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Gene Expression of Human Interleukin 3 and Human Interleukin 6 in Obese and Smokers of Covid-19 Patients in Wasit Province, Iraq

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ABSTRACT:
SARS-CoV-2, the novel coronavirus that causes COVID-19, emerged in China in late 2019 and rapidly reached pandemic status. The virus displays tropism for the respiratory system, causing symptoms that range from an acute respiratory syndrome, manifested in mild form in the vast majority of cases, and progressing in some cases to an extremely severe. Obesity and smoking are causing and increasing health problems all over the world. In combination with the current COVID-19 pandemic, led to increased risk of getting severe COVID-19. The aim of this study is determination of IL-6 and IL-3 gene expression and compare it with the IL-6 and IL-3 serum levels in smoking and obese patients with Covid-19 infection . So this study included 25 obese COVID-19 patients, 25 smokers with COVID-19, 25 non-obese & non-smoking COVID-19 patients (as control positive) and 25 healthy people (as negative control). Real-Time PCR used in quantification of IL-3 and IL-6 genes expression, and enzyme linked immunosorbent assay (ELISA) kits used to measure serum IL-3 and IL-6 levels. The expression & serum levels of IL-3& IL-6 was significantly increased a significant increasing ($p<0.0001$) in SARS-Cov-2 patients groups in comparison to control group (healthy individuals), also there is a significant increasing ($p<0.0001$) in IL-3 & IL-6 expression & levels of obese covid-19 patients group & smokers covid-19 Patients group in comparison with positive control group. Furthermore, There was a significant value of IL-6 with IL-3 serum levels and IL-6 with IL-3 gene expression and correlated positively

Introduction

Coronavirus Disease-19 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome-coronaviruses-2 (SARS-CoV-2) a highly pathogenic and transmissible coronavirus. Most cases of COVID-19 have mild to moderate symptoms, including cough, fever, myalgias, and headache, also coronavirus can lead to severe complications and death in some cases [1]. The first cases were described in late December 2019 in the Chinese city of Wuhan. Since then, the disease has rapidly spread over more than 200 countries and infected millions of cases with high mortality rates. This forced the World Health Organization (WHO) to announce COVID-19 as pandemic[2] .

Various host factors associated with an increased risk of disease include older age, male gender, diabetes, obesity, cardiovascular disease and malignancy[3,4] . Much clinical research suggests a strong relationship between body mass index (BMI) defined obesity and increased risk of testing positive for SARS-CoV-2, as well as increased risk of severe disease among patients with COVID-19[5]. Furthermore, evidence indicates that smokers are more likely to suffer more severe outcomes of COVID-19, such as admission into intensive care units and death, than never smokers[6]. Cytokines are the most important factors of immunity because of their involvement in mediating and controlling immune responses against various infectious agents, as well as inflammation[7]. In

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COVID-19 patients, the term cytokine storm has been used to describe the uncontrolled excessive production of inflammatory markers in COVID-19 patients [8] .

Many studies reported that Interleukin 6 (IL-6) is one of the important cytokines that is relevant to the outcome of COVID-19, including disease duration and severity [9]. The potential for the hyper activation of IL-6 in the host immune pathway contributes to the development of long-term symptoms of COVID-19, which in turn trigger uncontrolled inflammatory responses known as ‒cytokine storms‖ [10] . From other hand, Some study reported that IL-3 may be an independent prognostic marker for the outcome of severe SARS-CoV-2 infections. High plasma IL-3 levels in SARS-CoV-2 infections are associated with increased T cell activation [11] .

Aim of Study

Investigation role (IL-6 and IL-3) in covid-19 patient in groups of smokers and obese covid-19 patients a cross-sectional study. By Determination of IL-6 and IL-3 gene expression and compare it with the IL-6 and IL-3 serum levels in smoking and obese patients with Covid-19 infection .

Material and Methods

1. The study subjects

This study included One hundred blood samples were collected from individuals for a cross-sectional study of COVID-19 infection, 100 samples were divided into (75 patients infected covid-19 and 25 samples of healthy individuals as a negative control group) from Al-Hayat Center in Al-Zahra Teaching Hospital and External laboratories after fixing their infection, in Wasit provinces/Iraq. This study was carried out during the period between February 2022 to March 2023.

