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THE FUNCTION OF INTERLEUKIN-6 IN PREDICTING COVID-19 SEVERITY: A CORRELATION STUDY BETWEEN GENE EXPRESSION AND PROTEIN CONCENTRATION

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Abstract:

This study aimed to examine Interleukin-6 (IL-6) as predictive biomarker for COVID-19 severity, to achieve this aim, the protein concentration degree and gene expression was tested in 90 samples according to COVID-19 severity which had been classified as (Control, Mild, Moderate, and Severe COVID-19). The IL-6 gene expression was assessed by means of qPCR and the protein concentration changed into analyzed via ELIZA and Western Blot. The consequences revealed that, there was a positive correlation amongst IL-6 gene and protein levels to severity of COVID-19. Accordingly, the IL-6 play essential role as predictive biomarker for COVID-19 severity and play a pivotal function in COVID-19 prediction. Additional studies are wanted to verify these findings in larger cohorts and to analyze the precise mechanisms of IL-6 gene regulation across the various stages of COVID-19 infections.

Keywords: COVID-19, IL-6, qPCR, gene expression, elisa, IL-6 concentration.

Introduction

Interleukin-6 (IL-6), a multifunctional cytokine, possesses crucial role in the immune response against viruses so it has substantial interest in the study of COVID-19[1]. IL-6 is known to mediate fever and acute phase responses, indicating its involvement in the systemic inflammatory response syndrome that takes place in severe COVID-19 cases [2]. There was a strong relation between drastically increased levels of IL-6 and severe clinical results in patients with COVID-19 [3]. Specifically, cytokine release syndrome (CRS) drive by IL-6 that was observed in individuals experiencing severe manifestations of COVID-19, this contributing to multiple organ failure and acute respiratory distress syndrome [4]. This suggests that IL-6 represents a plausible therapeutic target in the context of COVID-19 management, so its protein concentration levels and gene expression might play role in COVID-19 patients and serve as prognostic markers. COVID-19 and the severe acute respiratory syndrome coronavirus 2

(SARS-CoV-2), have stimulate a huge global effort to understand the clinical manifestation and the pathogenesis of this novel virus [5]. Among the key findings emerging from these efforts in determining disease severity is the significant role played by cytokine storm, which is represented the hyperactive immune response [6]. Within this context, the multifunctional cytokine Interleukin-6 (IL-6), has drawn considerable attention due to its critical involvement in the inflammatory and immune response. Worse clinical outcomes in patients who had severe COVID-19 have been associated with increased levels of IL-6 [7]. Illness development may be associated with increased gene activity, and there is obvious evidence that IL-6 gene overexpression may be linked to severity of the disease [8]. Larger study is needed to validate and further investigate this connection because these results are based on small samples. The primary goal of this study is to acquire a more in-depth knowledge of the correlation between IL-6 gene expression and protein level in individuals with varied degrees of COVID-19 severity. Our goal is to advance the understanding of COVID-19 etiology and to pave the way for future treatment strategies that specifically target IL-6 through this work.

2. Materials and Methods

2.1 Sample Collection and Processing

Participants in this study were divided into four distinct groups: Control, Mild COVID-19, Moderate COVID-19, and Severe COVID-19. The severity of COVID-19 was determined based on the patients' clinical symptoms and medical history, following the guidelines set by the World Health Organization.

All samples were collected from public health laboratory in Al Diwaniyah. By using venipuncture, peripheral blood was collected from each participant, while venous blood was collected into Vacutainer tubes without addition anticoagulant (Becton Dickinson, USA) for serum preparation and total RNA isolation.

The collected specimens were allowed to undergo coagulation at room temperature for a period of 30 minutes for serum samples extraction. The separation of the serum from other constituents was done by centrifugation at 1,500 × g for 10 minutes, then aliquoted into 1.5 mL Eppendorf tubes, and immediately stored at -20°C until further analysis. The blood collected in the anticoagulant-containing tubes for total RNA isolation Samples collection and processing were conducted in accordance with the ethical guidelines of the Declaration of Helsinki and approved by the relevant ethical committee.

