Specific changes of erythroid regulators and hepcidin in patients infected by SARS-COV-2

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ABSTRACT

Iron metabolism is tightly linked to infectious and inflammatory signals through hepcidin synthesis. To date, iron homeostasis during SARS-CoV-2 infection has not yet been described. The aim of this study is to characterize the hepcidin and erythroid regulators (growth differentiation factor 15 (GDF-15) and erythroferrone (ERFE)) by measuring concentrations in plasma in context of COVID-19 disease.

We performed a single-center observational study of patients with COVID-19 to evaluate concentrations of main regulatory proteins involved in iron homeostasis, namely: hepcidin, ERFE and GDF-15. SARS-CoV-2 infection (COVID-19$^+$) was defined by a positive RT-PCR. Sixteen patients with COVID-19$^+$ were gender-matched and age-matched to 16 patients with a sepsis unrelated to SARS-CoV-2 (COVID-19$^-$) and were compared with non-parametric statistic test.

Clinical and hematological parameters, plasma iron, transferrin, transferrin saturation, ferritin, soluble transferrin receptor and C reactive protein were not statistically different between both groups. Median plasma hepcidin concentrations were higher in the COVID-19$^+$ group (44.1 (IQR 16.55–70.48) vs 14.2 (IQR 5.95–18.98) nmol/L, p=0.003), while median ERFE and GDF-15 concentrations were lower in the COVID-19$^+$ group (0.16 (IQR 0.01–0.73) vs 0.89 (IQR 0.19–3.82) ng/mL, p=0.035; 2003 (IQR 1355–2447) vs 4713 (IQR 2082–7774) pg/mL, p=0015), respectively) compared with the COVID-19$^-$ group.

This is the first study reporting lower ERFE and GDF-15 median concentrations in patients with COVID-19$^+$ compared with patients with COVID-19$^-$, associated with an increased median concentration of hepcidin in the COVID-19$^+$ group compared with COVID19$^-$ group.

Significance of this study

What is already known about this subject?

- ► Hepcidin and ferritin synthesis are increased during exacerbated inflammatory state.
- ► Disturbances of iron metabolism has been associated with the severity of COVID-19 disease.

What are the new findings?

- ► Erythroferrone and growth differentiation factor 15 concentrations are decreased during COVID-19 disease, while hepcidin concentrations are increased.

How might these results change the focus of research or clinical practice?

- ► These results support the investigation of both hepcidin and pro-inflammatory cytokines like interleukin-6 in the context of COVID-19 disease.
- ► This work suggests the interest of exploring iron metabolism and inflammation status in patients with COVID-19$^+$ in a prospective longitudinal study to assess their putative role in long-term outcome and possibly improve patient management.

INTRODUCTION

The SARS-CoV-2 virus responsible for COVID-19 is mainly associated with mild respiratory tract symptoms.[1] However, 0.25%–3% patients develop an acute respiratory distress syndrome and multiorgan failure.[2] The mechanisms of clinical severity have not been fully determined yet but, high concentrations of cytokines have been largely reported in COVID-19 and may be associated with tissue injury.[3]

Interestingly, a high serum ferritin concentration has been described as a feature that predicted with specificity and sensitivity the increased mortality risk.[4] During exacerbated inflammatory state, cytokines, in particular interleukin-6 (IL-6), increase ferritin and hepcidin synthesis.[5 6] High hepcidin during systemic inflammation, by reducing serum iron concentrations, leads to anemia.[7] Previously, Zhao *et al* found the severity and mortality of the disease was closely correlated with serum iron levels.[8]

Systemic iron homeostasis is orchestrated by the hepcidin-ferroportin axis, that is regulated by (i) inflammation through IL-6; (ii) iron storage via the circulating and tissue iron or (iii) erythroid regulators.[9–12] Here, we focused on growth and differentiation factor 15 (GDF-15) and erythroferrone (ERFE), two erythroid regulators as putative key actors of the mechanism of hepcidin deregulation. ERFE, a member of tumors necrosis factor-α proteins and GDF-15, a member of transforming growth factor-β superfamily, have been reported to repress hepcidin expression, both acting through the bone morphogenetic protein (BMP)-Smad pathway.[7 13] Thereafter, iron regulator proteins need to be evaluated in patients with COVID-19 to improve the understanding of these mechanisms and to suggest new therapeutic perspectives.

The aim of this study is to characterize the hepcidin and erythroid regulators (GDF-15 and ERFE) by measuring concentrations in plasma in the context of COVID-19 disease.

