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Atteration of D-dimer, Ferritin and cytokines levels in Iraqi COVID-19 recovered patients

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ABSTRACT— Corona Virus Disease-2019 (COVID-19) is a globally a main healthcare threat disease, millions of people dead with it, so it is important to understanding the role of D-dimer and ferritin levels as a result of the response to disease. Both proteins mostly rely on severity of the COVID-19 disease. COVID-19 induced hyper inflammation which are a chief reason of disease severity and death via "cytokine storm" elevated level of Interleukin-6 (IL-6), Interleukin-10(IL-10) and Tumor Necrosis Factor Alpha (TNF-α) associated with decreased Interferon gamma (IFN-γ) due to decrease in T-cell count [1]. Follow up D-dimer level and ferritin level for six months, in addition to the association between D-dimer & ferritin with COVID-19 severity in COVID-19 recovered patients. Follow up inflammatory cytokine (IL-6) level and regulatory cytokine (IL-10) level for three months after COVID-19 recovery and the connection between IL-6 and IL-10 levels in COVID-19 recovered patients. In addition to the correlation between cytokines levels COVID-19 with genders and COVID-19 severity. The nasopharyngeal swab to confirm recovery by reverse transcriptase real-time PCR technique. Detection of D-dimer levels via using ARCHITECT D- dimer auto analyzer, detection of Ferritin levels via using Maglumi Fully Auto Operator's Manual, while IL-6 and IL-10 levels via using Enzyme Linked Immune Sorbent Assay (ELISA). The results found that, comparison between studied groups show significant increased ($P \leq 0.01$) in D-dimer level (mg/L), serum ferritin (ng/mL) level, IL-6 & IL-10 (pg/ml) level in COVID-19 recovered patients groups as compared with apparently control group, also a significant differences ($P \leq 0.05$) in D-dimer, ferritin, IL-6 &IL-10 between studied groups. The comparison of D-dimer, ferritin, IL-6 & IL-10 level between mild, moderate and severe groups had shown significant differences at $P \leq 0.01$. Contrariwise, D-dimer levels decline after treatments, with highly correlation was observed between D-dimer & ferritin levels in patients groups, finally, highly significant correlation coefficient between IL-6 & Il-10 levels. Both gender affected with COVID-19. Markedly increased in D-dimer in COVID-19 recovered patients which may play as a risk factor for thrombosis. Interestingly, High levels of ferritin associated with COVID-19 recovered patients could be considered as an inflammatory factor. Finally, significant correlation between ferritin and D-dimer levels in studied groups and could be used as diagnostic biomarker for COVID-19. Elevated in serum concentration of IL-6, IL-10 in all age groups and both gender correlated with COVID-19 severity.

KEYWORDS: COVID-19, recovered patients, Ferritin, D-dimer, IL-6 & IL-10.

1. INTRODUCTION

Wuhan, China, it first place in the world that identified newly Severe Acute Respiratory Syndrome CoronaVirus called SARS-COV2 by International Committee on Taxonomy of Viruses (ICTV) since December 2019 [2- 4]. SARS-CoV-2, a novel strain of single strand RNA viruses from beta-coronavirus [5], it caused respiratory infection coupled with fever, dry cough, sore throat and dyspnea, like Middle East respiratory syndrome (MERS) symptoms [6], [7]. The case-fatality rate (CFR) was 2.3%, but in age group

70 to 79 years was 8.0% and was 14.8% in age group 80-89 and older [8]. In some severe cases the infection developed to respiratory failure and death, it had amplified D-dimer levels that associated with poor prognosis or suddenly death, although causes of thrombosis has not been determined [9], [10].

The anticoagulation medication necessary to use with carefully because its bleeding effect in patients [11]. Therefore, D-dimer is a biomarker used to identify thrombus formation at earlier stages and in follow up the response to treatment. It produce via fibrin degradation, it use as potentially biomarker for pulmonary embolism [12].

