Molecular Genetics and Metabolism Reports 31 (2022) 100877

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: www.elsevier.com/locate/ymgmr

A novel *COQ7* mutation causing primarily neuromuscular pathology and its treatment options

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ARTICLE INFO

Keywords:
Coenzyme Q
CoQ_{10}
Ubiquinone
COQ7
CoQ deficiency
2,4-dihydroxybenzoic acid

ABSTRACT

Coenzyme Q_{10} (CoQ_{10}) is necessary as electron transporter in mitochondrial respiration and other cellular functions. CoQ_{10} is synthesized by all cells and defects in the synthesis pathway result in primary CoQ_{10} deficiency that frequently leads to severe mitochondrial disease syndrome. CoQ_{10} is exceedingly hydrophobic, insoluble, and poorly bioavailable, with the result that dietary CoQ_{10} supplementation produces no or only minimal relief for patients. We studied a patient from Turkey and identified and characterized a new mutation in the CoQ_{10} biosynthetic gene *COQ7* (c.161G > A; p.Arg54Gln). We find that unexpected neuromuscular pathology can accompany CoQ_{10} deficiency caused by a *COQ7* mutation. We also show that by-passing the need for COQ7 by providing the unnatural precursor 2,4-dihydroxybenzoic acid, as has been proposed, is unlikely to be an effective and safe therapeutic option. In contrast, we show for the first time in human patient cells that the respiratory defect resulting from CoQ_{10} deficiency is rescued by providing CoQ_{10} formulated with caspofungin (CF/CoQ). Caspofungin is a clinically approved intravenous fungicide whose surfactant properties lead to CoQ_{10} micellization, complete water solubilization, and efficient uptake by cells and organs in animal studies. These findings reinforce the possibility of using CF/CoQ in the clinical treatment of CoQ_{10}-deficient patients.

1. Introduction

Primary coenzyme Q_{10} (CoQ_{10}) deficiency is a rare inborn error of metabolism that is caused by defects in CoQ_{10} biosynthetic genes [1–3]. Coenzyme Q_{10}, aka ubiquinone (UQ), is an essential, endogenously synthesized, highly lipophilic molecule. It is composed of a benzoquinone ring that is attached to a polyisoprenyl tail with 10 repeats in humans. The most pivotal function of CoQ_{10} is as an electron carrier in the mitochondrial electron transport chain [4]. CoQ_{10} deficiency results in dysfunction of mitochondrial energy metabolism and its downstream consequences [5,6]. Thus, CoQ_{10} deficiency is primarily a mitochondrial disorder. In addition to its indispensable role in mitochondrial oxidative phosphorylation, CoQ_{10} is also known to have antioxidant properties and participates directly in several other cellular processes [7,8]. The involvement of these other functions of CoQ_{10} in the pathogenesis of deficiency is not yet understood.

The CoQ biosynthetic pathway is highly conserved [1,9]. 11 genes have been described to be required for CoQ biosynthesis in human cells (*PDSS1, PDSS2, COQ2, COQ3, COQ4, COQ5, COQ6, COQ7, COQ8A, COQ8B, COQ9*), which are collectively called *COQ* genes [5,6]. Studies with the yeast *Saccharomyces cerevisiae* have suggested that several COQ proteins are organized into a high molecular mass complex on the matrix side of the inner mitochondrial membrane. Some COQ proteins do not appear to act enzymatically in CoQ synthesis, suggesting that they act to facilitate the anchoring or assembly of the CoQ biosynthetic complex [10–12].

COQ7 catalyzes the penultimate step of the CoQ_{10} biosynthetic pathway, converting demethoxyubiquinone (DMQ) to 5-hydroxy-ubiquinone which is then turned into the finished product, CoQ, by *O*-methylation [13,14]. Loss of COQ7 activity results in loss of CoQ_{10} as well as accumulation of DMQ_{10} [15–20]. DMQ is the only intermediate capable of accumulating in mutants with *COQ* gene defects. Its possible role in the phenotypes of pathogenic *COQ7* variants has not yet been clearly established [18,21–26]. However, in CoQ-deficient mammalian cells, DMQ has been shown to be able to allow for a minimum of respiratory electron transport [23].

Mutations in *COQ* genes cause primary CoQ_{10} deficiency, a clinically heterogenous and rare disorder [27,28]. CoQ_{10} deficiency due to pathogenic COQ7 mutations is known as primary CoQ_{10} deficiency-8 (OMIM # 616733, COQ10D8). To date, five COQ10D8 patients have been

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https://doi.org/10.1016/j.ymgmr.2022.100877
Received 27 April 2022; Accepted 27 April 2022
Available online 5 May 2022

reported in publications. The first reported case carries a homozygous c.422 T > A [p.Val141Glu] mutation and presents with a complex multisystem disorder, including growth retardation, delayed motor development, hearing loss, and progressive muscle weakness [16]. CoQ_{10} levels were severely decreased in the skeletal muscle and fibroblasts of the patient [16,17]. The second case reported a homozygous pathogenic mutation c.332 T > C [p.Leu111Pro] as well as a benign polymorphism, c.308C > T [p.T103M] [17]. This patient exhibits fewer and milder symptoms, one of which is spasticity. Only a moderate loss of CoQ_{10} levels was observed in the patient's cells. Moreover, other genetic defects (m.1555A > G and a heterozygous stop-gain mutation in *C2ORF71*) were identified [17]. Therefore, the causative role of the COQ7(p.Leu111Pro) variant in all of the proband's phenotypes cannot be rigorously established. The other 3 cases of *COQ7* defect are compound heterozygotes. One carries a deletion insertion resulting in a frameshift (c.599_600delinsTAATGCATC, p.[K200Ilefs*56) and a missense substitution (c.319C > T, p.Arg107Trp), presenting with fatal neonatal-onset multisystem phenotypes [29]. The other two are heterozygous for c.197 T > A [p.Ile66Asn] and c.446A > G [p.Tyr149Cys], and the patients were reported to show axonal neuropathy and mild neurodegenerative disorder [30].

