Detection of Interleukin-10 and interferon-γ as biomarkers in COVID-19 patients

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Abstract
Background: Measurement of serum levels of IFN-Y and IL-10 can be used as biomarker in COVID-19 patients and assessment of disease severity.Objectives: To assess the levels of Interlukin-10 and Interferon-γ in serum of COVID-19 patients using ELISA test. Materials and Methods: We collect serum and nasopharyngeal (NP) swab from 100 COVID-19 suspects and 50 control healthy persons in an Al Fallujah Teaching Hospital, Anbar, Iraq from 29 November 2021 to 15 February 2022. The results from the patients were compared with control group and disease severity. Results: Among 100 suspects, 84 (84%) were positive while in control group 11(22%) persons were positive for IFN-Y, with (P-value<0.05). Out of 56 positive NP RT-PCR, there were 53(94%) positive IFN-Y and from 44 negative NP RT-PCR,31(70.5%) were positive IFN-Y with (p-value<0.05).All moderate to severs patients 11(100%) were positive IFN-Y, while from 89 mild patients, 73(82%) were positive IFN-Y with (p-value >0.05). Among 100 patients 72were positive for interleukin- 10, in control group 9 persons were positive with (P-value<0.05). Among 56 positive RT-PCR 50 patients, were positive IL-10. Conclusion: There is significant increase in serum IFN-γ and IL-10 in COVID-19 patients.
Keywords: COVID-19, IFN-γ, IL-10, Nasopharyngeal (NP) swab, SARS-COV-2, serum.

1. Introduction

In December 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused the coronavirus disease (COVID-19) started in Wuhan, China (1). Then it expanded outside of China, creating worldwide public health disaster (2–4). The WHO declared COVID-19 a pandemic on March 11, 2020. The majority of patients have few symptoms and a good prognosis. Severe pneumonia, ARDS, multiple organ failure, myocardial infarction, metabolic acidosis, and even death have all occurred in certain COVID-19 patients (5). COVID-19 is worldwide contagious illness (6). According to the WHO, there were 6,401,046 deaths and 577,018,226 confirmed cases as of 3 August 2022. According to clinical data from several nations, almost one-third of the patients had acute respiratory distress syndrome (ARDS)(7), which is a major cause of death and may lead to pulmonary fibrosis in survivors. Angiotensin-converting enzyme 2 (ACE2), a receptor expressed in airway and alveolar epithelial cells, is known to have a high affinity for residues in the receptor-binding domain (RBD) of SARS-CoV-2 (8,9). SARS-CoV-2 endocytosed in epithelial cells are liberated and undergo fast replication by ACE2-mediated endocytosis, which results in pyroptosis, a characteristic virus-linked programmed cell death (10). The establishment of ARDS and fibrosis is caused by the release of viral RNA and damage-associated molecular patterns from dead epithelial cells in the lung (11,12). The pathogenic condition known as cytokine release syndrome (CRS), which is defined by a fast and protracted systemic increase of more than 20 inflammatory cytokines and chemokines , develops in severe and critically ill COVID-19 patients(13). Inflammation-induced lung injury, severe pneumonia, and ARDS are all results of the cytokine storm, which can harm microvascular endothelial cells, lung epithelial cells. Pulmonary fibrosis will result from the lung tissue being destroyed if the cytokine storm is not promptly treated(14). IFN-γ possesses anti-fibrotic properties in addition to its antiviral effect. It has been observed that IFN-deficient mice or mice treated with IFN-γ blocking antibodies have a protective role for IFN-γ in renal fibrosis(15).Intensive care unit (ICU) COVID-19 patients had significantly greater peripheral IL-10 concentrations than non-ICU patients (16,17). Additionally, IL-10 levels showed a high correlation with IL-6 levels as well as other inflammatory indicators including C-reactive protein(CRP)(18). The rapid accumulation of pro-inflammatory cytokines as a negative feedback loop has been suggested to be the primary cause of the clinical significance of highly elevated IL-10 levels in the serum of COVID-19 patients as an anti-inflammatory or immune-inhibitory mechanism (and thus biomarker)(17,19). Additionally, some researchers have suggested using recombinant IL-10 to treat ARDS in COVID-19 patients due to its immunoregulatory and antifibrotic properties (20). Recent studies demonstrate immune activation and inflammation in COVID-19 patients supporting the hypothesis that IL-10 may play a proinflammatory and immune-activating effect in COVID-19 pathogenesis (21).

