Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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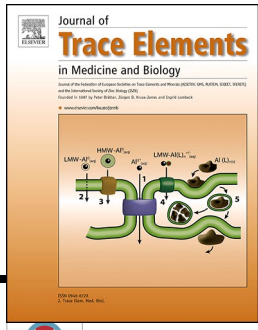

Evaluation of zinc, copper, and Cu:Zn ratio in serum, and their implications in the course of COVID-19

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A B S T R A C T

Background: The dynamics of essential metals such as Copper (Cu) and Zinc (Zn) may be associated with the novel coronavirus disease 2019 (COVID-19) that has spread across the globe.
Objectives: The aim of this study is to investigate the relationship between serum levels of Cu and Zn, as well as the Cu:Zn ratio in the acute phase of COVID-19 along with the assessment of their connection to other laboratory parameters (hematological, biochemical, hemostatic).
Methods: Serum levels of Cu and Zn were measured by atomic absorption spectrometry in 75 patients in the acute COVID-19 phase and were compared with those of 22 COVID-19 patients evaluated three months after the acute phase of the disease ('non-acute' group) and with those of 68 healthy individuals.
Results: In comparison with both the non-acute patients and the healthy controls, the acute patients had lower levels of hemoglobulin and albumin, and higher levels of glucose, creatinine, liver transaminases, C-reactive protein (CRP), and higher values of the neutrophils to lymphocytes ratio (NLR) at the hospital admission. They also exhibited increased levels of Cu and decreased of Zn, well represented by the Cu:Zn ratio which was higher in the acute patients than in both non-acute patients ($p = 0.001$) and healthy controls ($p < 0.001$), with no statistical difference between the last two groups. The Cu:Zn ratio (log scale) positively correlated with CRP (log scale; $r = 0.581$, $p < 0.001$) and NLR ($r = 0.436$, $p = 0.003$).
Conclusion: Current results demonstrate that abnormal dynamics of Cu and Zn levels in serum occur early during the course of COVID-19 disease, and are mainly associated with the inflammation response.

1. Introduction

Corona Virus Disease 2019 (COVID-19), caused by the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), is a rapidly evolving/mutating viral infection with 0.22 billion confirmed cases worldwide, resulting in more than 4.5 million deaths as of Sept 08, 2021 (https://covid19.who.int/). SARS-CoV-2 enters the host through its interaction with the Angiotensin-converting enzyme 2 (ACE2) receptor via its spike protein on type II pneumocytes [1]. COVID-19 predominantly affects the respiratory system causing viral pneumonia and pronounced cytokine storm resulting in widespread systemic immunopathological effects that cause damage to various organs and tissues ultimately leading to higher mortality rate in severe cases [2].

Physiological processes (e.g. aging), along with certain comorbidity like diabetes, cardiovascular diseases, hypertension, chronic kidney disease and chronic obstructive pulmonary disease (COPD) are thought to increase the risk of COVID-19 infection/severity [3,4].

Neutrophils to lymphocytes ratio (NLR) at the hospital admission has been suggested as a simple marker of the systemic inflammatory response in critical care patients and may represent a surrogate marker of disease severity [5,6]. Recently, NLR has been reported as an independent prognostic factor of the outcome in critically ill COVID-19 patients [6].

As robustly observed in other infections, systemic and chronic inflammatory responses are associated with significant biochemical and physiological alterations, thus affecting the concentration of plasma proteins, macro- and micro- nutrients [7,8].

Copper (Cu) and Zinc (Zn) are vital dietary nutrients for the human

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body, which has evolved an elaborate system to maintain their balance by regulating the concentrations in the circulation and in cell stores. During infections or inflammation, abnormal serum levels of certain trace metals and ratios between them can develop: one of the most common case is an elevation in serum Cu and a depression in serum Zn. These alterations can be expressed as Cu:Zn ratio, a clinically more useful index than only the concentration of either of the trace metals [9,10].

Up to date, several studies have been published on serum Zn and Cu levels in COVID-19 patients, demonstrating significant interferences of these metals on the disease pathogenesis and infection [11–15]. These trace elements are also strongly involved in the development of the immune system and mediate its effector functions to fight off infections through various mechanisms (Reviewed in [16]).

In severe COVID-19 patients, serum Zn levels have been also found to be lower [17] as compared with mild-to-moderate COVID-19 infection. Skalny et al., 2021 [12] also observed that increasing COVID-19 severity is associated with a significant gradual decrease in serum Zn levels (together with decreased serum levels of calcium, iron and selenium) when compared with controls. However, serum Cu and especially the Cu:Zn ratio are increased [12]. Increased Cu:Zn ratio has been used as a diagnostic and prognostic marker of inflammation in various pathological conditions like lymphoma, leukemia, breast and gastric cancer. It is pertinent to note that a higher Cu:Zn ratio has been demonstrated to be significantly correlated with oxidative stress, inflammation, undernutrition, and depressed immune function as pointed in other disease conditions [18,19].

