The effect of the current treatment protocol on some biochemical and inflammatory markers in severe COVID-19 patients

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ABSTRACT:
Treatment of coronavirus infections varied according to the severity of the symptoms, sites, and organs affected. COVID-19 severity ranged from mild, moderate, severe, and critical which may end in death. Disease treatment varied according to the stage, symptoms, and organs affected. Anti-virals, anti-inflammations, anti-bacterial, and anti-coagulants are commonly used in the treatment in addition to the tools helpful in improving respiration. To assess the efficacy of the current protocol used for the treatment of severe COVID-19 patients, forty severe COVID-19 cases admitted to al-Kindy hospital, Baghdad during the period from March 2021 to October 2021 were subjected to some biochemical and immunological tests thought to associate with the response to the therapy, disease progression, and mortality. After ten days of treatment, twenty patients died. When compared to the survivors, dead patients were of higher ages, had higher levels of urea, creatinine, C-reactive protein, interleukin-6, and D-dimer.
The correlation test revealed a significant positive correlation between mortality and IL-6 and D-dimer increase. Among all parameters tested in the study, ROC curve analysis showed that D-dimer has the largest area under the curve followed by IL-6 concerning mortality which indicated that these parameters are good predictors for disease progression and mortality outcome.

Keywords: corona virus, severe COVID-19, D-dimer, IL-6, urea, creatinin, ferritin, Mortality

Introduction:

In the late December 2019, a cluster of pneumonia cases of unknown origin reported in Wuhan, China developed later to a global pandemic. The researchers accused a new coronavirus with over 80% genetic similarities with SARS-CoV. However, the International Committee of the Coronavirus Study Group renamed the virus SARS-CoV2 (1). SARS-CoV-2 was expected to arise by the end of 2019 via zoonotic transmission through a bat reservoir and an unnamed interrelationship, leading to an epidemic (2). SARS-CoV2 is a recently found infection forwhich there is no known human resistance, and there may be risk factors that raise the severity of a patient's disease (3)

Factors increase the chance of severe COVID-19 include cardiovascular

illness, diabetes, lung infection, Leukemia, kidney illness, overweight, sickle cell diseases, transplant patients, and other immune-compromising (4).

Non-respiratory symptoms such as severe liver and renal failure, heart damage, and diarrhea, suggesting multiorgan involvement. SARS-CoV2 is assumed to start reproducing in the mucous membrane of the respiratory system, then disseminate to the lower respiratory tract and gastrointestinal mucosa, resulting in a mild viral load (5).

Symptoms range from mild-moderate infection of the upper respiratory tract in the form of fever associated with cough, sore throat, and fatigue, whereas non-respiratory symptoms like; head-ache, palpitation, abdominal pain, watery diarrhea, nausea, and vomiting precede respiratory symptoms in about 15% (6). Alveolar hemorrhage and pulmonary edema are two more lung diseases that have been observed. Furthermore, thrombo-emboli or central or peripheral pulmonary thrombi were observed throughout many COVID-19 patients, possibly showing a histological link with coagulopathies prevalent in this illness (7). Furthermore, 15–20 percent of COVID-19 individuals had abnormal liver enzymes (8). Patients with SARS-CoV-2 infection who develop cytokine storming syndrome often have harm to their central nervous system (CNS), resulting in neurological abnormalities. COVID-19 patients with CNS signs made up about 25% of the total. Inflammation is the most typical capacity during invasive infection. In most situations, both the innate immune responses, in their cellular and humeral, eliminate viral infections (9). Natural killer cells (NKs) cells, on the other hand, play a crucial role in the owner's defiance; they detect virus-infected cells in an antigen-independent manner, carry out cytotoxic actions, and quickly release significant amounts of IFN-, which aid in the activation of adaptive lymphocytes (10).

Interleukin-6 (IL6) has a pleiotropic role across the immune system. It is crucial in the formation of follicular helper T cells, TH17 subset deviation, and the formation of long-lived plasma cells. However, IL-6 can block CD8+ cytotoxic T cells by inhibiting the secretion of gamma interferon. Moreover, IL-6 by inducing suppression of cytokine signaling (SOCS-3) and increasing the expression of PD-1 can paralyze the cell-mediated antiviral response during a cytokine storm (11).