2. Material and Method

100 individuals who visited the acute respiratory infection clinic at Al-Fallujah Teaching Hospital in the Iraqi province of Al-Anbar between November 29 and February 15, 2022, were the subjects of a cross-sectional study. The inclusion criteria were suspects of both sexes with who experienced COVID-19 symptoms including fever, coughing, dyspnea, etc. for two to eight days and who were >15 years old with mean 35.95 year, 55 suspects was male. 50 healthy individuals with 28 (56%) men and 22 (44%) was women, >15years with mean 34.32 years from outside the hospital as control group had no symptom and confirmed negative for SARS-CoV-2 by NP RT-PCR with normal ESR and CRP. Depending on the severity of the illness, we divide the suspects into two groups: mild group were 89 and moderate to severe group were11.NP RT-PCR, serum IFN-γ and serum IL-10 was done.The Ethical Approval

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Committee of the University Of Anbar examined and approved the study protocol (reference number 38 on 13-9-2022) Informed consent was taken from every participant.

Sample collection: We collect five ml of blood by vein puncture using 5 ml syringe. Blood samples were placed into a gel tube, for 30 minutes at 37C° and centrifuged for 15 minutes at 3000 rpm. The resulting sera were then aspirated by automatic micropipette and putted in a clean test tube. Each tube was labeled and stored in deep freeze at -20C° for later immunological tests

Quantitative Measurement of Interferon-gamma (INF-γ) in serum by ELISA test

We fill the appropriate wells (96 wells) with fifty µl of each of the calibration, control, and sample and added 50µl of the conjugated anti-INF-HRP in each well. Next, we incubate for a further two hours at 18 to 28 C° on Dark a horizontal shaker set (DLabTECh) operating at 700 rpm.The liquid is then aspirated from each well after that the plate was washed three times with 199 ml of D.W. and 1 ml of wash buffer.

After the washing process, we added 100 µl of the chromogenic Solution (TMB) into each well within 15 minutes. A dark horizontal shaker set to 700 rpm was used to incubate the microtiter plate at (18–28) C° for 15 minutes. Then added 100 microliters of stop solution into each well. Finally, we read the absorbencies at 450 nm and 490 nm within 30 minutes and calculate the results.

The Demeditec INF-γ human ELISA (Enzyme-Linked Immunosorbent Assay) work

The Demeditec INF-γ- human ELISA (Enzyme-Linked Immunosorbent Assay) is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on a microtiterplate. The assay uses a monoclonal antibody (MAbs) directed against distinct epitopes of INF-Y. Calibrators and samples react with the capture monoclonal antibody (Mab1) coated on microtiter well and with a monoclonal antibody (Mab2) labeled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: coated Mab1-human INF-Y-Mab2-HRP, the microtiterplate is washed to remove the unbound enzyme-labeled antibody.

Quantitative Measurement of Interleukin-10 (IL-10) in serum by ELISA test.