In addition, Zn (reviewed in [20,21]) and other essential trace elements [21] play a critical and paramount role in antiviral immunity. In fact, Zn has been shown to inhibit replication of SARS-CoV-1 by suppressing RNA-dependent RNA polymerase activity of the virus in a dose dependent manner [22].

Various systematic reviews, meta-analysis and bioinformatics studies also reveal possible beneficial effects in COVID-19 when Cu or Zn are administered as supplements either alone or in combination with drugs/nutrients/vitamin C and vitamin D (reviewed comprehensively in [16,23,24]. In fact, a total of 55 clinical trials, evaluating the effect of Zn supplementation against COVID-19, are registered (https://clinicaltrials.gov/). Unfortunately, the trial results are contradictory or lack convincing evidence ([25,26], reviewed in [16]). Hence, imbalance in micronutrient status has been postulated as a potential modifiable risk factor for COVID-19 [27,28].

Therefore, we sought to investigate alterations in Cu and Zn serum levels and in the Cu:Zn ratio in the early stages of the COVID-19 disease, assessing the association of these trace elements with the biochemical, hematological and hemostatic laboratory indices of COVID-19 disease patients evaluated in the early phase of the infection (hereafter referred to as 'acute') and comparing them with those of clinically stable COVID-19 patients evaluated 3 months after the infection (hereafter referred to as 'non-acute') and of healthy controls.

2. Materials and methods

2.1. Subjects

The study included a total of 97 COVID-19 affected patients, directed to the University Hospital (UH) St. Ivan Rilski, Medical University,Sofia, Bulgaria, from January to April 2021. Diagnosis of COVID-19 was verified by RT-PCR, detecting SARS-CoV-2-positivity. The cohort of COVID-19 patients consisted of a group of 75 patients tested in the acute phase (blood sampling on the day of Hospital admission) and a group of 22 stable patients evaluated three months after the acute phase who were admitted at Rilski Hospital to execute a computed tomography scan, X-ray or other follow-up clinic visit.

The control group was made of 68 healthy volunteers from the Hospital staff or their family members, who consented to part take in the study: Their blood was collected after an overnight fast (Table 1).

All the Principles of Declaration of Helsinki (1964) and its later amendments were met. The study protocol was approved by the Institutional Hospital Ethics Committee. Written consent was obtained from all individuals. Sample collection was performed by a standard procedure. The analyses for COVID-19 patients were performed routinely during the disease treatment and monitoring, posing no extra sampling.

2.2. Laboratory evaluation

All analyses were carried out at the Clinical Laboratory Department of UH St. Ivan Rislki, Medical University, Sofia, Bulgaria.

The following laboratory tests were performed in all individuals: serum Cu, serum Zn, complete blood count (CBC), aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (Gluc), creatinine, total protein (TP), albumin (Alb), C-reactive protein (CRP). Ferritin, D-Dimer, procalcitonine (PCT) were assayed in all COVID-19 patients and blood gas parameters –only in the acute hospitalized patients.

Cu and Zn in serum were determined by flame atomic absorption spectrometry (FAAS; Perkin Elmer AAnalyst 400, USA). Cu and Zn were measured at 324.8 nm and 213.9 nm, respectively. Other instrumental parameters of the spectrometer were as follow: spectral bandwidth (Slit) (nm) – 0.7; Light source – hollow cathode lamp (HCL) with lamp current (mA) – 15 for Cu and electrode discharge lamp (EDL) with current 6 W for Zn; atomizer – flame air-acetylene; sample introduction into the atomizer – pulverization following preliminary dilution of samples 1:4 by distilled water. The reference ranges for serum Cu are as follows: Males: 12.3 − 22.4 μmol/L (78–142 μg/dL); Females: 13.2–24.3 μmol/L (84–155 μg/dL) [29]. The reference range for serum Zn is: 12–24 μmol/L (78 −157 μg/dL) for all adults [29].

Hematology analyser (Alinity q, Abbott, USA) was in use for CBC. Biochemical tests were done by a clinical biochemistry analyser (Alinity ci, Abbott, USA).

To rule out a thromboembolic disease, D-Dimer as a marker of deep vein thrombosis was measured in a citrate plasma by immunoturbidimetry (coagulometer Sysmex CS – 2000i, Germany).

Blood gas analyses were performed by ISE direct measurement of pH (Hydrogen ion activity), pCO_2 (partial pressure of carbon dioxide), and pO_2 (partial pressure of oxygen) devices (analyser Easy Blood Gas analyser, Medica, USA) using whole blood samples from syringes or glass capillary devices. Oxygen saturation (%$SatO_2$) was calculated automatically.

CRP and Ferritin were measured by immunoturbidimetry and procalcitonin by CMIA technique using Alinity ci, Abbott, USA.

