Iron and COVID-19: a prospective cohort study in the Emergency Department of Piacenza (Italy)

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Abstract. *Background and aim*: Dysregulation of iron metabolism and hyper-inflammation are two key points in the pathogenesis of coronavirus disease 2019 (COVID-19). Since high hepcidin levels and low serum iron can predict COVID-19 severity and mortality, we decided to investigate iron metabolism and inflammatory response in 32 COVID-19 adult patients with a diagnosis of COVID-19 defined by a positive result of RT-PCR nasopharyngeal swab, and admitted to an Italian emergency department for acute respiratory failure at different degree. *Methods*: Patients were stratified in 3 groups based on PaO_2/FiO_2 ratio at admission: 13 (41%) were normoxemic at rest and suffered from exertional dyspnea (group 1); 14 (44%) had a mild respiratory failure (group 2), and 5 (15%) a severe hypoxiemia (group 3). *Results*: White blood cells were significantly higher in group 3, while lymphocytes and hemoglobin were significantly reduced. Serum iron, transferrin saturation, non-transferrin-bound iron (NTBI) and ferritin were significantly increased in group 2. All the groups showed high hepcidin levels, but in group 3 this parameter was significantly altered. It is noteworthy that in group 1 inflammatory and oxidative indices were both within the normal range. *Conclusions*: We are aware that our study has some limitations, the small number of enrolled patients and the short period of data collection, but few works have been performed in the Emergency Room. However, we strongly believe that our results confirm the pivotal role of both iron metabolism dysregulation and hyper-inflammatory response in the pathogenesis of tissue and organ damage in COVID-19 patients. (www.actabiomedica.it)

Key words: COVID-19, Emergency, iron, ferritin, hepcidin, non-transferrin-bound iron

Background

The recent Sars-Cov-2 pandemic has put a strain on the worldwide healthcare system, leading to a new challenge in understanding the viral mechanism and the pathophysiology of coronavirus disease 19 (COVID-19). COVID-19 can have a wide spectrum of signs and symptoms, from fever and cough to multiple organ failure (MOF) and acute respiratory distress syndrome (ARDS) till death (1). The disease expression is associated with various risk factors, e.g., viral load, age, sex, presence of comorbidities (cardiovascular disease, diabetes mellitus and hypertension), innate and adaptive immune systems guest capacity (2). Unfortunately, the pathogenesis of COVID-19 has not been completely understood. Certainly, inflammatory cytokine storm and viral evasion of immune response play a central role in disease progression and severity (3). The clinical picture of the disease is dominated at different stages by significant

inflammation, hypercoagulopathy, respiratory damage with possible atypical ARDS (2,4). SARS-CoV-2 infected patients can die because of excess response of their immune system, characterized by abnormal high release of circulating pro-inflammatory cytokines, particularly IL-6 and IL-1b (3). In a meta-analysis by Henry et al., biomarkers of inflammation, cardiac and muscle injury, liver and kidney function and coagulation were significantly elevated in patients with both severe and fatal COVID-19, and IL-6, IL-10 and serum ferritin were strong discriminators for severe disease (5). IL-6 seems to play a relevant role in the COVID-19 pathogenesis: increased IL-6 levels are correlated with respiratory failure, ARDS and adverse clinical outcomes (6). IL-6 is a pleiotropic cytokine produced by activated macrophages, and involved in acute phase activation response, B cell proliferation and antibodies production, T cell differentiation and cytotoxicity, and synthesis of hepcidin in the liver (7). Overexpression of IL-6 is a key factor that causes organ damage through different mechanisms, such as maturing naïve T cells into effector T cells, inducing vascular endothelial growth factor expression, increasing vessel permeability, and reducing myocardium contractility (8). Post-mortem pathology has confirmed tissue necrosis, interstitial macrophage and monocyte infiltrations in the lung, heart and gastrointestinal mucosa, probably due to cytokine release syndrome (9).

Hepcidin is the main regulator of iron homeostasis (10). By degrading its target receptor ferroportin, hepcidin controls dietary iron uptake and iron release from iron-recycling macrophages (11). Hepcidin levels increase during infection or inflammation limiting the availability of iron in plasma leading to hypoferremia as a host defence mechanism (12). This process removes iron from microorganisms but can lead to inflammatory anemia (13). Anemia contributes to morbidity and mortality (14) acting with an immune-inflammation-driven mechanism, particularly in elderly patients (15). In a study conducted in 339 hospitalized elderly COVID-19 patients, mean hemoglobin values were lower than normal, and in a meta-analysis by Taneri et al. the prognosis of COVID-19 patients might depend on lower hemoglobin levels as severe cases had significantly lower hemoglobin levels than moderate cases (16). Indeed, iron is essential for both humans and bacteria, used in the erythropoietic process and in the pathogens' growth, respectively. Viruses need iron too, by using the host metabolic system to replicate (17). The innate immune system can limit iron availability to invading microbes via nutritional immunity. In this context, hepcidin is the major orchestrator of the hypoferremic response to infection (18). It has been reported that patients diagnosed with severe COVID-19 had higher hepcidin and serum ferritin levels, and hepcidin and serum ferritin tandem testing can predict COVID-19 severity with 94.6% specificity (19). Recently, Nai et al. confirmed these results in a cohort of COVID-19 hospitalized patients (20).

