**RESEARCH ARTICLE**

# Circulating Drivers of Pathophysiology in Post-COVID-19 Sequelae

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**Abstract:** ***Introduction****:* According to the WHO, post-COVID-19 sequelae impose an increased burden of illness and comorbidities among survivors. The present study evaluated systemic clinical complications associated with the dysregulation of circulatory cytokines in post-COVID-19 patients and compared them with those of healthy controls.

***Methods:*** This cross-sectional study included 147 participants divided into symptomatic post-COVID-19, asymptomatic post-COVID-19, and healthy control groups. After obtaining informed consent, peripheral blood samples were collected and analyzed using ELISA and RT-qPCR. Circulating levels of pro-inflammatory (IL-6, IL-1β, TNFα) and anti-inflammatory cytokine IL-10 were measured in relation to clinical sequelae.

***Results:*** Common symptoms were fatigue (48%), headache (48%), anxiety (64%), general weakness (70%), muscle pain (70%), joint pain (54%), chest pain (32%), dyspnea (36%), and post-activity tachypnea (40%) among post COVID-19 individulas. A higher prevalence of post-COVID-19 sequelae was observed in females aged >40 years and in those with a post-COVID-19 duration of <30 months. ELISA showed higher levels of IL-6, TNF-α, and IL-10 in post-COVID-19 patients than in controls ($p < 0.01$). RT-qPCR revealed upregulation of IL-6, TNF-α, IL-1β, and IL-10 mRNA, which correlated with post-COVID-19 sequelae ($p < 0.01$).

***Discussion:*** Older age and shorter duration of post-COVID-19 are associated with more symptom burden, implying incomplete immune recovery during early convalescence. Elevated IL-6, IL-1β, TNF-α, and IL-10 levels indicate persistent immune dysregulation, supporting the view that post-COVID-19 is a chronic inflammatory state with clinical effects.

***Conclusion:*** IL-6, TNFα, IL-1β, and IL-10 are dysregulated in post-COVID-19 systemic sequelae, and longitudinal studies are necessary to better understand and potentially reduce this dysregulation in post-COVID-19.

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## 1. INTRODUCTION

The COVID-19 pandemic caused immediate health problems worldwide and raised concerns about its lasting impacts on survivors. The World Health Organization [WHO] defines post-COVID-19 as symptoms and complications that persist for at least three months after the initial onset of COVID-19 [1, 2]. Post-COVID-19 health sequelae include increased socioeconomic burden, escalated comorbidities, and reduced quality of life. Multiple organ systems exhibit dysfunction that persists for short or prolonged periods of time [3]. Most clinical reports indicate that post-COVID-19 conditions include fatigue, joint pain, concentration impairment, anxiety, depression, dyspnea, and dry cough [4, 5]. The immune system is dysregulated during COVID-19 and post-COVID-19

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conditions [6, 7]. Immunity plays a key role in defending the body against pathogens. It shapes host–pathogen interactions during infection. If the immune system fails to respond effectively, it may contribute to disease progression [8]. In COVID-19, the cytokine storm escalates the production of various types of cytokines, contributing to systemic inflammation. Some immune cells that contribute to the cytokine storm and immune dysregulation include macrophages, neutrophils, eosinophils, basophils, monocytes, dendritic cells, and natural killer cells [9-11]. Consistent with this, COVID-19-infected human monocyte and leukocyte cell lines show increased production of pro-inflammatory cytokines with chemotactic activity [12]. Several other studies suggest that COVID-19 infection may cause long-term immune dysregulation through several mechanisms, including viral antigen persistence, autoimmunity, prolonged cytokine storm, immune cell exhaustion, altered immune cell populations, chronic inflammation, and dysbiosis [13-25]. Prolonged symptoms are often associated with sustained immune dysregulation, which may include a reduction in the number of T and B cells, pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and dysregulation of angiotensin converting enzyme 2 (ACE2) [26-28]. Emerging evidence indicates that persistent immune dysregulation and chronic inflammation are major circulating drivers of cytokine dysregulation in post-COVID-19 sequelae. Studies have revealed elevated levels of TNFα and IL-1β in individuals with early and persistent long COVID-19 condition [29, 30]. Longitudinal analyses have further shown that persistent dysregulation in IL-6, IL-2, IL-8, IL-18, and IL-1Ra cytokines has caused sustained immune activation following acute infection [31]. In individuals with post-COVID-19 syndrome, elevated concentrations of IL-1α, IL-1β, IL-6, MCP-1, and TNF-α, together with reduced anti-inflammatory responses of IL-10, further support the presence of a dysregulated and imbalanced cytokine milieu [32]. Additionally, DNA polymorphisms and virus-induced epigenetic modulation of cytokine-related gene expression may perpetuate chronic inflammation. Cytokine overexpression may also play a significant role in the development of systemic complications, particularly neuropsychiatric manifestations in long COVID-19 [33, 34]. Viral persistence activates the cytoplasmic nucleotide-binding oligomerization domain-like receptor 3 (NLRP3), which propagates inflammation and immune dysregulation [35, 36]. Together, viral persistence and sustained immune dysregulation might contribute to upregulation of several cytokines (IL-6, IL-1β, IL-10, ACE2, and TNFα) and chemokines (CCL2, CCL3). IL-1β, along with TNFα, promotes nuclear factor kappa B (NF-κB) nuclear translocation and p38 mitogen-activated protein kinase (p38-MAPK) phosphorylation, thereby activating cells and facilitating the cytokine storm and systemic inflammation [37, 38]. IL-6 is a vital cytokine and is associated with neuropsychological symptoms among COVID-19 survivors [39]. On the other hand, IL-10 has anti-inflammatory and anti-fibrotic effects and therefore, impairments in IL-10 may contribute to supporting persistent inflammation and immune dysregulation [40, 41]. Inflammatory cytokines like IL-6, TNFα, IL-1β, and interferons may also contribute to immune dysregulation in post-COVID-19 by promoting chronic inflammation [42, 43]. Thus, the present study analyzed the protein levels (pg/mL) and mRNA of pro-inflammatory cytokines IL-6, IL-1β, and TNFα, as well as the anti-inflammatory cytokine IL-10, in symptomatic and asymptomatic post-COVID-19 survivors, along with a control group. Secondarily, cytokine concentrations and gene expression were examined in relation to post-COVID-19 sequelae and systemic complications.

## 2. MATERIAL AND METHODS

The present study was approved by the Ethical Committee at the University of Lahore, Pakistan (REC-UOL-90-I-04-2023). The study was conducted between May 2023 and December 2024 in the outpatient medical clinics of local hospitals in Pakistan. All participants were informed verbally about the study, and written consent was obtained.

### 2.1. Sample Size and Population

The sample size was calculated as n = 147 using the formula for comparing three proportions at a 95% confidence interval, 80% power, and a 5% level of significance. Each group included 49 individuals (26 males and 23 females). Adult subjects aged >18 years who were >12 weeks from COVID-19 onset and had a negative RT-PCR test for COVID-19 were enrolled in the study by physicians from outpatient clinics at local hospitals. Post-COVID-19 patients were categorized into symptomatic and asymptomatic groups according to WHO guidelines and screened by a physician. The symptomatic post-COVID-19 group (n = 49) was defined as survivors with a confirmed history of RT-PCR testing for COVID-19 infection who developed symptoms related to the infection [44, 45]. Asymptomatic post-COVID-19 survivors (n = 49) had laboratory-confirmed COVID-19 infection by RT-qPCR but did not develop moderate to severe symptoms related to infection [46]. Healthy controls (n = 49) had no history of COVID-19 infection and had no current symptoms of disease. Healthy control subjects were recruited to serve as a comparison group with symptomatic and asymptomatic post-COVID-19 survivors. The detailed, inclusive, and exclusive criteria are as follows.

### 2.2. Inclusion Criteria

I. Symptomatic Post-COVID-19 Subjects

- Presence of at least one ongoing symptom (fatigue, anxiety, headache, depression, dyspnea, coughing, musculoskeletal pain) lasting more than 12 weeks post-acute infection.
- Evidence of previous COVID-19 infection confirmed by RT-PCR.

II. Asymptomatic Post-COVID-19 Subjects

- Absence of any symptoms persisting beyond 12 weeks after the onset of COVID-19 infection.
- Evidence of previous COVID-19 infection confirmed by RT-PCR report.

III. Healthy Control Subject

- The subjects had no signs or symptoms of illness used as controls. All physical examination findings and basic clinical assessments were within normal limits and confirmed by the physician's examination.
- Participants had evidence of recent negative diagnostic RT- PCR reports.