2.2 RNA Extraction

The QIAamp RNA Blood Mini Kit (QIAGEN, Germany) was utilized following manufacturer’s guidelines to extract total RNA from the whole blood samples. Initially blood samples lysed and homogenized using a guanidine-thiocyanate-based buffer with potent denaturing properties, effectively deactivating RNases to ensure the isolation of intact RNA. Subsequently, ethanol was introduced to the samples to create optimal binding conditions, and the resulting mixture was applied to the QIAamp spin column, enabling the selective binding of total RNA to the membrane while efficiently eliminating contaminants through subsequent

wash steps. Finally, high-quality RNA was eluted from the column using 30-60 µl of the supplied RNase-free water. Concentration and purity of the extracted RNA were determined by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and the RNA was stored at -20°C until further use for real-time quantitative PCR analysis.

2.3 Reverse Transcription

The extracted RNA was reverse-transcribed into cDNA using a reverse transcription kit (High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems™). Each reaction was set up as per the manufacturer's instructions. Typically, this includes a mix of extracted RNA, reverse transcriptase enzyme, oligo(dT) primers, dNTPs, and reaction buffers. The reaction mix was then incubated in a thermal cycler. The program generally begins with 10 minutes priming step at 25°C, then 120 minutes for reverse transcription at 37°C, and finally, 5 minutes for an enzyme inactivation step at 85°C. The resulting complementary DNA was either used immediately for qPCR or stored at -20°C for later use.

2.4. Quantitative Polymerase Chain Reaction (qPCR)

qPCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems™) on a QuantStudio 5 Real-Time PCR System (Applied Biosystems™) following the manufacturer's instructions. Each qPCR reaction mixture (20 µl) contained 7 µl of nuclease-free water, 1 µl of cDNA, 10 µl of SYBR Green Master Mix, 1 µl of forward primer and 1 µl of reverse primer. Primers for IL-6 and the housekeeping gene (GAPDH) were designed using Primer3 software and synthesized by a commercial provider (Table 1).

Table 1: Primer sequences used in this study.

Primers	Sequences	Annealing Temp. (°C)
IL-6 Forward	5'- TCATACCTCAGAGCCCACCA-3'	60°C
IL-6 Reverse	5'- CACCTAGTCCACGCCCAATT-3'	
GAPDH Forward	5'- GGTCACCAGGGCTGCTTTTA-3'	
GAPDH Reverse	5'- GACTCCACGACGTACTCAGC-3'	

The qPCR cycling parameters employed in this study included a preliminary denaturation phase including 10 minutes at 95°C, followed by denaturation at 95°C (40 cycles consisting of 15 seconds), finally the annealing/extension for 1 minute at 60°C. To verify the specificity of the reaction, a melting curve analysis was performed at the conclusion of the PCR process.

The obtained qPCR data was subjected to analysis using the $2^{-\Delta\Delta CT}$ method. This method is widely used in the studying gene expression. The CT (cycle threshold) value was used in the analysis. Calculation of ΔCT was done by subtracting CT value of the housekeeping gene from CT value of IL-6, this help in assessment of the target gene expression relative to the reference gene. This normalized the data for differences in the amount of total nucleic acid added to each

reaction. The relative expression level of IL-6 gene was calculated using $2^{-\Delta\Delta CT}$, where ΔΔCT equals the ΔCT of each COVID-19 patient sample minus the average ΔCT of the control group.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

Post the qPCR analysis, the serum IL-6 protein levels were quantified using a human IL-6 ELISA kit (*Thermo Fisher Scientific*), following the manufacturer's instructions. The experimental procedure involved the addition of serum samples and IL-6 standards to the wells of a microplate that had been incubated with a specific monoclonal antibody of human IL-6. Following an incubation period and subsequent washing to eliminate any unbound substances, an enzyme-bind to polyclonal antibody, designed to recognize human IL-6, was added to the wells. followed by further wash to eliminate any unlinked antibody-enzyme reagent. The solution of substrate was added for initiating a color development reaction that was directly proportional for the amount of IL-6 bound during the primary step. Subsequently, the color intensity was detected at a 450 nm wavelength by using a microplate reader. The quantification of IL-6 concentration in the serum samples was accomplished by correlating the $OD_{450\ nm}$ values of the samples with a previously constructed standard curve. (Figure 1). Each sample was assayed in duplicate, and the average value was used for quantification.