Studies with conditional *Coq7* knockout mice suggest that most CoQ deficiency symptoms are reversible when CoQ synthesis is restored [24]. However, there is currently no effective treatment available for primary CoQ_{10} deficiency patients that can significantly delay or reverse the disease course. Most patients are given oral CoQ_{10} supplementation following diagnosis, but there is lack of clear evidence for its efficacy. Many primary CoQ_{10} deficiency patients treated with CoQ_{10} showed little or no response [31–39]. Although, positive effects have been reported in some cases, the overall clinical benefit reported was limited [36,37,40–43]. Often effects were reported for a few symptoms and most of the other symptoms still persisted after CoQ_{10} treatment [33,44–47]. Better controlled and extensive studies are needed to clarify the efficacy of CoQ_{10} therapy. Recently, CF/CoQ_{10}, a water-soluble micellar formulation of CoQ_{10}, has been developed to improve the poor solubility and low bioavailability of CoQ_{10} [48]. It is based on the unexpected ability of the antifungal drug caspofungin (CF) to solubilize CoQ_{10} in aqueous solution. Compared to native CoQ_{10}, CF/CoQ_{10} was shown to exhibit much superior CoQ_{10} delivery efficiency including to the mitochondria [48]. However, its effect on CoQ_{10}-deficient human patient cells has not yet been tested.

For COQ10D8, 2,4-dihydroxybenzoic acid (DHB) is considered by some as a potential alternative treatment option. DHB is a structural analog of the native precursor of the CoQ benzoquinone ring, 4-hydroxybenzoic acid (4-HB). It differs from 4-HB only by already having a hydroxyl group at the position of the aromatic ring that COQ7 normally hydroxylates. Therefore, CoQ production using DHB obviates the need for COQ7 [12]. DHB administration has been demonstrated to reverse disease phenotypes in conditional *Coq7* knockout mice and $Coq9^{R239X}$ mouse model with COQ7 deficiency [24,26,49]. In patient skin fibroblasts, a significant elevation of CoQ_{10} level was observed in the COQ7 (p.Val141GluE) cells after treatment with DHB, which was accompanied by the rescue of respiratory deficiency. However, no change of CoQ_{10} level was seen upon DHB treatment in less severely affected COQ7(p.Leu111Pro) cells [17,50].

Here, we described a novel pathogenic *COQ7* allele causing mainly motor disorders including spasticity. This case further points to an association between *COQ7* defects and spasticity, or even CoQ_{10} deficiency in general and spasticity. Using the patient's fibroblasts, we explore further the possibility of using DHB or CF/CoQ_{10} to treat *COQ7* patients. Our findings suggest that DHB is unlikely to provide benefits in most or all cases, and that its use could be potentially exacerbating the condition. Conversely, the observed positive effects of CF/CoQ_{10} on the patient cells' respiratory capacity reinforces its potential as a treatment for CoQ_{10} deficiency.

2. Material and methods

2.1. Ethics statements

The experiments included in this study were performed in accordance with relevant guidelines and regulations, with human ethics approved by the Mugla Sitki Kocman University Ethics Committee. Informed consent was obtained from all subjects and their parents.

2.2. Materials and reagents

All cell culture reagents were obtained from Wisent BioProducts (Saint-Jean-Baptiste, QC, Canada), unless mentioned otherwise. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). CF/CoQ_{10} is a micellar solution of caspofungin (CF) and CoQ_{10}, prepared as described in the reference [48].

2.3. Whole-exome sequencing (WES)

Patient DNA was isolated from peripheral blood samples using Magpurix Blood DNA Extraction Kit 200 (Zinexts LSC, New Taipei City, Taiwan [R.O.C.]) according to the manufacturer's instructions. Whole exome sequencing was performed with the Nextera Rapid Capture Exome kit (Illumina) according to the manufacturer's instructions. We analyzed all variants affecting coding areas and ± 20 splicing regions. 98.8% of target regions had a coverage depth of more than 10× in the WES analysis. A novel, homozygous missense mutation in the gene *COQ7* was identified in the proband. The genome aggregation database (gnomAD) was used to determine the variant frequency. SIFT, PolyPhen2, and 1000 Genome databases were used to predict the functional impacts. We couldn't find this variant on 500 control chromosomes in our in-house database. These 500 control chromosomes include children with different genetic diseases and their parents. The relevant variant was verified by Sanger sequencing and analyzed using SeqScape Software 3 (Life Technologies Corporation, California, USA). After confirmation of the proband's mutation further Sanger sequencing was performed to identify the mutation in parents and siblings.

2.4. Cell culture

Skin fibroblasts were prepared from skin biopsies following standard procedures and were routinely cultured in standard DMEM (#319–005-CL, Wisent) supplemented with 10% fetal bovine serum (#080–150, Wisent) and 1% antibiotic-antimycotic (#450–115-EL, Wisent). Galactose medium was prepared with glucose-free DMEM (#11966025; Thermo Fisher) and by adding galactose at the final concentration of 10 mM, 1 mM sodium pyruvate, 10% dialyzed FBS (#26400044; Thermo Fisher), and 1% antibiotic/antimycotic. After galactose culture, cells were stained with crystal violet (0.05% crystal violet, 1% formaldehyde, and 1% methanol in phosphate buffered saline) at room temperature for 1 h before cell plate pictures were taken with a mobile phone. For CF/CoQ_{10} and 2,4-dihydroxybenzoic acid treatments, cells were collected for analyses after 1 and 4 days of treatment respectively.