### 2.3. Exclusion Criteria

I. Symptomatic Post-COVID-19 Subjects

- Participants with chronic fatigue syndrome, influenza, inflammatory bowel disease, fibromyalgia, any type of carcinoma, or multi-organ failure were excluded from the study.
- Pregnant female participants were excluded due to potential confounding symptoms and changes in immune response.

II. Asymptomatic Post-COVID-19 Subjects

- Severe pre-existing psychiatric conditions, such as schizophrenia or bipolar disorder, that may interfere with study participation.
- Inability to provide a reliable health history or participate in follow-up assessments.

III. Healthy Control Subjects

- Participants having signs or symptoms suggestive of COVID-19 and close contact with a confirmed COVID-19 patient within the past 3 months were excluded from the study.
- Pregnant females were excluded due to potential symptom overlap and physiological changes.

### 2.4. Experimental Procedure

Three months after the COVID-19 onset, peripheral blood samples were collected in sterile vacutainers for enzyme-linked immunosorbent assay (ELISA) and real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR). For protein estimation, plasma from each sample was separated and subjected to analysis of levels (pg/mL) of pro-inflammatory cytokines (IL-6, IL-1β, TNFα) and anti-inflammatory cytokine (IL-10) using the IL-6 ELISA Kit (BT Lab, catalog No. E0090H, China), IL-1β (BT Lab, catalog No. E0143Hu, China), TNF-α ELISA Kit (BT Lab, catalog No. E0082Hu, China) and IL-10 ELISA Kit (BT Lab, catalog No. E0102Hu, China). All procedures were conducted according to the manufacturer's instructions [47]. The assays were performed in duplicate for each sample to ensure the accuracy and reliability of the results. The absorbance was measured at 450 nm using a microplate reader (BIO-RAD, PR4100), and the concentrations (pg/mL) of IL-6, IL-1β, TNFα, and IL-10 were determined by comparing the optical densities of sample wells to a standard curve.

For mRNA expression analysis, serum was separated from whole blood samples, and RNA was extracted from each sample. Before cDNA synthesis, RNA quantification was confirmed using a microvolume spectrophotometer (NanoDrop™, Thermo Scientific, USA). For mRNA expression of IL-6, IL-1β, TNFα, and IL-10, primers were retrieved from published data and NCBI. (Supplementary I). The SYBR Green RT-qPCR method was used to verify the quantification of each sample. Cycle threshold (CT) values were recorded, CT graphs were analyzed, and relative quantification was performed to explore the association between cytokine expression and clinical outcomes [48]. The primers and product sizes are shown in Supplementary File 1. The assay cut-off value for cycle threshold (CT) was considered > 40 [49-50]. The protocol for RT-qPCR included an initial denaturation at 95°C for 2 minutes, followed by 45 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 20 seconds, and extension at 60°C for 30 seconds. The final step involved maintaining the final temperature at 4°C. The PCR products were run on a gel, and the desired band was identified, isolated, purified, and sent for sequencing. These sequences are presented in Supplementary File II as evidence.

### 2.5. Statistical Analysis

Independent variables (age, duration of post-COVID-19) and dependent variables (cytokine levels) were reported as mean ± standard deviation and compared using ANOVA. Gender and various systemic sequelae were presented as frequencies (percentages). Correlations between gender, age, duration, cytokine concentrations, gene expression, and post-COVID-19 sequelae were analyzed using SPSS. Statistical significance was defined as $p < 0.05$. All statistical analyses were conducted using SPSS version 24 and GraphPad Prism version 10.1.1.

## 3. RESULTS

The present study included 147 individuals aged 22 to 65 years. Blood samples and clinical histories were collected from each patient for ELISA and RT-qPCR analysis. Fatigue was reported more frequently in males (32%) than in females (16%) across all groups. The most common symptoms overall were weakness (86% in females vs. 64% in males) and muscle pain (86% in females vs. 64% in males). Headaches were reported by 71% of females compared with 39% of males, indicating a significant impact on women's health. Moderate symptoms such as fatigue (57% in females vs. 44% in males), chest pain (36% in both genders), and palpitations (36% in females vs. 22% in males) were also noted. Interestingly, females reported

higher rates of anxiety (28% vs. 21%) and depression (29% vs. 22%) than males. Conversely, lower prevalence rates were observed for discomfort (22% in males vs. 14% in females) and gastrointestinal issues like abdominal pain (36% in females vs. 17% in males) and diarrhea (29% in females vs. 11% in males), suggesting that these were milder symptoms. The average age was 48 ± 17 years for symptomatic post-COVID-19 survivors, 37 ± 13 years for asymptomatic survivors, and 33 ± 11 years in the control group. Participants were divided into two age groups (<40 and ≥40 years). Those aged 40 and above exhibited more symptoms than younger individuals. Among symptomatic individuals ≥40 years, higher rates of fatigue (24% vs. 18%), chest pain (22% vs. 10%), palpitations (16% vs. 10%), muscle pain (36% vs. 8%), and shortness of breath (20% vs. 16%) were observed compared with younger counterparts. Psychological symptoms, such as psychosocial distress (10% vs. 6%), anxiety (14% vs. 12%), and discomfort (12% vs. 8%), were more common in younger survivors (< 40 years). The duration of post-COVID-19 was categorized into two groups (<30 and ≥30 months) among both symptomatic and asymptomatic individuals. In the <30-month group, the median duration of post-COVID-19 symptoms was 16 months for symptomatic individuals and 10 months for asymptomatic individuals. While in the ≥30-months group, the median duration of post-COVID-19 symptoms was 45 months for symptomatic individuals and 10.5 months for asymptomatic individuals. Those with a shorter duration (<30 months) reported higher rates of fatigue (38% vs. 22%), chest pain (27.5% vs. 12.5%), and muscle pain (55% vs. 32.5%) compared with those with longer durations. Ground-glass opacification (GGOs) and gastrointestinal symptoms were also more frequent in the shorter duration group (< 30 months).

Overall, the data revealed that the most common symptoms among post COVID- 19 survivors were weakness (70%), muscle pain (70%), fatigue (48%), and headache (48%). A detailed comparison of symptom occurrence between symptomatic and asymptomatic individuals is shown in Fig. (**1**).

Common moderate symptoms included joint pain (54%), chest pain (32%), dyspnea (36%), and tachypnea (40%), indicating ongoing respiratory and musculoskeletal issues. Less common symptoms involved fever (18%), abdominal pain (22%), and psychosocial distress (16%). A radar chart showing the overall severity of each symptom in this study is presented in Fig. (**2**).

ANOVA analysis showed that the symptomatic group had the highest levels of IL-6, IL-1β, TNFα, and IL-10 (pg/mL) compared with asymptomatic post-COVID-19 individuals and controls (Fig. **3**).

