# Impact of Folic acid status or its metabolites and hypoxia on COVID-19

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## Abstract

Background: Folic acid is a hydro-soluble vitamin that classifies as part of the vitamin B complex. The coenzymes of folic acid are actively involved in the one- carbon metabolism. It was established that folate metabolism is involved in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).The study aimed to investigate that decreased folate levels with hypoxia may involve in patients with COVID-19
Methods: The study was performed between September 2021 to January 2022. A 75 subjects were enrolled in this study with COVID-19 (moderate =35, severe=45) with mean age (56 ± 1.6; 37 males, 38 female), and 45 subjects appeared to be normal healthy persons as the control group with mean age (41 ± 1.5; 17 males, 28 female) Folic acid levels was measured by colorimetric method. Serum 5-MTHF, HIF-1α, and TNF-α were measured by ELISA.
Results: : The results clearly showed low folic acid and its derivative 5-MTHF levels in patient groups with COVID-19 compared to control (P<0.01); a great effect was shown in the severe group. The finding also declared that serum level of TNF-α and HIF-1α were significantly increased in patients with COVID-19 infection compared to control groups (p <0.01).
Conclusions: The main findings may go some way towards establishing a link between folic acid status, a hypoxia and COVID-19 prevalence, with a possible association to the disease severity. Therefore, folic acid supplementation may protect against COVID-19

Keywords:Folic acid, Hypoxia, inflammation, COVID-19

## 1. Introduction

Coronavirus infection 2019 (COVID-19), a highly infectious disease caused by the coronavirus 2 (SARS-CoV-2) that causes severe acute respiratory syndrome, has spread over the world as a dangerous pandemic [1]. SARS-CoV-2 was initially identified in late December 2019 in Wuhan, Hubei Province, China, and quickly spread around the world, prompting the World Health Organization (WHO) to declare it a worldwide pandemic on March 11, 2020 [2]. SARS-CoV-2 viruses are encapsulated, non-segmented viruses with single-stranded RNA (ssRNA) ranging in length from 26 to 32 kb. The coronavirus genome is the longest among RNA viruses at this length. Negative-stained SARS-CoV-2 particles displayed a spherical morphology with a size varying from 60–140 nm and an external surface covered with unique 9–12 nm–long spikes that gave virions the appearance of a solar corona under electron microscopy (EM) [3].

SARS symptoms include flu-like symptoms that usually appear two to seven days after infection [4]. Low blood-oxygen levels have been a significant sign in COVID-19, which can be lethal in the most severe cases [5].

Folic acid is a water-soluble vitamin that belongs to the b - complex vitamins [6]. Folic acid is a synthetic version of folate that occurs in nature quite infrequently. It must first be converted into dihydrofolate (DHF) and then into its active form tetrahydrofolate (THF) before it can be used by the body after absorption in the colon [6]. Vitamin b12 (or folate), that plays an important part in one-carbon metabolism, is required for the production of a number of chemicals [7]. It assists in the creation of DNA and RNA, the genetic sequence of the organism, and is particularly vital during fast cell and tissue growth, such as during infancy, puberty, as well as pregnancy.

Recent study has connected low folate levels and its metabolites to COVID-19 infection. COVID-19 alters the host's folate and one-carbon metabolism at the post-transcriptional level to favor de novo purine synthesis, circumventing viral inhibition of host translation. In SARS-CoV-2-infected cells, intracellular glucose and folate are reduced, and viral replication is extraordinarily sensitive to inhibitors of folate and metabolism of one-carbon [8].

5-Methyltetrahydrofolate (5-MTHF) is the active form of folic acid in circulation. It is normally transported to peripheral tissue to be served as a coenzyme in cellular metabolism and functions. 5, 10- Methyltetrahydrofolate (5,10 MTHF) is intermediate in 1C metabolism, was converted to 5-MTHF, 5-formyltetrahydrofolate, and methenyltetrahydrofolate. It is predominantly produced via the interaction of tetrahydrofolate and serine, which is catalyzed by serine hydroxymethyltransferase. The enzyme serine hydroxy-methyl transferase uses it as a cofactor in the synthesis of serine from glycine. 5-MTHF serves as an enzyme precursor for Methylenetetrahydrofolate reductase [9]. 5,10-MTHF may also be employed as a cofactor in the thymidine biosynthesis.

