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Original Article

Evaluation of IL-6, IL-25 & IL-35 in the COVID 19 Patients and their Correlation to Demography Data in the Symptomatic Patients

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Abstract

The severe acute respiratory syndrome coronavirus 2, SARS-CoV-2, was first discovered in Wuhan, Hubei province, China. Cytokines play a critical role in COVID-19 infections through their inflammatory or anti-inflammatory activities. This study aimed to detect the diagnostic value of and the relationship between the interleukins under study, in addition to their relationship with demographic data in COVID-19 patients. Patients with a confirmed diagnosis of COVID-19 based on laboratory (PCR) results and the healthy control group were given their section of this investigation. The patient group had 120 COVID-19 patients, including 62 males and 58 females, while the control group consisted of 32 individuals (22 males and 10 females). The subdivision was then performed according to their vaccination status, chronic diseases, gender, and residence. Cytokine levels were detected using the ELISA technique. The immunological status of COVID-19 patients was determined by measuring interleukin (IL)-6, IL-25, and IL-35. During the research, it was found that IL-6 was highly significant in COVID-19 patients (0.001). However, its level was not significantly different (0.376) in patients regarding the type of chronic diseases, residence (0.353), and gender (0.574), but it was significantly different in vaccinated patients (0.029). It was also found that IL-6 is significantly correlated with IL-25 and IL-35. IL-25 was highly significant in COVID-19 patients (0.007), and there was a significant difference in its level in patients regarding the type of chronic disease (0.049). While there was no difference in terms of residence (0.421) and gender (0.681), corona vaccination showed a significant difference (0.047). IL-25 also had a significant correlation with IL-6 and IL-35. As for IL-35, it was significant in patients with COVID-19 (0.013) but not significantly different regarding chronic diseases (0.344), residence (0.877), or gender (0.800). However, it was significantly different in vaccinated patients, compared to the non-vaccinated ones. IL-35 was found to be significantly correlated with IL-25 and IL-6 (0.000). The examined interleukins increased in COVID-19 individuals. IL-6 remains an excellent marker for determining the immune state of patients with COVID-19. There were also strong correlations between the interleukins under study in COVID-19 patients. However, there was no relationship between age, residence, gender, and the concentration of studied cytokines. IL-25 increases significantly in COVID-19 patients suffering from chronic diseases. Therefore, it is more efficient in the follow-up of patients.

Keywords: Cytokines, Interleukins, SARS-CoV-2

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) originated in Wuhan, Hubei Province, China, and rapidly spread throughout the country (1). As of April 4, 2022, it is estimated that there had been 491,922,036 cases of coronavirus disease 2019 (COVID-19) and 6,176,684 resulting deaths. As of April 4, 2022, there had been 2,320,260 COVID-19

cases in Iraq, 25,173 deaths, and 2,284,245 recovered cases (2). Variable COVID-19 symptoms range from minor to severe (2). There are four families of coronavirus known as alpha, beta, gamma, and delta, and it is believed that alpha and beta variants originated in mammals, specifically in bats. Pigs and birds are also sources of gamma and delta variants. There are seven variants of coronavirus that can cause disease in humans. When beta coronavirus infects humans, it can cause a severe illness, while alpha coronavirus causes sickness and death infections (3). Interleukin (IL)-6 is a cytokine that can produce inflammation and functions as an anti-inflammatory myokine. It is a product of the human IL-6 gene, located on chromosome 7p21, and the GG genotype of the IL-6 promoter 174 C/G polymorphisms is correlated with higher IL-6 levels than the CC genotype (4). In the context of the coronavirus pandemic, some preliminary data suggest that IL-6 may be used as an inflammatory marker to identify a severe COVID-19 infection with a dismal outlook. This information comes from studies conducted in animals. IL-6 also plays a central role as a myokine, a cytokine generated by muscles and released in response to muscular contractions (5). IL-25 is a protein encoded in human chromosome 14 by the IL-25 gene, also referred to as IL-17E (6). It is composed of 177 amino acids discovered in 2001 (7). The essential function can activate NF-B and lead to the synthesis of IL-8 (also referred to as CXCL8), the primary chemotactic molecule discovered in neutrophils (6). IL-25 is also essential for the T helper (Th) 2 immune response, and it has been demonstrated that IL-25 stimulates the synthesis of IL-4, IL-5, and IL-13. IL-35 is an anti-inflammatory cytokine, recently discovered and belonging to the IL-12 family. A variety of regulatory lymphocytes are responsible for the production of IL-35, which is involved in the suppression of the immune system (8). It is also necessary to inhibit the growth of Th1 and Th17 cells in the early stages of Tregs (T-cells) development, and IL-35 reduces early T-cell proliferation. IL-35, which is produced by regulatory T-cells, regulatory B-cells, and even the cluster of differentiation (CD)8+ regulatory T-cells, can reduce the inflammation that occurs in immune cells (9). Although IL-35 is intermittently produced in tissues, it can be induced by inflammatory stimuli and subsequently transcribed by vascular endothelial cells, smooth muscle cells, and monocytes (10).

