

# Novel *MTND1* mutations cause isolated exercise intolerance, complex I deficiency and increased assembly factor expression

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## Abstract

Complex I (CI) is the largest of the five multi-subunit complexes constituting the human oxidative phosphorylation (OXPHOS) system. Seven of its catalytic core subunits are encoded by mitochondrial DNA (ND (NADH dehydrogenase)1–6, ND4L (NADH dehydrogenase subunit 4L)), with mutations in all seven having been reported in association with isolated CI deficiency. We investigated two unrelated adult patients presenting with marked exercise intolerance, persistent lactic acidemia and severe muscle-restricted isolated CI deficiency associated with sub-sarcolemmal mitochondrial accumulation. Screening of the mitochondrial genome detected novel mutations in the *MTND1* (NADH dehydrogenase subunit 1) gene, encoding subunit of CI [Patient 1, m.3365T>C predicting p.(Leu20Pro); Patient 2, m.4175G>A predicting p.(Trp290\*)] at high levels of mitochondrial DNA heteroplasmy in skeletal muscle. We evaluated the effect of these novel *MTND1* mutations on complex assembly showing that CI assembly, although markedly reduced, was viable in the absence of detectable ND1 signal. Real-time PCR and Western blotting showed overexpression of different CI assembly factor transcripts and proteins in patient tissue. Together, our data indicate that the mechanism underlying the expression of the biochemical defect may involve a compensatory response to the novel *MTND1* gene mutations, promoting assembly factor up-regulation and stabilization of respiratory chain super-complexes, resulting in partial rescue of the clinical phenotype.

**Key words:** assembly factor, complex I, exercise intolerance, mitochondrial, *MTND1*.

## INTRODUCTION

Isolated deficiencies of human complex I (CI) often cause severe early-onset progressive disease, but the spectrum of disorders associated with this biochemical abnormality is expanding. In particular, mutations in the ND1 subunit of CI [MIM #252010] are associated with Leigh syndrome [MIM #256000]; cardiomyopathy; epilepsy; encephalopathy; mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS)

[MIM #54000]; Leber hereditary optic neuropathy (LHON) [MIM #535000] and an overlap syndrome comprising clinical features of both LHON and MELAS [1–7]. Exercise intolerance is a common symptom of mitochondrial disorders that can occur in isolation or as part of a multi-system disorder and has been associated with mutations in many genes encoding subunits of various complexes [8–11]. However, the relationship between the

**Abbreviations:** <sup>31</sup>P-MRS, phosphorus magnetic resonance spectroscopy; ACTB,  $\beta$ -actin; AFG3L2, AFG3-like AAA ATPase 2; AT, anaerobic threshold; BN, blue native; CI/CII/CIII/CIV/CV, complex I/II/III/IV/V; CIII<sub>2</sub>, CIII dimer; COX, cytochrome c oxidase; CPET, cardiopulmonary exercise testing; H&E, haematoxylin and eosin; HSP60, heat shock protein 60; LHON, Leber hereditary optic neuropathy; LonP1, Lon peptidase 1; MELAS, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; MRC, Medical Research Council; NDUFA9, NADH dehydrogenase (ubiquinone) 1 $\alpha$  sub-complex 9; NDUFAL, NADH dehydrogenase (ubiquinone) complex I assembly factor; NDUFB8, NADH dehydrogenase (ubiquinone) 1 $\beta$  sub-complex 8; PCr, phosphocreatine; POTS, postural orthostatic tachycardia syndrome; RRF, ragged-red fibre; SDH, succinate dehydrogenase; SDHA, succinate dehydrogenase complex subunit A; VO<sub>2</sub> peak, peak oxygen consumption; VO<sub>2</sub>, oxygen uptake

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pathogenic mtDNA mutation and the biochemical and phenotypic expression of the defect remains poorly understood.

In the present paper, we describe two unrelated adult patients with severe isolated CI deficiency in skeletal muscle, progressive exercise intolerance, myopathy (without cardiomyopathy) and persistent lactic acidemia. Both patients harbour novel heteroplasmic *MTND1* (NADH dehydrogenase subunit 1) gene [MIM #516000] mutations. We have characterized  $\text{VO}_2$  (oxygen uptake) kinetics during graded aerobic exercise, assessed *in vivo* mitochondrial function using phosphorus MR spectroscopy and evaluated the molecular mechanisms underlying this purely muscular phenotype to understand the impact of both mutations on CI biogenesis.

## MATERIALS AND METHODS

### Study approval

Local study approval was granted (NRES Committee North East-Newcastle & North Tyneside 1) and written informed consent from both patients was obtained prior to study inclusion. All clinical investigations were evaluated according to the Declaration of Helsinki.

### Subjects

Patient 1 presented at age 16 years with mild exercise intolerance and prominent fatigue following a viral illness. She was diagnosed with chronic fatigue syndrome. At age 25 years she presented with progressive exertion-related dyspnoea and palpitations and was provisionally diagnosed with asthma. By 28 years of age, muscle weakness and fatigue with exercise-induced headache, vomiting, cardiac palpitations and syncope were prominent and a metabolic acidosis with elevated serum lactate was detected (Table 1). At this stage a neuromuscular opinion was sought. The clinical picture has progressed rapidly over the last 2 years with exercise tolerance reduced to less than 50 m. She has developed alcohol intolerance and postural orthostatic tachycardia syndrome (POTS). Patient 2 presented at age 22 years of age to a neurologist with life long indolent exertion-related muscle weakness and pain, dyspnoea, cardiac palpitations and syncope. There was no family history of muscle disease or parental consanguinity in either case.

### Cardiopulmonary exercise testing

Cardiopulmonary exercise testing (CPET) was performed in both subjects as described elsewhere [12]. Anaerobic threshold (AT) was determined using the V-slope method, as previously described [13].

### Phosphorus magnetic resonance spectroscopy

Muscle phosphorus magnetic resonance spectroscopy ( $^{31}\text{P}$ -MRS) was performed on the left calf, as detailed previously, in Patient 1 to evaluate *in vivo* mitochondrial function relative to an age matched reference group [14].

### Histochemical and biochemical analyses

Standard histological [H&E (haematoxylin and eosin), modified Gomori trichrome] and histochemical analyses of skeletal muscle biopsies were performed on fresh-frozen skeletal muscle sections (10  $\mu\text{m}$ ) [15]. Mitochondrial respiratory chain complex activities were determined in skeletal muscle homogenates and expressed relative to the activity of the matrix marker enzyme citrate synthase [16].

