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COVID-19 compromises iron homeostasis: Transferrin as a target of investigation

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ABSTRACT

Importance: Since the beginning of the COVID-19 pandemic, numerous metabolic alterations have been observed in individuals with this disease. It is known that SARS-CoV-2 can mimic the action of hepcidin, altering intracellular iron metabolism, but gaps remain in the understanding of possible outcomes in other pathways involved in the iron cycle.
Objective: To profile iron, ferritin and hepcidin levels and transferrin receptor gene expression in patients diagnosed with COVID-19 between June 2020 and September 2020.
Design, setting and participants: Cross-sectional study that evaluated iron metabolism markers in 427 participants, 218 with COVID-19 and 209 without the disease.
Exposures: The primary exposure was positive diagnose to COVID-19 in general population of Santo André and São Bernardo cities. The positive and negative diagnose were determinate through RT-qPCR.
Main outcomes and measures: Devido a evidências de alterações do ciclo do ferro em pacientes diagnosticados com COVID-19 e devido a corregulação entre hepcidina e receptor de transferrina, uma análise da expressão gênica deste último, poderia trazer insights sobre o estado de ferro celular. A hipótese foi confirmada, mostrando aumento da expressão de receptor de transferrina concomitante com redução do nível de hepcidina circulante.
Results: Serum iron presented lower values in individuals diagnosed with COVID-19, whereas serum ferritin presented much higher values in infected patients. Elderly subjects had lower serum iron levels and higher ferritin levels, and men with COVID-19 had higher ferritin values than women. Serum hepcidin was lower in the COVID-19 patient group and transferrin receptor gene expression was higher in the infected patient group compared to controls.
Conclusions and relevance: COVID-19 causes changes in several iron cycle pathways, with iron and ferritin levels being markers that reflect the state and evolution of infection, as well as the prognosis of the disease. The increased expression of the transferrin receptor gene suggests increased iron internalization and the mimicry of hepcidin action by SARS-CoV-2, reduces iron export via ferroportin, which would explain the low circulating levels of iron by intracellular trapping.

1. Introduction

In 2019, the first cases were reported of infection with the new coronavirus, called COVID-19 by the WHO on February 11, 2020 [1]. Symptoms of COVID-19 infection appear after an average incubation period of 5.2 days [2]. The most common initial symptoms are: fever, cough and myalgia or fatigue; the less common being sputum production, headache, hemoptysis and diarrhea [3]. The elderly and those with pre-existing comorbidities such as diabetes, high blood pressure, heart or lung disease seem to develop the severe type of COVID-19 more often compared to others [4]. Furthermore, due to the multiple interactive levels of viral attack, a set of several biochemical pathway imbalances

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such as iron dysmetabolism occurs in COVID-19. This is supposedly because SARS-CoV-2 mimics the action of hepcidin [5], which interacts with ferroportin, causing the internalization and degradation of this complex, favoring iron entry into the cell and decreasing its export from the interior of cells [6], hyperferritinemia and ferroptosis develop [5].

The worldwide mortality rate is indicative that the greatest cause of death from the disease is due to respiratory failure. However, other evidences link accelerated infection in critically ill COVID-19 patients to a hyperinflammatory state involving a cytokine storm. One component for increased inflammation is hyperferritinemia, which plays a crucial role in the pathogenesis and disease management strategies [7]. Therefore, the importance of iron metabolism dysregulation must be considered, which also promotes oxygen-starved blood disease. Systemic inflammation, normally associated with increased serum ferritin levels, releases cytokines, especially IL-6, and stimulates the synthesis of ferritin and hepcidin. Hepcidin, in turn, favors the intracellular increase of iron in cells by negatively regulating the transporter ferroportin, which is the main exporter of iron [5]. There are reports in the literature of altered serum ferritin levels in patients who did not survive COVID-19, levels that exceeded by up to two times those found in survivors, thus characterizing a state of hyperferritinemia in these patients who contracted the infection [7].