In addition to the iron key regulator hepcidin, circulating cytokines, such as IL-1 and tumor necrosis factor (TNF), increase the synthesis of the iron storage protein ferritin (7). Consequently, more iron is retained predominantly in the reticuloendothelial system. The iron amount is strictly regulated because of the potential formation of free radicals through the Fenton and Haber-Weiss reactions, leading to cell damage and organ failure. Non-transferrin bound iron (NTBI) is a highly toxic free form of iron, that contributes to organ damage, as documented in iron overload diseases (21). In healthy individuals, NTBI is rapidly cleared from the circulation by non-transferrin-bound iron transporters in the liver and other organs (e.g., pancreas, heart, and pituitary), resulting undetectable in their plasma (22). NTBI quantification can be useful in the diagnosis and management of iron-overloaded syndromes. Unfortunately, NTBI quantification is complex and restricted to just a few laboratories, limiting its use in the clinical practise (23). Thus, iron metabolism is expected to be impaired in SARS-CoV-2 infection. In severe COVID-19 patients hospitalized in intensive care unit, alterations of iron metabolism were associated with hypoxemia (24). In a meta-analysis study, a significant difference in mean ferritin levels has been found between COVID-19 survivors and non-survivors (16). Iron dysregulation can persist for at least two months after the onset of COVID-19 and is closely associated with non-resolving lung pathologies and impaired physical performance (25). However, studies on

iron metabolism in COVID-19 patients are limited, mainly aimed to measure ferritin as a marker of inflammation, but we believe that the altered mechanisms of iron metabolism are the key to understand the pathologic consequences of SARS-CoV-2 infection (26). The lack of knowledge is the main limit to an effective therapy for COVID-19. Liu et al. have proposed to use IL-6 blockade, e.g., tocilizumab, to treat COVID-19 induced cytokine release syndrome (27), but a recent study by Campochiaro et al. did not confirm a statistical difference between tocilizumab and standard treatment in a cohort of severe COVID-19 patients with hyper-inflammation (28). Iron chelators have been proposed as an adjunctive treatment of COVID-19 to attenuate ARDS and help control SARS-CoV-2 via multiple mechanisms (29).

Starting from the observation that overexpression of hepcidin and iron metabolism dysregulation might play a crucial role in COVID-19 (19), and as consequence, the understanding of altered iron metabolism and hyper-inflammation could be the key element to correctly treat and monitor COVID-19 patients, here we report our experience in a cohort of COVID-19 patients admitted to the Emergency Department of Piacenza (Italy) in order to investigate the relationship between iron, oxidative stress and pulmonary damage in COVID-19 patients.

Methods

Patients

Thirty-two COVID-19 adult patients consecutively admitted to the Emergency Room (ER) of Guglielmo da Saliceto Hospital, Emilia-Romagna, Piacenza, Italy, from 13rd January to 25th February 2021 have been recruited in this institutional observational protocol approved by the local ethics committee (Area Vasta Emilia-Nord; institutional review board approval number, 2020/0175903), according to STROBE guidelines. Iron metabolism and inflammatory mediators have been investigated at Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy. All patients gave written informed consent to data collection.

Inclusion criteria were:

- Age >= 18 years
- A confirmed diagnosis of SARS-CoV-2 infection defined as a positive result of real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay of nasopharyngeal swabs
- Respiratory failure, including exertional dyspnea.

Patients who had hematologic or solid malignancy on chemotherapy were excluded.

At admission, all patients were investigated in the COVID-19 area of the ER with arterial blood gas, laboratory tests, including iron metabolism and inflammatory mediators, lung ultrasound (LUS) and high-resolution CT scan of the chest (HRCT). Walking-test was performed in patients with normal peripheral oxygen saturation by pulse oximeter, who complained of exertional dyspnea. Demographics, comorbidities, symptom duration, clinical data, laboratory findings including blood gas analysis, and radiological investigations were recorded.

Laboratory findings

Arterial blood samples were analysed on a ABL90 FLEX Plus blood gas analyser (Radiometer Medical ApS, Brønshøj, Denmark). Full blood count, iron assessment (serum iron, ferritin, and transferrin), and serum values of creatinine, urea, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, triglycerides, fibrinogen, D-dimer, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) were quantified in all patients enrolled in the study. The formula used to calculate transferrin saturation is: Tf Sat = serum iron / (transferrin × 1.42) × 100.

Patients were divided in three groups based on their respiratory function, calculated using the partial pressure of arterial oxygen to fraction of inspired oxygen ratio (PaO_2/FiO_2). As stated by the 2012 Berlin definition (30) severity of ARDS was considered mild with PaO_2/FiO_2 201-300 mmHg, moderate with PaO_2/FiO_2 101-200 mmHg, or severe with PaO_2/FiO_2 <100 mmHg.

Iron metabolism and inflammatory parameters

Non-Transferrin Bound Iron (NTBI). Serum NTBI content was assayed by high performance liquid chromatography (HPLC) according to JB Porter et al. (31). Briefly, 450 μL of serum was added to 50 μL of nitrilotriacetic acid (NTA, N0128, Sigma-Aldrich) 800 mmoL/L (pH 7.0) to scavenge NTBI which was quantitatively converted to Fe–NTA complex. The ultrafiltrate (20 μL) containing the Fe–NTA complex was directly injected into the HPLC system (Perkin-Elmer series 200 IC titanium pump module; Perkin-Elmer Life Science, Boston, MA, USA). Normal individuals always show negative NTBI values because samples are measured in parallel with a corresponding blank formed by water and NTA. Water per se contains small amounts of iron not bound by transferrin, whereas in samples, the non-completely-saturated transferrin captures some iron from the Fe–NTA complex. Therefore, the blank subtraction makes the NTBI values negative in some samples.

Hepcidin. The EIA kit for competitive immunoassays from Peninsula Laboratories International (BMA Biomedicals, Augst, Switzerland) was used for the hepcidin-25 assay. The antiserum is captured by antibodies coated on a 96-well plate. A constant concentration of biotinylated tracer and varying concentrations of standard or serum peptide compete for specific binding to the antiserum. The captured biotinylate is subsequently bound by the streptavidin conjugated horseradish peroxidase, quantifiable at 450 nm with a plate spectrophotometer (Tecan infinite M200 pro).

Serum interleukin-6 (IL-6). IL-6 was evaluated with an ELISA kit (IBL International GMBH, Germany); Calibrators, controls or patient samples were incubated with the enzyme-labelled antibody and the biotin-coupled antibody in a streptavidin-coated microplate well. At the end of the reaction, the plate was read at 450 nm by Tecan infinite M200 pro spectrophotometer.