2. COVID-19 patients Groups

In this study, 75individuals infected with COVID-19 (41 women and 34 men) With ages ranging from over 20 Less than 80 years old. The patients were diagnosed as COVID-19 positive cases . For each patient, an information sheet was filled and written consent was obtained. The information included gender, age, body mass index(BMI), and presence and absence of chronic diseases. Besides, the following laboratory tests were also included: complete blood count (CBC) and C-reactive protein (CRP). Note\\All patients in this study do not suffer from any chronic diseases. This 75 patients were divided into three main groups; **Group 1 :** 25 Covid-19 patients suffering from obesity with a body mass index (BMI) higher than 25 kg/m2 (BMI ranging from 29 kg/m2 to 40 kg/m2). **Group 2 :** 25 smoking patients with Covid-19, with normal body mass index (BMI) less than 25 kg/m2. **Group 3:** 25 patients with COVID-19 were non-smokers, non-obese, as positive control group. This 75 patients infected with Covid-19 was compared with healthy people(25 samples) who do not have Covid-19, are non-smokers, and non- obese as a negative control group (**Group 4**) .

3. Methods

A- Samples Collection

Four milliliters of venous blood sample were collected by veni puncture from each subject (cases and controls) involved in the present study samples, Blood was divided into 2 parts:

The First part: (0.5-1 ml) of whole blood was put in tube sterilize microcenterfuge (1.5ml eppindrove tube) containing (0.5 ml RNA later) used for genomic RNA extraction, labeled and stored in -20 ℃.

The second part: (2- 3 ml) of whole blood were enabled in the gel tube for few minutes at the room temperature, and centrifuged at 3000 rpm for 15 min for a period of 3 minutes. The serum was isolated, labeled, and frozen at -20°C.

B- Serological Test (ELISA)

This test was used to evaluate the presence of Interleukin 3 (IL-3) and Interleukin 6 (IL-6)using enzyme-linked immunosorbent assay technique (ELISA). ELISA kits (E0093Hu, Human IL-3) & (E0090Hu, Human IL-6) for (Bioassay Technology Laboratory / China) according to the instructions of the manufacturer.

C- Quantitative Real-Time PCR (qPCR)

The quantitative Real-Time PCR used in quantification of IL-3 and IL-6 genes expression

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analysis that normalized by housekeeping gene (GAPDH) in patient and healthy blood samples by using Real-Time PCR technique. This method included several steps :

1- Total RNA Extraction : Total RNA were extracted from blood samples by using (FavorPrepTM RNA Kit) and done according to company instructions. The extracted RNA was checked by using Quantus™ Fluorometer (Promega. USA), using QuantiFluor® RNA Dye that check RNA concentration

2- cDNA synthesis : DNase-I treated RNA samples were also used in cDNA using EasyScript® One-Step cDNA Synthesis SuperMix and done according to company instructions.

3- RT- qPCR Primers : The Real Time PCR primer that used in gene expression of IL-3gene , IL-6 gene and housekeeping GAPDH gene[12,13] , they were designed in this study by using NCBI Genbank database and perimer 3 plus as following table (1).

Table (1): Primers Sequence Used In RT- qPCR

Primers	Sequence (5'-3')		Product Size (bp)
HumanIL-3	F	5'-GGACTTCAACAACCTCAATGGG-3'	184bp
	R	5'-TTGAATGCCTCCAGGTTTGG-3'	
HumanIL- 6	F	5'-AATTCGGTACATCCTCGACGG-3'	261bp
	R	5'-GGGCATGGATTTCAGACCC-3'	
Human GAPDH gene	F	5'-CCCATCACCATCTTCCAGGAGCG-3'	476 bp
	R	5'-CATGCCA GTGAGCTTCCCGTTCA-3'	

4- qPCR component preparation

qPCR master mix was prepared by using GoTaq® qPCR Master Mix kit based on SYBER green dye detection of target genes amplification in Real-Time PCR system and include: 5µL of cDNA template (100ng), 12.5µL of qPCR Master Mix, 1 µL of each primers (forward & reverse) then complete the volum to 25 µL with DEPC water.