The acute phase reaction was laboratory diagnosed on the base of the results for CRP, WBC, albumin, ferritin, procalcitonin.

2.3. Statistical analyses

Data are presented in terms of mean and standard deviation or, when appropriate, in terms of median and interquartile range (25–75th percentile). Logarithmic transformation was applied to CRP, AST and ALT.

Analysis of variance (ANOVA) was applied to assess the difference between the three diagnosis groups. Post comparisons were performed, and the Bonferroni method was applied to adjust the p value for multiple comparisons. ANCOVA analyses were used to take into account the differences for sex and age. Multiple comparisons were achieved applying Bonferroni adjustment. Mean differences and relative 95% Confidence Intervals (CIs) were described. If the logarithmic transformation was performed, the results were converted back into the original scale by means of the antilog. The chi-square test or, when appropriate, Fisher's exact test were applied to assess the association between the diagnosis and the biological variables under study, and to assess whether they were out of the normal range in the variables

Table 1
Demographic and clinical features of the patients with COVID-19 and healthy controls.

		Healthy controls	Non-acute	Acute		Post comparisons[a]		
	n	68	22	75	p	Non-acute vs healthy controls	Acute vs healthy controls	Non-acute vs acute
Age, yrs	mean (sd)	53.7 (12.84)	46.2 (14.81)	62.5 (14.91)	< 0.001	0.093	0.001	< 0.001
Sex								
Male	n (%)	32 (47%)	4 (18.2)	39 (52%)	0.019			
Female	n (%)	36 (53%)	18 (81.8%)	36 (48%)				
Outcome								
Recovered	n	n.a.	22	64				
Deceased	n	n.a.	0	11				
Length of stay, d	mean (sd)	n.a.	n.a.	11.5 (5.5)				
Blood routine laboratories								
Hemoglobulin, g/L	mean (sd)	144.7 (15.3)	135.3 (10.8)	130.5 (25.1)	p < 0.001			
WBC, x 10^9/L	median (25th-75th percentiles)	6.8 (5.86–7.8)	6.47 (5.81–8.90)	7.32 (4.27–9.84)	0.668[b,c]			
Lymphocytes, x 10^9/L	median (25th-75th percentiles)	2.39 (1.94–2.88)	2.09 (1.74–2.69)	1.04 (0.61–1.57)	< 0.001[b,c]	0.245	< 0.001	< 0.001
Neutrophils, x 10^9/L	median (25th-75th percentiles)	3.96 (3.16–5.29)	3.87 (3.52–4.86)	6.85 (3.27–8.39)	0.036[b,c]	0.999	0.037	0.451
Monocytes, x 10^9/L	median (25th-75th percentiles)	0.52 (0.38–0.68)	0.53 (0.40–0.54)	0.42 (0.24–0.68)	0.577[b,c]			
RBC, x 10^9/L	median (25th-75th percentiles)	4.87 (4.55–5.24)	4.76 (4.53–5.29)	4.8 (4.32–5.12)	0.290[b,c]			
Neutrophils to Lymphocytes ratio (NLR)	median (25th-75th percentiles)	1.57 (1.33–2.37)	1.96 (1.54–2.37)	5.35 (2.80–11.11)	< 0.001[b,c]	0.999	< 0.001	< 0.001
Biochemical indicators in serum								
Total protein, g/L	mean (sd)	70.3 (3.8)	58.7 (12.3)	70 (6.7)	< 0.001	< 0.001	0.999	< 0.001
Albumin, g/L	mean (sd)	45 (2.6)	42 (2.5)	40 (6.2)	< 0.001	0.004	< 0.001	0.999
Glucose, μmol/L	mean (sd)	5.7 (0.54)	5.5 (0.9)	7.5 (2.94)	< 0.001	0.002	< 0.001	0.746
Creatinine, μmol/L	mean (sd)	77.6 (13.8)	67 (8.6)	92.4 (37.6)	0.041	0.999	0.05	0.306
Aspartate Aminotransferase (AST), U/L	mean (sd)	15.13 (4.8)	17.68 (4.25)	38.5 (26)	< 0.001[b,c]	0.463	< 0.001	< 0.001
Alanine Aminotransferase (ALT), U/L	mean (sd)	18.4 (13.9)	21.22 (8.09)	43.9 (52)	< 0.001[b,c]	0.999	< 0.001	0.019
Ferritin, μg/L	mean (sd)	–	147 (164)	889 (1109)				

n.a.:non applicable; sd: standard deviation.
[a] Bonferroni correction was applied to the p value;
[b] logarithmic transformation was applied to analyzed data;
[c] Ancova model was applied adjusting for age and sex

considered.

Pearson's correlation was calculated to evaluate the association between the variables considered. Benjamini-Hochberg (BH) procedure was run to control the false discovery rate; an adjusted p value is reported.

