Zagazig University Medical Journal
www.zumj.journals.ekb.eg

Manuscript ID ZUMJ-2105-2237 (R2)
DOI **10.21608/zumj.2021.76794.2237**

ORGINAL ARTICLE

Cytokine Storm in COVID-19 Patients: Association between cytokines and disease severity

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Submit Date 2021-05-19
Revise Date 2021-06-27
Accept Date 2021-06-29

ABSTRACT

Objectives: To determine the cytokine storm profile in patients with COVID-19 infection in relation to the disease severity.

Methods: Serum levels of TNF-α, IFN-γ, IL-6 and IL-1β were measured by ELISA method and IL-1RII, TNFR1 and TNFR2 gene expression in peripheral blood mononuclear cells (PBMCs) were done using real time polymerase chain reaction (PCR) method in a total of 203 COVID-19 patients. The 1st group included 52 COVID-19 severe cases, the 2nd comprised 104 moderate cases and the 47 mild cases lied in the 3rd group.

Results: There was a significant association between degree of COVID-19 severity and IL6, TNF-α & IFN-γ serum levels ($p < 0.001$). IL-1β serum levels were not significantly correlated to the degree of COVID-19 severity ($p = 0.139$). There was a significant relation between the up regulation of IL-1RII, TNFR1 & TNFR2 gene expression and the disease severity ($p<0.001$ for each).

Conclusions: Our results suggested that IL6, TNF-α & IFN-γ cytokines serum levels and IL-1RII, TNFR1 & TNFR2 expressions were highly linked to the degree of severity in COVID-19 patients.

Keywords: COVID-19; Interferon-γ (IFN-γ); Tumor necrosis factor α (TNF-α); Interleukin 6 (IL-6); Interleukin 1β (IL-1β); Cytokine receptors.

INTRODUCTION

The World Health Organization had announced COVID-19 as a pandemic. By the beginning of July 2021, the number of deaths was 3,727,605 among 173,005,553 confirmed cases in WHO reports [1]. A new beta coronavirus has been identified as the causing pathogen for Severe Acute Respiratory Syndrome Coronavirus 2(SARS-CoV-2) [2]. The target organ for COVID-19 is the lung but affects other organs as well, causing multi-organ negative consequences [2, 3].

Three stages have been observed in COVID-19 patients, with reference to progression and extent: (i) “mild” which occurs in the vast majority of patients with minor symptoms and do not progress to severe disease; (ii) “moderate” these are hospitalized patients suffering from pneumonia on radiology accompanied with symptoms or laboratory lymphopenia or leukopenia; and (iii) “severe” who were admitted to the Intensive Care Unit due to systemic hyper-inflammatory status and ARDS and at risk of fatal outcome [4, 5]. Regardless of various targeted and non-targeted method for management, no specific

treatment has yet been proven effective in treating COVID-19.

Epidemiological studies have detected a rapid elevation of acute phase reactants in cases suffering from COVID-19, involving C-reactive protein (CRP), ESR, ferritin, and serum amyloid A, suggesting a rapid triggering of innate immunity [6-9]. As logic consequences, COVID- 19 cases show elevation of the circulatory levels of many cytokines as interleukin (IL)-1β, TNF-α, IL-6, IL-10, IL-17, IL-18, interferon- γ (IFN-γ), and monocyte chemoattractant protein-1 (MCP-1) [7,10].

The complex interplay between the host antiviral immune responses and the viral ability to modify these responses are critical for disease severity [11]. Previous studies elucidated a pivotal role for virus-associated immunopathological events in causing fatal pneumonia in Middle East Respiratory Syndrome (MERS) and SARS patients that are often associated with massive inflammatory cell infiltration and elevated proinflammatory cytokine responses called cytokine storm [12].

Despite of the possible hyper-activation of the innate immune system, more probably this cytokine storm is due to both defective first line of immunity and persistent cytokine dysregulation [IL-6, IL-1β, IFN-γ and tumour necrosis factor- α (TNF-α)], leading to generalized cytotoxicity. This culmination in cytokine release leads to coagulopathy, fever and acute respiratory distress syndrome (ARDS) (Figure 1) [13-15].

Certain types of these mediators can lead to vicious circle, including Natural Killer cell (NK) dysfunction by IL-6 [16] or macrophage cell stimulation via the H-chain of ferritin [17-19]. Moreover, hemo- phagocytosis was reported in lung tissues from SARS-CoV infected cases [19].

Two immune dysfunction patterns have been suggested in COVID-19 cases: (i) one of them suggests macrophage activation syndrome, which is mainly caused by IL-1β; and (ii) the other pattern of immune dysregulation is driven by IL-6, which is characterized by a combination of cytokine dysfunction, immunoparalysis, and lymphopenia (including NK cells and CD4+) [16].

An unanticipated progressive deterioration has been demonstrated in the late stages of COVID-19. This is often expressed as an unexpected exaggeration of symptoms (hyperthermia and dyspnea) and is associated with elevated levels of acute phase reactants (CRP, ESR, and ferritin) and coagulopathy (disseminated intravascular coagulation and increased d-dimers [6, 20-22]. In the most severe cases, signs, symptoms and laboratory findings are correlated to elevated levels of pro-inflammatory cytokines evoking a cytokine storm [21, 23, 24].