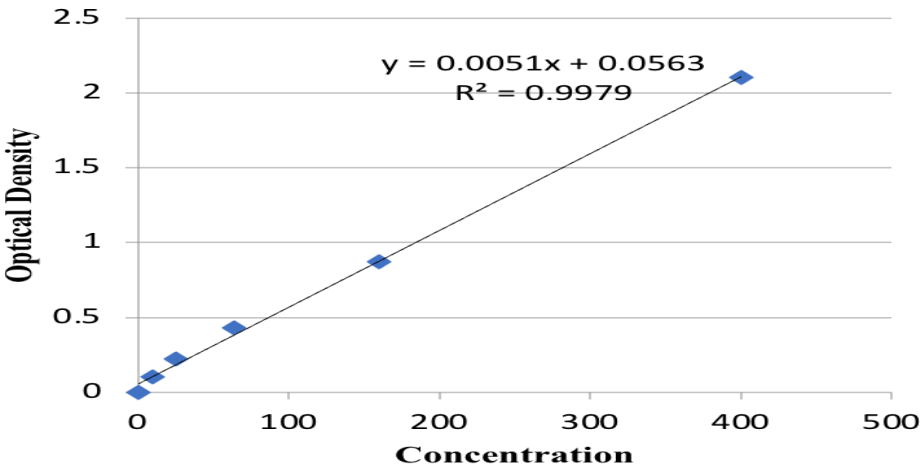

Figure 1: Standard Curve to Determine the Level of Interleukin-6.

2.6 SDS-PAGE electrophoresis and Western Blot

On gradient polyacrylamide gels (4%–20% SERVA), proteins were examined and separated based on their molecular weight. The sample buffer was combined with a desired quantity of protein before being heated for 10 minutes at 95°C. All samples and a protein marker (Geneflow) were loaded onto an SDS gel and run at 200 V for 55 minutes. In order to detect the bands of protein on scanner (GS 710 BIO-RAD). Gels were then used for the Western Blot assay using primary antibodies (Polyclonal anti-IL-6 antibody, Abcam, 1:5000 dilution). Western Blot was carried out to find separated proteins. an SDS-PAGE Gel was applied over an Amersham nitrocellulose membrane. Then, each side of three Whatman filter papers was

covered. The Western Blot Transfer System (BIO-RAD) was used to execute a protein-membrane transfer at 1mA per 1cm2 for two hours. Membranes were then shaken overnight at 4°C in 5% blocking buffer (skimmed milk or BSA). After washing with TBST buffer, membranes were coated with a primary antibody and blocked for three hours at room temperature. After that, membranes underwent three TBST washings for a total of 15 minutes. A membrane was coated with a secondary antibody (Alkaline-phosphatase conjugate, Aviva Systems, 1:2000) and then let remain at room temperature for three hours. Following a second TBST wash as described above, membranes were coated with BCIP®/NBT Liquid Substrate until protein bands appeared. Following a water wash to stop the reaction, the membranes were detected on scanner (GS 710 BIO-RAD scanner).

2.7 Statistical Analysis

The statistical analyses in this study were conducted using the SPSS (V.24). To assess the normality of the data distribution, the Shapiro-Wilk test was initially employed. Subsequently, a comparative analysis was conducted among the four distinct groups, namely the Control, mild COVID-19, moderate COVID-19, and severe COVID-19 groups, we utilized one-way analysis of variance (ANOVA), which is suitable for comparing the means of more than two independent groups. Considering that the assumption of homogeneity of variance was violated (as confirmed by Levene's test), the Games-Howell post hoc test was used for multiple comparisons between groups. This test is particularly suitable for datasets where the sample sizes and variances are unequal.