In the early stage of the disease, a normal or Increased values of liver enzymes, lactate dehydrogenase (LDH), muscle enzymes, and C-reactive protein can be detected. In critical patients, D-dimer value is increased, blood lymphocytes decreased persistently, and laboratory alterations of multiorgan imbalance (high amylase, coagulation disorders, etc.) are found (12).

Overproduction of proinflammatory cytokines, however, can give rise to what is called a “cytokine storm” which can harm the body through the production of a syndrome called “SIRS” (Systemic Inflammatory Response Syndrome) which leads to hypotension, pulmonary thrombosis, pulmonary edema, and hemorrhage, and if

not treated with appropriate therapy, it can lead to death **(13**).

Therefore, the treatment of COVID-19 includes symptomatic care and oxygen therapy. Patients with mild infections require early supportive management. For moderate to critically ill patients, anti-viral therapies can be administered as early as possible even if no strong evidence exists. Controversial results exist regarding lopinavir-ritonavir in treating severe COVID-19 patients (11, 14). However, this combined medication can be given to patients as a basic regimen. Hydroxychloroquine sulfate was found to produce an effective therapeutic role in the viral load reduction/disappearance in COVID-19 patients with an enforced effect by azithromycin.

Remdesivir, a broad-spectrum antiviral medicine, is also used to inhibit viral replication via terminating RNA transcription prematurely (13, 15). Considering the cytokine storm that happened during the progression of COVID-19, appropriate and short-term use of corticosteroids can be considered to inhibit the cytokine cascade for patients with severe COVID-19.
Anti-Coagulation and anti-bleeding drugs may be added according to the presence of bleeding and platelets count, and heparin is recommended in appropriate doses. Vitamins D, C, E, glutathione, and melatonin as strong antioxidants are also recommended.

This study aimed to assess the efficacy of the current treatment protocol in controlling severe COVID-19 through the investigation of the levels of certain biochemical and immunological parameters associated with the disease's severity and morbidity.

Subject, Materials, and Methods

Subject

This study was performed on severe COVID-19 patients during the period between March 2021 to October 2021 at Alkindy hospital in Baghdad /Iraq. The number of COVID-19 patients was 40 from the total number of 165 patients. The age ranges between 20 to 80 years. Of the 40 patients, 20 (50%) were men and 20 (50%) were women. 20 (50%) patients out of the 40 enrolled patients died.

One hundred and twenty-five patients were excluded for many reasons, including the death of the patient before the completion of the required number of samples, the difficulty of obtaining a sample from the patient, insufficient quantity of blood to conduct all the tests, blood sample loss during storage or transport and from patients admitted to hospitals before the launch of sample collection. All the enrolling patients (or their relatives) had to answer a questionnaire consisting of the following information: name, age, sex, education, residence area, chronic diseases, and admission date.

Materials & Methods:
Sample Collection

Two samples of blood had been taken from the enrolled patients. The first sample

was taken at the time of hospital admission and the second sample was taken after ten days of the treatment. A sample of 5 milliliters of venous blood was collected, then left to clot at room temperature for approximately 15-30 minutes, then centrifugated at 3000 rpm for 15 minutes. The resulting serum had been transferred carefully using automatic pipettes in to plan tube and one milliliter of serum was used for biochemical tests, the rest of the serum was stored in 0.2ml aliquots at -20°C until use in a serological assay for measuring the level of other parameters.

Methods:

- Human IL-6(Interleukin 6) ELISA Kit, supplied by BioVision, Inc.
- C-reactive protein was measured by finecare FIA meter.
- Urea and Creatinin concentrations were determined by an enzymatic assay using a Biomaghreb kit, Tunisia.
- Ferritin Human ELISA Kit, Invitrogen was supplied by Thermo Fisher Scientific.
- D-dimer Fluorescence ImmunoAssay (FIA) Test, CTK Biotech.