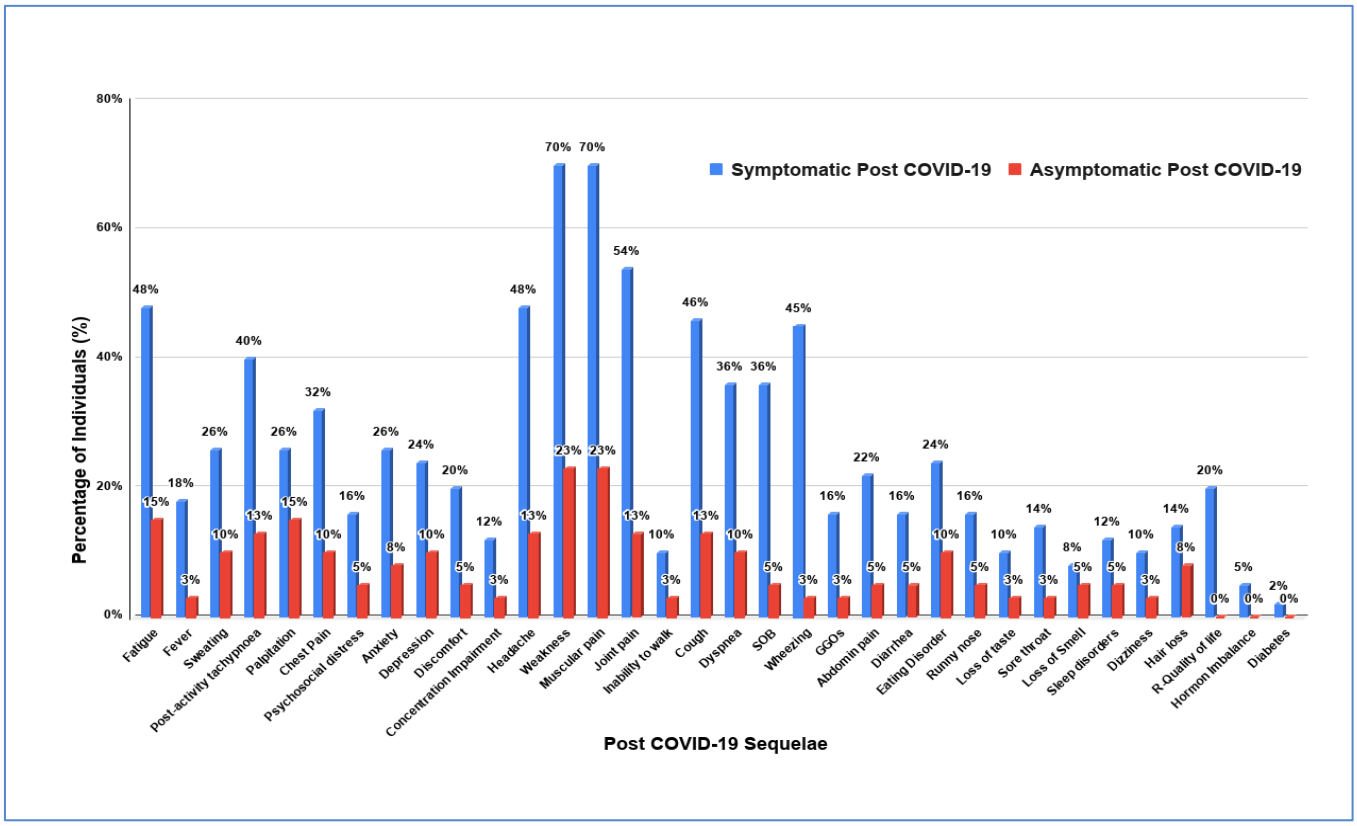


**Fig. (1).** A bar chart shows the distribution of symptoms in symptomatic and asymptomatic post-COVID-19 survivors. Musculoskeletal, cardiopulmonary, and neurological sequelae are more common among the population. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

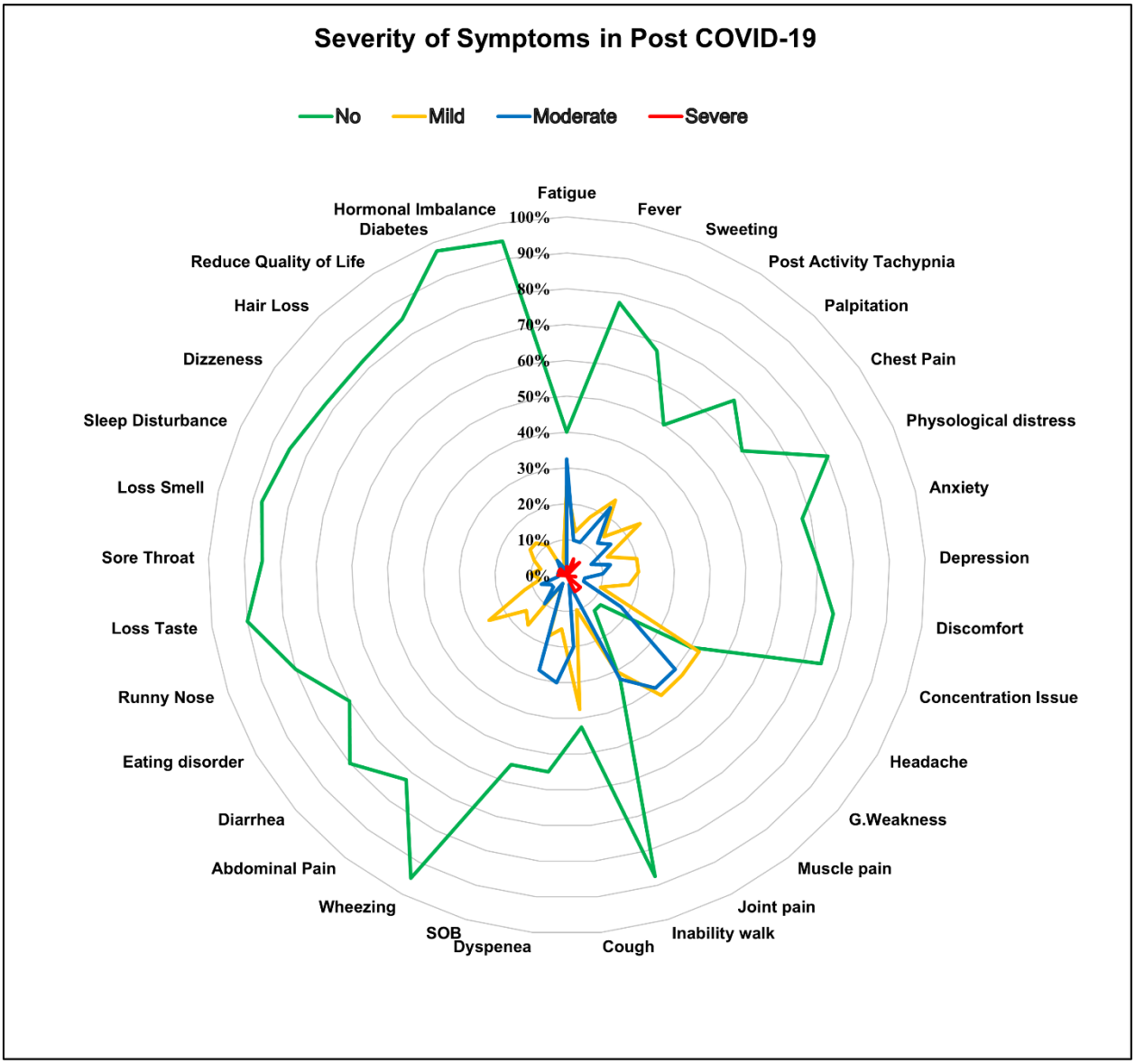


**Fig. (2).** A radar graph illustrating the severity of symptoms in post-COVID-19. The severity [%] of each symptom was calculated using Microsoft Excel or SPSS, and a radar chart was created. Green indicates no symptoms, yellow signifies mild symptoms, blue represents moderate severity, and red shows high severity on the radar chart. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

ELISA analysis showed that the mean IL-6 levels were 3.73 ± 0.60 pg/mL in symptomatic individuals, 2.49 ± 0.51 pg/mL in asymptomatic individuals, and 0.64 ± 0.08 pg/mL in controls. The average level of IL-1β was 0.37 ± 0.11 pg/mL in symptomatic individuals, 0.37 ± 0.06 pg/mL in asymptomatic individuals, and 0.14 ± 0.08 pg/mL in controls. For IL-10, the mean level was 0.33 ± 0.12 pg/mL in symptomatic individuals, 0.36 ± 0.13 pg/mL in asymptomatic individuals, and 0.11 ± 0.05 pg/mL in controls. TNFα levels were 0.88 ± 0.21 pg/mL in symptomatic individuals, 0.72 ± 0.14 pg/mL in asymptomatic individuals, and 0.11 ± 0.05 pg/mL in controls (Table **1**).

There was no statistical difference in protein levels between symptomatic and asymptomatic groups. Both post-COVID-19 groups (symptomatic and asymptomatic) exhibited significantly higher concentrations of IL-6, IL-1β, TNFα, and IL-10 compared to controls ($p$ <0.001) (Table **2**).