2.5. Measurement of mitochondrial oxygen consumption rate (OCR)

OCR was measured using a Seahorse XFe96 extracellular Flux analyzer (Agilent Technologies) as previously described [48,51], with small modifications. Skin fibroblasts were seeded in XF96 microplates with Seahorse assay media supplemented with galactose (10 mM), glutamine (2 mM) and pyruvate (1 mM). After baseline OCR measurements, oligomycin (1 μg/mL), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 1 μM), and a mix of rotenone (0.5 μM) and antimycin A (5 μM) were injected consecutively to determine different parameters of respiration. Data were collected with Wave software and exported to GraphPad Prism for graphical presentation. Final data was

normalized to protein per well (measured by a BCA protein assay; Thermo Fisher Scientific).

2.6. ATP assay

A luminescence-based kit (ATPlite 1step kit, PerkinElmer) was used to measure cellular ATP levels, according to the manufacturer's instructions. Luminescence was read on a TECAN Infinite M1000 plate reader.

2.7. CoQ extraction and quantification

CoQ quantification was carried out by HPLC, as previously described [17,48]. An Agilent 1260 Infinity LC system equipped with a quaternary pump (G7111A) and a variable wavelength detector (G7114A) was used. Cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 1% NP-40, 0.5% deoxycholate, 10 mM EDTA, 150 mM NaCl) before CoQ extraction with a mixture of ethanol and hexane (*v*/v: 5:2). Chromatography was carried out on a reverse-phase C18 column (2.1 × 50 mm, 1.8 μm, Agilent) with 70% methanol and 30% ethanol as the mobile phase at a flow rate of 0.3 mL/min. The detector was set at 275 nm. The final quantification of CoQ was normalized to the amount of protein measured with a BCA assay.

2.8. Western blotting

Cell lysates were prepared in RIPA buffer and protein concentration was assessed by a BCA assay. 60 μg protein per sample were loaded and separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. After blocking, all primary antibodies (rabbit anti-COQ7 15083–1-AP, anti-PDSS2 13544–1-AP, anti- VDAC1/Porin 55259–1-AP, PtgLab) were incubated in a 1:2000 dilution at 4 °C overnight, followed by HRP-conjugated secondary antibody (#7074, 1:2000, Cell Signaling) for 2 h at room temperature. Protein bands were visualized by ECL detection (NEL103E001EA, Froggabio Inc.) on X-ray films. Acquired images were cropped in Adobe Photoshop.

2.9. Statistical analysis

GraphPad Prism 9.0 (GraphPad Software, Inc.) was used for statistical analysis of the data. All quantitative results are expressed as mean ± standard error of the mean (S.E.M.) or ± the standard deviation (S.D.) as indicated. Analysis of variance was used to compare means across groups, and $p < 0.05$ was used for significance cut-off.

3. Results

3.1. Patient presentation

The patient is the third child of consanguineous parents who are first cousins. He was born at 39 weeks of gestational age, after a non-problematic pregnancy. He was healthy during the newborn period. While his early developmental stages were normal, symptoms became apparent with a global developmental delay at 15 months. By age 3, he was not yet capable of walking without help. The patient presented to the Hospital of Muğla Sıtkı Koçman University in February 2019 (at age of 4.5 years) with hypotonia, difficulty walking, motor developmental delay, getting tired quickly, speech delay, joint contractures, ataxia, and spasticity. On physical examination, he had normal height, weight, and head circumference. No major dysmorphic findings were noted. No abnormalities were observed in blood cell count, chemistry, liver and renal function, and urine and stool tests. Brain MRI showed increased T2A and Flair signal in the supratentorial bilateral periventricular white matter.

Since no abnormalities were observed in chromosome analysis and microarray analysis, WES analysis was performed. It identified a homozygous single base substitution (NM_016138: c.161G > A) in the *COQ7* gene. Parents and one sibling were found to be heterozygous for the variant but showed no significant clinical or laboratory manifestations. The other sibling is wild type. The proband did not receive any treatment during this two-year period. Since then, we have not noted any dramatic improvement or worthening in the patient's clinical signs. Of note, he can walk without help or support despite having a problem of balance.

3.2. Analyses of the patient skin fibroblasts

The *COQ7* mutation c.161G > A results in a replacement of arginine by glutamine at codon 54 in exon 2 (p.Arg54Gln) (Fig. 1A). GnomAD reports this variant at a frequency of <0.001% (3 carrier out of 251,488 alleles). Both SIFT and PolyPhen-2 predict the p.Arg54Gln mutation to affect protein function. And the amino acid change is predicted as destabilizing by SDM2 and DynaMut using AlphaFold predicted COQ7 structure [52,53].

We isolated skin fibroblasts from the patient and his unaffected sister carrying the heterozygous p.Arg54Gln mutation as well as from the non-carrier brother to be used as a control. The patient's cells showed no growth defect under normal culture condition compared to the cells from his non-carrier sibling which was used as a wild-type control (WT) (Fig. 1B). DMQ_{10} was detected in the patient's fibroblasts as well as a ≈ 45% reduction in CoQ_{10} level, confirming the pathogenicity of the mutation (NM_016138: c.161G > A; p.Arg54Gln) (Fig. 1C-D). The cells from the unaffected heterozygous sister showed no significant change of CoQ_{10} level and no detectable accumulation of DMQ_{10} (Fig. 1C). Western blot analysis revealed a decrease of COQ7 levels in both patient's cells and the cells from the heterozygous sibling. However, this was much more severe in the patient's cells, suggesting a severe effect of the mutation on protein stability (Fig. 2).

To determine the impact of the mutation on mitochondrial respiratory function, we measured oxygen consumption rates in the patient's cells using a Seahorse XFe96 analyzer and compared it to the WT control. As shown in Fig. 3A-B, while basal respiration rate was found unchanged, the patient' cells showed a decrease of the maximal respiratory rate under conditions of uncoupled respiration. To further characterize this effect, we treated the cells with CoQ_{10} by using the CF/CoQ_{10} formulation. A superior CoQ_{10} delivery efficiency has been demonstrated for the water soluble micelle form of CoQ_{10} in mouse embryonic fibroblasts and mice [48]. Here we show that CF/CoQ_{10} supplementation of the patient's cells can rescue the deficiency of the maximal respiratory rate, confirming directly that it is caused by CoQ_{10} deficiency.

