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From Mild Cases to Critical Cases of COVID-19: The Role of Genes in Inflammasome and Mitochondrial Dynamics

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ABSTRACT

The coronavirus disease 2019 (CVOID-19) has varied clinical manifestations including mild to severe acute respiratory symptoms. Inflammasome complex and mitochondria play an important role in initiating inflammatory responses and could potentially be affected by this infection. To study the inflammasome and mitochondrial fission and fusion gene expression levels in COVID-19 patients, we designed this experiment.

The inflammasome and mitochondrial gene expression profiles were determined by real-time polymerase chain reaction in the peripheral blood of 70 hospitalized CVOID-19 patients with mild to moderate symptoms (HOSP) and 30 ICU patients with severe symptoms (ICU) compared to 20 healthy controls (HC).

The results indicated that the expression of the dynamin-related protein-1 was extremely suppressed in HOSP while it came back to the normal range in the ICU group. However, the expression of fission 1 protein had a non-significant increase in HOSP and a decrease in the ICU group. The mitofusin-1 and dominant optic atrophy genes showed high expression levels (10-fold) and (70-fold), respectively, in the HOSP group. However, mitofusin-2 significantly decreased in both groups. Although leucine-rich–containing family, pyrin domain–containing-3 (NLRP3) and apoptosis-associated speck-like protein containing a caspase activating and recruitment domain genes dramatically increased in both groups (10 and 4-fold), other inflammasome genes declined

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in both groups. Finally, Nuclear factor kappa-light-chain-enhancer of activate d B cells (NF-κB) extremely decreased, and Intreleukine-1 showed high expression in ICU patients (3-fold).

CVOID-19 infection suppresses the fission genes and elevates the fusion gene expression in mitochondria, and it can cause activation of the inflammasome via the NLRP3 pathway.

Keywords: Corona disease 2019 (COVID-19); Inflammasome; Mitochondria

INTRODUCTION

The coronavirus disease 2019 (COVID-19) was recognized by the World Health Organization (WHO) as a respiratory viral infection in Wuhan, China in December 2019. By April 2022, nearly 50 million cases of illness and 6 million deaths from this disease have been reported.[1,2]

Multisystem inflammatory syndrome (MIS-C) is a hyperinflammation disorder caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and sometimes occurs 4–6 weeks after SARS-CoV-2 infection.[3-5] Moreover, a certain percentage (approx. 5-15%) of COVID-19 patients often develop acute respiratory distress syndrome (ARDS). In addition, systemic inflammatory response syndrome (SIRS) and multiorgan failure in some organs such as the heart, brain, spleen, liver, eyes, vasculature, and kidneys have been reported with high mortality rates among the patients. [6]

Besides the prominent cytopathic or cytotoxic effects of SARS-CoV-2, innate immunity and the arrays of immune response have been suggested to play a central role in the pathogenesis of the COVID-19 disease as well as its severity and mortality.[4,7] It seems that innate immunity has mediated the cytokine storm through the inflammasome pathway. Inflammasome as a part of innate immunity is a multiprotein platform that is activated upon infection or stress. The pathway is activated by the detection of pathogen-associated molecular patterns (PAMPs) via the pattern recognition receptors (PRRs) or by damage-associated molecular patterns.[8-10] PRRs play a crucial role in inflammatory responses and cytokine production. The activation of the cytosolic inflammasome complex triggers the induction of inflammatory cytokines as innate immune responses in viral diseases. The key role of the inflammasome complex is the activation of caspase-1 and the production of cytokines such as interleukin- 1(IL-1β), IL-18, and IL-37.[11,12]