Ferritin is an iron store intracellular protein; it plays an important role in inflammatory diseases. Some studies published that ferritin is an independent risk factor for COVID-19 severity [13- 15]. However, the connection of D-dimer & ferritin with mortality in COVID-19 patients is not clear. So it is important to focusing on this protein.

The inflammatory cytokines as interleukin IL-6, IL-10 & tumor necrosis factor (TNF) increased in severe cases then mild & moderate [16]. These cytokine associated with cytokine storm. COVID-19 is the highly explicative activity virus and a counteractive host immune response [17]. The excessive production of pro-inflammatory cytokines documented as a biomarker in severe case [18] and leading to deleterious effects and poor prognosis [19]. IL-6 considered inflammatory cytokine mostly produced by macrophages and T lymphocytes to controlling viral infections [20- 22]. IL-6 has a highly correlation with COVID-19 stages, need for mechanical ventilation and mortality [23]. IL-6 receptor antagonist (tocilizumab) use to block IL-6 receptors and inhibit IL-6 pathway in COVID-19. A recently reported meta-analysis found it was cumulative decline of mechanical ventilation but no effect on mortality, while in cohort study suggest an association between block IL-6 receptor and lower mortality. The IL-6 levels raise along with the disease stage and associated with respiratory failure [24]. COVID-19 patients also had a unique feature of cytokine storm, it is the dramatic elevation of IL-10 [25]. Besides, IL-10 levels highly linked with IL-6 [26]. Recent meta-analysis on severe and non-severe COVID-19 patients identified IL-6 and IL-10 as associated with COVID-19 severity [27]. IL-10 considered an anti-inflammatory biomarker that motivated via rapid accumulation of pro-inflammatory cytokines via negative feedback loop [28].

2. Material and methods

2-A: Study Design and Participants

The study involved 150 COVID-19 recovered Iraqi patients, age and gender matched to 70 healthy control, age ranged between 15-81 years. Swab and blood sample collected from patients in Department of Educational Laboratories/Clinical Immunology Section in Medical City in Baghdad, during the period from January 2021 to September 2021.

The recovery of patients confirmed by internal medicine specialist physician, remission symptoms and nasopharyngeal swab via Reverse Transcriptase–PCR (RT-PCR). Take four blood samples for each patient according to the time after recovery, as demonstrated below. Group 1: 0-14 days after recovery (n=150). Group 2: Two months after recovery (n=146). Group 3: Three months after recovery (n=142). Group4: 4-6 months after recovery (n=135).

2.1 Evaluation the levels of study parameters.

Detection of D-dimer by using ARCHITECT D-dimer auto analyzer, agglutination ammunoassay carry out by fully automated device normal value 0-0.5 mg/L and kit from Quanta D-dimer / Spain. Also, used the

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Maglumi auto analyzer to detect Ferritin level, it sandwich immunoluminometric assay, with normal value 13-350 ng/ml, kit from Roch /Switzerland

Detection of IL-6 and IL-10 by using ELISA system, sandwich ELISA immunoassay picograms per milliliter (pg/ml) and kit from Bio Legend / US & Canada.

2.2 Statistical analysis

The Social Science (SPSS) version 27 Statistical Program is used mean ± standard error to analyze quantitative data & frequency and percentage to qualitative data. T-test also used to comparing between two means, Analysis of Variance (ANOVA) used to comparing between over than two means.

3. Results

Age group: This study explained the association between COVID-19 recovered patients and age. These results clarified statistically significant differences P=0.001 at 0.01 presented in table 1. between studied groups according age.

Table 1: Participants characteristics.

Participants characteristics		COVID-19		Control		P-value
		No.	%	No.	%	
Age (years)	<20years	9	6.0			0.001**
	20---29	32	21.3			
	30---39	48	32.0			
	40---49	25	16.7			
	50---59	12	8.0			
	60---69	17	11.3			
	=>70years	7	4.7			
	Mean±SE (Range)	39.3±1.314 (7-81)				
Gender	Male	86	57.3			0.07
	Female	64	42.7			
Severity	Mild	45	30.0	-	-	0.37
	Moderate	58	38.7	-	-	
	Severe	47	31.3	-	-	
*Significant difference between proportions using Pearson Chi-square test at 0.05 level.						
#Significant difference between two independent means using Students-t-test at 0.05 level.						