Cells with severe mitochondrial dysfunction become often unable to grow in media containing galactose instead of glucose, which impairs the ability to produce ATP by glycolysis. However, the patient's cells showed no severe growth defect in media containing only galactose (Fig. 3C). In fact, measurement of whole cell ATP levels found no difference between the patient's cells and the WT control (Fig. 3D). These findings are consistent with the observation that the basal respiration rate was not significantly altered (Fig. 3A). Lastly, we tested the effect on CoQ_{10} levels of supplementing the patient's cells with DHB, but no significant change of CoQ_{10} levels was observed (Fig. 4). In contrast, the level of accumulated DMQ_{10} became very low with DHB treatment. Furthermore, in the wild-type control cells the same DHB treatment significantly lowered the total level of CoQ_{10} (Fig. 4).

4. Discussion

In recent years, with genome sequencing becoming faster and more affordable, the number of patients reported with primary CoQ deficiency is increasing [28,54]. The patient described in the present study harbors a homozygous mutation in the *COQ7* gene, in which the G to A substitution in codon 54 of exon 2 results in an arginine to glutamine change (p.Arg54Gln). In the patient's skin fibroblast, there is drastic loss

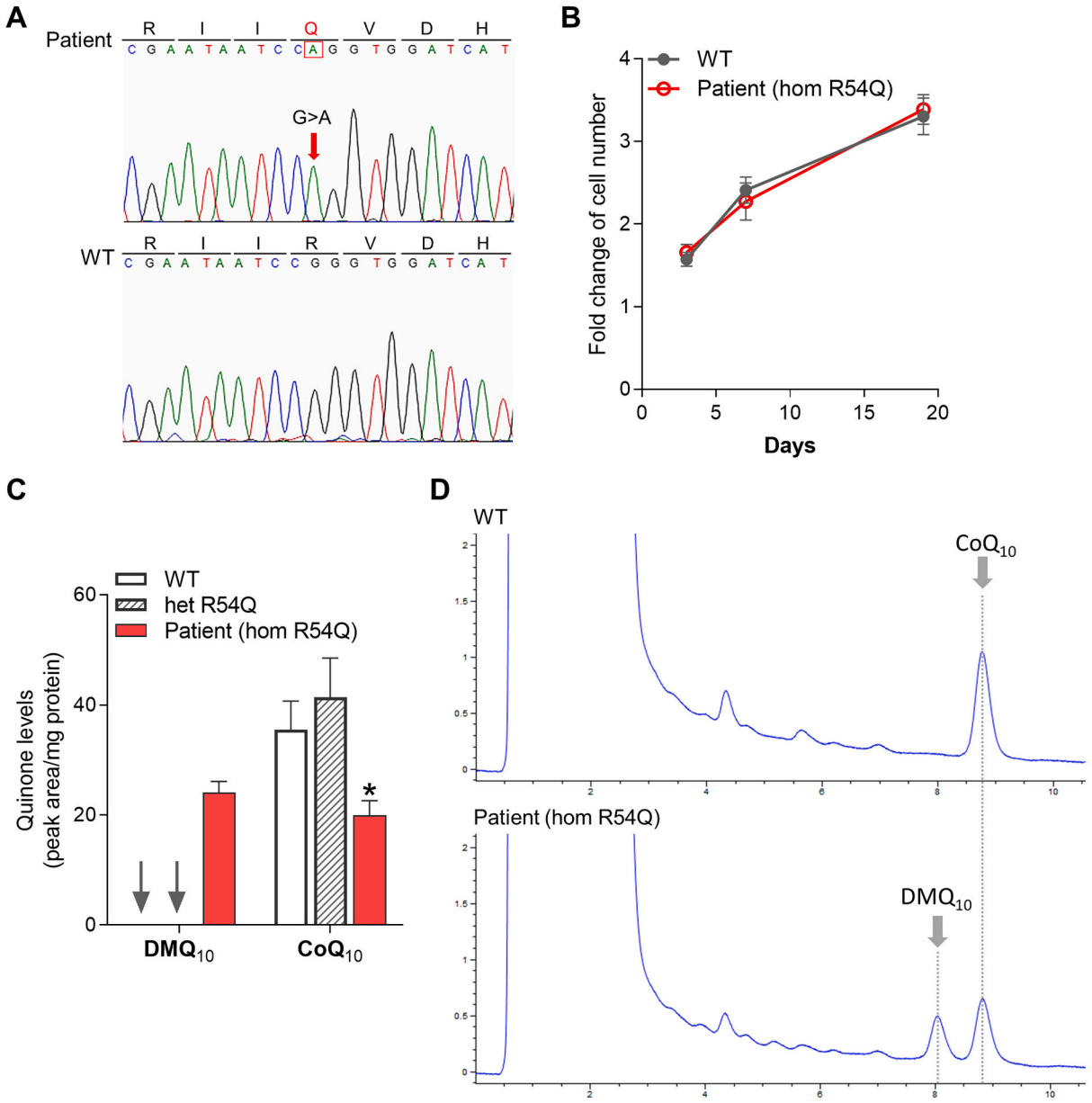

Fig. 1. Low CoQ_{10} level and accumulation of DMQ_{10} in the COQ7(p.Arg54Gln) patient skin fibroblasts. (A) Sanger sequencing chromatograms show a homozygous mutation at codon position 54 of exon 2 of COQ7 in the proband. His non-carrier sibling (WT) was used as a non-affected control. (B) Growth curves of skin fibroblasts derived from the proband and his non-carrier sibling (WT) ($n = 6$). (C) Quinone quantification in skin fibroblasts from the proband in comparison to the non-affected control. Cells were also obtained from the other healthy sibling, who is a heterozygous carrier for the variant, and were found to have comparable CoQ_{10} levels as the non-affected control. Hom: homozygous, Het: heterozygous. Values are shown as mean ± SEM ($n = 2$). *$p < 0.05$ (two-way ANOVA followed by Tukey's multiple comparison tests). (D) HPLC chromatograms of quinone extracts from human skin fibroblasts. The patient's cells show ≈45% decrease in CoQ_{10} level and accumulation of the biosynthetic precursor DMQ_{10}.