MATERIALS AND METHODS

Patients

As previously described,[14] patients hospitalized in the Tours University Hospital (Tours, France) for suspected COVID-19 from April 8 to April 20, 2020, who had a biochemical examination, including parameters of iron metabolism, and hematological exploration <7 days from COVID-19 diagnosis, were included. Suspicion of SARS-CoV-2 infection was based on clinical criteria including diarrhea, dyspnea, cough and fever. In this pilot study, 100 patients were included, of which 45 were COVID-19^{-} and 55 were COVID-19^{+} based on SARS-CoV-2. Consequently, out of the 55 patients with COVID-19^{+} included in this pilot study, 16 were randomly selected. Sixteen out of 45 patients with COVID-19^{-} were randomly preselected and are a part of the same cohort from a previous work.[14] On each preselection, age and sex matching was evaluated and the first selection of patients that respected age and sex matching was definitively approved. For this pilot study, no patient was excluded from either group. We used this biobank, from centrifuged samples preserved at –80°C, for iron metabolism exploration on the 32 patients. This exploration included iron, transferrin (allowing calculation of transferrin saturation), soluble transferrin receptor, hepcidin and some of its regulators, ERFE and GDF-15. Blood samples remaining after routine biological explorations were kept at −80°C after non-opposition of patients. This biobank containing samples of patients suspected from COVID-19 (including negative and positive patients with COVID-19) was available for further biological explorations, to note, the time of blood collection (in relation to the time of COVID-19 diagnosis) was also controlled by a standardization via limited delay between the symptoms and the date of PCR test.

SARS-CoV-2 RT-PCR

As previously described,[14] reverse transcriptase-PCR (RT-PCR) was performed on nasopharyngeal swabs in transport medium (UTM or Eswab) or bronchoalveolar lavage fluid. Samples were stored at +4°C before analysis. SARS-CoV-2 RNA was amplified by real-time RT-PCR targeting RdRp, E and/or N genes, using Allplex 2019-nCOV (Seegene), Abbott RealTime SARS-CoV-2 (Abbott) or Bosphore 2019-nCoV (Anatolia GeneWorks) assays. The negative RT-PCR patients were allocated to COVID-19^{-} group and patients with positive RT-PCR were allocated to COVID-19^{+} group. Demographic data such as gender, age and clinical parameters such as body mass index (kg/m^2), presence of high blood pressure, cardiovascular risk factors as well as treatments were collected.

Hematological exploration

Blood samples were drawn on EDTA K3 tubes (BectonDickinson) for hematological exploration (hemoglobin (Hb), mean corpuscular volume (MCV), platelet count (PC), white blood cells (WBC) and lymphocytes (Ly, relative number)) realized on a DxH analyzer (Beckman Coulter).

Iron metabolism exploration

Another blood sample was collected on lithium heparinate Barricor tubes (BectonDickinson) and centrifugated 3 min at 4000 g to measure the following parameters on Roche Cobas c501 and e601 analyzers : iron, transferrin, sTfR, ferritin and C reactive protein (CRP). To complete the exploration of iron metabolism, hepcidin and two of its regulators, ERFE and GDF-15, were assessed through ELISA methods (hepcidin-25 Enzyme Immunoassay kit (S-1337; Peninsula) for hepcidin; ERFE IE ELISA kit, ERF-001, Intrinsic LifeSciences for ERFE; Quantikine ELISA kit, DGD150, R&D Systems for GDF-15).

In both groups, the respiratory status on admission based on WHO ordinal scale for clinical improvement (0: uninfected, 1: no limitation of activities, 2: limitation of activities, 3: hospitalized, no oxygen therapy, 4: oxygen by nasal mask of prongs, 5: non-invasive ventilation of high-flow oxygen, 6: intubation and mechanical ventilation, 7: ventilation+additional organ support, 8: death) was used, ≤3 for moderate, ≥6 for critical.

Statistical analysis

Demographical, clinical and biological data were compared between both groups by a Wilcoxon test for continuous variables and a χ^2 test for categorial variables. Correlation between biological parameters was evaluated by Spearman's coefficient. We favored non-parametric tests in view of the small size of each group. Statistical analysis was performed using XLSTAT on Excel (Addinsoft (2020) Paris, France, https://www.xlstat.com). Level of statistical significance was $p<0.05$.