2. Materials and Methods

2.1. Study Design

This case-control study compared some cytokines of COVID-19 patients to those of healthy controls. Patients were selected from Rumaitha General Hospital and Martyr Youssef Hospital for Isolation (Al-Muthanna, Iraq), as well as Al-Hussein Teaching Hospital in (Thi-Qar, Iraq). The samples were collected from March 25, 2022, to June 1, 2022. The patients' and the control's demographic information included age, gender, residence, chronic disease, and vaccination status.

2.2. Sample Collection

A total of 152 blood samples were collected, including 32 from apparently healthy individuals as controls and 120 from COVID-19 patients. The patients were 10-90 years and were on medical observation, and the controls were 4-52 years. A total of 5 ml of blood was taken from COVID-19 patients and the healthy controls using disposable syringes, and the serum samples were kept at -20°C until use.

2.3. Determination of Interleukins

The ELISA was used as the analysis method to determine the interleukins according to the manufacturer's instructions (Bioassay, China).

2.4. Statistical Analysis

The data was statically analyzed using the SPSS software (version 26) and running One-Way ANOVA for mean variations, independent sample t-test, descriptive statistics, Chi-Squared test, and Pearson correlation at $P \leq 0.05$.

3. Results

The results recorded a significant increase in the concentration of IL-6 and IL-25. Furthermore, there was a

significant difference in IL-35 between COVID-19 patients and the control group ($P \leq 0.05$), as shown in table 1.

The findings also revealed significant differences in IL-25 levels among COVID-19 patients with different chronic diseases; however, no significant differences were observed in IL-6 and IL-35 levels among COVID-19 patients with different chronic diseases ($P \leq 0.05$), as shown in table 2.

The results revealed non-significant differences in the concentration of IL-6, IL-25, and IL-35 levels between COVID-19 male and female patients, as well as male and female controls ($P \leq 0.5$), as noted in table 3.

The findings indicated no significant differences in the concentration of all studied parameters in COVID-19 patients in terms of their residence area, as shown in table 4.

As indicated in table 5, the present study found a substantial rise in the concentration of IL-6, IL-25, and IL-35 in vaccinated individuals, while such a difference did not exist in the control group.

There was a highly significant difference between the studied interleukins in the positivity in patients with COVID-19 ($P \leq 0.05$), as shown in table 6. IL-6 and IL-25 elevated in high numbers in patients but in their normal range, while IL-35 had the lowest positivity.

The present findings demonstrated a significantly positive correlation between IL-6, IL-25, and IL-35 ($P \leq 0.05$ and 0.01), as demonstrated in table 7.

Table 1. The concentration of interleukins in patients and controls groups

Parameters	Cases	No.	Mean± SD	F of ANOVA	P
IL6	Case	120	127.974±157.111	11.218	0.001
	Control	32	34.662±11.161		
IL25	Case	120	946.185±1571.611	7.573	0.007
	Control	32	179.645±53.806		
IL35	Case	120	6.874±11.176	6.363	0.013

Table 2. Measurement of concentrations of interleukins in COVID-19 patients according to chronic disease

Chronic Diseases	Cases No.	IL6	IL25	IL35
		Mean ± SD		
Asthma.	10	151.203 ± 184.3	986.288 ± 1297.1	7.566 ± 9.54
B. P	11	67.252 ± 40.47	943.674 ± 1818.6	7.415 ± 13.47
DM	17	135.938 ± 187.3	784.25 ± 1465.9	5.496 ± 9.21
DM & B. P	26	148.793 ± 180.9	904.463 ± 1425.4	9.509 ± 14.47
LUK	1	75.141 ± 0.00	638.597 ± 0.00	4.181 ± 0.00
Non-chronic	55	119.325 ± 143.2	914.84 ± 1564.40	5.476 ± 9.58
p. value		.376	0.049	0.344

