Journal of Medical Virology

WILEY

JOURNAL OF MEDICAL VIROLOGY

RESEARCH ARTICLE OPEN ACCESS

Genetic Landscape and Mitochondrial Metabolic Dysregulation in Patients Suffering From Severe Long COVID

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Received: 13 August 2024 | **Revised:** 20 January 2025 | **Accepted:** 19 February 2025

Funding: This study was supported by Aarhus County Research Initiative, Undine, Horizon Europe 2021, and Novo Nordisk Fonden. Novo Nordisk Foundation grants (NNF21OC0066984), (NNF21OC0067157), (NNF20OC0064890 (LØ, THM), Horizon Europe 2021 grant (HORIZON-HLTH-2021-857), (DISEASE-04-07), UNDINE grant 101057100 (THM), Danish Innovation Fund (PASCAL-MID (8056-00010B) (THM)), Danish National Research Foundation (DNRF164 (CiViA)), and Aarhus County Research Initiative (JP, RKJO).

Keywords: bioenergetics | COVID-19 | inborn error og immunity | mitochondria | sequelae | whole genome sequencing

ABSTRACT

Long COVID represents a significant global health challenge with an unclear etiology. Alongside accumulating evidence of mitochondrial dysfunction in patients with acute SARS-CoV-2 infection, a symptomatic overlap exists between long COVID and mitochondrial disorders. However, the genetic underpinnings of mitochondrial dysfunction in long COVID have not been previously explored. We employed whole genome sequencing to analyze 13 patients with severe long COVID to identify genetic defects related to mitochondrial function. We performed extracellular bioenergetics flux analysis on peripheral blood mononuclear cells and proteomics to evaluate cellular bioenergetics and compared the results to those of healthy controls. Our investigation identified 10 variants classified as pathogenic or likely pathogenic and 83 variants of unknown significance affecting a wide range of mitochondria-associated biological functions. Bioenergetics flux analysis in peripheral blood mononuclear cells revealed an altered ATP production rate in four long COVID patients compared to healthy controls. This study presents initial evidence of a potential underlying genetic predisposition to mitochondrial dysfunction in long COVID while demonstrating altered cellular energy capacity in a subset of these patients. These findings open avenues for further research into the role of mitochondrial dysfunction and pathology in patients suffering from long COVID and may pave the way for targeted therapeutic strategies aimed at mitigating mitochondrial dysfunction.

1 | Introduction

Long COVID is defined as the continuation or emergence of new SARS-CoV-2-related symptoms 3 months after initial infection and affects up to 10% of all SARS-CoV-2-infected individuals [1]. The pathogenesis of long COVID and the biological features that distinguish individuals with long COVID from individuals with symptom-free recovery from COVID-19 remain incompletely

understood [2]. Prompted by the similarity of the clinical phenotype of long COVID and mitochondrial disorders and an increased focus on mitochondrial involvement in acute COVID-19 pathology, a hypothesis of prolonged mitochondrial dysfunction as a driver of long COVID has gained attention [3, 4].

Mitochondria play a critical role in cellular physiology and innate and adaptive immunity, and individuals with inherited mitochondrial defects display increased susceptibility to severe infections [5]. COVID-19 is associated with dysregulation of iron homeostasis, electrolyte imbalance, thrombosis, and excessive oxidative stress, all closely related to mitochondrial dysfunction [6, 7]. Congruently, SARS-CoV-2 polypeptides bind to mitochondrial proteins upon infection. Extensive evaluation of mitochondrial genes upon SARS-CoV-2 infection in nasal swaps and autopsy material from humans and a rodent model showed viral suppression of mitochondrial gene transcription. This led to the inhibition of oxidative phosphorylation and rewiring towards glycolysis, which may enhance conditions for viral replications while causing prolonged metabolic disturbances in the host [8]. The severity of acute COVID-19 has been shown to correlate with 15 mutations in mitochondrial DNA (mtDNA), and the mitochondrial function was impaired in the most severely affected patients [9]. Finally, examining membrane potential, morphology, and oxygen consumption, the presence of mitochondrial dysfunction in peripheral blood mononuclear cells in patients with acute COVID-19 has been established [10, 11].

While several genetic inborn errors of immunity (IEI), primarily impairing the production of response to type I interferon (IFN), have been described in patients with critical COVID-19 pneumonia, none have been found underlying development of long COVID [12]. IEI may instigate an impaired antiviral host response and lead to critical COVID-19, but most individuals who develop long COVID did not have severe acute illness [13]. Thus, taking into consideration the symptomatologic spectrum of long COVID and the ability of SARS-CoV-2 to interfere with mitochondrial function and disturb cellular homeostasis and metabolism, the undisclosed pathogenic features of long COVID could be sought in inborn errors of metabolism rather than in host immunity [4].

Few genetic investigations of predisposition to long COVID are available [14–16]. In this study, we hypothesized that mitochondrial dysfunction is a driver of long COVID and sought to investigate the pathogenesis of long COVID from a genetic perspective. For the first time, we here describe whole genome sequencing (WGS) analysis of individuals with severe long COVID, assessing single-gene inborn errors of metabolism.

2 | Materials and Methods

2.1 | Patient Cohort

The patient cohort consisted of 13 participants included at the long COVID Clinic at Aarhus University Hospital, Denmark. All participants had prior polymerase chain reaction (PCR) confirmed SARS-CoV-2 infection and were diagnosed with long COVID by infectious disease specialists. The participants were selected from a previous clinical trial cohort at our study site [17]. Whole blood for isolation of DNA and PBMCs was drawn upon ambulatory contact. The selection criteria for this cohort was severe clinical phenotype defined as symptoms from > 2 organ systems and decreased quality of life as measured by the EQ-5D questionnaire [18]. The EQ-5D provides a validated generic measure of overall health status addressing five dimensions: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression, which are graded from 1 (no problems) to 5 (extreme problems) (See supplementary). Subsequently, a country-specific health state index score ranging from 0 to 1 can be derived from the responses, with 0 representing the worst imaginable health and 1 representing optimal health. Patient symptom data was acquired from medical records and a long COVID-specific questionnaire comprising 32 questions addressing the most prevalent symptoms reported in a Danish cohort of patients [19]. Each response is divided into five levels of severity ranging from 0 to 4 (corresponding to the lowest severity to the highest severity) (See supplementary).

Sex- and age-matched healthy controls were included at Aarhus University Hospital. Blood was drawn for bioenergetic and proteomic analyses, and information about previous SARS-CoV-2 infection and vaccination was obtained.

