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Biomarkers for iron metabolism among patients hospitalized with community-acquired pneumonia caused by infection with SARS-CoV-2, bacteria, and influenza

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Ferritin, the central iron storage protein, has attracted attention as a biomarker of severe COVID-19. Few studies have investigated regulators of iron metabolism in the context of COVID-19. The aim was to evaluate biomarkers for iron metabolism in the acute phase response to community-acquired pneumonia (CAP) caused by SARS-CoV-2 compared with CAP caused by bacteria or influenza virus in hospitalized patients. A cross-sectional study of 164 patients from the Surviving Pneumonia Cohort recruited between January 8, 2019 and May 26, 2020. Blood samples were collected at admission and analyzed for levels of C-reactive protein (CRP), ferritin, soluble transferrin receptor, erythroferrone, and hepcidin. Median (IQR) hepcidin was higher in SARS-CoV-2 with 143.8 (100.7–180.7) ng/mL compared with bacterial and influenza infection with 78.8 (40.1–125.4) and 53.5 (25.2–125.8) ng/mL, respectively. The median ferritin level was more than 2-fold higher in patients with SARS-CoV-2 compared with the other etiologies ($p < 0.001$). Patients with SARS-CoV-2 had lower levels of erythroferrone and CRP compared with those infected with bacteria. Higher levels of hepcidin and lower levels of erythroferrone despite lower CRP levels among patients with SARS-CoV-2 compared with those infected with bacteria indicate alterations in iron metabolism in patients with SARS-CoV-2 infection.

Key words: COVID-19; community-acquired pneumonia; iron metabolism; hepcidin; ferritin; erythroferrone.

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During the ongoing severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic, ferritin upregulation has been a biomarker of SARS-CoV-2 disease (COVID-19) [1]. Ferritin is the major iron storage protein regulated by iron availability and inflammation [2]. Iron is crucial for the biochemical functions of most living cells and organisms [3], but limited availability results in competition for utilization between the host and pathogens. An effect of the innate immune response is limiting the access of pathogens to free iron [2]. As part of the acute phase response, the peptide hormone hepcidin is upregulated, mainly stimulated by the pro-inflammatory cytokine interleukin-6 [4]. Hepcidin is considered a master regulator of iron metabolism. Hepcidin acts by binding to the

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cellular iron exporter ferroportin, which is then depleted from cell membranes resulting in reduced absorption of dietary iron, iron sequestration in cells, and reduced flow of iron into tissue fluids [4]. In parallel to the action of increased hepcidin, early secreted pro-inflammatory cytokines such as tumor necrosis factor-α and interleukin-1 induce ferritin transcription to shift iron from the circulation to iron stores as measured by increased plasma (p)-ferritin and reduced p-iron [2]. In the presence of tumor necrosis factor-α and interleukin-1β, excess intracellular iron results in ferritin formation and secretion into plasma [5,6]. Hidden in cells, iron becomes unavailable to extracellular bacteria and fungi [3]. This redistribution may increase intracellular iron availability and enhance viral replication [7]. High concentrations of hepcidin result in decreased iron availability for erythropoiesis [8]. The hormone erythroferrone contributes to reestablishing the erythropoiesis through suppression of hepcidin [9]. Transferrin is responsible for iron transport in plasma and transfer of iron into cells. This action is carried out through interaction with transferrin receptors, which can be cleaved off the cells to form the soluble transferrin receptor. When the erythropoietic activity is reduced, the level of soluble transferrin receptor is low [10].

