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Optic neuropathy linked to *ACAD9* pathogenic variants: A potentially riboflavin-responsive disorder?

Naig Gueguen^{a,b,1}, Julie Piarroux^{c,1}, Emmanuelle Sarzi^d, Mehdi Benkirane^{e,f}, Gael Manes^g, Cécile Delettre^g, Pascal Amedro^{e,h}, Nicolas Leboucqⁱ, Michel Koenig^{e,f}, Pierre Meyer^{c,e}, Isabelle Meunier^j, Pascal Reynier^{a,b}, Guy Lenaers^{b,1}, Agathe Roubertie^{c,g,i,1,*}

^a Department of Biochemistry and Molecular Biology, CHU Angers, 49933 Angers, France

^b University of Angers, Unité Mixte de Recherche (UMR) MITOVASC, Centre National de la Recherche Scientifique (CNRS) 6015, Institut National de la Santé et de la Recherche Médicale (INSERM) U1083, 49933 Angers, France

^c CHU Montpellier, Département de Neuropédiatrie, Montpellier, France

^d NeuroMyoGene Institute-UCBL/CNRS UMR5310/INSERM U1217-Lyon, France

^e PhyMedExp, CNRS, INSERM, University of Montpellier, Montpellier, France

^f Laboratoire de Génétique Moléculaire, Institut Universitaire de Recherche Clinique, CHU de Montpellier, France

^g INM, University Montpellier, INSERM, Montpellier, France

^h Pediatric and Adult Congenital Cardiology Department, M3C Rare Cardiac Disease Reference Center, CHU Montpellier, France

ⁱ Département de Neuroradiologie, CHU Montpellier, Montpellier, France

^j National Center in Rare Diseases, Genetics of Sensory Diseases, University Hospital, Montpellier, France

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ABSTRACT

Mitochondrial complex I (CI) deficiencies (OMIM 252010) are the commonest inherited mitochondrial disorders in children. Acyl-CoA dehydrogenase 9 (ACAD9) is a flavoenzyme involved chiefly in CI assembly and possibly in fatty acid oxidation. Biallelic pathogenic variants result in CI dysfunction, with a phenotype ranging from early onset and sometimes fatal mitochondrial encephalopathy with lactic acidosis to late-onset exercise intolerance. Cardiomyopathy is often associated. We report a patient with childhood-onset optic and peripheral neuropathy without cardiac involvement, related to CI deficiency. Genetic analysis revealed compound heterozygous pathogenic variants in *ACAD9*, expanding the clinical spectrum associated to *ACAD9* mutations. Importantly, riboflavin treatment (15 mg/kg/day) improved long-distance visual acuity and demonstrated significant rescue of CI activity *in vitro*.

1. Introduction

Inherited Optic Neuropathies (ION) are a heterogeneous group of diseases, characterized by the loss of retinal ganglion cells, leading to bilateral loss of central vision. ION share common physiopathological mechanisms related to mitochondrial dysfunction affecting energy metabolism or network dynamics (Maresca et al., 2012).

Mitochondrial complex I (CI) or NADH:ubiquinone reductase (EC 7.1.1.2) is one of the four complexes forming the electron transport chain (ETC). CI deficiencies (OMIM 252010) represent up to one third of inherited mitochondrial diseases, and are caused by nuclear or mitochondrial genome pathogenic variants. Symptoms vary widely in nature

and severity, ranging from isolated sensorial diseases, as in Leber hereditary optic neuropathy or Dominant Optic Atrophy, to early onset multisystemic neurodegenerative phenotypes with poor outcome (Fiodorzuk and Sazanov, 2018; Koene et al., 2012).

Here, we report the case of a patient with childhood onset optic neuropathy associated with peripheral neuropathy, but without any cardiomyopathy, related to compound heterozygous pathogenic variants in the acyl-CoA dehydrogenase 9 (*ACAD9*) gene. Importantly, riboflavin treatment improved long distance visual acuity and demonstrated significant rescue of CI activity *in vitro*.

* Corresponding authors: Département de Neuropédiatrie, CHU Gui de Chauviac, 80 Avenue Fliche, 34295 Montpellier, France.

E-mail address: a-roubertie@chu-montpellier.fr (A. Roubertie).

¹ These authors contributed equally to the manuscript.