This project was approved by the Danish National Research Ethics Committee (Project ID 1-10-72-80-20) and the Data Protection Agency and conducted in accordance with the Helsinki Declaration. Following oral and written information, written consent was obtained from all patients before any study procedures.

2.2 | Genome Sequencing

Genomic DNA extracted from whole blood samples was subjected to WGS (P4-9 and P11-12) or whole exome sequencing (WES) (P1-3, P10, and P13) at the Department of Molecular Medicine at Aarhus University Hospital. WGS was performed as follows: Sequencing libraries were prepared with Illumina DNA PCR-Free Prep using 300 ng DNA input. The libraries were pooled and sequenced with Illumina NovaSeq. 6000. Sequencing reads were mapped to the hg38 reference genome using “bwa-mem.” Single nucleotide variants and indels were called with “GATK HaplotypeCaller.” WES was performed as follows: Sequencing libraries were prepared with Twist Library Preparation Enzymatic Fragmentation Kit and 50 ng DNA input. The libraries were pooled with equimolar DNA-input. The exome was captured with the Twist Comprehensive Exome probes with a spike-in probe with 17 additional targets using the Twist Hybridization and Wash kit. The captured libraries were sequenced with Illumina NovaSeq. 6000. Sequencing reads were mapped to the hg19 reference genome using “bwa mem” and PCR duplicates were identified with “Picard MarkDuplicates.” Single nucleotide variants and indels were called with “GATK HaplotypeCaller.”

Variant analysis and filtering were performed in VarSeq. 2.3.0 (Golden Helix Inc, Bozeman, MT). First, variants were filtered based on quality, keeping only high-quality variants. Variants were kept if they had either PASS or missing Variant Quality

Score Recalibration (VQSR) scores (not used on WGS data), variant allele frequency in the sample > 0.25, genotype quality ≥ 20, read depth > 30 (WES) or > 10 (WGS). Second, variants were kept if minor allele frequency < 0.001 in gnomAD exomes. Third, variants were filtered based on deleteriousness and only variants which were loss of function, missense or < 20 bp from splice sites, and fulfilled the American College of Medical Genetics and Genomics (ACMG) classification [20] as pathogenic, likely pathogenic or VUS, and had Combined Annotation Dependent Depletion (CADD) ≥ 15 or missing and Rare Exome Variant Ensemble Learner (REVEL) ≥ 0.25 or missing were kept. Finally, a filter for biological relevance to our hypothesis of mitochondrial dysfunction was applied by means of three gene lists: Human MitoCarta 3.0, the PanelApp Mitochondrial Disorders (v. 4.113), and an in-house list of muscle-related genes [21, 22]. Finally, only variants in genes with a gene damage index above 1 383 953 were kept [23] and variants found in uncertain reads in low-complexity areas were removed. All retained variants underwent thorough manual assessment regarding their impact on gene function and interference with relevant cellular and immune antiviral pathways. Their pathological potential, as documented in existing literature, was considered in a potential association with existing hypotheses on long COVID pathogenesis.

2.3 | Isolation of PBMCs

Blood samples were collected by venipuncture in EDTA tubes, and PBMCs were isolated by differential centrifugation in Ficoll (Cytiva Swedeb AB #17144003) using SepMate tubes according to manufacturer's instructions (STEMCELL Technologies, Catalog #85450). The purified PBMCs were cryopreserved in a solution containing 90% heat-inactivated fetal bovine serum (SERANA # S-FBS-SA-025) and 10% DMSO (Pan Reac Applichem cell culture grade #A3672) and stored in a liquid nitrogen freezer. For proteomics and bioenergetics measurements, cryopreserved PBMCs were thawed in Seahorse XF DMEM Medium, pH 7.4 (Agilent Technologies, MA, USA) and centrifuged at 400xg for 10 min. The yield and viability of PBMCs were determined using via-1 cassettes in image cytometer NucleoCounter-3000 (Chemometec, Allerod, Denmark) according to the manufacturer's instructions. The viability threshold for the inclusion of PBMCs was 90%.

2.4 | Liquid Chromatography-Mass Spectrometry (LC-MS)

Peptide-based proteomics analyses were performed to quantify proteins in PBMC lysates. The analyses were based on 18-plex TMT-labeled (Thermo Scientific) tryptic peptides and performed as previously described [24]. The resulting peptides were analyzed on nanoLC (Easy-nLC 1200, Thermo Scientific) coupled to tandem mass spectrometry (MS/MS) (Q-Exactive HF-X, Thermo Scientific, Bremen). Precolumn (Acclaim PepMap 100, 75 μm × 2 cm, nanoviper, Thermo Scientific) and analytical column (EASY-Spray column, PepMap RSLC C18, 2 μm, 100 Å, 75 μM × 25 cm) were used to trap and separate peptides using a 180 min gradient (4%–40% acetonitrile, 0.1% formic acid). Database searches were conducted in Proteome Discover 3.0 (Thermo Scientific) against the human database downloaded on August 22, 2022 from Uniprot.org. The expression level of each protein was normalized to the mean of a protein pool that was labeled with two different labels and run in each study in parallel. The protein pool was generated with equal amounts of isolated protein from randomly selected six controls and six patients, split into two to label with two different TMT labels, distributed into TMT studies and normalized expression values were used to compare samples across TMT studies. To control for the known ratio compression of TMT quantification, i.e., heterozygous levels are higher than 50%, the patient protein ratios were statistically compared with a cohort of 30 individuals with normal levels [25].

2.5 | Bioenergetic Profile

The PBMCs (220 000 cells/well) were resuspended in Seahorse XF DMEM Medium pH 7.4 (Agilent Technologies, MA, USA, #103575-100) supplemented with 10 mM glucose, 2 mM sodium pyruvate, and 2 mM glutamine (Agilent Technologies, MA, USA, #103577-100, #103578-100, #103579-100), and were seeded to poly-D lysine-coated (50 μg/mL; Sigma-Aldrich, #P6407) Seahorse XFe96 cell culture plates (Agilent Technologies, MA, USA). For comparison, the control PBMCs from age and sex-matched 13 healthy individuals (mean age of 50.1 years, range 24–65 years, 84.6% female) were included in the analysis. Bioenergetics analysis was performed using the Seahorse XF Real-Time ATP Rate Assay Kit (Agilent Technologies; #103592-100), with consecutive oligomycin (1.5 μM, inhibitor of complex V) and rotenone/antimycin A (1.25 μM/1.25 μM, inhibitor of complex I and III, respectively) injections. After the assay, results were analyzed using WAVE Software (Agilent Technologies, version 2.6). Wells with injection troubles and fluctuating measurements were excluded from the analysis per the manufacturer's recommendations. Curated data were exported from WAVE Software using the Seahorse XF Real-Time ATP Rate Assay Report Generator function, and the bioenergetic data were further handled in the resulting Excel sheet. Mitochondrial and glycolytic ATP production rates were calculated based on values from a minimum of 8 wells (median 13.5).

