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Mitochondrial Pathogenic Mutations and Expression Pattern of Oxidative Phosphorylation Genes in COVID-19 Patients

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ABSTRACT

Mitochondrial missense mutations and pathogenic variants have been implicated in the pathogenesis of COVID-19. This study evaluated the role of mitochondrial DNA (mtDNA) mutations and changes in gene expression in the progression of COVID-19 and their correlation with clinical characteristics.

Next-generation sequencing with high throughput was used to identify mtDNA mutations in 30 COVID-19 patients compared to 20 healthy controls. The potential impact of identified mutations on protein structure and stability was predicted using bioinformatic tools. Quantitative real-time polymerase chain reaction was employed to assess the expression levels of mtDNA-encoded genes involved in oxidative phosphorylation in COVID-19 patients and healthy controls. Correlations between gene expression levels, clinical parameters, including leukocyte, lymphocyte, neutrophil, and platelet count, as well as creatinine, alanine transaminase (ALT), aspartate transaminase (AST), and blood urea nitrogen (BUN) levels, and disease severity were analyzed.

We found 8 different mtDNA mutations in *ND1*, *ND5*, *CO3*, *ATP6*, and *CYB* genes, which were predicted to alter amino acids and decrease protein stability. Two missense unique mutations, C9555T in *CO3* and A12418T in *ND5* were identified and correlated with Complexes I and IV, respectively. This downregulation was correlated with age, elevated levels of leukocytes, lymphocytes, neutrophils, platelets, creatinine, ALT, AST, and BUN, as well as disease severity.

These findings suggest that mtDNA mutations and altered expression of oxidative phosphorylation genes contribute to mitochondrial dysfunction in COVID-19. Targeting mitochondrial dysfunction may represent a promising therapeutic strategy for COVID-19 treatment.

Keywords: COVID-19; Mitochondria; Mitochondrial DNA; Mutation; Oxidative stress

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a type of pulmonary infection caused by the SARS-CoV-2 virus, which has led to a deadly pandemic since its emergence in December 2019 and an effective cure for this illness has yet to be discovered.[1] The COVID-19infected individuals exhibit a diverse spectrum of symptoms

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ranging from an absence of clinical symptoms to mild, acute respiratory distress syndrome, pulmonary edema, fatal pneumonic complications, severe septic shock, to multiorgan failure.[2] The deleterious outcomes observed in the majority of COVID-19 patients are likely attributable to a wave of inflammatory processes known as the cytokine storm. This phenomenon is correlated with a variety of systemic events, such as iron homeostasis dysregulation, hypercoagulability, oxidative stress, electrolyte imbalance, and thrombus formation. The occurrence of adverse systemic events has been associated with mitochondrial dysfunction.[3-6] The examination of crosstalk between host and virus, in conjunction with intracellular signaling within a specific organelle, may be considered a potential avenue for therapeutic opportunities.[7,8]

Mitochondria as a key target in COVID-19 infection have a critical role in the immune response of the host against this viral infection. This is due to oxidative damage and the production of reactive oxygen species (ROS), which lead to mitochondrial DNA (mtDNA) damage.[9] The mtDNA displays a high degree of polymorphism and is responsible for encoding 13 distinct polypeptides. This genetic material is known for its propensity to undergo various types of mutations, including inversion, deletion, duplication, and point mutation. These alterations have been associated with the development of mitochondrial dysfunction, oxidative stress, and cell death, as well as increased susceptibility to various diseases.[10,11]

Mitochondria serve as the primary organelle for cellular energy production, with energy being primarily stored and transferred by adenosine triphosphate (ATP). The process of ATP generation is referred to as oxidative phosphorylation (OXPHOS). Beyond this fundamental role, mitochondria also function in Ca^{2+} storage, fatty acid oxidation, cell proliferation, stress management, autophagy, necrosis, apoptosis, innate immunity, biosynthesis of critical cofactors (such as heme, biotin, and iron-sulfur), as well as the production of lipids, amino acids, and carbohydrates.[12-14]

Patients with COVID-19 have been found to experience deleterious systemic events, including but not limited to oxidative stress, hypercoagulability, electrolyte imbalance, dysregulation of iron homeostasis, and thrombus formation, all of which are associated with mitochondrial dysfunction.[6,15] Interestingly, COVID-19 RNA and proteins are located within mitochondria and facilitate viral inflammation, survival, and replication by stealing mitochondrial function from the host cell. Consequently, mitochondria exhibit divergent metabolic capabilities, resulting in distinct responses towards both antiviral and inflammatory stimuli.[16,17]

Mitochondria infected with SARS-CoV-2 exhibit increased susceptibility to tissue dysfunction and severe pathological disorders. This is a notable observation that warrants further investigation. This elevated susceptibility may potentially amplify vulnerabilities to a variety of diseases, such as Parkinson's, bipolar disorder, Alzheimer's, and cancer [18]. This phenomenon is crucial for identifying genetic markers that can help manage and treat COVID-19 infection; however, more research is needed in this field.[15,19] Considering the heterogeneity of mtDNA in the biology of COVID-19, it is imperative to determine the role and probable correlation of various mtDNA variants resulting from mutations in SARS-CoV-2.

In this study, we aimed to explore the potential impact of the whole mitochondrial genome on the progression of COVID-19 severity from blood samples of Iranian patients, including both COVID-19–positive and healthy individuals through next-generation sequencing (NGS), with regards to the identification of mtDNA-related mutations or variants. Moreover, we evaluated the expression pattern of 13 mitochondrial genes from Complex I (including *ND1/2/3/4/4L/5/6*), Complex III (including cytochrome b [*CYB*]), Complex IV (including *CO1/2/3*), and Complex V (including *ATP6/8*) subunits of mtDNA in COVID-19 patients for a better understanding of the context-dependent regulation of these genes in COVID-19 patients to find the correlation between host's mitochondrial genome and the severity of COVID-19 infection.

MATERIALS AND METHODS

Genomic DNA Extraction and Amplicon Generation

Genomic DNA was isolated from peripheral blood samples (3–5 mL) in ethylenediaminetetraacetic acid (EDTA) tubes using the salting out method after cell lysis and proteinase K digestion (Kiagene Fanavar, Iran) in accordance with the manufacturer’s instructions. All genomic DNA samples were assessed for concentration and quality using a ScanDrop spectrophotometer (Analytik Jena, Germany) and agarose gel electrophoresis (Kiagene Fanavar, Iran). The mtDNA was assessed for 5 patients, including 3 men and 2 women. Then, a long-range polymerase chain reaction (PCR) (UltraRun

LongRange PCR Kit, Cat# 206402, Qiagen, Netherlands) with 2 sets of primers (Supplementary Table 1), was utilized for the library preparation and sequencing of all 37 genes or 16659 base pairs of mtDNA with a focus on analyzing mtDNA variants contributing to the disease, and targeted enrichment of mtDNA on an Illumina NovaSeq 6000 platform (Virtual Tour, USA). All 5 mtDNA sequences successfully fulfilled the predetermined criteria for quality control.