In addition to increased ferritin, studies point to the ability of SARS-CoV-2 to mimic the action of hepcidin, increasing circulating and tissue ferritin. This induces serum iron deficiency and lowered hemoglobin [5]. Excess iron interacts with molecular oxygen generating reactive oxygen species, which contributes to oxidative damage in different organs, such as the lungs, liver, kidneys and heart, which can lead to ferroptosis [7]. Regarding the extracellular environment, the combination of hemoglobinopathy and iron dysmetabolism impairs the ability of erythrocytes to transport O_2, resulting in hypoxia. Physiologically, anemic hypoxia induces pulmonary vasoconstriction and increased fibrin formation within this microvasculature. Thus, the dysregulated interaction of hepcidin and ferroportin can lead to pulmonary artery hypertension through smooth muscle proliferation [5], which perhaps helps elucidate death from respiratory failure in these COVID-19 patients.

Hepcidin is also upregulated by the transferrin/transferrin receptor complex. Transferrin is responsible for plasma iron transport and cellular iron internalization through interaction with TFRC [8]. In a state of iron deficiency, transferrin is upregulated, but during inflammation it is downregulated in order to decrease the availability of iron to pathogens. Additionally, in vitro studies have shown that TFRC may be an alternative receptor for SARS-CoV-2 [9].

Considering this information, this study evaluated the serum profile of iron, ferritin, hepcidin and TFRC gene expression in patients diagnosed with COVID-19 and treated in the public health system of São Bernardo do Campo during the second wave of COVID-19 in Brazil. The objective was to profile iron metabolism in this condition.

2. Material and methods

2.1. Design and eligibility criteria

This is a cross-sectional study in which biochemical variables from 218 patients diagnosed with COVID-19 (COVID-19) were evaluated and compared with 209 individuals without COVID-19 (CTL).

Inclusion criteria for the COVID-19 group: Patients diagnosed with COVID-19 and admitted to hospital wards in the municipality of São Bernardo do Campo and Santo André during the period from June 2020 to September 2020. Inclusion criteria for the group without CTL: patients admitted to the same hospitals for elective surgeries. Exclusion criteria: participants who did not meet the aforementioned inclusion criteria. The tests performed for the confirmation of SARS-CoV-2 infection (RT-qPCR) followed the precepts of the Epidemiological Surveillance Guide of the Brazilian Ministry of Health.

All samples were obtained before the start of the National Plan for the Operationalization of Vaccination against COVID-19 (PNO) of the Ministry of Health and precedes the start of Vaccination against COVID-19 in Brazil.

The participants' medical reports were collected through the 'Matrix FMABC' platform, in which it is possible to survey all the exams carried out in the Clinical Analysis Laboratory that provides services for the hospitals in the studied municipalities.

2.2. Sample collection

Samples were collected in hospitals in the municipalities of São Bernardo do Campo and Santo André(municipal hospitals, field hospitals or basic health units (UBS) or in the collection centers of Centro Universitário FMABC.

2.3. Ethical aspects

This study was carried out in accordance with Resolution No. 466 of 12/12/2012, from the National Health Council –CNS, which regulates research with human subjects. The project was approved by the Research Ethics Committee – CEP of FMABC. Approval number: 4.427.013. All participants received and signed the Free and Informed Consent Term, acknowledging and authorizing their participation in this study. The present study was carried out in accordance with the relevant guidelines and regulations/ethical principles of the Declaration of Helsinki.

2.4. Serum iron quantification

To determine the concentration of circulating iron the colorimetric method was performed, in which the addition of an acid precipitates the carrier protein and dissociates the iron. The released iron was then quantified with the addition of a chromogen, resulting in a color reaction. Serum iron quantification was performed in the Clinical Analysis Laboratory using the Ferrozine colorimetric method (Advia Chemistry Iron Ferrozine II Gen, Siemens Healthcare Diagnostics Inc. Tarrytown, NY, USA). The reference values used were: 30 – 150 μg/dL.