In terms of reporting, descriptive statistics (mean, standard deviation) were used to describe the main features of the IL-6 gene expression and protein concentration data for each group. The p-value threshold was set at less than 0.05 for all statistical tests to determine the level of significance. In addition, a Receiver Operating Characteristic (ROC) curve analysis was performed to assess the predictive value of IL-6 gene expression and protein concentration for COVID-19 severity. Calculations were performed to determine the area under the curve (AUC) for ROC, as well as negative predictive value (NPV), positive predictive value (PPV), specificity and sensitivity, to evaluate the diagnostic performance.

Graphs created with GraphPad Prism software was used to visualize IL-6 gene expression and protein concentration. The correlation between protein concentration level and IL-6 gene expression was evaluated with a Pearson correlation. Statistical significance was set at a (p-value) of less than 0.01.

3. Results and Discussions

3.1 Gene Expression

In this investigation, interleukin-6 (IL-6) expression levels were measured across various COVID-19 severity levels: Mild, Moderate, Severe, as well as a Control group. Each group comprised of different sample sizes: 30 for Control, 28 for Mild, 17 for Moderate, and 15 for Severe. A clear ascending trend in IL-6 expression was discerned in correlation with the rising

severity of COVID-19. This observation is shown in the (Table 2) and further illustrated in (Figure 2).

Table 2: IL-6 Expression Across Different Severity Groups

Group	Number of samples	IL-6 Expression (Mean ± SD)
Control	30	1.61 ± 1.31
Mild COVID-19	28	4.76 ± 3.32
Moderate COVID-19	17	12.21 ± 3.96
Severe COVID-19	15	19.51 ± 1.75

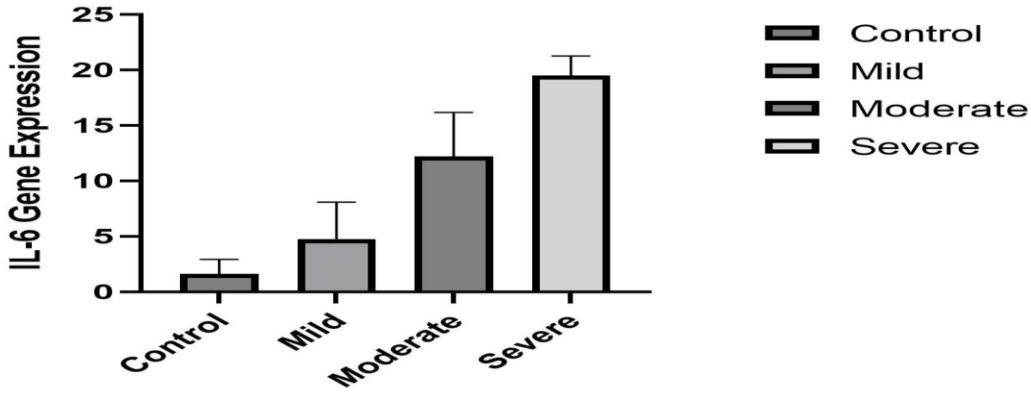

Figure 2: Comparative analysis of IL-6 expression levels in covid-19 patients and normal controls utilizing real-time PCR assay, with expression levels normalized to GAPDH.

Statistical analyses were conducted using a one-way ANOVA, which resulted in an F-value of 169.165, indicating a highly significant difference in IL-6 expression across the groups ($p<0.001$). The test for homogeneity of variances was also significant ($p<0.001$), indicating that the variances across the groups were not equal. Further analysis using post-hoc pairwise comparisons via the Games-Howell test revealed significant differences in IL-6 expression levels between all pairs of groups ($p<0.001$ for all comparisons). This observation suggests that there is considerable variation in the expression of IL-6 across persons with different levels of severity in COVID-19.