Table 3. Comparison between interleukins in male and female patients & control

Gender of patients& control	Cases No.	IL6	IL25	IL35
		Mean ± SD		
Male of patient	62	135.807±160.33	1003.478±1520.17	7.025±10.19
Female of patient	58	119.601±154.53	884.941±1635.88	6.714±12.213
P value		0.574	0.681	0.880
Male of control	22	35.516±9.57	168.469±56.09	1.902± 0.63
Female of control	10	32.725±14.44	204.227±40.63	1.817± 0.715
P value		0.530	0.081	0.739

Table 4. Based on a residence, interleukin concentrations were measured in COVID-19 patients

Residence	Cases No.	IL6	IL25	IL35
		Mean ± SD		
Urban	76	138.158 ± 164.8	1034.501 ± 1677.4	6.751 ± 10.56
Rural	44	110.383 ± 142.8	793.639 ± 1374.8	7.082 ± 12.26
P value		0.353	0.421	0.877

Table 5. Measurement of concentrations of Interleukins in patients and control according to vaccination and non-vaccination

Vaccination Status	Cases No.	IL6	IL25	IL35
		Mean ± SD		
Yes of patients	41	171.244 ± 199.5	1338.544 ± 1906.7	10.202 ± 14.36
No of patients	79	105.512 ± 125.4	742.55 ± 1334.5	5.188 ± 8.79
P value < 0.05		0.029	0.047	0.048
Yes, of control	15	35.698 ± 9.15	167.86 ± 63.78	2.103 ± 0.696
No of control	17	33.747 ± 12.88	190.03 ± 42.47	1.675 ± 0.556
P value		0.630	0.251	0.063

Table 6. Positive and negative interleukins result in Covid-19 patients

Result Status Interleukins	Positive		Negative	
	No.	%	No.	%
IL-6	95	79.2	25	20.8
IL-25	86	71.7	34	28.3
IL-35	52	43.3	68	56.7
P. value 0.000				

Table 7. A Pearson correlation between interleukins

Pearson		IL-6	IL-25	IL-35
IL-6	r		.771**	.683**
p. value			.000	.000
IL-25	r			.878**
p. value				.000

4. Discussion

The current investigation found a considerable increase in IL-6 and IL-25 concentration levels in COVID-19 patients. As demonstrated in table 1, there are also significant differences in IL-35 levels between COVID-19 patients and the control group (P<0.05).

4.1. IL-6

Our study revealed significant differences in IL-6 levels between patients and the control group, which was consistent with the findings of other studies (11). This difference occurs because the IL-6 proinflammatory cytokine increases in the early stages of various infections and inflammatory states, and the immune response to SARS-CoV-2 infection mobilizes several cytokines, mostly of proinflammatory nature. Changes in the concentration of cytokines are related to the existence of the illness and a worse prognosis. Regarding various coronavirus types, higher IL-6 levels have been identified in SARS patients, which correlates with the severity of symptoms, and in SARS-CoV-2 patients, which has been linked to a potential T cell dysfunction (12). It has been reported that SARS-CoV-2-induced cytokines may impair the ability of T-cells to

interact with dendritic cells, thereby reducing the viability of these cells and macrophages to eradicate the virus (13). COVID-19 patients have elevated IL-6 levels, which correlates with a serious prognosis (14).

4.2. IL-25

The findings indicated significant differences in IL-25 levels between patients and the control group, as shown in table 1. This result was similar to the immunological role of IL-25 detected in some studies, such as Barlow and McKenzie (15), as well as Goossens, Nakagawa (16). IL-25 may initiate and enhance Th2-type immune responses and plays a crucial role in the development of some allergic disorders. However, it remains unclear how IL-25 modulates the Th2 immune response. According to a number of studies, IL-25 increases the production of Th2-type cytokines and causes Th2-type immune responses primarily through two mechanisms. First, high doses of IL-25 can induce intrinsic lymphoid type 2 cells (ILC2s) to produce IL-4, IL-5, IL-13, and other cytokines. Second, low doses of IL-25 can induce Th cells to differentiate into Th2 cells with the participation of CD4+ T-cells and increase the expression of Th2-type cytokines (15). The current study demonstrated that IL-25 not only regulates type 2 immune responses, inflammation, cutaneous, and autoimmune illnesses but also functions in treating malignancies. Therefore, IL-25 offers fresh directions for research and can become a novel therapeutic target for these disorders (16).