2.5. Serum ferritin quantification

Quantitation of serum ferritin (ng/mL) was determined using the sandwich chemiluminescence method (kit Ref 33020 supplied by Beckman Coulter, Inc). Transferrin saturation was calculated as 100 × the serum concentration of iron/TIBC.

2.6. Serum quantification of hepcidin

Quantification of hepcidin was performed using the ELISA method from aliquots of serum and plasma samples obtained from peripheral blood, in collaboration with Dr. Mark Westerman and Dr. Gordana Olbina, from Intrinsic Life Sciences, La Jolla, California, USA, according to the protocol published by Ganz et al. in 2008.

2.7. TFRC expression

RNA extraction from whole blood: Total RNA was isolated using the TRIzol method (TRIzol LS Reagent, Thermo Fisher cat. no. 10296–010), according to the manufacturer's protocol. For total RNA extraction, blood samples were initially centrifuged at 4°C at 2500 rpm for 10 min to obtain the sediment. The supernatant was discarded and 1 mL of TRIzol was added to the cell pellet. The extraction process followed the standard protocol for TRIzol. Total RNA concentration was estimated through spectrophotometric reading using NanoDrop equipment (Thermo Fisher Scientific). cDNA synthesis: samples of total RNA (initial 1 μg) obtained from peripheral blood were converted into cDNA using

the SSIII First Strand qPCR Supermix (Invitrogen, cat no. 11752050), according to the manufacturer's protocol. TFRC expression was evaluated by RT-qPCR, according to the kit manufacturer's protocol. The specific primers for each selected gene were designed using Primer3 Input 0.4.0 software, available at <http://frodo.wi.mit.edu/primer3/> . The designed primer sequences were checked for specificity using the Primer-BLAST program, available at <http://www.ncbi.nlm.nih.gov/tools/primer-blast> .

To normalize the relative expression of the target gene, the expression value of the reference gene RPL13A was used. Initial standardization of real-time PCR amplifications took place on an Applied Biosystems 7500 qPCR Systems thermal cycler (Applied Biosystems, Foster City, CA, USA) in a final volume of 15 μL containing: 1X SYBR Green mix (Quantitec SYBR Green PCR kit, QIAGEN cat no. 204054), 10 pmol of each specific primer and 2 μL cDNA (initially diluted 10X). Starting cyclic parameters were an initial 14 hot start steps at 95 °C for 10 min, followed by 40 repetitions at 95 °C for 15 s and at 60 °C for 25 s. Forward TFRC primer sequence: gacaatgctgctttccctttc, Reverse: gttggctgttgtacctctcata, 241Kb amplicon. The ribosomal *RPL13A* gene was used as normalizing gene: Forward: gtggtcgtacgctgtgaag, Reverse: acagtgcgccagaaaatgc, 152 kb amplicon. Data were expressed as $2^{-\hat{}\Delta Cq}$.

2.8. Statistical Analyses

Sample size were estimated considering the heterogenous population of Santo André and São Bernardo cities, with 95% CI. Gene expression results were expressed as $2^{-\Delta ct}$ and biochemical values were expressed as mean ± standard deviation (SD). Differences between the studied groups were evaluated using Student's t-test (for parametric data) and the Mann-Whitney test (for non-parametric data). The analyses were performed using the GraphPad Prism computer program (GraphPad, version 7.0, USA). The level of significance was established at 5% (descriptive value of $p < 0.05$).

3. Results

A total of 427 participants were evaluated, with 209 participants in the CTL group (49%) and 218 in the COVID-19 group (51%). In the CTL group, 62% of participants were female and 38% male. In the group of participants with COVID-19. 5% of patients were male and 44% were female. The mean age of the CTL group was 43 ± 16 years and that of the COVID-19 group was 53 ± 19 years.