The RT-qPCR analysis of cytokine mRNA relative expression in symptomatic and asymptomatic post-COVID-19 individuals and controls, measured by SYBR Green, demonstrates that IL-6 is expressed at a level ten times greater in symptomatic individuals compared to asymptomatic ones, and fifteen times greater than in controls. For IL-1β, there is a 9-fold increase in symptomatic individuals relative to asymptomatic individuals and a 13-fold increase relative to controls. TNFα expression is 10 times higher in symptomatic individuals than in asymptomatic individuals and 16 times higher than in controls. Finally, IL-10 levels are six-fold higher in symptomatic

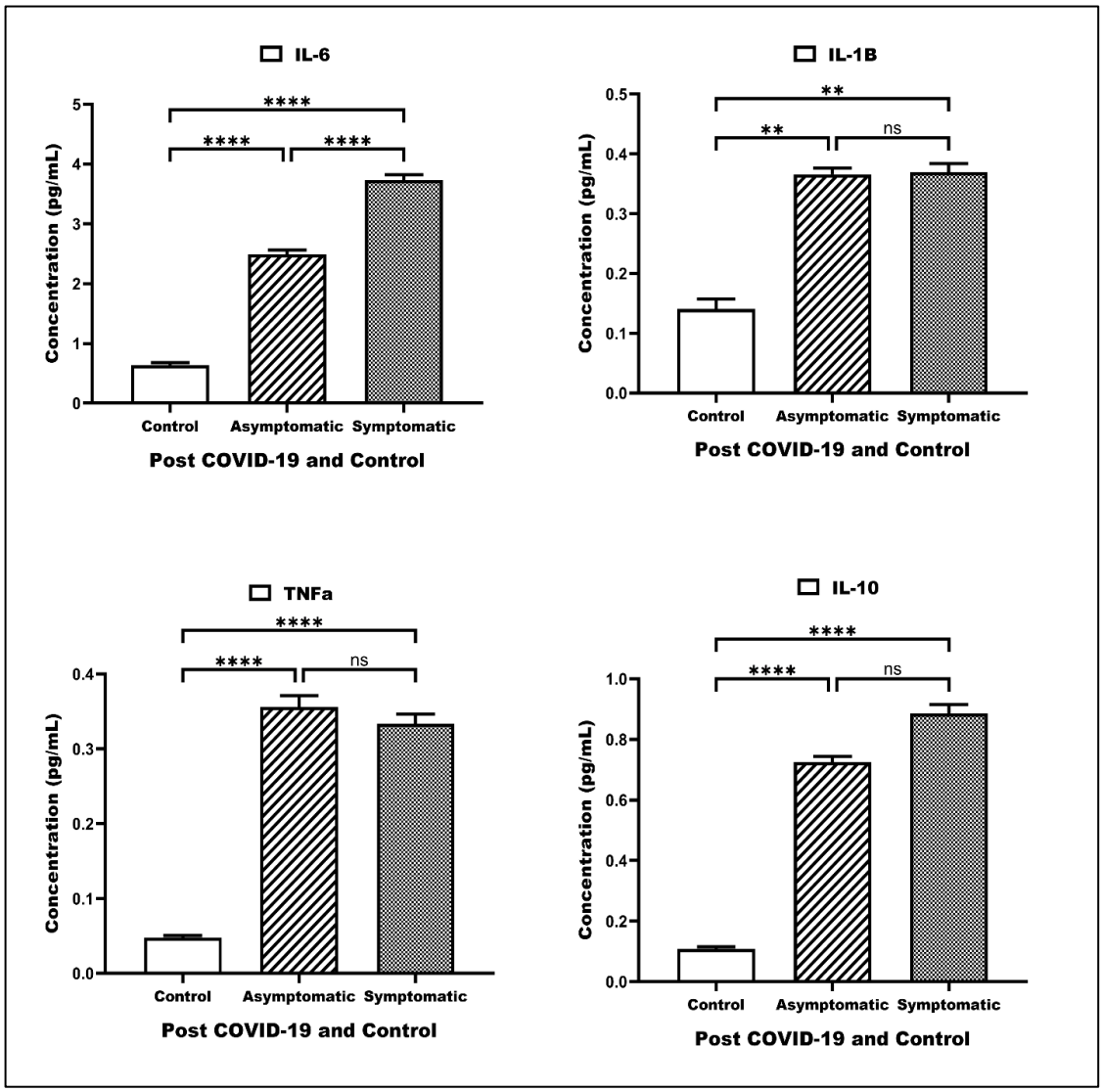


**Fig. (3).** A bar chart depicting the concentrations of IL-6, IL-1β, TNFα, IL-10, and SP (pg/L) in post-COVID-19 and control groups. The asterisk (A*****) indicates a statistically significant difference with $p < 0.001$. The levels of IL-6 and IL-10 were higher than those of IL-1β and TNFα. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

**Table 1. Descriptive STATISTICS OF CYTOKINES (pg/mL) in POST COVID-19 by ELISA.**

| Biomarkers | Study Groups | N | Mean | SD | Minimum | Maximum |
|---|---|---|---|---|---|---|
| IL6 (pg/mL) | Symptomatic | 49 | 3.73 | 0.60 | 2.49 | 4.85 |
| | Asymptomatic | 49 | 2.49 | 0.51 | 1.37 | 2.95 |
| | Control | 49 | 0.64 | 0.08 | 0.22 | 0.93 |
| IL-1β (pg/mL) | Symptomatic | 49 | 0.37 | 0.11 | 0.27 | 0.82 |
| | Asymptomatic | 49 | 0.37 | 0.07 | 0.23 | 0.49 |
| | Control | 49 | 0.14 | 0.08 | 0.05 | 0.46 |
| IL-10 (pg/mL) | Symptomatic | 49 | 0.33 | .009 | 0.10 | 0.46 |
| | Asymptomatic | 49 | 0.36 | 0.11 | 0.20 | 0.52 |
| | Control | 49 | 0.11 | 0.02 | 0.02 | 0.10 |
| TNFα (pg/mL) | Symptomatic | 49 | 0.88 | 0.21 | 0.40 | 1.31 |
| | Asymptomatic | 49 | 0.72 | 0.14 | 0.46 | 0.88 |
| | Control | 49 | 0.11 | 0.05 | 0.04 | 0.20 |

**Table 2. ANOVA Analysis of Cytokines (pg/mL) in Post COVID-19 by ELISA.**

| Biomarkers | | Sum of Squares | df | Mean Square | F | *p* |
|---|---|---|---|---|---|---|
| IL6 (pg/mL) | Between Groups | 237.62 | 2 | 118.81 | 504.79 | <.001* |
| | Within Groups | 33.89 | 144 | 0.23 | - | - |
| | Total | 271.52 | 146 | - | - | - |
| IL-1β (pg/mL) | Between Groups | 1.68 | 2 | 0.84 | 83.12 | <.001* |
| | Within Groups | 1.46 | 144 | .01 | - | - |
| | Total | 3.14 | 146 | - | - | - |
| IL-10 (pg/mL) | Between Groups | 2.89 | 2 | 1.45 | 215.10 | <.001* |
| | Within Groups | .97 | 144 | 0.01 | - | - |
| | Total | 3.87 | 146 | - | - | - |
| TNFα (pg/mL) | Between Groups | 16.50 | 2 | 8.25 | 368.06 | <.001* |
| | Within Groups | 3.23 | 144 | 0.02 | - | - |
| | Total | 19.73 | 146 | - | - | - |

**Table 3. Descriptive Statistics of Cytokines in Post COVID-19 by RT-qPCR.**

| Cytokines | Study Groups | N | Mean | SD | Minimum | Maximum |
|---|---|---|---|---|---|---|
| IL-6 | Symptomatic | 49 | 16.71 | 4.66 | 10.38 | 28.76 |
| | Asymptomatic | 49 | 6.67 | 1.59 | 4.68 | 9.82 |
| | Control | 49 | 1.65 | 0.21 | 1.37 | 2.02 |
| IL-1β | Symptomatic | 49 | 14.76 | 5.32 | 10.79 | 32.41 |
| | Asymptomatic | 49 | 5.24 | 1.69 | 2.66 | 8.77 |
| | Control | 49 | 1.60 | 0.37 | 1.20 | 2.61 |
| TNFα | Symptomatic | 49 | 17.53 | 4.13 | 12.03 | 26.71 |
| | Asymptomatic | 49 | 7.69 | 2.64 | 3.01 | 11.13 |
| | Control | 49 | 1.47 | 0.33 | 0.81 | 1.92 |
| IL-10 | Symptomatic | 49 | 19.12 | 4.57 | 10.63 | 28.00 |
| | Asymptomatic | 49 | 12.58 | 1.18 | 10.85 | 15.23 |
| | Control | 49 | 1.62 | 0.32 | 1.16 | 2.10 |

SD: Standard Deviation.

individuals than in asymptomatic individuals and seventeen-fold higher than in controls (see Table **3**).

The ANOVA analysis of the RT-qPCR data indicated that IL-6, IL-1β, TNFα, and IL-10 mRNA expression levels were significantly elevated in both symptomatic and asymptomatic post-COVID-19 survivors compared with controls ($p < 0.001$; Fig. **4**).

Further, post-hoc testing using the Tukey test showed relative expression differences present between the symptomatic and asymptomatic groups ($p > 0.001$), but both groups also showed statistically significant differences for IL-6, TNFα, IL-1β and IL-10 mRNA expression compared to the healthy control group ($p < 0.001$, Table **4**).