### Molecular genetics

Total DNA was extracted from available tissues including muscle, blood, buccal epithelia, urinary sediments, cultured myoblasts and fibroblasts. Muscle mtDNA rearrangements were investigated by long-range PCR strategies [17]. Direct sequencing of the entire mtDNA genome was undertaken [18]; alignment and variant calling were performed using SeqScape software (v2.1.1, Applied Biosystems) comparing changes to the GenBank reference sequence for human mtDNA (accession number NC\_012920.1).

### Assessment of mtDNA mutation load by quantitative pyrosequencing

Heteroplasmic levels of the m.3365T>C and m.4175G>A mutations were determined in homogenate tissue and individual laser-microcaptured COX-positive and COX-negative ragged-red fibres (RRFs) by quantitative pyrosequencing. Quantification of the heteroplasmy level of each variant was achieved using Pyromark Q24 software [19].

### Mitochondrial respiratory chain complex subunit immunohistochemistry

Complex subunit immunohistochemistry was carried out on frozen tissue sections as described previously [20]. Primary antibodies and their dilutions used were: CI ND1, 1:200 (gift from Dr Anne Lombes), CI NDUFB8 1:50, CII SDHA (succinate dehydrogenase complex subunit A; 70 kDa flavoprotein subunit) 1:1000, CIII UQCRC2 (ubiquinone: cytochrome *c* reductase core protein 2) (Core 2) 1:1000, CIV MTCO1 (mitochondrial cytochrome *c* oxidase subunit 1) 1:1000, CIV MTCO4, 1:1000, Porin, 1:1000; all commercially available antibodies were purchased from Abcam. Primary antibodies were detected using a polymer detection system (Menarini Diagnostics) as per the manufacturer's instructions and visualized with 3,3'-diaminobenzidine (DAB; Sigma).

### BN/PAGE and SDS/PAGE

For BN (blue native)/PAGE, frozen skeletal muscle (10–50 mg) was homogenized and solubilized with 2% dodecylmaltoside (individual complexes and smaller super-complexes) or 4% digitonin (super-complexes) [21] and electrophoresed through precast 4–16% (dodecylmaltoside) or 3–13% gels (Invitrogen). Activities of CI, CII and CIV were estimated by in-gel assays as described in [22]. For 2D analysis, 1D lanes were incubated for 15 min in 1% SDS and 1%  $\beta$ -mercaptoethanol and separated in a 16% Tricine/SDS gel (BN-SDS/PAGE). For SDS/PAGE, proteins were extracted from enriched membranes (muscle) and separated by standard SDS/PAGE procedures. Proteins were transferred onto PVDF membranes (GE Healthcare) and processed

for immunoblotting. Primary antibodies used were: NDUFB8 [NADH dehydrogenase (ubiquinone) 1 $\beta$  sub-complex 8], NDUFA9 [NADH dehydrogenase (ubiquinone) 1 $\alpha$  sub-complex 9], Core2 (complex III Core protein 2), COXI, COXII, SDHA, CV subunits, TOM20 (translocase of outer mitochondrial membrane 20 kDa subunit) and porin (MitoSciences); AFG3L2 (AFG3-like AAA ATPase 2; Proteintech); LonP1 (Lon peptidase 1) and NDUFAF3 [NADH dehydrogenase (ubiquinone) complex I assembly factor; Sigma Life Science]; HSP60 (heat shock protein 60; BD Biosciences); NDUFAF1 and NDUFAF2 (gifts from Professor Leo Nijtmans) and ND1 (gift from Dr A. Lombes). Antibodies were detected with appropriate horseradish peroxidase (HRP)-conjugated immunoglobulins (Dako) and chemiluminescence detection reagents (GE Healthcare).

### Quantitative real-time PCR

Total RNA from control ( $n=4$ ) and patient muscle was extracted using TRIzol Reagent (Life Technologies) and reverse transcribed (0.5–1  $\mu$ g) with random hexamers (Superscript III First Strand Synthesis System, Life Technologies). Levels of mRNA expression of *NDUFAF1* [MIM #606934], *NDUFAF2* [MIM #609653], *NDUFAF3* [MIM #612911] and *ACTB* ( $\beta$ -actin) [MIM #102630] were assessed in triplicate by real-time PCR using commercially-available TaqMan assays (assay IDs Hs00211245\_m1, Hs02380072\_u1, Hs00404252\_g1 and Hs01060665\_g1 respectively; Life Technologies). After normalization to *ACTB*, average expression levels of the CI assembly factor genes in patients were reported relative to control values, with differences assessed using a two-tailed Student's *t* test.

## RESULTS

### Clinical features

#### Patient 1

Patient 1 was of normal intelligence (Wechsler test of adult reading: thirty-seventh percentile), normal stature (1.7 m) and body habitus (68.7 kg). She had no ptosis and eye movements were normal. She did not have significant demonstrable clinical weakness at rest [Medical Research Council (MRC) 5/5]; however, subtle upper and lower limb proximal weakness (MRC 4+/5) was evident following minimal exertion (marching in place ten times). She also displayed marked evidence of increased ventilator rate (>30 breaths/min), sinus tachycardia (>120 beats/min) and bluish-hue discolouration to her digits. There was no evidence of a dysautonomia and no cardiac or respiratory muscle involvement. There was a persistent metabolic acidosis (serum bicarbonate 9–19 mmol/l; normal range 22–29 mmol/l), a high random serum lactate concentration [7.2–8.9 mmol/l; normal range <1.7 mmol/l], markedly increasing post exertion up to 15.5 mmol/l and normal total creatine kinase activity (52–76 units/l; normal range <200 units/l). At the age of 27 years, there was electrophysiological evidence of a significant proximal myopathy with no findings of neuropathy. Conventional echocardiogram and cardiac MRI revealed no cardiac muscle abnormalities. She was treated with riboflavin (50 mg three times a day), high dose

co-enzyme Q<sub>10</sub> (500 mg/day) and L-carnitine supplements with minimal symptomatic or objective improvement. Current treatment strategies include energy conservation with the use of a wheelchair and pharmacological management with ivabradine for POTS.

#### Patient 2

Patient 2 was of normal height (1.68 m) but thin body habitus (47 kg). He had subtle unilateral ptosis and eye movements were normal. His skeletal muscles were generally poorly developed but he did not have significant demonstrable clinical weakness at rest (MRC 5/5); however, subtle upper and lower limb proximal weakness (MRC 4+/5) was evident following moderate exertion (marching in place 20 times). There was no evidence of cardiac or respiratory muscle involvement. There was a persistently high resting serum lactate concentration (4.1–6.8 mmol/l; normal range <1.7 mmol/l), increasing post exertion [15.4 mmol/l; cycling for 5 min; peak power 53W; peak oxygen consumption (VO<sub>2</sub> peak) 14 ml/min per kg; AT 22% predicted VO<sub>2</sub> peak] and raised total creatine kinase activity (351–486 units/l; normal range <320 units/l). Electrophysiological changes at the age of 11 years were compatible with a myopathy with no findings of neuropathy.