When evaluating serum iron quantification, participants with COVID-19 were found to have statistically lower levels of iron when compared to CTL subjects (CTL: 28.4 ± 14.51 mcg/dL, COVID-19: 20.10 ± 12.96 mcg/dL, $p < 0.0001$, CI=95%) (Fig. 1A). Quantification of ferritin revealed that COVID-19 patients have 5 times the level of this protein in their blood compared with CTL participants (CTL: 163.6 ± 204.3 ng/dL, COVID-19: 883.8 ± 635.6 ng/dL, $p < 0.0001$ CI=95%) (Fig. 1B).

A relation was also found between sex of the individuals with COVID-19 and serum ferritin values. Men with COVID-19 were observed to have higher ferritin values than women (Men 1168 ± 618 ng/mL, Women 544 ± 469 ng/mL, *p = 0.001, CI=95%).

When assessing whether there was a relation between age and iron and ferritin levels, older individuals were found to have the lowest serum iron levels, characterizing a negative Pearson's coefficient between these parameters ($r = -0.2768$, *p = 0.006, CI=95%) (Fig. 2A). There was a positive Pearson's coefficient between age and ferritin quantification ($r = 0.2035$, *p = 0.04, CI=95%) (Fig. 2B).

When evaluating quantification data of hepcidin in serum samples from patients with a positive diagnosis for COVID-19 infection a reduction of this hormone was found in relation to CTL patients (1142 ± 1187 ng/mL vs. 2021 ± 1993 ng/mL, *p < 0.05, CI=95%) (Fig. 3). Of note, patients without COVID-19 infection were people who had been hospitalized with other infections or chronic diseases.

The study of TFRC gene expression in blood found that individuals infected with SARS-CoV-2 present higher expression of this gene in relation to uninfected individuals ($0.1381 \pm 0.6591^{2^{-\Delta Cq}}$, n = 90 vs $0.0738 \pm 0.2355^{\ 2^{-\Delta Cq}}$, n = 90, *p < 0.05, CI=95%), (Fig. 4).

4. Discussion

This study demonstrated that acute infection mediated by SARS-CoV-2 triggers an imbalance in iron homeostasis, with reduced serum iron and hepcidin concentrations, and increased ferritin and *TRFC* expression. The reduction in iron concentration observed in this study

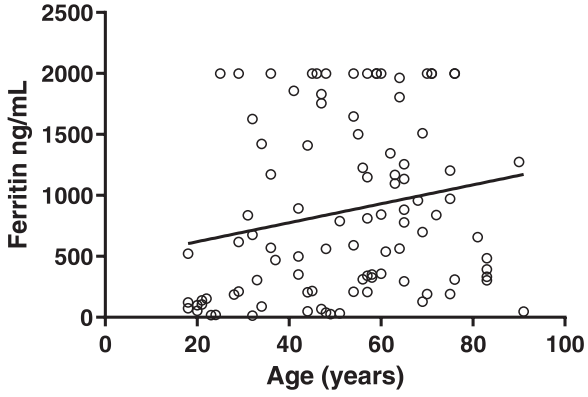

Fig. 2. Relation between age and iron and ferritin levels. Graphic representation of Pearson's coefficient test between age of the individuals with COVID-19 and quantifications of Iron (A) and Ferritin (B). Pearson's coefficient test.