Next Generation Sequencing of the Mitochondrial DNA

The libraries were prepared and purified according to the protocol provided by the manufacturer. In brief, PCR products that were amplified from the 2 sets of primers were combined. The KAPA HTP Library Preparation Kit (Roche NimbleGen, USA) was utilized for the creation of libraries for whole genome sequencing. Initially, the DNA was fragmented to approximately 250 base pairs using a Covaris M220 Focused-ultrasonicator, and the resulting sheared DNA was examined for fragment distribution on the TapeStation using the HSD1000 DNA ScreenTapes (Agilent, USA). Subsequently, the DNA underwent end repair and adapter ligation.

The products were then purified using Agencourt AmpureXP Beads (Beckman Coulter, USA), and then they were enriched under specific temperature conditions. Subsequently purified using Agencourt AmpureXP Beads, and the final libraries were assessed on the TapeStation using the D1000 DNA ScreenTapes (Agilent, USA). The created libraries were quantified using the Qubit HS Assay technique (Invitrogen, USA). The libraries that were acquired were pooled and diluted to achieve the optimal loading concentration. The cluster flow cell was then loaded onto the Illumina HiSeq X instrument in order to produce paired-end reads of 150 base pairs.

Data Analysis, Structural, and Functional Prediction Analysis

The sequencing data were exported to a FASTQ file and the quality of sequence reads was checked using FastQC4 v0.11.5. The Burrows-Wheeler Aligner was used to align the trimmed reads against the hg19 version of the genome, which is available at University of California, Santa Cruz. Then, the processed read data were aligned with the Cambridge Reference Sequence for sequence variants.

Human Mitochondrial DNA (NC_012920.1) was used as the reference mitochondrial sequence. We used multiple bioinformatics tools, including MutPred2 (mutpred.mutdb.org), MUTPro (https://mupro.proteomics.ics.uci.edu/), and I-Mutant2:0 (https://folding.biofold.org/i-mutant/i-mutant2.0.html) that predicted impactful missense variants, protein stability changes for single-site mutations from sequences, and stability changes upon mutation from the protein sequence or structure, respectively. Moreover, we applied several tools to predict sequence homology, protein structural information, and evolutionary conservation based on amino acid changes caused by mutations.[20] Polymorphism Phenotyping v2 (PolyPhen-2) software (http://genetics.bwh.harvard.edu/pph2/) is one of the tools that predicts the possible effect of missense mutations on the stability and function of proteins. Additionally, the Sorting Intolerant from Tolerant (SIFT) (http://sift-dna.org/dna.org/) and Protein Analysis THrough Evolutionary Relationship (PANTHER) (https://pantherdb.org/tools/csnpScoreForm.jsp) were applied to predict protein function based on amino acid substitution and identify the function of genes based on their evolutionary relationships, respectively.[21,22] The computation of the protein's secondary structure was performed, and the determination of the mutation's stability was achieved through the calculation of the alteration in free energy ($\Delta\Delta G$) (https://biosig.lab.uq.edu.au/dynamut/).[23]

Patients and Blood Samples

The present study involved 50 samples, including individuals who underwent PCR tests and indicated various severe symptoms, such as sore throat, fever, loss of taste, cough, shortness of breath, myalgia, and chest pain. PCR tests were administered to the participants in the research study. Based on the results, they were divided into 2 groups: a positive group consisting of 30 individuals infected with COVID-19, labeled as the sick group, and a negative group of 20 individuals not suffering from the disease, labeled as the healthy control group. It is noteworthy to mention that the samples were selected randomly and the individuals under study had not yet received any vaccination. The peripheral blood laboratory findings were obtained from both the patient and the control groups. These samples were obtained from individuals at 2 affiliated hospitals of the Zahedan University of Medical Sciences (ZUMS), namely Bu-Ali and Ali Ibne Abitaleb Hospitals, Zahedan, Iran. All blood specimens (3–5 mL) were collected in EDTA tubes to facilitate further experiments.

Relative Real-time Quantitative PCR Analysis

Total RNA was extracted from the peripheral blood samples using the Total RNA Extraction Kit (Parstous, Iran) following quantity, purity, and integrity of total RNAs were measured by a ScanDrop spectrophotometer (Analytik Jena), and the electrophoresis on 1% agarose gel, respectively. Then, to prevent DNA contamination, total RNA was treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA), and first-strand cDNA synthesis was performed using the Easy cDNA Synthesis Kit (Parstous, Iran) according to the manufacturer's instructions. The mRNA expression levels of *ND1/2/3/4/4L/5/6*, *ATP6/8*, *CO1/2/3*, and *CYB* in patient samples were compared with those in healthy control as reference samples by relative real-time PCR using SYBR Green (RealQ Plus 2x Master Mix Green, High Rox, AMPLIQON, Denmark) on an Applied Biosystems StepOne Real-Time PCR System thermocycler (Thermo Fisher Scientific, USA). To normalize the data, the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control, and the mRNA expression level of the gene of interest (GOI) was calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were carried out in duplicate. The optimal thermal profile, specific primer sequences, and amplicon size for each gene are displayed in Supplementary Table 2.

Statistical Analysis

All statistical analyses were performed using SPSS (IBM Corp, version 22; Armonk, NY, USA) and the R programming language (version 4.0.3). The Kolmogorov-Smirnov test was initially employed to assess the conformity of data to a normal distribution. The comparisons of variables and the differences in frequency of variants between individuals with the disease and those who are healthy were performed using chi-square, Mann-Whitney, or Fisher's exact tests. The probable correlation between variants in the 2 groups was assessed by the chi-square test. Differences were considered statistically significant at p values<0.05. The measurement of the predictive efficacy of independent factors was performed through the use of receiver operating characteristic (ROC) curves for COVID-19 severity. The area under the curve (AUC) value ranges from 0 to 1, indicating diagnostic power based on favorable (0.9<AUC≤1), appropriate (0.8<AUC≤0.9), acceptable (0.7<AUC≤0.8), weak (0.6<AUC≤0.7), and less (AUC≤0.6).