5- qPCR Thermocycler conditions: the qPCR plate was loaded following thermocycler protocol and the conditions included initial denaturation (95 °C for 5 min) following a 35 time cycles of denaturation at 95 °C for 30 s, annealing at (58.5°C for IL-3, 60°C for IL- 6) for 45s and extension at 72°C for 45 s each cycle; and final extension at 72°C for 5 min. The data results of q RT-PCR for target and housekeeping genes were analyzed by the relative quantification gene expression levels (fold change) (The ΔCT Method Using a reference gene).

Statistical analysis

SPSS 22.0 statistical software was used for statistical analysis. Count data were analyzed by the $\chi 2$ test. A P value < 0.05 indicates statistical significance.

Results & Discussion :

In this study, immune markers were studied for patients infected with Covid-19 who smoke and suffer from obesity, by testing IL-3 & IL-6 for 100 samples (cases and controls) using the ELISA test and Real-Time PCR (qPCR) .

1- Folding of IL-3 Expression :

IL-3 gene expression was elevated in response to covid-19 infection. IL-6 gene expression was significantly higher ($P < 0.0001$) in SARS-Cov-2 patients groups in comparison to control group (healthy individuals). Table (2)& Figure (1) Showed the expression of IL-3 in patients and control groups,

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the statistical analysis of data observed that there is a significant increasing ($p<0.0001$) in obese covid-19 patients group with each other groups and it's relatively higher in obese covid-19 patients than other groups, also there is a significant increasing ($p<0.001$) in IL-3 expression of smokers covid-19 patients group with obese covid-19 patients group.

Table (2) : Gene Expression of IL-3 In Patients and Control Groups

Parameter	Groups	Mean+S.E	P value
Folding of IL-3 expression	Control	0.578±0.021	<0.0001**
	Obese Covid-19 Patients	0.671±0.018	
	Smokers Covid-19Patients	0.603±0.005	
	Covid-19 patients	0.612±0.005	

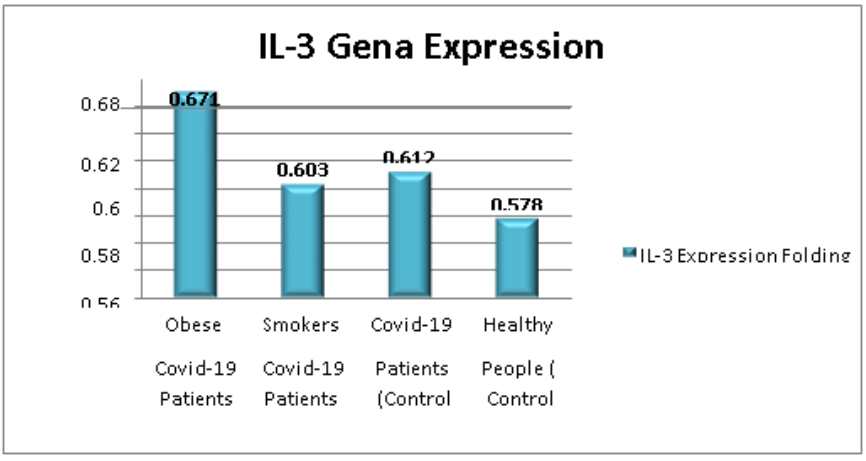

Figure (1): Mean Folding's of IL-3 Gene Expression In Patients and ControlGroups

Mechanistically, IL-3 increases innate antiviral immunity by promoting the recruitment of circulating plasmacytoid dendritic cells (pDCs) into the airways and byenhancing pDC-mediated T cell activation upon viral stimulation. Interestingly, the ability of IL-3 to improve adaptive immunity was confirmed in patients with SARS- CoV-2 infections.[11]

2- Folding of IL-6 Expression :

Also IL-6 gene expression was elevated in response to covid-19 infection in combination with the effect of other factors, obesity and smoking as shown in Table (3) & Figure (2). The results of the current study showed a significant increase (P= 0.009) in the expression of IL-6 in the group of obese Covid-19 patients compared to Smokers Covid-19 Patients and Control groups. While the results also showed that there was no significant difference in the expression of IL-6 in the group of smokers Covid-19 patients and non-smokers infected with Covid-19 (control positive), this may indicate that smoking does not affect the level of IL-6 expression in smoking Covid-19 patients in compared to those non-smokers infected with Covid-19.