of COQ7 protein expression, and a moderate decrease of CoQ_{10} level associated with accumulation of DMQ_{10}, the normal substrate of the enzyme. The findings strongly suggest that the p.Arg54Gln mutation is pathogenic.

In the patient's skin fibroblasts, we detected a ≈ 45% decrease of CoQ_{10} and lower mitochondrial respiratory capacity. The observed respiratory defect can be rescued by CoQ_{10} supplementation with CF/CoQ_{10}. CF/CoQ_{10} is a micellar formulation of CoQ_{10} with the water-soluble antifungal drug caspofungin [48]. Because CoQ_{10} is highly hydrophobic, it is only available in oral formulations, despite its very poor oral bioavailability. The water-soluble formation CF/CoQ_{10} makes it possible to deliver CoQ_{10} via other routes. It was shown in mice that CF/CoQ_{10} can be administrated intravenously without detectable toxicity [48]. The efficiency of CoQ_{10} delivery by CF/CoQ_{10} varies among tissues as expected, and uptake by the brain was the least efficient among the tissues examined (liver, heart, skeletal muscle, kidney, brain, lung, and spleen), which could be expected given that the brain-blood barrier is known to be an obstacle of drug delivery to the CNS. CoQ_{10} deficiency patients usually present with multiple and heterozygous symptoms [1,3]. While some symptoms such as encephalopathy, might be harder to treat, improvement of other disease pathologies could make a difference in the patients' quality of life. Furthermore, even a small increase in brain CoQ could partially, or even strongly, alleviate symptoms. In the present study, we showed rescue of the respiratory defect in the patient cells by CF/CoQ_{10}, which is the first demonstration of its efficacy in human CoQ_{10}-deficient cells and encourages further

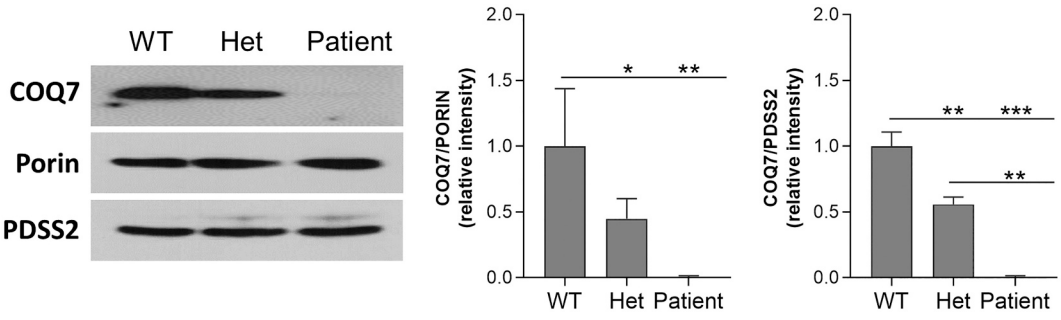

Fig. 2. Western blot analysis of expression of COQ7. In the skin fibroblasts from the p.Arg54Gln COQ7 patient, there is a severe reduction in COQ7 expression, in comparison to the cells from the non-carrier sibling. In addition, the heterozygous sibling's cell also showed lowered COQ7 levels. The level of the COQ biosynthetic enzyme PDSS2 is unchanged in all 3 genotypes. The mitochondrial outer membrane protein porin was used as a loading control. Western blots were cropped to show only relevant bands. Uncropped blots are presented in **Supplementary Fig. 1**. Western blot quantification was performed using Image J.

studies to evaluate its potential to treat CoQ_{10} deficiency disease.

Overall, our findings suggest that inadequate CoQ_{10} production in the COQ7(p.Arg54Gln) patient's fibroblasts compromises mitochondrial energy metabolism, and this potentially could affect various cellular processes. However, it is worth noting that caution is needed in interpreting results from in vitro fibroblasts studies, as effects of any *COQ* gene mutation may have different consequences in cultured cells versus particular animal tissues [44]. Cases have also been reported where decreased CoQ_{10} content was found in fibroblasts but not in muscle [55]. On the other hand, a normal CoQ_{10} level in fibroblasts should not rule out a diagnosis of CoQ deficiency.

Three COQ7 patients described in the literature so far harbor different COQ7 variants, and their clinical picture varies widely [16,17,29]. However, all have developmental delay, hearing impairment, and muscle phenotypes. The first case of COQ7 deficiency reported a homozygous missense mutation (p.Val141Glu) which might disrupt the enzyme's active di-iron binding site and was shown to severely impair COQ7 function and CoQ_{10} synthesis [16,17]. The patient never learned to stand or walk and presented with significant muscular hypotonia and peripheral sensorimotor polyneuropathy [16]. The most recent patient reported was found to carry compound heterozygous *COQ7* variants and was also severely affected, with a severe CoQ deficit scored in fibroblasts, and clinical manifestations including progressive multisystemic dysfunction and death at 1 year of age [29]. In contrast, the patient we describe here is more comparable to a Canadian patient we previously reported to carry a homozygous p.Leu111Pro mutation. As in the p.Leu111Pro patient, the skin fibroblasts show only a moderate loss of CoQ_{10} [17]. Furthermore, both patients present with relatively milder phenotypes compared to the two patients mentioned above, and one of their prominent phenotypes is spasticity. As mentioned in the introduction, the p.Leu111Pro patient also carries a mitochondrial DNA mutation (A1555G) and a heterozygous mutation in the *C2Orf71* gene in addition to the *COQ7* mutation [17]. Nonetheless, the finding of spasticity in both patients suggests that this is a symptom that is associated with the *COQ7* mutations. A clinical implication is that the possibility of a *COQ7* mutation or a CoQ_{10} deficiency of any etiology should be considered for young patients with spasticity in addition to other symptoms.