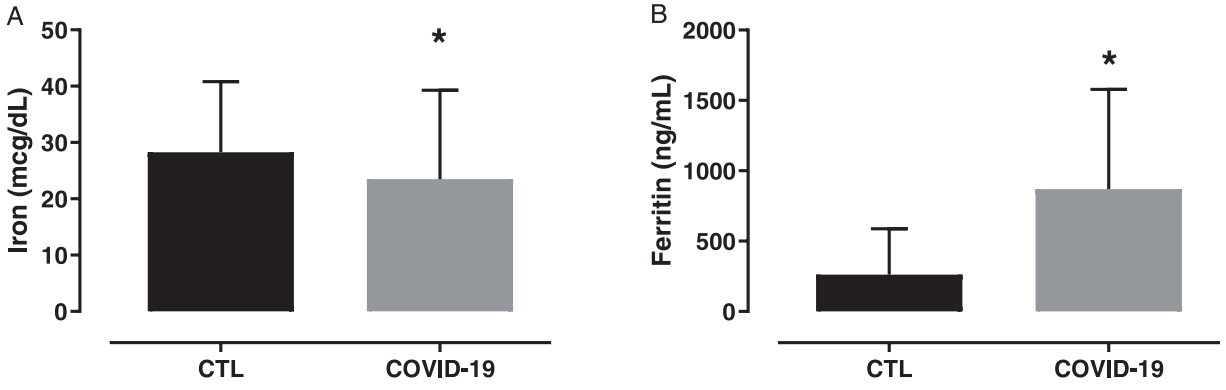

Fig. 1. Serum iron and ferritin quantification. Graphic representation of blood iron (A) and ferritin (B) quantification data from control patients and patients with a confirmed diagnosis of COVID-19. Mann-Whitney test versus CTL group.

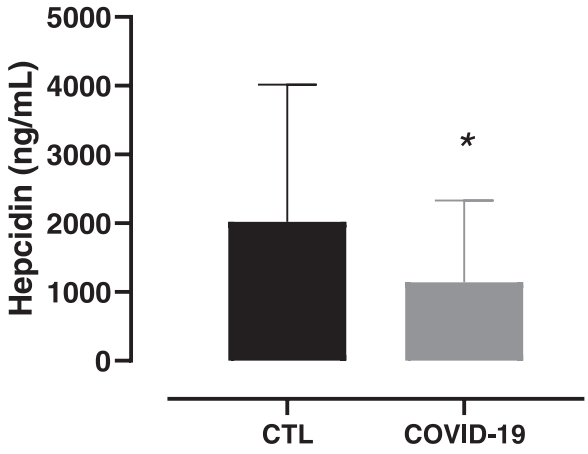

Fig. 3. Quantification of hepcidin. Hepcidin quantification values in serum samples from control patients and patients with a confirmed diagnosis of COVID-19. Mann-Whitney test versus CTL group.

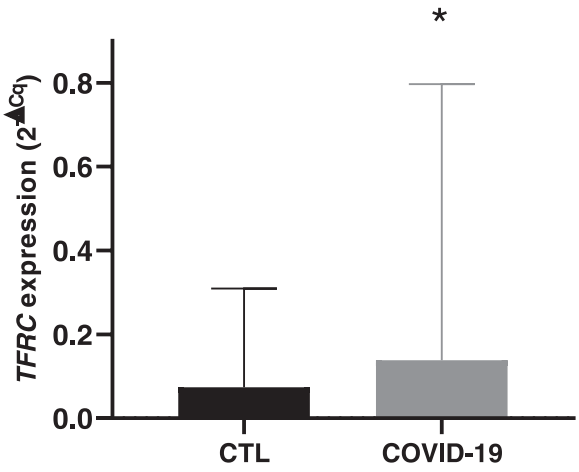

Fig. 4. TFRC gene expression. Graphic representation of the evaluation of transferrin receptor gene expression TFRC in the blood of control patients and patients with a confirmed diagnosis of COVID-19. Mann-Whitney test versus CTL group.

was found by German researchers, who showed iron deficiency in 80% of patients with COVID-19 at the time of admission to hospital, and this low serum level was directly associated with systemic inflammation and a prolonged hospital stay [10]. The infectious process per se favors the reduction of iron levels due to an adaptive mechanism of the innate immune response, one that limits the bioavailability of iron in the presence of pathogens; a response termed 'inflammation hypoferremia' [11,12].