The analysis of the Receiver Operating Characteristic (ROC) curve pertaining to IL-6 gene expression revealed a statistically significant ability to distinguish between the control group and the COVID-19 group ($p < 0.001$). The AUC value of 0.907 suggests a substantial level of accuracy across the entirety of the dataset. The present investigation yielded findings indicating that the analysis identified a cutoff value of 3.22 as best for IL-6 gene expression. This cutoff value was associated with a sensitivity of 76.7% and a specificity of 93.3%. Furthermore, the study yielded a positive predictive value (PPV) of 95.5% and a negative predictive value (NPV) of 67.2% as depicted in Figure 3. The results suggest that IL-6 gene expression could potentially serve as a predictive marker for COVID-19 severity.

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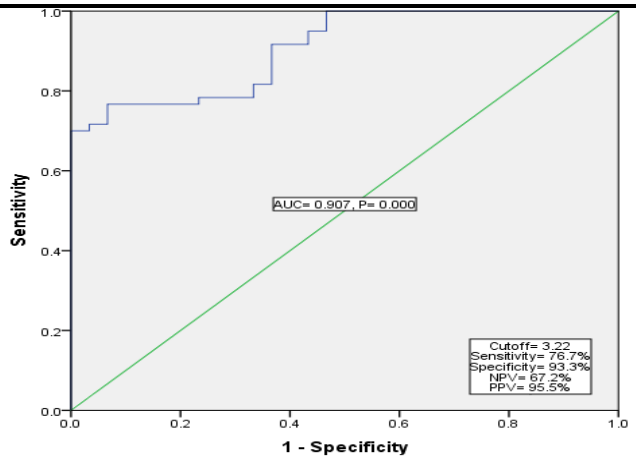

Figure 3: ROC analysis of IL-6 mRNA expression in COVID-19 patients compared to control.

In light of our findings, it becomes increasingly evident that IL-6 expression is largely tied to COVID-19 severity. The progressive increase of IL-6 expression levels across escalating severity groups corroborates with previous findings. It was found that IL-6 is markedly upregulated in patients with COVID-19 and specifically in the patients that exhibiting severe disease conditions [9,10]. Our results go a step further and delineate the expression levels not just for severe cases, but also for mild and moderate cases, suggesting a dose-response relationship.

The elevated IL-6 expression in the severe COVID-19 group, mostly 4-fold to 19-fold higher than the control group in our study, is consistent with previous research that found IL-6 to be a key marker of inflammation and severity in COVID-19 patients [11].

It's noteworthy that even in the mild COVID-19 group, IL-6 expression was considerably higher than the control group. This is in line with Abdelhafiz et al., [12] study that found that even non-severe COVID-19 patients possessed elevated levels of IL-6 in comparison to the healthy individuals.

Furthermore, our findings extend these observations by providing a more granular perspective, with statistically significant differences not only between the control and COVID-19 groups but also among different COVID-19 severity groups. This distinction is crucial for understanding the role of IL-6 at various stages of the disease that may guide a therapeutic use of IL-6 inhibitors, which are currently being investigated in numerous clinical trials [13].

The ROC curve analysis for IL-6 gene expression yielded a high AUC, sensitivity, and specificity in distinguishing between control and COVID-19 groups as mentioned previously. This suggests a good predictive accuracy for COVID-19 severity based on IL-6 gene expression levels. These results are compatible with earlier studies that found an association between increased IL-6 gene expression and the COVID-19 severity [14]. In a previous work, the PPV and NPV for IL-6 expression as a marker for COVID-19 were 62% and 93%, respectively, with a cutoff above 11.26 [12]. In our study, we found a higher PPV of 95.5% but a lower NPV of 67.2% with a lower cutoff of 3.22.

This higher PPV suggests our test more accurately predicts COVID-19 presence when IL-6 expression exceeds the cutoff. However, the lower NPV implies less reliability in excluding the disease when IL-6 expression is below the cutoff.

The lower cutoff could suggest earlier or less severe IL-6 upregulation in the disease. Yet, these differences might also be due to variations in populations, disease prevalence, or analytical methods across studies. More research is needed to clarify these findings and improve IL-6 expression as a biomarker for COVID-19.