Statistical Analysis

SPSS (Statistical Package for Social Science) version 26 program was used for the analysis of the data. Normally distributed data were expressed as mean ± standard deviation, non-normally distributed data were expressed as the median and interquartile range (5-95 percent), and categorical variables as percentages. Differences between independent groups were compared using Student's t-test for normally distributed data. In correlation analysis, the Pearson correlation test was used for variables with normal distribution, and the Spearman correlation test was used for variables with abnormal distribution. ROC curve analysis was used to select possibly optimal parameters significantly correlated with poor outcomes and to discard suboptimal ones independently from the context. *A p*-value of < 0.05 was considered statistically significant for all tests. Calculation of the odds ratio and relative risk was done by MedCalc® Statistical Software version 20.218 (MedCalc Software Ltd, Ostend, Belgium).

Results:

This cross-sectional study included forty severe and critical COVID-19 patients with a mean age of 46.18 years old. According to their outcome, the lived patients had a significantly lower mean age of 42.3 years compared to dead patients 50.05 years old (p=0.004) as displayed in table 1.

Table 1: Descriptive analysis of the age of COVID-19 patients.

Parameter	Outcome	Mean	SD	Median	Min.	Max.	F	P
Age (yrs)	**Dead**	50.05	8.882	49.5	38	72	9.413	0.004
	Live	42.30	6.634	42	29	55		
	Total	46.28	8.696	44	29	72		

C-reactive protein significantly increased in dead patients compared to the survivors at baseline and after days of treatment, however, the treatment did not alter the level of CRP significantly in both groups of patients. Ferritin levels did not differ significantly between the two groups either before or after treatment as shown in table 2.

Table 2: comparison between live and dead patients in the levels of ferritin and CRP before and after treatment.

Parameter	Treatment	Outcome						p-value
		LIVE			DEAD			
		Median	5-95% percentile		Median	5-95% percentile		
FERRITIN	Before	548.9	50.2	1650	583.4	26.4	1650	0.587
	After	612.9	26.4	1650	587.8	238.2	1650	0.631
p-value		0.638			0.934			
CRP	Before	54.1	3.7	292.5	102.25	19.7	260.53	0.004*
	After	63.75	3.7	292.5	91.31	6.85	260.53	0.009*
p-value		0.378			0.704			

Kidney function tests:

Kidney functions may be affected seriously due to many infections or certain therapies. In this study, Urea and Creatinin levels were assessed to evaluate the effect of covid-19 infection or drugs used for treatment on kidney functions. As shown in table 3, Urea levels increased significantly in patients after ten days of treatment (p = 0.00). Analyses of the differences between dead and live patients revealed no significance either before (p = 0.489) or after treatment (p = 0.431) as displayed in table 4.

Table 3: Descriptive statistics of the Urea and creatinin levels of patients before and after treatment.

Parameter	**Treatment**	**Mean**	**SD**	**Median**	**Min.**	**Max.**	**F**	**P**
Urea	**Before**	42.55	17.804	34.00	22.50	98.00	15.347	.000
	After	63.67	28.570	60.00	29.00	210.00		
Creatinin	Before	0.79	0.324	0.79	0.12	1.74	23.149	.000
	After	1.28	0.543	1.17	0.50	3.00		

Creatinin also significantly increased in the patients after treatment (p = 0.00) with no significance between dead and live patients before treatment (p = 0.473) or after treatment (p = 0.586) as shown in table 4.

Table 4: Comparison between dead and live patients in the Urea and creatinine levels before and after treatment

Paramete	**Treatmen**	**Outcom**	**Mean**	**SD**	**Media**	**Min.**	**Max.**	**F**	**P**

r	t	e			n				
Urea	**Before**	**Dead**	40.60	14.254	36.50	22.50	74.00	0.488	0.489
		Live	44.61	21.118	34.00	23.00	98.00		
	After	**Dead**	60.11	13.21	57	45	98	0.634	0.431
		Live	67.43	38.85	61	29	210		
Creatinine	**Before**	**Dead**	0.83	0.307	0.83	0.36	1.74	0.525	0.473
		Live	0.75	0.345	0.70	0.12	1.55		
	After	**Dead**	1.32	0.598	1.17	0.60	3.00	0.301	0.586
		Live	1.23	0.489	1.10	0.50	2.50		

The interleukin-6 level decreased non-significantly (p = 0.586) in the patients after ten days of treatment as shown in table 5.