2.6 | Statistics and Bioinformatics

Questionnaire statistics were performed using STATA (v17.0). For the bioenergetics analysis, control, and patient groups were compared using two-tailed unpaired *t*-tests on GraphPad Prism (v.10.2.3). For the proteomics analysis, control and patient groups were compared using two-tailed unpaired *t*-test on Excel (p-val < 0.05 and |log2FC| > 0.20). Individual patients were compared to the control group using the t-dist function in Excel. The enrichment analysis of differentially expressed proteins for the patient group and for each individual patient was performed using (Database for Annotation, Visualization and Integrated Discovery) compared to the background proteome of 2045 quantified proteins [26]. Heatmaps with hierarchical clustering using Euclidean distances were generated in R with the pheatmap function [27]. Principal component analysis was performed using GraphPad Prism (v10.2.3). The fold change distributions of mitochondrial proteins were tested using the

Wilcoxon signed-rank test on the median fold change value against the hypothetical median of 1 in GraphPad. Patients with a 5% change in median values and p-val < 0.05 were considered presenting a regulated mitochondrial proteome.

3 | Results

3.1 | Patients

This cohort consisted of 13 Danish patients of white European descent, who were all directed to the long COVID Clinic upon referral from their general practitioners owing to persistent long COVID symptoms affecting two or more organ systems. The median age within the cohort was 52 years (range 23–66), and 11 out of 13 patients (84.6%) were female. The infection timeline for these patients stretched from March 2020 to November 2021, indicating that the infections were predominantly due to the original Wuhan strain of SARS-CoV-2 and the subsequent Alpha variant, and none had been vaccinated at the time of infection [28]. Notably, despite the long-term complications, none of these patients experienced severe symptoms during the acute phase of the infection, and none required hospitalization. Due to severe muscular fatigue and, in some cases, paresthesia, five of 13 patients were referred to electromyography at the Department of Neurophysiology at Aarhus University Hospital. All five patients had patterns of abnormality leading to a diagnosis of myopathy [29]. Demographic cohort data is summarized in Table 1. Age- and sex-matched healthy controls were included to compare bioenergetic profile and mitochondrial proteome. The median age of this group was 49 years (IQR 44–61), and the mean time from the first SARS-CoV-2 infection to blood draw was 624.9 days (95%CI: 522.5–727.4) (Table S6). This differs slightly from the time from infection to blood drawn in the 13 long COVID patients, which was 774.2 days (95% CI: 655.5–892.8) ($p = 0.12$).

The predominant symptoms in this cohort were physical and mental fatigue, short-term memory loss, hypersomnia and headache, which were all reported in 12 of 13 (92.3%) of the patients. Second, 11 of 13 (84.6%) patients reported concentration difficulties and muscle fatigue. Finally, dyspnea at rest, a frequently reported symptom in the literature, only occurred in three of 13 (23.1%) patients (Figure 1 and Supporting Information S1: Table 1). The mean EQ-5D-5L health index was 0.70 (95% CI: 0.65;0.75), which is significantly lower than the Danish population average of 0.90 ($p < 0.0001$) [30]. The most affected dimension was the ability to perform usual activities, in which all patients had moderate to extreme problems. Only three (23%) reported symptoms of anxiety or depression, making this the least affected dimension. Overall, the EQ-5D revealed significantly reduced quality of life in the patients.

3.2 | Genetic Variants Identified by WGS/WES and Bioinformatics Analysis

WGS/WES analysis of these 13 patients with severe long COVID identified 83 unique genetic variants across 74 genes related to mitochondrial function, of which 10 variants, including *ACOT9, SDHAF3, ALG14, PTCD3, ANTKMT, ATP2A1, FA2H, RARS2, LAMA2*, and *PDF* were classified as pathogenic or likely pathogenic (Table 2 and Supporting Information S1: Table 2). The 10 variants predicted to be pathogenic or likely pathogenic were detected in genes spanning a wide range of mitochondrial functions. Overall, they were categorized in the mitochondrial functions of OXPHOS, fatty acid metabolism and myogenic metabolism (Table 2). The filtering process of genetic variants is described in detail in the methods section and depicted in Figure 2. Within the cohort, an additional search for previously described IEIs associated with severe COVID-19 identified two dominantly inherited, missense variants in *TBK1* and *IRF3* with high REVEL and CADD-scores. While defects in these genes have been associated with severe COVID-19, none of the specific variants identified here have previously been described in relation to severe viral infection (Supporting Information S1: Table 3) [31].

3.3 | Proteomics Characterization of Patients

To assess whether the genetic alterations impacted the proteome, we performed mass spectrometry-based proteomics of PBMCs of patients compared to 13 healthy controls. Sixteen proteins for which genetic variation had been found could be quantitated in the PBMCs. However, no significant alterations were found on the protein level for these genes (Supporting Information S1: Table 4). A complete list of all quantified proteins (total and mitochondrial) can be found in (Supporting Information S1: Table 8)

Principle component analysis of the proteome did not indicate separate clustering of patients and controls (Supporting Information S1: Figure 1), and the functional enrichment analysis did not result in significantly enriched biological processes in the combined patient group compared to the control individuals. These findings were in agreement with the heterogeneity of the cohort. However, a subgroup of patients, P2, P4, and P13, showed significant regulation of the RNA splicing and binding-related pathways (Supporting Information S1: Table 5).

To assess the mitochondrial content of the patient individuals compared to the control group, we analyzed the median fold changes of the 243 quantified mitochondrial proteins (Table 3 and Supporting Information S1: Figure 2A–N) [21]. Overall, the mitochondrial proteomes of the patient and control group were not significantly different. However, on the individual patient level, we detected a downregulation of the mitochondrial proteome in P1, P2, P3, and P13 based on median fold change distributions, an upregulation in P9 (Table 3 and Supporting Information S1: Figure 2).

Statistical analyses of differential regulation of mitochondrial proteins in the patient group compared to the control group yielded three statistically significant mitochondrial proteins (p-val < 0.05 and $|\log2FC| > 0.20$). Interestingly, one of the three differentially regulated proteins was the upregulation of the major mitochondrial antioxidant protein SOD2 (Superoxide Dismutase 2) (Fold change: 1.195, p-value:0.002) in the patient group compared to the control group (Supporting Information S1: Figure 2A). The two other significantly regulated mitochondrial proteins were IVD (Isovaleryl-CoA dehydrogenase), involved in Leucine metabolism, which was upregulated (Fold change: 1.156, *p*-value: 0.034), and COMT

TABLE 1 | Demographics and clinical information of the Long COVID cohort.