Malondialdehyde (MDA). MDA assay was carried out by a colorimetric method (BIOXYTECH LPO-586 TN OXYS int., USA) based on the formation of the chromophore starting from the chromogen N-methyl-2-phenylindole (R1), detected at 586 nm wavelength (spectrophotometer Beckman DU 640). This method only measures serum free MDA, because the assay conditions do not allow MDA release by proteins through Schiff base. After having found the regression line and consequently r (> 0.999), A, B, just apply the equation of the line X = (Y-A) / B, knowing that Y = (Abs sample-White).

Chitotriosidase enzyme. Chitotriosidase enzyme activity was measured according to Hollack et al. (32). Briefly, fluorescence of 4-methylumbelliferone obtained by incubating 5 **μ**L of serum with 100 **μ**L of 22 **μ**mol/L 4-methylumbelliferyl-**β**-d-N′,N′,N″-triacetylchitotrioside (M5639, Sigma-Aldrich) was detected by Glomax Discover (Promega) fluorimeter (excitation 360 nm, emission 450 nm) after adding 900 **μ**L of NaOH-glycine stop solution (71686, Fluka and G7126, Sigma-Aldrich, respectively). Enzyme activity was expressed as nmoL/h/mL.

Radiological findings

LUS. All the patients were investigated using a rapid and simple LUS protocol to explore all the pulmonary fields adopting the 12-region model, 6 on each side, as previously proposed by Via et al. (33). LUS score was calculated for each region, using the following criteria, as reported by Manivel et al. (34): A-lines or 1-2 B-lines with smooth thin pleura line = 0; 2 or more B-lines with irregular/thickened pleura = 1; "white lung" (e.g., coalescent B lines) = 2; subpleural consolidations = 3. LUS score can range from 0 (lowest) to 36 (highest). According to CLUE protocol (34) the severity of the lung damage can be classified as follows: mild (score 1-5), moderate (score > 5, < 15) and severe (score > 15). A normal lung has a total score of 0. LUS was performed by an emergency physician, using an ultrasound machine Philips Affiniti 70 (Philips, Amsterdam, Holland) with a convex array probe (1– 8 MHz). The machine was dedicated solely to COVID-19 patients.

HRCT. HRCT was performed with a 16-slice scanner (Emotion 16; Siemens, Forchheim, Germany), moving to the apex to the lung bases, to investigate the lung damage. Visual scoring was calculated as percentage of well-aerated lung (WAL) parenchyma (% WAL), as reported by Colombi et al. (35).

Statistical analysis

The normal distribution of variables was analysed using the ANOVA test; means, medians, standard deviation (SD), interquartile range (IQR) have been calculated when appropriate. Correlations were analysed with the Pearson test; p-values lower than 0.05 were considered significant.

Results

We enrolled 32 (13 females, 19 males) COVID-19 patients. The mean age was 65 years (range 28-91 years). All patients were Italian, except one who came from Albania. Patients' demographics, clinical and laboratory findings are reported in table 1.

Lymphocytes were reduced while liver function was slightly altered. Serum iron and transferrin saturation % were lower than normal values but NTBI was detected in a few patients. Ferritin, inflammatory and oxidative stress parameters were higher with respect to healthy subjects.

All patients presented to the ER were complaining of dyspnea at different degree after a mean symptom duration of 7 days (range 2 – 28 days). Patients have been stratified in 3 groups according to PaO_2/FiO_2 ratio at admission: 13/32 (40%) patients with exertional dyspnea resulted normoxemic at rest (PaO_2/FiO_2 > 300, Group 1), 14/32 (44%) had a mild respiratory failure (PaO_2/FiO_2 201-300 mmHg, Group 2), and 5/32 (16%) had a severe respiratory failure (PaO_2/FiO_2 <100 mmHg, Group 3). None of the enrolled patients had a moderate respiratory failure (PaO_2/FiO_2 101-200 mmHg). Patients' demographics, clinical and laboratory parameters registered at admission are reported in table 2 for each group.

Table 1. Demographic, Hematological, Iron Metabolism and Inflammatory Parameters

Demographics		
Subjects [n]	32	
Sex [M/F]	13/19	
Age [years] (min-max)	63.5 (28-91)	
Laboratory findings		
	Median value (IQR)	**Normal range**
White blood cells [× 10^9 per L]	6.8 (3.8-9.8)	4.0-10.0
Neutrophils [× 10^9 per L]	5.7 (3.0-8.4)	2.0-8.0
Lymphocytes [× 10^9 per L]	0.8 (0.5-1.2)	1.5-4.0
Eosinophils [× 10^9 per L]	0 (0.-0.01)	0.10-0.50
RBC [x 10^6 per mcL]	4.71 (4.27-5.16)	4.00-5.40
Hemoglobin [g/dL]	13.9 (13.0-14.8)	12.0-16.0
Mean cell volume [fL]	86 (84-88)	82-98
Mean hemoglobin concentration [pg]	29 (28-30)	27-32
Creatinine [mg/dL]	0.84 (0.70-0.99)	0.60-1.00
Urea [mg/dL]	36 (25-48)	10-50
AST [U/L]	**32 (26-39)**	10-31
ALT [U/L]	26 (13-39)	10-31
Iron parameters		
Serum iron [mcg/dL]	30 (0-20)	49-151
Transferrin saturation [%]	9 (6-13)	20-45
Ferritin [ng/mL]	**446 (237-655)**	12-120
NTBI [μM]	**-0.060 (-0.40- 0.29)**	-0.60- 0.001

(*Continued***)**

Table 1. Demographic, Hematological, Iron Metabolism and Inflammatory Parameters (*Continued*)

