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Article

SERUM LEVEL OF CERULOPLASMIN, ANGIOTENSIN CONVERTING ENZYME AND TRANSFERIN AS MARKERS OF SEVERITY IN SARS-COV-2 INFECTION IN PATIENTS WITH TYPE 2 DIABETES

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Abstract: The aim of this study was to analyze other possible new markers of severity that can be assessed in patients with type 2 diabetes and SARS-CoV-2 (Severe acute respiratory syndrome coronavirus 2) infection. Our study included 90 subjects: 45 patients with type 2 diabetes mellitus and infected with the SARS-CoV-2 virus, and 45 healthy persons as control. The serum level of ceruloplasmin at hospital admission was statistically significant directly correlated with the severity of COVID-19 (Coronavirus disease) (p=0.037) and with the serum level of IL-6 (Interleukine 6) (p=0.000). Ceruloplasmin was statistically significant inversely correlated with serum iron level (p=0.000). However, we observed that ACE (Angiotensin converting enzyme) decreased in severe forms of the infection with SARS-CoV-2 virus in patients with type 2 diabetes (p=0.001).   Moreover, the decrease in ACE level was correlated with an increase in IL-6 level in these patients (p=0.001). IL-6 increase was statistically significant inversely correlated with serum iron, transferrin and ACE levels. There was a noticed a decreasing tendency of the transferrin depending on the severity of the COVID-19 infection (p=0.000). In addition to the known severity factors in the context of infection with the new coronavirus, decreased concentrations of ceruloplasmin, ACE and transferrin may represent new markers of COVID-19 severity in patients with type 2 diabetes. These parameters analyzed at the hospital admission could show the evolution towards the severe forms of infection with the SARS-CoV-2 virus.

Keywords: ceruloplasmin; Angiotensin Converting Enzyme; IL-6; transferrin; COVID-19; type 2 diabetes; SARS-CoV-2; serum iron

1. Introduction

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2 of 20

In SARS-CoV-2 virus infection there are certain parameters, presented in the specialized literature, that can be used as markers of severity in infected patients [1]. Since the beginning of the COVID-19 pandemic, advanced age has been established among the risk factors, as well as the association of certain comorbidities, including type 2 diabetes [2-7]. Other severity parameters in the infection with the new coronavirus were: hyperferritinemia, increased d-dimers, increased CRP (C reactive proteine) and decreased number of lymphocytes [8-17]. In addition to increased ferritin, regarding iron metabolism in COVID-19, decreased serum iron was associated with the severity of infection [18-20]. The link between decreased transferrin, hyperferritinemia and increased CRP was associated with proinflammatory status and progression to severe forms of the disease [21-23]. The study of ceruloplasmin in SARS-CoV-2 virus infection is a topic of interest because there are studies that describe the fact that ceruloplasmin can act within the human body, in healthy people, both with an antioxidant role, but also with a pro-oxidant role [24-26]. Ceruloplasmin is a glycoprotein synthesized in the liver and has 6 copper atoms in its composition, with a particularly important role in iron metabolism, oxidizing ferrous iron to ferric iron, the ferric iron form being the only one that can be bound to transferrin [27, 28]. It is known that ceruloplasmin can also act as an acute phase reactant, and ceruloplasmin levels are increased in inflammation, being an independent risk factor for complications associated with diabetes [29,30]. Most of the copper in the body, *i.e.*, 90%, is bound to ceruloplasmin and 10% is bound to albumin [31]. Type 2 diabetes is a metabolic condition characterized by a chronic inflammatory state in which serum ceruloplasmin is elevated, but ceruloplasmin is not correlated with diabetes severity [32-35]. In type 2 diabetes, insulin resistance occurs due to endothelial dysfunction, which is associated with increased ROS (Reactive oxygen species) production [36-38]. The imbalance between ROS and antioxidants is represented by oxidative stress, one of the most important factors in the pathogenesis of type 2 diabetes [39,40]. Oxidative stress affects pancreatic beta-cell function and apoptosis [41-43]. Iron excess is toxic and pro-oxidant, and its accumulation causes inflammation of pancreatic beta cells and is a risk factor for diabetes development and evolution, and a risk of mortality [44-46]. Iron homeostasis is regulated by hepcidin and ferroportin, which modulates the flux of iron into the systemic circulation [47-49]. Iron deposits are mainly represented by ferritin, which can also play a role as an acute phase reactant, hyperferritinemia being significantly associated with the severity and unfavorable prognosis in the COVID-19 infection. [50,51]. Decreased transferrin level was strongly associated with severe forms of SARS-CoV-2 infection, with transferrin representing the transport of circulating iron [52].

The aim of this study was to identify other possible biomarkers of severity in SARS-CoV-2 virus infection, in patients with type 2 diabetes as an associated comorbidity.

2. Material and Methods

2.1. Study design

The study was based on a total of 90 subjects, divided in a group of 45 patients hospitalized at the Clinical Hospital "Gavril Curteanu" Oradea, between October 21, 2021 and December 31, 2021, randomly selected, having as the main inclusion criteria the positive RT-PCR (Reverse transcription polymerase chain reaction) rapid antigen test for viral infection and the diagnosis of type 2 diabetes. And the control group of 45 healthy persons randomly chosen, with a negative RT-PCR/rapid antigen test as the inclusion criterion and who were not known with diabetes or other associated pathologies. The present study was conducted in accordance with the Declaration of Helsinki. Before taking part in this study, each participant gave a written informed consent for inclusion. The study protocol was approved by the Ethics Committee of the Clinical Hospital "Gavril Curteanu" Oradea (No. 32652/16.11.2020) and by the Ethics Committee of the University of Oradea (No. 5/A, 21.09.2020).