Our study provides a quantitative framework for understanding the upregulation of IL-6 in COVID-19. Future research should delve deeper into the mechanisms underlying this differential expression and its implications for patient outcomes.

3.2 IL-6 Concentration Levels across Different Severity of COVID-19

In an investigation of IL-6 concentration levels in different COVID-19 severity groups, noticeable disparities were identified. The average IL-6 values (mean ± SD) for the control group, mild, moderate, and severe cases were documented in (Table 3) and illustrated in (Figure 4 and 5) Post hoc multiple comparisons indicated statistically significant differences in IL-6 concentration between all pairs of severity levels (all $p < 0.001$).

Table 3: IL-6 Concentration Levels Across COVID-19 Severity Levels

Group	Number of samples	IL-6 Concentration (mean ± SD) pg/mL
Control	30	11.05 ± 4.09
Mild COVID-19	28	19.16 ± 7.17
Moderate COVID-19	17	35.05 ± 9.66
Severe COVID-19	15	97.33 ± 9.81

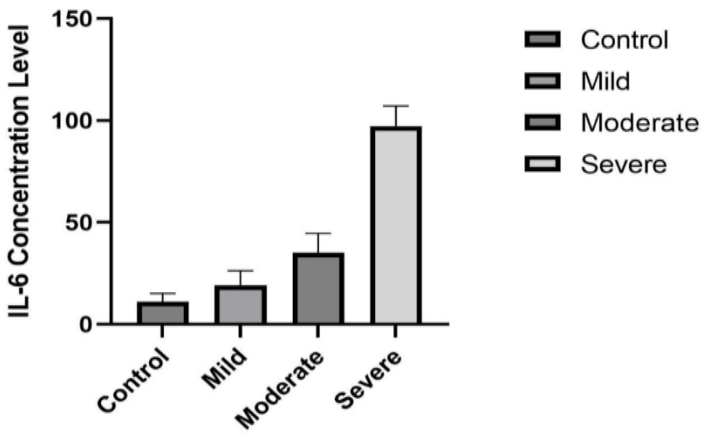

Figure 4: Analysis of IL-6 concentration levels in COVID-19 patients compared to normal controls using ELISA.

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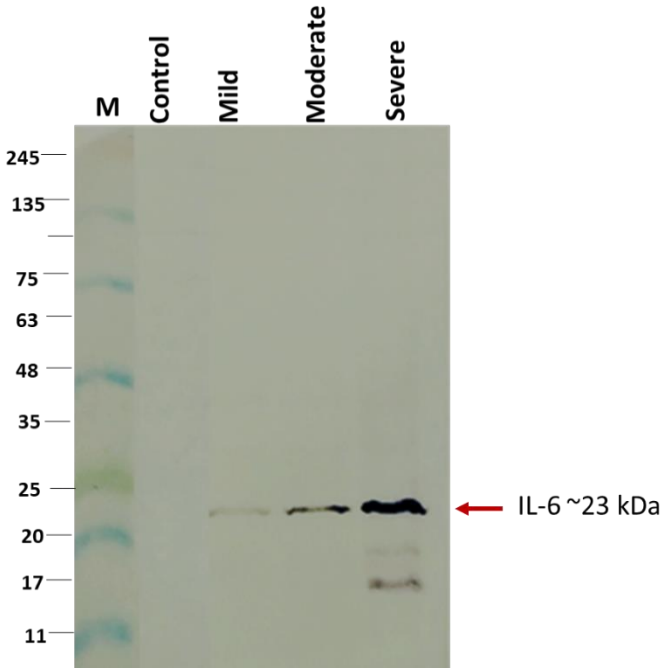

Figure 5: IL-6 levels were detected by Western Blot by using polyclonal anti-IL-6 antibodies, the result displays IL-6 bands at a molecular weight of ~23 kDa.