Cytokines bind to their cognate transmembrane receptors and initiate downstream signaling leading to multiple functions including induction of immune responses. Cytokine receptors dysregulation is implicated in mediating the immune responses of these cytokines [25].

In the present study, we demonstrated the change in cytokines and their receptors in COVID-19 patients with variable disease severity. Identifying the distinct cytokine profile of COVID-19 has practical implications, as it can be prognostic of clinical deterioration.

SUBJECTS AND METHODS

Patients

The study was conducted in Faculty of Medicine, Zagazig University, Egypt. The total number of COVID-19 patients was 203, comprised 89 males and 114 females, of mean age ± SD (43 ± 14.9) ranging from 42–74 years. They were sub grouped according to severity in reference to Ministry of Health & Population (MOHP), Egypt protocol 2020 into: 1st GROUP (Severe cases): with 52 patients, who were admitted to the ICU (Intensive Care Unit) due to COVID-19 serious consequences. 2nd GROUP (Moderate cases): included 104 patients, these are hospitalized patients suffering from pneumonia on radiology accompanied with symptoms or laboratory lymphopenia or leukopenia. 3rd GROUP (Mild cases): comprised 47 patients, the patients were considered as mild cases if they show symptoms with no radiological evidence for

pneumonia with lymphopenia or leukopenia. And they were asked for home quarantine with follow up.

All patients had no history of hypertension, diabetes or other diseases. Written informed consent was obtained from all participants, the study was approved by the research ethical committee of Faculty of Medicine, Zagazig University. The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Laboratory examination

Routine blood tests were measured by the XS-1000i hematology analyzer (Sysemx, Japan). Biochemical indictors were tested by the ADVIA2400 Chemistry System (Siemens, Germany). Coagulation tests were analyzed by the ACLTOP750 automatic coagulation analyzer (Instrumentation Laboratory, USA).

Cytokines estimation

Serum levels of interleukin- 1 β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interferon- γ (IFN-γ) were measured by double antibody sandwich Enzyme-Linked ImmunoSorbent Assay ELISA kits (Assaypro., LLC, USA; Biosource Europe S.A., Belgium; Ray Biotech, USA) according to the instructions supplied by manufacturer. The minimum detectable concentrations were 0.1 pg/mL for IL-1β, 7.5 pg/mL for IL-6, 0.5 pg/mL for TNF-α, and 2 pg/mL for IFN-γ.

IL-1RII, TNFR1 and TNFR2 Gene expression

RNA extraction

We collected 3 mL of whole blood from each participant in the anti-coagulant EDTA tubes. According to manufacturer's instructions, and by density gradient centrifugation on Ficoll-Paque solution (lympholyte, Cedarlane, Sweden), we isolated Peripheral Blood Mononuclear Cells (PBMCs). RNeasy Mini Kit- Qiagen was used for RNA extraction. Estimation of RNA purity was indicated at a ratio of absorbance (260 & 280 nm).

Reverse Transcription

Reverse transcription of RNA was done via (QuantiTect Reverse Transcription Kit, QIAGEN, Germany) Quantiscript reverse transcriptase.

Real Time-PCR

Specific RNA amplification was done in a 20 µl reaction mixture including 100 pmol/ul primers, 5 µl of cDNA template and 10 µl Eva Green mix (Jena Bioscience). The amplification was done using (RT-PCR) - (StratageneMx3005P-qPCR System). The primers sequence was: forward: 5′-ACCACAGTCCATGCCATCAC-3′, reverse: 5′-TCCACCACCCTGTTGCTGTA-3′ for glyceraldhyde 3 phosphate dehydrogenase (G3PHD), forward 5'TGCAAAGTGTTTCTGGGAAC-3', reverse 5'-ATATTGCCCCCACAACCAAG-3' for IL 1 receptor type 2 (IL1-RIl), forward: 5′-GAACCAGCCACAGGCACCA-3′, reverse: 5′-ACGATGCAGGTGACATTGAC-3′ for TNFR2 and forward: 5′-TATTGGACTGGTCCCTCACC-3′, reverse: 5′-GTCATTGTACAAGTAGGTTC-3′ for TNFR1. Regarding qPCR reactions, we performed initial denaturation & polymerase activation at 95 °C for thirty seconds, then forty cycles of denaturation at 95 °C for fifteen seconds; elongation & annealing at 58 °C for sixty seconds. We employed ΔCT method to calculate relative gene expression changes in relation to G3PDH (reference gene).

Statistical Analysis

Data were analyzed using the IBM SPSS for Mac version 23. Data were presented as mean ± standard deviation (SD). Student's t test, ANOVA test and chi-squared test were used when appropriate. Serum cytokines levels and expressions were analyzed using the normality test. $p < 0.05$ was considered statistically significant.

RESULTS

General patient characteristics

COVID-19 diagnostic criteria were met in all of the enrolled participants. The median ages were: 65 (55-74) in 1st group, 56 (45-64) in 2nd group, and 54 (42-65) in 3rd group. Females to males ratio was 1.16:1. The common comorbidities in all patients of different groups were: malignancy (5 [2.4%]), digestive diseases (26 [12.8%]), chronic lung disease (27 [13.3%]), cardiovascular disease

(35 [17.2%]), diabetes (37 [18.2%]), and hypertension (65 [32.0%]).

