Research article

Cytokine profile as biomarker of COVID-19 and its correlation with liver function enzymes and other markers of inflammationGita Nepal¹, Maitreyee D. S.², Akshatha Rao Aroor³, Shivashankara A. R.⁴¹M.Sc. MLT Student in Biochemistry, ²Associate Professor of Biochemistry, ³Associate Professor of General Medicine,⁴Professor of Biochemistry, Father Muller Medical College, Mangalore, Karnataka India

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ABSTRACT

Introduction and Aim: Various hematological and biochemical parameters are assessed as biomarkers of COVID-19, caused by the SARS-CoV-2. In this study, efforts were made to identify the correlation of cytokines (IL-6, TNF- α , IL-1 α , and IL-1 β) with the liver function enzyme markers, LDH, CRP, Ferritin, and D-dimer. We also assessed the correlation of cytokines with severity of COVID-19.

Materials and Methods: We analyzed 53 serum samples of RT-PCR-positive patients admitted to the hospital. Cytokines (IL-6, TNF- α , IL-1 α , and IL-1 β) were analyzed with sandwich ELISA. The levels of cytokines were compared between mild, moderate, and severe cases of COVID-19, and the correlation among cytokines, liver function marker enzymes, LDH, CRP, D-dimer, and ferritin were analyzed.

Results: Along with CRP, LDH and ferritin, IL-6 showed significant difference between mild, moderate, and severe COVID groups. significant correlation between IL-6 & LDH (p -.002), IL-6 & D-Dimer (p- .010), IL-6 & IL-1 β (p-.027), IL-1 β & D-Dimer (p- .010), IL-1 β & LDH (p-.027), and IL-1 β & TNF- α & (p-.000).

Conclusion: Cytokines especially IL-6 correlated with disease severity. Assaying the profile of cytokines could be of immense value in diagnosis, prognosis, and management of COVID-19.

Keywords: Biomarker; COVID-19; cytokines; inflammation; interleukins.

INTRODUCTION

The COVID-19 is caused by the SARS-CoV-2. These positive single-stranded large RNA viruses belong to the beta-coronaviruses subfamily. COVID-19, an acute respiratory infection has become pandemic and a serious threat to the whole world since 2019, has led to millions of deaths and hospitalization worldwide (1). Along with the respiratory symptoms, various degree of liver and cardiac damages are seen in hospitalized patients(2,3). COVID-19 infection shows an aggressive inflammatory response, releasing a large amount of pro-inflammatory cytokines known as “cytokine storm”. Cytokines are produced by immune cells like innate macrophages, dendritic cells, natural killer cells T and B lymphocytes. The cytokine stimulates the activity of the genes which are involved in the process of inflammation and immunity, and called “pro-inflammatory”(4). Most important pro-inflammatory cytokines of the innate immune response are IL-1 α , TNF- α , and IL-6. COVID-19patients show a wide range of clinical severity from asymptomatic to fatal conditions. Advanced age, underlying comorbidities weakened immune system, cytokine release syndrome and lymphopenia are risk factors for higher severity of illness and death from COVID-19(4). Cytokine storm is a maladaptive cytokine release in response to infection and other stimuli. The pathogenesis is complex but includes loss of regulatory control of pro-inflammatory cytokine production, both at local and

systemic levels. Evidence shows that, in COVID- 19 epidemic, severe deterioration in some patients is closely associated with dysregulated, excessive cytokine release phenomenon (5).

Several biomarkers are being used for the diagnosis of COVID-19along with the RT-PCR. Such biomarkers include, C-reactive protein (CRP), ferritin, D-dimer and lactate dehydrogenase (LDH). Ferritin and CRP not only act as an acute-phase reactants but, also is responsible for the synthesis of cytokines and causes cytokine storm(6). Some studies have suggested to have a high level of TNF- α in the patient with inflammatory disease and hence suggested to use anti-TNF antibody in the treatment of COVID-19to hospitalized patients to prevent the progression of the disease(7). Clinical studies suggested that high levels of cytokines particularly IL-6, TNF- α , IL-1 α , and IL-1 β are seen in SARS- COVID-19with severe patients requiring ICU(8). There is paucity of studies on the interleukin profile in COVID-19positive patients. We aimed to determine the levels of cytokines namely, Interleukin-1 α (IL-1 α), Interleukin-1 β (IL-1 β), Interleukin-6 (IL-6), Tumor necrosis factor- α (TNF- α) in serum and to correlate these cytokine markers with routinely done biochemical tests of liver function (AST, ALT, ALP), D-Dimer, LDH and C-reactive protein and ferritin in hospitalized COVID-19. We also aimed to compare the cytokine levels between mild, moderate, and severe COVID-19infection positive patients.