Hepcidin and ferritin have been used as markers of COVID-19 severity [1,11,12] and poor outcomes [11–13]. In a retrospective study, hepcidin levels were 25.5 ± 5.8 and 31.7 ± 8.9 ng/mL for mild and severe COVID-19 and ferritin levels were 135.6 ± 20.7 and 207.8 ± 45.2 ng/mL for mild and severe COVID-19 [1]. Another retrospective study among hospitalized patients found elevated median (IQR) ferritin levels among severe and non-severe COVID-19 cases, but with no statistically significant difference between the two groups. However, the serum iron level was lower among severe COVID-19 cases with median (IQR) of 2.3 (2.2–2.5) μmol/L compared with 4.3 (3.3–5.2) μmol/L in non-severe cases [14]. A study compared biomarkers in SARS-CoV-2 infected hospitalized individuals with SARS-CoV-2 infected non-hospitalized individuals. They reported elevated ferritin levels among hospitalized individuals with median (IQR) of (777,341–1339) μg/L, whereas the median (IQR) ferritin level among non-hospitalized patients was within the normal range with 227 (83–569) μg/L [15]. In contrast, one study reported lower hepcidin levels in critical patients compared with healthy controls and found no difference in hepcidin levels between survivors and non-survivors [16]. In a systematic review, Taneri and colleagues reported a overall mean (95% CI) ferritin level of 777 (701–853) ng/mL in patients with COVID-19 and that survivors had 606 (462–751) ng/mL lower mean (95% CI) ferritin levels compared with COVID-19 non-survivors [17]. Though, elevated ferritin levels have also been reported in asymptomatic and mild COVID-19 [18]. Belllmann-Weiler *et al.* investigated predictive value of anemia and dysregulated iron metabolism in COVID-19. They reported that initial anemia was associated with higher risk of death and that alterations in iron metabolism indicated by high ferritin/transferrin ratio was associated with increased inflammation and higher risk of admission to intensive care unit [19]. A study onCAP investigating differences in hepcidin and ferritin levels across etiologies reported that hepcidin were higher in bacterial compared with viral CAP, whereas ferritin levels were similar [20]. COVID-19 have increased the interest in CAP caused by viral infections and several studies have focused on the biomarkers of iron metabolism in patients with COVID-19. Though the results are conflicting and to our knowledge no other studies have compared biomarkers of iron metabolism across different etiologies in the context of COVID-19. Therefore, we aimed to evaluate biomarkers for iron metabolism in the acute phase response to CAP caused by SARS-CoV-2 compared with CAP caused by bacteria and influenza virus in hospitalized patients.

MATERIALS AND METHODS

This cross-sectional study involving patients admitted between January 8, 2019, and May 26, 2020 was nested in the Surviving Pneumonia Cohort, a prospective study among patients admitted with CAP to Nordsjællands Hospital, Denmark (ClinicalTrials.gov: NCT03795662). Inclusion criteria were ≥ 18 years with CAP. CAP was defined as new infiltrate on chest X-ray and ≥1 of the following symptoms: fever (≥38·0°C), cough, pleuritic chest pain, dyspnea, or focal chest signs on auscultation. Patients were excluded if there was no pathogen detection in blood, airways, or urine (*Legionella pneumophila* antigen or *Streptococcus pneumoniae* antigen). Results regarding bacterial etiologies, body composition, physical function, immune function, and metabolism are described elsewhere [21].

Clinical data were recorded on admission. Venous blood samples were collected <48 after admission and analyzed at the local, accredited Clinical Biochemistry Department for levels of C-reactive protein (CRP), ferritin, iron, hemoglobin, transferrin, transferrin saturation, reticulocytes, lactate dehydrogenase and creatinine. For analysis of interleukin-6, sTfR, erythroferrone, and hepcidin, EDTA blood were kept on ice until centrifuged at 3000 *g* for 15 min at 4°C. Plasma was stored in Eppendorf Lobind tubes (Sigma Aldrich, Denmark) at −80°C until analysis according to the manufacturers' instructions using immunoassays (V-PLEX Viral Panel 3 Human Kit, catalog no. K15347D, Meso Scale Discovery, MD, USA),

Human sTfR ELISA (Biovendor, Czech Republic), and Intrinsic ERFE IE ELISA Kit (Intrinsic LifeSciences, San Diego, CA, USA) [22], respectively. Hepcidin was measured by MALDI-TOF mass spectrometry.