To analyze the association of each metal with the overall survival, a Cox regression model was performed. A multivariable model was performed considering only the variables with a $p < 0.10$ at the univariable analysis. The best subset was select by the Stepwise forward selection method. Results were presented as Hazard Ratio (HR) and 95% Confidence Interval (95% CI). A p value < 0.05 was considered statistically significant.

All statistical analyses were performed by applying R (version 4.1.1) and R Studio (version 2021.09.0) software.

3. Results

The present study included a total of 97 patients confirmed with COVID-19, comprising 75 hospitalized patients in the acute phase and 22 clinically stable patients evaluated at the Hospital three months after their acute phase. Five patients had Type 2 diabetes. The data of the laboratory tests were compared with those of 68 healthy controls (Table 1). The three groups differed for age and sex distribution, so the statistical analyses were adjusted for these confounders. In comparison with the non-acute patients and the healthy controls, the acute patients had a lower level of hemoglobulin [$F(2, 153) = 8.584$, $p < 0.001$], and Alb [$F(2, 147) = 20.655$, $p < 0.001$], and higher levels of Gluc [$F(2, 150) = 14.859$), $p < 0.001$)], creatinine [$F(2, 155) = 8.254$, $p < 0.001$], as well as liver transaminases [AST ($F(2, 144) = 41.95$, $p < 0.001$; ALT ($F(2, 144) = 18.12$, $p < 0.001$), Table 1) and CRP [($F(2, 160) = 138.57$, $p < 0.001$)] (Table 2; Fig. 1).

A significant difference in NLR was also observed, after adjusting for sex and age (logarithmic scale) [$F(2160) = 20.85$, $p < 0.001$], with NLR being at about three times higher in the acute patients than in both non-acute patients and healthy controls (Table 1). D-Dimer was not changed between acute and non-acute groups [$F(1,69) = 1.424$, $p = 0.237$].

Adjusting for sex and age, Cu, Zn and the Cu:Zn ratio were significantly different across the groups (Table 2); $F(2, 160) = 12.38$, $p < 0.001$)]. Acute patients had on average higher Cu values than both non-acute patients ($p = 0.012$) and healthy controls ($p < 0.001$), while the difference between non-acute patients and healthy controls was not significant ($p = 0.999$; Fig. 1). Also, Zn concentrations differed across the groups [$F(2, 160) = 3.69$, $p = 0.027$]. Acute patients had the lowest Zn levels of all groups, with a significant difference from non-acute patients ($p = 0.023$), who did not differ from the healthy controls ($p = 0.999$; Fig. 1). The Cu:Zn ratio was different across the groups [$F(2, 160) = 11.7$, $p < 0.001$]. Specifically, acute patients presented higher mean value of Cu:Zn ratio than both non-acute patients ($p = 0.001$) and healthy controls ($p < 0.001$), who instead showed no difference from each other (Table 2; Fig. 1).

We then explored in each group the percentage of individuals who had values that fell outside the normal reference ranges of the biochemical indicators under investigation (Table 3). The percentage of patients with abnormal CRP values differed across the groups: 88% of

Table 2
Serum concentrations of the biochemical indicators under study in the patients with COVID-19 and in the healthy controls.

		Healthy controls	Non-acute	Acute		Post comparisons[a]		
	n	68	22	75	p	Non-acute vs Healthy controls	Acute vs Healthy controls	Non-acute vs acute
Age		53.7 (12.84)	46.2 (14.81)	62.5 (14.91)	< 0.001	0.093	0.001	< 0.001
Sex, Male	n(%)	32 (47%)	4 (18.2%)	39 (52%)	0.019			
C-Reactive Protein (CRP), serum,mg/L	Mediana (25–75th percentiles)	2.42 (1.64–3.25)	2 (1–4)	50 (15.2–86)	< 0.001[b,c]	0.646	< 0.001	< 0.001
Cu, serum, µmol/L	mean (sd)	15.8 (2.78)	16.2 (2.41)	19 (4.54)	< 0.001[c]	0.999	< 0.001	0.012
Zn, serum, µmol/L	mean (sd)	12.8 (1.71)	14.9 (3.72)	12 (3.71)	0.027[c]	0.063	0.999	0.023
Cu:Zn ratio, serum, adimentional	Median (25–75th percentiles)	1.22 (1.10–1.35)	1.06 (0.91–1.33)	1.72 (1.21–2.35)	< 0.001[b,c]	0.999	< 0.001	0.001

sd: standard deviation
[a] Bonferroni correction was applied to the p value.
[b] logarithmic transformation was applied to analyzed data.
[c] Ancova model was applied adjusting for age and sex.