4.3. IL-35

Our investigation demonstrated a substantial difference in IL-35 levels between patients and the control group, as shown in table 1. This outcome was comparable to the immunological function of IL-35 in previous investigations, such as Turnis, Sawant (17). It is believed that IL-35 is an essential modulator of the host's immune response and tumor survival with specialized immunosuppressive actions via influencing local inflammatory responses and anti-tumor immunity in the tumor microenvironment (TME) (17). In addition to its suppressive role, IL-35 is also implicated in tumor growth and immune surveillance, and several human cancers, including acute myeloid leukemia, pancreatic ductal adenocarcinoma, and colorectal cancer, have been reported to have elevated amounts of circulating IL-35 (18).

According to some immunological investigations, T-cells that release IL-35 and have a protective role have an immunosuppressive effect on inflammations induced by T-cells and protect Th1 and Th17 cells (19). Previous studies (20) agree that IL-35 was the inhibitor cytokine when it appeared in various chronic inflammatory illnesses and parasitic/bacterial infections. Li, Tian (20) showed that CD4+ T-cells in patients with chronic HBV infection have considerably higher amounts of EBI3 mRNA and protein than healthy individuals and patients whose HBV infection has disappeared. In addition, it has been demonstrated that IL-35 inhibits the proliferation of HBV antigen-specific cytotoxic T-lymphocytes *in vitro* and interferon production (20). A high proportion of COVID-19 patients (120) under study suffered from chronic diseases (65 samples, 54.2%), 22.5% (37 samples) had diabetes mellitus and hypertension, followed by 14.2% having diabetes only (17 samples from total), and 8.3% having asthma (10 samples from total). The cause of this may be that chronic illnesses cause an immune-compromised state that has some characteristics of infectious disorders and associated complications, including endothelial dysfunction, the proinflammatory state, innate immune system abnormalities, diabetes, hyperglycemia, increased systemic oxidative stress, and vascular inflammation. Vascular inflammation and excessive oxidative stress are major hallmarks of phenotypical endothelial dysfunction, and vascular endothelial dysfunction associated with chronic illnesses is related to both local vascular inflammation and systemic inflammation (21). Elements of both innate and adaptive immune responses have new roles in regulating endothelial function in hypertensive

circumstances. According to previous research, activation of the complement system of innate immunity may adversely influence vascular endothelial function in hypertension (22). As the SARS-CoV-2 virus attaches to ACE2 on cell surfaces, the viral infection may interact with diabetes, making SARS-CoV-2 patients with diabetes more susceptible to severe illness and death (23).

As seen in table 2, this analysis showed that COVID-19 patients had no significant variations in IL-6 and IL-35 levels, but there were significant differences in IL-25 levels among patients regarding their chronic disease. In table 3, we note that all concentrations of the parameters are non-significant between male and female COVID-19 patients, where we notice an increase in women than in men in the control group. As shown in table 4, the present research found a significant increase in IL-25 levels in females in the control group. We noted that IL-6, IL-25, and IL-35 have non-significant differences in males and females, as shown in table 3. The findings disagree with Han, Yang (23) claiming a difference between genders for the following reasons set by the same publisher. First, all publications describing the differential expression of cytokines in males and females originated in China. More clinical studies should investigate the difference in COVID-19 cytokines between genders. Second, the median (IQR) is often employed as a statistical model in investigations. We had to use a computation formula (10, 11) to translate these to mean and standard deviation, which may differ from the accurate clinical data. Thirdly, our meta-analysis focused mostly on English-language research, which may have led to language bias (23). As seen in table 4, this research found no statistically significant variations in the concentration of any investigated parameters in COVID-19 patients based on their residence. Based on the findings, there was a considerable increase in IL-6, IL-25, and IL-35 in vaccinated patients, compared to non-vaccinated ones. According to the findings of this study, there was a statistically significant rise in the concentration of IL-35 in the vaccinated control group; however, the other parameters did not record any statistically significant differences according to vaccination, as shown in table 5. The results of these investigations were consistent with Zinkernagel (24). Recent research into COVID-19 has revealed that the immune system plays a critical role in determining the severity of the disease. The SARS-CoV-2 virus is highly infectious to cells in the lower respiratory system, where it quickly triggers a local immune response that destroys this vital and delicate organ. Treatments aiming at the virus alone are not enough to prevent more severe manifestations; the immune system must also be targeted and controlled (24). The results of this investigation were consistent Grifoni, Weiskopf (25) mentioning that SARS-CoV-2-unexposed people were only able to *in vitro* establish a CD4+ T-cell response to SARS-CoV-2-derived peptides in 50% of cases and a CD8+ T-cell response in 20% of cases. The response rate in COVID-19 convalescent patients was much higher, 100% for CD4+ and 70% for CD8+ T-cells (25).