Tumor necrosis factor-alpha is a member of the (TNF-α) is a potent pro-inflammatory cytokine and immune-modulator produced mainly by macrophages, monocytes, T lymphocytes, and natural killer cells, in addition, it is the first cytokine to be created in minutes of every damage or

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stress caused by pro-inflammatory stimulation [10].

Higher expressions of TNF-α cytokines levels in the blood appeared to indicate the intensity as well as outcome of covid-19 infected individuals [11]. Covid-19 viral burden was shown to be positively linked with these cytokines [12]. The onset of many clinical symptoms such as flu-like symptoms, fever, weariness, and lethargy is strongly linked to the production of this cytokine, but it may also contribute to pulmonary damage, vascular leakage, cardiac failure, as well as the creation of acute-phase protein [13]. TNF-α is cytokines that thought to be detrimental and are implicated in the development of cytokine storms following Covid-19 invasion [14]. TNF-α inhibitors and other key signaling system inhibitors were also mentioned as potential therapy options for severe covid-19 infections [15].

Hypoxia inducible factor-1alpha (HIF-1α), is the transcription controller of metabolic adaptability to variations in the oxygen atmosphere, It has an 826-amino acid polypeptide chain includes an N-terminal transactivation domain (N-TAD) as well as a C-terminal transactivation domain (C-TAD).it is not only involves in several normal physiological process in the body systems, but also is strongly linked to the pathophysiology of many disorders [16] A variety of post-translational changes, including hydroxylation, acetylation, and phosphorylation, influence HIF-1α action. HIF-1 is a crucial controller of physiological activities such as metabolism, cell growth, and angiogenesis. HIF-1α also is a key promoter of glycolysis as well as the inflammatory reaction, suggesting that HIF-1α may have a role in the pathophysiology of Covid-19.

HIF-1α regulates immunological and inflammatory reactions in the course of a viral illness [17]. The pathophysiology of SARS-CoV-2 is initiated by specific identification of ACE2 on the membrane of ACE2 positive cells, such as alveolar type II cells (AT2) as well as capillary endothelial. As a result, those cells are invaded by the viral, which causes inflammation plus hypoxia, which activates HIF-1α expression [18].

In serious forms of Covid-19, HIF-1 stimulation can cause a cytokine storm by activating and stabilizing immune cells such as macrophages as well as neutrophils, starting to cause such cells to produce huge quantities of proinflammatory cytokines, vascular leakage (via VEGF upregulation), as well as damage of the alveolar-interstitial-endothelial epithelial complex barriers [18].

Moreover, the potential influence of HIF-1α on Covid-19 development and ARDS manifestations in patients might be described by its critical function in these other immune system components, such as the complement cascade, where the C3a plus C5a fractions play key roles in disease pathology [18]. Therefore, inhibiting the activity of this transcription factor or disrupting its linked signaling pathway may reduce COVID-19 manifestations and patient death [19].

The study aimed to address the folic acid status and hypoxia in patients with COVID-19 infection.

## 2. Materials, methods and Subjects

### Subjects

Seventy five (moderate =35, severe=45) with mean age (56 ± 1.6; 37 males, 38 female) clinical evidence of COVID-19 was detected depending on the physicians' diagnosis. Subjects were enrolled in this study between September 2021 and January 2022 at the Al-Amal specialized hospital. Forty five appearing normal healthy subjects with mean age (41 ± 1.5; 17 males, 28 female) who visiting the hospital for a routine check-up without any history of COVID-19, with no other endocrine problem or metabolic renal diseases, acute illness or infection were selected as control subjects. All of the cases were classified as moderate and severe based on clinical symptoms, laboratory testing, and chest computed tomography (CT) scans,

All laboratory tests analysis was performed in the clinical biochemistry Research Lab, College of Medicine, University of Al- Qadisiyah. Body mass index (BMI) was calculated as body weight (kg) divided by squared height (in meters). General data: age, gender, history of other diseases was recorded. All subjects signed a written informed consent and all study methods were approved by the Ethical Committee of College of medicine, university of al-Qadisiyah and the Ministry of Health.