2. Material and methods

Informed consents were obtained from the parents to perform genetic, biochemical and molecular analyses and to publish all data. The study was carried out in accordance with the Declaration of Helsinki and

was approved by Montpellier University Hospital Institutional Review Board.

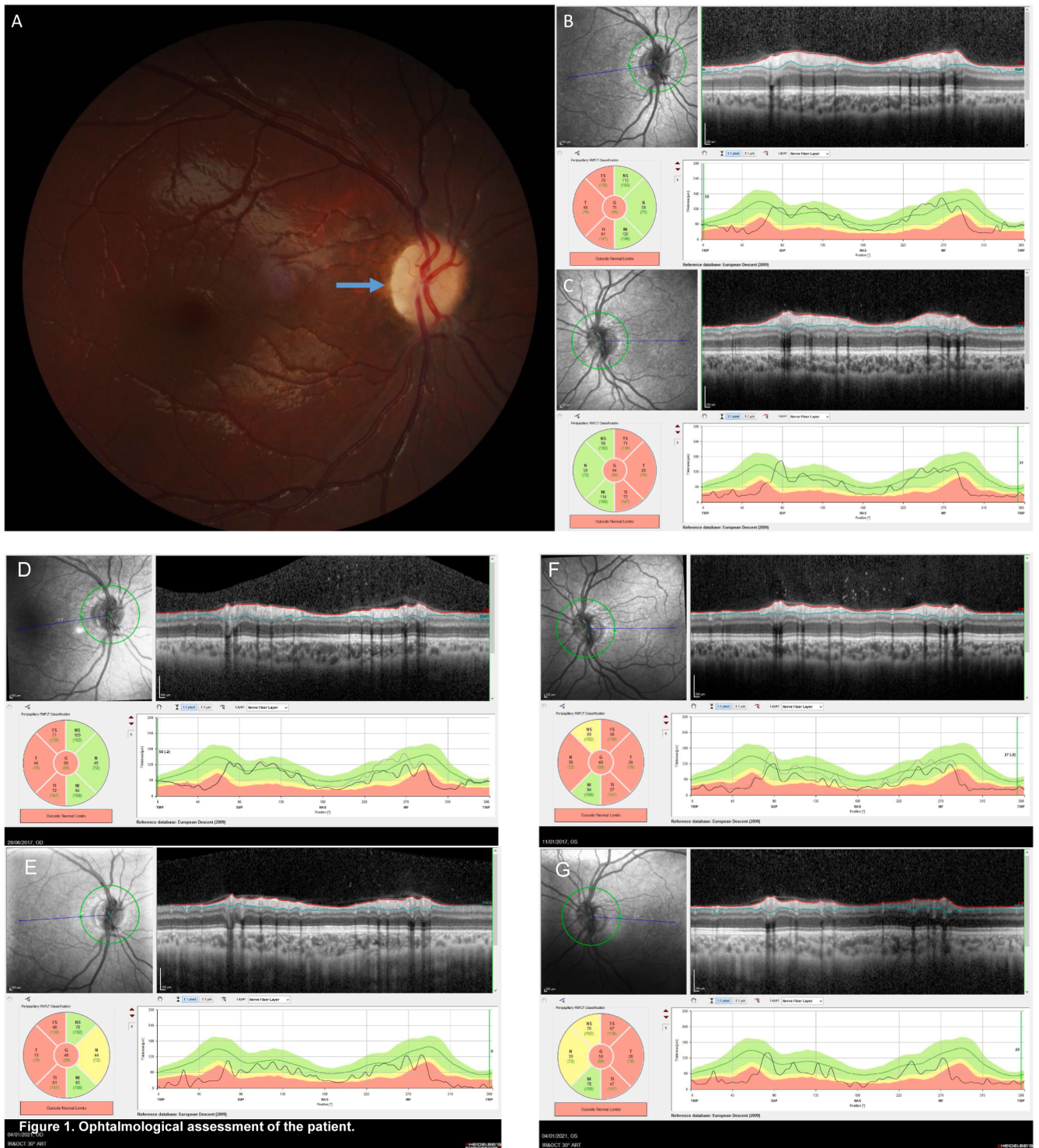


Fig. 1. Ophthalmological assessment of the patient at the age of 7 years. Visual acuity was 20/125 P5 in the right eye and 20/200 P5 left eye. A fundus picture of the right eye. Note the temporal pallor (blue arrow). B-G: Retinal Nerve Fiber Layer (RNFL) analysis. Significant thinning of the temporo-macular bundle in both eyes. B: initial RNFL, right eye; temporal thickness 46 µm, visual acuity 20/125 P5. C: initial RNFL, left eye; temporal thickness 28 µm, visual acuity 20/200. P5. D: RNFL before treatment, right eye, temporal thickness 46 µm, visual acuity 20/400 P6. E: last follow-up, right eye; temporal thickness 13 µm, final visual acuity 20/100 P10. F: RNFL before treatment, left eye; temporal thickness 27 µm, visual acuity 20/400 P6. G: left eye, last follow-up; temporal thickness 28 µm, final visual acuity 20/100 P10.