The inclusion criteria in the group of patients were the diagnosis of type 2 diabetes and SARS-CoV-2 virus infection confirmed by positive RT-PCR/rapid antigen test. Exclusion criteria from the group of patients included: absence of type 2 diabetes, absence of infection with the SARS-CoV-2 virus by negative RT-PCR/rapid antigen test.

Inclusion criteria in the control group were healthy individuals, absence of any diseases or medication, including absence of type 2 diabetes and negative RT-PCR/rapid antigen test.

2.2. Data collection

The data were collected in an EXCEL file including the following biomedical parameters: iron biomarkers (ceruloplasmin, serum iron, sideremia, transferrin), anthropometric parameters - age (years), parameters regarding the severity of COVID-19 (absent=0, mild=1, moderate=2, severe=3), Angiotensin-converting enzyme (ACE) and interleukin 6 (IL-6). In the group of patients with type 2 diabetes mellitus and SARS-CoV-2 virus infection, we also evaluated the relationship between certain parameters of iron metabolism (including serum iron, transferrin, ceruloplasmin, hemoglobin, number of erythrocytes, ferritin), coagulation parameters (INR - International normalized ratio, platelets number) and inflammation parameters (CRP, fibrinogen, LDH - Lactate dehydrogenase and lymphocytes number). We analyzed the regression models that reflected the connection and influence of independent parameters such as coagulation and inflammation on serum iron levels at hospital admission. We also evaluated if the gender of the subjects influenced the level of the biomedical parameters considered in our study.

2.3. Methods

Ceruloplasmin determination was achieved by using the nephelometric method (reagent N Antiserum to Human Ceruloplasmin, ref. 10446451, BN ProSpec analyzer, Siemens Healthineers). Spectrophotometric method was employed for sideremia measurement (Iron_2 reagent, ref.10377510, Advia 1800 analyzer, Siemens Healthineers). Transferrin was determined by using the nephelometric method (reagent N Antiserum to Human Transferrin, ref. 10446309, BN ProSpec analyzer, Siemens Healthineers). The ACE biomarker was determined by the spectrophotometric method (Angiotensin Converting Enzyme (ACE) reagent, ref. 12796, analyzer A15, BioSystems SA, Spain). Interleukin 6 was quantified by the electrochemiluminescence method (ECLIA = electrochemiluminescence immunoassay), (IL-6 reagent, ref. 05109442190, Cobas E4111 analyzer, Roche Diagnostics GmbH). All these biomarkers were analyzed from blood samples collected at the time of hospital admission.

2.4. Statistical analysis

In the statistical processing of the data, both descriptive statistics procedures were used to calculate averages, standard deviations (for quantitative data), frequencies (for qualitative data), correlation coefficients, graphic representations of the data, as well as statistical analysis procedures. Concerning the data statistical analysis, statistical tests appropriate to the analysis performed were used (Student test, ANOVA test, Student test for paired samples, the chi square test, normality tests (Kolmogorov-Smirnov), non-parametric tests (Kruskal-Wallis), as well as the regression analysis. In order to evaluate the intensity of the correlation of two variables (quantitative and/or order), the following empirical Colton rule was used: for the interpretation of the correlation coefficient value for two quantitative variables, both the parametric Pearson (R - used when both variables are approximately normally distributed), as well as the non-parametric Spearman (Ro - used when at least one of the variables is not normally distributed). The threshold of statistical significance of the tests used in the statistical analyses was the one usually applied in medical research p=0.05 (=5%). The significance threshold p=0.01 was also used for the case when the result of the analysis was strongly significant (p<0.01). To perform the statistical calculations, the EXCEL data file which contained the study data, was converted into an SPSS file, the statistical processing was performed with the statistical software SPSS version 20.

3. Results

In the comparative analysis between infected patients and healthy persons, we denoted with 1 the group of patients with type 2 diabetes mellitus and SARS-CoV-2 virus infection, and with 2 the control group of healthy subjects. In group 1 were 5 mild, 13 moderate and 27 severe forms of SARS-CoV-2 viral infection on CT (Computer tomography) at hospital admission.

We identified a significant statistical difference between the means of the quantitative parameters in the group of infected patients, compared to the group of healthy subjects. The means of IL-6 and ceruloplasmin parameters in patients infected with the SARS-CoV-2 virus were statistically significantly higher than in the control group. Regarding the following parameters such as: serum iron, ACE and transferrin, the means for patients infected with the novel coronavirus were statistically significantly lower than in healthy subjects (Table 1).

Table 1. Comparison between patients with COVID-19 and healthy subjects for the following parameters (age, IL-6, serum iron, ACE, ceruloplasmin, transferrin).