Correlation analyses associated circulating cytokine levels and mRNA expression with post-COVID-19 symptoms, revealing distinct inflammatory patterns, as illustrated in Tables **5** and **6**. IL-6 and TNFα showed strong correlations with multisystem symptoms. IL-6 showed positive associations ($p < 0.05$) with fatigue, cough, dyspnea, muscle pain, weakness, joint pain, headache, anxiety, and depression, with moderate correlation coefficients ($r \approx 0.38–0.74$). Its association with loss of taste and smell, sleep disturbances, hormonal imbalance, wheezing, and diabetes was not statistically significant ($p > 0.05$). TNFα also showed substantial correlations ($r \approx 0.70$; $p < 0.05$) with fatigue, cough, dyspnea, muscle and joint pain, headache, anxiety, depression, chest, and abdominal pain, indicating a role in inflammatory responses. Conversely, its association with wheezing, diabetes, or loss of smell was not significant. IL-1β demonstrated moderate positive correlations ($r \approx 0.65$; $p < 0.05$) with

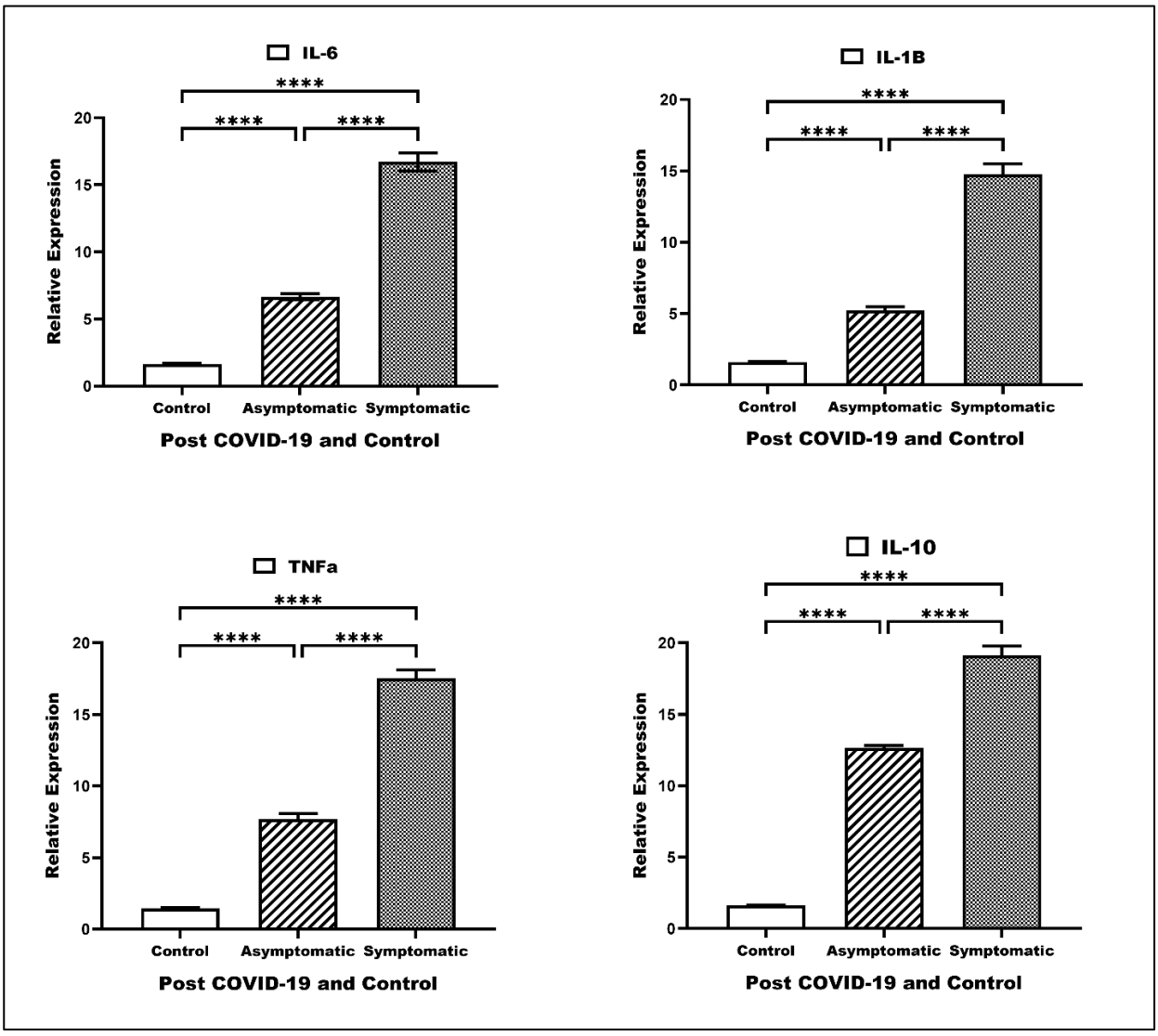


**Fig. (4).** Presents a bar chart depicting the relative expression levels of IL-6, IL-1β, TNFα, IL-10, and TAC1 in both post-COVID-19 and control groups. The symbol A***** indicates a statistically significant difference with p < 0.001. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

**Table 4. ANOVA Analysis of Cytokines in Post COVID-19 by RT-qPCR.**

| Biomarkers | | Sum of Squares | df | Mean Square | F | *p* |
|---|---|---|---|---|---|---|
| IL-6 | Between Groups | 5759.68 | 2 | 2879.84 | 355.97 | <.001* |
| | Within Groups | 1164.98 | 144 | 8.09 | - | - |
| | Total | 6924.67 | 146 | - | - | - |
| IL-1β | Between Groups | 4521.65 | 2 | 2260.82 | 216.95 | <.001* |
| | Within Groups | 1500.63 | 144 | 10.42 | - | - |
| | Total | 6022.28 | 146 | - | - | - |
| TNFα | Between Groups | 6428.82 | 2 | 3214.41 | 400.32 | <.001* |
| | Within Groups | 1156.25 | 144 | 8.03 | - | - |
| | Total | 7585.08 | 146 | - | - | - |
| IL-10 | Between Groups | 7666.91 | 2 | 3833.46 | 513.52 | <.001* |
| | Within Groups | 1074.97 | 144 | 7.47 | - | - |
| | Total | 154.19 | 146 | - | - | - |

**Table 5. Correlation Cytokines (pg/mL) and Post COVID-19 sequelae.**

| S. No | Symptoms | IL-6 (pg/mL) | | IL-1β (pg/mL) | | TNFα (pg/mL) | | IL-10 (pg/mL) | |
|---|---|---|---|---|---|---|---|---|---|
| | | r | *p* | r | *p* | r | *p* | r | *p* |
| 1. | Fatigue | .55 | .31 | <0.01 | .42 | <0.01 | .53 | <0.01 | <0.01 |
| 2. | Fever | .32 | .08 | .342 | .20 | .017 | .22 | .008 | <0.01 |
| 3. | Sweating | .28 | .25 | .003 | .21 | .012 | .31 | <0.01 | .001 |
| 4. | Tachypnea | .53 | .21 | .010 | .22 | .007 | .44 | <0.01 | <0.01 |
| 5. | Palpitation | .32 | .42 | <0.01 | .20 | .013 | .16 | .048 | <0.01 |
| 6. | Chest Pain | .33 | .39 | <0.01 | .32 | <0.01 | .30 | <0.01 | <0.01 |
| 7. | Psychosocial distress | .38 | .09 | .257 | .16 | .050 | .34 | <0.01 | <0.01 |
| 8. | Anxiety | .37 | .29 | <0.01 | .10 | .209 | .25 | .002 | <0.01 |
| 9. | Depression | .28 | .35 | <0.01 | .08 | .337 | .31 | <0.01 | <0.01 |
| 10. | Discomfort | .30 | .37 | <0.01 | .11 | .192 | .17 | .045 | <0.01 |
| 11. | Concentration Impairment | .38 | .03 | .741 | .19 | .023 | .24 | .004 | <0.01 |
| 12. | Headache | .57 | .52 | <0.01 | .48 | <0.01 | .53 | <0.01 | <0.01 |
| 13. | Weakness | .73 | .60 | <0.01 | .51 | <0.01 | .66 | <0.01 | <0.01 |
| 14. | Muscular pain | .73 | .58 | <0.01 | .48 | <0.01 | .66 | <0.01 | <0.01 |
| 15. | Joint pain | .66 | .30 | <0.01 | .39 | <0.01 | .57 | <0.01 | <0.01 |
| 16. | Cough | .56 | .27 | .001 | .42 | <0.01 | .56 | <0.01 | <0.01 |
| 17. | Dyspnea | .54 | .20 | .017 | .21 | .010 | .41 | <0.01 | <0.01 |
| 18. | Shortness of Breath | .47 | .22 | .007 | .18 | .030 | .42 | <0.01 | <0.01 |
| 19. | Wheezing | .12 | .05 | .546 | .07 | .382 | .24 | .003 | .155 |
| 20. | GGOs CXR | .28 | .13 | .109 | .23 | .005 | .29 | <0.01 | .001 |
| 21. | Abdominal pain | .44 | .22 | .007 | .34 | <0.01 | .33 | <0.01 | <0.01 |
| 22. | Diarrhea | .23 | .34 | <0.01 | .28 | <0.01 | .20 | .015 | .005 |
| 23. | Eating Disorder | .37 | .19 | .020 | .33 | <0.01 | .36 | <0.01 | <0.01 |
| 24. | Runny nose | .17 | .26 | .002 | .13 | .125 | .24 | .003 | .036 |
| 25. | Loss of taste | .25 | -.02 | .831 | .15 | .078 | .15 | .079 | .002 |
| 26. | Sore throat | .26 | .30 | <0.01 | .17 | .043 | .23 | .006 | .001 |
| 27. | Loss of Smell | .14 | .06 | .504 | .16 | .052 | .08 | .345 | .093 |
| 28. | Sleep disorders | .14 | .40 | <0.01 | .11 | .179 | .12 | .146 | .095 |
| 29. | Dizziness | .17 | .37 | <0.01 | .01 | .951 | .11 | .172 | .039 |
| 30. | Hair loss | .34 | .18 | .027 | .31 | <0.01 | .36 | <0.01 | <0.01 |
| 31. | Diabetes | .09 | -.08 | .318 | .116 | .162 | .06 | .458 | .302 |
| 32. | Hormonal Imbalance | .17 | .073 | .38 | .170 | .040 | .14 | .09 | .044 |