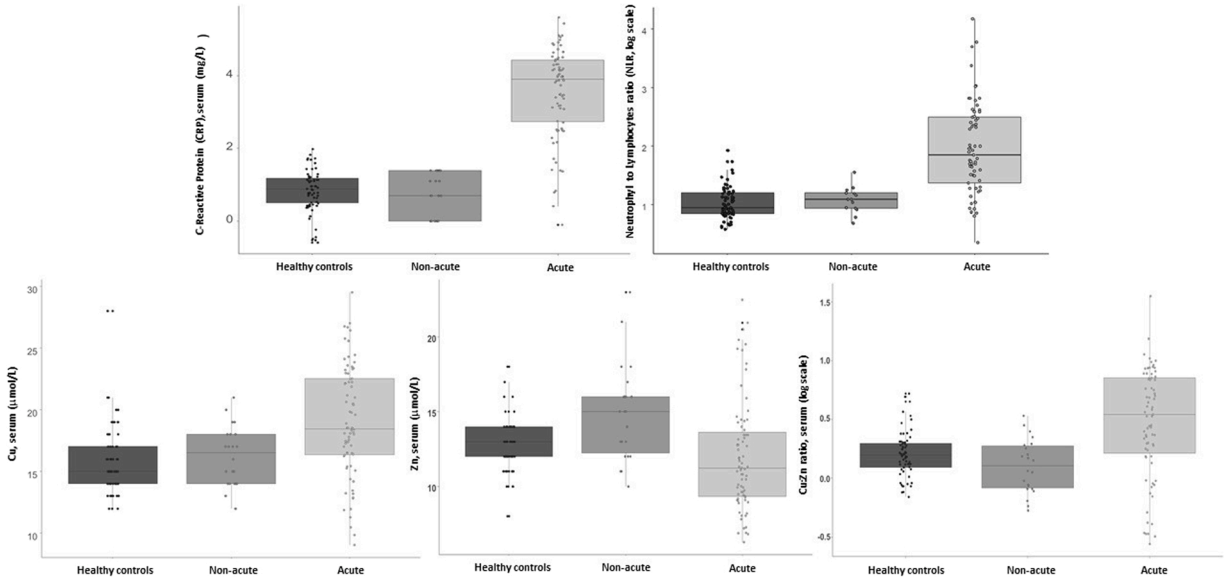

Fig. 1. C-Reactive Protein (CRP), neutrophil to lymphocyte ratio (NLR), serum metal levels, and Cu:Zn ratio in COVID-19 patients and healthy controls in relation to the disease state. Data are expressed as mean ± standard deviation (sd).

Table 3
Association between diagnosis groups and the number of individuals with values outside of the reference range in CRP, Cu and Zn.

Number of individuals with		Healthy controls 68	Non-acute 22	Acute patients 75	p value
CRP values outside of the reference range	n (%)	2 (2.9%)	0 (0%)	66 (88%)	< 0.001
Cu outside of the reference range	n (%)	13 (19.1%)	2 (9.1%)	17 (22.7%)	0.398[a]
Zn values outside of the reference range	n (%)	21 (30.9%)	5 (22.7%)	44 (58.7%)	< 0.001

[a] Fisher exact test

the acute patients, none of the non-acute patients and 3% of the healthy controls had CRP values exceeding the upper reference limit (Chi-square $p < 0.001$). Furthermore, 58.7% of the acute patients had values of Zn lower than the reference range, thus being the highest percentage among the three groups, with 30.9% in the healthy controls and 22.7% in the non-acute patients (Table 3). According to the Recommendation for Analysis in Trace Element SAS Handbook [30] cut-off value for zinc deficiency is set to < 7.7 µmol/L, while possible deficiency with no clinical significance is fixed in the range 7.7–10.7 µmol/L. On the basis of these criteria for Zn deficiency, in the present study 0% of both non-acute patients and healthy controls had Zn levels less than 7.7 µmol/L vs. 8% of the acute patients. Conversely, 8.8% of healthy controls had values in the range 7.7–10.7, vs. 31.8% and 33.3% of the patients in the non-acute and in the acute phase, respectively.

Considering only the acute patients, we evaluated the number of individuals with values outside the reference range of the laboratory variables of interest (Table 4).

We sought evidence for association between routine laboratory indicators (hematological, biochemical, hemostasis) and the metal profile in the COVID-19 acute phase (Fig. 2). Zn associated with Cu ($r = -0.44$, p and BH adjusted $p < 0.001$), NLR ($r = -0.43$, $p = 0.004$; BH adjusted $p = 0.007$) and CRP ($r = -0.49$, p and BH adjusted $p < 0.001$). Cu was significantly associated with CRP ($r = 0.44$, p and BH adjusted $p < 0.001$). Of note, an associations between NLR and D-Dimer, Cu:Zn and CRP also appeared. Finally, Ferritin was significantly correlated with D-Dimer and CRP (Fig. 2).

Age was negatively correlated with Zn ($r = -0.37$, $p = 0.001$) and positively with the ratio Cu:Zn (logarithmic scale) ($r = 0.34$, $p = 0.003$), NLR ($r = 0.415$, $p = 0.003$), D-Dimer ($r = 0.24$, $p = 0.036$) and CRP (logarithmic scale) ($r = 0.29$, $p = 0.008$).