As was the case with reduced serum iron levels, hyperferritinemia was present in many cases of critically ill patients hospitalized with COVID-19, this being a very common finding in this disease, and which indicates a worse prognosis for the patient, therefore being a reason for ferritin being used as a marker for COVID-19 severity [13]. Associated with this, serum ferritin values are related to admission to intensive care units and increased mortality rates (concentrations greater than 500 ng/mL predict mortality by up to 58%) [14]. Zhou et al. suggest that patients with COVID-19 had 5 times the levels of ferritin compared to patients without the infection, and severe disease can be predicted when serum ferritin is greater than 162 ng/mL (86.9% sensitivity, 70.3% specificity) [14–16], giving this protein an important role in the stratification of risk for these patients.

Considering the results of the present study, in relation to iron and ferritin, it can be said they support the hypothesis that SARS-CoV-2, by mimicking the action of hepcidin, increases the circulation of ferritin while inducing serum iron and hemoglobin level deficiency. In this dynamic, the patient develops a state of anemia, even though they are hyperferritinemic [5]. The mimetic action of the viral protein on hepcidin can lead to ferroportin blockade causing intracellular iron trapping and hypoferremia. There is evidence that during inflammation, the accumulation of intracellular iron also increases ferritin, further increasing the concentration of intracellular iron, evidencing metabolic dysregulation that can lead to early multicellular ferropoptosis [9].

Biologically, when considering the hypoferremia of inflammation, the common mechanism in this scenario is the increase of hepcidin mediated by cytokines (mainly IL-6). This increase in hepcidin during infectious processes has been well described in the literature, especially in bacterial infections. However, there is a gap regarding the real role of hepcidin in viral infections, such as by SARS-CoV-2 [11]. It is worth mentioning that COVID-19 clinical cases are highly heterogeneous and for this reason controversial data can be found on the function of this protein in the course of infection. To better understand the role of this defensin, one must consider the amount of iron that was compromised, or 'sequestered', during SARS-CoV-2 infection, as well as the underlying comorbidities of each infected individual. What is known is that hepcidin, being a key factor in iron absorption, is protective against infections, therefore, its decrease corroborates with the reduction of iron absorption found during COVID-19 and with a worse prognosis [17,18].

This study showed that older individuals have lower serum iron levels, while ferritin levels increase. This finding corroborates a meta-analysis that showed that ferritin levels were higher in the older population and in patients who did not survive the infection [16]. This is because ferritin plays a pathogenic role through its binding to T cell immunoglobulin and mucin domain 2 (TIM-2), promoting the expression of multiple pro-inflammatory mediators, which causes prolonged inflammation; the higher the levels of ferritin, the greater the amount of inflammatory cytokines, which can lead to death [14].

Hematopoiesis is a dynamic and intermittent process that occurs from the moment of embryo implantation until the last days of the individual's life, however, from 65 years of age onwards, senescence causes this process to fail. Considering that iron directly interferes with hematopoiesis, iron deficiency in this age group is linked to the worsening of the outcome of the infection, especially because it is in this age group that individuals with comorbidities such as type 2 diabetes mellitus, hypertension, among others are found. The association between alterations in iron homeostasis and senescence point to a worse prognosis for the infected patient [16].

Regarding sex and serum ferritin levels, it was possible to observe that men had higher ferritin levels than women. According to the Qin et al. study, men had higher mortality rates than women (22.2% vs. 10.4%), with an HR of 1.923 (95% CI, 1.181–3.130); This is being due to an excess of inflammatory reaction related to the severity of COVID-19. Male patients had an exacerbated inflammatory reaction, with high levels of IL-10, TNF-α, LDH, ferritin and CRP [19]. An exacerbated response to infection is believed to be mediated by hyperferritinemia, which may be related to effects of sex hormones on innate immune responses. As an example, men produced more IL-10 than women after virus stimulation, which was positively related to the higher concentration of androgens in men [19].