3. Results

3.1. Case report

The patient was the second child of healthy and non-consanguineous parents. Family history, pregnancy and perinatal period were uneventful. Psychomotor development was normal (walk unaided at 12 months of age, first sentences at 3 years of age). He was reported since the age of 3 as clumsy, with coordination difficulties, awkward gait and walking fatigability. The patient was referred at 6.8 years of age for progressive visual disability. Visual acuity was 20/125 P5 in the right eye and 20/200 P5 in the left eye, with dark colors dyschromatopsia. Fundus examination revealed bilateral optic disc pallor with temporal predominance (Fig. 1A) and spectral-domain optical coherence tomography (SD-OCT) disclosed a marked decrease in the thickness of the retinal nerve fiber layer on the temporal area (Fig. 1B, D). Neurological examination showed steppage gait, distal muscular weakness, discrete calf atrophy and abolished deep tendon reflexes on the four limbs. General examination was normal, height and weight parameters were below 1.5 SD, and cognitive function was normal.

Electrophysiological recording disclosed the absence of sensory potentials, in agreement with sensory neuropathy of the four limbs. Auditory evoked potentials and audiometry, transthoracic echocardiography and electrocardiogram (ECG) were normal.

Laboratory parameters disclosed slightly elevated levels of lactate (3.1 mmol/l, normal range 0.5–2.2) and alanine (554 micromol/l, normal range 168–510) with normal bicarbonate levels at 6.8 years of age; creatine phosphokinase, ammonia, acyl carnitine profile, renal and hepatic functions were normal. Cerebro-medullar magnetic resonance imaging (MRI) did not reveal any structural abnormality, while cerebral spectroscopy disclosed an elevated lactate peak.

Orthoptic and physical therapy was initiated, and the patient benefited from school facilities.

At 7.5 and 8.5 years of age, visual acuity had decreased to 20/400 P10 in the right eye and 20/400 P14 in the left eye; bilateral thinning of the retinal fiber layer was more obvious on the OCT; neurological examination remained unchanged. Ophthalmological worsening was confirmed by brain MRI that showed discrete optic tract atrophy (data not shown). Biological analysis of lactate and amino-acids were normal.

At 8.5 years of years, given the visual acuity worsening and the mitochondrial dysfunction together with the molecular diagnosis (see below), riboflavin 15 mg/kg/day and ibedenone 22 mg/kg/day supplementations were initiated. The treatment was well tolerated after reducing idebenone supplementation to 15 mg/kg/day, due to digestive disturbances. Idebenone was discontinued after one year, whereas riboflavin was maintained.

A couple of months after treatment initiation, the parents noticed a better appetite and improvement of lower limb fatigue and visual function. At 9.5 years, visual acuity was 20/100 P10 in both eyes. At last follow-up at 11 years of age, the patient was stable in terms of visual acuity except for near vision difficulties; thickness of the retinal nerve fiber layer remained unchanged on the left eye (Fig. 1D–G). Clinical examination and growth parameters were stable. The patient was independent for all daily living activities and was able to walk a distance of 3 km. Academic achievements were normal. The patient underwent a complete cardiac assessment, including echocardiography, cardiac MRI, 24-hour ECG monitoring and cardio-pulmonary exercise test, which was normal. Brain MRI was unchanged.

3.2 Genetics

Gene panel sequencing identified that the patient harbored two *ACAD9* variants: c.358delT p.(Phe120Serfs*9) inherited from his mother, resulting in a frameshift and occurrence of a premature stop codon, and c.1562A > G p.(Lys521Arg) inherited from his father, substituting an amino-acid that is invariant among all metazoans. Both

variants were present in GnomAD with a frequency of 10^{-4} and 8×10^{-6} , respectively. According to Varsome analysis, the c.358delT variant was classified pathogenic (PVS1, PM2, PP3 and PP5), and the c.1562A > G variant likely pathogenic (PP2, PP3 and PM2) according to the ACMG classification. No mutation was identified in genes involved in inherited optic atrophies or in riboflavin transport or metabolism (Mosegaard et al., 2020).