Fever was the most prominent symptom at disease onset (155 [75.6%]), cough came second (133 [65.3%]), then fatigability (110 [49.3%]), then dyspnea (106 [52.2%]), lastly muscular pain and anorexia were (92 [45.3%]) and (58 [28.6%]) respectively. While pharyngitis, expectoration, and dizziness all were in (32 [15.8%]). Less common symptoms according to system affected were diarrhea (28 [13.8%]), vomiting

(7 [3.4%]), then headache & syncope (9 [4.4%%]) and (16 [7.9%]) respectively, coryza (8 [3.9%]), at last oliguria & hematuria (3 [1.5%]) and (6 [3.0%%]) respectively.

Overall, ARDS represented 100 cases (49.3%), abnormal liver function represented 81 cases (34.9%), impaired renal function 68 cases (33.5%), acute cardiac injury 45 cases (22.2%), and shock 13 cases (6.4%).83 (40.9%) patients were discharged, 84 (41.4%) were improved, 16 (7.9%) remained in the ICU, and the total death number was 20 (9.8%) by the end of August, 2020. **(Table 1)**

Laboratory parameters:

In comparison with the mild patients group, the critically severe patients group had significantly higher levels of neutrophils (NEUT) as well as the whole white blood cells (WBCs), procalcitonin (PCT), D-D dimer (D-D), C-reactive protein (CRP), lactate dehydrogenase (LDH), alanine aminotransferase (ALT) & aspartate aminotransferase (AST), prothrombin time (PT), , blood urea nitrogen (BUN), creatinine, creatine kinase-MB (CK-MB),HSTNI, and glucose ($P < 0.05$), while significantly lower levels of total protein (TP), platelet (PLT), lymphocytes, , albumin (ALB), CO2, Ca2+, K+, Na+ and C1q ($P < 0.05$) **(Table 2).**

Our study showed that the critically severe patients group had high infection indexes, hepato-renal function abnormalities and abnormal myocardial zymo-grams ($P < 0.05$). In comparison with sever patients, the critically severe patients had significantly higher NEUT, WBC, HSTNI, PT, and PCT, while had markedly lower CO2 ($P < 0.05$). In comparison with the mild group, sever group had significantly higher levels of ALT, GLU, CRP and CK-MB while had lower lymphocytes level, ALB, Ca2+, and TP ($P < 0.05$) **(Table 2).**

Cytokine and cytokine receptors profile

The IL-6 concentration in the severe group was 7-fold higher than in the mild group and 2.4-fold higher than in the moderate group at disease onset. IL-6 in the moderate type was 2.9-fold higher than in the mild group ($P < 0.05$) **(Fig. 1a).** The IFN-γ level in the severe group was 3.7-fold higher than in the mild group and 2.2-fold higher than in the moderate group. IFN-γ in the moderate group was 1.7-fold higher than in the mild group ($P < 0.05$) **(Fig. 1b).**

Moreover, for TNF-α level in the severe group was 3.3-fold higher than in the mild group and 2.6-fold higher than in the moderate group. TNF-α in the moderate group was 1.3-fold higher than in the mild group ($P < 0.05$) **(Fig. 1c).** There was no significant difference in IL-1β among the three groups ($P > 0.05$**) (Figure 2d)** & **(Table 3).**

Degree of COVID-19 severity was significantly associated with upregulation of IL-1RII, TNFR1 and TNFR2 gene expression ($p < 0.001$ for each) **(Table 4).**

Correlations among all of the parameters

Close association between IL-6 levels and: IFN-γ ($r = 0.56$, $P < 0.01$), TNF-α ($r = 0.42$, $P < 0.01$), NEUT($r=0.43$,$P=0.01$), PT ($r=0.40$,$P=0.01$), BUN ($r=0.47$,$P=0.01$), PCT ($r=0.63$,$P<0.01$), and CRP ($r = 0.48$, $P < 0.01$). Close association between IFN-γ levels and: IL-6, TNF-α ($r = 0.46$, $P < 0.01$), NEUT ($r = 0.31$, $P = 0.05$) and D-D ($r = 0.29$, $P = 0.02$). Close association between TNF-α levels and: IL-6, IFN-γ, dyspnea ($r = 0.323$, $P < 0.01$), fatigability ($r = 0.326$, $P < 0.01$), anorexia ($r = 0.266$, $P = 0.025$) and diarrhea ($r = 0.267$, $P = 0.024$).

Table (1) Baseline characteristics of patients infected by SARS-CoV-2 with various severities.