RESULTS

Distribution of Variants in Mitochondrial DNA

The genome sequence of the human mitochondria that was employed for detecting mutations was the Cambridge Reference Series (https://mitomap.org). The mutations, nucleotide positions and exchanges, amino acid alterations, and type of mutations detected in mitochondrial-encoded genes are shown in Table 1. In total, 8 missense mutations in the *ND1*, *ND5*, *CO3*, *ATP6*, and *CYB* genes were detected in COVID-19 patients. The T4216C and A8860G mutations were missense types, causing amino acid alteration in the *ND1* and *ATP6* genes, respectively. Six mutations were nonsynonymous substitution types in *CO3* (G9942A and C9555T), *ND5* (G13477A and A12418T), and *CYB* (C14766T and A15326G), which alter the amino acid sequence in COVID-19 patients.

Results of In Silico Analysis Predicting the Effects of Human Mitochondrial-encoded Gene Variants

We utilized various in silico variant prediction tools, such as MutPred2, MUTPro, I-Mutant2:0, PolyPhen-2, SIFT, PANTHER, and ΔΔG, to predict the functional impacts of the mitochondrial-encoded genes in COVID-19 patients. As a result of the in silico analysis, we found 2 novel mutations, including C9555T and A12418T in *CO3* and *ND5* genes, respectively, that have various *CYB* effects on COVID-19 disease (Table 2 and Figures 1 and 2). These mutations were predicted to be deleterious and tolerated variants in *CO3* and *ND5* genes, respectively, which cause amino acid alteration in these genes.

Study Population

The clinical and demographic information of all the COVID-19-positive and healthy control individuals are shown in Table 3.

The gender ratio of individuals comprised 20 females and 30 males. The mean age±standard deviation (SD) of patients was 55.4±21.10 years and ranged from 15 to 87 years. The mean age±SD of healthy controls was 48.75±21.28 years and ranged from 13 to 80 years. There was no statistically significant difference in terms of age between the 2 groups (p>0.05).

Table 1. Distribution of mitochondrial DNA gene mutations in COVID-19 patients

Gene	Nucleotide Position	Nucleotide Exchange	Amino Acid Position	Amino Acid Change	Mutation Type
MT-ND1	4216	T → C	304	Y → H	Missense
MT-ATP6	8860	A → G	112	T → A	Missense
MT-CO3	9556	C → T	117	P → L	Missense
MT-CO3	9942	G → A	246	D → N	Missense
MT-ND5	12418	A → C	28	K → Q	Missense
MT-ND5	13477	G → A	381	A → T	Missense
MT-CYB	14766	C → T	7	T → I	Missense
MT-CYB	15326	A → G	194	T → A	Missense

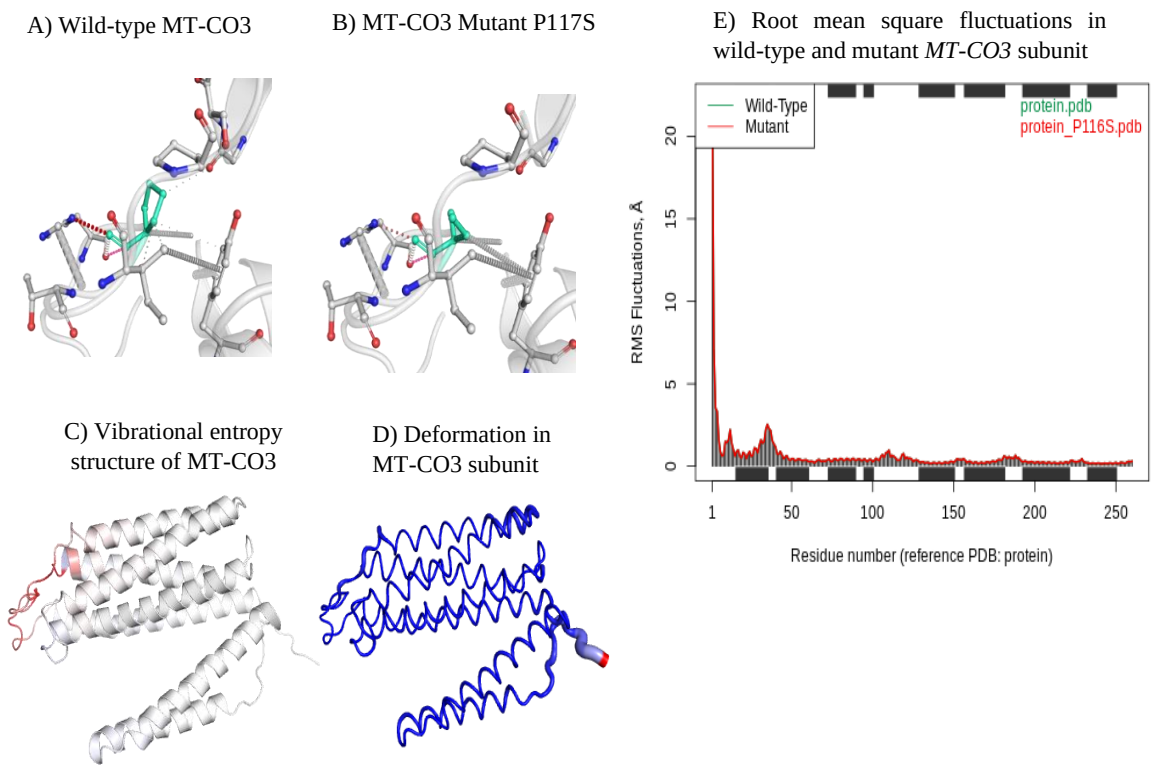


Figure 1. Computational effect of mutation C9556T in the *CO3* gene. (A) Three-dimensional structure of the wild-type MT-CO3 protein subunit. (B) Three-dimensional structure of P117S mutant MT-CO3 protein subunit. The light green area highlights the conformational change in the mutant MT-CO3 in comparison with the wild type. (C) Difference (Δ) in vibrational entropy between wild type and P117S mutant MT-CO3 protein. The blue portion represents rigidification of the structure and the red portion represents increased flexibility. (D) Deformation in MT-CO3 subunit showing disruption of hydrogen, halogen, and hydrophobic bonds at the colored sites. (E) Root mean square fluctuations (RMSF) plot of molecular dynamic simulations comparing the mutant and wild-type proteins.