Table 4
Number (%) of the acute COVID-19 patients with values of the studied biological variables outside the reference range.

Subjects with	Acute patients n = 75 (%)
CRP values outside of the reference interval	66 (88%)
Cu values outside of the reference interval	
less than the lower limit of the reference interval (<12.3 **μmol/L**)	9 (12%)
higher than the upper limit of the reference interval (>24.3 **μmol/L**)	8 (10.7%)
Zn values outside of the reference interval (12–24 μmol/L)	
less than the lower limit of the reference interval **(<12 μmol/L)**	44 (58.7%)
higher than the upper limit of the reference interval **(>24 μmol/L)**	- (0%)
%SatO_2cut off < 95%	52 (89.7%) n = 43
PCT low risk (<0.5 ng/mL)	37 (86%) n = 67
D-Dimer values outside of the reference interval (reference value to exclude deep vein thrombosis and pulmonary embolism <0.55 mg/L)	39 (58.2%)
NLR outside of the reference interval (2.5–3)	n = 44
less than the lower **lower** limit of the reference interval (<2.5)	9 (20.5%)
higher than the upper **upper** limit of the reference interval (>3)	29 (65.9%)
Ferritin outside of the reference interval (M:40–280 μg/L; F:30–140 μg/L)	n = 64
less than the lower lower **lower** limit of the reference interval	1 (1.6%)
higher than the upper **upper** limit of the reference interval	51 (79.7%)
Ferritin values> 500 μg/L	34 (53.1%)

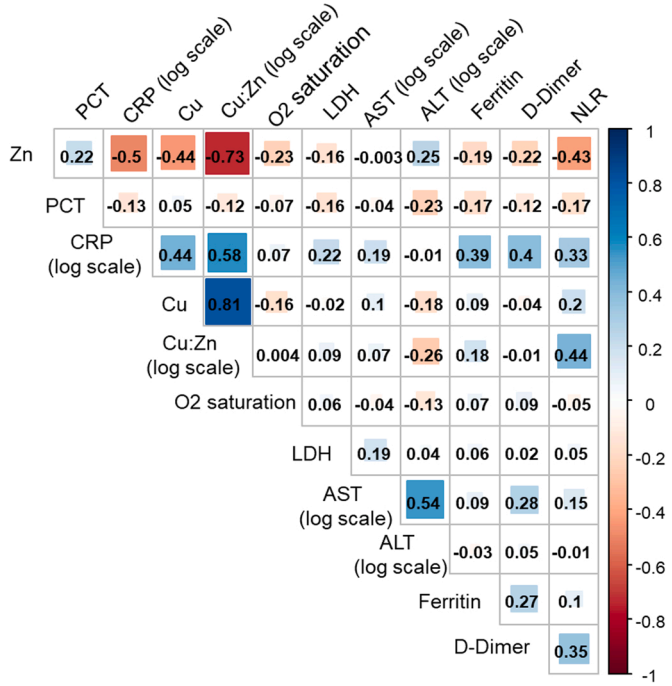

Fig. 2. Correlogram of the associations among the biochemical indicators and the metals under study. Spearman correlations coefficients of significant correlations are reported in the correlogram, Blue and red color represent positively and negatively correlations, respectively. Non-significant correlation coefficients are reported in blank.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Partial correlations were also performed, checking for the effect of age: while the correlations between Zn and NLR ($r = -0.34$, $p = 0.023$), Zn and CRP ($r = -0.44$, $p < 0.001$), Cu and CRP ($r = 0.363$, $p < 0.001$), Cu and NLR ($r = 0.387$, $p < 0.001$), Cu:Zn ratio and NLR ($r = 0.579$, $p < 0.001$) and Cu:Zn ratio and CRP ($r = 0.509$, $p < 0.001$) were maintained or even increased, those between NLR and D-Dimer ($r = 0.26$, $p = 0.118$), and NLR and CRP ($r = 0.24$, $p = 0.128$) were not. D-Dimer was significantly correlated with CRP ($r = 0.385$, $p = 0.001$).

Interestingly, none of the associations assessed in the acute patients were evident in the non-acute ones and in the healthy controls (data not shown, all $p > 0.1$). For the acute patients, we assessed the association between the prolongation of the hospital stay (days) and the outcome in terms of recovery or death. The information was available for 69 patients. The mean prolongation of stay in the hospital was 11.5 days (sd=5.51) (Table 1), median equal to 10 days (25–75th percentiles: 8–15).

Setting a cut-off of 10 days (the estimated number of days for the treatment of uncomplicated pneumonia), 52% (n = 39) of patients stayed in the hospital for less than or equal to 10 days and the remaining 40% (n = 30) more the 10 days.

15% of patients (n = 11/75) died.