**Table 6. Relative expression of Genes and Post COVID-19 sequelae.**

| S. No | Symptoms | IL-6 | | IL-1β | | TNFα | | IL10 | |
|---|---|---|---|---|---|---|---|---|---|
| | | r | *p* | r | *p* | r | *p* | r | *p* |
| 1. | Fatigue | .47 | <0.01 | .39 | <0.01 | .44 | <0.01 | .52 | <0.01 |
| 2. | Fever | .35 | <0.01 | .31 | <0.01 | .30 | <0.01 | .24 | .004 |
| 3. | Sweating | .33 | <0.01 | .37 | <0.01 | .36 | <0.01 | .29 | <0.01 |
| 4. | Tachypnea | .52 | <0.01 | .55 | <0.01 | .55 | <0.01 | .44 | <0.01 |

**(Table 6) Contd….**

| S. No | Symptoms | IL-6 | | IL-1β | | TNFα | | IL10 | |
|---|---|---|---|---|---|---|---|---|---|
| | | r | *p* | r | *p* | r | *p* | r | *p* |
| 5. | Palpitation | .36 | <0.01 | .31 | <0.01 | .37 | <0.01 | .36 | <0.01 |
| 6. | Chest Pain | .31 | <0.01 | .25 | .002 | .35 | <0.01 | .32 | <0.01 |
| 7. | Psychosocial distress | .46 | <0.01 | .40 | <0.01 | .40 | <0.01 | .32 | <0.01 |
| 8. | Anxiety | .50 | <0.01 | .43 | <0.01 | .46 | <0.01 | .44 | <0.01 |
| 9. | Depression | .42 | <0.01 | .42 | <0.01 | .38 | <0.01 | .44 | <0.01 |
| 10. | Discomfort | .38 | <0.01 | .35 | <0.01 | .34 | <0.01 | .34 | <0.01 |
| 11. | Concentration Impairment | .27 | .001 | .30 | <0.01 | .26 | .001 | .27 | .001 |
| 12. | Headache | .53 | <0.01 | .45 | <0.01 | .50 | <0.01 | .55 | <0.01 |
| 13. | Weakness | .73 | <0.01 | .65 | <0.01 | .69 | <0.01 | .77 | <0.01 |
| 14. | Muscular pain | .74 | <0.01 | .65 | <0.01 | .70 | <0.01 | .77 | <0.01 |
| 15. | Joint pain | .66 | <0.01 | .62 | <0.01 | .63 | <0.01 | .64 | <0.01 |
| 16. | Cough | .60 | <0.01 | .57 | <0.01 | .63 | <0.01 | .60 | <0.01 |
| 17. | Dyspnea | .57 | <0.01 | .55 | <0.01 | .59 | <0.01 | .47 | <0.01 |
| 18. | Shortness of Breath | .58 | <0.01 | .56 | <0.01 | .59 | <0.01 | .52 | <0.01 |
| 19. | Wheezing | .08 | .329 | .07 | .367 | .09 | .291 | .16 | .057 |
| 20. | GGOs CXR | .40 | <0.01 | .35 | <0.01 | .32 | <0.01 | .29 | <0.01 |
| 21. | Abdominal pain | .33 | <0.01 | .25 | .002 | .35 | <0.01 | .26 | .001 |
| 22. | Diarrhea | .02 | .780 | .05 | .572 | .04 | .623 | .21 | .011 |
| 23. | Eating Disorder | .25 | .002 | .27 | .001 | .25 | .002 | .28 | .001 |
| 24. | Runny nose | .27 | .001 | .13 | .107 | .27 | .001 | .29 | <0.01 |
| 25. | Loss of taste | .16 | .058 | .15 | .071 | .17 | .039 | .18 | .030 |
| 26. | Sore throat | .38 | <0.01 | .44 | <0.01 | .34 | <0.01 | .35 | <0.01 |
| 27. | Loss of Smell | .23 | .006 | .16 | .048 | .18 | .028 | .13 | .106 |
| 28. | Sleep disorders | .22 | .008 | .16 | .049 | .19 | .022 | .20 | .013 |
| 29. | Dizziness | .21 | .010 | .22 | .008 | .19 | .024 | .29 | <0.01 |
| 30. | Hair loss | .19 | .024 | .18 | .033 | .17 | .045 | .23 | .005 |
| 31. | Diabetes | -.06 | .49 | -.012 | .889 | -.032 | .69 | .06 | .49 |
| 32. | Hormonal Imbalance | .16 | .055 | .16 | .056 | .129 | .12 | .15 | .08 |

headache, muscle and joint pain, cough, dyspnea, abdominal complaints, diarrhea, and psychosocial distress, but was not significantly correlated with fatigue, fever, nasal symptoms, hormonal imbalance, or diabetes. The IL-10 correlations were weaker but positive, suggesting a compensatory anti-inflammatory response, as depicted in Fig. (**5**).

The relative gene expression analysis showed the strongest correlation (r ~ 0.20–0.55) with key post-COVID-19 symptoms, including fatigue, cough, dyspnea, and pain as shown in Fig. (**6**). Psychological symptoms like anxiety and depression also showed moderate correlations with IL-6, TNFα, and IL-1β. Symptoms such as anosmia, ageusia, wheezing, and diabetes did not demonstrate significant correlations, indicating limited cytokine influence.

## 4. DISCUSSION

Cytokines are small secreted proteins (<40 kDa) that are key mediators of inflammation and immune responses. Their dysregulation or abrupt production can lead to clinical symptoms and systemic complications [51]. Our study investigated symptomatic and asymptomatic post-COVID-19 survivors and compared them with control individuals. Post-COVID-19 symptom patterns varied between genders, with males reporting higher rates of physical discomfort, muscular pain, and joint pain. Conversely, females in the control group showed higher rates of chest pain, psychosocial distress, and anxiety.