### Methods

A blood sample (5 mL), was collected from all study groups and was allowed to clot for 30 min, then serum was separated by centrifugation at (4000 rpm) for 15- 20 min at room temperature 37°C. The separated serum was preserved using Eppendorf tubes at - 20 ˚C for biochemical analysis. Folic acid was measured by colorimetric method. 5-MTHF, HIF-1α, TNF-α and D. dimer analysis were quantified using ELISA following the manufacturer's recommendations (Bioassay, China).

## 3. Statistical Analysis

Data are expressed as means ± standard error of the mean (SEM). Statistical analysis was carried out by statistical package for social sciences (SPSS) version 23. Qualitative (categorical) variables were expressed as numbers and percentages and Chi-square test was used to show the difference between groups The Andersen-Darling test was used to check normality. The significant difference between control and experimental subjects were determined by Student's t-test, while significant differences between groups were determined using one-way ANOVA followed by post hoc analysis using Tukey's test. A P value of ≤ 0.05 was considered significant throughout.

## 4. Results

120 subjects were included, 75 patients with COVID-19, 40 patients with severe Covid-19 infection (Female= 21, Male = 20) with mean age (55.5±2.1), and 35 patients with moderate covid-19 infection, (Female=17, Male =17), with mean age (56.24±2.6). 45 healthy subjects were included in the study as a healthy control group (Female=28, Male=17), whose average age was (41.8±1.57) years. All clinical and hemodynamic variables are summarized in (table 1).

The clinical and biochemical variables of patients with

moderate and severe were compared with control as shown in (Table 2).

There have been no significant variations were observed in BMI, Diastolic & Systolic blood pressure, (P >0.05) in patient groups compared to control. The levels of D. dimer and CRP (C - reactive protein) were significantly increased in the serum of patient groups compared to the control (Table 2).

**Table 1: Comparison of the clinical and hemodynamic variables study groups.**

| Variables | Groups | | | P-value |
|---|---|---|---|---|
| | COVID-19 | | Control | |
| | moderate | sever | | |
| Total Number | 35 | 40 | 45 | |
| Gender | | | | |
| Females, n (%) Males, n (%) | 16 (46%) 19 (54%) | 22 (55%) 18 (45%) | 28 (62%) 17 (38%) | P>0.05 |
| Age(years) | | | | |
| mean ± SEM | 56.24±2.6 | 55.5±2.1 | 41.8±1.57 | P>0.05 |
| BMI (Kg) | | | | |
| mean ± SEM | 31.1±0.79 | 31.4±0.11 | 29.6±0.57 | P > 0.05 |
| Family history with Other diseases | | | | |
| Cardiovascular diseases Diabetes Hypertension | 0(0% 6 (17%) 19 (54%) | 2(5%) 10 (25%) 21 (53%) | 0(0%) 3(6.6%) 2(4.4%) | P<0.05 |
| BMI: Body Mass Index | | | | |

**Table 2: Comparison of the biochemical parameters in study groups.**

| Parameters (Mean ± SEM) | Groups | | Control | P-value |
|---|---|---|---|---|
| | COVID-19 | | | |
| | moderate | Sever | | |
| Diastolic blood pressure (mmHg) | 84.6±2.4* | 84.15 ±2.3* | 77.4±0.571 | P < 0.05 |
| Systolic blood pressure (mmHg) | 151.5±2.8* | 146.6.±3.0* | 119±0.431 | P < 0.05 |
| CRP(mg/l) | 100.5±2.2** | 96.55±2.6** | 3.7±0.13 | P < 0.01 |
| Oxygen saturation% | 92.0±0.45 | 87.3±3.1* | 96.2±0.11 | P < 0.05 |
| D. dimer (ng/ml) | 889.9±15.6** | 1459±325** | 124.6±13.2 | P<0.001 |
| CRP: C - reactive protein, * significant between patents group and control P<0.05 ** significant between patents groups and control P<0.01 | | | | |

## Folic acid and 5-MTHF levels in COVID-19

Recently, it was suggested that an association between folate deficiency and COVID-19 infection. The results were showed low folic acid levels in patient groups with COVID-19 in comparison to the control (P < 0.01, figure 1). A Significant decreases were found in the patient groups (severe and moderate) compared to control, a high decreased levels were indicated in the group with severe COVID-19 infection (P < 0.01). There were significant differences between the patient groups (figure 2).