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Table (3) : Gene Expression of IL-6 In Patients and Control Groups

Parameter	Groups	Mean+S.E	P value
Folding of IL-6	**Control**	0.590±0.031	0.009**
expression	**Obese Covid-19 Patients**	0.786±0.060 [ac]	
	Smokers Covid-19 Patients	0.628±0.027 [c]	
	Covid-19 patients	0.689±0.043	

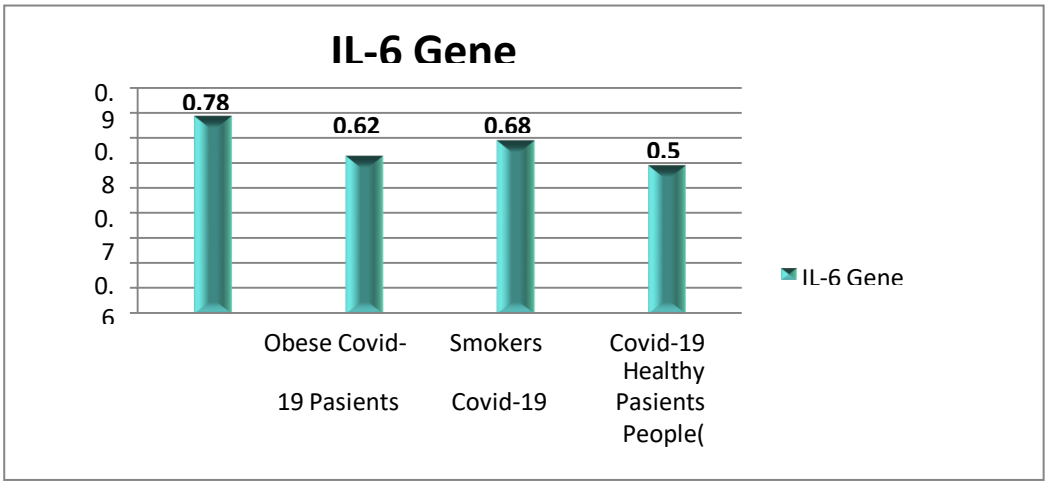

Figure (2): Mean Folding's of IL-6 Gene Expression In Patients and ControlGroups

The IL-6 cytokine‘s mRNA expression was observed to be higher in SARS patients‘ peripheral blood mononuclear cell (PBMC)[14,15]. The level of IL-6 expression was found to be greater in SARS patients and was associated with the severity of their sickness[16] .

Moreover, the current study have shown agreement with several COVID-19 studies, that were showed elevated IL-6 levels in patients with COVID-19[17,18,19,20].

From Other hand , Lagunas-Rangel and Chavez-Valencia, (2020) have shown that patients with severe COVID-19 had a greater IL-6/IFN ratio than those with mild COVID-19, which might be due to a stronger cytokine storm promoting lung injury[21].

SARS-CoV-2 stimulates the innate immune system, leading macrophages and other innate immune cells, including IL-6, to generate a large number of cytokines and chemokines. Antigen-presenting cells may also initiate adaptive immunity (mainly dendritic cells). T cells and B cells are antiviral cells that indirectly or directly stimulate the generation of proinflammatory cytokines. In addition, when inflammatory chemicals stimulate the alveoli, a large amount of inflammatory exudate and erythrocytes enter the alveoli, resulting in dyspnea and respiratory arrest[22].

Also, Memoli *et al.*, 2007, investigated IL-6 mRNA was expressed in subcutaneous and omental fragments of adipose tissue from all subjects. Furthermore, a

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significant expression of both components of IL-6 receptor complex was demonstrated in all adipose tissue samples[23]. Likewise Sindhu *et al.*, 2015, also agree with this result by showed that the IL-6R/IL-6 expression correlated positively with the adipose tissue expression and obese individuals showed higher IL-6R expression (103.8±4.807) in the adipose tissue as compared with lean/overweight (68.06±4.179) subjects (P<0.0001). The elevated IL-6R expression correlated positively with body mass index (BMI) and the increased IL-6R expression in obesity was also confirmed by RT-PCR[24].