NOD-like receptor (NLR) family pyrin domain containing 3 (NLRP3) is a necessary component in inflammasome complex assembly and activation. NLRP3 inflammasome activation consists of two modes, priming and activation.[13] In the first step, PAMPs are identified by Toll-like receptors (TLRs) resulting in the activation of Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). The cytokine has an upregulating role in the transcription of inactive inflammasome components such as NLRP3, procaspase-1, IL-1β, and IL-18. In the second step, the activation of NLRP3 results in the pyroptosis process.[10,11] In COVID-19 disease, pyroptosis is a process of cell death caused by inflammasome-dependent pathways. Following the entry of SARS-CoV-2 into epithelial cells via angiotensin-converting enzyme 2 (ACE2), the viral genome is translated and spread in host cells. NLRP3 is activated through mechanisms such as viral N protein, ATP, reactive oxygen species (ROS) accumulation, lysosomal rupture, K+ and Cl- efflux, and Ca2+ flux. The result is dead cells and damaged mitochondrial structure (dsDNA) which activates Absent-in-melanoma 2 (AIM2). Then, the assembly of NLRP3 and AIM2 is identified as a caspase-1 promoter signal.[14,15]

Current data show that upon the virus enters the cell, mitochondria play a crucial role in inducing the immune responses against the virus.[16,17] Inside the mitochondria, there are several keys signaling proteins such as NLR family members, mitochondrial antiviral signaling (MAVS), and stimulation of interferon genes (STING). These proteins cause a close relationship between the mitochondria and the innate immune system.[18,19] Furthermore, mitochondria are involved in cell metabolism, ATP production, modulation of programmed cell death (PCD) pathways, regulation of various biochemical pathways, and cell cycle control. Wu et al, determined the genomic RNA of the SARS-CoV-2 virus replicates both in the host mitochondria and nucleus. Also, the 5’ and 3’ non-coding regions can cause the localization of RNA in mitochondria. This process is called mitochondrial hijacking by SARS-CoV-2.[20-22] Furthermore, the mitochondria have fission and fusion processes, and the balance between them is

determined as a proper function of this organelle. Using master regulator analysis, a recent study reported a downregulation in a member of the mitochondrial complex I in cells infected by SARSCoV-2. It suggests the virus may cause the host cell death by disrupting the mitochondrial function.[23]

On the other hand, the serious damage of mitochondria resulted to releasing of its contents into the inter and intracellular microenvironments which led to the production of proinflammatory cytokines such as IL-1β via activating nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP3) inflammasome, which is composed of NLRP3 caspase1and apoptosis-associated Speck-like protein containing a caspase recruitment domain (ASC).[24-26]

The present study aimed to investigate the simultaneous expression patterns of 12 key genes involved in the NLRP3 and mitochondrial process and to determine their correlation at the expression level within different phenotypes of the COVID-19 disease, including mild to moderate, and severe patients compared to the control group.

MATERIALS AND METHODS

The patients were male and their ages were between 18–60 years, and all of them were diagnosed with SARS-CoV-2 based on the positive real-time polymerase chain reaction (RT-PCR) test. Under symptoms intensity, the cases were grouped into three groups: 30 cases in the intensive care unit (ICU group) with severe symptoms, 70 cases with mild to moderate symptoms (HOSP group), and finally, 20 cases with negative results of COVID-19 infection which considered the healthy controls (HC group).

A total of 100 patients' cDNA samples was harvested from the Biobank of Baqiyatallah University of Medical Sciences, Tehran, Iran. The cDNAs of blood specimens were gathered from patients with COVID-19 admitted to Baqiyatallah Hospital from 2020 March to April 2020. The extraction of RNA and cDNA synthesis were accomplished based on the kit protocol (BioBasic, Canada).

The blood samples were obtained to analyze the laboratory parameters. The effects of COVID-19 infection on the expression level of inflammasome pathway genes and mitochondria were studied. To measure gene expression, the real-time PCR was done using SYBR green master mix reagents (Gene All, Korea) and Rotor-gene real-time PCR system (Qiagen) with specific primers from previous studies [10,19] (Supplementary Table 1). The PCR was carried out by using a Master mix (12.5 µL), 50 ng of the total RNA, 1 µL of the forward and reverse primers, and 8.5 µL of ddH2O. PCR was initialized by enzyme activation at 95ºC for 5 minutes followed by 37 amplification cycles (95ºC for 30 sec, annealing temperature for 30 sec, and 72º C for 30 sec) and finalized by melting curve analysis. Threshold cycle values were normalized by GAPDH expression and the changes in gene expression were analyzed with the 2^(-ΔΔCT) method.