The results were demonstrated that 5- MTHF levels were decreased significantly in the patient with COVID-19 infection compared to control (P < 0.01, figure 3). There has been a marked decline in moderate and severe patients groups compared to the control, moderate group declared a significant decrease (P < 0.01, figure 4).

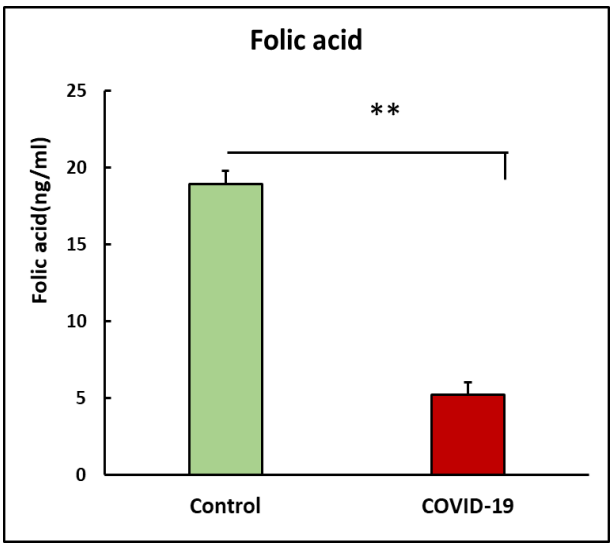


*Figure (1): Serum folic acid levels in patients with covid-19 infection and control. Data are expressed as means ± SEM, **indicates a significant change between patients and control (P<0.01).*

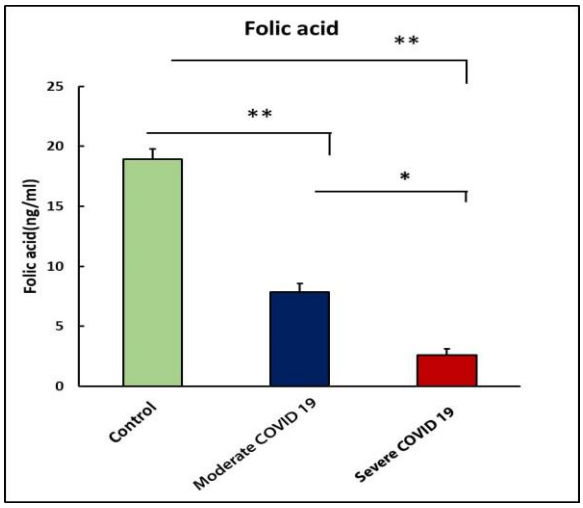


*Figure (2): Serum folic acid levels in patients with moderate COVID-19, severe-COVID, and control. Data are expressed as mean ± SEM. **indicates a significant change between patients groups and control (P < 0.01),* significant changes between moderate and severe groups (P < 0.05)*

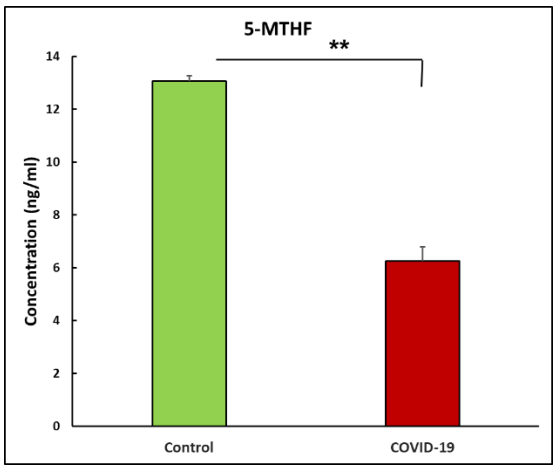


*Figure (3): Serum 5-MTHF level in patients with covid-19 and control. Data are expressed as means ± SEM, **indicates a significant change between patients and control (P < 0.01).*