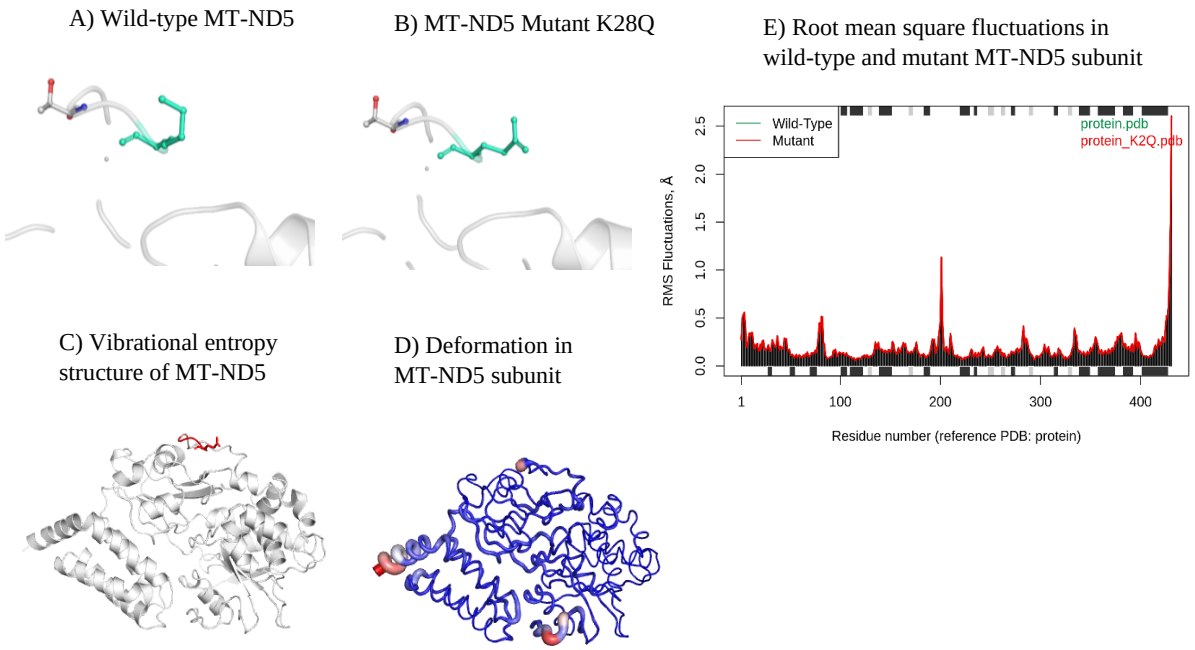


Figure 2. Computational effect of mutation A12418C in the *ND5* gene. (A) Three-dimensional structure of the wild-type MT-ND5 protein subunit. (B) Three-dimensional structure of the K28Q mutant MT-ND5 protein subunit. The light green area highlights the conformational change in the mutant MT-ND5 compared with the wild type. (C) Difference (Δ) in vibrational entropy between the wild type and K28Q mutant MT-ND5 protein. The blue portion represents rigidification of the structure and the red portion represents increased flexibility. (D) Deformation in the MT-ND5 subunit showing disruption of hydrogen, halogen, and hydrophobic bonds at the colored sites. (E) Root mean square fluctuations (RMSF) plot of molecular dynamic simulations comparing the mutant and wild-type proteins.

Table 2. Results of in silico analysis predicting the effects of human mitochondrial DNA variants in COVID-19 patients

Gene	Nucleoid Position	Amino Acid Change	Polyphen-2 Human Probability	SIFT Prediction	PANTHER Probability	I-mutant Prediction	MutPred2 Predication	MUTPro	ΔΔG
MT-ND1	4216	Y → H	Benign	Tolerated low confidence	Probably Damaging	Decrease	Benign	Decrease Stability	−1.046
MT-ATP6	8860	T → A	Benign	Tolerated	Probably Damaging	Decrease	Benign	Decrease Stability	−0.398
MT-CO3	9556	P → L	Probably Damaging	Deleterious	Probably Damaging	Decrease	Potentially Pathogenicity	Decrease Stability	−0.723
MT-CO3	9942	D → N	Probably Damaging	Deleterious low confidence	Probably Damaging	Decrease	High Pathogenicity	Decrease Stability	−1.016
MT-ND5	12418	K → Q	Possibly Damaging	Tolerated low confidence	Probably Benign	Decrease	Benign	Decrease Stability	−1.172
MT-ND5	13477	A → T	Benign	Tolerated	Probably Benign	Decrease	High Pathogenicity	Decrease Stability	−0.898
MT-CYB	14766	T → I	Benign	Deleterious low confidence	Probably Benign	Increase	Benign	Decrease Stability	−0.146
MT-CYB	15326	T → A	Benign	Tolerated low confidence	Probably Benign	Decrease	Benign	Decrease Stability	−0.427

PolyPhen-2: polymorphism phenotyping v2, SIFT: sorting intolerant from tolerant, PANTHER: protein analysis through evolutionary relationships, I-Mutant: I-Mutant prediction, MutPred2: mutation prediction, MUTPro: mutation probability

Table 3. The diagnostic value of blood routine test and general biochemical results in baseline laboratory findings of all included COVID-19-positive and healthy controls

Parameters (reference range)	COVID-19 (+) patients (n=30) (mean±SD)	Control groups (n=20) (mean±SD)	*p*
Age (years)	55.4 ± 21.108	48.75 ± 21.28	0.371
SpO_2	87.46 ± 4.64	98.24 ± 0.909	**0.000**
RBC (4–5.8 million/mm^3)	4.02 ± 0.745	4.93 ± 0.237	**0.000**
MCV (80–95 fL)	83.67 ± 1.993	87.22 ± 2.274	**0.000**
MCH (26–34 pg)	28.87 ± 1.912	30.01 ± 1.482	**0.029**
MCHC (32–36 g/dL)	33.24 ± 1.382	34.295 ± 0.92	**0.004**
Hemoglobin (12–16 g/dL)	10.03 ± 0.973	12.62 ± 0.544	**0.000**
WBC (4–10.6 * 1000/mm^3)	16.69 ± 4.075	6.22 ± 0.891	**0.000**
Lymphocytes (% of WBC)	16.13 ± 2.87	33.02 ± 3.477	**0.000**
Neutrophils (% of WBC)	77.40 ± 2.002	64.35 ± 3.588	**0.000**
Platelets (15–440 * 1000/mm^3)	195.86 ± 61.692	235.0 ± 36.896	**0.007**
Creatinine (0.6–10.4 mg/dL)	1.43 ± 0.31	0.894 ± 0.145	**0.000**
ALT (Up to 40 IU/L)	38.83 ± 10.866	19.15 ± 5.33	**0.000**
AST (Up to 38 IU/L)	40.566 ± 10.63	20.6 ± 5.461	**0.000**
BUN (7–18.6 mg/dL)	22.1 ± 5.867	13.13 ± 3.014	**0.000**

ALT: alanine aminotransferase, AST: aspartate aminotransferase, BUN: blood urea nitrogen, MCV: mean corpuscular volume, WBC: white blood cell count, Cr: creatinine, MCHC: mean corpuscular hemoglobin concentration, SpO_2: oxygen saturation, RBC: red blood cell. *p* values of < 0.05 are considered statistically significant.