A ROC analysis further highlighted the potential of IL-6 as a predictor for COVID-19 severity, yielding an AUC of 0.927 ($p < 0.001$). An optimal cutoff value for IL-6 concentration was identified at 18.56 pg/mL, providing a sensitivity of 83.3%, specificity of 93.3%, PPV of 94.4%, and NPV of 73.6% in specifying the COVID-19. However, the test of homogeneity of variances indicated unequal variances (Levene's test, $p = 0.007$), which necessitates caution in interpreting these results (Figure 6).

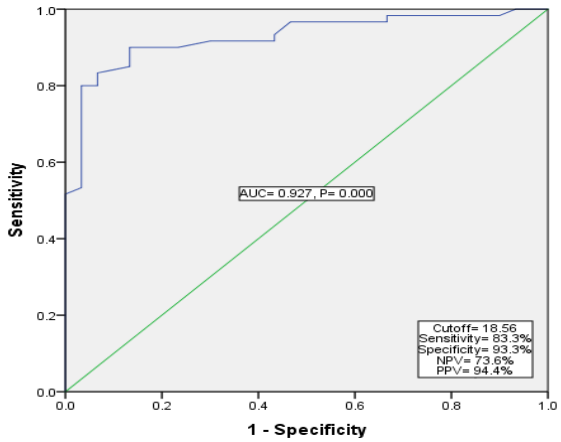

Figure 6: ROC curve of the IL-6 concentration level in COVID-19 infected patients.

Our findings revealed significant differences in IL-6 concentrations across varying severity levels of COVID-19. This aligns with previous literature in Iraq suggesting an association

between elevated IL-6 levels and increased disease severity [15]. The ROC analysis in our study supported the potential of IL-6 as a predictor for COVID-19 severity, which corroborates with other studies that highlight the predictive power of IL-6 [16].

While our study determined an IL-6 cutoff point of 18.56 pg/ml, another study has suggested a slightly higher cutoff value of 19.03 pg/ml [17]. This minor discrepancy might be due to differences in the sampled populations, the disease stages represented, or the specific measurement methods used in each study.

A significantly higher Positive Predictive Value (PPV) of 94.4% has been revealed by our study compared to previous study which reported the PPV is 77.3% [18] ensuring that our test has a stronger ability to correctly identify patients with elevated IL-6 levels. However, our test might be slightly less efficient in identifying individuals without elevated IL-6 concentration levels because we noticed a lower Negative Predictive Value (NPV) of 73.6%, compared to the 87.8% from the same study. This observation variations between our study and the earlier investigation conducted by Taha et al. (18) in positive predictive value (PPV) and negative predictive value (NPV) may be due to some reasons including criteria for patient selection, stage of disease in the study, or differences in disease prevalence.

3.3 The Relationship Between IL-6 Gene Expression and Concentration Levels

Given the data obtained from both qPCR and ELISA, we investigated the relationship between the IL-6 gene expression and its protein concentration. This allowed us to observe the correlation between genetic activity and its resultant protein expression.

Analysis of our data revealed a strong and statistically significant correlation between IL-6 gene expression levels and protein concentration as measured by ELISA (Pearson correlation coefficient, $r = 0.925$, $p < 0.001$). This association was consistent across all groups (control, mild, moderate, and severe) as mentioned in (Table 4) and clarified in (Figure 7).

Table 4: Correlation between IL-6 (Gene Expression and Protein Concentration)

		Gene Expression	Protein Concentration
Protein Concentration	**Pearson Correlation**	.925**	1
	Significant (2-tailed)	.000	
	NO of Samples	90	90
GeneExpression	**Pearson Correlation**	1	.925**
	Significant (2-tailed)		.000
	NO of Samples	90	90
Significant correlation between IL-6 Protein concentration and IL-6 gene expression at the 0.01 level (2-tailed).			