SAMPLE		N	Mean	Std. Deviation	p	Test
Age (years)	1	45	69.600	11.727	0.000	T
	2	45	55.000	3.126		
IL-6 (pg/mL)	1	45	77.843	158.320	0.000	M-W
	2	45	2.052	1.448		
Serum iron (µg/dL)	1	45	56.156	31.321	0.000	T
	2	45	93.155	28.035		
ACE (U/L)	1	44	20.891	17.312	0.018	T
	2	45	28.099	9.616		
Ceruloplasmin (g/L)	1	44	0.325	0.081	0.000	T
	2	45	0.273	0.040		
Transferrin (g/L)	1	44	1.761	0.486	0.000	T
	2	45	2.707	0.254		

1 = the group of patients with type 2 diabetes and SARS-CoV-2 virus infection

2 = control group of healthy subjects.

N = number of evaluated persons.

p = The threshold for statistical significance of the tests used in statistical analyses is the one usually applied in medical research p=0.05,.and p < 0.001 is strongly significant.

The serum ceruloplasmin was statistically significant inversely correlated with the severity of SARS-CoV-2 infection, but also with IL-6 and serum iron levels. However, ceruloplasmin did not statistically significantly correlate with transferrin, ACE or age (Table 2). We identified an increasing tendency of the ceruloplasmin concentration depending on the level of infection with COVID-19 (Figure 1).

Table 2. Spearman correlations (Ro) of ceruloplasmin.

Evaluated parameters	Ceruloplasmin		
	Ro	p	N
COVID-19 severity	**.222***	**.037**	89
Age (years)	.162	.130	89
IL-6 (pg/mL)	**.425****	**.000**	89
Serum iron (µg/dL)	**-.530****	**.000**	89
ACE (U/L)	-.105	.329	89
Transferrin (g/L)	-.055	.610	89

Ro = Spearman type correlations (used when at least one of the variables is not normally distributed) and minus means inverse correlation.

* = correlation significance < 0.05.

** = correlation significance <0.01.

p = The threshold for statistical significance of the tests used in statistical analyses is the one usually applied in medical research p=0.05 (=5%).

N= number of evaluated persons.

IL-6= interleukin 6.
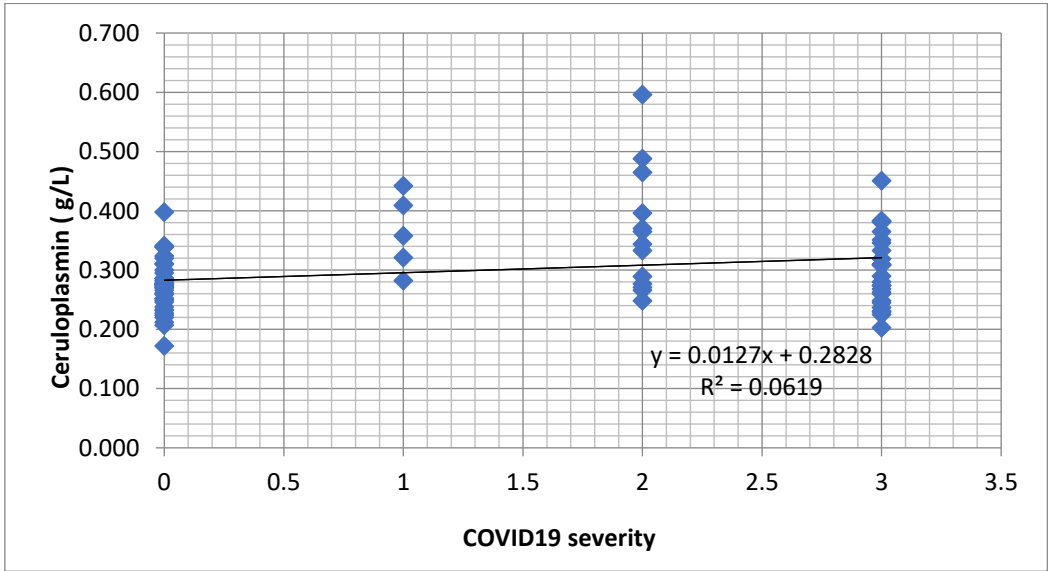
ACE= angiotensin-converting enzyme.

Figure 1. Regression function associated with the scatter plot for ceruloplasmin and severity of COVID-19 (an increasing tendency of the ceruloplasmin concentration depending on the level of infection).

In the case of serum iron level (sideremia), this parameter correlated strongly significantly (p < 0.001) and inversely with the severity of COVID-19, ceruloplasmin, age and IL-6 (Table 3). A decreasing tendency of serum iron values was observed in dependence with the severity of the COVID-19 infection (Figure 2).

Table 3. Spearman correlations (Ro) of the serum iron level.

Evaluated parameters	Serum iron		
	Ro	p	N
COVID-19 severity	-.476**	.000	90
Age (years)	-.358**	.001	90
IL-6 (pg/mL)	-.617**	.000	90
Ceruloplasmin (g/L)	-.530**	.000	89

Ro = Spearman type correlations (used when at least one of the variables is not normally distributed) and minus means inverse correlation.