The white blood cells (WBC), neutrophils, blood urea nitrogen (BUN), creatinine (Cr), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels of COVID-19 patients were higher than those of healthy control individuals (*p* value>0.05). While, the levels of oxygen saturation (SpO_2), red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), hemoglobin (Hb), lymphocytes, and platelets were higher in healthy control individuals compared to COVID-19 patients (*p*>0.05).

The mRNA Expression of Mitochondrial-encoded Genes in COVID-19 Patients

We investigated the mRNA expression level of 13 mitochondrial-encoded genes in Complexes I, III, IV,

and V of oxidative phosphorylation to test the correlation between COVID-19 infection with altered mitochondrial regulation in healthy individuals and COVID-19 patients. The relative qRT-PCR of *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *CYB*, *CO1*, *CO2*, *CO3*, *ATP6*, and *ATP8* in all COVID-19 patients and healthy control individuals are demonstrated in Figure 3 as a dot plot based on the relative mRNA expression. The mean of the mRNA expression levels is demonstrated in Table 4. The mean fold change of *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, and *ND6* in Complex I in COVID-19 patients were 0.78±1.51, 0.85±1.04, 0.86±0.76, 0.93±1.15, 1.25±1.33, 0.96±1.26, and 0.95±0.87, respectively. As a result, the mean mRNA expression levels were decreased for all genes in Complex I in COVID-19 patients compared to healthy control individuals, suggesting that mRNA expression is altered in COVID-19 patients. Interestingly, it can be observed that the reduction in fold change was greater for *ND4* in comparison to all other subunits that were examined. This observation implies that *ND4* plays a crucial role in the process of oxidative phosphorylation. Moreover, the average fold change of *CYB* in Complex III was 0.93±0.91 in COVID-19 patients compared to healthy control individuals (1.28±0.91).

Additionally, the mean mRNA fold changes were decreased for all genes in Complex IV, including *CO1* (0.82±0.76), *CO2* (0.98±0.62), and *CO3* (0.61±0.51) in COVID-19 patients compared to healthy control individuals. Finally, the average expression levels of *ATP6* and *ATP8* in Complex V were 0.57±0.82 and 0.96±1.21, respectively, in COVID-19 patients compared to 1.51±1.28 and 1.36±1.05, respectively, in healthy control individuals. According to analysis, there was a trend for mRNA expression levels of mitochondrial-encoded genes in Complexes I, III, IV, and V to decrease in COVID-19 patients compared to healthy individuals, suggesting a reduced demand for mitochondrial gene expression during COVID-19 infection progresses.

Table 4. Statistical analysis of mRNA fold changes in COVID-19 patients and healthy control individuals

Gene	COVID-19 patients (n=30) (mean ± SD)	Control group (n=20) (mean ± SD)	*p* value
ND1	0.7890 ± 1.512	1.5518 ± 1.837	**0.001**
ND2	0.8583 ± 1.047	2.2173 ± 3.143	**0.044**
ND3	0.8680 ± 0.766	1.3478 ± 1.033	0.061
ND4	0.9361 ± 1.155	1.5901 ± 1.794	**0.018**
ND4L	1.2515 ± 1.339	1.7309 ± 1.827	0.337
ND5	0.9621 ± 1.266	1.2663 ± 0.815	**0.016**
ND6	0.9522 ± 0.879	1.2311 ± 0.656	**0.047**
ATP6	0.5791 ± 0.820	1.5186 ± 1.282	**0.001**
ATP8	0.9615 ± 1.217	1.3618 ± 1.059	0.089
CO1	0.8270 ± 0.765	1.2996 ± 0.836	**0.038**
CO2	0.9899 ± 0.623	1.4353 ± 1.590	0.482
CO3	0.6179 ± 0.514	1.3759 ± 1.336	**0.005**
CYB	0.9372 ± 0.9101	1.2831 ± 0.916	0.102