Adipose tissue is currently recognized as a rich milieu and source of interleukin-6 (IL-6). Circulating levels of IL-6 may be increased in obese individuals and linked with coronary artery disease[25] .

3- IL-3 level in the studied samples

Table (4) presented the serum level of IL-3 in patients and control groups, the statistical analysis of data observed that there is a significant increasing (p<0.0001) in IL-3 levels in SARS-Cov-2 patients in comparison to control group (healthy individuals) as shown in figure(3). The means were (56.506±6.775) ng/mL in control group, (121.331±6.83) ng/mL in obese Covid-19 Patients, (139.782±8.456) ng/mL in smokers covid-19 patients and (87.780±5.482) ng/mL in covid-19 patients (control positive)

Table (4) : The mean value of IL-3 in patients and controls

Parameter	Groups	Mean+S.E	P value
IL-3 levels	Control	56.506±6.775	<0.0001**
	Obese Covid-19 Patients	121.331±6.833	
	Smokers Covid-19Patients	139.782±8.456	
	Covid-19 patients	87.780±5.482	

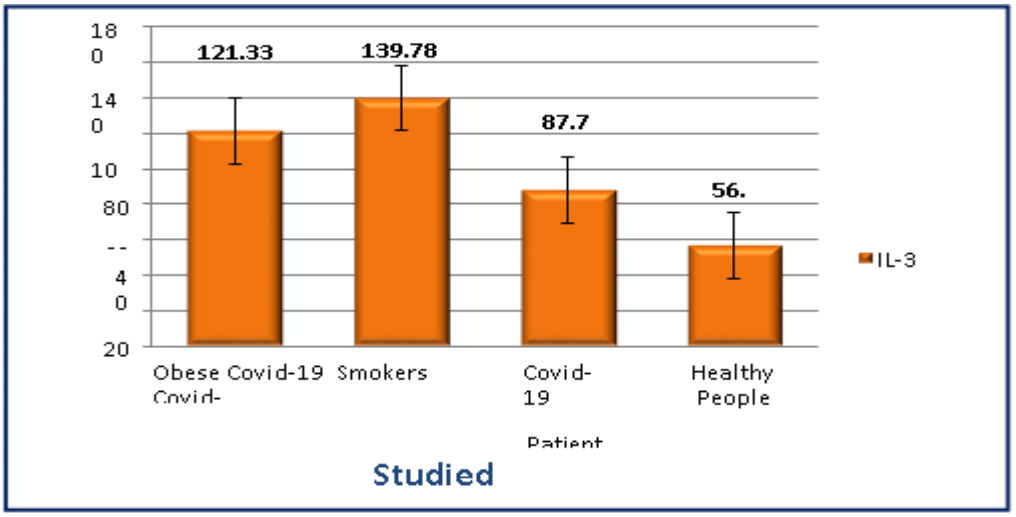

Figure (3) : Mean Value of IL-3 levels In Patients and Control Groups

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There is a significant increasing ($p<0.0001$) in IL-3 levels of obese covid-19 patients group with control negative and control positive, also there is a significant increasing ($p<0.001$) in IL-3 levels of smokers covid-19 patients group with control negative and control positive.

The results of the current study showed that the percentage of IL-3 in smokers covid-19 Patients group was higher than that of other groups. While the results showed a low level of interleukin 3 in healthy people (control negative).

Bénard *et al.*, 2023 agree with result, by showed high plasma IL-3 levels in SARS- CoV-2 infections are associated with increased T cell activation, likewise, SARS-CoV- 2+ patients with high plasma IL-3 levels exhiéited higher plasma IFNγ levels, and suggested that IL-3-stimulated Plasmacytoid dendritic cells pDCs induce T cell activation during SARS-CoV-2 infections [11] .

From other hand , In another study to Bénard *et al.*, 2021 identify IL-3 as an independent prognostic marker for the outcome during SARS-CoV-2 infections. Specifically, low plasma IL-3 levels is immunity by promoting plasmacytoid dendritic cells (pDC) recruitment into the lungs[11] .