** = correlation significance <0.01.

p = The threshold for statistical significance of the tests used in statistical analyses is the one usually applied in medical research p=0.05 (=5%), and p < 0.001 = strongly significant.
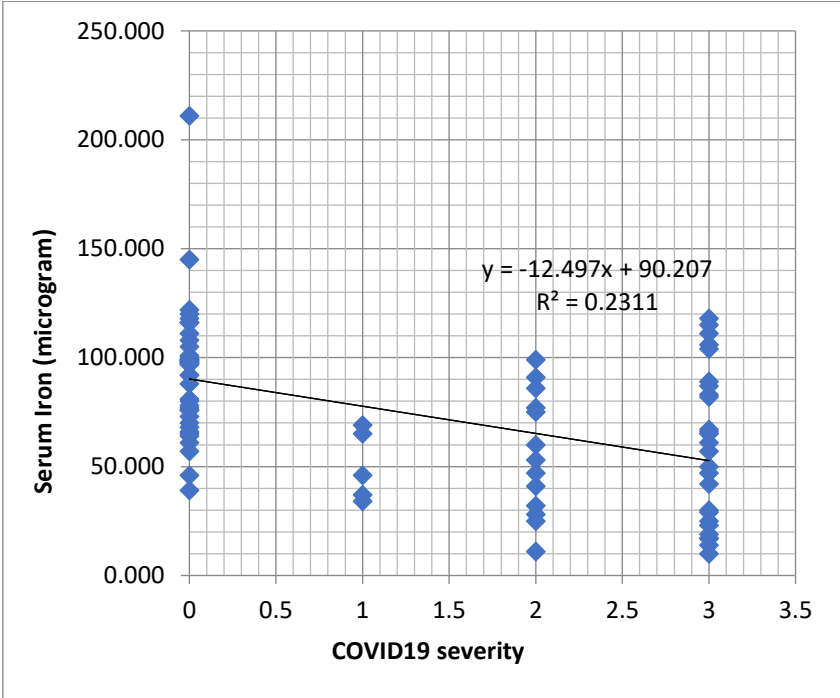
N = number of evaluated persons.

Figure 2. Regression function associated with the scatter plot for serum iron and the severity of COVID-19 (a decreasing tendency of serum iron values in dependence with the severity of the infection).

A strongly statistically significant, but inverse correlation with the severity of COVID-19, IL-6 and age was observed in this study (p=0.001). Transferrin did not correlate significantly with serum ceruloplasmin (Table 4). However, there was a decreasing tendency of the transferrin level depending on the severity of the COVID-19 infection (Figure 3).

Table 4. Spearman correlations (Ro) of the transferrin level.

Evaluated parameters	Transferrin		
	Ro	**p**	**N**
COVID-19 severity	**-.791****	**.000**	89
Age (years)	**-.504****	**.000**	89
IL-6 (pg/mL)	**-.668****	**.000**	89
Ceruloplasmin (g/L)	-.055	.610	89

Ro = Spearman type correlations (used when at least one of the variables is not normally distributed) and minus means inverse correlation.

** = correlation significance <0.01.

p = The threshold for statistical significance of the tests used in statistical analyses is the one usually applied in medical research p=0.05 (=5%), and p < 0.001 = strongly significant.

N = number of evaluated persons.

IL-6= interleukin 6.
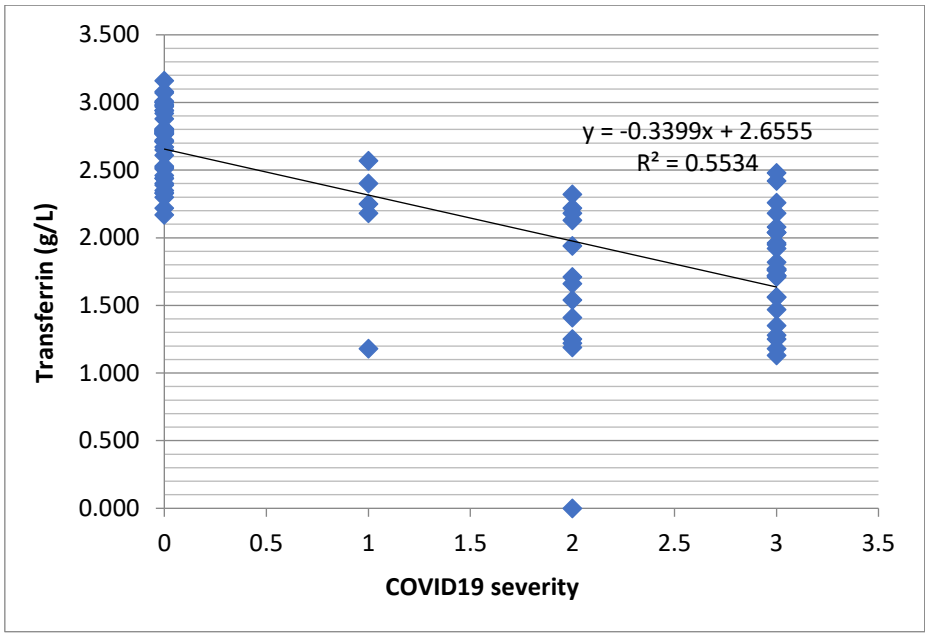
Figure 3. Regression function associated with the scatter plot for transferrin and severity of COVID-19 (the decreasing tendency of transferrin level depending on the severity of infection).

Age was strongly significant directly correlated with IL-6. However, age correlated strongly significantly, but inversely with ACE, transferrin and serum iron (Table 5).

Table 5. Spearman correlations (Ro) of age.