Statistical Analysis

The results were indicated as the mean±standard error of the mean (SEM). Paired-sample t-test analysis and ANOVA in Graph Pad Prism 8.0 software (San Diego, CA, USA) were used for statistical analysis. (statistically significant: $p<0.05$).

RESULTS

In this experiment, the gene expression status of both the inflammasome system and the mitochondrial organelle were evaluated by RT-PCR. The clinical laboratory parameters of both patient groups are shown in Table 1.

No significant elevation was shown in the expression level of genes like mitochondrial fission 1 protein (fis1) in hospitalized cases (~1.2-fold, $p=0.584$), but it demonstrated a bit reduction in ICU cases (~0.8-fold, $p=0.1033$) (Figure 1A). However, regarding dynamin-related protein 1 (drp1), a marked suppression was indicated in both groups; more than 90% in hospitalized cases ($p<0.0001$) compared to 40% in the ICU group ($p<0.0001$) (Figure 1B).

There was a significant enhancement in the expression level of the mitofusin 1 (mfn1) gene in hospitalized (~9-fold, $p=0.0002$) and ICU cases (~10-fold, $p<0.0001$) (Figure 1C). Furthermore, dominant optic atrophy (opa1) as a fusion gene showed a high expression level in hospitalized (~76-fold, $p<0.0001$) compared to ICU cases (~13-fold, $p<0.0001$) (Figure 1D). Meanwhile, mitofusion 2 (mfn2) gene was significantly repressed in hospitalized cases (~ 0.2-fold, $p=0.0002$) and fully suppressed in ICU cases (~0.02-fold, $p<0.0001$) (Figure 1E).

Table 1. The laboratory parameters

Parameters	Normal Range	Non -ICU patients	ICU patients	Confidence interval	Bonferroni correction (adjusted *p*)
Red Blood Cell Count	4.3-5.7x10^6/µL	4.25	3.70	0.04	0.000
Hemoglobin (Hb)	13.5-17.5 g/dL	12.29	10.86	0.12	0.000
Hematocrit (Hct)	31-45%	36.17	32.19	0.34	0.000
Platelet (Plt)	145-420x1000/µL	231.77	188.26	4.61	0.000
White Blood Cell Count	3.5-10.5x1000/ µL	9.03	12.54	0.40	0.000
%Polymorphonuclear	50-70%	69.53	80.20	0.73	0.000
%Monocyte	4-11%	6.43	5.32	0.17	0.000
%Eosinophil	1-6%	2.22	1.34	0.15	0.000
%Total Lymph	11-49%	20.95	33.30	3.90	0.000
%Basophil	0-2%	0.41	0.48	0.14	0.342
Partial Prothrombin Time Test (PTT)	22-36 Sec	35.67	41.22	0.95	0.000
Prothrombin Time Test (PT)	Up to 14.5 Sec	16.16	17.76	0.34	0.000
International Normalized Ratio (INR)	0.8-1.2	1.27	1.39	0.03	0.000
Blood Glucose	74-100 mg/dL	140.36	165.78	5.15	0.000
Hemoglobin (Hb. A1c)	4-6%	7.20	6.57	0.74	0.123
Albumin	3.4-5.4 mg/dL	3.45	2.92	0.13	0.000
Ferritin	10-120 mg/ dL	405.05	896.93	265.13	0.001
Total Iron-Binding Capacity (TIBC)	240-450 ug/dL	247.88	217.08	55.24	0.308
Serum Glutamic Oxaloacetic Transaminase (SGOT)	<35 U/L	59.44	84.58	40.52	0.236
Serum Glutamic Pyrovic Transaminase (SGPT)	<45 U/L	56.54	74.37	24.20	0.163
Alkaline Phosphatase (ALP)	Up to 270 U/L	283.26	300.68	38.90	0.433
Blood Urea Nitrogen (BUN)	7-19 mg/dL	20.52	26.77	0.89	0.000
Direct Bilirubin	Up to 3 mg/dL	0.92	1.10	0.29	0.261
Total Bilirubin	0-2 mg/dL	2.91	9.78	0.86	0.000
Phosphor	2.5-4.5 mg/dL	3.63	3.43	0.28	0.178
High Density Lipoproteins (HDL)	>35 mg/dL	36.93	31.75	18.41	0.620
Cholesterol	Up to 200 mg/dL	164.20	147.75	63.18	0.646
Low-Density Lipoproteins (LDL)	<100 mg/dL	100.26	100.55	42.44	0.990
Lactate Dehydrogenase (LDH)	207-414 U/L	727.33	1231.25	278.84	0.005
Uric acid	3.5-7,2 mg/dL	5.64	5.78	1.12	0.830
C-Reactive Protein (CRP)	5 mg/L	18.45	18.61	3.56	0.929
Vitamin D3 level	>30 ng/mL Sufficient	32.68	31.13	7.36	0.700