Patient ID	Age	Sex	Medical history	Time from acute infection to blood sampling (days)	EQ-5D-5L health index	Predominant symptoms	COVID-19 PCR date	Long COVID diagnosis date
P1	40	F	Mb. Crohn	634	0.568	Hypersomnia, fatigue, myalgia	06.03.2020	18.09.2020
P2	44	F	Healthy	360	0.723	Cognitive impairment, fatigue, headache	27.11.2021	29.03.2021
P3	58	F	Healthy	537	0.732	Insomnia, fatigue, paresthesia	31.05.2020	22.09.2020
P4	50	F	Healthy	906	0.745	Cognitive impairment, myalgia, arthralgia	02.01.2021	28.04.2021
P5	48	F	Hypertension, sleep apnea	900	0.573	Paresthesia, dyspnea, hypersomnia	09.01.2021	28.05.2021
P6	52	F	Migraine	904	0.659	Insomnia, mental fatigue	05.01.2021	11.05.2021
P7	54	F	Rheumatoid arthritis, hypertension	959	0.629	Cognitive impairment, fatigue, pain	05.11.2020	12.05.2021
P8	52	M	Healthy	907	0.788	Mental fatigue, hypersomnia, headache	02.01.2021	21.05.2021
P9	45	M	Healthy	937	0.838	Mental fatigue, impaired memory, headache	02.12.2020	27.04.2021
P10	23	F	Healthy	605	0.680	Fatigue, sleep disturbances, dyspnea	24.04.2020	11.05.2021
P11	56	F	Healthy	911	0.788	Mental fatigue, impaired memory and concentration	29.12.2020	14.06.2021
P12	66	F	Healthy	891	0.639	Cognitive impairment, fatigue, myasthenia	12.01.2021	02.06.2021
P13	52	F	Healthy	613	0.723	Cognitive impairment, myalgia, paresthesia	23.05.2020	06.10.2020

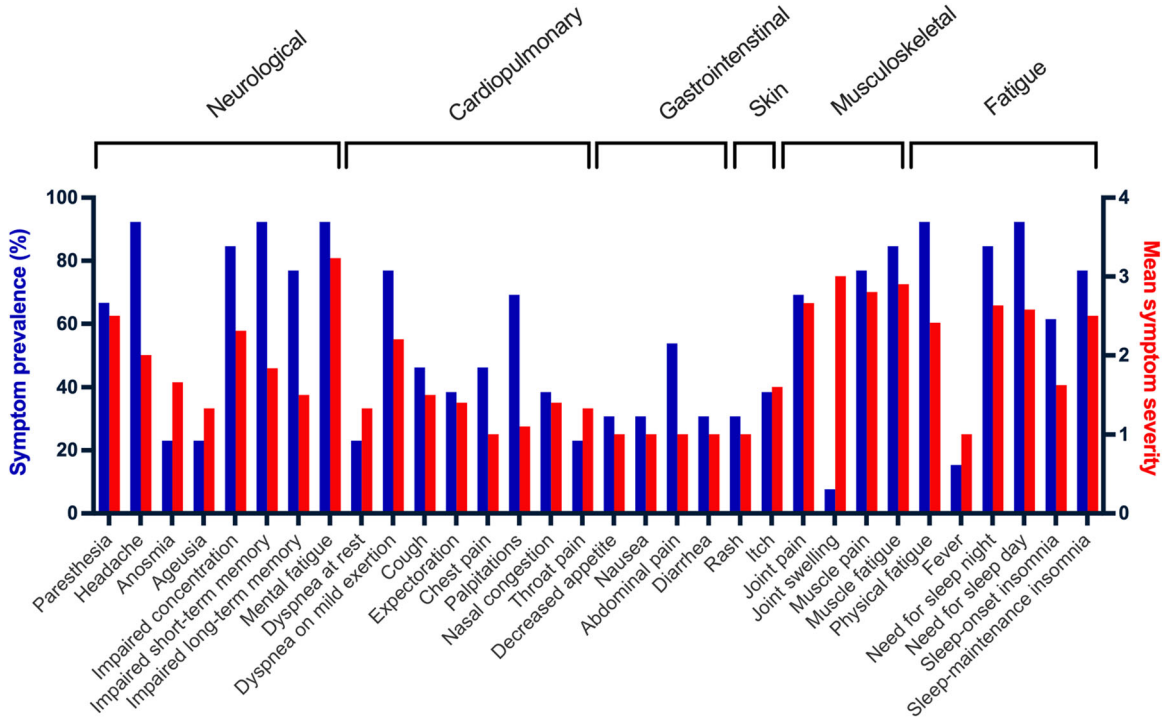

FIGURE 1 | Overview of symptomatology of the Long COVID cohort. The X-axis displays 32 frequent symptoms in Long COVID, which constitutes our Long COVID-specific questionnaire. All patients have responded to the questionnaire, grading the severity of their symptoms from 0 to 4 (0 signifies no symptom, 4 the most severe degree). The left Y-axis depicts the presence of a given symptom in any severity degree. The right Y-axis shows the mean severity degree of symptoms reported for the entire cohort.

(Catechol O-methyltransferase), involved in catecholamines metabolism, which was downregulated (Fold change: 0.857, *p*-value: 0.006) (Supporting Information S1: Figure 2A).

At an individual protein level, several mitochondrial electron transport chain (ETC) subunits were downregulated in P1 (NDUFA10 and UQCRB), P2 (SDHB and NDUFA10), P4 (UQCR10), and P13 (COX5A and UQCRB). GARS1(Glycine--tRNA ligase) and HEBP1 (Heme-binding protein 1) were commonly regulated among patients. GARS1, which is involved in cytosolic and mitochondrial protein translation, was downregulated in P1, P5, and P6; HEBP1 involved in heme detoxification was downregulated in P11 and P12 and upregulated in P6 (Supporting Information S1: Figure 2B–N).

Protein expression levels of quantified immune cell surface or cluster of differentiation CD markers indicated no substantial differences in PBMC composition between the control and patient groups Supporting Information S1: Figure 3). Collectively, these data reflect individual changes in mitochondrial proteins mainly affecting proteins involved in mitochondrial energy function and oxidative stress.