Table 5: Descriptive statistics of the interleukin-6 level in patients before and after treatment.

Treatment	Mean	SD	Median	Minimum	Maximum	F	P
Before	0.634	0.545	0.492	.185	2.780	.299	.586
After	0.563	0.594	0.336	.068	2.784		
Total	0.599	0.568	0.390	.068	2.784		

Analyzing the differences in IL-6 levels between dead and live patients revealed a significant increase in this marker in the dead group either before (p = 0.014) or after treatment (p = 0.029) as shown in Table 6.

Table 6: Comparison between dead and live patients at the interleukin-6 level before and after treatment

Treatment	**Outcome**	**Mean**	**SD**	**Median**	**Minimum**	**Maximum**	**F**	**P**
Before	**Dead**	0.839	0.649	0.612	0.19	2.78	6.632	.014
	Live	0.419	0.296	0.291	0.20	1.22		
After	**Dead**	0.764	0.770	0.415	0.07	2.78	5.185	.029
	Live	0.352	0.167	0.328	0.14	0.72		

D-dimer test which is used to check for blood clotting problems revealed a significant increase in the patients after days of treatment (p = 0.007) as shown in table 7.

Table 7: Descriptive statistics of the D-dimer marker in patients before and after treatment.

Treatment	Mean	SD	Median	Minimum	Maximum	F	P
Before	388.82	245.532	320.00	100.00	780.00	8.222	.007
After	917.05	718.781	680.00	120.00	2100.00		
Total	652.94	592.956	450.00	100.00	2100.00		

The comparison between dead and live patients revealed a significant increase in the non survived group (p = 0.00) before and after treatment as seen in table 8.

Table 8: Comparison between dead and live patients in the D-dimer marker before and after treatment

Treatment	Outcome	Mean	SD	Median	Minimum	Maximum	F	P
Before	**Dead**	561	160.170	565	300	780	44.144	.000
	Live	142.85	47.858	130	100	210		
After	**Dead**	1376	586.632	1540	390	2100.	24.350	.000
	Live	261.42	94.767	250	120	400.		

Moreover, t-test analysis showed that this marker significantly increased within the same groups (live and dead) during the days of follow-up (p = 0.000 and 0.026 respectively) as shown in table 9.

Table 9: T- test result for the comparison of D-dimer level in live and dead patients before and after treatment.

Outcome	Treatment	Mean	SD	Median	minimum	Maximum	T	P
Live	**Before**	142.85	47.858	130	100	210	5.644	.000
	After	261.42	94.767	250	120	400		
Dead	**Before**	561	160.170	565	300	780	2.932	.026
	After	1376	586.632	1540	390	2100		

Correlation between the parameters used in the study:

The Pearson correlation coefficient was used to measure the closeness of association between variables studied in COVID-19

patients. The correlation coefficient is positive if the two variables under test tend to be high or low together, and the larger its value the closer the association. Conversely, the correlation coefficient is negative if high values of one variable tend to go with low values of the other, and vice versa. Results of the correlations between parameters used in this study are displayed in the following tables.

Table 10: The correlations between renal function indices and the parameters used in this study.

		UREA	CREAtinin
Age	Pearson Correlation	.047	.053
	Sig. (2-tailed)	.774	.750
Fatal Outcome	Pearson Correlation	-.110	.085
	Sig. (2-tailed)	.338	.458
IL-6	Pearson Correlation	-.073	-.002
	Sig. (2-tailed)	.524	.983
D – dimer	Pearson Correlation	.120	.323
	Sig. (2-tailed)	.501	.063

As shown in table 10, renal function parameters have no significant correlations with the age of the patients, fatal outcome, IL-6, and D-dimer level in this study.

Table 11: The correlations between the age, fatal outcome, IL-6, and D-dimer parameters used in this study.