3.3 Respiratory chain complex maximal activities and maximal phosphorylating respirations (Fig. 2A, B): Complex 1 deficiency

The specific CI activity (NUR activity normalized to CS, NUR/CS, Fig. 2A) was decreased in the patient and in the positive control (Ctr+) *ACAD9*-mutated cell lines (-51%, > 3 SD; -30%, > 2SD for patient and Ctr+ cells, respectively), while activities of the complexes II, III, IV and the F1-ATPase were unchanged, demonstrating an isolated CI deficiency (Fig. 2A). By contrast, the maximal CI-linked respirations (Fig. 2B, State 3 MP and MPG) were mostly preserved (-30% and -16% in both patient and Ctr+ cells, respectively < 2SD).

3.4 Impact of the *ACAD9* variants on CI assembly (blue native PAGE, Fig. 2C, D)

CI holoenzyme level was slightly decreased in both *ACAD9* mutated cells, compared to controls ($66 \pm 18\%$ and $72 \pm 20\%$ in patient and Ctr+ cells, respectively, Fig. 2C). To further analyze the blockade step in CI assembly, CI intermediates were revealed with assembly factors targeted antibodies (Fig. 2D). The ~400 kDa Pp-b intermediate assembly is assisted by the MCIA complex, which comprises *ACAD9*, (Fig. 2D, middle panel, lanes 1–3 and 5, marked with *) (Formosa et al., 2020). This intermediate assembly was present in control cells. However, in *ACAD9* mutated cells (patient and Ctr+ cells), the Pp-b intermediate could not be detected (Fig. 2D, middle panel), resulting in accumulation of the matrix intermediates Q and mostly Q/Pp-a (Fig. 2D, upper panel). Neither Q nor Pp-a intermediates require *ACAD9* for their assembly. Inversely, in cells mutated in the CI NDUFS7 subunit, located in the Q module, Q and Q/Pp-a matrix intermediates were lacking (Fig. 2D, upper panel) and a consistent accumulation of the membrane Pp-b intermediates was evidenced. These results indicate a major CI assembly defect linked to a specific impairment of assembly and/or stabilization of the *ACAD9*-containing submodule.

3.5 Riboflavin treatment enhanced CI activity

In the patient cells, 48 h riboflavin treatment dose-dependently increased CI activity, as measured by NUR activity (Fig. 2E) with a significant effect at 10 μ M (+81%, $p = 0.049$). This result is reinforced by CI IGA analysis (Fig. 2F), indicating an enhanced NADH-NTB reduction in patient cells treated with riboflavin. While riboflavin treatment stimulated CI activity, it did not alleviate the CI assembly defect: neither the CI holoenzyme level (Fig. 2G), nor the Pp-b assembly intermediate (Fig. 2H) was increased, while the Q/Pp-a submodule showed marked accumulation.

4 Discussion

We report the case of a patient with normal cognitive function who developed childhood-onset rapidly progressive optic neuropathy associated to sensory neuropathy, lactic acidosis and lactate peak on brain spectroscopy. This phenotype suggested the diagnosis of a defect of energy metabolism and gene panel sequencing identified two *ACAD9* variants.

Classically, the clinical presentation of *ACAD9* deficiency (OMIM 611126) is mainly represented by cardiomyopathy. Other features include lactic acidosis, myopathy and developmental delay (Dewulf et al., 2016; Lagoutte-Renosi et al., 2015; Repp et al., 2018). Age of onset