The days of hospitalization (on a logarithmic scale) were neither correlated with age ($r = 0.05$, $p = 0.677$; B-H adjusted $p = 0.894$) nor with the biochemical variables under study (all $p > 0.2$) except Ferritin ($r = 0.32$, $p = 0.012$); however, the correlation did not survive when the BH correction of the p-value was applied (BH adjusted $p = 0.12$).

A univariable analysis to assess the association between mortality rate (dependent variable) and biological variables under study (independent variables) was run (Table 5). Overall, a survival of 41% was observed (SE=29.2%). The model revealed that the mortality rate was significantly associated with increasing age (HR=1.07, 95% CI 1.02–1.14), higher NLR values (HR=1.09, 95% CI 1.03–1.16), lower Zn values (HR=0.63, 95% CI 0.44–0.88), higher Cu/Zn ratio values (HR=3.89, 95% CI 1.25–12.07) and increased levels of D-Dimer (HR=1.48, 95% CI 1.004–2.17). Among these variables, only the NLR was individuated by the stepwise forward selection method (HR= 1.09, 95% CI 1.03–1.16, $p = 0.005$) as significantly associated with the rate of mortality in the multivariable model.

4. Discussion

In this study, we sought possible associations among clinical outcomes, routine clinical laboratory indicators, and serum levels of Cu and Zn and their ratio Cu:Zn, in acute patients and in clinically stable COVID-19 patients evaluated 3 months after their first admission to the hospital. The main result of the study is that both Cu levels and the Cu:Zn ratio increased while Zn levels decreased in the acute patients, which was the group with the lowest levels of Zn. Even though the mean and median values of Zn in the acute phase did not reach the statistical threshold with respect to the healthy controls, the number of acute patients with values of Zn lower than the reference range doubled. This

Table 5
univariable and multivariable analyses to assess the association between the mortality rate and the biological variables under study.

Independent variable		Univariable		
		HR	95% CI	p
Sex	M	Reference		
	F	2.14	0.53–8.55	0.283
Age	Years	1.07	1.02–1.14	0.011
CRP	Serum, mg/L	1.01	0.99–1.02	0.162
NLR	Blood, adimentional	1.09	1.03–1.16	0.005
Cu	Serum, μmol/L	1.03	0.89–1.19	0.673
Zn	Serum, μmol/L	0.63	0.44–0.88	0.007
Cu/Zn ratio	Serum, Adimentional	3.89	1.25–12.07	0.019
D-Dimer	Plasma, mg/L	1.48	1.004–2.17	0.048
AST	Serum, U/L	1.01	0.98–1.03	0.694
ALT	Serum, U/L	0.97	0.92–1.01	0.159
Ferritin	Serum, μg/L	1	0.99–1.00	0.93

observation suggests skewed Zn values in the acute COVID-19 phase with approximately 60% of the patients showing values below the reference range. The comparatively high number (30%) of healthy controls with Zn values less than the lower reference limit can be ascribed to certain regional variations in the Bulgarian population as the city of Sofia and its surroundings are among the regions with the lowest values of serum Zn [31]. However, none of the healthy controls had values below the cut-off for Zn deficiency (< 7.7 μmol/L), and only about 9% of them showed values in the range of possible deficiency (7.7–10.7μmol/L) but with no clinical significance [30]. On this basis, it can be said that our result of an increased percentage of acute patients with serum Zn levels lower than the reference interval is in line with earlier studies reporting an association between Zn deficiency and COVID-19: Zn-deficient patients were typified by higher rate of COVID-19 complications and longer hospital stays [32]. Furthermore, lower Zn levels were observed in SARS-CoV-2 positive pregnant women and the alteration of the biometal profile was clearly associated with the disease severity [12,13,23].

We noted that in patients of the non-acute group, Zn levels were higher than in healthy controls. In this regard, we speculate that affected individuals might start taking supplements with zinc, selenium and vitamin D when they tested positive for COVID-19, which has been a common practice during the pandemic.

It must be remembered that Cu and Zn are also crucial essential trace elements with a major role in the antioxidant defense system [9,10,33].

Regarding the Cu:Zn ratio, it has been shown that it is associated with a severer disease state, and it has been advocated as a predictor of a lower oxygen saturation [12]. We extended this knowledge, showing that Cu and Zn alterations occur very early during the COVID-19 disease course and are mainly associated with the inflammation response. In fact, another result of the current research is the association of Cu, Zn and Cu:Zn ratio with routine clinical laboratory indicators during the acute phase of the disease. Specifically, the present study demonstrates an association between serum Zn, Cu and Cu:Zn ratio with CRP and NLR, even after correcting for the effect of age, different from the correlation between NLR and CRP that was lost after correcting for age. Interestingly all the associations described were not present in the in clinically stable non-acute patients.