NO. Number; S.E.: Standard Error; P-value: Probability value.

3.1 Gender groups.

Table 1 shows the distribution of COVID-19 according gender; non-significant differences P= 0.07 (P>0.05) between COVID-19 recovered patients with gender.

3.2 Detection of D-dimer and ferritin level.

D-dimer be in high level in G1 (0.8±0.03) followed by G2(0.33±0.015), G3(0.19±0.007) and G4 (0.23±0.01). A significant increment P=0.0001(P≤ 0.01) in D-dimer level in COVID-19 recovered patients G1 studied group as compared with apparently healthy control group as clarified in table 2. Comparison within studied groups shows significant differences P=0.0001, P=0.0001, P=0.0001 consequently at (P≤0.01) between G1 vs. G2, G3 & G4. Also significant differences P=0.0001, P=0.0001(P≤0.01) in that order between G2 vs. G3 & G4. In addition significant differences P=0.006 at 0.01 level between G3 vs. G4 as demonstrated in table 2.

Table 2: The distribution in D-dimer levels between groups.

Time after recovery/	D-	COVID-19	P value compared with

dimer(0-0.5 mg/L)	Mean±SE	G1	G2	G3	G4
G1 (n=150)	0.80±0.03				
G2 (n=146)	0.33±0.015	**0.0001#**			
G3 (n=142)	0.19±0.007	**0.0001#**	**0.0001#**		
G4 (n=135)	0.23±0.01	**0.0001#**	**0.0001#**	**0.006#**	
Control	0.253± 0.015	**0.0001#**			

#Significant difference between two dependent means using Paired-t-test at 0.05 level.

NO. Number; S.E.: Standard Error; P-value: Probability value.mg/L: milligrams per liter.

The statistical analysis show significant increased in ferritin level in G1 (206.27±11.3) then G2 (99.49±4.44), G3(43.36±1.13) and G4 (45.35±1.75). Results of the current study found a significant elevation P=0.0001(P≤0.01) in ferritin level regarding COVID-19 recovered patients studied G1 group as compared with apparently healthy control group as donated in table 3.

In addition significant differences P=0.0001, P=0.0001, P=0.0001 consequently at (P≤0.01) between studied groups (G1 vs. G2, G3 & G4). Moreover significant differences P=0.0001, P=0.0001(P≤0.01) in that order between G2 vs. G3 & G4. No significant differences P=0.944 at P>0.05 level between G3 vs. G4 as clearly presented in table 3.

Table 3: The distribution of ferritin levels between groups.

Time after recovery/ Ferritin level (13-350ng/ml)	COVID-19	P value compared with			
	Mean±SE	0-14d	1m	2m	4-6m
G1 (n=150)	206.27±11.30				
G2 (n=146)	99.49±4.44	0.0001#			
G3 (n=142)	43.36±1.13	0.0001#	0.0001#		
G4 (n=135)	45.35±1.75	0.0001#	0.0001#	0.944	
Control	44.75± 2.98	0.0001#			

#Significant difference between two dependent means using Paired-t-test at 0.05 level.

NO. Number; S.E.: Standard Error; P-value: Probability value; ng/mL: Nanograms per milliliter.

3.3 Correlation of D-dimer and ferritin level with severity.

The level of ferritin was high with significant differences P=0.0001 (P≤0.01) level between mild, moderate & severe sub groups in G1, but, it was within normal level with non significant differences P=0.831, P=0.961 and P=0.039 at 0.05 level respectively in G2, G3 & G4. Highly significant differences P=0.0001, P=0.0001 & P=0.0001 at 0.01 level in D-dimer level respectively in G1, G2 & G3 between severity sub groups. D-dimer decreased to normal level with non significant differences in G4 P=0.135 at 0.05 level, as presented in table 4.