3.4 | Fold Enrichment Analysis of Genetic Variants

The total list of the genes that were detected to have genetic variation in at least one patient contained 1012 genes. Approximately 65% of these genes were only detected in one patient, and there is a high probability that many of these are variants without disease significance. A total of 355 gene variants were carried by two or more patients, and these genes were queried for functionally enriched pathways using the total human genome as a comparative background. The DAVID, Database for Annotation, Visualization, and Integrated Discovery, resource was applied, and 12 functional clusters were overrepresented (Supporting Information S1: Table 7). Figure 3 depicts the fold enrichments of the seven top clusters. Of the seven clusters, the ATP-binding cluster contained the highest number of genes [32] however with the lowest fold enrichment. The genes in the top cluster, Mucin-related genes, had a median detection in five patients, and cluster three with Basement membrane-related genes had a median detection in four patients. The other clusters only had a median detection in only two patients indicating that those genes/pathways are not likely to be main drivers of the phenotype in the studied patient group.

3.5 | Mitochondrial Energy Function Assessment

To investigate cellular bioenergetics, i.e., the relative rate of ATP production from glycolysis and mitochondrial OXPHOS, Seahorse XF real-time ATP rate analysis of PBMCs was performed (Figure 4). As a group, the patients did not differ from the controls in terms of total ATP production, glycolytic ATP production rate, and mitochondrial ATP production rate. Next, a bioenergetic map reflecting mitochondrial-generated ATP versus glycolysis-generated ATP in individual samples was created. P4 shows an evident decrease in both mitochondrial and glycolytic ATP production rates. A small subgroup of

TABLE 2 | Genetic variants related to mitochondrial function predicted to be pathogenic or likely pathogenic. All variants were heterozygous and followed autosomal recessive inheritance. NA = not applicable.

Patient ID	Gene symbol	Gene name	Protein function	Biological process	Transcript ID	Transcript variant	ACMG class	Translation impact	CADD; REVEL scores	gnomAD frequency (%)
P2	*ACOT9*	Acyl-CoA thioesterase 9	Acyl-CoA thioesterase	Fatty acid metabolism	NM_001037171.2	c.1168 C > T	Likely Pathogenic	Stop gained	33; NA	0.000011
P3	*SDHAF3*	Succinate dehydrogenase complex assembly factor 3	Succinate dehydrogenase	OXPHOS	NM_020186.3	c.39 C > G	Likely Pathogenic	Stop gained	34; NA	NA
P5	*ALG14*	UDP-N-acetylglucosaminyl-transferase subunit	Glycosyltrans-ferase	Protein glycosyl-ation	NM_144988.4	c.136+1 G > C	Pathogenic	Splice donor	35; NA	0.000077
P5	*PTCD3*	Pentatricopeptide repeat domain 3	Mitochondrial translation	OXPHOS	NM_017952.6	c.1407delA	Likely Pathogenic	Frameshift	33; NA	NA
P7	*ANTK-MT*	Adenine nucleotide translocase lysine methyltransferase	Methyltrans-ferase	OXPHOS	NM_023933.3	c.102delC	Likely Pathogenic	Frameshift	20,6; NA	0.000000
P9	*ATP2A1*	ATPase sarcoplasmic/ endoplasmic reticulum Ca2+ transporting 1	SERCA ATPase	Myogenic	NM_173201.5	c.100 G > T	Pathogenic	Stop gained	40; NA	0.000045
P11	*FA2H*	Fatty acid 2-hydroxylase	Hydroxylation	Fatty acid metabolism	NM_024306.5	c.1119 A > T	Likely Pathogenic	Stop lost	16,64; NA	0.000031
P12	*RARS2*	Arginyl-tRNA synthetase 2	Transfer of L-arginine to its cognate tRNA	OXPHOS	NM_020320.5	c.442 A > G	Likely Pathogenic	Missense	26,3; 0,565	0.000543
P12	*LAMA2*	Laminin subunit alpha 2	Basement membrane component	Myogenic	NM_000426.4	c.7732 C > T	Pathogenic	Stop gained	47; NA	0.000053
P13	*PDF*	Peptide deformylase, mitochondrial	Peptide deformylation	Protein maturation	NM_022341.2	c.309dupG	Likely Pathogenic	Frameshift	32; NA	NA

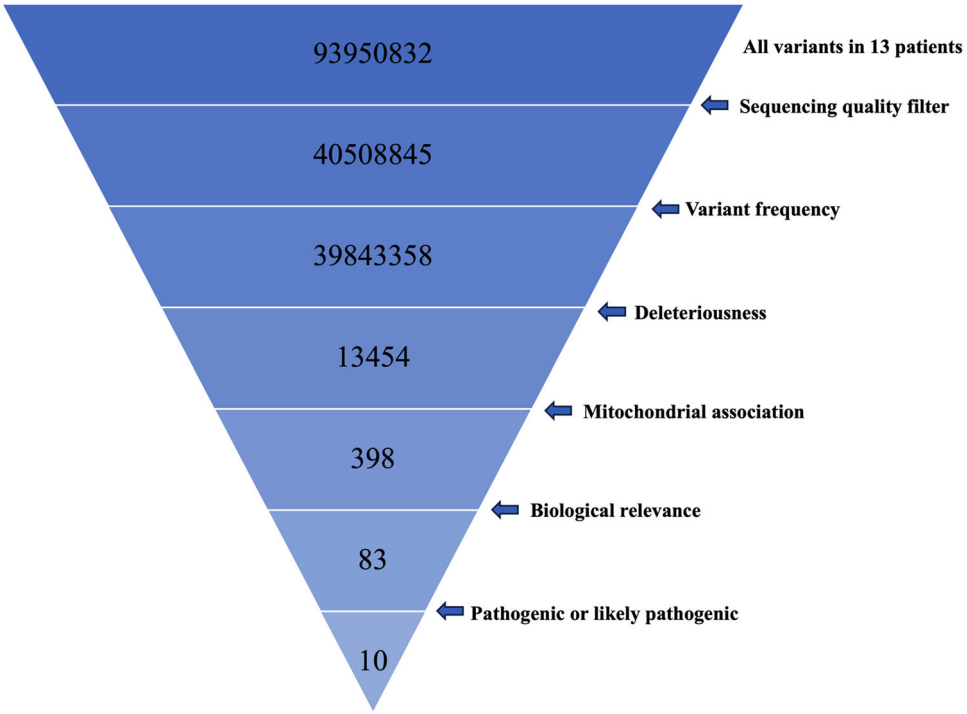

FIGURE 2 | Filtering cascade in whole genome sequencing. This figure provides an overview of the filtering process from the initial total number of variants in the 13 patients suffering from long COVID. Chronologically, we only included variants of high quality, with a frequency $< 0.01\%$, classified to pathogenic, likely pathogenic, VUS, VUS/conflicting, VUS/weak benign, VUS/weak pathogenic or missing in the ACMG sample classifier, with a CADD score > 15 and a REVEL score > 0.25 for missense variants. Only variants related to the mitochondria as defined by the MitoCarta 3.0 and PanelApp mitochondria gene lists were selected, and subsequently employed to manual evaluation for biological relevance (uncertain reads in low-complexity areas removed, variants with high gene damage index removed, see methods) before finally excluding all variants not ACMG-classified as *pathogenic* or *likely pathogenic*.