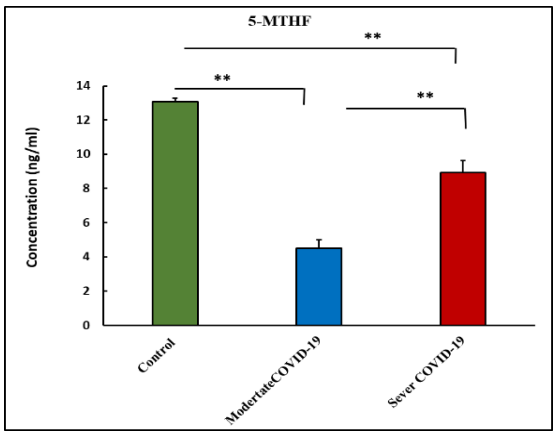


*Figure (4): Serum 5- MTHF levels in patients with moderate COVID-19, severe COVID-19, and control groups. Data are expressed as mean ± SEM. **indicates a significant change between patients and control, and significant change between moderate and severe groups (P < 0.01).*

## TNF-$\alpha$ levels in COVID-19

Inflammation has been suggested to be the main contributor in the COVID-19 pathogenesis, the circulatory pro-inflammatory cytokines are associated with COVID-19. The present study showed that serum TNF-$\alpha$ levels were significantly increased in patient with COVID-19, compared to control group (P<0.01, figure 5). A significant increase was observed between patient groups compared to control, with a high increase was indicated in the severe COVID-19 group (P<0.01, figure 6).

Inflammation has been suggested to be the main contributor in the COVID-19 pathogenesis, the circulatory pro-inflammatory cytokines and micro -environmental hypoxia associated with covid-19. In the present study, tissue hypoxia was examined in patient and control. The results clarified an increase in levels of HIF-1α in patient with COVID-19, compared to control group (P<0.01, figure 7). A significant increase was observed between moderate and severe patient groups compared to control, with a high increase was indicated in the severe COVID-19 group (P<0.05, Figure 8). Non- significant changes were indicated between the patient groups.

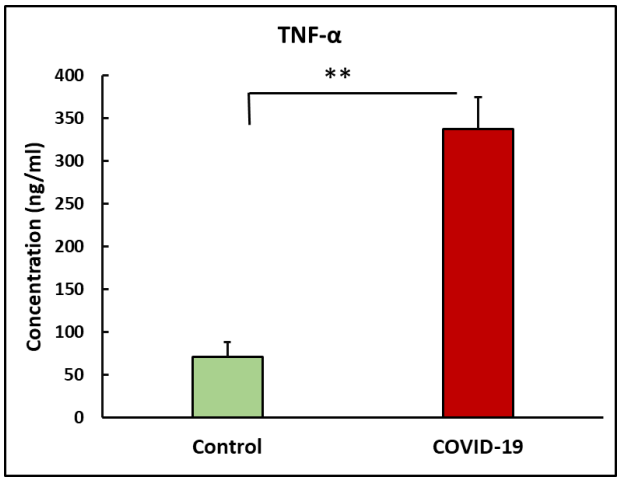


*Figure (5): Serum TNF-**α** level in patient with covid-19 infection and control. Data are expressed as means ± SEM, **indicates a significant change between patients and control (P < 0.01).*