RESULTS

Patients

Thirty-two patients were randomly included in this study (16 COVID-19^{+} and 16 COVID-19^{-}). On admission, in the COVID-19^{+} group, seven (43.8%) patients had moderate (SpO2 ≥94% on room air), five (31.2%) had severe (oxygen therapy) and four had (25%) critical (mechanically ventilated) illness. In the COVID-19^{-} group, 10 (62.5%) patients had SpO2 ≥94% on room air, 3 (18,75%) had oxygen therapy, 3 (18.75%) had mechanical ventilation and 9 patients had a documented bacterial infection. Matching criteria were not significantly different between the two groups. The time between plasma sampling and SARS-CoV-2 RT-PCR was comparable in the two groups (2.5±1.3 vs 3.5±2.5 days in COVID-19^{-} and COVID-19^{+},

Table 1 Demographical and biological characteristics of patients with SARS-CoV-2⁻ and SARS-CoV-2⁺

	Patients with COVID-19⁻ Median (IQR: Q1–Q3)	Patients with COVID-19⁺ Median (IQR: Q1–Q3)	P value
Age (years)	80 (59.6–87.6)	76.8 (56–86.3)	0.64
Gender (%male)	50	50	1
BMI (kg/m²)	28.5 (24.9–34.1)	24.5 (22.4–26.2)	0.067
Oxygen (RA/OT/MV)	10/3/3	7/5/4	0.55
Hemoglobin (g/L)	116 (100.5–123.5)	116.5 (102–129.3)	0.816
RBCs (T/L)	3.9 (3.2–4.2)	3.9 (3.3–4.5)	0.59
MCV (fL)	92.3 (89.7–96.6)	90.2 (88.4–93.5)	0.112
PC (10^9/L)	245.5 (120–321.8)	205 (165.3–286.8)	0.926
WBC (10^9/L)	9.7 (6.5–11.6)	6.3 (4.4–8.8)	0.088
Lymphocyte (10^9/L)	1.1 (0.7–1.8)	1 (0.8–1.5)	0.759
Iron (µmol/L)	9.5 (7.5–11)	6.5 (5–13.5)	0.191
Transferrin (g/L)	1.9 (1.1–2.3)	1.6 (1.2–2)	0.446
Transferrin saturation (%)	23.6 (16–31.5)	21.4 (14.2–27.1)	0.545
Ferritin (µg/L)	450 (237–761)	600 (338–1495)	0.346
sTfR (mg/L)	3.5 (2.9–4.7)	3.2 (2.6–4.8)	0.574
CRP (mg/L)	24.3 (5.1–75.2)	38.8 (4.7–149)	0.417
Hepcidin (nmol/L)	14.2 (5.95–18.98)	44.1 (16.55–70.48)	0.003
ERFE (ng/mL)	0.89 (0.19–3.82)	0.16 (0.01–0.73)	0.035
GDF-15 (pg/mL)	4713 (2082–7774)	2003 (1355–2447)	0.015

BMI, body mass index; CRP, C reactive protein; ERFE, erythroferrone; GDF-15, growth differentiation factors 15; MCV, mean corpuscular volume; MV, mechanical ventilation; OT, oxygen therapy; PC, platelet count; RA, room air; RBC, red blood count; sTfR, soluble transferrin receptor; WBC, white blood cell.

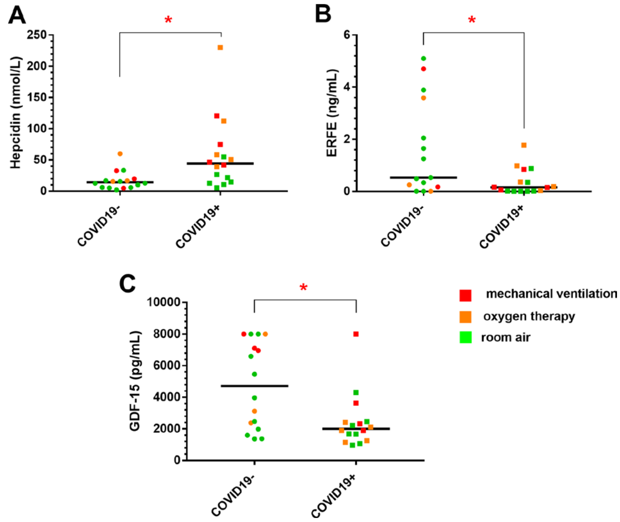

Figure 1 Dot plot between COVID-19-infected (COVID-19⁺) or COVID-19 no-infected (COVID-19⁻) patients. (A) Higher hepcidin level in COVID-19⁺ group (44.1 (IQR 16.55–70.48) vs 14.2 (IQR 5.95–18.98) nmol/L, p=0.003). (B) Lower erythroferrone (ERFE) level in COVID-19⁺ group (0.16 (IQR 0.01–0.73) vs 0.89 (IQR 0.19–3.82) ng/mL, p=0.035). (C) Lower growth differentiation factor 15 (GDF-15) in COVID-19⁺ group (2003 (IQR 1355–2447) vs 4713 (IQR 2082–7774) pg/mL, p=0015). Data value: median (IQR Q1–Q3). *p <0.05

respectively, p=0.276). Clinical parameters were comparable between groups, respiratory function was heterogeneous within each group but not different between both groups (p=0.547) (table 1). Hematological parameters including Hb, MCV, PC, WBC, Ly were not significantly different between both groups, despite a tendency for MCV and WBC to be lower in patients with COVID-19⁺ (table 1).