4- IL-6 level in the studied samples

IL-6 serum level also significantly elevated ($p<0.0001$) in SARS-Cov-2 patients groups as compared to control group, Where the results showed that , the mean of IL-6 was (65.7±6.246) ng/mL in healthy people, (144.261±8.743) ng/mL in obese covid-19 patients, (168.386±7.467) ng/mL in smokers covid-19 patients, and (101.381±8.317) ng/mL in covid-19 patients group (control positive) (Table 5) & (Figure 4):

associated with increased severity, viral load, and mortality during SARS-CoV-2 infections[26]. ·

The results of the current study showed high level of IL-3 in smokers & obese covid-19 Patients groups were higher than that of other groups due to, Monocyte-

derived macrophages (mono-Mc), monocyte-derived dendritic cells (DCs), and plasmacytoid DCs (pDCs) were significantly enriched in smokers‘ tissue . Among these clusters, pDCs, specifically enriched in the tissue of smokers [27] . Also the adipose tissue is the primary and first organ affected by inflammation and sustains obesity-induced inflammation[28] , and Various immune cell types, including macrophages, dendritic cells (DCs), and T and B cells, have been identified in the adipose tissue and implicated as key players in obesity-associated immune responses. IL-3 produced mainly by immune cells (CD4+ T cells are the main producers of IL-3, CD8+ memory T cells ,basophils , mast cells and some B cells) that plays a key role during inflammatory diseases[29] . Also during some viral infection , IL-3 improves innate antiviral

Table (5) : The mean value of IL-6 in patients and controls

Parameter	Groups	Mean+S.E	P value
IL-6 levels	Control	65.7±6.246	<0.0001**
	Obese Covid-19 Patients	144.261±8.743	
	Smokers Covid-19Patients	168.386±7.467	
	Covid-19 patients	101.381±8.317	

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In the comparison between groups, the statistical results showed a significant value (0.001), to obese covid-19 patients group with smokers covid-19 patients group , control positive and control negative. The same comparison results for smokers covid-19 patients group with others groups.

Therefore, there is a significant value for the level of interleukin 6 between the studied groups in general, and between each group separately with the other.

The results of the current study showed that the percentage of IL-6 in smokers covid-19 Patients & obese covid-19 patients groups was Significantly higher than other groups.

Most studies reached results consistent with the results of this study, as the results of Banerjee *et al.*, 2020 ; Maurya *et al.*, 2021; Mal *et al.*, 2022 and Nikkhoo *et al.*, 2023

, They showed a significant increase (p=0.001) of IL- 6 level in obese covid-19 patients [30,31,32,33] .

Because SARS CoV2 enters host cell via Angiotensin Converting Enzyme 2 receptor, expression of which is upregulated in visceral fat tissue in obese people, underscoring the fact that adipose tissue is a potential reservoir for virus, adipose tissue is also a source of many proinflammatory mediators and adipokines[30] .

On the other hand, results of Liu *et al.*, 2020; Al-tameemi *et al.*, 2022 & Haghgoo *et al.*,2023 , showed agreement of a significant increase (p=0.01) of IL- 6 level in smokers covid-19 patients [34,35,36]. Tobacco-smoke airway exposure is also characterized by a chronic inflammation with activation of inflammatory cells and cytokine release including interleukin-6 (IL-6). A high release of cytokine in response to viral infection is documented in COVID-19 patients with adverse clinical outcomes and IL-6 is a key element of the cytokine storm syndrome leading to multi-organ damage[37].