TABLE 3 | Median fold changes of detected mitochondrial proteins ($n = 243$) in patients ($n = 13$) compared to control individuals ($n = 13$). Changes that correspond to more than a 5% difference compared to the control cohort are indicated in bold. Mitochondrial proteins have an overall tendency to be downregulated in Patient 1, Patient 2, Patient 3 and Patient 13, whereas an upregulation in Patient 9.

Fold changes	Median	25% Percentile	75% Percentile	Range
Patients vs Controls	0.9894	0.9657	1.015	0.3989
Patient 1 vs Controls	**0.9359**	0.8505	1.018	0.9005
Patient 2 vs Controls	**0.918**	0.84	1.017	1.488
Patient 3 vs Controls	**0.9374**	0.8774	1.006	1.119
Patient 4 vs Controls	1.024	0.9489	1.108	1.661
Patient 5 vs Controls	1.005	0.9134	1.114	0.9718
Patient 6 vs Controls	1.012	0.9066	1.128	1.453
Patient 7 vs Controls	1.035	0.9646	1.102	0.687
Patient 8 vs Controls	1.011	0.9612	1.068	0.7698
Patient 9 vs Controls	**1.051**	0.9869	1.119	0.8536
Patient 10 vs Controls	1.004	0.9522	1.047	0.52
Patient 11 vs Controls	0.9964	0.9501	1.048	0.7327
Patient 12 vs Controls	1	0.9319	1.085	0.7764
Patient 13 vs Controls	**0.8639**	0.8012	0.9729	1.396

patients (P6, P8, P13) shows a slightly increased mitochondrial ATP production rate, while the rest of the patients (P1, P2, P3, P4, P5, P7, P9, P10, P11, P12) show ATP production rates similar to the control individuals (Figure 4). A common characteristic among the patients is that they carry one or more rare variants in genes controlling the synthesis or maintenance of ETC proteins. While individual heterozygous variants might each have mild or benign effects on mitochondrial function, it is

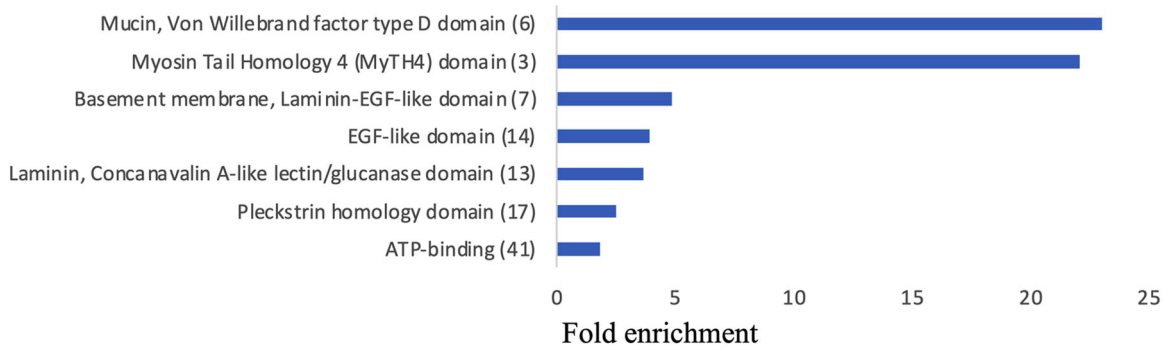

FIGURE 3 | Fold enrichment of the genes with genetic variants in two or more patients. The 355 genes with detected gene variants in two or more patients were searched for functional overrepresentation in DAVID (https://davidbioinformatics.nih.gov/). The number within the bracket is the number of genes in the respective cluster.

possible that the combined impact can decrease ATP production due to severe functional impairments, as seen in patient P4, who carries individual missense mutations in the *POLG*, and *MIPEP* genes, which control the replication and maturation of ETC proteins, respectively (Supporting Information S1: Table 2). In others, the gene defects may be milder or trigger compensatory mechanisms, such as enhanced mitochondrial biogenesis or optimized ETC function, that maintain or even increase ATP production, as seen in patients P6, P8, and P13, who carry variants in one or two genes essential for ETC integrity and function. Both hypo- and hypermetabolic states of cellular ATP rate can lead to dysfunction by impairing the cell's metabolic flexibility and adaptability to environmental changes [33].

4 | Discussion

In this effort to establish patterns of underlying genetic predisposition for the development of long COVID, we performed comprehensive genomic analysis with a focus on genes related to mitochondrial function on 13 patients with a severe long COVID phenotype dominated by neurological and muscular symptoms/myopathy. We identified 10 heterozygous variants classified as pathogenic or likely pathogenic and categorized these genes into OXPHOS-, fatty acid metabolism-, and muscle function-related genes. To obtain functional evidence of disturbed mitochondrial metabolism, we performed Seahorse XF real-time ATP rate analysis of PBMCs, which revealed changes in bioenergetic function in 4 of the 13 patients. Notably, in P4, who has severely impaired mitochondrial function with a very low mitoATP production, we found two variants related to OXPHOS (in *MIPEP* and *POLG*), revealing that the patient with cumulative hits in different OXPHOS-related genes displays the lowest mitoATP production. Proteomic analysis revealed changes in proteins involved in mitochondrial antioxidant and energy function with P1, P2, P4, and P13 showing downregulation of ETC proteins. The mitochondrial proteome was downregulated in P1, P2, P3, and P13, whereas P9 showed signs of an upregulated mitochondrial proteome. Altogether, we here provide a list of multiple potentially pathogenic variants in genes related to mitochondrial function and metabolism together with functional data, which together support a hypothesis of underlying minor mitochondrial abnormalities that may become functionally important during and after cellular stress implied by acute SARS-CoV-2 infection in susceptible individuals.

Only a few other studies have addressed a genetic disposition to develop long COVID. In one study, WGS was performed to assess blood DNA methylation data in correlation with long COVID symptoms [16]. While they report that blood DNA methylation levels may identify and stratify long COVID severity, none of the variants detected in our cohort are directly linked to DNA methylation. Another study identified 11 genetic variants in *POLG* through next-generation sequencing for 494 genes in long COVID patients, as in our P4 with severely compromised mitochondrial function [14]. Finally, a recent study applied combinatorial analysis to detect 73 genes associated to long COVID phenotype. While no variants in the 73 genes have been detected in our cohort, there is an interesting overlap, as the study identifies *RYR3* and *ACOT12* genes associated with long COVID [15]. *RYR1* variants were found in P6 and P13 and a likely pathogenic variant of *ACOT9* was detected in P2. Thus, while the literature in this area is still scarce, few functional overlaps already exist.