Evaluated parameters	Age		
	Ro	p	N
IL-6 (pg/mL)	.497**	.000	90
Serum iron (µg/dL)	-.358**	.001	90
ACE (U/L)	-.346**	.001	89
Ceruloplasmin (g/L)	.162	.130	89
Transferrin (g/L)	-.504**	.000	89

Ro = Spearman type correlations (used when at least one of the variables is not normally distributed) and minus means inverse correlation.

** = correlation significance <0.01.

p = The threshold for statistical significance of the tests used in statistical analyses is the one usually applied in medical research p=0.05 (=5%), and p < 0.001 = strongly significant.

N = number of evaluated persons.

IL-6= interleukin 6.

ACE= angiotensin-converting enzyme.

The ACE concentration strongly correlated statistically significantly (p=0.001) and inversely with the severity of COVID-19, age, and IL-6 (Table 6).

Table 6. Spearman correlations (Ro) of ACE level.

Evaluated parameters	Angiotensin-converting enzyme

	Ro	p	N
COVID-19 severity	**-.348****	**.001**	89
Age (years)	**-.346****	**.001**	89
IL-6 (pg/mL)	**-.349****	**.001**	89
Ceruloplasmin (g/L)	-.105	.329	89

Ro = Spearman type correlations (used when at least one of the variables is not normally distributed) and minus means inverse correlation.

** = correlation significance <0.01.

p = The threshold for statistical significance of the tests used in statistical analyses is the one usually applied in medical research p=0.05 (=5%), and p < 0.001 = strongly significant.

N = number of evaluated persons.

IL-6= interleukin 6 .

IL-6 correlated non-parametrically (Spearman) strongly statistically significant and directly with the severity of the COVID-19 infection, age and ceruloplasmin (p<0.001). An inverse correlation was noticed between IL-6 and transferrin, ACE and serum iron. (Table 7). An increasing tendency of the IL-6 concentration was observed in dependence with the severity of the COVID-19 infection (Figure 4).

Table 7. Spearman correlations (Ro) of the IL-6 level.

Evaluated parameters	IL-6		
	Ro	p	N
COVID-19 severity	**.819****	**.000**	90
Age (years)	**.497****	**.000**	90
Serum iron (µg/dL)	**-.617****	**.000**	90
ACE (U/L)	**-.349****	**.001**	89
Ceruloplasmin (g/L)	**.425****	**.000**	89
Transferrin (g/L)	**-.668****	**.000**	89

Ro = Spearman type correlations (used when at least one of the variables is not normally distributed) and minus means inverse correlation.

** = correlation significance <0.01.

p = The threshold for statistical significance of the tests used in statistical analyses is the one usually applied in medical research p=0.05 (=5%), and p < 0.001 = strongly significant.

N = number of evaluated persons.
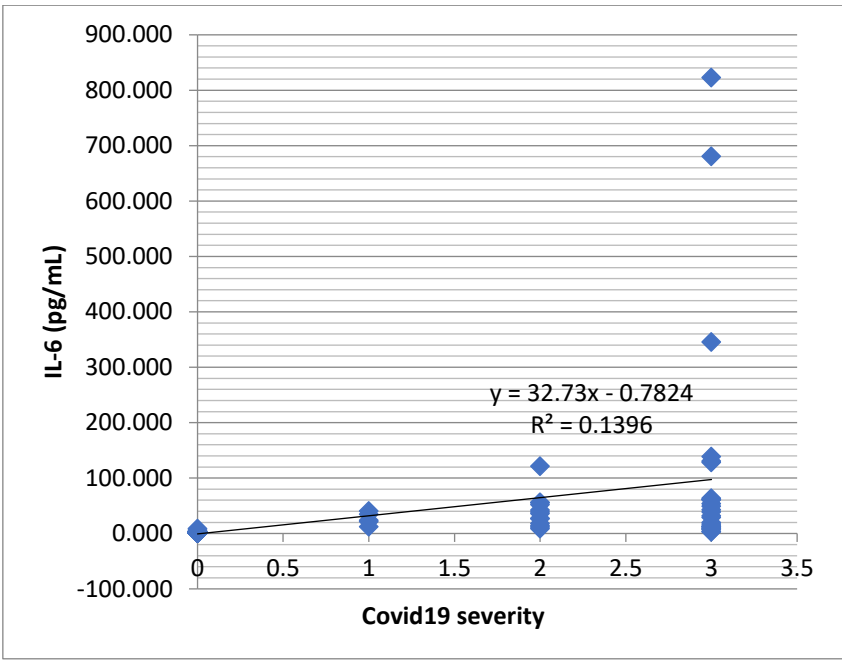
ACE= angiotensin-converting enzyme.

Figure 4. Regression function associated with the scatter plot for IL-6 and severity of COVID-19 (the increasing tendency of the IL-6 level depending on the severity of the infection).

The severity of COVID-19 correlated statistically significantly with all quantitative parameters of the study. Good ($|Ro|>0.5$) and very good ($|Ro|>0.75$) and strongly statistically significant correlations were established between the COVID-19 severity and IL-6, age, and transferrin level. Also, the severity of COVID-19 correlated directly ($Ro>0$) with IL-6, age, and ceruloplasmin level. Inverse correlation ($Ro<0$) appeared between the COVID-19 severity and serum iron, ACE and transferrin levels (Table 8).

Table 8. Spearman correlations (Ro) of the clinical form of SARS-CoV-2 disease and the quantitative parameters.