We put 100 µL of Incubation Buffer into in each well and we put 100 µL of each Calibrator, Control, and sample into the appropriate wells. After that we incubate the wells for 2 hours at (18-28) C° on Dark - a horizontal shaker set (DLabTECh) at 700 rpm.Then we aspirate the liquid from each well then The plate was washed 3 times with washer Solution (199 ml D.W+1mlwash buffer).we put 100µL of specimen diluent and then 50 µL of anti-IL-10-HRP conjugate into all the wells after that. We incubate for 2 hours at (18-28) C° on a horizontal shaker set at 700rpm.Then we aspirate the liquid from each well. The plate was washed three times again before we Put 100µL of the TMB Solution into each well within 15 minutes following the washing step. The microtiterplate was incubated for 15 minutes at (18-28) C° on a horizontal shaker set at 700rpm, after the incubation, we added 100 µL of stop solution into each well. Finally, we read the absorbencies at 450 nm and 490 nm within 30 minutes and calculate the results. Immunosorbent Assay) work

The Demeditec IL-10 human ELISA is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiterplate. The assay uses a monoclonal antibody (MAbs) directed against distinct epitopes of IL-10. Calibrators and samples react with the capture monoclonal antibody (Mab1) coated on microtiter well and with a monoclonal antibody(Mab2) labeled with horseradish peroxidase(HRP).After an incubation period allowing the formation of a sandwich: coated Mab1-human IL-10-Mab2-HRP, the microtiterplate is washed to remove the unbound enzyme-labeled antibody

3. Statistical Analysis

The data were entered and analyzed using IBM SPSS statistics version 26 (32 bit edition) for the window. For continuous variables, the means and standard deviation (SD) were used. While the categorical data were shown in simple tables as frequencies and percentages. The Chi-Square test was used to compare the category variables. The reference normal value were 0.03ng/l,1.6ng/l for IFN-γ and IL10 respectively. Kappa coefficient was used to measure the agreement between the patients sample and control sample. P-value of less than 0.05 is considered statistically significant difference.

4. Results

There are 150 participants in this study, 50 from the control group (healthy individuals) and 100 COVID-19 suspects. **Table 1** shows that the patients group contained 55 (55%) men and 45 (45%) women, 60 (60%) ≤ 35-year and40 (40%) > 35 year. compared to 28 (56%) men and 22 (44%) women, 33 (66%) ≤35year and 17 (34%)> 35 year in the control group. There was a mild agreement between patients and control group (kappa coefficient 0.475, 95% CI 0.723e0.979; P-value>0.05).

Table 1: Distribution of the patients and control group according to gender, age group

Variable	Patient	Control
sex		
Male	55 (55%)	28 (56%)
Female	45 (45%)	22 (44%)
Total	100 (100%)	50 (100%)
Age		
≤35	60 (60%)	33 (66%)
>35	40 (40%)	17 (34%)
Total	100 (100%)	50 (100%)

The level of IFN-γ

84 (84%) of the 100 patients had positive result, while 16 (16%) had negative result. control group consisted of 4(8%) were positive and 46(92%) were negative for IFN-γ with a P-value <0.05 **(Table 2).**

: ITabe2IFN-γ Distribution according to patients and control (P-value=0.000)				
	IFN-Y		Total N (%)	
	Positive N (%)	Negative N (%)		P-value
Case				0.000
Patient Count	84 (84.0%)	16 (16.0%)	100 (100.0%)	
Control Count	4 (8%)	46(92.0%)	50 (100.0%)	
Total Count(% within Case)	88 (58.7%)	62(41.3%)	150 (100.0%)	

53 (94%) of the 56 positive NP RT-PCR results were positive for IFN-Y, while only 3 (5.4%) were negative. Of the 44 negative NP RT-PCR results, 31 (70.5%) were positive for IFN-Y, while 13 (29.5%) were negative (p-value <0.05).

IFN-Y was positive in 11 out of 11 moderate-to-severe patients (100%) while out of 89 mild patients 73 (82%) were positive, 16(18%) patients were negative (P-value >0.05), as indicated in **(Table 3).**

Table3: Distribution of IFN-Y according to NP RT-PCR and COVID-19 severity				
	IFN-Y			
	Positive N (%)	Negative N (%)	Total N (%)	P-value
NP swab for RT-PCR for SARS-COV-2				0.001
Positive	53 (94.6%)	3 (5.4%)	56 (100.0%)	
Negative	31 (70.5%)	13 (29.5%)	44 (100.0%)	
Severity				0.125
Moderate to sever	11 (100.0%)	0 (0.0%)	11 (100.0%)	
Mild	73 (82.0%)	16 (18.0%)	89 (100.0%)	