The varying clinical manifestations among the COQ7 patients, or more generally among most primary CoQ_{10} deficiency patients, are likely mainly attributable to varying severities of CoQ_{10} deficiency, although genetic modifiers acting on mitochondrial function cannot be excluded [8]. Skin fibroblasts from patients are often the only material available for direct measurement of CoQ_{10} levels. In general, the severity of CoQ_{10} loss found in isolated patient fibroblasts positively correlates with the severity of the disease [1,31]. In addition to COQ7 defects, pathogenic COQ9 variants also cause accumulation of DMQ due to a decrease in the levels of COQ7 [18,56]. In mice, complete loss of *Coq7* gene function results in embryonic lethality, as is also observed with several other *Coq* genes and with genes necessary for the proper assembly of ETC complexes, and this despite the presence of DMQ [1,14,19,57–60]. A more recent study with mammalian cells indicates that DMQ can function as an electron carrier in the mitochondrial respiratory chain similar to CoQ but much less efficiently [23]. Therefore, in *COQ7* or *COQ9* patient cells that still have residual CoQ_{10} biosynthesis, DMQ is unlikely to play a significant role in the phenotypic presentation at the level of mitochondrial respiration scored in cultured cells. On the other hand, there is the possibility that DMQ might compete with CoQ and thus exert a potentially inhibitory effect on mitochondrial respiration. In *C. elegans* the orthologue of *COQ7* is *clk-1*. Addition of a pentane extract from *clk-1* mutant mitochondria (containing DMQ_9) to CoQ_9-replete mitochondria was shown to partially inhibit Complex I-III activity [22]. Whether any such effect exists in vivo is not yet known. However, several studies suggest a low likelihood for significant toxicity of DMQ. Indeed, upon treatment with DHB, significant phenotypic rescue was observed in an inducible *Coq7* knockout model and a *Coq9* knock-in (R239X) mouse model. But in both models, DMQ accumulation persisted in the treated mice [24,26,49]. Liver-specific *Coq7* knockout mice were shown to be indistinguishable from the wild type, despite large accumulation of DMQ in the hepatocytes [21]. Furthermore, in *C. elegans*, suppressors of the missense mutation *clk-1(e2519)* were found to restore the ability to synthesize CoQ, but only in exceedingly small amounts, and still accumulate large amounts of DMQ. Yes, these small amounts of CoQ were sufficient to suppress the mutant phenotypes [25]. Finally, it is noteworthy that the degree of loss of CoQ_{10} in every patient tissue is necessarily unknown. Some specific cell types in the patients might suffer much more severe CoQ_{10} deficiency than cultured fibroblasts, let alone because cultured cells are exposed to atmospheric oxygen levels. Therefore, some in vivo cell types might benefit more or less from the large accumulation of DMQ, for its limited ability to carry electrons or maybe other properties.

Although in the patient's fibroblasts there was only a moderate reduction in CoQ_{10} level, we observed a drastic loss of COQ7 protein expression, confirming that the p.Arg54Gln mutation disturbs protein stability. We also found that in the cells from the healthy heterozygous sibling there is also a significant loss of COQ7 expression, yet CoQ_{10} levels are not affected. It is not yet known below what threshold a decrease of COQ7 abundance decreases CoQ_{10} levels and causes CoQ_{10} deficiency symptoms. Previously published works on COQ7 patients did not report on the expression of COQ7 in the patients' skin fibroblasts or biopsy tissues [16,17,29]. However, we previously have shown that when COQ7(p.Leu111Pro) was expressed in a heterologous system, a very low mutant COQ7 protein level was associated with an only moderate CoQ_{10} deficiency [17]. Taken together, these findings indicate that a minimal amount of COQ7 protein may be sufficient to maintain enough activity for synthesis of most CoQ in the cell. However, although $Coq7^{+/-}$ mouse mutants were shown not to be haplo-insufficient and to have wild-type level of CoQ, the distribution of CoQ was found to be