Inflammation parameters		
ESR [mm/h]	**55 (35-76)**	<30
CRP [mg/dL]	**3.4 (0-6.8)**	0-0.5
Hepcidin [ng/mL]	**95 (72-119)**	12-25
IL6 [pg/mL]	**12.0 (1.00-23.0)**	2.0-15.0
Chitotriosidase [nmol/h/mL]	**96 (43-149)**	30-80
Fibrinogen [mg/dL]	**477 (362-592)**	15-400
D-dimer [ng/mL]	631 (244-1019)	<1000
Oxidative stress		
MDA [μM]	0.49 (0.21-0.77)	0.10-0.35
LDH [U/L]	**313 (255-371)**	0-247
Respiratory function		
PaO2/FiO2 >300 mmHg [n (%)]	13 (41)	
PaO2/FiO2 > 200 < 300 mmHg [n (%)]	14 (44)	
PaO2/FiO2 > 100 <200 mmHg [n (%)]	0	
PaO2/FiO2 < 100 mmHg [n (%)]	5 (15)	

M: male, F: female. RBC: red blood cells, AST: aspartate aminotransferase, ALT: alanine aminotransferase, NTBI: non-transferrin-bound iron, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, IL-6: interleukin 6, MDA: malondialdehyde, LDH: lactate dehydrogenase, are reported as median and IQR value.

Table 2. Demographics, laboratory and radiological results of the 3 groups of patients stratified according to PaO_2/FiO_2 ratio

	Group 1 (G1) PaO_2/FiO_2 > 300 mmHg	**Group 2 (G2) PaO_2/FiO_2 > 200 < 300 mmHg**	**Group 3 (G3) PaO_2/FiO_2 <100 mmHg**	**P<0.05**
N° patients (M/F)	13 (6/7)	14 (11/3)	5 (2/3)	
Mean Age [years] (min-max)	54 (13)	68 (15)	*88 (5)	*p=0.001 vs G1-G2
Mean symptom duration [day]	8	7	4	
Comordities (n, %)				
HP	2 (15)	9 (64)	5 (100)	
DM	1 (7)	5 (35)	1 (20)	
CAD	0	2 (14)	2 (40)	
Dyslipidemia	1 (7)	7 (50)	1 (20)	
Overwight/obesity	1 (7)	3 (21)	1 (20)	
White blood cells [× 10^9 per L]	4.8 (3.9-5.7)	7.7 (5.1-10.4)	*18.2 (16.8-19.7)	*p=0.001 vs G1-G2
Lymphocytes [× 10^9 per L]	0.9 (0.5-1.3)	0.8 (0.5-1.1)	*°0.5 (0.2-0.8)	*p=0.001 vs G1 ° p=0.04 vs G2
Hemoglobin [g/dL]	14.3 (13.5-15.1)	14.0 (13.0-15.0)	*11.7 (10.9-12.6)	*p=0.001 vs G1-G2
Serum iron [mcg/dL]	31 (22-41)	*°31 (20-42)	23 (12-34)	*p=0.001 vs G1 ° p=0.04 vs G3

Transferrin saturation [%]	9 (7-12)	*9 (3-15)	8 (1-16)	*p=0.001 vs G1
Ferritin [ng/mL]	275 (72-479)	*417 (198-637)	559 (455-664)	*p=0.001 vs G1-G3
NTBI [μM]	-0.34 (-0.79-0.11)	0.11 (0.18-0.40)	-0.06 (-0.77-0.65)	
ESR [mm/h]	38 (27-49)	63 (50-76)	*120 (113-128)	*p=0.001 vs G1-G2
CRP [mg/dL]	1.3 (0.7-1.9)	5.1 (2.3-7.9)	^*14.0 (9.2-18.9)	^p=0.01 vs G2 *p=0.001 vs G1
Hepcidin [ng/mL]	95 (79-111)	95 (58-133)	*110 (49-172)	*p=0.001 vs G1-G2
IL-6 [pg/mL]	5.6 (0.85-10.4)	12.0 (2.0-23.0)	°50.0 (2.2-97.8)	° p=0.04 vs G1-G2
Chitotriosidase[nmol/h/mL]	63 (26-100)	*117 (56-178)	117 (62-173)	*p=0.001 vs G1
Fibrinogen [mg/dL]	402 (360-444)	536 (466-606)	*754 (629-879)	*p=0.001 vs G1-G2
D-dimer [ng/mL]	409 °(276-543)	763 (332-1195)	3614 (79-7150)	°p=0.04 vs G2
MDA [μM]	0.40 (0.09-0.72)	0.49 (0.24-0.75)	^0.53 (0.20-0.87)	^ p=0.01 vs G1-G2
LDH [U/L]	251 (192-311)	320 (278-362)	^462 (277-648)	^p=0.01 vs G1-G2
LUS [Score] mean +/-SD	8+/-6	11+/-5	21+/-4	
HRCT [Visual Score] mean +/-SD	15+/-5	24+/-9	56+/-19	

M: male, F: female. HP: hypertension, DM: diabetes mellitus, CAD: cardiovascular disease, NTBI: non-transferrin-bound iron, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, IL-6: interleukin 6, MDA: malondialdehyde, LDH: lactate dehydrogenase. LUS: lung ultrasound, HRTC: high-resolution chest tomography. Laboratory tests are reported as median (IQR) value.

White blood cells were significantly higher in Group 3 respect to Group 1 and Group 2, while lymphocytes and hemoglobin were significantly reduced. Serum iron, NTBI, transferrin saturation and ferritin were significantly increased in Group 2. All the Groups showed high hepcidin levels, but in Group 3 this parameter was significantly altered respect to Group 1 and 2. It is noteworthy that in Group 1 inflammatory and oxidative indices were within the normal range.

Two positive correlations regarding iron metabolism were found between NTBI and transferrin saturation, and between NTBI and chitotriosidase (Fig. 1A). A positive correlation between hepcidin and fibrinogen and between hepcidin and CRP were detected (Fig. 1B).

Moreover, a positive correlation regarding imaging techniques was found between HRCT and LUS (Fig. 2).