Table 4: The correlation of Ferritin and D-dimer level according severity.

Time after recovery groups	Mild		Moderate		Severe		P value
	No	Mean±SE	No	Mean±SE	No	Mean±SE	

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G1 (n=150)							
Ferritin (ng/mL)	45	117.03±6.59	58	191.58±7.59	47	307.70±28.22	**0.0001^**
D dimer (mg/L)	45	0.51±0.03	58	0.72±0.03	47	1.19±0.04	**0.0001^**
G2 (n=146)							
Ferritin (ng/mL)	43	91.73±8.17	58	101.20±7.20	45	104.95±7.76	0.831
D dimer (mg/L)	43	0.19±0.02	58	0.32±0.01	45	0.48±0.02	**0.0001^**
G3 (n=142)							
Ferritin (ng/mL)	41	43.95±1.13	57	43.26±0.67	44	42.95±1.04	0.961
D dimer (mg/L)	41	0.15±0.01	57	0.19±0.01	44	0.24±0.01	**0.0001^**
G4 (n=135)							
Ferritin (ng/mL)	38	47.05±3.47	54	44.31±3.04	43	45.07±3.26	0.039
D dimer (mg/L)	38	0.19±0.019	54	0.24±0.01	43	0.24±0.01	0.135

^Significant difference among three independent means using ANOVA-test at 0.05 level.

NO. Number; S.E.: Standard Error; P-value: Probability value.mg/L: milligrams per liter; ng/mL: Nanograms per milliliter.

3.4 Evaluation of IL-6 and IL-10 levels.

Quantitative assay used to measured IL-6 & IL-10 quantification. Statistical analysis shown a significant increase P=0.0001 (P≤0.01) in IL-6 level in COVID-19 recovered patients groups as compared to healthy control group. Also significant differences P=0.0001, P=0.0001(P≤0.01) in G 2 (7.88±0.35) and G 3(7.55±0.24) vs. G1 (37.29±5.36). While non-significant differences P=0.55 (P>0.05) between G2 (7.88±0.35) vs. G 3(7.55±0.24) as clearly shown in table 5.

Regarding the main regulatory cytokine IL-10, the present study found significantly increased P=0.0001(P≤0.01) in IL-10 level in COVID-19 recovered patients as compared to healthy control group. In addition to, a significant differences P=0.0001(P≤0.01) between G2 (3.06±0.01) vs. G1 (7.01±0.33). Likewise, there are a significant differences P=0.0001, P=0.0001(P≤0.01) between G3(1.79±0.14) vs. G1(7.01±0.33) and G2(3.06±0.01) respectively as explained in table 6.

Table 5: The concentration of IL-6 in studied groups

Studied groups	IL-6 (pg/ml)	P-value		
	Mean±SE	G1	G2	G3
G1	37.29±5.36			
G2	7.88±0.35	**0.0001#**		
G3	7.55±0.24	**0.0001#**	0.550	
CON.	6.39±0.4		**0.0001#**	

#Significant difference between two dependent means using Paired-t-test at 0.05 level.

N. Number; S.E.: Standard Error; P-value: Probability value; pg/ml: Picograms per milliliter. G1: Group 1, after 1 month of recovery; G2: Group 2, 2-3 months after recovery. G3: Group 3, 4-6 months after recovery. G4: Group4, over 6 months after recovery. CON.: Apparently healthy control group.

Table 6: The concentration of IL-10 in studied groups.

Studied groups	IL-10 (pg/ml)	P value		
	Mean ±SE	G1	G2	G3

G1	7.01±0.334		
G2	3.06±0.098	**0.0001#**	
G3	1.79±0.138	**0.0001#**	**0.0001#**
CON.	1.82±0.142		**0.0001#**

#Significant difference between two dependent means using Paired-t-test at 0.05 level.