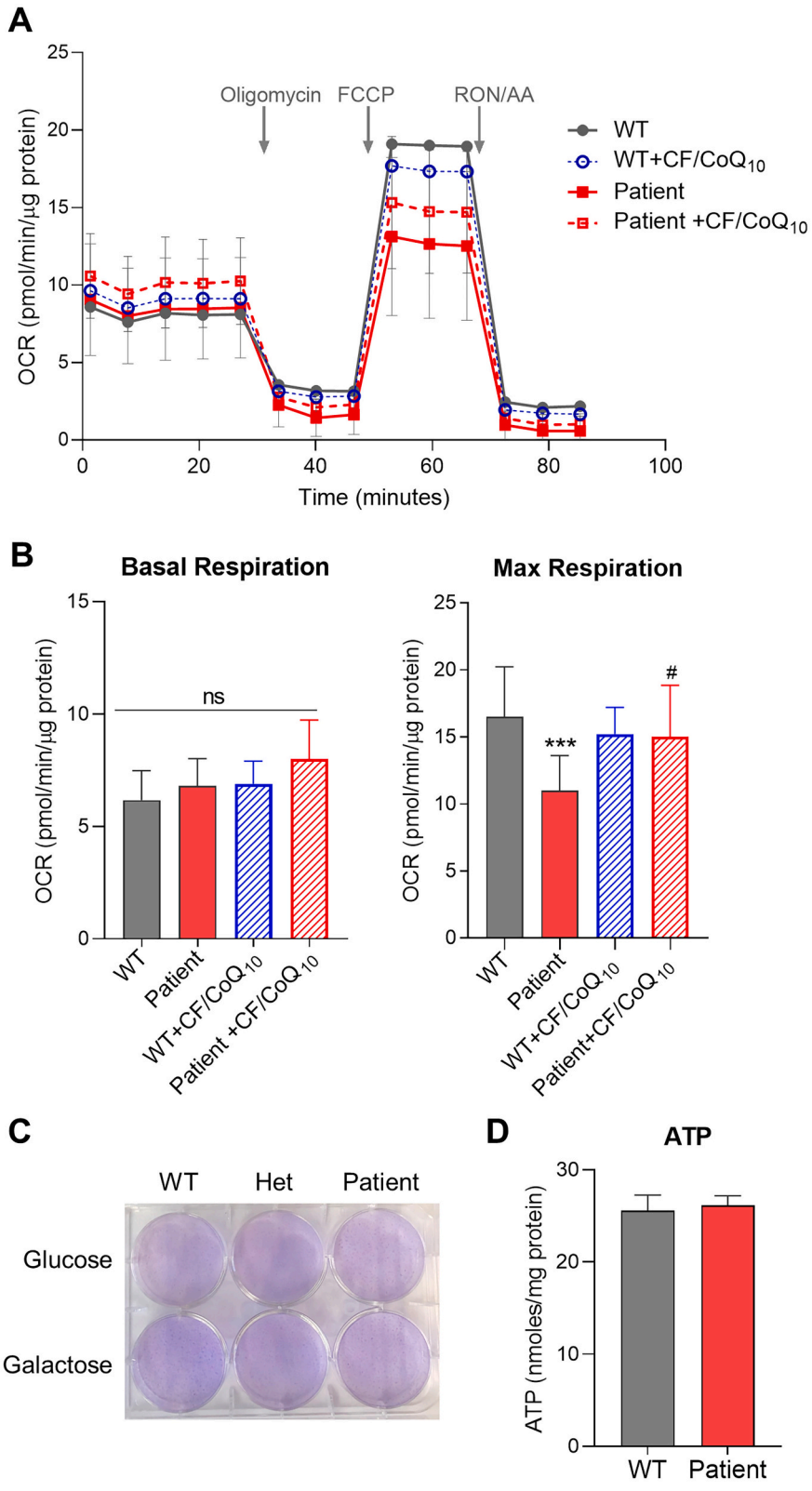

Fig. 3. Measurement of mitochondrial respiratory function by Seahorse Flux Analyzer. (A) Oxygen consumption rate (OCR) traces of skin fibroblasts from the proband or its non-carrier sibling (WT) measured on a Seahorse XFe96 Analyzer. For the CF/CoQ_{10} treatment groups, OCR was measured after 2 days of culture in medium supplemented with CoQ_{10} at the final concentration of 1 µM. Arrows indicate injections of test compounds into the chamber. Compounds were added in the following order: oligomycin, carbonyl cyanide p-(tri-flur-omethoxy)phenyl-hydrazone (FCCP), a mixture of rotenone (RON) and antimycin A (AA). (B) Bar graphs showing the quantifications of basal respiration and maximal respiratory capacity. The final OCR values were normalized to total protein content and presented as mean ± SEM ($n = 12$). ns: not significant. $^{***}p < 0.001$ compared to untreated WT, and $^{\#}p < 0.05$ compared to untreated patient cells (one-way ANOVA followed by Tukey's multiple comparison tests). (C) Cell staining pictures taken after culture in glucose or galactose medium for 96 h. Crystal violet staining was used to visualize the surviving cells. Positive crystal violent staining (purple) is indicative of surviving cells at the end of the culture under various conditions. (D) ATP level in the patient's cells is comparable to that of WT control cells. Values shown are mean ± SEM ($n = 5$).

altered within mitochondrial membranes in *Coq7*$^{+/-}$ tissues, suggesting an additional function of COQ7 in CoQ membrane distribution [58]. Whether the mitochondrial CoQ distribution is affected in patient cells with a *COQ7* defect is not known.

We found that DHB treatment does not increase CoQ_{10} levels in the COQ7(p.Arg54Gln) patient cells. A similar effect was observed in the p.Leu111Pro patient cells [17]. There are reasons to believe that this is because the patient's cells show only a moderate loss of CoQ_{10}, indicating significant COQ7 activity still remains. Indeed, the native CoQ biosynthetic pathway using 4-HB as the aromatic ring precursor and CoQ synthesis from DHB compete for the same CoQ pathway enzymes. Thus, CoQ production from DHB must lead to a reduced rate of CoQ