		Fatal Outcome	IL-6 level	D dimer level
Age	Pearson Correlation	.450**	.213	.678**
	Sig. (2-tailed)	.004	.193	.003
Fatal Outcome	Pearson Correlation		.368**	.646**
	Sig. (2-tailed)		.001	.000
IL-6 level	Pearson Correlation			.476**
	Sig. (2-tailed)			.004
**. Correlation (r) is significant at the 0.01 level (2-tailed).				
*. Correlation (r) is significant at the 0.05 level (2-tailed).				

Table 11 shows that the fatal outcome has a significant positive correlation with the age of the patients, IL-6, and D-dimer. Finally, a significant positive correlation between IL-6 and D-dimer was found.

To select possibly optimal models and to discard suboptimal ones independently from the cost context, parameters significantly correlated with mortality outcome were analyzed by the ROC curve as shown in Figure 1.

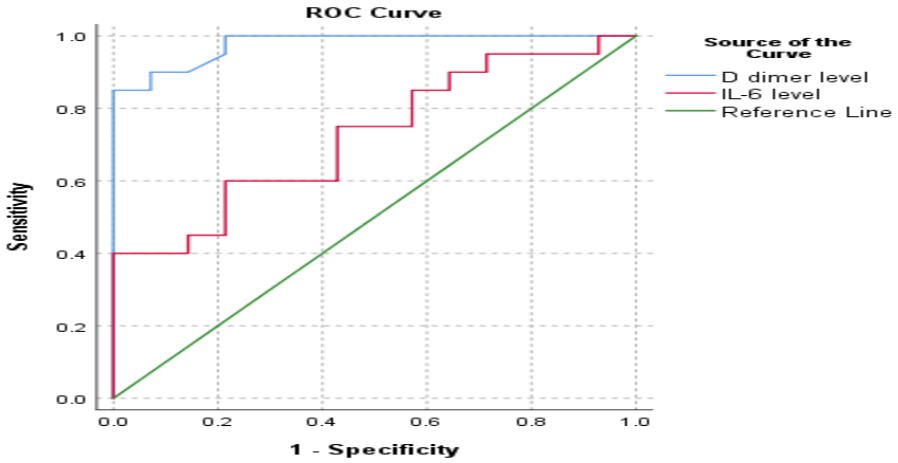

Figure 1: ROC curve of the parameters significantly correlated with poor outcomes.

D-dimer occupied the largest area under the ROC curve followed by IL-6 as shown in table 12.

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Table 12: The area under the ROC curve of the parameters in correlation with poor outcomes

Area Under the ROC Curve					
Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
IL-6 level	.725	.086	.009	.556	.894
D dimer level	.977	.020	.000	.938	1.016
a. Under the nonparametric assumption					
b. Null hypothesis: true area = 0.5					

Cut off values for IL-6 and D-dimer were calculated from the ROC curve as 0.46 pg\ml for IL-6 and 3.25 mg\L for the D-dimer. The association between mortality and increased IL-6 level is high since the odds ratio is 4.466 (95% CI= 1.778 to 12.24, p= 0.0018). A significant higher increase in IL-6 level than cut off value in non survivors than survivors, as seen in the figure 2, with a relative risk=2.279, 95% CI= 1.2919 to 4.0207 at p=0.0045. on the other hand, 90% of the dead patients had D-dimer level more than the cut off value with a high odds ratio (54, 95% CI= 6.6678 to 437.322, p= 0.0002) and a high relative risk (RR= 8.57, 95% CI= 2.2622 to 32.4766, p= 0.0016) as seen in the figure 3. The results of odds ratio and relative risk are seen in the table 13.

Table 13 :Association analysis of cutoff values for IL-6 and D-dimer according to the mortality outcome.

Parameter Cut off Value		Outcome	
		LIVE	DEAD
Serum IL-6 (pg/ml)	< 0.46	28	15
		74%	37.5%
	≥ 0.46	10	25
		26%	62.5%
Total		38 (48.7%)	40 (51.3%)
Odds ratio		4.6667	
95% CI		1.7780 to 12.2484	
z statistic		3.129	
Significance level		P = 0.0018	
Relative risk		2.2791	
95% CI		1.2919 to 4.0207	
z statistic		2.844	
Significance level		P = 0.0045	
Serum D-dimer (mg\L)	< 3.25	12	2
		85.7%	10%
	≥3.25	2	18
		14.3%	90%
Total		41.2%	58.8%
Odds ratio		54.0000	
95% CI		6.6678 to 437.3227	
z statistic		3.738	
Significance level		P = 0.0002	
Relative risk		8.5714	
95% CI		2.2622 to 32.4766	
z statistic		3.161	
Significance level		P = 0.0016	