### CPET

VO<sub>2</sub> peak, peak work capacity (power; W), AT and absolute AT (expressed as a percentage of predicted peak VO<sub>2</sub>; ATVO<sub>2</sub>), were markedly decreased in both subjects (Table 1). Plasma lactate concentrations were concomitantly raised during CPET (Patient 1: resting lactate: 4.4 mmol/l, peak lactate: 13.2 mmol/l; Patient 2: resting lactate: 6.8 mmol/l, peak lactate: 15.4 mmol/l), with slow lactate decay time to baseline (Patient 1: 60 min, Patient 2: 40 min).

### <sup>31</sup>P-MRS shows significant mitochondrial energy defects associated with isolated CI deficiency

<sup>31</sup>P-MRS examination showed that the ability of Patient 1's muscle to reduce phosphocreatine (PCr) and accumulate ADP during exercise was normal. However, in recovery from muscle contraction, the main indicators of mitochondrial function demonstrated a severe impairment in oxidative metabolism (Table 1).

### Characterization of isolated complex I deficiency in skeletal muscle biopsies

Oxidative enzyme reactions [succinate dehydrogenase (SDH) and cytochrome *c* oxidase (COX)] revealed numerous fibres with increased activity at the fibre periphery, confirmed by modified Gomori trichrome staining which showed sub-sarcolemmal accumulations typical of 'ragged-red' changes affecting >25% of all fibres in both patients (Figure 1A). Mitochondrial respiratory chain enzyme studies revealed isolated CI deficiency in skeletal muscle from both patients (Table 1), including a repeat muscle biopsy obtained from Patient 1 taken 2 years after her initial diagnostic biopsy. The biochemical defect in this patient was not expressed in cultured myoblasts (results not shown). Muscle analyses of Patient 1, shown in the present study, were performed on the first muscle biopsy. The activities of all other respiratory

**Table 1** Peak exercise parameters, <sup>31</sup>P-MRS examination on calf evaluating *in vivo* mitochondrial function and assessment of respiratory chain complex activities in skeletal muscle homogenates

BPM, beats per min; DCPIP, 2,6-dichlorophenol-indophenol; n/a, not available; t<sub>1/2</sub> PCR (s), half time for PCR recovery from end exercise to baseline concentrations; VO<sub>2</sub>, oxygen uptake. Enzyme activities are expressed as nmol of NADH oxidized/min per unit of citrate synthase (CS) for CI, nmol of DCPIP reduced/min per unit of CS for CII (succinate:ubiquinone-1 reductase) and the apparent first-order rate constant/s per unit of CS for CIII and CIV ( $\times 10^3$ ).

Parameter	Patient 1		Patient 2	Reference data (means $\pm$ S.D.)
CPET				
Peak exercise data	Absolute value (% of predicted)		Absolute value (% of predicted)	n/a
Heart rate (BPM)	172 (89)		180 (92)	n/a
VO <sub>2</sub> (ml/min)	0.575 (27)		0.638 (22)	n/a
VO <sub>2</sub> (ml/min per kg)	8 (27)		14 (22)	n/a
VO <sub>2</sub> (% of predicted VO <sub>2</sub> )	27		22	n/a
Power (W)	35 (21)		53 (27)	n/a
AT				
ATVO <sub>2</sub> (ml/min per kg)	6		10	n/a
ATVO <sub>2</sub> (% of predicted peak VO <sub>2</sub> )	20		16	n/a
ATVO <sub>2</sub> (% of recorded peak VO <sub>2</sub> )	75		74	n/a
<sup>31</sup> P-MRS				[14]
End exercise data				
t <sub>1/2</sub> PCr	52.5		n/a	29.2 $\pm$ 3.1
t <sub>1/2</sub> ADP	31.4		n/a	20 $\pm$ 1
Respiratory chain complex activities				
	Biopsy 1	Biopsy 2	Biopsy	Controls (n = 25)
CI/CS	0.018	0.009	0.001	0.104 $\pm$ 0.036
CII/CS	0.092	0.127	0.070	0.145 $\pm$ 0.047
CIII/CS	0.673	1.790	0.634	0.554 $\pm$ 0.345
CIV/CS	0.834	1.394	1.216	1.124 $\pm$ 0.511

chain complexes were normal, confirming an isolated CI deficiency (Table 1). This was further confirmed by muscle immunohistochemistry in Patient 1 in which expression of both mtDNA-encoded (ND1) and nuclear-encoded (NDUFB8) CI structural components was markedly decreased in or absent from a vast number (>80%) of all muscle fibres (see Figures 1B,i and 1B,ii); protein components of CII, CIII and CIV showed normal expression (Figure 1B).