The expression level of inflammasome genes indicated that the NLRP3 pathway was active in both hospitalized and ICU patients and NLRP1 and AIM2 pathways were suppressed. nlrp3 gene was highly expressed close to 10-fold in hospitalized patients (p<0.0001) however, it declined to 5-fold in ICU patients (p=0.0002) (Figure 2A). Another part of the NLRP3 pathway (i.e., asc gene) displayed an augmentation in both hospitalized (4-fold, p< 0.0001) and ICU patients' groups (20-fold, p<0.0001) (Figure 2B). Results suggested that the aim2 gene was suppressed and its expression level fell to 40% and 20% in hospitalized and ICU patients, respectively (p<0.0001, Figure 2C). The full suppression of nlrp1 gene expression was shown in both hospitalized and ICU cases.

NF-κB as an expression factor involved in the inflammasome pathway was highly affected by virus infection and tended to decline significantly in both groups with a reducing trend from 40% in hospitalized cases to about 10% in ICU cases (p<0.0001, Figure 2D). Although IL-1β, which is an inflammatory cytokine, did not show a significant decrease in hospitalized patients, it indicated a much higher expression level (3-fold, p=0.0003) in ICU patients (Figure 2E).

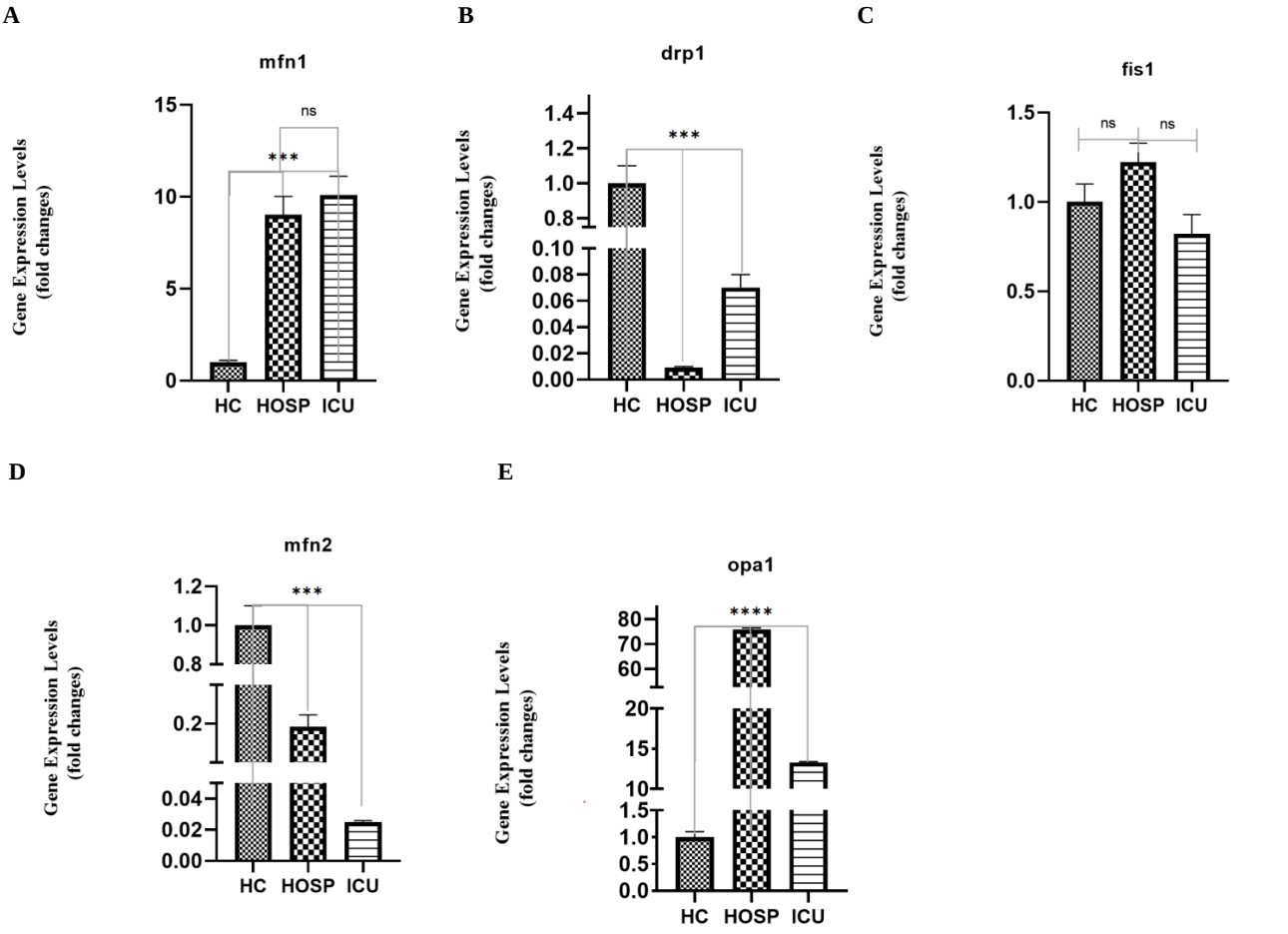

Figure 1. Alterations in mitochondrial fission (A & B), and fusion (C, D, & E) gene expression levels in hospitalized (HOSP) and ICU cases (ICU). In fission genes (A & B), the fis1 gene was not affected during the COVID-19 disease but the drp1 gene was completely suppressed by SARS-CoV-2. In infusion genes (C, D, & E), the mfn1 gene was overexpressed (10-fold), and the opa1 gene was vigorously overexpressed in the HOSP group (70-fold) and came back to 10-fold change in ICU cases. The mfn2 gene was completely suppressed during the disease. Data were expressed as Mean±SEM. Error bars represent the standard deviation of each experimental group, respectively. ns: not significant, ***p<0.001 and **** p<0.0001. HOSP: mild to moderate cases and ICU: critical cases which admitted to the intensive care unit.