MATERIALS AND METHODS

The cross-sectional study was conducted in the Department of Clinical Biochemistry at Father Muller Medical College Hospital and the Father Muller Research Centre. This study was approved by the Institutional Ethics. As the ethics committee granted us waiver of consent due to use of left-over samples in the lab for analysis of cytokines, Informed consent was not required. Fifty-three confirmed COVID-19 positive patients by RT-PCR who were admitted to the hospital during India's first wave were included in this research. Asymptomatic patients admitted to the hospital due to coronavirus infection were excluded. Data of routinely done biochemical tests for managing COVID-19 positive patients (liver function tests, D-dimer, CRP, Ferritin, LDH) and the clinical history of disease severity-mild, moderate, severe; were collected from the database. Cytokines (IL-6, TNF- α , IL- α , IL-1 β) were assayed by Sandwich ELISA using ELISA kits of Diaclone and ELISA reader of Biorad. The procedure was followed as per the manufacture's instruction. Standards and control specimen were run along with samples in each batch of analysis.

Ethical approval

All procedures performed in studies involving human participants were as per the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or

comparable ethical standards. The study was approved by Father Muller Institutional ethics committee. (FMMC/CCM/538/2020)

Statistical analysis

Analysis was done using SPSS version 23.0 software. The descriptive statistics median and IQR, Kruskal–Wallis test, Bonferroni test were used for the comparisons between groups. Pearson Correlation statistical test was used to assess the association between Cytokines (IL6, IL-1 α , 1 β , TNF- α) with AST, ALT, D-dimer, ferritin, CRP, LDH markers. Value of $P < 0.05$ was considered statistically significant.

RESULTS

Results of our study are presented in tables 1 to 3. There was significant difference in serum levels of CRP, ferritin, LDH and IL-6 when the mild, moderate, and severe cases were compared. Serum CRP was significantly higher in moderate and severe cases of COVID-19 when compared to mild cases. The serum CRP levels showed increase with progression of the disease. Similar observations were seen with regard to LDH, ferritin and IL-6 (Table 2). Among the cytokines IL-6 showed significant positive correlation with LDH, D-dimer and IL-1 α among the COVID-19 patients; while serum IL-1 α showed significant positive correlation with LDH, D-dimer and TNF- α (Table 3).

Table 1: IQR of IL 6, IL-1 α , IL 1 β , and TNF- α , AST, ALT, ALP, LDH, Ferritin, CRP.

Biochemical markers (n=53)	Percentile		
	25 th	50 th (Median)	75 th
AST	27.50	43.00	63.50
ALT	19.00	33.00	55.00
ALP	67.00	78.00	108.00
LDH	233.50	331.00	441.00
D-DIMER	324.50	522.00	1033.00
CRP	14.6600	45.1000	111.910
Ferritin	208.0000	410.1000	866.900
IL-6	6.350	21.700	74.300
TNF- α	15.450	17.900	21.350
IL-1 α	70.23350	75.77100	106.690
IL-1 β	33.50200	35.49200	44.082

Table 2: Difference between mild, moderate, and severe COVID-19 infections with regard to various biomarkers (Kruskal Wallis test was done to find out the association between severity of COVID-19 infections with the biochemical markers)

Group		N	Median (IQR)	Kruskal Wallis test p value
AST	Mild	18	43(20.75--56)	0.086
	Moderate	21	36(26.5--57.5)	
	Severe	14	56.5(36.75--109)	
ALT	Mild	18	32.5(16.75--58.5)	0.132
	Moderate	21	27(15.5--45.5)	
	Severe	14	48(24.25--58.25)	
ALP	Mild	18	76.5(61.25--108)	0.808
	Moderate	21	88(68--112)	
	Severe	14	79(64.5--94.5)	
LDH	Mild	18	276.5(197--359.5)	0.001***
	Moderate	21	272(193--394)	