### Identification of novel pathogenic *MTND1* mutations

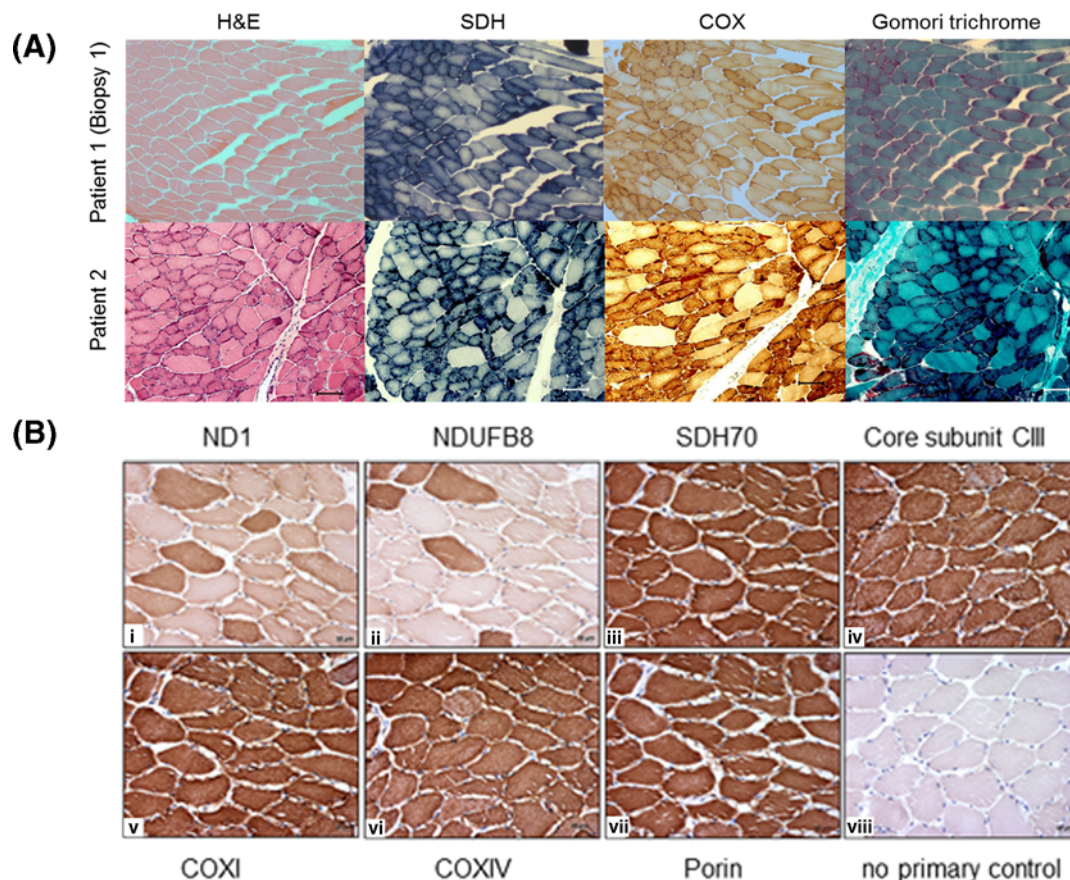
Having excluded mtDNA rearrangements, we determined the mtDNA sequence in muscle from both patients identifying novel candidate pathogenic *MTND1* mutations; Patient 1 harboured a novel m.3365T>C variant [predicting p.(Leu20Pro)] which was present at high levels of heteroplasmy (82% in biopsy 1; 86% in biopsy 2) in skeletal muscle, but undetectable in all other tissue samples tested (Figures 2A and 2B). Patient 2 was shown to harbour a novel m.4175G>A *MTND1* variant predicting p.(Trp290\*) and premature truncation of the ND1 protein. Quantitative pyrosequencing showed that the m.4175G>A mutation was present at high levels of heteroplasmy in skeletal muscle (90% mutant load); at low levels (5% mutant load) in a urinary sediment-derived DNA sample, but undetectable in all other DNA samples (Figure 2C). Concurrent studies in the mothers

of both patients failed to detect the respective mtDNA mutation suggesting *de novo* mutation events.

The m.3365T>C and m.4175G>A mutations were not previously reported on online databases of pathogenic mtDNA mutations [23,24] or within our own database of >980 human mtDNA sequences. Using quantitative pyrosequencing, we detected a trend towards higher levels of the m.3365T>C mutation in RRF (72.2  $\pm$  24.4%, n = 10) than non-RRF (56.5  $\pm$  28.8%, n = 10, P = 0.2049, two-tailed Student's *t* test). For the m.4175G>A mutation, significantly higher levels of mutation were detected in COX-positive RRF (89.0  $\pm$  24.2% n = 21) than in COX-positive non-RRF (39.6  $\pm$  44.2%, n = 11) (P = 0.0025, two-tailed Student's *t* test), confirming segregation of the m.4175A genotype with a histopathological abnormality.

### *MTND1* mutations are associated with loss of immunoreactive CI subunits and impaired CI assembly

The m.3365T>C; p.(Leu20Pro) mutation is located within the first transmembrane domain of the ND1 protein, whereas the m.4175G>A; p.(Trp290\*) mutation occurs within the hydrophilic loop that faces the mitochondrial matrix between transmembrane domains G and H (Figure 2D). Western blot analysis of muscle from both patients failed to detect ND1 protein and identified markedly decreased levels of the nuclear-encoded CI



**Figure 1** (A) Histological and histochemical assessment of patient muscle biopsies and (B) immunohistochemical analyses of muscle mitochondrial respiratory chain components in Patient 1

(A) Serial cryosections from both patients were assessed histologically (H&E and modified Gomori trichrome) and histochemically for oxidative enzyme activities including SDH and COX. Histological analysis of Patient 2's biopsy revealed moderate variation in fibre size with abundant, slightly hypotrophic, fibre clusters showing sub-sarcolemmal and granular sarcoplasmic basophilia and occasional fibres with internal nuclei. Both the histochemical reaction and the modified Gomori trichrome staining confirmed sub-sarcolemmal mitochondrial accumulation typical of RRF 'ragged-red' fibres in both subjects. (B) i, CI ND1 subunit; ii, CI NDUFB8 subunit; iii, CII 70 kDa flavoprotein subunit; iv, CIII core protein 2; v, CIV subunit 1; vi, CIV subunit 4; viii, porin; viii, no primary antibody control. Immunocytochemical studies confirm very low expression of both CI-encoded subunits (NDUFB8 and ND1), whereas there was normal immunoreactivity to the structural components of all other mitochondrial respiratory chain complexes.

subunit NDUFB8, whereas steady-state levels of other respiratory chain complex structural subunits were unaffected (Figures 3A and 3B). BN/PAGE analysis with subsequent Western blot analyses showed a fully assembled CI could be detected in both patients, albeit at very low levels, whereas steady-state levels of fully-assembled CII, CIII and CIV appeared unchanged (Figure 3C). This observation was accurately reflected in the in-gel activity assays which indicated some residual CI activity (Figure 3D), in agreement with immunocytochemical studies (Figure 1B).