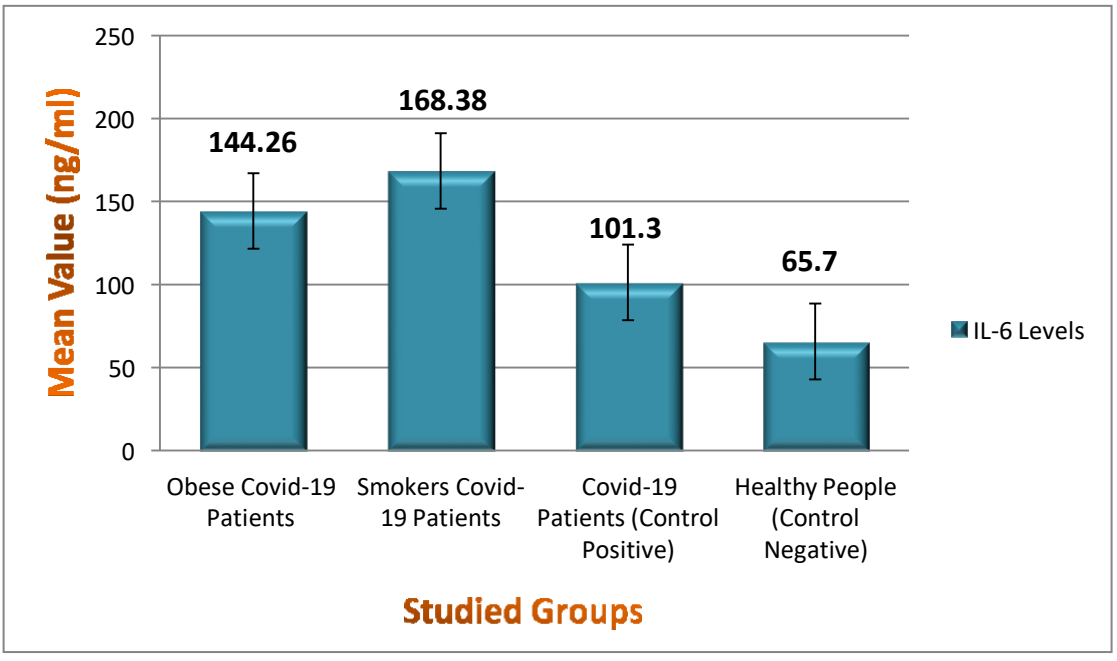

Figure (4) : Mean Value of IL-6 levels In Patients and Control Groups

5- Correlation Between Expression and Levels Of IL-3 &IL-6

The correlation between the expression and levels of IL-3 & IL-6 for the samples studied, as it was shown that there was a highly significant value (P=0.001) of IL-6& IL- 3 serum levels with a strong positive correlation, and also there was a significant value (P=0.001)IL-6& IL-3 gene expression with a strong positive correlation .While on the other hand, there was a moderate positive correlation of the IL-3 serum level with the IL-3 gene expression, with a significant

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value (P=0.04). But on the contrary, there was no significant value of IL-3 gene expression with IL-6 serum levels and It's weakly positively correlated. While, the results also showed that there was no significant value of IL-6 gene expression with IL-3 & IL-6 serum levels and there was weakly positive correlated, as shown in the Table(6) :

Table (6): Correlation Between Expression and Levels Of IL-3 &IL-6

Parameter		**IL-3 Levels**	**IL-6 Levels**	**Foldingof IL-3 expression**	**Foldingof IL-6 expression**
IL-3 Levels	Pearson Correlation	1			
	Sig. (2-tailed)				
IL-6 Levels	Pearson Correlation	0.596**	1		
	Sig. (2-tailed)	0.001			
Folding of IL-3 expression	Pearson Correlation	0.203*	0.176	1	
	Sig. (2-tailed)	0.043	0.079		
Folding of IL-6 expression	Pearson Correlation	0.14	0.166	0.370**	1
	Sig. (2-tailed)	0.163	0.099	0.001	

The protein and mRNA abundances are determined by the relationships between the rates of the processes producing and degrading the participating molecules. In mammalian cells, mRNAs are produced at a much lower rate than proteins are; on average, a mammalian cell produces two copies of a given mRNA per hour, whereas it produces dozens of copies of the corresponding protein per mRNA per hour. Similarly, mRNAs are less stable than proteins (with an average half-life of 2.6–7 hours versus 46 hours, respectively)[38]. The long half-lives of proteins have been confirmed by independent studies in other systems and suggest a potentially large role of protein dilution‘ — that is, the decrease in protein concentration owing to cell division[39] .

Recent studies suggest a perhaps undervalued role for post-transcriptional,translational and degradation regulation in the determination of protein concentrations, contributing at least as much as transcription itself[40].

Conclusions:

1- Obesity and smoking are risk factors that increase the severity of Covid-19 infection.

2- Increased expression & serum levels of some cytokines, such as IL-3 and IL-6, in smokers

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Covid-19 patients & obese Covid-19 patients than in patients with Covid-19 who are non-smokers and non-obese .

3- There was a significant value of IL-6 with IL-3 serum levels and IL-6 with IL-3 gene expression and correlated positively .

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