An analysis of the clinical and biological aspects was carried out for each group bringing out some particular behaviours. Thirteen patients (7 females, 6 males) with mean age 54 years (range 28-71 years) were classified as group 1. They presented to our ER after a mean time of 8+/-7 days since the diagnosis of SARS-CoV-2 infection. Nine out thirteen (70%) had no comorbidities. Two (15%) patients suffered from hypertension, and one (7%) patient was diabetic and dyslipidemic. Mean LUS and HRCT visual scores were 8 +/- 6 and 15+/-5 %, respectively, ranging from mild to moderate lung damage. Five (38%) patients had a positive walking-test and were hospitalized to be enrolled in an experimental COVID-19 protocol. All these patients were discharged after 2 weeks of hospitalization with normal oxygen saturation at room ambient. Seven (54%) patients were discharged directly from the ER without oxygen need, only one (8%) patient required low-dose oxygen therapy (1 Lt/min) via nasal prongs. None of them has been readmitted to ER. At time of writing, all patients are in good medical condition.

Patients classified as group 2 were 14 (11 males, 3 females) with mean age 68 years (range 46-87 years), admitted to the ER after a mean symptom duration

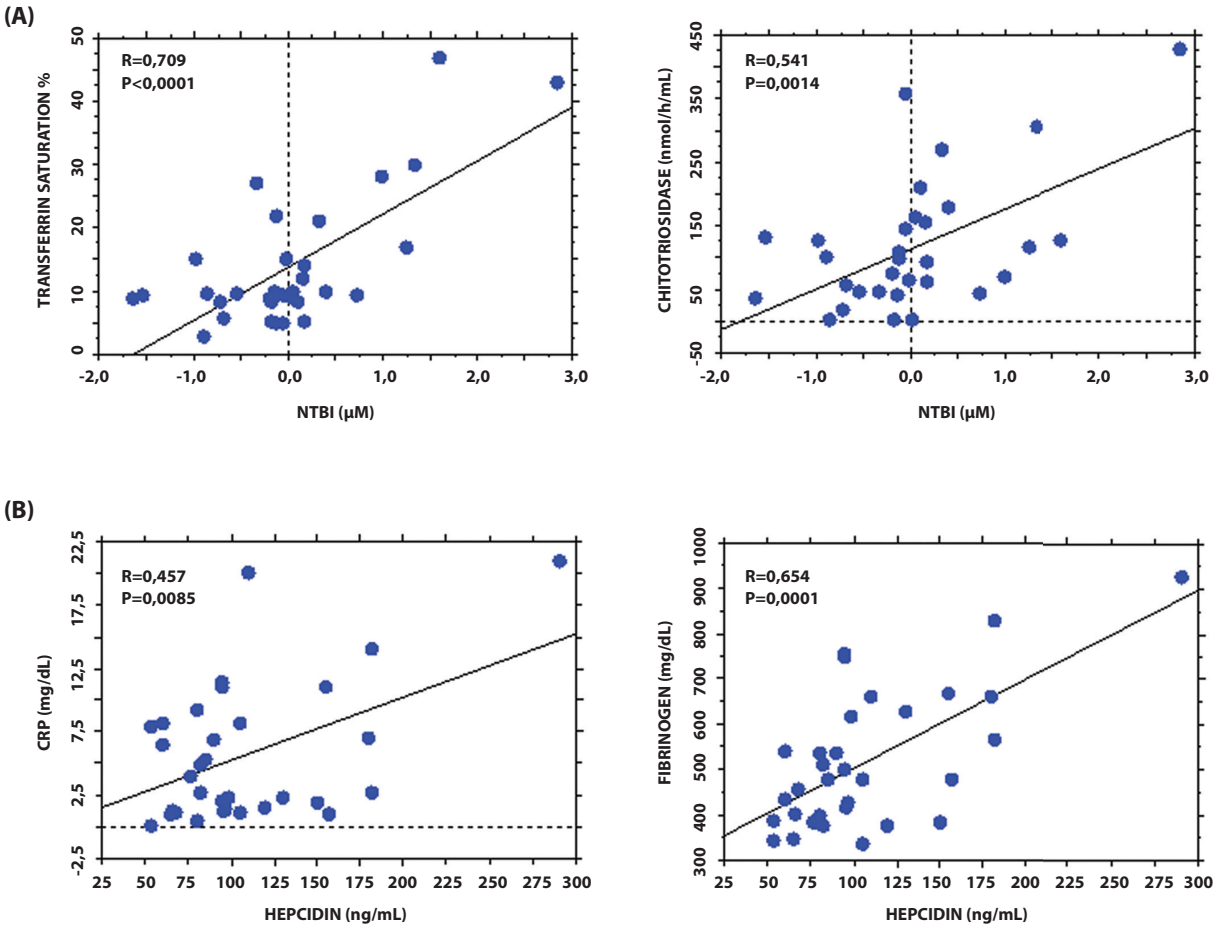

Figure 1. Iron metabolism and inflammation status: (A) NTBI correlations with Transferrin Saturation % and Chitrotriosidase respectively; (B) correlations between Hepcidin and CRP and between Hepcidin and Fibrinogen.

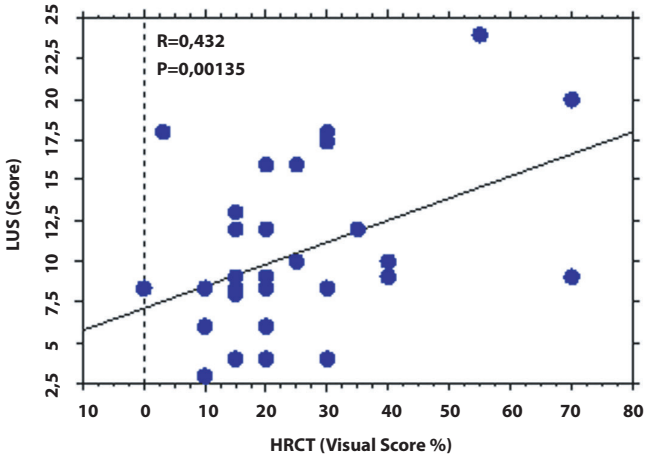

Figure 2. Correlation between imaging techniques: HRCT and LUS.