	NO.(%) Total N =203	Group I Severe cases (N = 52)	Group II Moderate cases (N = 104)	Group III Mild cases (N = 47)	P		
					1 vs 2	1 vs 3	2vs3
Characteristics							
Age, median, years	60 (42–74)	65 (55–74)	56 (45–64)	54 (42–65)	0.28	0.05	1.00
Sex							
Female	109 (53.7)	28 (53.8)	58 (55.8)	23 (48.9)	0.03	0.05	0.03
Male	94 (46.3)	24 (46.2)	46 (44.2)	24 (51.1)	0.22	0.05	1.00
Signs and symptoms							
T, median, °C	38.5(38.0–39.4)	38.5(38.0–39.4)	38.5(38.0–39)	38(38.0–39)	0.25	0.01	1.00
HR, median	82.0(75.0–110.0)	100.0(78.0–110.0)	94.0(76.0–108.0)	90.0(75.0–107.0)	0.06	0.30	0.54
RR, median	20.0(18.0–26.0)	22.0(18.0–26.0)	20.0(18.0–24.0)	19.0(18.0–25.0)	**0.01**	**<0.01**	0.67
Fever	155(75.6)	50(96.1)	65(62.5)	40(85.1)	1.00	0.62	1.00
Coug h	133(65.5)	47(71.1)	48(46.1)	38(79.1)	0.54	0.46	0.38
Dyspnea	106(52.2)	37(52.1)	35(33.7)	34(70.8)	1.00	**<0.01**	**<0.01**
Fatigue	110(49.3)	51(98.1)	31(29.8)	28(59.6)	1.00	0.13	0.05
Myalgia	92(45.3)	24(46.1)	58(55.8)	10(21.3)	0.55	0.76	1.00
Anorexia	58(28.6)	22(42.3)	24(23.0)	12(25.5)	1.00	1.00	0.33
Expectoration	32(15.8)	10(19.2)	12(11.5)	10(21.3)	0.54	0.54	1.00
Diarrhea	28(13.8)	7(13.5)	12(11.5)	9(19.1)	1.00	**0.01**	0.44
Pharyngalgia	32(15.8)	4(7.7)	24(23.0)	4(8.5)	1.00	1.00	0.54
Dizziness	32(15.8)	4(7.7)	14(13.5)	14(13.5)	1.00	0.66	1.00
Syncope	16(7.9)	4(7.7)	12(11.5)	0	1.00	0.58	-
Headache	9(4.4)	5(9.6)	3(3.0)	1(0.49)	0.65	**0.04**	0.30
Coryza	8(3.9)	5(9.6)	3(3.0)	0	1.00	0.46	0.33
Vomiting	7(3.4)	5(9.6)	2(2.0)	0	0.54	0.46	0.30
Hematuria	6(3.0)	4(7.7)	2(2.0)	0	1.00	0.24	-
Oliguria	3(1.5)	2(3.8)	1(1.0)	0	1.00	0.47	-

Comorbidities				7 (14.9)	0.06	0.04	1.00
Hypertension	65 (32.0)	26 (50.0)	32 (30.8)	2 (4.2)	1.00	0.23	0.29
Diabetes	37 (18.2)	10 (19.2)	25 (24.0)	3 (6.4)	**0.03**	**0.01**	1.00
Cardiovascular disease	35 (17.2)	22 (42.3)	10 (9.6)	3 (6.4)	0.64	1.00	1.00
Chronic lung disease	27 (13.3)	4 (7.7)	20 (19.2)	2 (4.3)	0.84	1.00	1.00
Malignancy	5 (2.4)	2 (3.8)	1 (0.1)	3 (6.4)	1.00	1.00	0.44
Digestive diseases	26 (12.8)	6 (11.5)	15 (14.4)				
Complications							
ARDS	100 (49.3)	50 (96.2)	50 (48.0)	0	**<0.01**	**<0.01**	**<0.01**
Abnormal liver function	81 (34.9)	31 (59.6)	43 (41.3)	7 (14.9)	0.17	**<0.01**	**<0.01**
Acute cardiac injury	45 (22.2)	42 (80.8)	2 (1.9)	1 (2.1)	**<0.01**	**<0.01**	1,00
Acute kidney injury	68 (33.5)	44 (84.6)	22 (21.1)	2 (4.2)	**<0.01**	**<0.01**	**0.03**
Shock	13 (6.4)	12 (23.0)	1 (0.9)	0	0.66	**0.01**	**-**
Clinical outcome							
Died	20 (9.8)	20 (38.5)	0	0	**<0.01**	**<0.01**	**-**
Remained in ICU	16 (7.9)	16 (30.8)	0	0	**0.02**	**<0.01**	**-**
Condition improve	84 (41.4)	10 (19.2)	56 (53.8)	18 (38.3)	0.32	**<0.01**	0.23
Hospital discharge	83 (40.9)	6 (11.5)	48 (46.7)	29 (61.7)	**0.02**	**0.02**	0.44

Data: n/N (%), n (%), mean, and median. Comparing the mild, sever, and critically severe patients: one-way analysis of variance, Fisher's exact test, or chi-squared test - (P-value < 1.5) considered significant. ARDS: Acute Respiratory Distress Syndrome; HR: heart rate; RR: respiratory rate; T: temperature

 Doi:610.21608/zumj.2021.76794.2237

Table (2) Laboratory findings of the COVID-19 patients with various disease severities.