The mean concentration was (2.301ng/l ± 1.615) for patients and (0.154 ng/l ± 0.547) for control as shown **in figure 1**

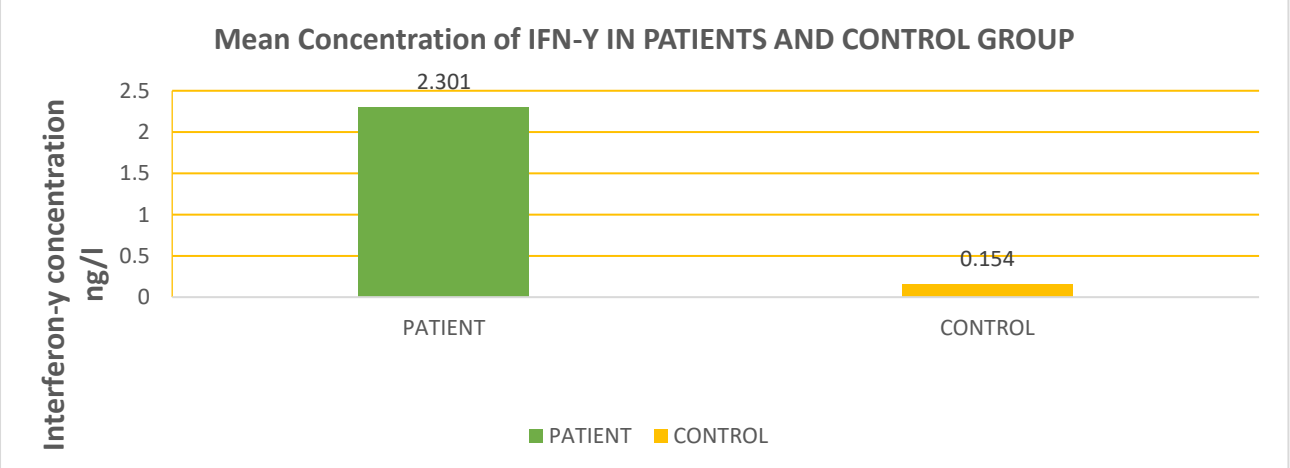

Figure 1. Mean concentration of IFN-Y between patients and control Data on optical density (450 nm) on the concentration scale are shown in **Figure 2**, with values ranging from (Zero to 30). Most of the time, the two dimensions' interaction is nonlinear.