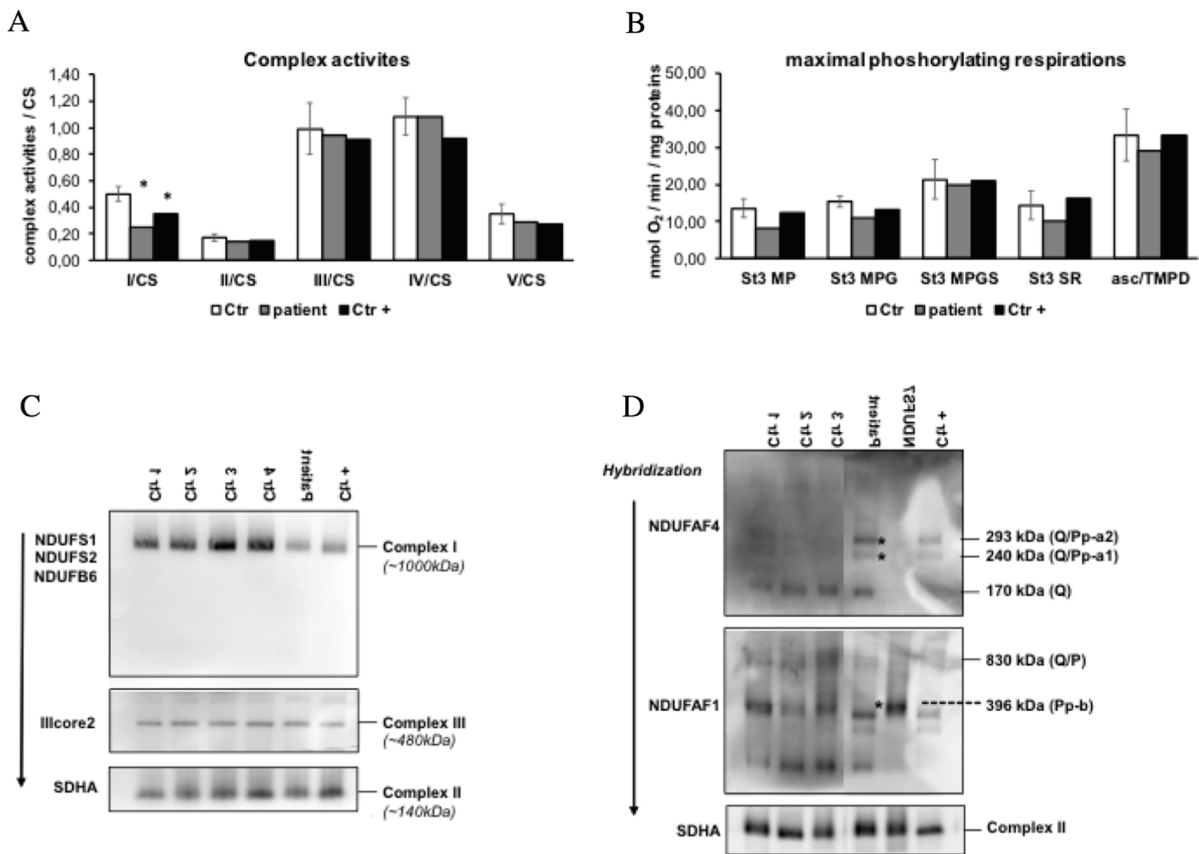


Fig. 2. A–D: impact of the *ACAD9* variants on the CI activity and assembly. E–H: impact of the riboflavin supplementation on the CI activity and assembly. **A.** Maximal enzymatic activity of Complexes I, II, III, IV and V, normalized to the citrate synthase (CS) one. **B.** Maximal respiration rates in phosphorylating conditions, determined on permeabilized cells with: CI substrates, malate, pyruvate (St3 MP) and malate, pyruvate and glutamate (St3 MPG); CI and CII substrates malate, pyruvate, glutamate and succinate (St3 MPGS); CII substrate succinate and complex I inhibited with rotenone (St3 SR) and the artificial CIV substrate TMPD reduced by ascorbate (asc/TMPD). **C.** Representative blots of CI holoenzyme level analyzed by Blue Native PAGE. Membranes were sequentially hybridized with antibodies targeting CI structural subunits NDUFB6, NDUFS2, NDUFS1, IIIcore2 and SDHA antibodies. CI holoenzyme quantity was normalized to the CII one, used as a reference. **D.** Representative blots of CI Assembly intermediates, analyzed by Blue Native PAGE. Membranes were sequentially hybridized using antibodies targeting CI assembly factor anti-NDUFAF4, NDUFAF1 and CII structural subunit SDHA. NDUFAF4 antibody detected the Q-containing intermediates, i.e. Q at 170 kDa (1) and Q/Pp-a (at ~240 kDa and ~290 kDa, intermediates). The detection of NDUFAF1 revealed a main band at ~400 kDa matching with the size described for the Pp-b intermediate and two higher faint bands below and above 830 kDa matching the Q/Pp and Q/P intermediates. **A, B.** Analyses were performed at least on two biological replicates for each cell line of each group. Results are presented as means \pm SD. * indicates a significant difference compared with Ctr group. **C, D.** Analyses were performed on two biological replicates for each cell line. Ctr: control fibroblast cells. Ctr+: *ACAD9*-mutated cells. NDUFS7: NDUFS7-mutated cells. **E.** Effect of riboflavin supplementation on CI maximal enzymatic activity. **F.** Representative CI in-gel activity analysis on control (Ctr) and patient cells treated for 48H with either vehicle (NaOH 0.1 M) or riboflavin (3 and 10 μ M). **G.** Effect of riboflavin supplementation on CI holoenzyme level. Upper panel: Representative blots of CI holoenzyme level analyzed by blue-native PAGE. Membranes were hybridized with antibodies targeting CI structural subunits NDUFS1 and CII subunit SDHA. Lower panel: CI holoenzyme quantity. CI holoenzyme quantity was normalized to the CII one, used as a reference. **H.