D-Dimer	Severe	14	504(343.5--832.25)	0.054
	Mild	18	428.5(225--751)	
	Moderate	21	460(337.5--896)	
	Severe	14	915(397.25--3104.5)	
CRP	Mild	18	10.425(3.865--15.1425)	0.000***
	Moderate	21	45.29(41.095--72.185)	
	Severe	14	203.305(143.97--287.5825)	
Ferritin	Mild	18	254(187.275--577.775)	0.003***
	Moderate	21	304.7(201.85--651.95)	
	Severe	14	976.5(423.9--1597.75)	
IL-6	Mild	18	9.1(3.55--23.275)	0.024*
	Moderate	21	31(11.45--69.45)	
	Severe	14	118.6(3.675--191.3)	
TNF-α	Mild	18	18.65(15.85--23.125)	0.167
	Moderate	21	16.2(13.7--20.35)	
	Severe	14	18.7(16.875--25.625)	
IL-1α	Mild	18	71.57(65.36525--135.247)	0.365
	Moderate	21	78(70.4375--92.79)	
	Severe	14	76.352(71.37775--129.69)	
IL-1β	Mild	18	35.18(32.414--41.0535)	0.386
	Moderate	21	35.492(33.9055--37.9765)	
	Severe	14	36.951(33.87475--67.782)	

p***<0.001(very highly significant), p** <0.01(highly significant), p* <0.05(significant).

Table 3: Correlation between various biomarkers in COVID-19(n=53)

Strength of association between the cytokine and other markers (n=53)		r (Pearson Correlation)	P
Cytokines & LDH	IL-6&LDH	.416	.002
	TNF- α & LDH	.030	.833
	IL-1 α & LDH	-.066	.637
	IL-1 β & LDH	.303	.027
Cytokines & AST, ALT, ALP	IL-6 & AST	.188	.177
	TNF- α & AST	.049	.725
	IL-1 α & AST	-.046	.746
	IL-1 β & AST	-.101	.472
	IL-6 & ALT	.005	.974
	TNF- α & ALT	-.047	.740
	IL-1 α & ALT	.002	.988
	IL-1 β & ALT	-.170	.224
	IL-6 & ALP	-.064	.654
	TNF- α & ALP	.191	.179
	IL-1 α & ALP	-.071	.622
	IL-1 β & ALP	-.006	.969
Cytokines & CRP	IL-6 & CRP	.226	.104
	TNF- α & CRP	-.021	.880
	IL-1 α & CRP	-.058	.679
	IL-1 β & CRP	.021	.883
Cytokines & Ferritin	IL-6 & Ferritin	.077	.582
	TNF- α & Ferritin	-.056	.692
	IL-1 α & Ferritin	-.107	.444
	IL-1 α & Ferritin	-.106	.448
Cytokines and D-dimer	IL-6 & D-Dimer	.350	.010
	TNF- α & D-Dimer	-.022	.874
	IL-1 α & D-Dimer	-.028	.841
	IL-1 β & D-Dimer	.350	.010
Strength of association between Cytokines	IL-6 & TNF- α	-.020	.886
	IL-6 & IL-1 α	-.074	.596
	IL-6 & IL-1 β	.303	.027
	TNF- α & IL-1 α	-.009	.948
	TNF- α & IL-1 β	.561	.000
	IL-1 α & IL-1 β	.079	.574

DISCUSSION

Our study showed significant difference with regard to the biochemical markers CRP, ferritin, LDH and IL-6 among mild, moderate, and severe COVID-19 cases. There was significant correlation of IL-6 and IL-1 β with the conventionally used biomarkers LDH and D-dimer. The findings of various studies on cytokines in COVID-19 are summarized in table 4.