of 7 days (SD 3). All patients needed oxygen via nasal prongs (1-4 Lt/min), presented a moderate/severe lung damage as documented by LUS score (mean value 11, SD 5) and HRCT visual score (mean value 24%, SD 9). Among them, 4 (28%) had a dysmetabolic syndrome, 5 (35%) hypertension, 1 (7%) diabetes, 2 (14%) cardiovascular diseases, and 3 (21%) dyslipidemia. Three (21%) out fourteen had BMI > 30. Based on D-Dimer value over 1000 ng/mL, CT pulmonary angiography was performed in 5/14 (36%) patients and resulted negative for pulmonary embolism. Six (43%) patients were discharged with low flow oxygen therapy, followed and treated at home by USCA (special COVID-19 medical units) with good clinical response. The other patients (8, 57%) were hospitalized, 2 (14%) of them died for ARDS. One patient was a 78-year-old male affected by hypertension and severe aortic stenosis who was admitted to the ER for exertional dyspnea in the last 5 days under treatment with oral steroids and azithromycin. LUS and HRCT score were respectively of 13 and 15%, documenting a moderate lung involvement. Blood tests showed elevated WBC (20810/mm^3) with lymphocytopenia (750/mm^3) and increased inflammatory markers (ESR 34 mm/h, CRP 1.55 mg/dL, hepcidin 120 ng/mL, IL-6 10.3 pg/mL) and serum ferritin (690 ng/mL) with normal transferrin saturation (12%) and serum iron (35 ng/mL). Oxidative damage was revealed by increased levels of LDH (365 U/L), chitotriosidase (157 nmoL/h/mL), MDA (0.47 μM) and the presence of NTBI (0.16 μM). Hemoglobin, D-Dimer, fibrinogen, liver and renal function were all in the normal range. The patient developed rapidly ARDS treated with C-PAP NIV, high-dose intravenous corticosteroids and low molecular weight heparin (LMWH). He died after 7 days of hospitalization because of MOF. Interestingly, the patient developed mild inflammatory anemia (Hb 11.6 g/dL, MCV 86.5 fL, MCH 28 pg) with hyperferritinemia (5219 ng/mL) and elevated CRP (9.97 mg/dL). Repeated blood cultures were negative, and procalcitonin resulted always in the normal range, excluding a bacterial infection.

The other patient was a 69-year-old man suffering from a dysmetabolic syndrome, who was admitted to the ER complaining of exertional dyspnea, fever, myalgias and fatigue in the last 8 days under treatment with oral corticosteroids and LMWH at prophylactic dose. HRCT showed a bilateral COVID-19 pneumonia with visual score of 25-35%. LUS score resulted 12, confirmed a moderate lung involvement. Laboratory tests documented increased inflammatory markers, e.g., CRP 1.03 mg/dL, ESR 65 mm/h, IL-6 30 pg/mL, hepcidin 157 ng/mL and fibrinogen 477 mg/dL. Full blood count, D-Dimer, liver and renal function were normal. Serum ferritin was only slightly increased (325 ng/mL) with normal transferrin saturation (22%). NTBI, chitotriosidase and MDA were all in the normal range. After 3 days, the patient developed an ARDS, requiring intubation and mechanical ventilation. He died of multiorgan failure (MOF) after 23 days of hospitalization. Interestingly, blood tests documented the progressive development of moderate/severe inflammatory anemia (Hb 8.2 g/dL, MCV 97.4 fL, MCH 30.1 pg) with the raising of all the inflammatory markers, e.g., CRP (13.46 mg/dL), IL-6 (56.44 pg/mL), and fibrinogen (806 mg/dL).

Finally, an interesting case is a 72-year-old male patient with a history of hyperuricemia, hypertension, atrial fibrillation in oral anticoagulant therapy (warfarin) and cardiac valvular disease, who was admitted to the ER for exertional dyspnea under a 6-day oral steroid therapy. HRTC showed a bilateral COVID-19 pneumonia with visual score of 35-40%. Blood tests revealed a marked inflammatory status with elevated CRP (7.98 mg/dL), ESR (51 mm/h), IL-6 (16.86 pg/mL), reduced serum iron (17 μg/dL), slightly increased serum ferritin (309 ng/mL) and normal transferrin saturation (5%) with a slight increased hepcidin value (54 pg/mL). NTBI, chitotriosidase and MDA resulted normal. Full blood count, fibrinogen, D-Dimer were in the normal range. During the hospitalization, the patient developed a progressive worsening dyspnea, firstly treated with High Flow Nasal Cannula oxygen therapy, and then with CPAP NIV with the complete resolution of respiratory failure. During the hospitalization, D-Dimer increased significantly (4272 ng/mL) and a normochromic normocytic moderate anemia occurred (Hb 10.7 g/dL, MCV 92.3 fL, MCH 30.6 pg). Surprisingly, the patient was discharged eupnoeic at room ambient with a rapid amelioration of hemoglobin value (12.1 g/dL) after 30 days of hospitalization.