** Effect of riboflavin supplementation on CI Assembly intermediates. Representative blots of CI Assembly intermediates, analyzed by blue-native PAGE. Membranes were sequentially hybridized using antibodies targeting CI assembly factor anti-NDUFAF4, NDUFAF1 and CII subunit SDHA. **E, F, G, H.** Ctr and patient cells were treated with riboflavin (3 or 10 μ M) or with NaOH 0.1 M in DMEM-F12 (vehicle condition, "0 μ M"), for 48H. Treatments: n = 3, performed on independent biological samples. Results are presented as means \pm SD. * indicates a significant difference compared with Ctr group; # indicates a significant riboflavin effect ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and level of severity may vary, but usually early-onset is associated with poor survival. Severe intellectual disability is not common, and most patients who survive beyond the first 2 years are independent in daily living activities (Repp et al., 2018); mild phenotypes limited to myopathic features have been described (Repp et al., 2018; Schrank et al., 2017). Optic atrophy was reported in one patient with early-onset cardiomyopathy (Collet et al., 2015). Although we cannot exclude that optic nerve involvement was underestimated in previously published clinical cases, as funduscopic examination was not performed, the phenotype of our patient is singular and enlarges the phenotypic spectrum associated to *ACAD9* pathogenic variants.

Acyl-CoA dehydrogenase 9 is a mitochondrial flavoenzyme with

structural similarities to very-long-chain acyl-CoA dehydrogenase, both enzymes catalyzing the initial step of the fatty acid oxidation. *ACAD9* is also crucial for oxidative phosphorylation (Nouws et al., 2010). Indeed, it plays a central role among the core subunits that compose the mitochondrial CI assembly (MCIA) complex, involved in assembly of membrane intermediate Pp-b (Formosa et al., 2020; Nouws et al., 2010, 2013), and *ACAD9* pathogenic variants leads to decreased CI assembly and activity (Haack et al., 2010).

The patient described here harbors compound heterozygous *ACAD9* variants, one pathogenic resulting in a stop codon already reported (Repp et al., 2018), the other predicted to be likely pathogenic. Biochemical and cell biology studies demonstrated reduced quantity of