Comparisons between CAP caused by infection with SARS-CoV-2 and bacteria were of primary interest, whereas comparisons with CAP caused by influenza virus were included as a reference viral infection. For normally distributed variables, comparisons were done with one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. Non-normally distributed variables were compared using Kruskal–Wallis test, followed by the Dunn-Bonferroni test for pairwise comparisons. Results are shown as means ± SD or medians (IQR), as appropriate. p-values <0.05 were considered statistically significant. Data analyses were carried out using STATA/IC version 17.0 (StataCorp LP, Texas, USA), while Fig. 1 was conducted with IBM SPSS Statistics for Windows.

Ethical considerations

The study was approved by the scientific Ethics Committee at the Capital Region of Denmark on August 21, 2018 (H-18024256), registered on ClinicalTrials.gov (NCT03795662), and conducted in accordance with the Declaration of Helsinki. Oral and written informed consent was obtained from all patients before enrolment.

RESULTS

We included 164 patients hospitalized with CAP, of which 40 were infected with SARS-CoV-2, 99 with bacteria, and 25 with influenza virus. As previously reported, *Haemophilus influenzae* (n = 30, 30.3%) and *S. pneumoniae* (n = 18, 18.2%) were the two most common pathogens among patients with bacterial CAP. Other pathogens included *Escherichia coli*, *Staphylococcus aureus*, *Mycoplasma pneumoniae*, and *L. pneumophila* [21]. Patient characteristics are shown in Table 1. There was no difference in age or sex distribution between the three groups. Patients infected with SARS-CoV-2 had less comorbidity than the two other groups, and fewer received supplementary oxygen upon admission. The CURB-65 score of pneumonia severity did not differ between the groups [21]. Median (IQR) hepcidin were higher (143.8 (100.7–180.7) ng/mL) in patients infected with SARS-CoV-2 than in patients infected with bacteria (78.8 (40.1–125.4) ng/mL), $p < 0.001$) or influenza virus (53.5 (25.2–125.8), $p = 0.003$) and the median ferritin level was more than twice as high in patients infected with SARS-CoV-2 with a median (IQR) of 775 (426–1460) compared with bacteria and influenza virus with median (IQR) of 322 (191–569) and 305 (230–384), respectively. The reticulocyte counts were lower in patients infected with SARS-CoV-2 with median (IQR) of 27.5 (20.5–40.0) compared with patients infected with bacterial infection and influenza with median (IQR) of 50 (39.0–64.5) and 52 (29–71).). Bilirubin levels were higher in patients infected with SARS-CoV-2 compared with patients infected with bacteria and influenza with median (IQR) of 10.0 (8.5–12.0), 8.0 (6.0–13.0), and 6.5 (5.0–10.5),