Heterozygous genetic variants can lead to haploinsufficiency, implying that a single copy of a given gene is inadequate to maintain the proper function of this gene at the level of the cell and organism. All variants in our analysis are heterozygous, which is unsurprising, as the majority of the described genetic defects in this study would be incompatible with life (or cause neuromuscular syndromes with high penetrance and early disease manifestations) if homozygous. Overall, the patients in this cohort present very few comorbidities and have generally lived lives free of serious disease up until COVID-19. This suggests a metabolic disturbance, potentially genetically determined, in a susceptible individual, triggered during viral infection. SARS-CoV-2 causes downregulation of host antioxidant pathways, resulting in excess of reactive oxygen species (ROS). While a certain level of ROS is necessary for mitochondrial energy production and host immunity, excess ROS has the opposite effect and may lead to organelle and cell damage [34]. Thus, oxidative stress during SARS-CoV-2 infection could potentially exacerbate or trigger a predisposition to metabolic dysfunction, leading to the manifestation of clinical disease. Accordingly, global proteomic investigations of immune cells in our patient cohort showed SOD2 encoding the mitochondrial superoxide dismutase 2 to be the most significantly regulated protein. SOD2 converts superoxide radicals (O_2^-), mainly released from the respiratory

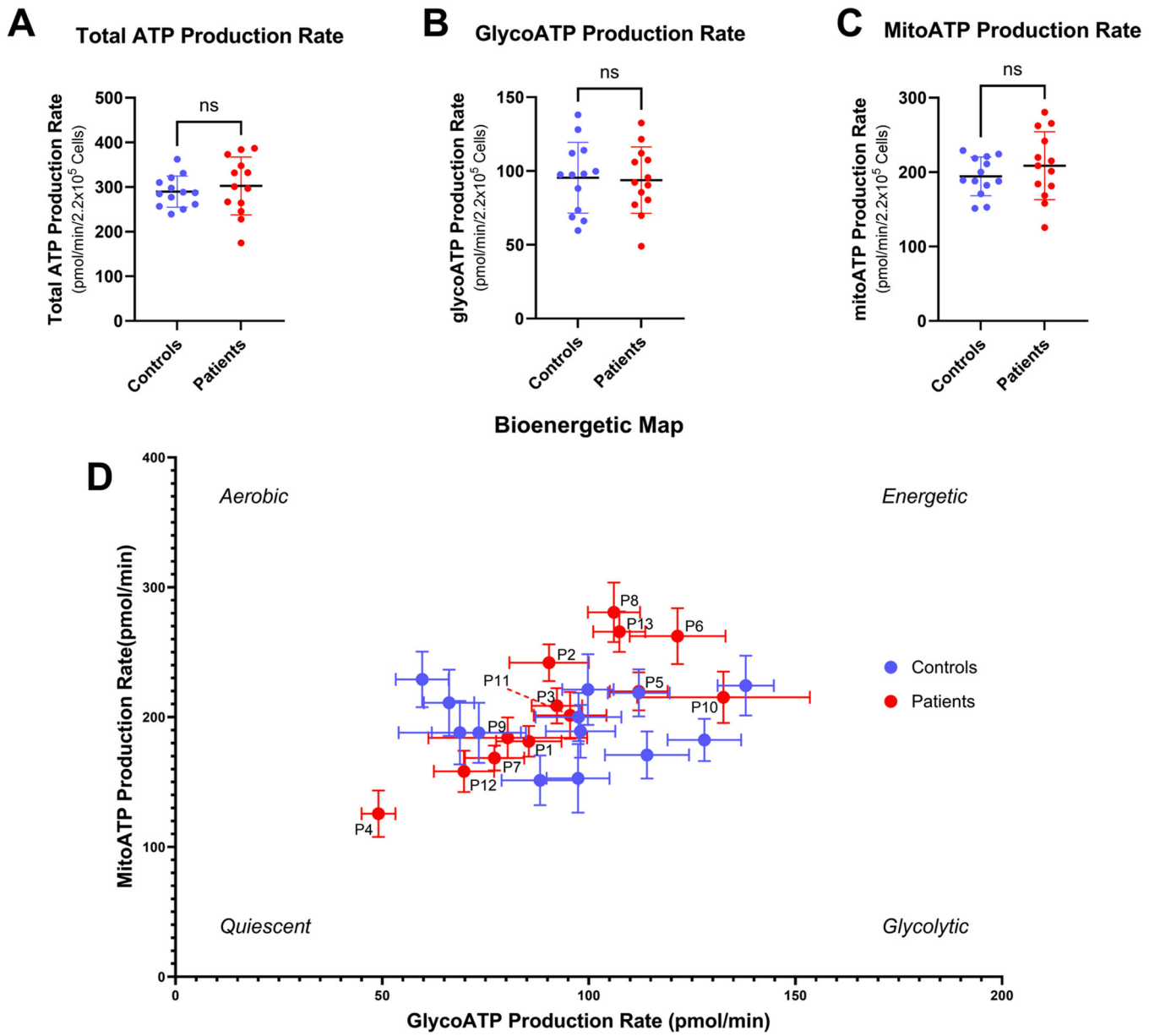

FIGURE 4 | Seahorse XF real-time ATP rate analysis of PBMCs. ATP production rate in the patient group (n = 13) as compared to the control group mean ($n = 13$), represented as (A) Total ATP production, (B) Glycolytic ATP production, and (C) Mitochondrial ATP production. Lines indicate mean, and error bars are shown as ±SD values. The control individuals (blue) and patients (red) were measured in at least eight replicates. An unpaired *t*-test was performed between the patient and the control values. (D) Bioenergetic map with mitochondrial ATP production versus glycolytic ATP production. Shown are means ± SD of at least eight replicates of individual patient samples as compared to the individual control values. (SD = standard deviation, ns = not significant, P# = Patient ID).

chain during energy synthesis, into hydrogen peroxide (H_2O_2), preventing mitochondrial oxidative damage. While SOD2 is essential for protecting immune cells from oxidative stress, excessive SOD2 activity can cause an imbalance in ROS levels that impair immune cell activation, signaling, and pathogen killing. This disruption can weaken immune responses, delay infection resolution, and contribute to immunosuppressive conditions [35–37]. A recent study on patients developing myalgic encephalomyelitis following prolonged recovery from mononucleosis showed that they, before infections, had dysregulated metabolic and antioxidant function that might have impaired their ability to manage the virus effectively [38]. A possible link between genetics and pre-illness disturbances was not investigated.