CRP is an important clinical evaluation index in the early stage of COVID-19: it can reflect the extent of disease severity and damages, which is especially useful for patients unsuitable to be referred to other facilities or in critical condition [34].

Immune activation and damaged endothelial cells often lead to inflammation and stimulated CRP synthesis. Due to this reason, in clinical practice, CRP is the most commonly assayed biomarker of acute and chronic inflammation [35]. In agreement with the proinflammatory activity of Cu, the present study exhibited an association between serum Cu levels and COVID-19 severity, including circulating CRP level [36].

NLR has been shown to be a systemic inflammatory marker, a potential predictor of clinical risk and outcome in many diseases and a significant prognostic biomarker of outcomes in critically ill COVID-19 patients [6]. COVID-19 infection, especially in the acute phase, is associated with a severe dysregulation of inflammatory processes by overactivation and outbreak of an immunological/cytokine storm. Cu and Zn have an impact on processes involved in cell-mediated immune reactions, of both innate and adaptative immune system [9,10,33]. The typical depletion of Zn from circulation after stress can be associated with an increase in liver Zn content (review in [37–39]), which in turn facilitates an increase of the biosynthesis of metallothioneins (MTs), i.e., low-molecular cysteine-rich metal-binding proteins. It is known that liver protein synthesis is induced by Interleukin (IL) − 1, IL-6, interferon, Tumor necrosis factor in response to inflammation and stress. MTs act as reservoir for zinc in intracellular deficiency and as a buffering agent in zinc excess to prevent possible toxicity. MTs regulate maturations of diverse immune cells and the signaling pathways. As acute-phase reactants, they are also involved in inflammation (review in [38,39]), coping with the oxidant challenge induced by the infection. MTs antioxidant properties are mainly due to the ability of the sulfhydryl groups to react easily with oxidants and electrophiles. Also, the binding of metals with Fenton reactivity (Fe, Cu) can reduce oxidative stress. Stimulated expression of MTs increases the resistance of tissues and cells to the highly toxic free radicals. The reactivity and coordination dynamics of MTs with Zn^{2+} and Cu^{+} are highly dependent on the regulation of metal intracellular distribution according to the biological requirements [39]. MTs present a unique adaptive mechanism of physiological buffering of the most competitive ions of the essential trace elements Zn and Cu [39].

Current results are in line both with the proposed role in Zn-mediated immune modulatory response and with the role of Cu during inflammation. The Cu:Zn ratio increase in the acute COVID-19 patients suggests Cu and Zn mobilization and redistribution starting in the very early stages of COVID-19 infection and likely involving the utilization of metals from depots in the liver in processes of activation of pro-inflammatory cytokines and of induction of acute phase reactants (for specialized literature refer to [23]), induced by IL-1 [40]. The role of ceruloplasmin during infections can be related to reduction of oxidative stress, via scavenging reactive oxidant species. Higher levels of Cu can, in fact, cause oxidative stress due to involvement of Cu^{2+}in Fenton-type chemistry, producing highly damaging hydroxyl radicals [41], which can further exacerbate the COVID-19 severity. Fenton reaction can be triggered by excess of Fe as well that is generally high during infections. In this condition ferritin concentrations increase and reduce free Fe, as also supported by our result of high percentage of acute patients with ferritin levels higher than the upper value of the reference range in the acute patient group. Ferritin was also the sole biological variable associated with the prolongation of the hospitalization, even though the association was weak and did not survive at the BH correction. In the same line, the study of the association with mortality rate revealed a four-fold increase of likelihood of mortality associated with Cu:Zn ratio and a 9% increase with the NLR. However, these results should be considered explorative and must be taken with caution. In fact, our study has a number of limitations, consisting primarily in the small sample size and in the lack of a follow-up. Nevertheless, by confirming previously published data and by assessing a Cu and Zn mobilization in the very early stages of the disease, this research supports the opinion of a possible beneficial effect of Zn supplementation in COVID-19 [16,23,42].

CRediT authorship contribution statement

The content of the paper has not been yet published or submitted for publication elsewhere. Authors also confirm that all authors have contributed significantly, and that all authors are in agreement with the content of the manuscript. Specifically, **Irena Ivanova:** Conceptualization, Methodology, Writing – original draft, and Writing – review & editing. **Ilaria Simonelli:** Software, Data curation, Statistical analyses, Visualization. **Amit Pal:** Writing – original draft, Writing – review & editing. **Mauro Rongioletti:** Writing – original draft, Writing – review & editing. **Mariacarla Ventriglia:** Writing – original draft, Writing – review & editing. **Bisera Atanasova:** Writing – review & editing. **Rosanna Squitti:** Conceptualization, Writing – original draft, Writing – review & editing, Visualization.

Conflicts of interest

RS is Chief Scientific Officer of IGEA Pharma N.V.; she has some shares in IGEA Pharma N.V. Other authors declare no commercial or noncommercial conflicts of interest relating to this work.

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