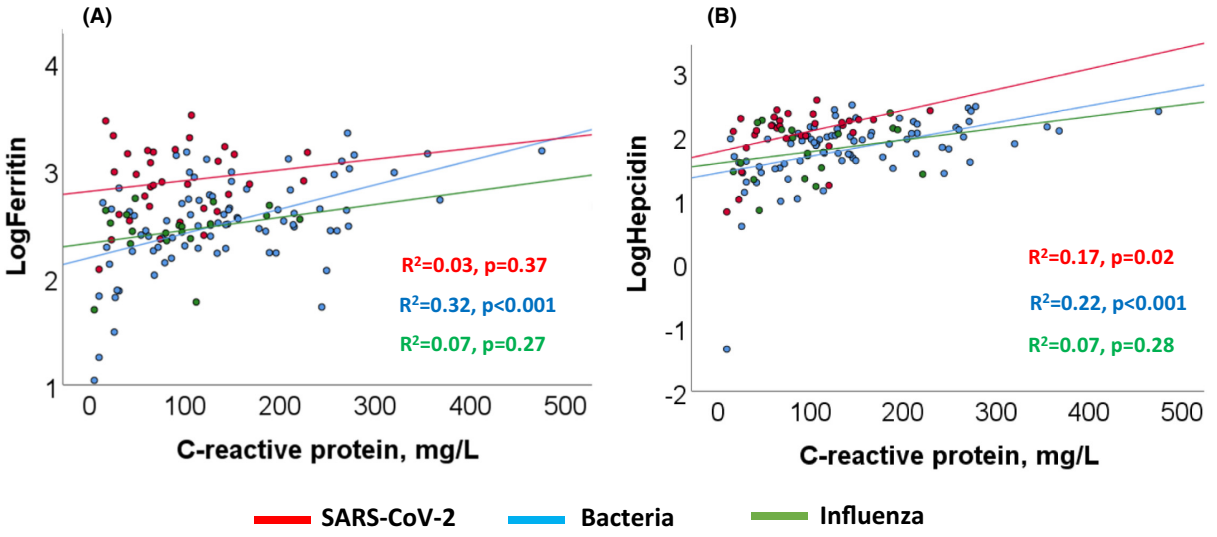

Fig. 1. Association between logFerritin and CRP level (A) and loghepcidin and CRP level (B) in patients hospitalized with community-acquired pneumonia infected with SARS-CoV-2, bacteria, or influenza virus. (A) Shows that in those with bacterial infection, CRP was associated with ferritin level. It also show that in some infected with SARS-CoV-2 high ferritin levels were seen at a modestly elevated CRP. (B) Shows that hepcidin increased with increasing CRP in those infected with bacteria and SARS-CoV-2, whereas no association was seen in those infected with influenza.

respectively. Patients with COVID-19 had lower levels of erythroferrone compared with those with bacterial infection, whereas levels of lactate dehydrogenase and hemoglobin were higher. Level of CRP was lower in patients with COVID-19 compared those with bacterial infection, whereas the level of interleukin-6 was similar. Comparing those with viral infections, those with SARS-CoV-2 infection had higher interleukin-6 level compared with those infected with influenza, whereas the level of CRP was similar. To express the level of ferritin and hepcidin for a given level of inflammation, we calculated ferritin:CRP and hepcidin:CRP ratios. Both ferritin:CRP ratio and hepcidin:CRP were higher in patients with COVID-19 compared with bacterial infection ($p < 0.001$) and influenza ($p = 0.001$). CRP was positively associated with ferritin in patients with bacterial infection ($p < 0.001$), while there was no association between CRP and ferritin in those infected with SARS-CoV-2 and influenza virus ($p > 0.05$). Of note, some patients with COVID-19 had high ferritin levels at a modestly elevated CRP, suggesting a partly inflammation-independent upregulation of ferritin. Hepcidin increased with increasing CRP in patients infected with SARS-CoV-2 ($p = 0.02$) and bacteria ($p < 0.001$), while no association was seen between hepcidin and CRP in those infected with influenza **(**Fig. 1**)**.

DISCUSSION

We observed differences in several biomarkers of iron metabolism across the etiologies in the acute phase among patients hospitalized with CAP. The findings may indicate that SARS-CoV-2 play a role in iron regulation.