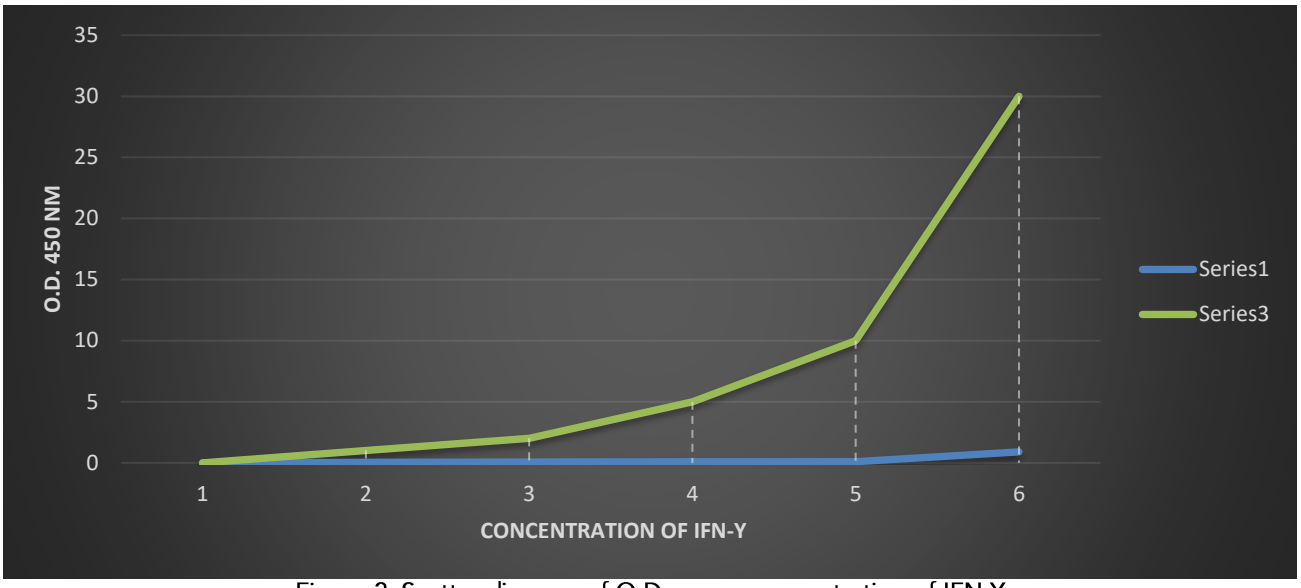

Figure 2. Scatter diagram of O.D. versus concentration of IFN-Y

As shown in **(Table 4)**, among 100 patients, 72(72%) were positive and 28(28%) were negative for interleukin-10, while in the control group, 3(6%) were positive and 47(94%) were negative with P-value<0.05.

Table 4: Distribution of IL-10 according to patients and Control group				
	IL-10		Total N (%)	P-value
	Positive N (%)	Negative N (%)		
Case				0.000
Patient count	72 (72.0%)	28 (28.0%)	100 (100.0%)	
Control count	3(6.0%)	47 (94.0%)	50 (100.0%)	
Total count(% within case)	75 (50.0%)	75 (50.0%)	150 (100.0%)	

In the group of 56 positive RT-PCR, 50 (89.3%)patients had positive IL-10 and 6(10.7%) had negative IL-10, while in the group of 44 negative RT-PCR, 22 (50%) patients had positive IL-10 and 22(50%) patients had negative IL-10. In the group of 11 patients with moderate to severe symptoms, all of the patients were positive IL-10, while in the group of 89 patients with mild symptoms, 61(68.5%) patients had positive IL-10 and 28(31.5%) patients had negative IL-10 **(Table 5)**.

Table 5: Distribution of IL-10 according to NP RT-PCR and COVID-19 severity				
	IL-10			
	Positive N (%)	Negative N (%)	Total N (%)	P-value
NP swab for RT-PCR for SARS-COV-2				0.000
Positive	50 (89.3%)	6 (10.7%)	56 (100.0%)	
Negative	22 (50.0%)	22 (50.0%)	44 (100.0%)	
Severity				0.028
Moderate to sever	11 (100.0%)	0 (0.0%)	11 (100.0%)	
Mild	61 (68.5%)	28 (31.5%)	89 (100.0%)	

For patients, the mean IL-10 concentration was (1.86 ng/l± 0.565), compared to (1.11 ng/l ±0.227) for control group **(Figure 3)**.

