. Dynamic changes in ferritin showed a progressive rise among COVID-19 patients, which was not observed among septic patients. An unexpected increase of hepcidin at T2 was seen in the septic subjects only and could be explained by the fact that the 3 highest hepcidin values out of 6 samples measured belonged to nonsurvivors. This is further supported by the strong reduction of hepcidin at discharge and at 3-month follow-up in survivors. Finally, the progressive increase of sTfR and sTfR index in the sepsis cohort, in contrast with a reduction in the COVID-19 pneumonia cohort, could be explained by the higher prevalence of anemia in sepsis. Indeed, the resolution of inflammation could have unveiled iron deficiency, which in some patients could contribute to the persistence of anemia after sepsis. To the best of our knowledge, this is the first study comparing the dynamics of a wide range of biochemical, including iron-related, parameters in cohorts of patients infected by different pathogens. Taken together, our findings may reflect specific patterns of inflammation triggered by the SARS-CoV-2 virus and bacteria. Therefore, we performed a logistic analysis to test the hypothesis that the differences in the biochemical profile at hospital

admission could predict infection etiology. Despite a baseline difference in age and number of comorbidities between the cohorts, with COVID-19 patients being elder and more comorbid, these variables did not contribute to etiology identification at multivariable analysis. Conversely, anemia, high CRP and WBC count, and low hepcidin values were significantly associated with a higher risk of sepsis. Intriguingly, also in a study performed on patients affected by CAP before the COVID-19 pandemic outbreak, increased hepcidin was associated with a higher risk of atypical bacterial and a lower risk of viral etiology [28]. These findings are of great importance as they might have clinical implications on contributing to reducing delays in the application of appropriate antimicrobial therapy [56].

Our study has some limitations. The design of the study does not allow causal inference. We did not take into consideration the treatment regimens that are likely to have influenced the patients' outcomes, in particular the mortality rate, which was higher, even though not statistically significant, for the COVID-19 cohort. Several factors could have influenced the higher mortality rate, such as the novelty of the disease and the lack of treatment knowledge, together with dramatically limited hospital capacity and experience in managing COVID-19 during early pandemic waves compared to sepsis. The small size of the sepsis cohort prevented multivariable analysis, and some iron-related parameters were measured in a few patients. Follow-up time points were missing for the COVID-19 cohort at discharge (T3) and 3 months later (T4), and long-COVID patients were not included in the study; therefore, comparisons between cohorts were limited to baseline and disease in-hospital evolution. For the sepsis cohort, some differences in blood sample timing due to T3 and T4 time-point definitions could have influenced the parameters analysed. Moreover, the comparative analyses were restricted to common inflammatory biomarkers, as cytokine assays were performed in the COVID-19 pneumonia cohort only. Finally, despite the known influence of sex on iron metabolism, inflammation, and erythropoiesis, analyses were not stratified by sex, as this aspect was outside the aims of the study, and no sex-related patterns were found at exploratory analyses. The small size of the sepsis cohort further limited the possibility of gender analysis.

Despite these limitations, our findings contribute to a better understanding of the complex pathophysiology of the host response to infections from different etiologies and provide some novel observations on AI in the acute and post-acute setting, deserving future confirmation on independent cohorts.

6. CONCLUSIONS

This study of well-characterized patients affected by severe infections points out a profound alteration of iron homeostasis during acute COVID-19 pneumonia and sepsis, showing important differences between the two groups, possibly reflecting different inflammatory patterns.

Different factors were associated with in-hospital mortality in the two cohorts.

In COVID-19 pneumonia, inflammation and erythropoiesis, rather than the hypoxic drive, seem to upregulate hepcidin at baseline.

Specific baseline hematological parameters, including hepcidin, have been identified as possible predictors of infection etiology.

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