Disturbance of iron homeostasis

The following parameters were modified without reaching significance: CRP and ferritin were higher in patients with COVID-19⁺ but iron and transferrin were lower (table 1). Median plasma hepcidin concentrations were significantly higher in the COVID-19⁺ group (44.1 (IQR 16.55–70.48) vs 14.2 (IQR 5.95–18.98) nmol/L, p=0.003), while median ERFE and GDF-15 concentrations were significantly lower in the COVID-19⁺ group (0.16 (IQR 0.01–0.73) vs 0.89 (IQR 0.19–3.82) ng/mL, p=0.035; 2003 (IQR 1355–2447) vs 4713 (IQR 2082–7774) pg/mL, p=0015, respectively) compared with the COVID-19⁻ group (figure 1).

In both groups, hepcidin and CRP concentrations were significantly positively correlated (COVID-19⁺: r=0.81, p=0.003; COVID-19⁻: r=0.51, p=0.027). In the COVID-19⁺ group, hepcidin presented a significant correlation with ERFE and ferritin (r=0.32, p=0.025; r=0.478, p=0004, respectively) that was not found in COVID-19⁻ group.

Interestingly, in the COVID-19⁺ group, patients with oxygen therapy or mechanical ventilation (n=9) presented higher hepcidin concentrations versus room air patients (n=7) (58.1 (IQR 44.1–116.4) vs 18.2 (IQR 12.9–39.5) nmol/L, p=0.002), and not in the COVID-19⁻ group.

One result from patients with COVID-19⁺ emerge with a very high value for hepcidin, we did not find any pre-analytical or analytical problem that could explain this result. If this result is considered at outlier and removed, even after correction for multiple tests, the significant difference in hepcidin measurement persists and does not bias our results.

DISCUSSION

Our findings revealed that compared with non-COVID-19-infected patients, COVID-19-infected patients presented higher median hepcidin concentrations and lower median ERFE and GDF-15 concentrations. To our knowledge, this preliminary study is the first to explore hepcidin and these two erythroid iron regulatory proteins during the first days of COVID-19 infection compared with matched controls with inflammation.

The increase of hepcidin concentrations in COVID-19 infection

Comparable levels of CRP and iron parameters in the two groups indicated the presence of an inflammatory syndrome. As previously described, we observed a significant correlation between CRP and hepcidin concentrations whatever the SARS-CoV-2 infection status (COVID-19⁺ or COVID-19⁻).[15] The novelty of our findings is that the increase in hepcidin concentrations in patients with COVID-19⁺ was reported in comparison to matched controls with inflammation and not to healthy controls as previously reported.[16] As CRP was not significantly different between COVID-19⁻ and COVID-19⁺, we are convinced that CRP is not a confusion bias in this cohort of 32 subjects. Even if hepcidin is correlated with CRP, we can assume that the difference in hepcidin concentrations between COVID-19$^-$ and COVID-19$^+$ is independent from CRP values. Thus, in the context of COVID-19, the higher concentrations of hepcidin suggest the involvement of other independent factors that should be further explored such as IL-6 with formal investigation in a larger cohort.

Relation between hepcidin deregulation and severity of COVID-19 infection

We observed a higher hepcidin concentration in severe/critical patients versus moderate illness thus confirming that hepcidin is associated with morbidity and outcome in COVID-19 disease.[17] The absence of significance for some features (ferritin, iron, transferrin, CRP, MCV and WBCs) is likely due to a lack of statistical power. However, these parameters, routinely available, are now well characterized in the context of SARS-COV disease. Although the COVID-19$^+$ and COVID-19$^-$ groups were similar for age, sex and disease severity, the high heterogeneity within each group suggests a modification of the methodology for future studies. For example, a stratification of patients based on disease severity in a larger cohort may be informative.