Group 3 patients were significantly older with mean age 88 years (range 81 – 90 years). All of them complained progressive severe dyspnea with a mean delay of 4 days from symptom onset and required oxygen via high-flux reservoir mask at admission. All patients had a severe lung damage with elevated mean LUS and HRCT visual scores, respectively of 21 +/-4 and 56 +/-19 %. Among them, 2 patients with a previous history of cardiac ischemic disease also complained acute chest pain with ischemic ECG changes and elevated troponin I (1449 ng/mL and 2719 ng/mL, normal value < 31). Considering comorbidities, hypertension was present in all patients (100%), cardiac diseases in 2/5 (40%) patients, diabetes mellitus and overweight only in one (20%) patient. All patients had elevated inflammatory markers (ESR, CRP, fibrinogen, hepcidin, IL-6), significantly increased WBC and D-Dimer, and a moderate inflammatory anemia (Table 2). Four out five patients died from MOF after a mean time of hospitalization of 6+/-4 days. Surprisingly, a 90-year-old woman patient with a history of hypertension and depression, admitted to the ER for a severe respiratory distress syndrome to about 13 days since COVID-19 diagnosis (PaO_2/FiO_2 64 mmHg, LUS score 24, HRCT visual score 70%), is still alive and discharged after 33 days of hospitalization with a complete recovery from ARDS. Even in presence at admission of elevated inflammatory markers (CRP 9.46 mg/dL, ESR 88 mm/h, fibrinogen 436 mg/dL) and D-Dimer (3614 ng/mL), lymphocytopenia (720/ mm^3) and mild inflammatory anemia (Hb 11 g/dL, MCV 87.3 fL, MCH 28.4 pg) with increased serum ferritin (479 ng/mL), normal transferrin saturation (16%) and serum iron (27 pg/mL), serum hepcidin and IL-6 were only slightly increased, respectively 50 ng/mL and 8.2 pg/mL. Oxidative damage was documented by increased values of LDH (665 U/L), NTBI (1.25 μM), chitotriosidase (117 nmoL/h/mL) and MDA (0.89 μM). During the hospitalization, the patient has been treated with intravenous corticosteroids, low molecular weight heparin, azithromycin and oxygen therapy with a progressive and complete resolution of ARDS and a consensual amelioration of inflammatory markers (CRP 1.06 mg/dL), LDH (292 U/L) and D-Dimer (736 ng/mL) at discharge. In addition, the degree of anemia was unchanged during the hospitalization.

Finally, in the hypothesis of macrophage activation syndrome, an acquired form of hemophagocytic lymphohistiocytosis characterized by a fatal MOF due to an uncontrolled activation and proliferation of T lymphocytes and macrophages, high levels of IL-6, hyperferritinemia, and evidence of intravascular coagulation, as demonstrated by elevated D-Dimer and low fibrinogen, described also in association with the influenza A H1N1 virus during 2009 pandemic (36), we measured the triglyceride levels, that were normal in all patients (data not shown).

Conclusions

In this report, we evaluated iron metabolism and inflammatory markers in COVID-19 patients. It is important to point out that based on the current literature, this prospective study is the first study to investigate both iron metabolism and inflammatory response in COVID-19 patients admitted to an ER. All the patients enrolled in the study have been investigated before being treated with steroids, antiviral agents and/or antibiotic therapies. The inflammatory status and the iron metabolism can be considered a clear picture of COVID-19, not influenced by any pharmacological treatment.

This study confirmed high ferritin levels in COVID-19 patients. This hyper-ferritinemia may be due to inflammation considering that serum iron e transferrin saturation levels were within the normal range. Cytochine cascade induces hepcidin, CRP and fibrinogen during inflammation and infection, this is confirmed by hepcidin correlation with CRP and fibrinogen found in our study (Fig.1B) (37).

It has been reported that hyper-inflammation in association with iron homeostasis dysregulation may play a key role in pathogenesis of disease including viral infections (23). It may be hypothesized that hyperferritinemia is associated with iron toxicity because of ferritin leakage and free iron released by damaged tissues. This idea is supported by the evidence that all the inflammation parameters were altered in patients with severe ARDS (Group 3) and by the detection of a positive correlation between NTBI and chitotriosidase as macrophage activation sign.

In inflammation, the local expression of the hepcidin induces to lock iron into macrophages. The iron-sequestrating capacity of tissue macrophages is very much powerful, but it could be overwhelmed (35).

Moreover, free iron can often increase virulence and prooxidant reactions and contribute to alveolar oxidative damage through promoting ROS formation (18) responsible for oxidative stress with alteration of proteins, lipids, and DNA.

In our study a severe degree of inflammation in group 3 subjects is well documented by very high levels of white blood cells, ferritin, NTBI, hepcidin, CRP, IL-6, fibrinogen and D-Dimer. These patients showed also advanced age and severe anemia and lung damage. It is noteworthy that only one 90-year-old woman of this group survived: she had lower hepcidin levels and mild anemia persisting throughout the entire period of hospitalization respect to the other patients of the group. In group 2 the inflammatory values were lower than in group 3 except for the two deceased patients who reported high hepcidin, high IL-6, NTBI and MDA at the time of admission. During the hospitalization, the two patients became anemic. Data observation permit to hypothesize that high hepcidin levels and worsening of anemia degree during hospitalization constitute the leitmotif for these COVID-19 non survived subjects.

Based on these results, mortality was associated with older age, shorter symptom duration, increased white blood cell count, lower hemoglobin level with normal MCV and MCH as occurred in inflammatory anemia, high CRP and ESR, and D-Dimer.

After discussing hematological data, it should be absolutely pointed out that in our study a positive correlation between LUS and HRCT parameters was found. This data confirms the fundamental role of the utilization of ultrasound in discriminating cases of interstitial pneumonia already at the access to the emergency room, as previously hypothesized (38). In this situation LUS should be considered a reliable and complementary tool to the CT scan, useful in cost-benefit terms, saving time and human resources.

We are aware that our study has some limitations, including the small number of enrolled patients, the short time period of data collection and the collection of the blood samples only at admission in the ER, but very few works have been performed in the ER. However, we strongly believe that our results confirm the pivotal role of both iron metabolism dysregulation and hyper-inflammatory response in the pathogenesis of tissue and organ damage in COVID-19 patients.

In summary, we believe that iron and inflammation are two key elements to understand the pathogenetic mechanisms of COVID-19, and both of them play a central role in the development of organ damage, particularly for the respiratory function. For all these reasons, we strongly suggest to investigate iron metabolism and inflammatory markers in all hospitalized COVID-19 patients to monitor the clinical course of this life-threatening disease, promptly predict negative prognosis, and timely identify the target elements for a successfully patient-tailored therapy. In conclusion, our results highlight the possibility that a therapeutic strategy for severe COVID-19 could be based on the combination of anti-inflammatory drugs and iron chelators. Further large-scale studies are needed to confirm this hypothesis.