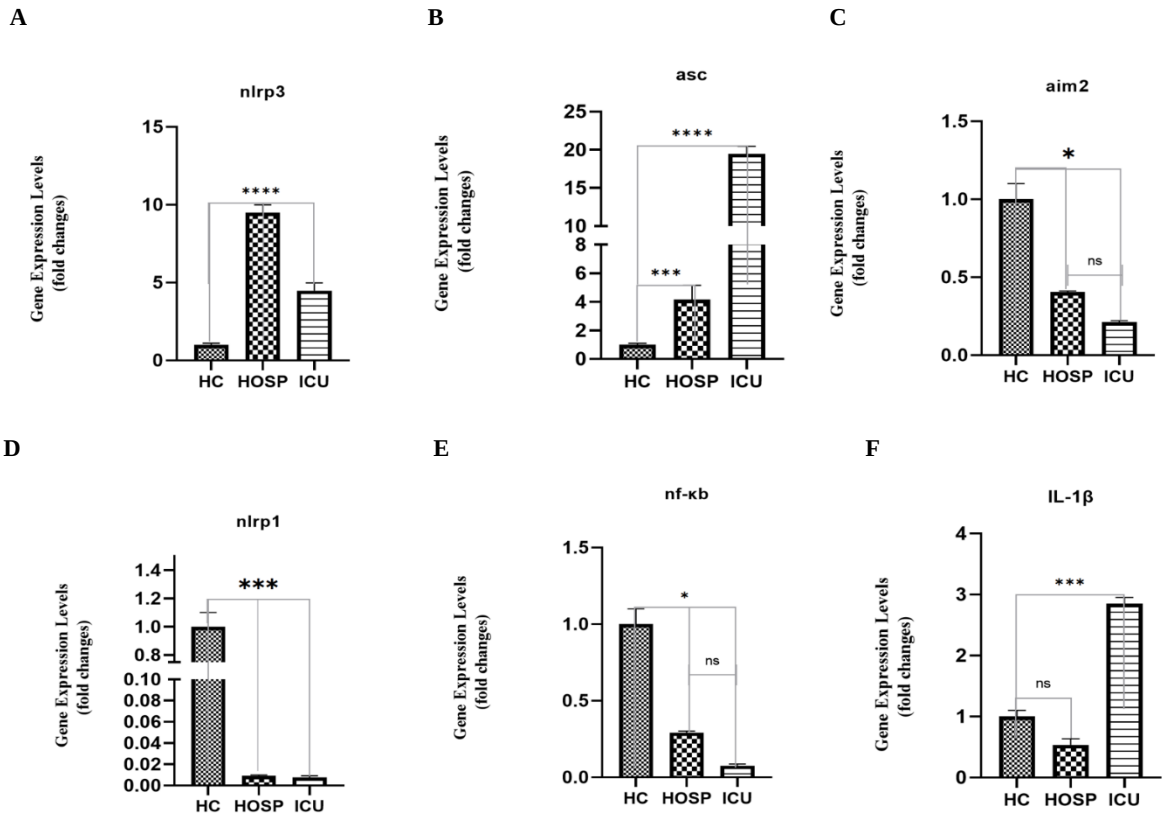

Figure 2. Alterations in inflammasome gene expression levels in COVID-19 hospitalized and ICU cases. (A) The nlrp3 expression level was significantly increased in hospitalized patients (10-fold) and returned to a 5-fold expression level in ICU patients. (B) The ASC gene was vigorously affected by the SARS-CoV-2 virus, reached 4-fold changes in the HOSP group, and elevated to the maximum level (25-fold increase) in ICU patients compared to the controls. (C, D, and E) The aim2, nlrp1, and nf-kb genes were fully suppressed in HOSP and ICU groups. (F) The interleukin-1β (IL-1β) gene did not show an increase in hospitalized cases but had a 3-fold increase in ICU patients. Data were expressed as Mean±SEM. Error bars represent the standard deviation of each experimental group, respectively. ns: not significant, ***p<0.001 and **** p<0.0001. HOSP: mild to moderate cases and ICU: critical cases which admitted to the intensive care unit.