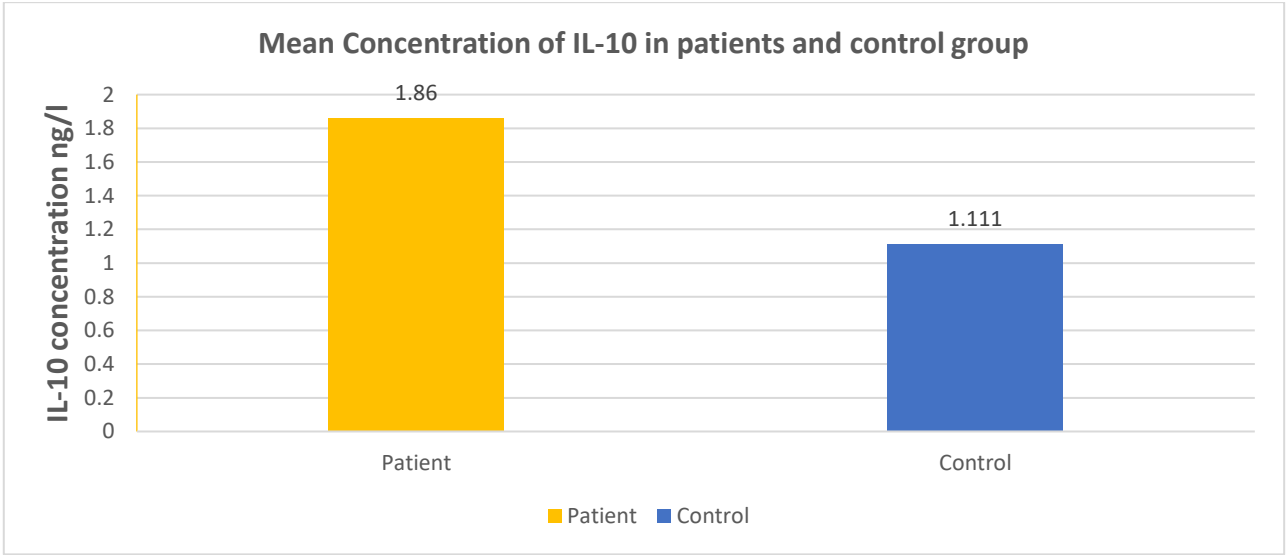

Figure 3. Mean concentration of IL-10 between patients and control

On the concentration scale in **Figure 4**, the optical density (450 nm) data are shown, with values ranging from (zero to2.5).

This Figure clearly illustrates how the relationship between the two dimensions is nonlinear.

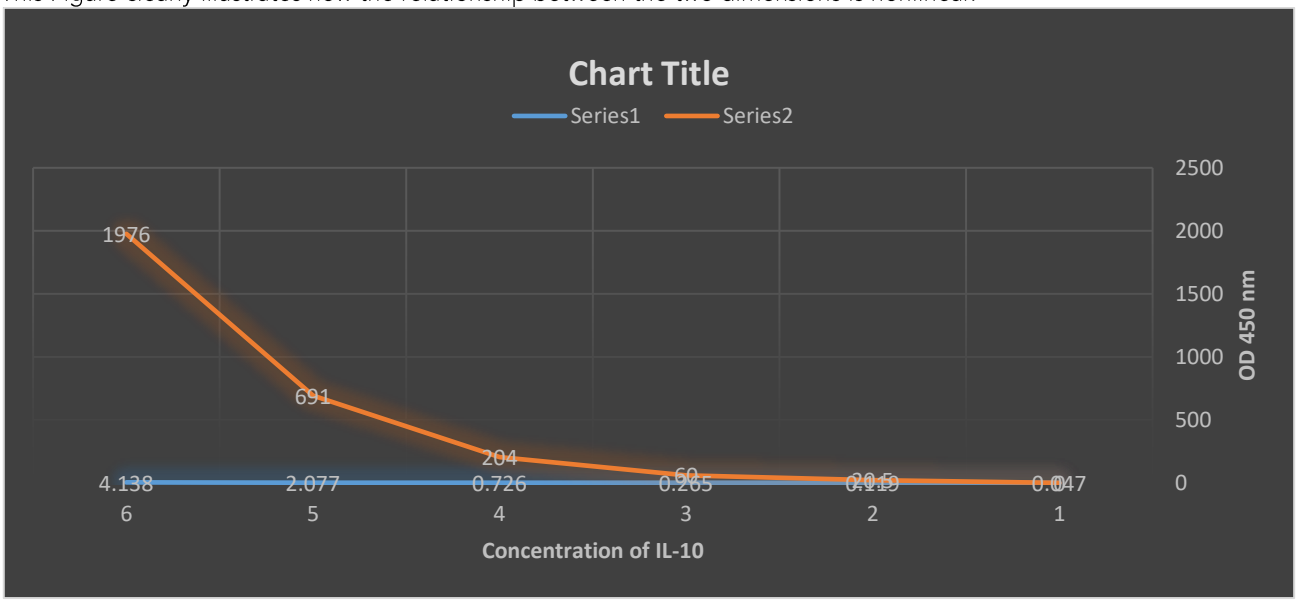

Figure 4. Scatter diagram of O.D. versus concentration of IL-10.