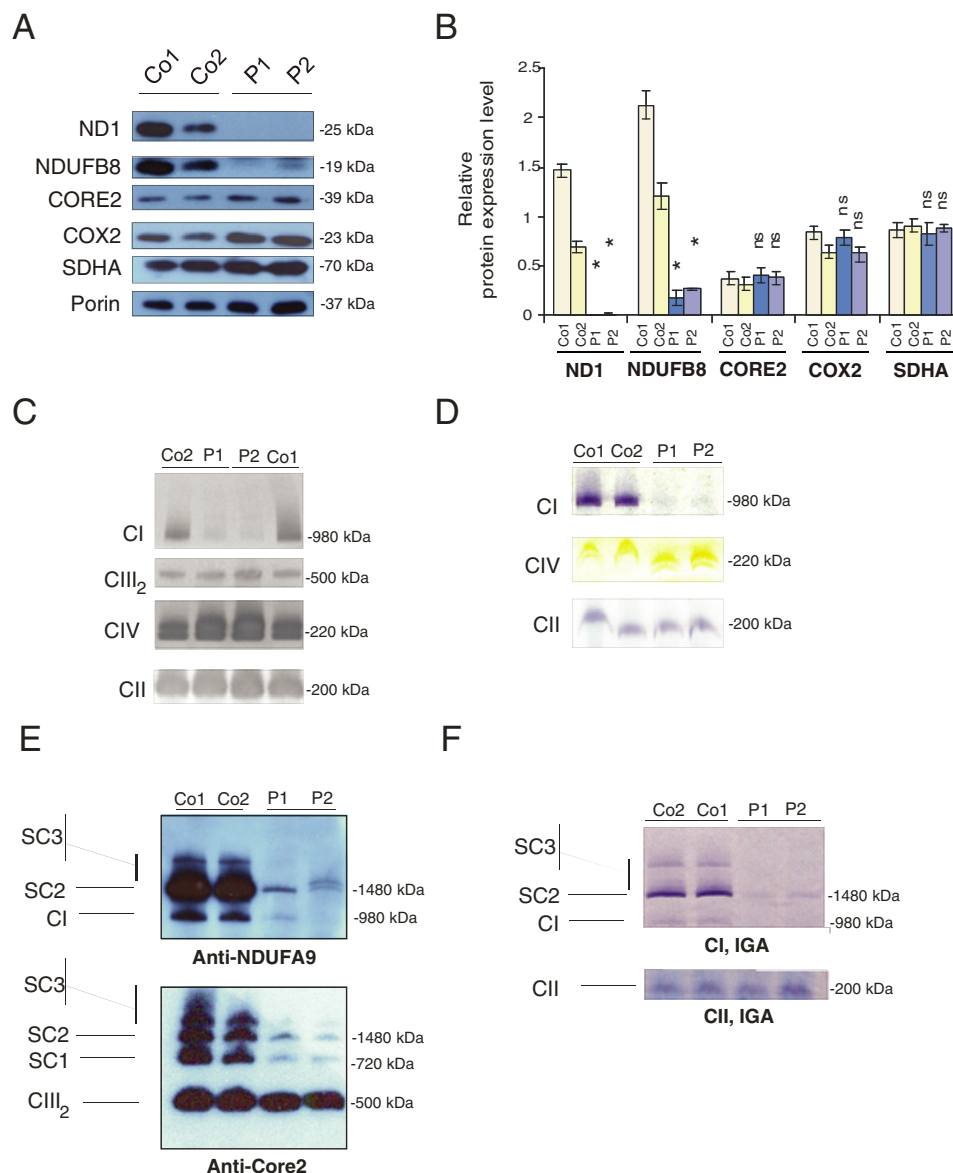
BN/PAGE, followed by Western blot and in-gel-activity analyses of control muscle samples, showed a small amount of fully assembled CI in its monomeric form, whereas the majority of assembled complex was associated with other complexes building the SC2 (supercomplex 2) [CI + CIII<sub>2</sub> (CIII dimer)] and SC3 (CI + CIII<sub>2</sub> + CIV<sub>(1-4)</sub>) super-complexes (Figure 3E). Levels of fully assembled CI were strongly decreased in patient

samples, with residual CI associated, in the most part, with CIII in the SC2 (CI+CIII<sub>2</sub>) super-complex (Patient 1) and with CIV in the SC3 (CI + CIII<sub>2</sub> + CIV<sub>(1-4)</sub>) super-complex (Patient 2; Figures 3E and 3F).

### ***MTND1* mutations stimulate an increase in CI assembly factor expression**

We evaluated the steady-state levels of different CI assembly factors including NDUFAF3 (early-stage factor), NDUFAF1 (involved in the middle stages) and NDUFAF2 (involved in the late stages of CI assembly) and mitochondrial proteases including AFG3L2, LonP1 and HSP60. We observed no changes in the expression levels of any proteases; however, steady-state levels of all three CI assembly factors were markedly increased in patient muscle samples suggestive of a compensatory response to the ND1 defect (Figure 4A). Confirmatory real-time PCR analyses showed increased *NDUFAF1* [MIM #606934], *NDUFAF2*