In this study, symptomatic individuals had a higher average age (48±17 years) than the asymptomatic

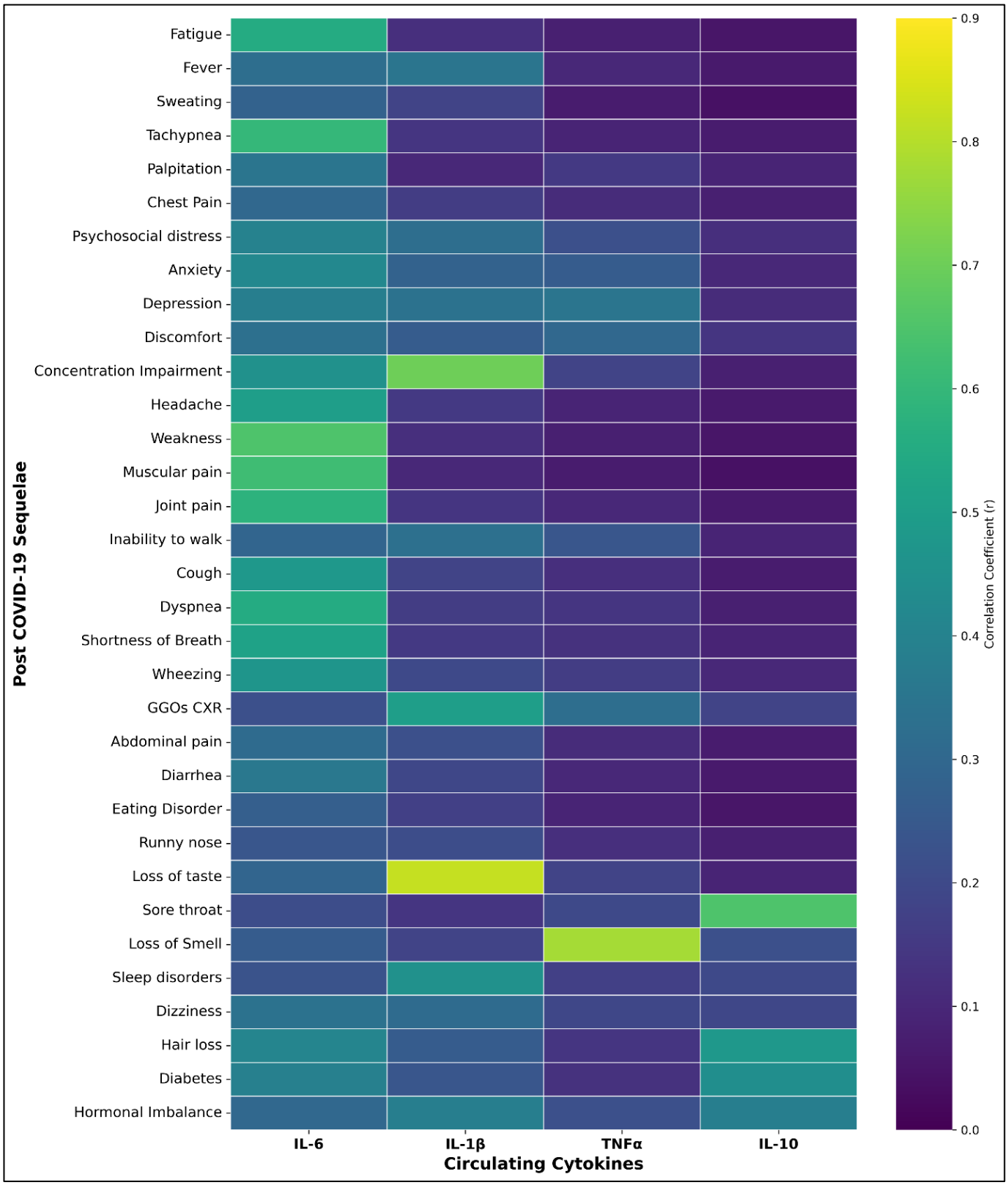


**Fig. (5).** The heatmap color gradient graph displays the strength of correlations between circulating cytokines (pg/mL) and post-COVID-19 sequelae. Darker purple shades indicate weak or no correlation (r close to 0.0), while colors transitioning through blue and teal represent mild to moderate positive correlations (r ≈ 0.2–0.4). Stronger associations are shown by green shades (r ≈ 0.5–0.7), and bright yellow regions reflect the highest correlation values (r ≥ 0.8), indicating very strong positive relationships. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

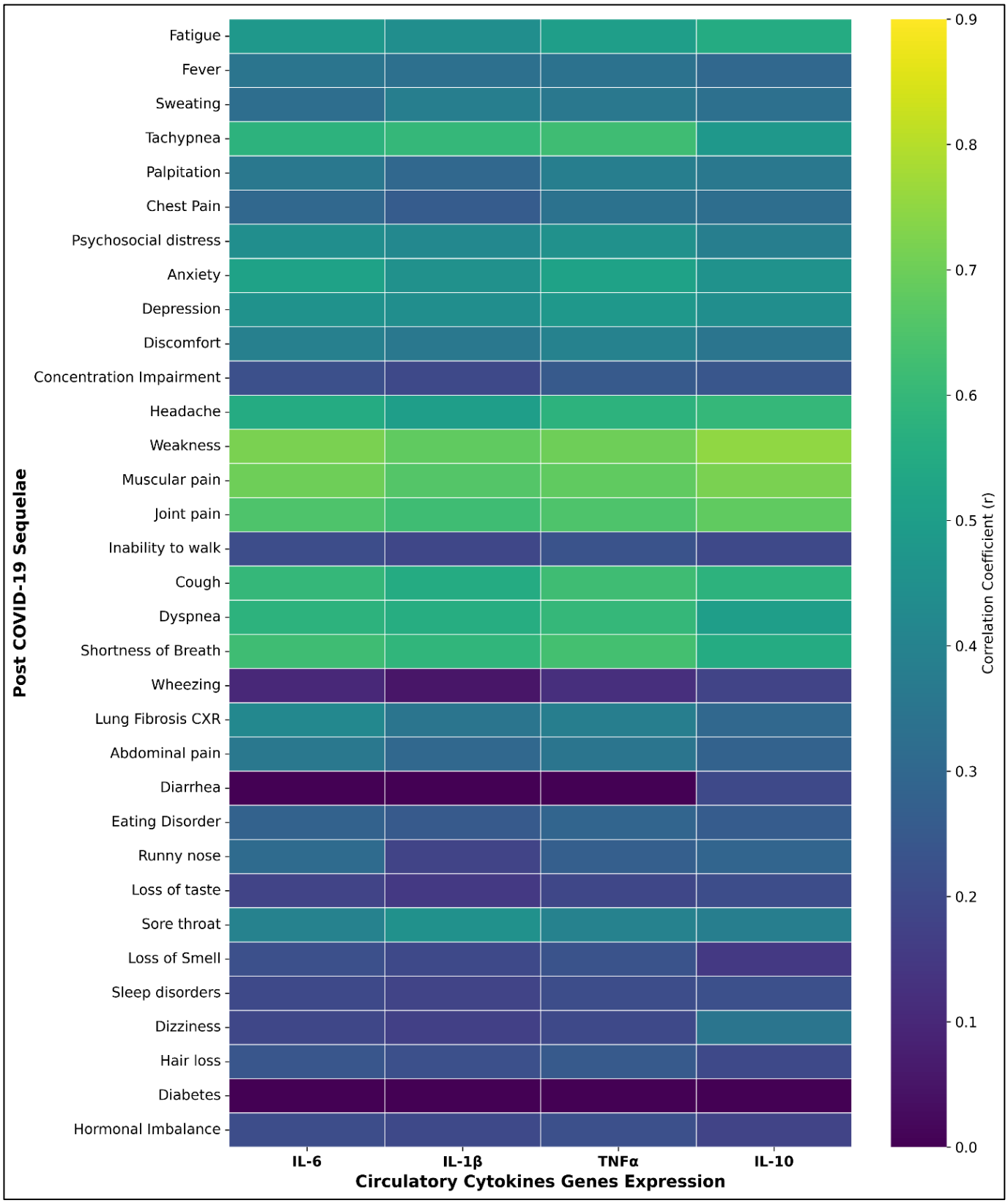


**Fig. (6).** The heatmap color scale indicates the strength of correlation between gene expression and post-COVID-19 sequelae. Dark purple shows very weak or no correlation (r ≈ 0.0–0.1), while blue to teal represents mild to moderate correlations (r ~0.2–0.4). Green indicates stronger correlations (r ≈ 0.5–0.6), and yellow signifies the highest positive correlations (r ≥ 0.7). Therefore, warmer colors (green to yellow) correspond to stronger cytokine–symptom relationships, whereas cooler colors (purple to teal) suggest minimal or weak associations. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

(37±13 years) and control (33±11 years) groups. Additionally, our data indicated that older patients (≥40 years) showed more symptoms than younger individuals (<40 years). Post-COVID-19 symptom patterns showed that older survivors experienced higher rates of symptoms such as fatigue, chest pain, palpitations, and dyspnea compared to younger survivors. Psychological symptoms like psychosocial distress were more common among younger individuals. The present study revealed that older patients (≥40 years) experienced more symptoms and this aligns with previous studies on COVID-19 severity and long-term outcomes [52]. Mansell *et al.* (2021) similarly found that age was associated with a higher risk of developing long-term COVID symptoms and an increase in symptom burden due to immune senescence and a higher baseline inflammatory state in older individuals [53]. Another study reported that older patients with COVID-19 had a higher risk of hospitalization and chronic conditions, including cardiovascular, neuromuscular, and neurodegenerative conditions [54]. The persistence of symptoms in certain individuals indicated that post-COVID-19 might be a manifestation of a chronic inflammatory syndrome and the immune system may fail to fully reset after the initial phase of the illness. As a result, comprehensive long-term monitoring and management of post-COVID-19 in older patients increases the health burden. In this study, the average duration of post-COVID-19 was longer in symptomatic individuals than in asymptomatic individuals. Those with a shorter duration of post-COVID-19 experienced more symptoms, such as increased fatigue, chest pain, and muscular pain, compared to those with a longer duration (≥30 months). These findings are consistent with those reported by Groff *et al* (2021) and de Oliveira *et al* (2022) [55, 56].