In the present study, patients infected with SARS-CoV-2 had higher levels of hepcidin and ferritin compared with the two other etiologies. The median ferritin level of 775 ng/mL observed among patients with SARS-CoV-2 in the present study is similar to the median ferritin level observed in another study on hospitalized patients with a level of 777 μg/L [15]. A systematic review and meta-analysis reported a similar overall ferritin level based on 54 studies with a pooled mean of 777 ng/mL [17]. Oppen and colleagues investigated ferritin and hepcidin levels across etiologies in CAP patients and reported similar ferritin levels among patients with CAP caused by bacteria and viral infection [20]. In the present study, ferritin levels were also similar in CAP patients infected with bacteria and influenza, whereas the median ferritin level was 2-fold higher in those infected with SARS-CoV-2 compared with the two other etiologies and high ferritin levels was also observed in some with only modestly elevated CRP levels. Hyperferritinemia are multifactual and cytokine level, cellular damage, and site of infection (intracellular or extracellular) may all play a role [23]. The two most common bacteria in the group with bacterial CAP were a mix of intracellular (*H. influenzae*) and extracellular (*S. pneumoniae*) pathogens. Viruses are obviously exclusively intracellular pathogens. Therefore, the higher level of ferritin in those infected with SARS-CoV-2 compared with bacterial CAP could partly be explained by a higher level of intracellular invasion. The level of interleukin-6 did not differ between CAP caused by SARS-CoV-2 or bacteria, though other cytokines could play a role. As reported previously from this population, interleukin-10 and interferon-γ were higher in those infected with SARS-CoV-2 compared with those with bacterial CAP [21]. The level of interleukin-6 was higher in those with SARS-CoV-2 compared with those with influenza. Interferon-γ, interleukin-4, and interleukin-5 were also higher in those with SARS-CoV-2 compared with those with influenza [21]. As COVID-19 has been associated with multiorgan involvement [24] further cellular damage may also contribute to higher ferritin levels in those with SARS-CoV-2 compared with CAP caused by bacterial infection and influenza as a result of secondary organ involvement. Oppen and colleagues also reported that CAP caused by bacteria had higher hepcidin levels compared with CAP caused by a viral infection [20]. In the present study, we found similar hepcidin levels in patients with CAP caused by influenza and bacteria, whereas median hepcidin was higher in patients with CAP caused by SARS-COV-2 compared with patients with CAP caused by influenza and bacteria. Hepcidin synthesis during infections result in depletion of extracellular iron which is a defense mechanism to withhold iron from invading pathogens. This may partly explain the difference in hepcidin between those with bacterial infection compared with those with COVID-19, since the entrapment of iron within cells may prevent growth of extracellular bacteria and simultaneously promote survival and growth of intracellular located pathogens. Conversely, it has also been suggested that ferroportin expression can be downregulated by invading pathogens independent of the level of hepcidin and iron [25]. It has also been suggested that the main purpose of hepcidin synthesis during infection is to avoid formation of nontransferrin-bound iron which can occur as a result of infection-related destruction of tissue and enterocytes. This group of iron species may be

Table 1. Demographic and laboratory characteristics for 164 patients admitted with community-acquired pneumonia (CAP)

	SARS-CoV-2 CAP (N = 40)	Bacterial CAP (N = 99)	Influenza CAP (N = 25)
Age, median (IQR), years	72 (59–77)	73 (61–83)	72 (65–79)
Sex, male, n (%)	24 (60)	52 (53)	11 (44)
Laboratory characteristics			
Hemoglobin, mmol/L	8.0 ± 0.9	7.4 ± 1.0*	7.7 ± 1.0
Iron, µmol/L	4.5 (4–10)	5.0 (4–9)	7.0 (4–11)
Ferritin, ng/mL	775 (426–1460)	322 (191–569)*	305 (230–384)*
Hepcidin, ng/mL	143.8 (100.7–180.7)	78.8 (40.1–125.4)*	53.5 (25.2–125.8)*
Transferrin, g/L	1.5 ± 0.3	1.7 ± 0.5	1.8 ± 0.4
Transferrin saturation, %	18.5 (9–22.5)	13 (9–21)	15.5 (10–22)
Reticulocytes, ($\times 10^9$/L)	27.5 (20.5–40.0)	50 (39.0–64.5)*	52 (29–71)*
Soluble transferrin receptor 2, mg/L	0.64 (0.5–0.8)	0.74 (0.6–1.1)	0.82 (0.6–1.2)
Erythroferrone, ng/mL	0.63 (0.3–2.0)	1.60 (0.6–3.1)*	1.16 (0.7–2.2)
Erythrocytes (MVC), fl	91.2 ± 6.7	93.0 ± 6.7	91.8 ± 5.0
Hemoglobin (MCHC), mmol/L	20.8 ± 0.7	20.4 ± 1.1	20.2 ± 1.0
Haptoglobin, g/L	3.4 ± 0.9	3.2 ± 1.1	3.3 ± 0.9
Lactate dehydrogenase, u/L	276 (229–354)	189 (164–219)*	245 (189–306)
Bilirubin, µmol/L	10 (8.5–12.0)	8.0 (6.0–13.0)*	6.5 (5.0–10.5)*
C-reactive protein, mg/L	76 (49–134)	126 (76–196)*	87.5 (44–128.5)
Interleukin-6, ng/L	10.7 (4.5–19.1)	6.4 (2.9–17.5)	3.6 (1.7–8.2)*
Creatinine, µmol/L	76.0 (62–89)	74 (58–86)	69.5 (56.5–87.5)
Hepcidin:ferritin, ng/mL/ng/mL	0.15 (0.1–0.3)	0.18 (0.1–0.3)	0.20 (0.1–0.4)
Ferritin:CRP, ng/mL/mg/L	10.20 (6.4–18.6)	2.71 (1.8–4.7)*	3.24 (2.5–9.8)*
Hepcidin:CRP, ng/mL/mg/L	1.36 (1.1–2.6)	0.56 (0.4–1.0)*	0.67 (0.3–1.5)*