DISCUSSION

In this study, we aimed to show and compare the mitochondria and inflammasome status in both COVID-19 hospitalized and ICU patients. Investigating the expression status of genes in one or more molecular pathways can give a comprehensive scheme of the activity or inhibition status of the signaling pathways. This perspective can help in understanding the pathogenesis and severity of the disease and also be fruitful in identifying disease biomarkers and choosing treatment approaches. Mohebi et al, suggested that surveying the status of mitochondrial genes of blood immune cells has a pivotal role as a biomarker in the prognosis and diagnosis of Covid-19.[27]

The results indicated that fusion genes that contribute to mitochondria repair, including mfn1 (10-fold) and opa1 (70-fold), had vigorous overexpression. Adversely, the mfn2 gene expression level almost has been suppressed, especially in ICU patients. On the other hand, the fission genes that are involved in mitochondria fragmentations either showed no alteration in their expression level (fis1) or were completely suppressed (drp1). Inflammasome as a sensor complex plays a crucial role in sensing PAMPs leading to the release of inflammatory cytokines.[9] Besides, mitochondria are now known to play a key role in innate immune system responses and provide a close relationship with the inflammasome complex. They have a critical role in controlling oxidative stress and inflammation through fission and fusion processes,

mitophagy mechanisms, and sources of reactive oxygen species.[19,28]

It is crucial to mention that other studies have observed suppression in genes involved in the fission process and an increase in the expression of fusion genes in SARS-CoV-2 infection. "SARS-CoV-2 hijacks mitochondria and localizes its RNA within this organelle, utilizing it for both concealment and replication purposes." Meanwhile, the SARS- CoV-2 open reading frame 9b (Orf9b) protein is placed in the mitochondria and other accessory proteins (e.g. Orf3b, Orf7a, and Orf8a) are in close contact with the organelle.[21,29] Studies have reported that coronavirus translocates its ORF-9b protein into mitochondria and can destroy dynamin-like protein (Drp1) resulting in mitochondrial fission..[25,27,30] In other words, the virus replicates itself by preventing the expression of fission genes to inhibit the fragmentation of mitochondria, and on the other hand, by increasing the expression level of fusion genes promotes mitochondrial survival. Shi et al, and Singh et al, reported that SARS-CoV-2 by reducing the expression of genes involved in the fission process (i.e. drp-1, up to 70% reduction) can disrupt the mitochondrial fission and leading to hyperfused mitochondria.[30,31] To sum up, the virus by increasing the expression of fusion genes and enlarging mitochondria, prevents apoptosis and makes microenvironment conditions suitable for self-replication in the host cells.[32]

Moreover, the fission and fusion genes contribute to the regulation of mitochondrial antiviral signaling (MAVS) which is a necessary cellular antiviral defense system. Reducing profusion gene expression of mfn2 and adversely by increasing mfn1 and opa1 expression levels, the cells could attempt to enhance the MAVS activity.[25,33] It seems, in this study, that by regulating mfn2, mfn1, and opa1 genes, both in the early and late stages of COVID-19 disease, the cells try to augment the MAVS function. Despite this phenomenon, the virus finally appeared to be successful in hijacking the mitochondria, causing the dysfunction of this vital organelle, suppressing the MAVS activity, and escaping innate immunity.[33]

Generally, the inflammasome is known as the first line to sense PAMPs and release proinflammatory cytokines such as IL-1β. The results revealed that among inflammasome pathways, the NLRP3 pathway is activated in both hospitalized and ICU patients. In this regard, nlrp3 and asc genes raised to 10- and 20-fold changes during hospitalization and subsequently in ICU. Although nlrp3 showed a decrease in ICU patients, it did not return to the normal level. Meanwhile, other inflammasome genes such as aim2 and nlrp1 were completely suppressed. Accumulated data support the implication of the NLRP3 inflammasome in mediating inflammation during lung injury and acute respiratory disorder syndrome (ARDS). Although the SARS-CoV-2 virus with ORF-9 tries to reduce MAVS and NLRP-3 gene expression, the virus genome encodes 3 virulence proteins, E, open reading frame 3a (ORF3a), and ORF8a. These proteins promote the activation of the inflammasome by the NLRP3 pathway.[14] Junqueira et al, showed that SARS-CoV-2 can attack 10% of the blood monocytes of patients and by activation of NLRP3 and AIM2 can induce pyroptosis in these cells.[34]