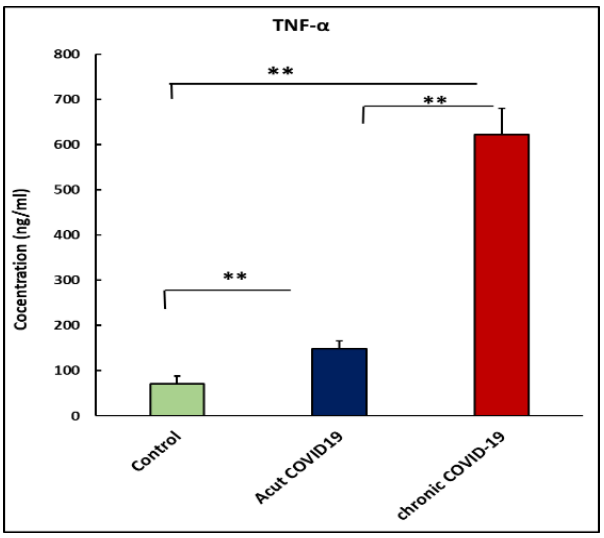


*Figure (6) Serum TNF-**α** levels in patients with moderate COVID-19, severe COVID-19, and control groups. Data are expressed as mean ± SEM. **indicates a significant change between patients groups and control (P < 0.01).*

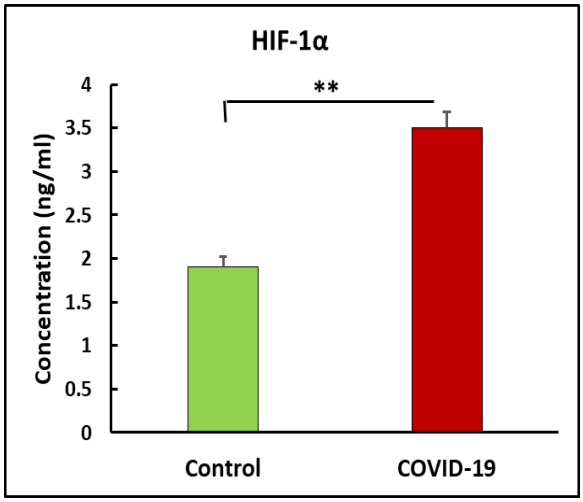


*Figure (7): Serum HIF-1**α** level in patients with covid-19 and control. Data are expressed as means ± SEM, **indicates a significant change between patients and control (P < 0.01).*

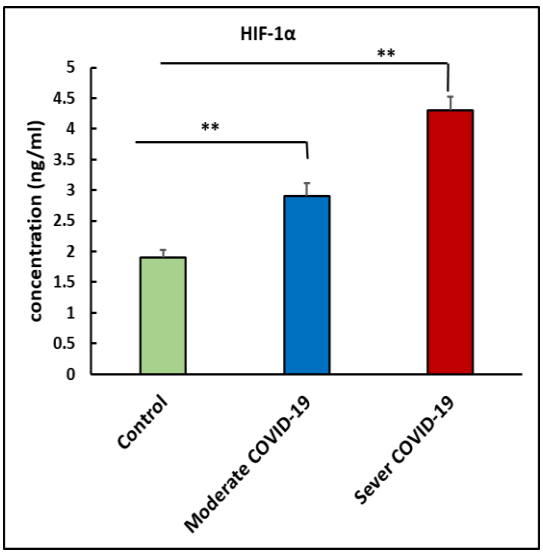


*Figure (8): Serum HIF-1**α** levels in patients with moderate COVID-19, severe COVID-19 and control. Data are expressed as mean ± SEM. **indicates a significant change between patients groups and control (P < 0.01), NS indicates non-significant change between*

*moderate and severe groups of patients.*

## 5. Discussion

Folate, often known as vitamin B9, is a necessary component for DNA as well as RNA production enzymes. By addition to purine as well as pyrimidine production and carbon transfer events of amino acid metabolism, it is necessary for hematopoiesis as well as red blood cell generation [20]. 5-MTHF and 5, 10-MTHF serve as a precursor for several enzymes throughout the body. DNA synthesis requires 5,10-MTHF at a rate of just one equivalent every four nucleotides [21].

In the present investigation, 5-MTHF levels were significantly lower in the patients group than in the control group. A study by Meisel et al. [22] found no connection between hospitalized COVID-19 patients' folate levels and illness outcomes. Inadequate nutritional intake may be a probable cause in these people.