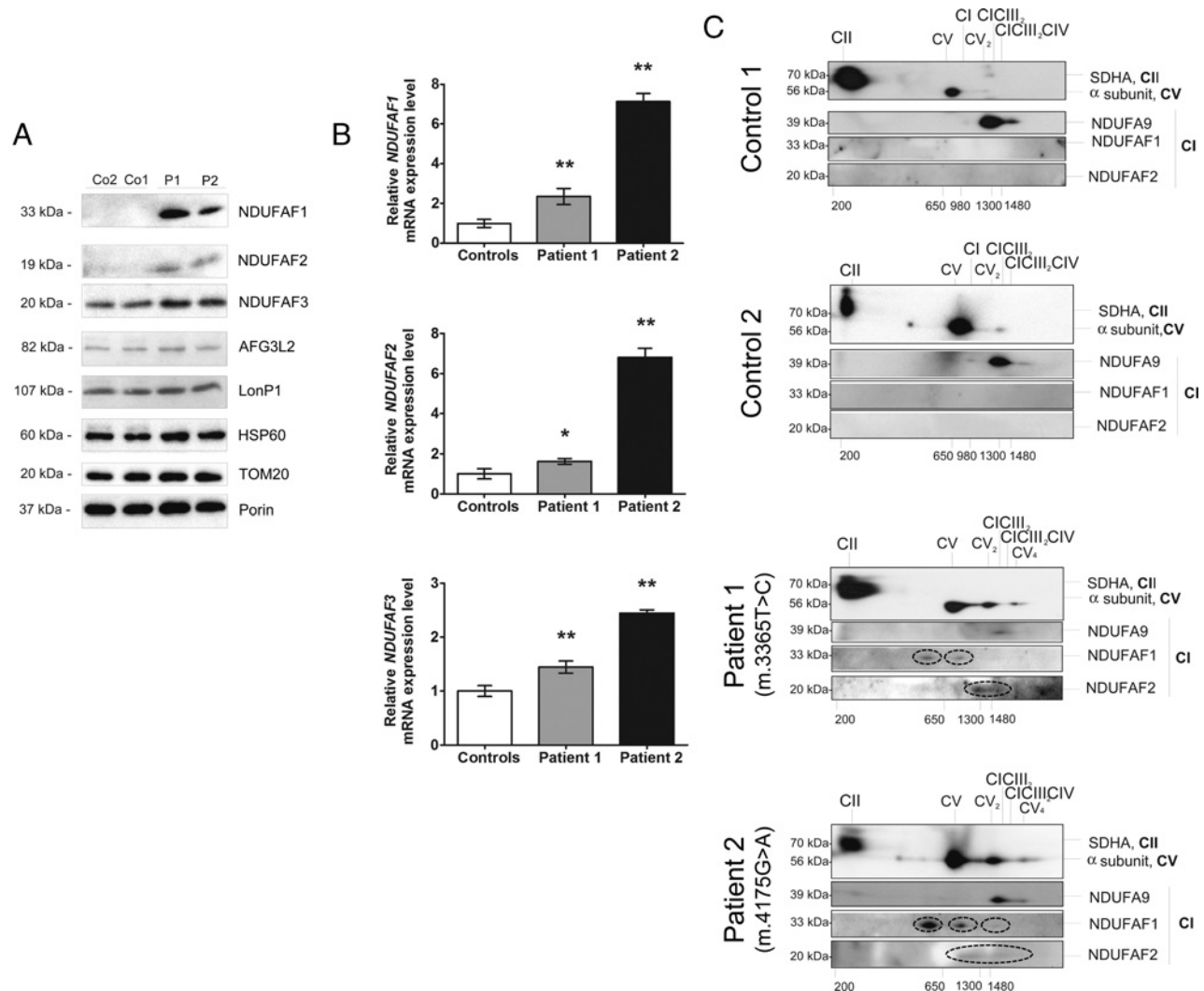


**Figure 3** *MTND1* mutations lead to a loss of immunoreactive CI subunits and impair CI assembly

(A) SDS/PAGE and Western blot analysis of muscle lysates (20  $\mu$ g) from two controls (Co1, Co2) and two patients (P1, m.3365T>C mutation; P2, m.4175G>A mutation). Membranes were probed with antibodies directed against mitochondrial respiratory chain subunits and porin as a loading control. (B) Relative protein expression levels. Band intensity of indicated protein is normalized to band intensity of the loading control porin. Shown are mean values of three independent experiments  $\pm$  S.D. \* $P$  < 0.05 relative to controls; ns, not significant. (C) BN/PAGE of dodecyl maltoside (DDM)-solubilized muscle samples (75  $\mu$ g) from two controls (Co1, Co2) and both patients with *MTND1* mutations (P1, P2) followed by Western blot analysis. Membranes were probed with anti-NDUFA9 for CI, SDHA for CII, CORE2 for CIII<sub>2</sub> and COX4 for CIV. Blots are representative of two independent experiments. (D) In-gel enzyme activity (IGA) assay of respiratory chain complexes. BN/PAGE was performed on DDM-solubilized muscle samples (150  $\mu$ g) from two controls and both patients. Enzyme activities for CI, CIV and CII were examined. (E) BN/PAGE of digitonin-solubilized muscle samples (100  $\mu$ g) from two controls (Co1, Co2) and both patients (P1, P2) followed by Western blot analysis. Membranes were probed with anti-NDUFA9 and with anti-CORE2. SC1, CIII<sub>2</sub> + CIV; SC2, CI + CIII<sub>2</sub>; SC3, CI + CIII<sub>2</sub> + CIV. Blots are representative of two independent experiments. (F) IGA assay of mitochondrial super-complexes. BN/PAGE was performed on digitonin-solubilized muscle samples (200  $\mu$ g) from two controls and the two patients with *MTND1* mutations (P1, P2). Enzyme activities for CI and CII were examined. SC1, CIII<sub>2</sub> + CIV; SC2, CI + CIII<sub>2</sub>; SC3, CI + CIII<sub>2</sub> + CIV.

Smaller CI subunits were also detectable with several intermediate complexes clearly seen in association with CI assembly factors. Accumulation of stalled intermediates of ~500 kDa and ~800 kDa has previously been reported in patients with various

CI assembly pathway mutations who also exhibit reduced assembly of the holoenzyme complex [26–31]. Steady-state levels of the mitochondrial quality-control machinery including the AAA protease AFG3L2, LonP1 protease and HSP60 remained



**Figure 4** (A and B) *MTND1* mutations stimulate an increase in CI assembly factor expression and (C) 2D BN/PAGE analysis of muscle mitochondria identifies smaller CI sub-complexes associated with *MTND1* mutation

(A) SDS/PAGE and Western blot analysis of muscle lysates (20  $\mu$ g) from two controls (Co1, Co2) and both patients (P1, m.3365T>C mutation; P2, m.4175G>A mutation). Membranes were probed with antibodies directed against the CI assembly factors NDUFAF1, NDUFAF2 and NDUFAF3, the mitochondrial AAA protease AFG3L2, the LonP1 protease, the HSP60 and porin as a loading control. Blots are representative of two independent experiments. (B) Relative mRNA expression levels of three CI assembly factor genes. The mRNA expression levels of *NDUFAF1*, *NDUFAF2* and *NDUFAF3* were measured by real-time PCR in Patients 1 and 2 and controls ( $n = 4$ ) and normalized to *ACTB* mRNA levels. Average patient data from three replicate experiments are expressed relative to control values  $\pm$  S.D. \* $P < 0.015$ ; \*\* $P < 0.001$  relative to controls. (C) Digitonin-solubilized mitochondrial complexes were separated by 1D BN/PAGE, followed by 2D Tricine-SDS/PAGE and Western blot analysis. Membranes were probed with SDHA for CII,  $\alpha$  subunit for CV, NDUFA9 for CI and NDUFAF1 and NDUFAF2. SC1, CII<sub>2</sub> + CIV; SC2, CI + CIII<sub>2</sub>; SC3, CI + CIII<sub>2</sub> + CIV. The smaller sub-complexes observed in both patients are highlighted by the ellipses; NDUFAF1 was associated with two CI intermediates of  $\sim$ 500 and  $\sim$ 800 kDa, whereas NDUFAF2 was detected within higher-molecular-mass associations corresponding to complexes of 800 and 1500 kDa in the muscle of Patients 1 and 2.

unchanged in association with up-regulation of assembly factor for intermediate-stage (NDUFAF1) and late-stage (NDUFAF2) CI assembly in patient muscle, suggesting a shift in this dynamic equilibrium state that favours assembly and stability of respiratory complex formation over degradation.

We speculate that mutation load and tissue-specific effects in CI formation and stability, mediated through assembly factor up-regulation, act as a compensatory response to the

deleterious *MTND1* genetic defect, with an absence of secondary defects in CII, CIII and CIV further facilitating complex stabilization. Taken together, our findings all support assembly factor up-regulation in isolated CI deficiency as an adaptive process to stabilize the respiratory complexes, a phenomenon not previously reported with *MTND1* mutations [32] and that the pathogenic role of these mutations at a molecular level is dependent on the stage of CI assembly. Mitotic segregation has long been recognized



to contribute to phenotypic variability that may explain the isolated muscle defects in these patients, although we concede that definitive exclusion of CNS involvement cannot be definitively made based on current evaluations.

In summary, we report two novel *MTND1* mutations that clinically manifest as isolated exercise intolerance and at a molecular level cause assembly factor up-regulation and sub-complex assembly acting to stabilize respiratory chain complexes and salvage assembly of the holoenzyme complex.