Evaluated parameters	COVID-19 severity		
	Ro	p	N
Age (years)	**.605**[**]	**.000**	90
IL-6 (pg/mL)	**.819**[**]	**.000**	90
Serum iron (µg/dL)	**-.476**[**]	**.000**	90
ACE (U/L)	**-.348**[**]	**.001**	89
Ceruloplasmin (g/L)	.222[*]	.037	89
Transferrin (g/L)	**-.791**[**]	**.000**	89

Ro = Spearman type correlations (used when at least one of the variables is not normally distributed) and minus means inverse correlation.

* =   correlation significance < 0.05.

** = correlation significance <0.01.

p = The threshold for statistical significance of the tests used in statistical analyses is the one usually applied in medical research p=0.05 (=5%), and p < 0.001 = strongly significant.

N = number of evaluated persons.

IL-6= interleukin 6.

ACE= angiotensin-converting enzyme.

Within the group of the diabetic patients with SARS-CoV-2 virus infection, we evaluated possible correlations between the serum level of CRP, LDH, D-dimers, number of lymphocytes, INR, ceruloplasmin and the COVID-19 severity. Ceruloplasmin and lymphocyte count had a statistically significant decreasing tendency in severe forms of SARS-CoV-2 infection (the Spearman correlation coefficient Ro<0), meanwhile for the other parameters, there was a significantly increasing tendency in severe forms of SARS-CoV-2 infection (Ro>0). Moreover, the severity of COVID-19 correlated strongly significantly (p<0.01) with d-Dimers and lymphocytes number (Table 9).

Table 9. Correlation between COVID-19 severity and ceruloplasmin, CRP, LDH, D-Dimer, Lymphocytes and INR.

Evaluated parameters	COVID-19 severity		
	Spearman Ro	**p**	**N**
Ceruloplasmin (g/L)	-.380*	.011	44
CRP (mg/L)	.375*	.013	43
LDH (U/L)	.370*	.015	43
D-Dimer (ng/mL)	.393**	.009	43
Lymphocytes number (*1000/μL)	-.471**	.001	43
INR	.313*	.043	42

Ro = Spearman type correlations (used when at least one of the variables is not normally distributed) and minus means inverse correlation.

* = 　correlation significance < 0.05.

** = correlation significance <0.01.

p = The threshold for statistical significance of the tests used in statistical analyses is the one usually applied in medical research p=0.05 (=5%), and p < 0.001 = strongly significant.

N = number of evaluated persons.

CRP= C-reactive protein.

LDH= Lactate dehydrogenase.

INR= International normalized ratio.

For the patients with COVID-19, we observed that IL-6 correlated non-parametrically (Spearman) significantly (p<0.05) with the numbers of neutrophils, platelets, and serum iron level. Neutrophils, platelets, and serum iron shown a significantly decreasing tendency when IL-6 increases (Spearman correlation coefficient Ro<0). IL-6 correlated strongly significantly (p<0.01) with CRP (Table 10).

Table 10. Correlation between IL-6 and CRP, neutrophils and platelets numbers.

Evaluated parameters	IL-6		
	Spearman Ro	**p**	**N**
Serum iron (µg/dL)	-.512**	.000	45
CRP (mg/L)	.395**	.009	43
Neutrophils number (*1000/μL)	-.364*	.016	43
Platelets number (*1000/μL)	-.343*	.026	42

Ro = Spearman type correlations (used when at least one of the variables is not normally distributed) and minus means inverse correlation.

* =   correlation significance < 0.05.

** = correlation significance <0.01.

p = The threshold for statistical significance of the tests used in statistical analyses is the one usually applied in medical research p=0.05 (=5%), and p < 0.001 = strongly significant.

N = number of evaluated persons.

IL-6= interleukin 6.

CRP= C-reactive protein

Serum iron, ACE, ceruloplasmin and transferrin did not differ statistically significantly for the female gender compared to the male gender (p>0.05) (Table 11). Also, ferritin, hemoglobin, RBC (Red blood cell), glycemia, CRP, LDH and fibrinogen did not differ statistically significantly for the female gender compared to the male gender (p>0.05) (Table 12).

Table 11. Serum iron, IL-6, ACE, ceruloplasmin and transferrin variation according to the patients gender.

Parameters	Gender	N	Mean	Std. deviation	Test t, p
Serum iron (µg/dL)	W	29	56.310	33.495	0.965
	M	16	55.875	27.985	
IL-6 (pg/mL)	W	29	94.028	187.189	0.245
	M	16	48.508	81.807	
ACE (U/L)	W	29	19.462	17.039	0.453
	M	15	23.653	18.097	
Ceruloplasmin (g/L)	W	29	0.321	0.070	0.670
	M	15	0.332	0.100	
Transferrin (g/L)	W	29	1.789	0.536	0.603
	M	15	1.70733	.383321	

N = number of evaluated persons,.

W= women, M= men, .

IL-6= interleukin 6,.

ACE= angiotensin-converting enzyme.

p = The threshold for statistical significance of the tests used in statistical analyses is the one usually applied in medical research p=0.05 (=5%), and p < 0.001 = strongly significant.

t   =T (Student) test.

Table 12. Other parameters variation according to the patients gender.