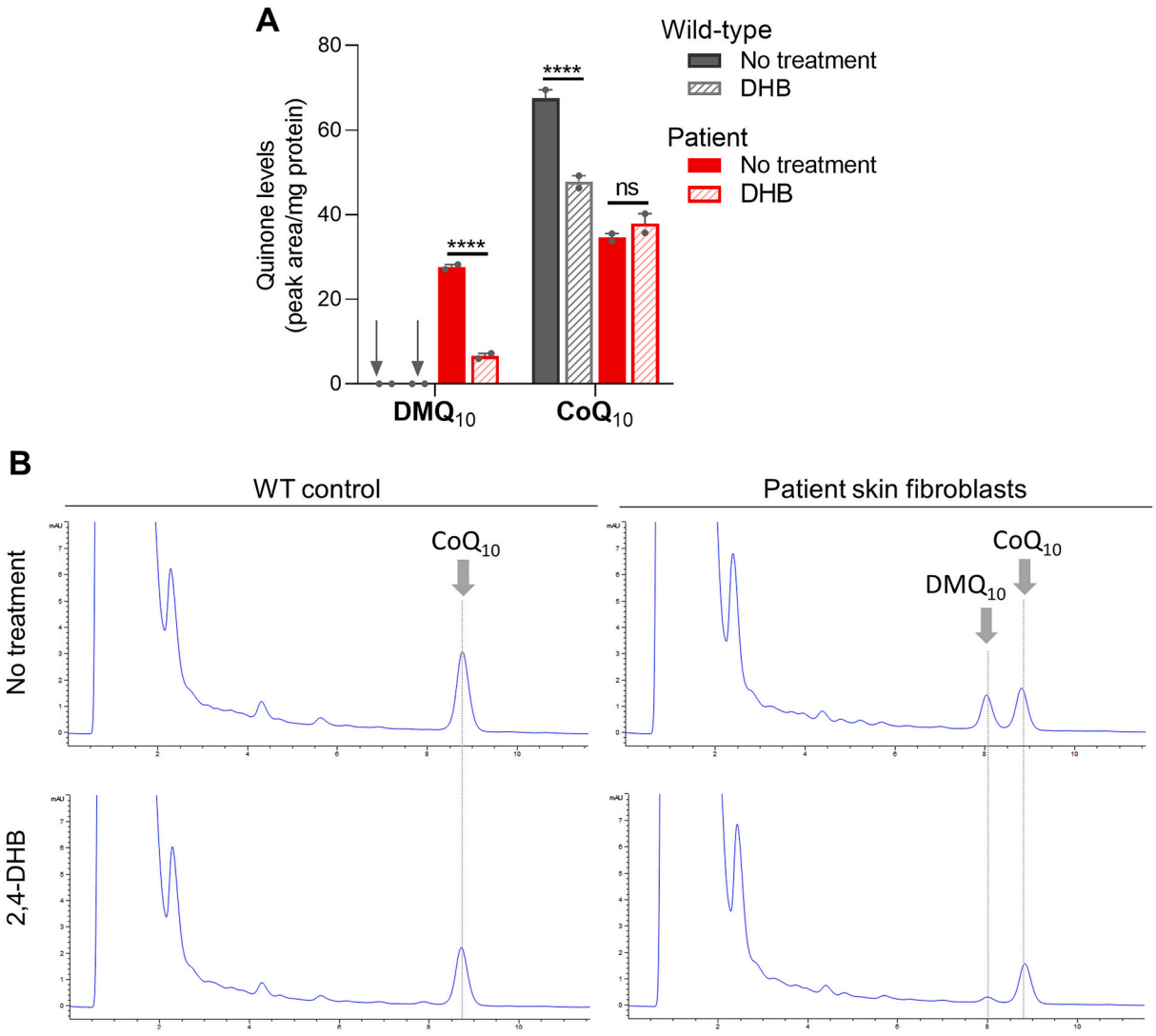

Fig. 4. Effects of 2,4-dihydroxybenzoic acid (DHB) supplementation on quinone levels in the COQ7(p.Arg54Gln) skin fibroblasts. Quinone quantitation is shown in (A). Values shown are mean ± SEM ($n = 2$). The treated cells were collected after 4 days of culture in 0.75 mM DHB supplemented medium. ns: not significant. $^{****}p < 0.0001$ (two-way ANOVA followed by Tukey's multiple comparison tests). Representative HPLC chromatograms of quinone determination are shown in (B).

synthesis from the native pathway. This explains why after DHB treatment, a significant reduction of DMQ levels was observed in all *COQ7* mutant patient cells tested, as well as in mouse cells with *Coq7* defects [17,24,26,50]. In other words, the total net CoQ production does not necessarily increase in *COQ7* mutant cells provided with DHB because lower CoQ production from 4-HB might offset the gain from CoQ produced via DHB. Moreover, as shown in this and previous studies, CoQ_{10} concentration decreased in normal cells after DHB treatment, clearly indicating that CoQ biosynthetic enzymes are less efficient when using DHB-derived biosynthetic intermediates [16,17,24].

Despite the considerations in the previous paragraph, DHB treatment was found to increase the level of CoQ_{10} in skin fibroblasts from the COQ7(p.Val141Glu) patient, which is more severely affected than the Leu111Pro and Arg54Gln patients [16,17]. Thus, as we have also observed in mice, a very severe loss of COQ7 activity can be partially alleviated by DHB. Of note, while a similar effects of DHB were observed in the Leu111Pro and Arg54Gln patient fibroblasts on the levels of DMQ and CoQ, a small increase in the uncoupled maximal mitochondrial respiration rate was observed in the COQ7(p.Leu111Pro) patient cells, whereas in the COQ7(p.Arg54Gln) patient cells, no detectable effect was found [17]. The exact cause of this difference of DHB effect on mitochondrial respiration in the two different patient-derived fibroblast lines, and how other cell types respond to DHB treatment remain to be explored. In sum, these observations suggest that DHB likely would benefit only very severe COQ7 patients with very little remaining COQ7 activity. But such cases might not be able to survive development. The findings with the COQ7(p.Arg54Gln) patient cells we report in this study also stresses that the possibility of DHB treatment must be approached with extreme caution as DHB could lead to reduced net CoQ_{10} synthesis and further aggravate the deficiency. Meanwhile, though the potential mechanisms are unknown, benefits of DHB treatment on other CoQ deficiency models have been reported more recently [49,61,62]. Thus, more future studies are warranted to explore the possibility of using DHB as a treatment.

Funding

This work is supported by a Foundation grant from the Canadian Institutes of Health Research: FDN-159916. S. Hekimi is Campbell Chair of Developmental Biology.

Declaration of Competing Interest

SH and YW have received royalty payment from Clarus Therapeutics Holdings. SH also consults for Clarus Therapeutics Holdings. EG declares no potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2022.100877.

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