IFN-Y and IL-10 were elevated in COVID-19 patients especially in severe cases with mean concentration of IFN-Y 4.871ng/l and 1.983NG/L in severe and mild cases respectively, for IL-10 the mean concentration was 2.409 ng/l and 1.801ng/l in severe and mild cases respectively as shown in figure 5.

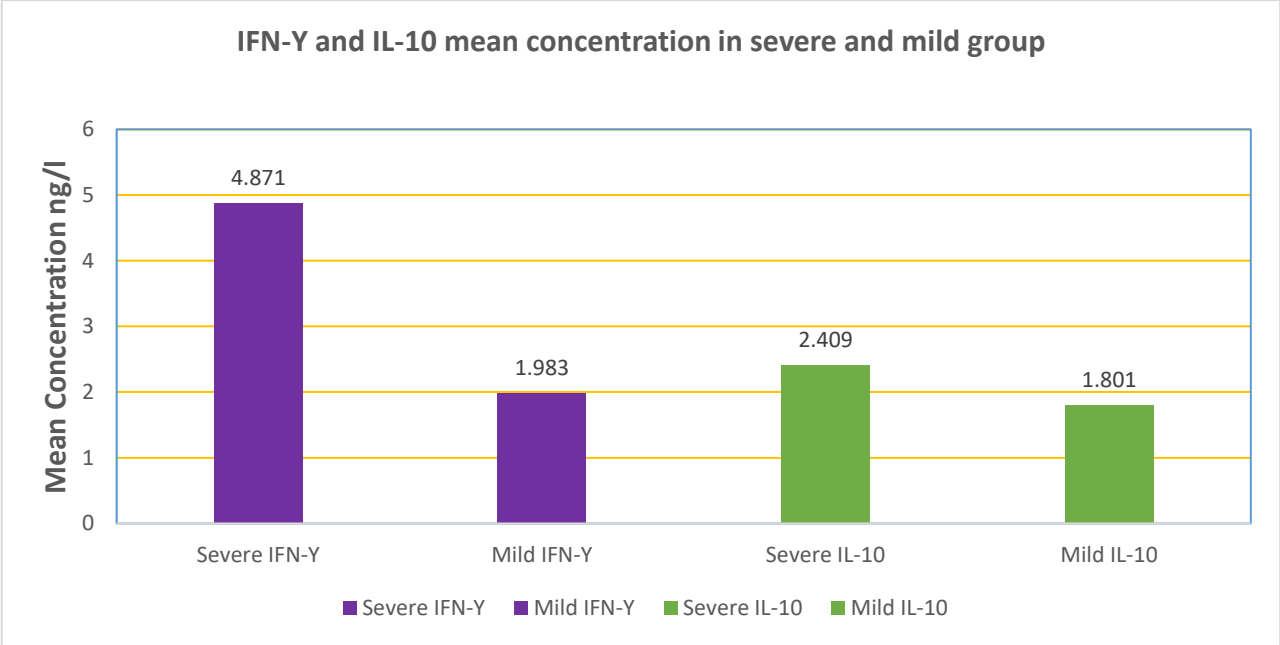

Figure 5. IFN-Y and IL-10 mean concentration in severe and mild group

Discussion

This study show significant elevation of serum level of IFN-Y in COVID-19 suspects in comparism to control group with P- value 0.000, there is significant elevation in NP RT-PCR positive patients in comparism to NP RT-PCR negative patients with P-value 0.001. Although the P value between severe and mild groups (0.125), all severely ill patients show elevated serum IFN-Y with mean concentration 4.871ng/l and1.983ng/l for severe and mild cases respectively.Also this study show significant elevation of serum level of IL-10 in COVID-19 suspects in comparism to control group with P- value 0.000, there is significant elevation in NP RT-PCR positive patients in comparism to NP RT-PCR. Negative patients with P value 0.000. Although the P value between severe and mild groups (0.028), all severely ill patients show elevated serum IL-10 with mean concentration 2.409ng/l and1.801ng/l for severe and mild cases respectively.In addition to its antiviral activities, IFN-g has anti-fibrotic qualities. IFN-, and type II IFN produced by NK cells, T lymphocytes, and cells from the innate and adaptive phases of the immune response, is essential to all immune response stages. IFN-system protection against viruses is vital. IFN- prevents the virus from multiplying and induces the production of cytokines by T cells, increasing the capacity of cytotoxic T lymphocytes to destroy the virus. On the other hand, persistently elevated IFN- levels worsen organ failure, progressive tissue damage, and systemic inflammation. Because the unclear function of IFN- in the outcome, understanding the serum pattern of this and other cytokines in COVID-19 patients is essential (22). Patients with a severe type of COVID-19 exhibited higher levels of IFN-Y and IL-10 compared to those with a mild variant (23). In contrast, patients with a severe form of COVID-19 had lower levels of IFN-Y than those with a moderate form of the condition according to a different study involving 21 patients (24). A longer time of follow-up is required because our study was conducted at the early stages of the