The quantification of hepcidin in this study showed that patients with COVID-19 show a reduction in this peptide against infection, which is in line with another study that showed a reduction in hepcidin levels among patients considered to be in critical health, compared to healthy individuals. Yagci and colleagues (2021) found that hepcidin levels were lower in intubated patients, confirming the hypothesis that hepcidin may play a protective role in COVID-19 [20]. However, other data diverge and indicate that hepcidin is found in higher concentrations in 61.3% of patients, without mentioning disease severity [21]. Zhou et al. (2020) reinforce that patients diagnosed with severe COVID-19 had

higher levels of hepcidin and serum ferritin, and affirm that the severity of COVID-19 can be predicted with the hepcidin/serum ferritin ratio with a sensitivity of 95.7% [15].

With regard to TFRC gene expression, this is the first study to profile the expression of this mRNA, within the studied conditions. With the reduction of iron bioavailability, it is believed that transferrin receptor up-regulation occurs to facilitate the transport of this component to all tissues and maintain cellular homeostasis. A Chinese study evaluated the expression of different genes against cellular hypoxia at different times of exposure to hypoxia, and showed that there is a difference in TFRC expression at different levels of cellular hypoxia, ranging from normal to low expression under conditions of reduced O_2 (3%). The present study did not assess the levels of hypoxia of participants with COVID-19, so there is no way to compare this with in vitro studies [22]. LV and colleagues investigated patients with severe COVID-19 and found that they had lower serum levels of serum transferrin, which were inversely associated with risks of negative outcomes, i.e., high transferrin was associated with low risks of severe disease (aRR 0.21, 95% CI: 0.08–0.52) and ARDS (aRR 0.24, 95% CI: 0.10 –0.56) and low transferrin was associated with increased risk of COVID-19 severity [23]. Therefore, it can be suggested that transferrin receptor synthesis is regulated by circulating levels of iron, in addition, it is possible that transferrin is important in preventing aggravation of the infection.

The data found show a high variability in the age of the participants and the patients were not grouped according to comorbidities and sex at the time of data analysis, therefore the lack of these data is considered an experimental limitation.

5. Conclusion

COVID-19 interferes both in the synthesis and expression of proteins involved in iron homeostasis. It can be observed that altered iron and ferritin levels were consistent with the inflammatory state of those infected with SARS-CoV-2 and the monitoring of these markers reflects the state of the infection and its evolution, as well as the prognosis of the disease. The reduction of hepcidin, this being the main factor for iron absorption, corroborates with the reduction of systemic iron absorption. Increased TFRC gene expression suggests increased iron internalization and the mimicry of hepcidin action by SARS-CoV-2, reducing iron export by ferroportin, which would explain low circulating levels of iron due to intracellular trapping. Furthermore, as there is in vitro evidence that TFRC may be an alternative receptor for SARS-CoV-2, this receptor could be considered as a target of studies in order to better understand its diagnostic and therapeutic potential.

Findings

Serum hepcidin presented lower serum levels and transferrin receptor expression presented higher levels in COVID-19 patients, regardless of disease severity.

Author statements

All authors participated in the study development and are in agreement about the publication.

Question

Are transferrin expression altered in COVID-19 patients?

Meaning

This study suggests that changes in transferrin receptor gene expression are associated with iron dysmetabolism in the pathophysiology of COVID-19, showing that this parameter may provide insights about cells iron status.

CRediT authorship contribution statement

Ana Carolina Macedo Gaiatto: Formal analysis, Data curation, Writing – review & editing. **Thaciane Alkmim Bibo:** Formal analysis, Data curation, Writing – review & editing. **Nicolle de Godoy Moreira:** Formal analysis, Data curation, Writing – review & editing. **Joyce Regina Santos Raimundo:** Data curation, Writing – review & editing. **Beatriz da Costa Aguiar Alves:** Data curation, Writing – review & editing. **Thaís Gascón:** Data curation, Writing – review & editing. **Fernando Luiz Affonso Fonseca:** Formal analysis, Data curation, Writing – review & editing. **Glaucia Luciano da Veiga:** Formal analysis, Data curation, Writing – review & editing.

Conflict of interest

The authors declare no conflicts of interesting.

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