Our hypothesis does not rely on one common gene defect in the vast long COVID population with an incidence of up to approximately 10% in all SARS-CoV-2-infected individuals, which is why extremely rare, single genetic defects are unlikely to be the main cause of the disease [1]. IEI as well as inborn errors of metabolism are traditionally considered to be "individually rare and collectively numerous." With the discovery of enhanced diagnostic tools, the prevalence of inborn errors of metabolism is rising in children and young adults [39]. This is relevant in light of the multifactorial nature of long COVID with diverse symptomatology and differing recovery trajectories, where the common denominator may be one of many inborn errors of metabolism—whereby individuals with genetic

predisposition develop symptoms after severe cellular oxidative stress caused by SARS-CoV-2-infection [6]. An emerging theory termed "synergistic heterozygosity" further supports this. In this complex pathogenesis, multiple heterozygous variants related to the same metabolic pathway cause mitochondrial flux impairment and thus mimic a monogenic disease [40]. First, inherited mitochondrial disease may be the result of not only one but several genetic hits. Second, it is possible that development of long COVID results from a combination of one or more genetic hits together with environmental factors, such as microbiome composition, hormonal status, comorbidity, and psychiatric/psychological profile etc. All of these possible etiological factors unfortunately can not be addressed in the present study, where we focused on the influence of genetics and mitochondrial metabolic disturbances. Our findings resonate with this, as several patients exhibited multiple hits in the electron transport chain, suggesting that such cumulative defects could be a risk factor for developing long COVID.

Only a subset of individuals exposed to SARS-CoV-2 develop long COVID, indicating a heightened vulnerability to dysregulated immune cell responses following infection. We found no patient- or control-specific clustering based on detected CD markers. Cell pathways affected in these patients with relation to mitochondrial alterations might include oxidative stress, cell death and apoptosis, NF-kB activation, and inflammation mediated by cytokines and interferons. Alternatively, factors secreted by infection-affected cells across various tissues may amplify or prolong the host's immune responses, or cause metabolic decompensation with tissue-specific functional consequences. In either case, individual genomic variants, which become functional when an individual is exposed to a new virus, could play a role. As mitochondria are essential for controlling the proper function and responsiveness of the immune system, we hypothesized that genetic variants in mitochondrial genes could play a role. In support of this theory, patients with inborn errors of metabolism have dysregulated immune responses and are less able to tolerate an infection [32, 41, 42].

The present study is not without limitations. Studies applying WGS/WES often contain small sample sizes due to analysis complexity and cost intensity. However, while providing valuable insight, our cohort of 13 patients with severe long COVID disease presentations does not capture the full genetic diversity and the range of mitochondrial genetic defects associated with the long COVID. Further, while our cohort selection was based on a broad clinical phenotype to address severe long COVID, it may not be representative for the large body of long COVID patients worldwide. This study is also limited by the general technical limitations of WGS/WES, making us unable to address e.g. epigenetic changes. Moreover, important insights might be overlooked by excluding genetic variants in pathways beyond the defined search parameters, potentially obscuring a more complete genetic profile of long COVID. Finally, we performed a Seahorse analysis as a common functional assessment. Seahorse analysis provides a snapshot of mitochondrial function, which might not capture the complexity of how a specific gene variant affects the broader aspects of mitochondrial biology. Moreover, a cell can compensate for changes in ATP, and variants might have different impacts on bioenergetics in different cell types due to varying metabolic environments and demands.

While the identified genes present compelling candidates for further study, optimally, they should undergo individual functional assessment and evaluation to define causality and explore interplay with other variants within the given patient. While this thorough individual functional evaluation is extremely resource and time-consuming, this is the optimal procedure for verifying a pathogenic defect in a given condition. The overall frequency of inborn errors of metabolism in long COVID patients should be addressed in a larger cohort study. Furthermore, evaluation of inheritance patterns of mitochondrial-related genetic variants in family members of patients with long COVID or in family clusters could provide valuable insight. Looking to the future, large-scale studies are needed to explore the therapeutic potential of targeting mitochondrial dysfunction in long COVID. Longitudinal studies could also provide valuable insights into the dynamics and progression of mitochondrial pathology and dysfunction in long COVID.

In conclusion, our study addresses two highly debated areas of long COVID pathogenesis: genetic susceptibility to developing the disease and mitochondrial dysfunction as the driver of fatigue and neurological symptoms. While we have provided initial insight into the genetic landscape of long COVID, a complete map of the pathophysiological mechanisms in long COVID is still needed to improve understanding of the pathogenesis of long COVID. We hope this research paves the way for more targeted and effective interventions to aid those suffering from the long-term effects of COVID-19.

Author Contributions

Kristoffer Skaalum Hansen, Johan Palmfeldt, RKJ and Trine H. Mogensen designed the study. Kristoffer Skaalum Hansen included participants. Sofie Eg Jørgensen, Cagla Cömert, Johan Palmfeldt, Rikke Katrine Jentoft Olsen and Kristoffer Skaalum Hansen conducted the experiments and analyzed the data with participation from Rikke Katrine Jentoft Olsen and Trine H. Mogensen. Kristoffer Skaalum Hansen and Trine H. Mogensen wrote the first draft of the manuscript. All authors critically read, revised, and approved the final version of the manuscript.

Acknowledgments

We warmly thank the patients for participating in the study. We thank the Department of Molecular Medicine, Aarhus University Hospital for the assistance with whole genome sequencing. We thank Stine Christensen from Research Unit for Molecular Medicine, Aarhus University for her assistance with bioenergetic analyses. Novo Nordisk Foundation grants NNF21OC0066984, NNF21OC0067157, NNF20OC0064890 (LØ, THM), Horizon Europe 2021 grant HORIZON-HLTH-2021-857, DISEASE-04-07 UNDINE grant 101057100 (THM), Danish Innovation Fund, PASCAL-MID (8056-00010B) (THM), Danish National Research Foundation, DNRF164 (CiViA), and Aarhus County Research Initiative (JP, RKJO).

Conflicts of Interest

The authors declare that the research was conducted without any commercial or financial relationships that could potentially create a conflict of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due

to privacy or ethical restrictions. The datasets in this article cannot be openly distributed due to restrictions imposed by Danish GDPR regulations. These regulations prevent the dissemination of complete human genetic sequences in a public repository. Data set and sequences will be shared with relevant individuals and according to ethical permission by contact to the corresponding author.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.