	Normal Range	Group I Severe cases (N = 52)	Group II Moderate cases (N = 104)	Group III Mild cases (N = 47)	P		
					1 vs 2	1 vs 3	2vs3
WBC, $\times 10^9$/L	3.5–9.5	12.30 ± 1.40	7.06 ± 1.34	5.67 ± 1.34	0.01	<0.001	1.00
NEUT, $\times 10^9$/L	1.8–6.3	11.04 ± 1.24	6.09 ± 1.34	3.34 ± 1.06	0.01	<0.001	0.57
NEUT,%	40–75	88.59 ± 1.42	78.57 ± 1.72	70.43 ± 1.66	0.16	<0.001	0.04
LY, $\times 10^9$/L	1.1–3.2	0.64 ± 0.06	0.51 ± 0.03	1.49 ± 0.09	1.00	<0.001	<0.001
LY, %	20–50	6.71 ± 1.05	12.01 ± 0.09	24.11 ± 0.08	0.33	<0.001	0.03
HGB, g/L	115–150	125.24 ± 4.29	135.34 ± 8.90	137.35 ± 8.94	0.23	0.04	1.00
RBC, $\times 10^{12}$	3.8–5.1	3.92 ± 0.12	4.09 ± 0.08	4.13 ± 0.01	0.65	0.28	1.00
HCT,%	35–45	36.33 ± 1.12	39.21 ± 1.99	38.11 ± 1.90	0.31	0.22	1.00
PLT, $\times 10^9$/L	125–350	173.56 ± 9.50	197.09 ± 9.53	232.06 ± 8.56	0.67	0.01	0.29
PT, s	9.4–12.5	13.40 ± 0.44	12.66 ± 0.34	12.08 ± 0.04	0.06	0.01	0.69
APTT, s	25.1–36.5	41.90 ± 8.01	29.65 ± 9.05	32.07 ± 9.09	0.24	0.39	0.31
TT-E, s	10.3–16.6	33.60 ± 9.68	15.44 ± 7.69	15.32 ± 4.11	0.33	0.21	0.99
FIB, mg/dL	238–498	444.37 ± 27.13	494.44 ± 37.19	434.07 ± 17.18	1.00	1.00	0.59
DD, ng/mL	0–500	5433 ± 2001.99	1789 ± 1067.07	1996 ± 786.14	0.13	<0.001	1.00
ALT, U/L	7–45	51.87 ± 7.42	65.07 ± 13.09	35.12 ± 7.16	1.00	0.01	0.02
AST, U/L	13–35	73.45 ± 13.66	42.66 ± 14.06	32.11 ± 9.44	0.14	0.01	0.22
TP, g/L	65–85	63.80 ± 0.24	70.82 ± 0.86	76.78 ± 0.99	0.99	<0.001	<0.001
ALB, g/L	40–55	32.77 ± 0.80	39.73 ± 0.55	43.54 ± 0.21	1.00	<0.001	<0.001
GLB, g/L	20–30	28.88 ± 0.99	30.88 ± 0.93	28.43 ± 0.64	0.67	0.87	0.88
GGT, U/L	8–57	62.66 ± 14.50	87.69 ± 12.56	39.99 ± 9.43	0.69	0.38	0.23
ALP, U/L	30–120	77.66 ± 5.01	84.45 ± 3.13	92.05 ± 3.99	1.00	0.47	1.00
TBA, umol/L	0–15	4.11 ± 0.46	5.66 ± 0.34	4.01 ± 0.07	0.97	0.18	0.44
BUN, mmol/L	2.8–7.6	7.41 ± 0.02	6.21 ± 0.06	4.41 ± 0.09	0.24	<0.001	0.05
CREA, umol/L	49–90	82.99 ± 7.22	70.93 ± 6.26	65.75 ± 6.44	0.29	0.05	0.53
UA, umol/L	155–357	280.06 ± 33.22	232.06 ± 13.29	308.12 ± 8.25	0.31	0.98	0.08

CO2, mmol/L	21–29	20.88 ± 0.87	25.18 ± 0.07	25.99 ± 0.05	<0.001	<0.001	1.00
CYSC, mg/L	0–1.2	1.20 ± 0.05	1.16 ± 0.09	1.02 ± 0.01	1.00	0.13	0.60
K^+, mmol/L	3.5–5.3	4.01 ± 0.43	4.58 ± 0.44	4.77 ± 0.54	0.25	<0.001	0.51
Na^+, mmol/L	137–147	136.44 ± 1.24	138.94 ± 1.29	138.99 ± 1.33	0.34	0.03	1.00
Cl^-, mmol/L	99–110	103.42 ± 1.13	104.92 ± 0.19	102.67 ± 0.04	0.89	1.00	0.83
Ca^{2+}, mmol/L	2.11–2.52	1.99 ± 0.04	2.03 ± 0.06	2.25 ± 0.07	0.66	<0.001	<0.001
CK, U/L	<145	438.99 ± 191.90	158.99 ± 54.92	122.43 ± 69.04	0.46	0.28	0.81
CKMB, U/L	0–25	54.99 ± 18.60	44.05 ± 17.62	12.40 ± 15.89	1.00	<0.001	0.07
LDH, U/L	125–243	528 ± 55.90	327 ± 54.93	222 ± 53.88	0.34	<0.001	0.23
MYO, ng/mL	<140.1	247.99 ± 72.23	197.90 ± 62.29	123.92 ± 66.23	0.27	0.13	1.00
HSTNI, pg/mL	0–26.2	967.31 ± 801.22	35.45 ± 46.29	8.32 ± 8.21	0.03	0.01	1.00
GLU, mmol/L	3.9–6.1	12.24 ± 1.12	8.27 ± 0.15	6.34 ± 0.03	1.00	<0.001	0.04
C1q, mg/L	159–233	177.32 ± 9.23	197.39 ± 11.63	220.04 ± 3.12	1.00	0.01	0.23
PCT, ng/ml	<0.05	1.82 ± 0.77	0.83 ± 0.67	0.08 ± 0.61	0.01	<0.001	0.50
CRP, mg/L	0.0–3.0	107.99 ± 14.32	77.79 ± 13.31	30.70 ± 8.30	0.46	<0.001	0.02