N. Number; S.E.: Standard Error; P-value: Probability value;pg/ml: Picograms per milliliter. G1: Group 1, after 1 month of recovery; G2: Group 2, 2-3 months after recovery. G3: Group 3, 4-6 months after recovery. G4: Group4, over 6 months after recovery. CON.: Apparently healthy control group.

3.5 The differences in IL-6 & IL-10 levels according to COVID-19 severity.

It is interesting to note that there were a significant difference P=0.0001 (P≤0.01) in IL-6 levels between mild (14.76±0.42), moderate (20.93±0.64) and severe (79.04±15.51) sub groups in G1. While non-significant differences P=0.676, P=0.081(P>0.05) between severity sub groups respectively in G 2 and G 3 as shown in table 7.

As depicted in table 8, there were a significant differences P=0.0001 (P≤0.01) in IL-10 levels between mild (5.46±0.26), moderate (6.29±0.22) and severe (9.36±0.9) subgroup in G 1. While non-significant differences P=0.201, P=0.235 (P>0.05) between severity subgroups in G2 and G3.

Table-7: The differences in IL-6 levels according COVID-19 severity.

Studied groups	IL-6 (pg/ml)						P-value
	Mild		Moderate		Severe		
	N.	Mean±SE	N.	Mean±SE	N.	Mean±SE	
G1	45	14.76±0.423	58	20.93±0.64	47	79.04±15.51	0.0001 ^
G2	43	7.85±0.632	58	8.21±0.57	45	7.46±0.63	0.676
G3	41	8.40±0.513	57	7.21±0.32	44	7.21±0.42	0.081

^Significant difference among three independent means using ANOVA-test at 0.05 level.

N.: Number; S.E.: Standard Error; P-value: Probability value; pg/ml: Picograms per milliliter. G1: Group 1, after 1 month of recovery; G2: Group 2, 2-3 months after recovery. G3: Group 3, 4-6 months after recovery. G4: Group4, over 6 months after recovery.

Table 8: The differences in IL-10 levels according COVID-19 severity.

Studied groups	IL-10 (pg/ml)						P value
	Mild		Moderate		Severe		
	N.	Mean±SE	N.	Mean±SE	N.	Mean±SE	
G1	45	5.46±0.26	58	6.29±0.22	47	9.36±0.9	**P1=0.0001^**
G2	43	3.03±0.22	58	2.89±0.09	45	3.31±0.19	P2=0.201

G3	41	1.96±0.29	57	1.94±0.21	44	1.44±0.20	P3=0.235

^Significant difference among three independent means using ANOVA-test at 0.05 level.

N. Number; S.E.: Standard Error; P-value: Probability value; pg/ml: Picograms per milliliter. G1: Group 1, after 1 month of recovery; G2: Group 2, 2-3 months after recovery. G3: Group 3, 4-6 months after recovery. G4: Group4, over 6 months after recovery.

3.6 The association between D-dimer and ferritin level.

Interestingly, highly significant association between D-dimer & ferritin level in group 1, r=0.732, P=0.0001 at the 0.01 level. Also positive relationship with highly significant correlation P=0.0001(P≤0.01) between IL-6 level and IL-10 level (r = 585). In addition, a positive correlation P=0.0001(P≤0.01) between ferritin and IL-6 (r=0.550) as shown in table 9.

Table 9: The correlation between D-dimer and ferritin level.

G1		Ferritin	D-dimer	IL6	IL10
Age (years)	**r**	0.050	-0.038	-0.016	0.001
	P	0.542	0.640	0.842	-
Ferritin	**r**	-	**0.732****	**0.550****	0.319**
	P	-	0.0001	**0.0001**	0.0001
D-dimer	**r**	**0.732****	-	0.367**	0.274**
	P	0.0001	-	0.0001	0.001
IL6	**r**	**0.550****	0.367**	-	**0.585****
	P	**0.0001**	0.0001	-	0.0001
IL10	**r**	0.319**	0.274**	**0.585****	-
	P	0.0001	0.001	0.0001	-

*Correlation is significant at the 0.05 level **Correlation is highly significant at the 0.01 level.