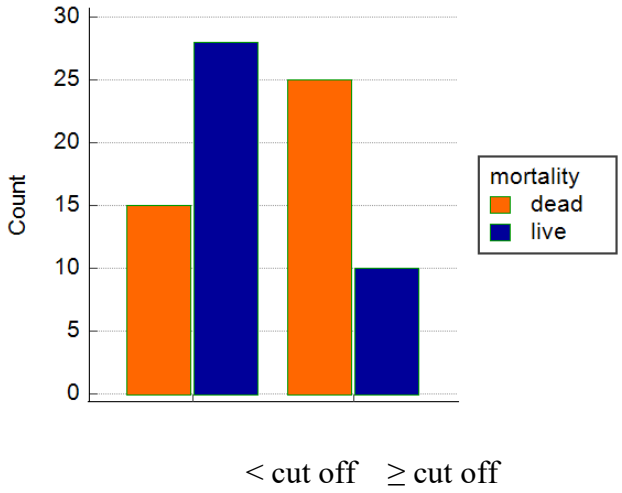

Figure 2: Mortality outcome according to the cut off value of Interleukin-6

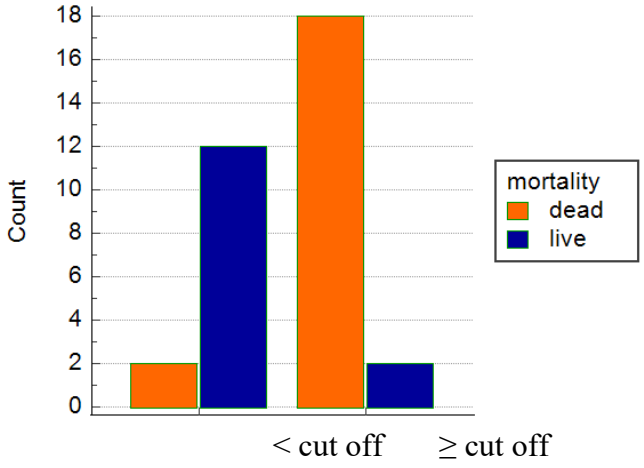

Figure 3:Mortality outcome according to the cut off value of D-dimer

Discussion:

Covid-19 is primarily a respiratory tract disease with a wide range of tissues and organs that could be infected wherever they express ACE-2 receptors. This image is obvious in severe and critical cases of the disease as different signs and symptoms are observed including inflammation of the lungs, liver, and kidneys dysfunctions, anemia, coagulopathies, gastrointestinal disorders, etc. The most prominent features of severe and critical symptoms are cytokine storm and coagulopathies.

Cytokine storm is a condition in which the immune system is over-reactivated flaring uncontrolled inflammatory response (16). Criteria that distinguish cytokine storm and commonly used in the diagnosis are fever, the elevation of C-reactive protein, neutrophils, ferritin, fibrinogen, LDH, IL-6 (17, 18, 19). Other parameters of severity

include a D-dimer increase, raised neutrophils to lymphocytes ratio, and decreased lymphocytes to CRP ratio.
Many multi-variate analyses studies reported that severe COVID-19 is associated with older age and male gender (20, 21, 22, 23). The younger ages were weaned successfully in those who received mechanical ventilation (24). In this study, the mean age of the non-survivors was significantly higher than survivor patients. Moreover, the age parameter had a significant positive correlation with mortality outcomes.

A meta-analysis by Wu et al included forty-one studies and more than five thousand patients found a significant increase in CRP and D-dimer in severe COVID-19 cases (25). Alnore et al reported that 45 studies found high CRP and D-dimer levels are associated with severe cases (26).