To evaluate the impact of SARS-Co V-2 on cytokine levels we evaluated IL-6, IL-1 α , IL-1 β , and TNF- α ; we observed significant change in IL-6 but, other cytokines did not show any significant difference between the mild, moderate, and severe COVID-19 cases. However, the levels of all cytokines were significantly higher than the biological reference intervals reported with healthy population, in various studies, indicating cytokine storm(9). The increases in cytokines IL-6 is in concordance with the findings of

previous studies. The change observed has been suggested to be due to the cytokine storm, inflammation and tissue injury.

The findings of IL-1, IL-1 β and TNF- α not differing as per severity of the disease is a salient finding of our study. Previously, Chen et al. reported higher levels of IL-6 and TNF- α in severe cases of COVID-19 when compared to moderate cases and these authors also reported that levels of IL-1 β did not differ between the severe and moderate cases (12). We assessed the correlation of cytokines with liver function marker enzymes, and the conventional COVID biomarkers LDH, ferritin, D-dimer and CRP. We observed significant correlation of IL-6 and IL-1 β levels with LDH and D-dimer, and significant correlation between IL-1 β with IL-6 and TNF- α . Previously, Effenberger *et al.*, showed a significant association between liver markers and IL-6 (14,15).

Table 4: Previous studies on cytokines and COVID-19

Authors	Sample size n Type of study	Groups	Findings
Han <i>et al.</i> , (14)	102 COVID-19 patients. Longitudinal study	Classified into moderate, severe, and critical.	Higher serum levels of cytokines (TNF- α , IFN- γ , IL-2, IL-4, IL-6, and IL-10) and CRP than control individuals. IL-10 positively correlated with CRP levels
Zeng <i>et al.</i> , (10)	317 laboratory-confirmed COVID-19 patients. Retrospective study.	Moderate, severe, or critically ill form of the disease	Critically ill patients had higher concentrations of soluble interleukin (IL)-2 receptor, IL-6, IL-8, IL-10, TNF- α , ferritin, procalcitonin, LDH, hsCRP, and hsCRP/L than patients with severe or moderate disease
Zhang <i>et al.</i> , (16)	901 patients with SARS-CoV-2 infection. Cohort study	COVID-19 patients - grouped as Common cases, severe cases, and critical cases.	Significant difference in IL-6 levels between common, severe, and critical groups. IL-6 level of >37.65 pg/ml was predictive of in-hospital death with a 91.7% sensitivity and 95.7% specificity.
Chen <i>et al.</i> , (12)	21 patients COVID-19 were analyzed. Retrospective study	Classified as severe (11 cases) and moderate (10 cases)	Severe cases - had markedly higher levels of IL-2R, IL-6, IL-10, and TNF- α , when compared to moderate cases.
Del Valle <i>et al.</i> , (9)	Cohort n = 1715	Mild, moderate, severe, and severe with end-organ damage	High serum IL-6, IL-8 and TNF- α levels at the time of hospitalization were strong and independent predictors of patient survival. Elevated IL-6 and TNF- α serum levels at presentation were strong predictors of disease severity and survival.
Keddie <i>et al.</i> , (13)	203 samples Cross-sectional observational study	WHO COVID-19 ordinal severity scale	IL-6, strongly correlated with the WHO ordinal scale of illness severity. IL-6 levels of ≥ 3.27 pg/ml provided a sensitivity of 0.87 and specificity of 0.64 for a requirement of ventilation
Effenberger <i>et al.</i> , (15)	655 patients with suspected COVID-19. Cross-sectional design.	No groups made	Liver injury correlated with systemic IL-6 level

CONCLUSION

In conclusion, amongst the cytokines, IL-6 has consistently shown correlation with tissue injury marker LDH, disease severity marker D-dimer, and inflammatory marker IL-1 β . Other cytokine marker IL-1 β has shown a significant correlation with TNF- α , IL-6 and LDH. With the high levels of cytokines along

with the biomarkers CRP, ferritin, LDH may be of importance to correlate with disease severity of SARS-CoV2. Assaying the profile of cytokines could be of immense value in diagnosis, prognosis, and management of COVID-19. Further studies with larger sample size correlating cytokines with disease severity and various other routine markers of COVID-

19 are required to establish cytokines as reliable prognostic markers of COVID-19.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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