## CLINICAL PERSPECTIVES

- We sought to assess *in vivo* and *in vitro* mitochondrial function and evaluate the molecular mechanisms underlying a purely muscular phenotype in two adults whose clinical pictures were dominated by progressive exercise intolerance, lactic acidemia and severe isolated mitochondrial CI deficiency in muscle.
- We describe the detailed clinical, physiological, biochemical and molecular characterization of these patients who we show to harbour novel heteroplasmic mutations in the mtDNA-encoded *MTND1* gene.
- We demonstrate for the first time that mitochondrial super-complex reorganization occurs as a response to a compensatory mechanism to partially extricate the clinical phenotype involving up-regulation of CI assembly factors, a phenomenon not previously reported with *MTND1* mutations.

## AUTHOR CONTRIBUTION

The study was conceptualized and designed by Grainne Gorman, Emma Blakely, Hue-Tran Hornig-Do, Doug Turnbull, Robert McFarland and Robert Taylor. Acquisition of data was performed by Grainne Gorman, Emma Blakely, Hue-Tran Hornig-Do, Helen Tuppen, Laura Greaves, Langping He, Angela Baker, Gavin Falkous, Jane Newman, Michael Trenell, Bryan Lecky, Richard K. Petty, Doug Turnbull, Robert McFarland and Robert Taylor. Analysis and interpretation of data was done by Grainne Gorman, Emma Blakely, Hue-Tran Hornig-Do, Helen Tuppen, Laura Greaves, Langping He, Angela Baker, Gavin Falkous, Jane Newman, Michael Trenell, Bryan Lecky, Richard K. Petty, Doug Turnbull, Robert McFarland and Robert Taylor. The manuscript was drafted by Grainne Gorman, Emma Blakely, Hue-Tran Hornig-Do, Helen Tuppen, Laura Greaves and Robert Taylor. Critical revision of the manuscript for important intellectual content was done by Grainne Gorman, Emma Blakely, Hue-Tran Hornig-Do, Helen Tuppen, Laura Greaves, Michael Trenell, Bryan Lecky, Richard K. Petty, Doug Turnbull, Robert McFarland and Robert Taylor. The study was supervised by Grainne Gorman, Robert McFarland and Robert Taylor.

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## REFERENCES

- 1 Kirby, D.M., McFarland, R., Ohtake, A., Dunning, C., Ryan, M.T., Wilson, C., Ketteridge, D., Turnbull, D.M., Thorburn, D.R. and Taylor, R.W. (2004) Mutations of the mitochondrial ND1 gene as a cause of MELAS. *J. Med. Genet.* **41**, 784–789 [CrossRef PubMed](#)
- 2 Malfatti, E., Bugiani, M., Invernizzi, F., de Souza, C.F., Farina, L., Carrara, F., Lamantea, E., Antozzi, C., Confalonieri, P., Sanseverino, M.T. et al. (2007) Novel mutations of ND genes in complex I deficiency associated with mitochondrial encephalopathy. *Brain* **130**, 1894–1904 [CrossRef PubMed](#)
- 3 Delmiro, A., Rivera, H., Garcia-Silva, M.T., Garcia-Consuegra, I., Martin-Hernandez, E., Quijada-Fraile, P., de Las Heras, R.S., Moreno-Izquierdo, A., Martin, M.A., Arenas, J. and Martinez-Azorin, F. (2013) Whole-exome sequencing identifies a variant of the mitochondrial MT-ND1 gene associated with epileptic encephalopathy: west syndrome evolving to Lennox-Gastaut syndrome. *Hum. Mutat.* **34**, 1623–1627 [CrossRef PubMed](#)
- 4 Moslemi, A.R., Darin, N., Tulinius, M., Wiklund, L.M., Holme, E. and Oldfors, A. (2008) Progressive encephalopathy and complex I deficiency associated with mutations in *MTND1*. *Neuropediatrics* **39**, 24–28 [CrossRef PubMed](#)
- 5 Blakely, E.L., de Silva, R., King, A., Schwarzer, V., Harrower, T., Dawidek, G., Turnbull, D.M. and Taylor, R.W. (2005) LHON/MELAS overlap syndrome associated with a mitochondrial *MTND1* gene mutation. *Eur. J. Hum. Genet.* **13**, 623–627 [CrossRef PubMed](#)
- 6 Patsi, J., Maliniemi, P., Pakanen, S., Hinttala, R., Uusimaa, J., Majamaa, K., Nystrom, T., Kervinen, M. and Hassinen, I.E. (2012) LHON/MELAS overlap mutation in ND1 subunit of mitochondrial complex I affects ubiquinone binding as revealed by modeling in *Escherichia coli* NDH-1. *Biochim. Biophys. Acta* **1817**, 312–318 [CrossRef PubMed](#)
- 7 Valentino, M.L., Barboni, P., Ghelli, A., Bucchi, L., Rengo, C., Achilli, A., Torroni, A., Liguori, A., Lodi, R., Barbiroli, B., Dotti, M., Federico, A., Baruzzi, A. and Carelli, V. (2004) The ND1 gene of complex I is a mutational hot spot for Leber's hereditary optic neuropathy. *Ann. Neurol.* **56**, 631–641 [CrossRef PubMed](#)
- 8 Downham, E., Winterthun, S., Nakkestad, H.L., Hirth, A., Halvorsen, T., Taylor, R.W. and Bindoff, L.A. (2008) A novel mitochondrial ND5 (*MTND5*) gene mutation giving isolated exercise intolerance. *Neuromuscul. Disord.* **18**, 310–314 [CrossRef PubMed](#)
- 9 Andreu, A.L., Tanji, K., Bruno, C., Hadjigeorgiou, G.M., Sue, C.M., Jay, C., Ohnishi, T., Shanske, S., Bonilla, E. and DiMauro, S. (1999) Exercise intolerance due to a nonsense mutation in the mtDNA ND4 gene. *Ann. Neurol.* **45**, 820–823 [CrossRef PubMed](#)