(P value < 0.05) was considered statistically significant. ALB: albumin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; APTT: activated partial thromboplastin time; AST: aspartate aminotransferase; BUN: blood urea nitrogen; C1q: complement C1q; CK: creatine kinase; CKMB: creatine kinase-MB; CREA: creatinine; CRP: C-reactive protein; CO2: carbon dioxide; CYSC: cystatin C; DD: D-dimer; FIB: fibrinogen content; GGT: alpha-glutamyl transpeptidase; GLB: globin; GLU: glucose; HCT: hematocrit; HGB: hemoglobin; HSTNI: hypersensitive troponin I; LDH: lactate dehydrogenase; LY: lymphocytes; MYO: myoglobin; NEUT: neutrophils; PCT: procalcitonin; PLT: platelet; PT: prothrombin time; RBC: red blood cell; TBA: total bile acid; TP: total protein; TT-E: thrombin time (extended); UA: uric acid; WBC: white blood cell;

Table (3) Serum levels of different measured serum cytokines and their correlation to the degree of severity.

	Group I Severe cases (N = 52)	Group II Moderate cases (N = 104)	Group III Mild cases (N = 47)	P
IL6 serum (pg/ml) Mean ±SD	289.8 ± 56.4	123.2 ± 47.9	41.8 ± 21.0	**0.000**
INF-γ serum (pg/ml) Mean ±SD	68.1 ± 8.9	31.6 ± 11.8	18.2 ± 7.5	**0.000**
TNF-α serum (pg/ml) Mean ±SD	78.3 ± 15.5	30.2 ± 11.9	23.4 ± 8.2	**0.000**
IL-1 β serum (pg/ml) Mean ±SD	4.3 ± 7.8	2.5 ± 5.1	2.7 ± 2.6	0.139

IFN-γ: Interferon Gamma, TNF-α: Tumor Necrotizing Factor Alpha, IL: Interleukin, SD: Standard Deviation

Table (4) Comparison different groups regarding studied cytokines receptors gene expression

	Group I Severe cases (N = 52)	Group II Moderate cases (N = 104)	Group III Mild cases (N = 47)	**P value**
TNFR1 relative expression Mean ±SD	2.99±0.25	1.61±0.14	1.19±0.63	<0.001
TNFR2 relative expression Mean ±SD	2.63±0.35	1.43±0.12	1.24±0.43	<0.001
IL1RII relative expression Mean ±SD	3.89±0.63	2.51±0.37	1.86±0.16	<0.001

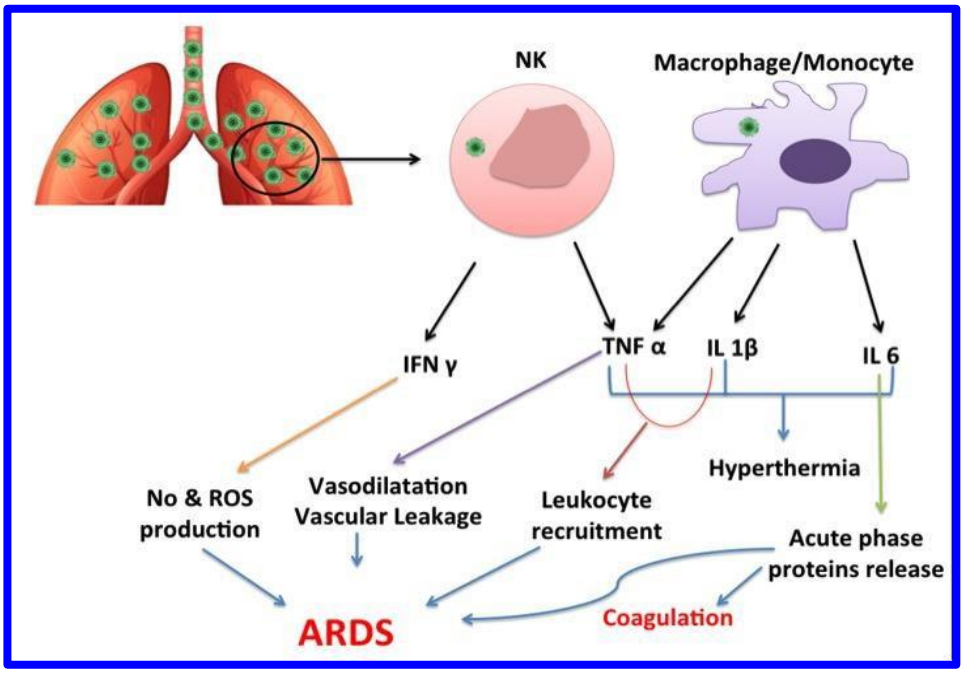
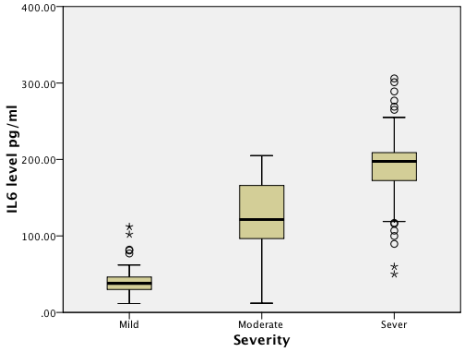
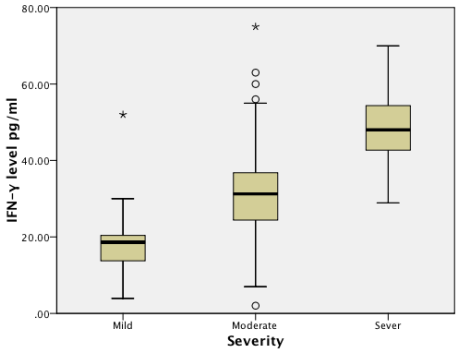