Shrotri, van Schalkwyk (26) claimed that although vaccination can elicit robust T-cell responses (memory and effector function have been shown against multiple viral epitopes), the importance of T-cell responses for protection and susceptibility at the population level, apart from memory B cell responses, is still up for debate, and vaccinated patients have a solid secondary immune response to the virus, which is developed faster than the primary immune response of non-infected and non-vaccinated persons, which requires a long time to develop (26).

Through our study of randomly selected samples of COVID-19 patients (Table 6), we found that IL-6 has a high positivity percentage (79.2%) among patients, which indicates that it is a good sign for the follow-up of patients, especially in severe cases. IL-6 was followed by IL-25 (71.7%), whose high concentration helps determine the inflammatory state of patients, represents an essential indicator for the immune state of the patient, and refers to the presence of a high inflammatory response in symptomatic COVID-19

patients with enhancing humoral immune response. The IL-25 induced the production of Th2 cytokines which down-regulate cell-mediated immune responses, which is necessary to control viral infections and leads to the progression of the signs of disease. IL-35 increased more than the rest of the cytokines in the control group and decreased in the patients' group, which indicates the importance of IL-35 due to its protective role and an immunosuppressive effect on inflammation, which is induced by T-cells and protects Th1 and Th17 cells (19). Previous studies (27) also found that IL-35 was the inhibitor cytokine when it appeared in various chronic inflammatory illnesses and parasitic/bacterial infections.

Table 7 shows a significant positive correlation between IL-6, IL-25, IL-35, and CRP. Additionally, the present data demonstrated a significant positive correlation between IL-6 and IL-25. There was a substantial, strong positive link between IL-25 and IL-35 and a significantly positive correlation between IL-6 and IL-35.

The results of this study are consistent with that of Pedersen and Ho (11). The immune response to COVID-19 infection mobilizes several cytokines, mainly of a proinflammatory nature, and changes in their concentrations are related to the presence of the illness and a worse prognosis. IL-6 was a proinflammatory cytokine that increases in the early stages of a variety of infections and inflammatory states (11).

IL-25 is a key player in the emergence of several allergy disorders and has the capacity to both trigger and exacerbate Th2-type immune responses. Numerous studies have shown that IL-25 enhances the production of Th2-type cytokines and triggers Th2-type immunological responses, although it is yet unknown how IL-25 controls the Th2 immune response (15). In addition to its suppressive function, IL-35 is connected to tumor development and immune surveillance. Acute myeloid leukemia, pancreatic ductal adenocarcinoma, and colorectal cancer are only a few human tumors that have been shown to have higher levels of circulating IL-35 (18). The correlation between IL-6, IL-25, and IL-35 was significant. These findings are consistent with Turnis, Sawant (17), and IL-35 is regarded as a critical regulator of the host's immune response and tumor survival, influencing local inflammatory reactions and anti-tumor immunity in the TME (17).

Authors' Contribution

Study concept and design: L. F. M. H. A.
Acquisition of data: S. A. A. A.
Analysis and interpretation of data: L. F. M. H. A.
Drafting of the manuscript: S. A. A. A.
Critical revision of the manuscript for important intellectual content: L. F. M. H. A.
Statistical analysis: L. F. M. H. A.
Administrative, technical, and material support: S. A. A. A.

Ethics

The current study was performed under the instruction of the Directorate of Health's Al-Muthanna Committee (No. 131 on March 15, 2022) after obtaining the participants' acceptance.

Conflict of Interest

The authors declare that they have no conflict of interest.

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