illness (for a period of 2 to 8 days). Interleukin (IL)-10, a pleiotropic cytokine, is widely known for its potent anti-inflammatory and immunosuppressive characteristics. IL-10 was formerly assumed a product of T helper 2 cells but is now known to be produced by a range of myeloid and lymphoid-derived immune cells engaged in both innate and adaptive immunity. One of the primary functions of IL-10 is to inhibit the host immune system's reaction to pathogens during infection, which aids in preventing immunopathology and tissue damage. In order to accomplish this, IL-10 inhibits the synthesis of cytokines that promote inflammation as well as the antigen presentation by activated monocytes, macrophages, and dendritic cells. Additionally, it regulates the overactive T lymphocytes' growth and activation. The principal method through which IL-10 has anti-inflammatory effects is through contact with the IL-10 receptor, which is most abundantly expressed on monocytes and macrophages (25). IL-10 levels in individuals with COVID-19 predict worse outcomes, according to numerous studies (26,27). After a severe SARS-CoV-2 infection, a significant early rise in the anti-inflammatory cytokine IL-10 appears to be a defining characteristic of hyper inflammation (28). Given its well-established roles as an anti-inflammatory and immunosuppressive cytokine, the large increase in IL-10 may be interpreted as an effort to limit tissue damage and hyper inflammation (25,29). The concurrent elevations of IL-10 and several pro-inflammatory cytokines raise concerns about whether IL-10 is effectively suppressing inflammation as is the case in other inflammatory conditions or whether it is acting differently than it usually does as an anti-inflammatory molecule.This is true even though there is a correlation between elevated IL-10 levels and disease severity(30).n fact, one explanation for the odd finding of high IL-10 and pro-inflammatory cytokine levels is the ability of IL-10 to function in some situations as both an immunostimulatory and pro-inflammatory molecule(18,19). Another strong and previously unstudied factor is the potential escape of activated immune cells from IL-10's anti-inflammatory effect (i.e., IL-10 "resistance"), which leads to abnormally pro-inflammatory cytokine responses (165,166

Conclusion:

This study shows significant rise in serum level of IFN-γ and IL-10 in COVID-19 patients especially in severe cases. Both of them also increase in mild cases as part of immunomodulatory action of IL-10 and antiviral with antifibrotic activity of IFN-γ. Unacceptable rise in serum IFN-γ and IL-10 usually occurs in cytokine storm with poor prognosis.

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