Overall, in our study, symptomatic individuals showed higher rates of fatigue, headache, anxiety, muscle pain, and joint pain compared to asymptomatic and control individuals. Other studies also reported that fatigue, joint or muscular pain, dyspnea, and depression persisted after COVID-19 infection [57, 58]. Another study also reported that fatigue, myalgia, and arthralgia were common in COVID-19 survivors [59]. Others reported that neuropsychological, respiratory, cardiac, and musculoskeletal complications were more common after COVID-19 [60, 61]. In the present study, the ELISA method was used to estimate cytokines (IL-6, IL-1β, IL-10, and TNFα) concentration in symptomatic and asymptomatic post-COVID-19 individuals and in controls. IL-6, a key mediator of inflammation and a marker of severe COVID-19, was elevated in both symptomatic and asymptomatic individuals. The role of IL-6 in driving the cytokine storm during acute COVID-19 is well documented, and its elevated levels in post-COVID-19 patients may reflect ongoing low-grade inflammation [62, 63]. Therefore, in the present study, high IL-6 levels were observed in asymptomatic individuals. IL-1β, another key pro-inflammatory cytokine, also showed elevated levels in both symptomatic and asymptomatic individuals compared to controls. IL-1β plays a central role in activating the inflammatory pathway, which has been linked to severe COVID-19 [64]. Persistent elevation of IL-1β after COVID-19 may contribute to the long-term systemic inflammation seen in these patients, further supporting our hypothesis of ongoing immune dysregulation. TNFα, a hallmark of acute inflammatory responses, was elevated in both symptomatic and asymptomatic groups compared to controls. Elevated TNFα levels may explain the musculoskeletal and neuropsychological symptoms observed in post-COVID-19 cases [65, 66]. More detailed studies are necessary to block TNFα expression in post-COVID-19 patients to reduce systemic inflammation. IL-10, an anti-inflammatory cytokine, was elevated in both symptomatic and asymptomatic individuals compared to controls. The rise in IL-10 may indicate a compensatory response that counteracts ongoing inflammation. However, IL-10's inability to fully resolve inflammation suggests an imbalance between pro-inflammatory and anti-inflammatory signals in post-COVID-19 patients [67]. This cytokine imbalance might contribute to the chronic symptoms and immune dysregulation seen in these individuals.

The present study showed significantly higher protein levels of IL-6, IL-1β, and TNFα in post-COVID-19 individuals, especially in symptomatic cases, indicating prolonged immune response activation. This aligns with the findings of Phetsouphanh *et al*. (2022), who reported that dysregulation of pro-inflammatory cytokines remained in long COVID-19 patients up to 8 months post-infection [68]. The present study also showed a statistically significant difference between the symptomatic and asymptomatic post-COVID-19 groups and the control group. The present study generally supports the idea that individuals may develop “post-viral syndrome, ” in which the immune system remains hyperactive for a long time after viral clearance, leading to chronic symptoms [69-71].

RT-qPCR enables quick and accurate measurement of gene expression [72, 73]. The present study used the relative expression method for RT-qPCR data to examine mRNA levels of cytokines in symptomatic, asymptomatic post-COVID-19 individuals and controls [74, 75]. In the current study, RT-qPCR showed that relative IL-1β mRNA expression was similar between symptomatic and asymptomatic post-COVID-19 survivors and controls. The mRNA levels of IL-6 and TNFα were higher in both symptomatic and asymptomatic individuals compared to controls ($p < 0.05$). Interestingly, IL-10 mRNA levels were also higher in both groups than in controls, which aligns with the ELISA results of the current study. Although IL-10 is normally considered an anti-inflammatory cytokine, its continued expression in post-COVID-19 individuals was insufficient to reduce persistent inflammation [76, 77]. Thus, the ongoing transcriptional activity of IL-10, despite high levels of pro-inflammatory cytokines, might suggest a dysregulated immune feedback loop in which both inflammatory and anti-inflammatory responses are activated but insufficient to restore normal systemic functions. This suggests that even patients without

obvious post-COVID-19 symptoms may face risks of long-term health issues. Routine monitoring of cytokine profiles in post-COVID-19 patients could help identify those at risk of chronic inflammatory conditions, enabling early interventions such as anti-inflammatory therapies or immunomodulatory treatments. Moreover, the absence of significant differences between symptomatic and asymptomatic groups indicates that symptomology alone might not reliably reflect underlying immune dysregulation. Also, to develop a more comprehensive understanding of circulatory biomarkers and their implications for post-COVID-19 conditions, much elaborated studies are needed at the organ or tissue level. Receptor expression of biomarkers can also provide a more complete picture of the underlying signaling mechanisms. There are several limitations to this study. First, the study duration was short, and patient information was limited to outpatient services and discharge records. Second, the small sample size may limit the generalizability of the findings. Third, the analysis was performed using blood samples, which may not fully reflect biological processes at the histological level. Further cohort studies incorporating combined circulatory and tissue-based analyses are needed to better clarify the roles of circulating cytokines and their implications for post-COVID-19 sequelae.

## CONCLUSION

In conclusion, severe symptoms and systemic complications following COVID-19 are found to be linked with female gender, older age (≥ 40 years), and shorter duration (< 30 months). Whereas IL-6, IL-1β, TNFα, and IL-10 were expressed in both symptomatic and asymptomatic post-COVID-19 survivors, indicating immune dysregulation long after the acute phase of infection and showing the most widespread correlations with post-COVID-19 symptoms at both protein and gene expression levels, identifying them as primary inflammatory drivers. Thus, IL-6, IL-1β, TNFα, and IL-10 may serve as potential biomarkers for post-COVID-19 sequelae. The present study was conducted over a relatively short period. Therefore, further research is needed to develop a more comprehensive understanding of circulatory biomarkers and their implications for post-COVID-19 conditions.

## AUTHORS' CONTRIBUTIONS

It is hereby acknowledged that all authors have accepted responsibility for the manuscript's content and consented to its submission. They have meticulously reviewed all results and unanimously approved the final version of the manuscript. MA, AN and LR conceived the core ideas and designed the outlines of the manuscript. XZ contributed to the project administration and supervision. JQ, AA, MU, AO, AS, XZ and TM carried out the critical analysis of literature. All authors made significant contributions to writing and revising the manuscript. All authors read and approved the submitted version of the manuscript.

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| ACE2 | = | Angiotensin converting enzyme 2 |
| cDNA | = | Complementary Deoxyribonucleic Acid |
| CT | = | Cycle threshold |
| DAMP | = | Damaged-associated molecular patterns |
| ELISA | = | Enzyme-linked immunosorbent assay |
| IL-10 | = | Interleukin 10 |
| IL-1β | = | Interleukin 1 beta |
| IL-6 | = | Interleukin 6 |
| mRNA | = | Messenger Ribonucleic Acid |
| NF-κB | = | Nuclear factor kappa B nuclear translocation |
| p38-MAPK | = | p38 mitogen-activated protein kinase |
| PAMP | = | Pathogen-associated molecular patterns |
| RT-qPCR | = | Real-time reverse transcription quantitative polymerase chain reaction |
| TNFα | = | Tumor necrosis factor alpha |

## ETHICS APPROVAL AND INFORMED CONSENT

All experiments involving clinical samples were conducted in accordance with the protocol approved by the Research Ethics Committee of The University of Lahore, Pakistan (Ethics Code: REC-UOL-90-I-04-2023).

## HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All procedures performed in studies involving human participants were in accordance with the ethical standards of institutional and/or research committee and with the 1975 declaration of Helsinki, as revised in 2013.

## CONSENT FOR PUBLICATION

Informed consent was obtained from all individual participants included in the study.

## STANDARD OF REPORTING

STROBE guidelines were followed.

## AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

## FUNDING

None.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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## SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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