Figure (1) Cytokine storm of COVID-19

The entry of SARS-CoV-2 in lung tissue cells, leads to necrotizing and apoptotic effects resulting in lung injury and the recruitment of large amounts of immune cells within the lungs. Natural Killer cell (NK) and monocytes, along with alveolar macrophages and neutrophils, secreting pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, Interferon Gamma (IFN-γ), and tumor necrosis factor (TNF)-α. These mediators result in cytokine storm, coagulopathy, fever and acute respiratory distress syndrome (ARDS).



(a)



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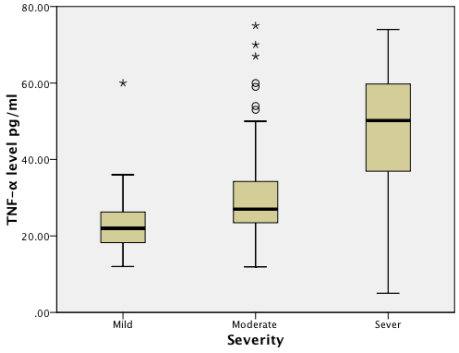
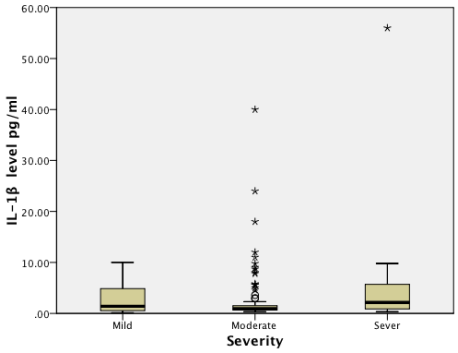


(c)



(d)

DISCUSSION

A notable feature, that high viral replication of in SARS-cov 2 cases leads to higher levels of proinflammatory cytokine production by infected epithelial cells **[26].** In spite of the probability of being a part of necessary initial body immune response to pathogens, exacerbation of chemokines and proinflammatory cytokines expression is associated with acute respiratory distress syndrome (ARDS) and immunopathology **[25].** Therefore, illustration of cytokine expression role in disease severity is fundamental. Identifying immune correlates of COVID-19 disease severity is an urgent need for clinical management, vaccine evaluation, and drug development.

Our study results showed that the degree of COVID-19 severity was significantly associated with IFN-γ, TNF-α and IL6 serum levels ($p < 0.001$), however this association does not exist with serum IL-1 β values.

Yang et al found that serum concentrations of some of these cytokines can help to distinguish between severe, moderate, and mild cases (mainly IL-1 β, IL-6, IL-7, and TNF-α) **[10].** Moreover, these cytokines levels in moderate and mild cases are generally lower than the levels observed in severe cytokine release syndrome (CRS) **[11, 12].** Accordingly, cytokine dysregulation could be considered as a general sign for SARS-CoV-2 infection, and the term 'cytokine storm' can describe critical COVID-19 situations, as disseminated intravascular coagulation, multiple organ failure or ARDS. In the most severe cases, signs, symptoms and laboratory findings are correlated to elevated levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, IFN-γ), evoking a cytokine storm **[21,23-25].** **Liu et al** results suggested that COVID-19 infection stimulates huge changes in an array of cytokines, with association to disease severity **[27].**

Our results were in agreement with **Giamarellos-Bourboulis et al** and **Li et al** as they found that the unique pattern of immunological dysregulation in cases of severe COVID-19 is presented by overexpression of IL-6 and TNF-α, with persistent cytokine production and hyper-inflammation, also **Zhang et al** found that IL-6 serum levels are much higher in severe cases of COVID-19 infection **[16, 28, 29].**