Our findings are consistent with recent reports evaluating the parameters of infection severity. For example, Shah *et al* showed that patients with COVID-19$^+$ with severe hypoxemia recruited at the time of admission in the intensive care unit (ICU) had significantly lower concentrations of serum iron (median 2.3 (IQR 2.2–2.5) vs 4.3 (IQR 3.3–5.2) µmol/L, $p<0.001$) than patients with non-severe hypoxemia.[18] They also reported that hypoferremia was more severe than in previously reported cohorts of non-COVID-19 ICU patients, including those with sepsis.[19,20] These data indicated that hypoferremia may be a specific feature of severe COVID-19 disease.

The pathophysiological mechanisms explaining the link between hepcidin deregulation and infection severity may involve inflammatory actors and oxidative stress associated with intracellular iron overload. The increased levels of pro-inflammatory cytokines, in particular IL-6, IL-1α and IL-1β during inflammation are associated with hepcidin overexpression and ferroportin downregulation. Consequently, iron export from cells is impeded, thus resulting in intracellular iron overload.[21] The toxicity of iron overload is mainly based on Fenton reactions involved in ferroptosis.[22] This mechanism could induce an immune response after release of damage-associated molecular patterns and alarmins, which is associated with increased cell death.[23]

First exploration of ERFE, GDF-15 with hepcidin in COVID-19 infection

Systemic iron homeostasis is orchestrated by the hepcidin-ferroportin axis, which is regulated by (i) inflammation through IL-6; (ii) iron storage via the circulating and tissue iron or (iii) erythroid regulators.[9,24] Here, we focused on two erythroid regulators as putative key actors of the mechanism of hepcidin deregulation. Although the role of GDF-15 in hepcidin regulation is still debated, its investigation in this context might contribute to a better knowledge of the mechanism. Indeed, few groups have focused their exploration on GDF-15 and ERFE in a viral infection context.[25–27] A previous study demonstrated that increasing of hepcidin secretion after hepatitis C virus eradication was linked to a decrease of ERFE.[27] Another group reported that low levels of HIV-1 viremia were associated with significant higher levels of GDF-15 compared with patients with virus eradication.[26] In the COVID-19 context, rare studies showed a relation between GDF-15 and prognosis in COVID-19 infection through an association between GDF-15 concentrations and SARS-CoV-2 viremia, hypoxemia and worse outcome.[25] To our knowledge, there is no study exploring ERFE in SARS-CoV-2 infection.

Our findings revealed that the median concentrations of two erythroid regulators were lower in patients with COVID-19$^+$ compared with the COVID-19$^-$ group. It should be noted that the GDF-15 and ERFE values of our two groups are different from those obtained in a healthy population.[28] Moreover, hepcidin concentrations were positively correlated with ERFE and ferritin in patients with COVID-19$^+$. This positive correlation was unexpected: ERFE, a member of tumor necrosis factor-α proteins and GDF-15, a member of transforming growth factor-β superfamily, have been reported to repress hepcidin expression, both acting through the BMP-SMAD pathway.[7,13] Our observation due to the kinetic of hepcidin regulation may be potentially different between both groups: negative correlation in patients with COVID-19$^-$ (not significant) and positive in patients with COVID-19$^+$. It might involve the evolution of ERFE, starting to rise again in response to the elevation of hepcidin in patients with COVID-19$^+$. The lower GDF-15 concentrations in patients with COVID-19$^+$ are interesting on another aspect. GDF-15 could inhibit the recruitment of infiltrating pro-inflammatory cells by interfering with chemokine signaling and β2-integrin/lymphocytes function-associated antigen activation,[29] and temper inflammation-induced damage.[30]

CONCLUSION

This study reports for the first time lower ERFE and GDF-15 median concentrations in patients with COVID-19$^+$ compared with patients with COVID-19$^-$. Even if inflammation and increased concentrations of hepcidin may be observed in many other viral diseases, this increase is a major observation in the COVID-19$^+$ group and is associated with a decrease in regulators of hepcidin metabolism.

This preliminary study merits to be followed by a prospective longitudinal study of iron metabolism and inflammation status in patients with COVID-19$^+$ to evaluate the evolution of these early disturbances and their putative role on long-term patient outcome.

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Patient consent for publication Not applicable.

Ethics approval All patients included in this study were informed in writing regarding the collection of their samples remaining from routine biological analyses for research aims and were given the right to refuse such uses. In addition, all patients were informed about the data obtained and about their right to access these data, according to articles L.1121-1 and R1121-2 of the French Public Health Code. All experimental protocols were approved by the University Hospital of Tours ('cellule de recherches non interventionnelles'). Participants gave informed consent to participate in the study before taking part.

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