SD: standard deviation

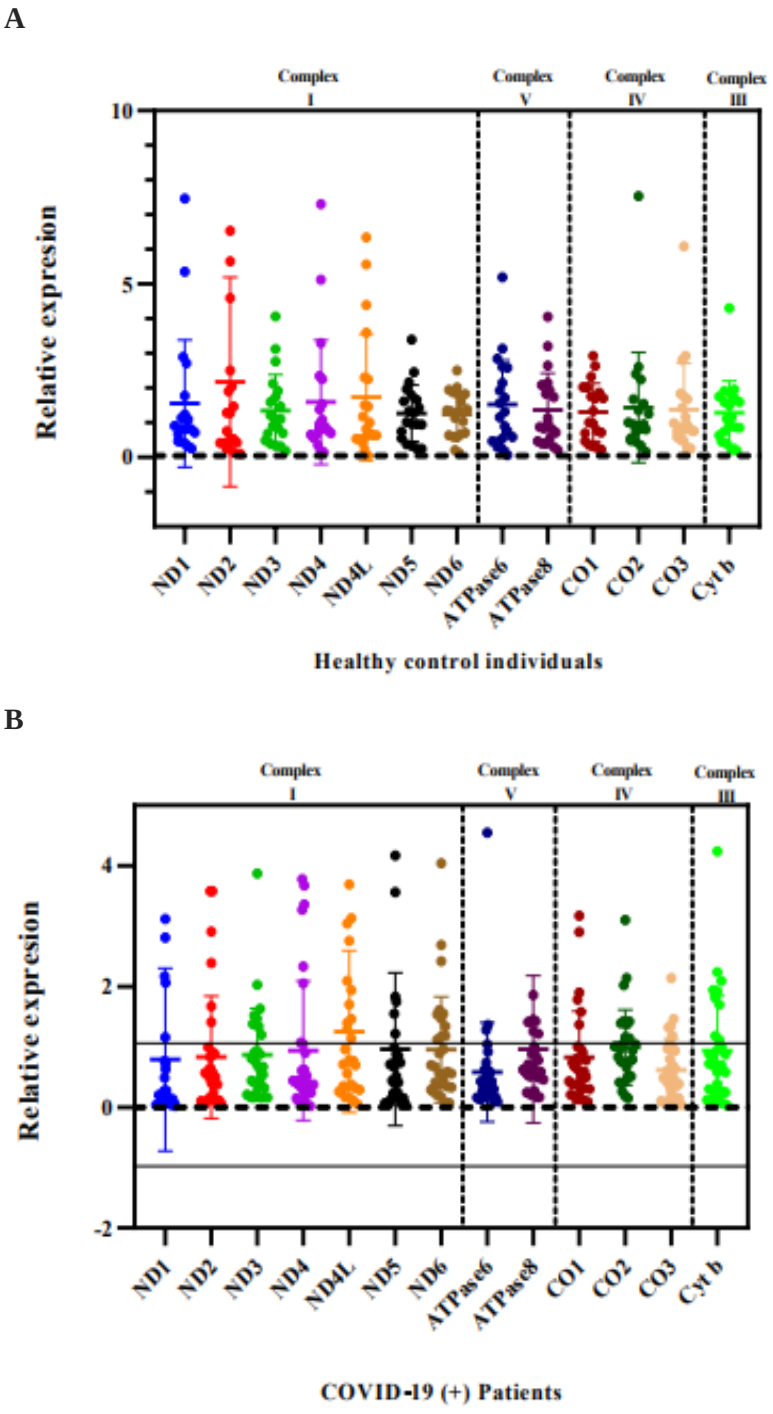


Figure 3. Dot plot represents descriptive analysis of relative gene expression of *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *CYB*, *CO1*, *CO2*, *CO3*, *ATP6*, and *ATP8* in the healthy control individuals (A) and COVID-19 patients (B). The black lines indicate the thresholds for the over- and under-expression. The range between over- and under-expression shows the cases with normal mRNA gene expression.

Clinicopathological Relevance of Mitochondrial-encoded Genes Expression in COVID-19 Patients

The correlation of mitochondrial-encoded transcript expression with COVID-19 patients' clinicopathological features indicated the effect of gene dysregulation on the severity of COVID-19 infection in patients. There were some significant associations between mitochondrial-encoded transcript expression with hematology and biochemistry tests in patients (Table 5).

Association of COVID-19 Patients' Clinicopathological Features Regarding mRNA Expression of Mitochondrial Complex 1-encoded Genes

There was a significant correlation between the mRNA expression of *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, and *ND6* and SpO_2, RBC, MCV, MCHC, Hb, lymphocytes, BUN, and total WBC (p<0.05).

Association of COVID-19 Patients' Clinicopathological Features Regarding the mRNA Expression of Mitochondrial Complex V-encoded Genes

The mRNA expression level of *ATP6* was significantly correlated with SpO_2, RBC, MCV, MCHC, WBC, BUN, lymphocytes, neutrophils, Cr, platelets, and Hb (p<0.05), while the mRNA expression level of *ATP8* was significantly associated with BUN.

Association of COVID-19 Patients' Clinicopathological Features Regarding the mRNA Expression of Mitochondrial Complex III-encoded Genes

There was a significant association between *CYB* expression in COVID-19 patients with MCHC (p<0.05).

Association of COVID-19 Patients' Clinicopathological Features with the mRNA Expression of Mitochondrial Complex IV-encoded Genes

The mRNA expression of *CO1*, *CO2*, and *CO3* was significantly associated with SpO_2, MCHC, WBC, BUN, lymphocytes, neutrophils, platelets, and Cr (p<0.05).

The results showed that the mRNA expression of *CO3*, *ATP6*, and *ND2* has the most correlation with COVID-19 patients' clinicopathological features.

Association of Severity, Age, and Sex of COVID-19 Patients Regarding mRNA Expression of Mitochondrial-encoded Genes

The mRNA expression of most mitochondrial-encoded genes was remarkably associated with disease severity. The expression pattern of *ND1/2*, *ATP6/8*, and *CO3* was significantly associated with the age of COVID-19 patients. Additionally, the expression level of *CO2* showed a significant correlation with the sex of COVID-19 patients.

Prognostic Value of Mitochondrial Markers in Assessing Risk for COVID-19 Patients

Among patients with different severity of COVID-19, the mRNA expression levels of *ND1/2/4/5/6*, *CO1/3*, and *ATP6* were significantly different in COVID-19 patients compared to healthy control individuals (p<0.05) (Table 5). ROC curves were constructed to evaluate the diagnostic value of potential mitochondrial-encoded markers in COVID-19 patients (Figure 4). Among the mitochondrial-encoded markers, *ND1*, *ND2*, *ND3*, *ND4*, *ND5*, *ND6*, *CO1*, *CO2*, *CO3*, and *ATP6* indicated the highest area under the ROC curve (AUC between 0.6 and 0.7, p<0.05).

Table 5. Comparison between the correlation of Mitochondrial-encoded genes with clinicopathological features of COVID-19 patients

Clinicopathological features	Mitochondrial-encoded genes												
	ND1	***ND2***	***ND3***	***ND4***	***ND4L***	***ND5***	***ND6***	***ATP6***	***ATP8***	***CO1***	***CO2***	***CO3***	***CYB***
Severity		0.297[*1] 0.036[2]	0.288[*] 0.042	0.280[*] 0.049			0.378[**] 0.007	0.482[**] 0.000	0.298[*] 0.036	0.305[*] 0.031		0.399[**] 0.004	0.377[**] 0.007
Gender											0.286[*] 0.044		
Age	−0.687[**] 0.000	−0.290[*] 0.041						−0.401[**] 0.004	−0.366[**] 0.009			−0.317[*] 0.025	
SpO_2	0.409[**] 0.003	0.327[*] 0.021	0.301[*] 0.034	0.309[*] 0.029				0.435[**] 0.002				0.382[**] 0.006	
RBC								0.330[*] 0.019					
MCV		0.316[*] 0.025						0.395[**] 0.005					
MCHC	0.315[*] 0.026			0.278 0.051		0.280[*] 0.049		0.369[**] 0.008				0.328[*] 0.020	0.417[**] 0.003
Hemoglobin		0.307[*] 0.030											
WBC		−0.290[*] 0.041						−0.415[**] 0.003				−0.334[*] 0.018	

Table 5. Continued...