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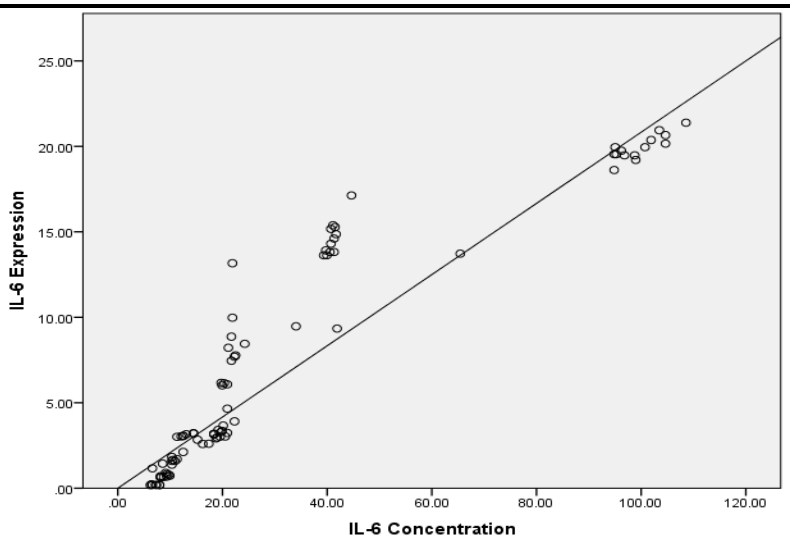

Figure 7: Correlation between IL6 gene expression and IL6 protein concentration level

The present investigation has revealed a noteworthy association between the concentration of the protein (IL-6) and the expressions of the gene (IL-6), highlighting the potential usefulness of IL-6 as a prognostic biomarker for the severity of COVID-19 in patients. The high correlation coefficient (r = 0.925) indicates a substantial association between molecular responses, specifically gene expression levels, and physiological responses, specifically protein concentration. Nevertheless, it is imperative to acknowledge the necessity for future research endeavours in order to substantiate and go deeper into these aforementioned discoveries. Subsequent investigations should prioritise the examination of this association within more extensive sample sizes and among heterogeneous populations. Additionally, it is imperative to explore the possible consequences of this link for predictive modeling and the management of patients. Furthermore, it is advisable to evaluate the association between IL-6 levels and additional clinical and laboratory markers in order to enhance our understanding of the illness progression in individuals affected by COVID-19.

This finding supports the understanding that the body's immune response, as indicated by IL-6 protein levels, is driven by the genetic response to the virus [19]. Consequently, our results further emphasize interleukin-6 pivotal role in COVID-19 progression as well as severity as mentioned by other studies [20,21]. Future therapeutic interventions targeting IL-6 gene expression may hold potential in controlling the cytokine storm and severe implications linked to COVID-19.

However, additional investigations are required to elucidate the intricate mechanisms governing IL-6 gene regulation throughout the various stages of COVID-19 infection, as well as to ascertain the exact role of this cytokine in the pathogenesis of the disease.

4. Conclusion

In conclusion, this study provides compelling evidence to support a crucial role of IL-6 in the progression and severity of COVID-19. We have demonstrated significant differences in both

the gene expression and protein concentration of IL-6 across varying severity levels of the disease and established a robust correlation between these two measures. Our findings also suggest a potential for IL-6 as a predictive biomarker for severity of COVID-19, as indicated by the strong predictive accuracy shown in the ROC analyses.

The progressive increase of IL-6 expression and concentration in relation to disease severity emphasizes the role of this cytokine in the immune response to COVID-19. Our delineation of IL-6 levels across varying disease severities, and the finding of a strong correlation between interleukin-6 protein concentration and IL-6 gene expression are significant additions to the understanding of the disease's molecular pathogenesis.

Nonetheless, we recognize the limitations inherent in our study, such as the need for validation in larger cohorts and diverse populations. The unequal variances across groups suggest the potential for more complex underlying relationships. Our results should therefore be interpreted with some caution until further research can verify these findings.

Ultimately, our findings underscore the interleukin-6 role in COVID-19 that may guide development of therapeutic strategies, including those that target IL-6 expression or activity. Further Investigation is required to understand the detailed mechanisms of IL-6 gene regulation, as well as its precise role in COVID-19 pathology as well as its potential utility in predictive modeling in addition to patient management.

5. Acknowledgements

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6. Disclosure and conflict of interest

The authors declare that they have no conflicts of interest.

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