C-reactive protein significantly increased in non-survivor patients compared to the survivors whereas no significant difference in ferritin level between the two groups. Similar results were obtained with others (20, 27) indicating the inflammation development at least during the period of the study. Ferritin was highly elevated in severe cases in many studies (27, 28) whereas no significant difference in titer between survivors and non-survivors in this study which may suggest that there is no significant depletion in iron storage, the function of ferretin.

IL-6 is an inflammatory marker found to increase in many diseases such as cytokine storm, certain renal diseases, and asthma. In this study, this marker significantly increased in non survived patients at admission and after ten days of hospitalization compared to survived ones. Moreover, it is positively correlated with D-dimer, the other most important marker of severity and mortality.

Analysis of the parameters significantly correlate with mortality by ROC curve approved that IL-6 is the second parameter after D-dimer that predict poor outcome.

Renal function parameters associated with disease severity:

COVID-19 is a multi-organ infectious disease. It is recommended that renal function tests are mandatory in severe coronavirus disease. Renal functions were negatively affected due to coronavirus infection in this study. Urea level increase accuses coronavirus or hospitalization.

However, kidney treatment should be taken as a priority in parallel with other critical signs of COVID-19 infection.

Parameters associated with coagulopathy:

D-dimer marker, which is closely associated with coagulopathies, was significantly higher in non-survivals than survivals either before or after treatment. On the other hand, treatment did not correct the level of D-dimer in both survival and non-survival patients. The D-dimer level increased in the survival group between 3 to 8-folds before treatment and slightly decreased after treatment (between 3 to 6-fold) while non-survival showed about 7 to 30-fold in D-

dimer level at baseline and the level slightly decreased between 7 to 23-folds after treatment.

Many studies reported that elevated D-dimer and decreased platelet count are associated with COVID-19 coagulopathy (30, 31) and severe cases (32). Price *et al.* reported that prevalence of thromboembolism in these patients is underestimated because of the types of detection tools used (33). A work by Bilaloglu *et al.* supported this conclusion as he found in a study on 3334 COVID-19 patients a percent of 3.2% incidence of pulmonary imbolism diagnosed by routine clinical care but when imaged by computed tomography pulmonary angiography revealed 24% incidence of this type of embolism (34). It is known now that thromboembolism will induce stronger cytokine storm and inflammation development that lead, even in mild cases, to progression of hypoxemia and poor clinical outcome (35). Formation of other types of thromboembolism in other organs will worsen the disease and develop into critical and increase the mortality. Moreover, a study found that recovered patients have a reduction in D-dimer level whereas a continuous increase in its level was a predictive of adverse outcome (29, 36). Selection of possibly optimal parameters significantly correlated with poor outcomes and to discard suboptimal ones, ROC curve analysis used in this study revealed that D-dimer, which has the greatest area under curve and high odds ratio and relative risk, followed by IL-6, are good predictors for worst outcome and mortality suggesting the need for an administration of a suitable anticoagulant for treatment of severe COVID-19 cases. This conclusion is supported by Lemos *et al.* work who found that anticoagulant could reduce in-hospital mortality and intubation rate if taken therapeutically or prophylactically with no significant difference between the two precesses. Moreover, he found that the anticoagulant enoxaparin could improve gas exchange and decrease the need for mechanical ventilation in severe COVID-19 cases (27).

Conclusion:

In a detailed study, Samprathi & Jayashree wrote that none of the proposed anti-viral, anti-inflammatory, anticoagulant and anti-fibrotic therapeutic strategies have been proven to be conclusively beneficial (37). This is due, at least partially, to the presence of different variants and phenotypes of SARS-COV-2 and, consequently, different hematological, biochemical, and immunological responses. However, Gao *et al.* reported that socioeconomic status, diet, lifestyle, geographical differences, ethnicity, exposed viral load, day of initiation of treatment, and quality of health care have been reported to influence individual outcome (38).

This study which was conducted to assess the efficacy of the current protocol of treatment of severe COVID-19 cases concluded that mortality outcome is significantly associated with an increase of D-dimer and IL-6 levels.

Accordingly, administration of a proper anticoagulant and anti-inflammatory

medicines capable of preventing or decreasing D-dimer and IL-6 elevation is of the first priority in treatment protocol of severe COVID-19 cases.

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