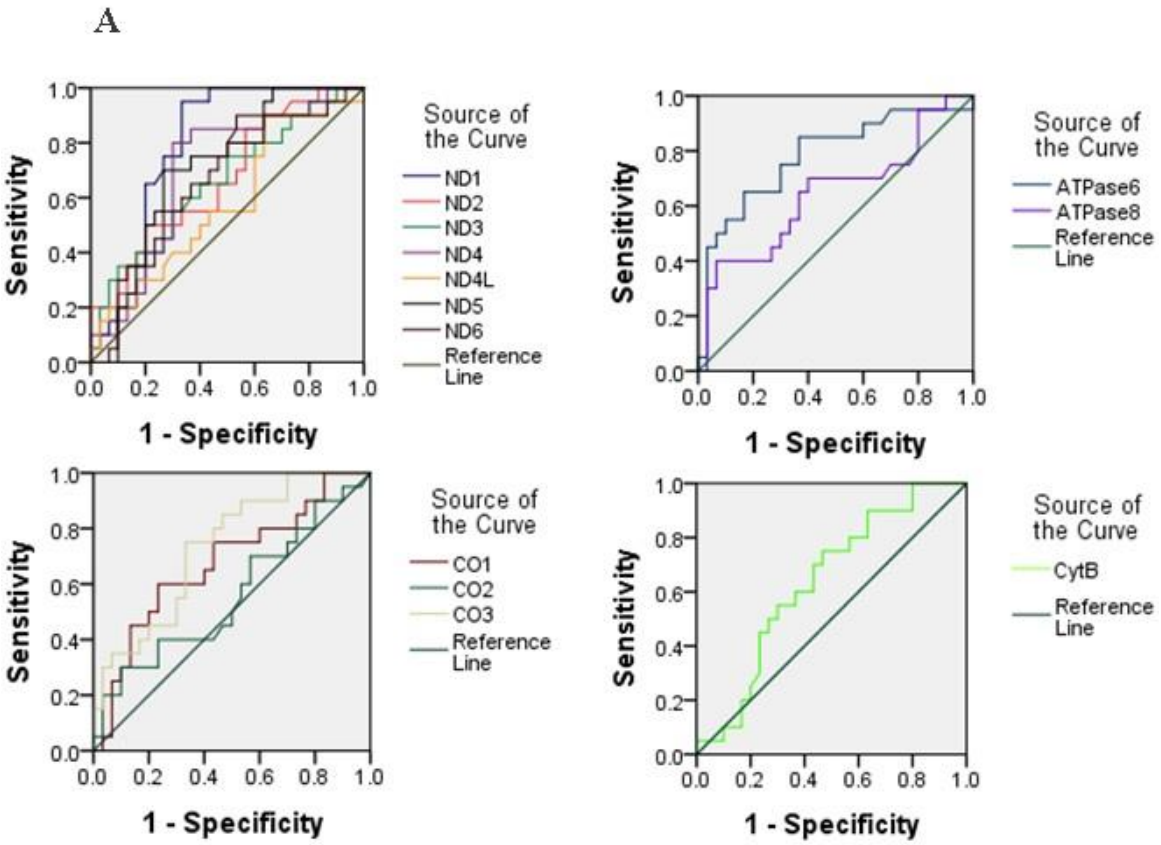
Clinicopathological features	Mitochondrial-encoded genes											
	ND1	***ND2***	***ND3***	***ND4***	***ND4L***	***ND5***	***ND6***	***ATP6***	***ATP8***	***CO1***	***CO2***	***CO3***	***CYB***
Lymphocytes	0.291*	0.424**	0.290*					0.422**		0.344*		0.443**	
	0.040	0.002	0.041					0.002		0.014		0.001	
Neutrophils								−0.284*				−0.288*	
								0.046				0.043	
Platelets								0.415**				0.327*	
								0.003				0.020	
Cr								−0.450**				−0.289*	
								0.001				0.041	
BUN	−0.616**	−0.345*		0.294*	−0.300*		−0.281*	−0.494**	−0.424**	0.388**		0.420**	
	0.000	0.014		0.038	0.034		0.048	0.000	0.002	0.005		0.002	

BUN: blood urea nitrogen, Cr: creatinine, LYM: lymphocytes, MCHC: mean corpuscular hemoglobin concentration, MCV: mean corpuscular volume, RBC: red blood cell count, SpO_2: oxygen saturation, WBC: white blood cell count

*Correlation is significant at the 0.05 level.

**Correlation is significant at the 0.01 level.

Significant correlation is based on [1]Pearson correlation and [2]Sig. (two-tailed)



	Gene	AUC	Std	*p*
Complex I	*ND1*	0.789	0.065	0.001
	ND2	0.669	0.077	0.044
	ND3	0.658	0.079	0.061
	ND4	0.698	0.077	0.018
	ND4L	0.581	0.083	0.337
	ND5	0.703	0.074	0.016
	ND6	0.668	0.078	0.047
Complex V	*ATP6*	0.781	0.070	0.001
	ATP8	0.643	0.083	0.089
Complex IV	*CO1*	0.675	0.079	0.038
	CO2	0.559	0.086	0.482
	CO3	0.735	0.070	0.005
Complex III	*CYB*	0.638	0.079	0.102

Figure 4. Receiver operating characteristic (ROC) curve of mitochondrial markers for diagnosis of COVID-19 patients. The area under the curve (AUC) value ranges from 0 to 1, which indicates the diagnostic power: favorable ($0.9 < AUC \leq 1$), appropriate ($0.8 < AUC \leq 0.9$), acceptable ($0.7 < AUC \leq 0.8$), weak ($0.6 < AUC \leq 0.7$), and less ($AUC \leq 0.6$).

DISCUSSION

Mutations in human mtDNA are associated with mitochondrial dysfunction, aberrations in oxidative energy metabolism, and modifications in the encoded proteins.[24,25] Mitochondrial dysfunction has been shown to impact various cellular processes, including metabolism, calcium regulation, gene and protein balance, oxidative stress, apoptosis, homeostasis, and senescence.[26,27] A variety of viruses have a significant effect on the function of mitochondria, metabolic processes, and signaling of innate immunity.[26] Considering the undeniable role of mitochondria in innate antiviral immunity and inflammation, its function has also been proven in COVID-19 pathogenesis.[28] Therefore, we have investigated the significance of mtDNA mutations and variants and the expression level of mitochondrial-encoded genes in blood samples of COVID-19 patients compared to healthy control individuals, which may hold diagnostic and prognostic value for COVID-19 patients.

In this study, we identified 8 variants in several regions of the mitochondrial genome, including *ND5*, *CO3*, *ATP6*, and *CYB* among COVID-19 patients. Among these variants, C9555T and A12418T mutations in *CO3* and *ND5* genes, respectively, were distinct, unique variants. These variants were deleterious for *CO3* and tolerated for *ND5*, which caused amino acid changes in these genes. Additionally, the altered expression of mitochondrial-encoded transcripts, including *ND1/2/4/5/6*, *CO1/3*, and *ATP6* was indicated in all COVID-19 patients compared with healthy control individuals. First, our data demonstrated the decreased mRNA expression levels of mitochondrial transcripts in Complexes I, III, IV, and V during the progression of COVID-19 infection in individuals. Second, there was a significant correlation between altered expression of mitochondrial transcripts with some hematology and biochemistry tests in patients. Taken together, these results can suggest that mitochondrial-encoded genes play an important role in the detection of COVID-19 patients.