Data are presented as mean ± SD, median (IQR), or % (n), *$p < 0.05$, different from SARS-CoV-2. For normally distributed variables, one-way ANOVA models were fitted, followed by pairwise comparisons and Bonferroni adjustment of p-values. For non-normally distributed variables, Kruskal–Wallis tests were applied. For pairwise comparisons, Nemelyi–Dunn tests were applied.

more easily to utilize by pathogens, and they may also produce toxic substances [26]. The host response to various invading pathogens is complex and evidence is still lacking on various mechanisms involved in iron metabolism during viral and bacterial infections. Many studies regarding the mechanisms involved in iron metabolism are based on *in vitro* or animal studies and the evidence on humans is still sparse. IL-6 level was higher in patients infected with SARS-CoV-2 compared with those infected with influenza and hepcidin levels were higher in those with SARS-CoV-2 compared with those infected with bacteria and influenza indicating that the classic pathway of anemia of inflammation was more prominent in COVID-19 patients. Furthermore, erythroferrone, known to ameliorate anemia of inflammation [9] was lower in patients with SARS-CoV-2 compared with CAP. Collectively, this could explain the large difference in reticulocyte counts between patients infected with SARS-CoV-2 and bacterial infection despite a modest though significant difference in hemoglobin. Patients infected with influenza virus had similarly raised erythroferrone, and reticulocyte counts as patients with bacterial infection for similar hemoglobin levels. Patients with CAP caused by bacterial infection had lower hemoglobin level than CAP caused by SARS-CoV-2 but higher hepcidin level. It has been reported that comorbidity is an independent predictor of bacterial pneumonia [27] and one may therefore speculate if underlying diseases may contribute to lower hemoglobin levels in those with bacterial infection. In addition, it is expected that persistent high concentrations of hepcidin would result in decreased iron availability for erythropoiesis and result in iron-restricted anemia in SARS-CoV-2 infections of longer duration [8]. The high level of bilirubin in those infected with SARS-CoV-2 was a surprising finding. COVID-19 affect multiple organs including the endothelium [28]. Endothelium dysfunction has been associated COVID-19 is related to development of atherosclerosis and cardiovascular diseases [29]. Bilirubin are up-regulated to improve endothelial function [29] which may explain the high level of bilirubin in patients with COVID-19.

A strength of the study includes the wide range of biomarkers of iron metabolism (*i.e.* erythroferrone, sTfR, hepcidin) – added to the more commonly used ferritin, hemoglobin *etc*. The cross-sectional nature of this study is a limitation, however, because it precludes inferences of causality

and we are not able to assess the dynamics from onset of infection to iron metabolic changes. Another limitation is the small number of severe cases indicated by few admissions to ICU (7%) and few non-survivors (5.5% with-in hospital and 30-days after discharge) as reported previously [21]. A higher number of severe cases would provide a more diverse population since more severe CAP might influence iron regulation differently across etiologies. The small number of patients with CAP caused by influenza is also a limitation. Though the amount, of patients admitted with CAP caused by influenza has been very limited during the COVID-19 pandemic.

CONCLUSION

We observed higher levels of hepcidin and lower levels of erythroferrone despite lower CRP levels among patients infected with SARS-CoV-2 compared with those infected with bacteria indicating alterations in iron metabolism in patients with SARS-CoV-2 infection. The role of SARS-Cov-2 on iron regulation should be investigated in future studies including the effect on disease severity and adverse outcomes.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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