Conflict of Interest: Each author declares that he has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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Invited Commentary

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Is it a high time to focus on iron-mediated pathology initiated by COVID-induced inflammation?

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The paper published in the current issue of Acta Biomedica by Duca et al. clearly demonstrate a link between deregulated of iron homeostasis (FeH) and hyperinflammation in non-treated COVID-19 patients. The authors found essentially altered physiologically critical parameters of FeH (serum ferritin, hepcidin, iron, NTBI and transferrin saturation) in concert with enhanced indexes of inflammation (IL6, CRP, chitotriosidase enzyme etc) and oxidative stress (MDA, LDH). Importantly, an essential alteration of the parameters which stay at a cross-road between inflammation and FeH (ferritin and hepcidin) are shown in their work. All these alterations were enhanced by disease severity. These observations are in line with other clinical reports (1-3) while Duca et al. applied quantitative analysis of interaction between two physiological systems – immune response and FeH. Notably, despite more than 240,000 publications related to COVID-19 are currently available in PubMed, the precise mechanisms of abnormal SARS-Cov2-induced pathogenesis still is not completely understood. Recently, it was hypothesized that deregulated FeH is a presumable core of Sars-Cov2-induced pathology (4,5). Nevertheless, just 0,01-0,02% of the papers related to COVID-19 demonstrated a significance of FeH disturbance for the SARS-Cov-2 pathology and the results of Duca et al. adjust to this pool of research. Among iron-related parameters serum ferritin is generally accepted as one of the additional markers of SARS-Cov-2 infection. Elevated ferritin levels are usually interpreted as a marker of inflammation (1). Mechanistically, an elevation of serum ferritin levels mainly results from inflammation followed by local ferroptosis while subsequent disturbance of local FeH amplifies both inflammation and the FeH deregulation. Data regarding serum hepcidin levels are a bit contradictory among publications. Basically, physiological role of hepcidin, a major FeH hormone, is protecting

(4). Mechanistically, hepcidin down-regulates the only iron exporter ferroportin to lock the iron inside cells, thus, blocking local iron recycling to prevent tissue injury. This hepcidin function makes local hepcidin levels extremely important and it may be expected to be as pronounced as other FeH parameters (ferritin, non-heme iron, hemoglobin, haptoglobin) shown in infected RDS patients in comparison to health subjects (6).

Intriguingly, Duca et al. also revealed a correlation between some parameters of the two systems (NTBI versus both transferrin saturation and chitotriosidase as well as hepcidin versus CRP). In addition, a correlation between lung ultrasound (LUS) and high-resolution CT scan of the chest (HRCT) opens a possibility to monitor the disease progression and treatment by LUS.

Importantly, a positive correlation between hepcidin and fibrinogen points on a third system linked to the cross-talk between inflammation and FeH. In patients with COVID-19 , fibrinolysis shutdown is accompanied with markedly elevated D-dimer concentrations, a marker of hyperfibrinolysis (7). Thus, stable fibrin clots persist despite activated fibrinolysis. This paradox can simply be explained, given that ferric ions are able to induce a formation of proteolytically insoluble fibrin clots (8) due to continuous local iron influx via ferroptosis. Despite some limitations of the research as mentioned by Duca et al., the authors should be prized for an attempt to consider a link between deregulated FeH and abnormal coagulation in patients infected by SARS-Cov-2. Surely, this work will inspirate researchers to extent the task and to look for updated designs of both experimental approaches and data analysis.

At least three directions may extend the research of Duca et al. to reveal the mechanism of iron-dependent pathology initiated by SARS-Cov-2 infection.

First, a correlation between local characteristics of inflammation and the FeH in SARS-Cov-2 infected lungs needs an evaluation as they could be more pronounced in compare to systemic one. Remarkably, Duca et al. operate with systemic parameters of the interacting physiological systems while they interpret their results at the level of local changes in iron metabolism mediated by tissue iron-sequestrating cells.

Second, as it is quite difficult to measure many parameters both in lavage and serum in statistically relevant cohort of patients, case reports regarding individual patients may reveal a link between local and systemic characteristics in the course of disease progression and a recovery during treatment.

Third, as abnormal coagulation seen as D-dimer levels enlargement in concert with ferritin may result from iron-induced fibrin clots stabilization (5,9), then, in line with a correlation between fibrinogen and hepcidin found by Duce at al. a correlation between D-dimer levels and local NTBI may be supposed. In tissues, even partial blocking of small vessels by fibrin clots suppress two main ways of iron efflux from the infected organs (4) and, thus, it may enhance the local ferroptosis and subsequent local NTBI elevation.

In lungs, free iron may catalyze stabilization of fibrin clots (8). This link between disturbed local FeH and abnormal fibrin clotting in COVID-induced pathology hypothesized earlier (5, 9) still needs clinical and experimental verification. Remarkably, a bit elevated levels of serum NTBI observed by Duce et al. illustrates the extension of the local FeH disturbance into systemic level and explains the abnormal fibrinolysis observed in blood of patients with COVID-19 as well as it presumably unravels the way how local FeH disturbance in respiratory tract may lead to post-COVID complications in cardiovascular and neurological systems, kidney failure and Kawasaki-like disease in children via iron-mediated fibrin clots formation in small vessels.

Finally, it should be noticed that covidin, recently identified by a computational approach, if its availability and hepcidin function mimicking is confirmed experimentally and clinically, could explain enormous amplification of iron-mediated COVID-induced lungs damage as well as the disease complications (10).

In conclusion, a cross-talk between SARS-Cov-2 induced hyper-inflammation, dysregulation of FeH and fibrin clots formation seems to be a key point of the pathogenesis of the coronavirus disease. More precisely, FeH

presumably stays at a cross-road between two generally accepted COVID-initiated pathological events: hyperinflammation and abnormal fibrin clotting. If so, intensive research is needed to look for interventions able to support and recover FeH in SARS-Cov-2 infected patients.

Conflict of Interest: Author declares that she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article

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