The laboratory results were utilized to explain patients' prognosis, although they were nonspecific in relation to the COVID-19 infection. The disease severity is related to the increase of liver enzymes, WBC, creatine kinase, C-reactive protein, troponin, lactate dehydrogenase, lymphopenia, thrombocytopenia, and cytokine secretion.[29,30] In total, there were statistically significant differences in laboratory findings between COVID-19 patients and healthy individuals, as all of them could be introduced as risk factors for disease severity.[31-33] Our laboratory findings indicated higher WBC, lymphocyte, neutrophil, and PLT counts and lower SpO_2, RBC, MCV, MCHC, and Hb counts in COVID-19 patients. Some liver and renal test biomarkers, such as troponin, AST, ALT, and Cr are candidates for predicting the severity of the COVID-19 disease.[34,35]

Consistent with previous studies, our COVID-19 patients had higher levels of laboratory biochemical markers, including Cr, ALT, AST, and BUN than healthy individuals. The results of previous studies provide evidence for the possible role of liver enzymes as independent factors in predicting COVID-19 severity.[36,37] It is noteworthy that these laboratory findings can be utilized for adjunctive diagnosis of COVID-19 patients. Based on the increased levels of AST ALT, BUN, and Cr in our COVID-19 patients, we can propose that the laboratory findings were associated with the disease and can be used as predictors for the severity of the disease. Overall, a significant number of comorbidities and biomarkers are correlated with the severity and manifestations of COVID-19, which affects morbidity, disease severity, mortality, and poor outcomes in patients.[31,38]

Common genetic variants in human mtDNA can disrupt mitochondrial functions, such as transcription, ROS production, and age-related diseases.[39-41] Given that COVID-19 elicits an indirect generation of ROS, it is conceivable that cells from the geriatric population could potentially be subjected to a greater degree of ROS when infected with this virus compared to cells from their younger counterparts.[42]

Many silent mutations do not affect the sequence, structure, and function of proteins.[43] However, it has been revealed that synonymous DNA alterations resulting in silent mutations can have a significant impact on the stability, folding, and gene expression of mRNA, translation rate as well as post-translational modification of proteins.[43-46] It has been reported that there is an association between the frequency of mtDNA mutations and COVID-19 disease.[47,48] According to our data, 2 different mutations in each of the *CO3*, *ND5*, and CYB genes as well as 1 mutation in each of *ND1* and *ATP6* genes were found in COVID-19 patients. It has

been proven that mtDNA mutations affect metabolic parameters and the progression of some diseases; consequently, mutations in *ND1/2/4/5/6*, *CO1/3*, and *ATP6* genes may be involved in COVID-19 infection in patients. The missense mutations were identified in these genes and included G9942A and C9555T (in *CO3*), G13477A and A12418T (in *ND5*), C14766T and A15326G (in *CYB*), T4216C (in *ND1*), and A8860C (in *ATP6*). These data can confirm the impact of COVID-19 infection on mtDNA, where mtDNA mutations and clinical tests can reflect the severity of the disease. Interestingly, 2 unique missense mutations of C9555T and A12418T were observed in patients associated with mitochondrial Complex I and IV genes. The C9555T mutation is potentially pathogenic, probably damaging, and deleterious based on analysis of MutPred2, PolyPhen2, and SIFT scores. While the A12418T mutation is benign and tolerated low confidence based on in silico analysis. Deficiency of mitochondrial respiratory Complexes I and IV leads to changes in morphology, membrane potential, and intracellular calcium homeostasis of mitochondrial, which result in apoptosis, enhanced ROS production, decreased nicotinamide adenine dinucleotide (NADH) enzyme activity, defect in proton pumping process, hypoxic condition, electron leak, and increased Hypoxia-inducible factor 1-alpha (HIF-1α).[15,49,50] As a result of our study along with previous studies, it can be proposed that mtDNA mutations in Complexes I and IV of this organelle in patients with COVID-19 can lead to mitochondrial respiratory chain dysfunction and oxidative damage. All identified mutations have the potential to alter amino acids and secondary conformation of *ND1/2/4/5/6*, *CO1/3*, and *ATP6* proteins, leading to reduced enzymatic functionality and stability as well as effects on ROS and disease severity. Our data are consistent with previous studies that changes in amino acids can potentially cause modifications in proteins that lead to increased or decreased susceptibility towards COVID-19.[51] Moreover, the free energy change was negative for all mutations, suggesting protein instability.[15,48]

Except for the investigation of mtDNA mutations, our study examined mtDNA-encoded gene expression in COVID-19 patients. Our data demonstrated a significant difference in mtDNA-encoded gene expression between COVID-19 patients and healthy individuals, indicating a reduction in mtDNA gene expression in response to COVID-19 infection. The upregulation of nuclear DNA-encoded OXPHOS genes has been observed in COVID-19 patients.[52] These results together with our data propel us to hypothesize that there may be a probable change in OXPHOS gene co-regulation. Alteration in OXPHOS function leads to upregulation of genes encoding glycolysis enzymes and production of a glycolytic environment, production of ROS, and increased cytokine storm in COVID-19 patients.[53] These results can provide evidence for the state of pre-apoptosis and cell death. Moreover, disruption in mtDNA and altered expression of mtDNA-encoded gene can lead to the release of mtDNA into the cytoplasm of the cell, resulting in the secretion of pro-inflammatory cytokines and interleukin (IL)-1β/IL-18 as well as the response of type I interferon.[54] Additionally, our findings indicated that the downregulation of mtDNA gene expression was associated with laboratory findings and the severity of the disease. We also performed an ROC curve analysis to indicate whether the mtDNA gene can be used as an indicator to determine COVID-19 patients from healthy individuals. The cutoff values for *ND1/2/3/4/4L/5/6*, *CO1/2/3*, *ATP6/8*, and *CYB* were significant for the diagnosis of patients. However, the AUC values were between 0.7 and 0.6, indicating weak diagnostic power. Taken together, our data propose the key role of mitochondria in the severity of COVID-19.

In summary, to the best of our knowledge, no publication has focused on the correlation between mtDNA-encoded gene mutations and expression in Iranian COVID-19 patients. The pathogenic mtDNA mutations and altered expression of mtDNA-encoded complex genes in the OXPHOS system in COVID-19 patients alter amino acid structures and subsequently affect protein stability and patients' energy metabolism. Subsequently, these pathogenic variants and altered expression levels of genes along with the laboratory data can provide the key role of mitochondrial reprogramming in COVID-19 patients. Moreover, it could be a viable indication of susceptibility to COVID-19 and provide a way for therapeutic interventions.

STATEMENT OF ETHICS

Procedures were approved by the Sistan and Baluchestan University, Zahedan, Sistan and Baluchestan province, Iran, and according to the 1964 Helsinki Declaration. (Ethics Code: IR.USB.REC.1399.030).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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