Compatible with our data, **McGonagle et al** assured the role of elevated IL-6 in COVID-19 cases progression and deterioration, with high-lightening the role of anti-IL6R administration in tissue remodeling **[30].** In their study**, Tang et al** concluded that there was a significantly higher level of IL-6 and IL-10 in the critically severe patients compared to severe and mild cases **[31].** IL-6 has a significant role in integrated immunity through defense ability against viral infections **[32].** Yet, in some respiratory infections, like respiratory syncytial virus infection and influenza, elevated IL-6 levels has been linked to worse outcomes and increased lung damage **[32,33].**

Zheng et al also found that IFN-γ expression in COVID-19 patients was much higher than healthy controls **[34].** In viral infections, antiviral IFN acts not only to control viral infections but also to program the adaptive immune response to promote viral clearance **[35].**

Contrary to our finding, **Zheng et al** found no statistical differences in TNF-α and IL-6 plasma levels among the three groups of their study, which was also divided according to severity. They also reported decrease cases in IFN-γ serum levels in severe cases **[36].** This could be attributed to a smaller study sample size, as **Zheng et al** study group was 22 subjects.

Yeleswaram et al stated that cytokine storm is a common manifestation in severe 2019-nCoV infections. As in comparison with healthy subjects, patients suffering from COVID-19 showed elevated levels of IL-1β; which doesn't match our findings; IL-6, IFN-γ, and TNF-α; which come in agreement with our results **[37].**

Moreover, **Ye et al** review article showed that providing a background on the strategies of the 2019-nCoV cytokine elevations could be of great benefit for future guidance in clinical treatment **[38].**

In the current study, TNF-α levels TNFR1 and TNFR2 expressions were significantly related to disease severity. TNF production

may be increased with macrophages and monocytes exposure to microbial products, then enhancing their activation status, increasing procoagulant molecules expression and promoting adhesion molecule expression on endothelial cells. Our believe is that evidence is sufficiently support clinical trials in patients with COVID-19 receiving anti-TNF therapy. TNF-α effects are done through interacting with the TNFR superfamily receptors.

Patients with severe COVID-19 were reported to have plasma sTNFR1 elevation **[39]. Mortaz et al** detected association between mortality of COVID-19 patients in the ICU and elevated serum levels of soluble TNF-α receptor. **Bowman et al** found that TNFR1 and TNFR2 were increased in COVID-19 patients at the time of hospitalization and before treatment beginning. Moreover, these markers levels were increased in cases with critical disease who died in comparison with cases with critical disease who recovered **[40].** They suggested that there is association between disease severity and TNFR1 & TNFR2 and these markers may be predictors of mortality in critically ill patients. The signaling cascade of TNF/TNFR may be an effective goal to reduce patients' mortality in critical COVID-19 cases **[40].**

Interleukin-1 receptor (IL-1R) is a cytokine receptor which binds interleukin 1. Two forms of the receptor exist. The type I receptor is primarily responsible for transmitting the inflammatory effects of IL-1 while type II receptors may act as a suppressor of IL-1 activity by competing for IL-1 binding **[41].** Our results found an upregulation of IL-1R type II indicating that it may be a defense mechanism to reduce the cytokine storm effects. It is the first paper studying the expression of IL-1RII in COVID-19 patients. **Zhao et al** found that early production of inhibitory mediators including IL-1RA were significantly associated with disease severity and it might be useful prognostic biomarkers to guide treatment strategies **[42].**

All these findings are needed to be interpreted in a larger study. COVID-19 symptoms & severity and association with cytokines profiles and end organ involvement are needed to be explored as this may enhance clinical care.

Our results suggest that a wide variety of cytokines are triggered by COVID-19 infections. These could be used as effective biomarkers of COVID-19 degree of severity. We also suggest that once the mechanisms of the involved cytokines have been characterized, modulators of their responses could play an essential role in fighting this infection.

5. Conclusion

Our results suggested that serum levels of IL6, TNF-α & IFN-γ cytokines and expressions of IL-1RII, TNFR1 & TNFR2 were highly linked to COVID-19 patients' degree of severity.

6. RECOMMENDATIONS

As cytokines could be effective biomarkers of the degree of severity of COVID-19 infection, more detailed studies about pathways and their correlation with COVID-19 is recommended.

6. Footnotes:

Figure (1):

The entry of SARS-CoV-2 in lung tissue cells, leads to necrotizing and apoptotic effects resulting in lung injury and the recruitment of large amounts of immune cells within the lungs. Natural Killer cell (NK) and monocytes, along with alveolar macrophages and neutrophils, secreting pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, Interferon Gamma (IFN-γ), and tumor necrosis factor (TNF)-α. These mediators result in cytokine storm, coagulopathy, fever and acute respiratory distress syndrome (ARDS).

Figure (2):

Box plot graphics of the serum levels of (a) IL-6, (b) IFN-γ, (c) TNF-α and (d) IL-1β according to severity of COVID-19 (◦:outliers)

The authors declare that they have no competing interests

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How to Cite

Sarhan, W., Shalaby, S., Zidan, N., El-Hawary, A., Ismail, N., Makani, V., Abd elnour, H. Cytokine Storm in COVID-19 Patients: Association between cytokines and disease severity. *Zagazig University Medical Journal*, 2021; (1640-1653): -. doi: 10.21608/zumj.2021.76794.2237