P-value: Probability value; r = correlation coefficient.

4. Discussion

In this analysis of our data of COVID-19 recovered patients, D-dimer was significantly higher levels, which may be caused by action of the coagulation system, it then decrease in levels due to action of anticoagulant therapy. While it has been reported that D-dimer level is significantly increased in COVID-19 recovered patients [29], [30], so this study focused on follow up of ferritin level in COVID-19 recovered patients which differs during disease development. D-dimer is produced via fibrin degradation, so it acts as an indicator of fibrinolytic action [31], [32]. High level of D-dimer which may be increased risk of thrombus [33]. In addition there is a relationship between D-dimer levels and the markers of inflammation [29], [30] This explains the link between D-dimer and some symptoms (fever, extreme sweating) lead to increase blood viscosity and hypercoagulable state as a result of action of coagulation system [34], so D-dimer associated with disease severity and mortality [35]. Other study reported that elevated D-dimer level associated with increased risk of COVID-19 severity and mortality [36], [37]. Furthermore, high D-dimer levels in COVID-19 recovered patients require for anticoagulant therapy which decreases the level to normal [35].

Present study of ferritin level in recently COVID-19 recovered patients, as study of Rasyid (2020) in Indonesia that documented a higher levels of ferritin associated with COVID-19 patients [38], also associated with several autopsies and with higher mortality than those with lower ferritin values [39]. High ferritin levels can be used as a biomarker to identification and management of COVID-19 patients [40]. Hyperferritinemia was a critically case in stable hospitalized patients [41], this may lead to acute liver failure in COVID-19 patients [42]. In Meta analysis study, the hyperferritinemia in COVID-19 in terms of

mortality, comorbidities, and severity [43] as other study that reported ferritin levels were strongly linked to the severity of COVID-19 [44].

Present study about a main role of IL-6 in COVID-19 patient's cytokine storm, its highly correlation to disease severity, emergency case or death, so, IL-6 can be used as a pharmacological target [45], [46]. The present results revealed that increasing the levels of IL-6 in COVID-19 recovered patient, this elevation correlated to disease severity. It consider as a potential prognostic marker has positive correlation with other inflammatory marker as CRP [16]. Some previous studies showed that the IL-6 level is useful to imagine if patients needing mechanical ventilation during hospitalization [16], [47]. On the other hand, other study in Spain found a mortality risk model based on IL-6 level, neutrophil/lymphocyte ratio, Lactate dehydrogenase (LDH) level, and age [48]. Also, other study in Italia recommended IL-6 as a useful predictor to endpoint of severe COVID-19 [49]. Other studies published the association between IL-6 level and development which evaluated by CT scan [16], [44].

Present study found the dramatic early rise of anti-inflammatory cytokine IL-10 be a distinguishing feature of hyper-inflammation during severe COVID-19, it is anti-inflammatory and immunosuppressive cytokine [46]. The height of IL-10 could be help in stop tissue damage. Though, the relationship between elevated IL-10 levels and COVID-19 severity, may be due to either IL-10 is acting as a different traditional role (anti-inflammatory molecule). On other hands, other studies suggested that IL-10 act as a pro-inflammatory molecule [50]. The classical role of IL-10 as inhibitor of the beginning of an adaptive T cell response [46], [51] It may also inhibits antiviral protection in COVID-19. Some authors have published that hyper-inflammation in severe COVID-19 may due to mast cell activation syndrome so it be a goal for therapeutic inhibition [52].

5. Conclusion

Noticeably increased in D-dimer & ferritin level in COVID-19 recovered patients and could be used as diagnostic biomarker for COVID-19. COVID-19 severity associated with elevation of IL-6 and IL-10 level.

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