Other Parameters	Gender	N	Mean	Deviatia std.	Testul t, p
Ferritin (ng/ml)	W	26	538.081	379.858	.079
	M	16	836.506	696.056	
Hemoglobin (g/dL)	W	27	12.733	1.684	.126
	M	16	13.606	1.914	

		N			
RBC(*100^3/µL)	W	27	4.444	0.568	.572
	M	16	4.551	0.642	
Glycemia (mg/dL)	W	26	224.192	87.761	.678
	M	16	211.875	100.572	
CRP (mg/L)	W	27	101.613	76.581	.837
	M	16	96.806	67.662	
LDH (U/L)	W	27	382.259	146.793	.569
	M	16	416.625	247.522	
Fibrinogen (mg/dL)	W	27	470.300	119.607	.435
	M	16	504.463	163.552	
d-Dimer (ng/mL)	W	27	1135.111	2241.639	.546
	M	16	1959.875	3820.193	
Lymfocytes number (*1000/µL)	W	27	0.933	0.604	.440
	M	16	1.169	1.111	

N = number of evaluated persons, .

W= women, M= men,.

IL-6= interleukin 6,.

CRP= C-reactive protein, .

LDH= Lactate dehydrogenase,   .

RBC= Red blood cell.

p = The threshold for statistical significance of the tests used in statistical analyses is the one usually applied in medical research p=0.05 (=5%), and p < 0.001 = strongly significant.

t   =T (Student) test.

The multiple linear regression model between serum iron (as dependent parameter) and ceruloplasmin, hemoglobin, RBC, platelets number and fibrinogen (as independent parameters) can be expressed by the following equation:

(Serum iron) = - 164.297 x (Ceruloplasmin) + 15.281 x (Hemoglobin) - 26.98 x RBC + 0.186 x (Platelets number) – 0.186 x (Fibrinogen).

The multiple correlation coefficient (R=0.942, R_{square}=0.871) in this model was strongly statistically significant (p<0.001) indicating that the serum iron variation was due in a proportion of 87.1% to predictor parameters such as ceruloplasmin, hemoglobin, RBC, platelets number. Therefore, this regression model gave a good enough estimation of serum iron parameter (Table 13, Table 14).

Table 13. The multiple linear regression model between serum iron (as dependent parameter) and ceruloplasmin, hemoglobin, RBC, platelets number and fibrinogen (as independent parameters).

Model	Unstandardized Coefficients	Standardized Coefficients	p	95.0% Confidence Interval for B	
	B	Beta		Lower Bound	Upper Bound
Ceruloplasmin (g/L)	-164.297	-.850	**.001**	-252.774	-75.819
Hemoglobin(g/dL)	15.281	3.066	**.001**	6.911	23.652
1 RBC (*100^3/µL)	-26.980	-1.864	**.031**	-51.324	-2.636
Fibrinogen(mg/dL)	-.016	-.127	.545	-.070	.038
Platets number (*1000/µL)	.186	.680	**.000**	.097	.275

p = The threshold for statistical significance of the tests used in statistical analyses is the one usually applied in medical research p=0.05 (=5%), and p < 0.001 = strongly significant.

RBC= red blood cell.

Table 14. The coefficient of multiple correlation in the linear regression model.

Model	R	R Square[b]	Adjusted R Square	Change Statistics	
				R Square Change	Sig. F Change
1	**.942[a]**	**.887**	.871	.887	.000

R^2 = the proportion of the variation of the dependent variable (serum iron) due to the variation of the predictor variables ceruloplasmin, hemoglobin, platelets number and RBC.

4. Discussions

During the pandemic, certain markers of poor prognosis and increased mortality were identified in patients infected with the SARS-CoV-2 virus [53-56]. Among the first common markers of severity recognized in COVID-19 infection were: advancing age, presence of metabolic and cardiovascular comorbidities, increased ferritin, high levels of pro-inflammatory cytokines, especially increased IL-6 [57, 58]. In the studies of patients with newly diagnosed type 2 diabetes and SARS-CoV-2, hyper-ferritinemia, increased CRP and decreased lymphocyte counts were more evident than in patients without comorbidity (without type 2 diabetes) [59-61]. Associations between significantly elevated levels of serum ferritin, d-dimers and CRP in patients with COVID-19 requiring transfer to intensive care units have been reported in the literature [62, 63]. In other studies, the link between hyperfer-ritinemia, decreased serum transferrin and increased CRP levels in the pro-inflammatory state from the COVID-19 infection was presented [64, 65].

Decreased serum transferrin and decreased transferrin saturation, but also increased hepcidin levels were associated with severe forms of COVID-19 infection [66-68]. High levels of ferritin, as an acute phase reactant, have been reported in many studies as a negative prognostic marker [69]. In some studies, the role of transferrin has been suggested as a possible central element of pathogenicity in SARS-CoV-2 infection [70].

The mechanism of action of serum iron in patients with COVID-19 is not fully elucidated, in the literature it has been assumed that a decrease in serum iron may be associated with an increase in hepcidin level and with hyperferritinemia [71]. Concerning iron metabolism, there are certain studies regarding the decrease in serum iron as a marker associated with the increased risk of mortality due to SARS-CoV-2 infection [72, 73]. Decreased serum iron has been shown to be a predictor of severe forms of COVID-19 infection [74-76].

In the present study, we evaluated other new possible markers of COVID-19 severity and their associations by comparing serum levels of ceruloplasmin, IL-6, serum iron, transferrin, and ACE in 45 healthy control subjects versus 45 type-2 diabetic patients infected with SARS-CoV-2 virus, randomly chosen. The subjects' gender did not significantly influence the level of the evaluated biomedical parameters.