Although the previous studies have indicated that the other inflammasome genes such as nlrp1 have remained uncharacterized during SARS-CoV-2 infection[35], surprisingly, our results demonstrated NLRP1 inflammasome was completely suppressed suggesting that dsRNA is not activated during COVID-19 disease. In contrast to our finding, Ferriera et al, reported that Aim2 involved in sensing intracellular dsDNA was unexpectedly activated in monocytes. It seems that AIM2 activation was due to the release of ROS and oxidization of mtDNA into cytoplasm.[36]

Our findings indicated that the NF-κB gene expression level was downregulated in both hospitalized and ICU patients. In the cytosol, NF-κB was inhibited by IκB (its inhibitor), and IκB kinase can dissociate IκB from NF-κB upon need, and then NF-κB was transferred into the nucleus and can induce some inflammatory cytokine genes such as IL-18 and IL-1 β.[37] In this study, expression levels of the IL-1β gene were stable in hospitalized patients but started to increase in ICU patients (up to 3-fold). The decreased expression of the NF-kB gene in hospitalized and ICU patients suggests that SARS-CoV-2 may trigger IκB kinase activation, leading to the dissociation of NF-κB from IκB. Subsequently, NF-κB translocates into the nucleus to enhance the expression of the IL-1β gene.In a clinical study has been demonstrated that the expression level of IL-1β and IL-6 in serum and alveolar lavage fluid are elevated in SARS-CoV-2 infected patients.[38] Pan et al, indicated that SARS-CoV-2 proteins, particularly the N protein, play a crucial role in regulating the secretion of mature IL-1β through the NLRP3 inflammasome system. qRT-PCR analyses revealed significant

induction of IL-1β, IL-6, and TNF mRNAs in mice. However, it appears that the N protein was not involved in the regulation of the NF-κB gene signaling pathway. Furthermore, the N protein did not directly interact with the ASC protein; instead, the protein interacted with endogenous ASC in the presence of NLRP3.[39] Nagaraja et al, reported that the SARS-CoV-2 ORF3a, ORF7a, E and S proteins promote activating the NLRP3 inflammasome. These proteins also stimulate NF-kB activation, leading to the upregulation of NLRP3, IL-1β, and IL-18 gene expression .[40]

As shown in Table 2, there is a significant increase in the levels of ferritin among hospitalized and ICU patients. Ferritin is an important regulator of iron that is present in the blood and the main cell organelles such as mitochondria. The major role of ferritin is to protect the system against ROS (reactive oxygen species) in various physiological and pathological conditions. In COVID-19 patients, hyperferritinemic syndrome appears, which directly correlates with the disease's severity. It seems in response to tissue damage and cytokine stimulation, defense proteins such as ferritin and CRP are produced and secreted by the liver in acute phase response. Ferritin gene expression and its translation into protein is induced by IL-1 and IL-6 cytokines.[41-43]

During the study, it is important to consider that both patient groups may have underlying diseases or be taking their routine medications, which could potentially result in pharmacological interactions affecting the inflammasome and mitochondrial gene expression levels. Our findings support the outcomes of previous research in this area, indicating that SARS-CoV-2 not only activates the NLRP3 inflammasome but also appears to inhibit the fission process while promoting the fusion mechanism to facilitate its replication within the mitochondria.

STATEMENT OF ETHICS

The local ethical committee of Baqiyatallah Hospital approved this study (IR.BMSU.REC.1399.039). The project was described as an overview for each patient and patients' participation was voluntary. Patients' consent was taken consciously at the beginning of the project, and all of them were persuaded about data confidentiality all the patients were assured that none of the trials and tests had any risk for them.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders also had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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