Several viral and bacterial illnesses, including influenza, mycoplasma pneumonia, parvovirus, Epstein-Barr virus, and respiratory tract infections in young children, have been related to reduced folate levels. Possible scientific explanations include folate's crucial role in developing the innate and adaptive immune systems by sustaining natural killer cell (NK) cytotoxic activity, T-helper 1 (Th1)-mediated immunological response, as well as immunoglobulins formation [22].

The proton-coupled folate transporter is the primary carrier involved in delivering folate into intestinal cells after folate digestion, which is the alternative reason for low folate levels, this facilitative folate transporter with high affinity is predominantly located in the proximal jejunum as well as duodenum, PCFT seems to be the major folate as well as folic acid transporter into small intestinal cells. Vitamin D influences PCFT gene expression, among other transcription factors. As vitamin D insufficiency was related with a greater infection risk and poor outcomes in COVID-19 patients, we expected that folate levels would be lowered in COVID-19 patients [22].

Low folic acid is accombiened by low 5-MTHF which may be due to decreased availability of folate and, as a consequence, decreased its metabolite, 5-MTHF.

TNF-α is essential in virtually every acute inflammatory responses, functioning as an inflammatory amplifier. More than 10 distinct autoimmune inflammatory illnesses have been treated with TNF-α inhibition, indicating that this might be a feasible treatment strategy to minimize organ damage in individuals with COVID-19 infection [19]. The present results showed that TNF-α levels were significantly higher in the COVID-19 patient group than in the control group. These result in consistent with A et al. [23] findings, which demonstrate the same outcome. Kempuraj et al. [24] demonstrated that the intensity of Covid-19 symptoms correlates with a rise in peripheral levels of cytokines. TNF-α involved in the various biological function such as accelerated secretion of other pro-inflammatory/chemotactic mediators, the up-regulation of adhesion molecules, as well as the increased migration of eosinophils and neutrophils. TNF-α was identified at elevated levels in the sera of SARS individuals [25]. These findings suggest that TNF-α is a strong inflammatory cytokine involved in the pathophysiology of SARS.

TNF-α increases oxidative stress by increasing the generation of reactive oxygen species; one of the origins of generation of these ROS may be the virus-induced mitochondrial malfunction. COVID-19 describes a "cytokine storm" with the generation of Il-2, TNF-α, Il-6, Il-7, TNF-α, etc. [26]. These result in a cytokine burst with hyper-inflammation and hyper-ferritinemia, which would be known to produce ROS through the Fenton reaction (Fe2+ + H2O2 Fe3+ + HO + HO). furthermore, cytokines with endotoxins activate one of the isoforms of nitric oxide synthetase (NOs), the inducible isoform iNOs, that stimulates the generation of nitric oxide NO, which reacts with the superoxide ion to produce the highly oxidizing peroxynitrite radical ($ONOO^-$) [27].

TNF-α increases leukocyte recruitment by activating vascular cell adhesion molecule-1 (VCAM-1) expression on endothelial cells and enhances monocyte chemotaxis by raising endothelial cell synthesis of Monocyte chemoattractant protein-1 (MCP-1) [28]. Furthermore, TNF-α is one of the primary reasons of endothelial dysfunction, which is believed to be a common characteristic of Covid-19 [29].

Additionally, TNF-α may have a function in acute thrombosis. Similarly, TNF-α stimulation of human macrophages in vitro led to an increase in matrix-degrading metalloproteinases synthesis. Furthermore, TNF-α affects hemostasis by promoting the suppression of ACE-2, which causes an increase in angiotensin II levels (AT-II). Both TNF-α and AT-II have been involved in increasing tissue factor (TF) expression in platelets as well as macrophages. Furthermore, the production of antiphospholipid immunoglobulins linked with COVID-19 may potentially stimulate TF production. Future research should focus on TF, which may be a crucial mediator in the development of thrombotic events in COVID-19 by mediating thrombin production and subsequent clot formation [30].

The present study was reported the overexpression of HIF-1α in patients with COVID-19 which highlight its critical functions as an activator for both SARS-CoV-2 infection as well as inflammatory response, making it a promising therapeutic target for virus-induced inflammatory disorders and COVID-19 (20).