We identified that serum ceruloplasmin increased in severe forms of infection, being a possible new marker of COVID-19 severity. We also found that there was a strong connection between increased serum ceruloplasmin and increased IL-6 levels. Thus, a possible explanation could be the fact that the increased level of ceruloplasmin could also signal a possible negative evolution, with cytokine storm in these patients, through the correlation with the high level of IL-6. Increased IL-6 level in patients with COVID-19 has been associated in other studies with the cytokine storm, increased levels of pro-inflammatory cytokines such as IL-6, IL-7, IL-10, IL-1B being present in patients with severe forms and with a negative impact on the endothelial function [77-80]. In our research, increased IL-6 was statistically significant correlated with decreased serum iron, decreased serum ACE,

and decreased transferrin. Julian Hackler *et al.* presented a possible connection between survival in COVID-19 infection, elevated serum copper levels, and a slight ceruloplasmin increase tendency [81].

Analysis of serum iron in our study revealed a statistically significant correlation between decreased serum iron and severity of SARS-CoV-2 infection. We also found that the decrease in sideremia was correlated with the increase in serum ceruloplasmin, IL-6 level, and age. IL-6 is involved in the production of hepcidin which is a peptide hormone that ensures iron homeostasis [82]. Marco Ciotti *et al.* presented the association between hepcidin values and the transfer to intensive care in patients infected with SARS-CoV-2 virus, but they did not identify significant differences in serum iron levels between survivors and deceased [68].

At the patients included in our study, we observed that the decrease in the serum level of ACE was significantly correlated with severe forms of infection and with the increase of IL-6, so the low level of ACE could represent a new possible marker of severity in patients with COVID-19.   We have not identified in the literature reliable data related to the role of ACE in patients with COVID-19 or in patients with type-2 diabetes and SARS-CoV-2 virus infection, although the role of ACE 2 (Angiotensin converting enzyme 2) in the infection with the new coronavirus has been intensively studied and presented [83-85]. ACE is produced by both kidneys and lungs, being present in vascular structures and being a component of the renin-angiotensin-aldosterone system together with ACE2, important in cardiovascular and renal homeostasis. ACE and ACE2 are metallopeptidases, glycoproteins that metabolize circulating peptides, but ACE and ACE2 are functionally different. ACE mainly acts as a carboxypeptidase, converting angiotensin I into angiotensin II, by removing the terminal dipeptide C; also, through ACE, Angiotensin 1-9 can be converted to Angiotensin 1-7. ACE2 has an enzymatic role by regulating the renin-angiotensin-aldosterone system, but also the kinin-kallikrein system; ACE2 also has a non-enzymatic function representing the entry receptor of the SARS-COV2 virus into the human body. The serum level of ACE did not correlate statistically significantly with the level of serum ceruloplasmin at the patients included in this study [86,87].

By analyzing new possible negative prognostic markers, and their association with the others severity markers in the infection with the new coronavirus, we identified a significant inverse statistical correlation between the tendency of decreasing ceruloplasmin (part of iron metabolism), severe forms of COVID-19 and the number of lymphocytes. Lymphopenia, as evidence of immune suppression and a marker of severity in COVID-19 infection, was identified in the patients from our study.

Other studies shown the connection between the decrease in serum iron, hemoglobin and hypoxia in patients with COVID-19, but also the connection between hyperferritinemia and the stimulation of the expression of pro-inflammatory cytokines by macrophages including IL-6 [88].

One of the finding of our study was the tendency of decreased number of platelets (as coagulation parameters), neutrophils count and association with increased IL-6 cytokine level. On the other hand, the level of IL-6 was directly influenced by the increase in CRP level.

Data from the literature regarding patients with diabetes and COVID-19 infection also mentioned that the predictors of mortality such as lymphopenia, ferritinemia, increase of CRP and D-dimers were more evident than in nondiabetic patients [89-91].

Another important finding of our study was that ceruloplasmin and transferrin (as parameters of iron metabolism) and their connection with ACE could show the evolution in the direction of severe forms of COVID-19.

In this research, the regression model for serum iron and ceruloplasmin parameters was also analyzed and it was observed that serum ceruloplasmin, hemoglobin, erythrocytes and platelets count strongly significantly influenced sideremia in patients with SARS-CoV-2 virus at hospital admission.

Among the limitations of this study, it can be mentioned: quite low number of patients, single-center study, parameters evaluated at hospital admission. Future studies on much larger groups are needed.

5. Conclusions

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15 of 20

Regarding the severe forms of infection with SARS-CoV-2 virus, we identified a direct relationship between the COVID-19 severity and the level of ceruloplasmin as an antioxidant biomarker, and also a direct relationship between the infection severity and IL-6 levels as a pro-inflammatory cytokine. Moreover, the COVID-19 severity can be influenced by decreased iron deposits, decreased transferrin and decreased ACE levels.

Thus, in patients with type-2 diabetes and viral infection with the new coronavirus, other possible markers independent of the COVID-19 severity such as serum ceruloplasmin, ACE and transferrin levels can be determined at hospital admission. These possible new severity markers may serve to complement all other severity markers already established in COVID-19 infection in patients with type 2 diabetes.

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