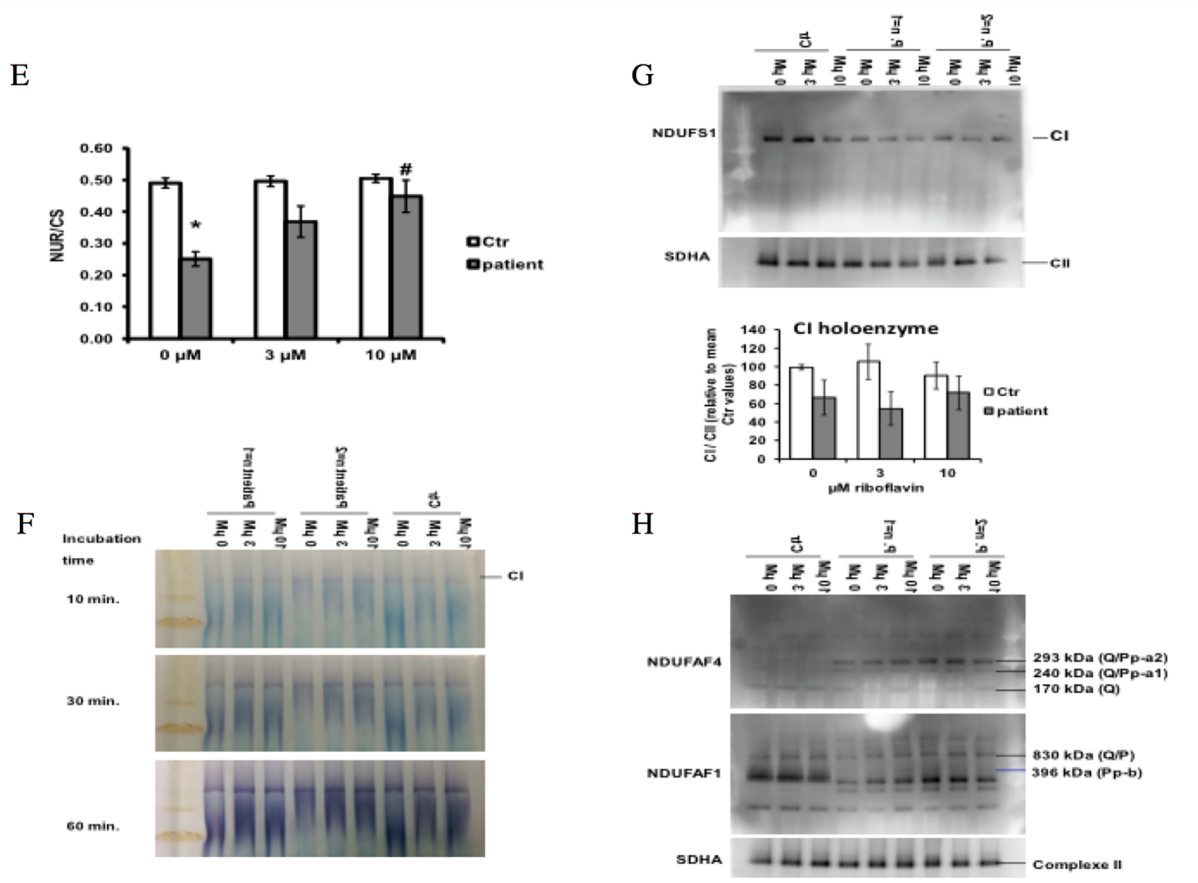


Fig. 2. (continued).

the CI Pp-b intermediate and disruption of CI assembly leading to decreased CI activity in the patient's fibroblasts, as well as in cells from a control *ACAD9* mutated patient. This result highlights the pathogenic effects of the patient's variants and their functional significance, comparable to published cases (Nouws et al., 2010; Repp et al., 2018).

A major finding of this case is the improvement of the long distance visual acuity by riboflavin and idebenone treatment, even after eliminating idebenone supplementation due to secondary deleterious effects. Supporting this observation, we found that riboflavin supplementation improved CI activity in fibroblasts from our patient. Clinical benefit and improvement of CI activity by riboflavin supplementation have already been previously reported in most *ACAD9* presentations (Gerards et al., 2010; Haack et al., 2010; Kadoya et al., 2019; Repp et al., 2018). Riboflavin is the vitamin precursor of FAD moiety, a cofactor of *ACAD9*. It has been hypothesized that riboflavin might increase *ACAD9* activity and/or stability, resulting in improvement of CI assembly (Gerards et al., 2010). However, recent studies showed that bound-FAD cofactor is essential for *ACAD9* acyl-CoA dehydrogenase activity, but is released through deflavination, when *ACAD9* switches to its CI assembly function (Giachin et al., 2021). Accordingly, we found that riboflavin supplementation did not restore CI assembly in the fibroblasts from the patient described here, although CI activity was increased. Therefore, effects of riboflavin supplementation on CI activity might involve a more subtle cellular process unrelated to CI assembly, which could be linked to *ACAD9* fatty acid oxidation activity (Schiff et al., 2015).

5 Conclusion

Therapeutic options for inherited optic neuropathies remain limited, although gene therapy appears to represent a promising approach (Gilhooley et al., 2021). Here, we enlarged the phenotypic spectrum

associated to *ACAD9* pathogenic variants, by describing a new form of syndromic optic neuropathy related to CI deficiency, which hopefully was responsive to riboflavin supplementation. We propose to screen *ACAD9* gene in patients with optic neuropathy, especially when associated to sensory peripheral neuropathy, and propose to consider riboflavin administration.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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