Hypoxia-responsive transcription factors HIF-1α play a major regulatory function in these homeostatic alterations at the systemic as well as cellular levels [31]. During euoxic circumstances, HIF-1α is constantly expressed and swiftly destroyed, but its degradation is delayed under hypoxic conditions, resulting in stability and buildup of HIF-1α. In the present investigation, COVID-19 patients showed a significant increase in HIF-1α compared to healthy control. This result was consistent with the findings of McElvaney, Oliver J. McEvoy, and Natalie L. McElvaney et al. [28] PHDs (prolyl hydroxylases) hydroxylate HIF-1a at preserved proline residues in healthy resting cells, designating it for ubiquitination and fast proteasomal destruction. PHDs are oxygen dependent. In a situation of normoxia, HIF-1a normally

has a short cytoplasmic half-life, a fast turnover rate, and low baseline levels, while it is preserved in a state of relative hypoxemia. This is due to the fact that inflammatory sites are characterized by hypoxia, which is also present in severe pneumonia and respiratory distress after SARS-CoV-2 infections.

HIF-1α is an essential factor that is induced by hypoxia. It has a pro-inflammatory impact by regulating the high rate of IFNI generated by cytokines including Interleukin 6 (IL-6) as well as TNF-α and by activating the signal transducer and activator of transcription 3 (STAT3) pathway to promote the inflammatory response. Therefore, in this microenvironment with hypoxia and HIF-1α activity, decrease of HIF-1α transcription or inhibition of its activity may be useful in decreasing viral-induced inflammation in organs such as the lung in COVID-19 [18].

## 6. Conclusions

The study findings provides crucial evidence for future studies evaluating the impact of folic acid statues and supplementation on COVID-19-associated morbidity and mortality. Folic acid deficiency result in impaired host resistance to COVID-19 infection and enhanced hypoxia-induced events as indicated by increase TNF-α and HIF-1α. Therefore, further studies involving the impact of folate status and folic acid supplementation on susceptibility to COVID-19 and its fatal complications are warranted.

References

M et al. [17] found that HIF-1α and inflammatory cytokines were induced in COVID-19 patients relative to healthy individuals, significantly induced in elderly patients relative to young patients, and elevated in elderly healthy individuals relative to young healthy individuals.

HIF-1α and inflammatory cytokines were increased in COVID-19 patients compared to healthy persons, considerably increased in old patients compared to young patients, as well as raised in elderly healthy individuals compared to young healthy individuals. generation by triggering mitochondrial damage as well as reactive oxygen species produced from mitochondria (Mito-ROS) production. Ultimately, HIF-1α boosted viral infection and pro-inflammatory responses. In addition, HIF-1α may encourage the widespread infection of many other viruses. Thus, we hypothesized that, during infection with SARS-CoV-2, ORF3a promotes Mito-ROS generation to stimulate HIF-1α, which then in turn exacerbates viral infection with inflammatory responses.

The CRP levels of patients on Covid-19 were higher than those of the control group. The findings of the current research align with those of Yufei et al. [32] investigation [32]. With response to viral infection, the liver produces substantial quantities of CRP. This CRP is an indicator of infection, inflammation, but also tissue damage. Following inflammatory reactions, the CRP concentration rises [33].

In this research, there was a statistically significant ($p<0.001$) increase in D-dimer levels between COVID-19 patients and healthy controls. The findings of this investigation are consistent with those of earlier studies [34] [34], Zhang et al. [35] that demonstrated higher D-Dimer levels in COVID-19 patients. In such a patient having Covid-19, an elevated D-dimer level suggested a condition of hyper-coagulability, which might be due to a variety of causes. First, viral infections are often accompanied by a robust pro-inflammatory response and inadequate anti-inflammatory response regulation [13]. D-dimer promotes endothelial cell dysfunction, leading in an overproduction of thrombin. The other is that severe Covid-19 hypoxia may activate thrombosis via a hypoxia-inducible transcription factor-dependent signaling pathway in addition to an increase in blood viscosity [36].

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