- 10 Andreu, A.L., Hanna, M.G., Reichmann, H., Bruno, C., Penn, A.S., Tanji, K., Pallotti, F., Iwata, S., Bonilla, E., Lach, B., Morgan-Hughes, J. and DiMauro, S. (1999) Exercise intolerance due to mutations in the cytochrome b gene of mitochondrial DNA. *N. Engl. J. Med.* **341**, 1037–1044 [CrossRef PubMed](#)
- 11 McFarland, R., Taylor, R.W., Chinnery, P.F., Howell, N. and Turnbull, D.M. (2004) A novel sporadic mutation in cytochrome c oxidase subunit II as a cause of rhabdomyolysis. *Neuromuscul. Disord.* **14**, 162–166 [CrossRef PubMed](#)
- 12 Bates, M.G., Newman, J.H., Jakovljevic, D.G., Hollingsworth, K.G., Alston, C.L., Zaleski, P., Klawe, J.J., Blamire, A.M., Macgowan, G.A., Keavney, B.D. et al. (2013) Defining cardiac adaptations and safety of endurance training in patients with m.3243A>G-related mitochondrial disease. *Int. J. Cardiol.* **168**, 3599–3608 [CrossRef PubMed](#)
- 13 Beaver, W.L., Wasserman, K. and Whipp, B.J. (1986) A new method for detecting anaerobic threshold by gas exchange. *J. Appl. Physiol.* **60**, 2020–2027 [PubMed](#)
- 14 Trenell, M.I., Hollingsworth, K.G., Lim, E.L. and Taylor, R. (2008) Increased daily walking improves lipid oxidation without changes in mitochondrial function in type 2 diabetes. *Diabetes Care* **31**, 1644–1649 [CrossRef PubMed](#)
- 15 Old, S.L. and Johnson, M.A. (1989) Methods of microphotometric assay of succinate dehydrogenase and cytochrome c oxidase activities for use on human skeletal muscle. *Histochem. J.* **21**, 545–555 [CrossRef PubMed](#)
- 16 Kirby, D.M., Thorburn, D.R., Turnbull, D.M. and Taylor, R.W. (2007) Biochemical assays of respiratory chain complex activity. *Methods Cell Biol.* **80**, 93–119 [CrossRef PubMed](#)
- 17 Blakely, E.L., He, L., Taylor, R.W., Chinnery, P.F., Lightowlers, R.N., Schaefer, A.M. and Turnbull, D.M. (2004) Mitochondrial DNA deletion in “identical” twin brothers. *J. Med. Genet.* **41**, e19 [CrossRef PubMed](#)
- 18 Tuppen, H.A., Hogan, V.E., He, L., Blakely, E.L., Worgan, L., Al-Dosary, M., Saretzki, G., Alston, C.L., Morris, A.A., Clarke, M. et al. (2010) The p.M292T NDUFS2 mutation causes complex I-deficient Leigh syndrome in multiple families. *Brain* **133**, 2952–2963 [CrossRef PubMed](#)
- 19 White, H.E., Durston, V.J., Seller, A., Fratter, C., Harvey, J.F. and Cross, N.C. (2005) Accurate detection and quantitation of heteroplasmic mitochondrial point mutations by pyrosequencing. *Genet. Test.* **9**, 190–199 [CrossRef PubMed](#)
- 20 Greaves, L.C., Barron, M.J., Plusa, S., Kirkwood, T.B., Mathers, J.C., Taylor, R.W. and Turnbull, D.M. (2010) Defects in multiple complexes of the respiratory chain are present in ageing human colonic crypts. *Exp. Gerontol.* **45**, 573–579 [CrossRef PubMed](#)
- 21 Wittig, I., Braun, H.P. and Schagger, H. (2006) Blue native PAGE. *Nat. Protoc.* **1**, 418–428 [CrossRef PubMed](#)
- 22 Calvaruso, M.A., Smeitink, J. and Nijtmans, L. (2008) Electrophoresis techniques to investigate defects in oxidative phosphorylation. *Methods* **46**, 281–287 [CrossRef PubMed](#)
- 23 Ruiz-Pesini, E., Lott, M.T., Procaccio, V., Poole, J.C., Brandon, M.C., Mishmar, D., Yi, C., Kreuziger, J., Baldi, P. and Wallace, D.C. (2007) An enhanced MITOMAP with a global mtDNA mutational phylogeny. *Nucleic Acids Res.* **35**, D823–D828 [CrossRef PubMed](#)
- 24 Ingman, M. and Gyllensten, U. (2006) mtDB: Human Mitochondrial Genome Database, a resource for population genetics and medical sciences. *Nucleic Acids Res.* **34**, D749–D751 [CrossRef PubMed](#)
- 25 Flaherty, K.R., Wald, J., Weisman, I.M., Zeballos, R.J., Schork, M.A., Blaivas, M., Rubenfire, M. and Martinez, F.J. (2001) Unexplained exertional limitation: characterization of patients with a mitochondrial myopathy. *Am. J. Respir. Crit. Care Med.* **164**, 425–432 [CrossRef PubMed](#)
- 26 Ogilvie, I., Kennaway, N.G. and Shoubridge, E.A. (2005) A molecular chaperone for mitochondrial complex I assembly is mutated in a progressive encephalopathy. *J. Clin. Invest.* **115**, 2784–2792 [CrossRef PubMed](#)
- 27 Petruzzella, V., Vergari, R., Puzifferri, I., Boffoli, D., Lamantea, E., Zeviani, M. and Papa, S. (2001) A nonsense mutation in the NDUFS4 gene encoding the 18 kDa (AQDQ) subunit of complex I abolishes assembly and activity of the complex in a patient with Leigh-like syndrome. *Hum. Mol. Genet.* **10**, 529–535 [CrossRef PubMed](#)
- 28 Scacco, S., Petruzzella, V., Budde, S., Vergari, R., Tamborra, R., Panelli, D., van den Heuvel, L.P., Smeitink, J.A. and Papa, S. (2003) Pathological mutations of the human NDUFS4 gene of the 18-kDa (AQDQ) subunit of complex I affect the expression of the protein and the assembly and function of the complex. *J. Biol. Chem.* **278**, 44161–44167 [CrossRef PubMed](#)
- 29 Ugalde, C., Vogel, R., Huijbens, R., Van Den Heuvel, B., Smeitink, J. and Nijtmans, L. (2004) Human mitochondrial complex I assembles through the combination of evolutionary conserved modules: a framework to interpret complex I deficiencies. *Hum. Mol. Genet.* **13**, 2461–2472 [CrossRef PubMed](#)
- 30 Ugalde, C., Janssen, R.J., van den Heuvel, L.P., Smeitink, J.A. and Nijtmans, L.G. (2004) Differences in assembly or stability of complex I and other mitochondrial OXPHOS complexes in inherited complex I deficiency. *Hum. Mol. Genet.* **13**, 659–667 [CrossRef PubMed](#)
- 31 Hornig-Do, H.T., Tatsuta, T., Buckermann, A., Bust, M., Kollberg, G., Rotig, A., Hellmich, M., Nijtmans, L. and Wiesner, R.J. (2012) Nonsense mutations in the COX1 subunit impair the stability of respiratory chain complexes rather than their assembly. *EMBO J.* **31**, 1293–1307 [CrossRef PubMed](#)
- 32 McKenzie, M. and Ryan, M.T. (2010) Assembly factors of human mitochondrial complex I and their defects in disease. *IUBMB Life* **62**, 497–502 [CrossRef PubMed](#)

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