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Identification of novel mutations in five patients with mitochondrial encephalomyopathy

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ABSTRACT

MELAS, MERRF, LHON and NARP, are well-established mitochondrial syndromes associated with specific point mutations of mitochondrial DNA (mtDNA). However, these recurrent mtDNA mutations account for only a minority of mitochondrial disease cases. To evaluate the impact of novel mtDNA mutations, we performed mtDNA sequence analysis in muscle and other tissues of 240 patients with different mitochondrial neuromuscular syndromes. We identified a total of 33 subjects with novel, private or uncommon mutations. Among these, five novel mutations were found in both paediatric and adult cases. We here report on the clinical description of these patients, as well as the biochemical and molecular genetic characterization of the corresponding mutations. Patients 1 and 2 showed changes in *ND* genes, patient 3 carried a heteroplasmic deletion in the *COI* gene, patients 4 and 5 carried heteroplasmic mutations in $tRNA^{Trp}$ and $tRNA^{Phe}$, respectively. Altogether, these data indicate that mtDNA analysis must become part of the routine screening for mitochondrial disorders.

1. Introduction

The clinical spectrum of mitochondrial disorders is extremely heterogeneous, affecting young and adult patients at any age, with involvement of a wide variety of tissues. Recent epidemiological studies assign to the five most frequent pathogenic mitochondrial DNA (mtDNA) mutations a prevalence as high as 1 in 5000 live births [1].

An accurate and complete diagnostic workout is of course essential, and requires a systematic approach that combines and integrates clinical investigation and laboratory studies. DNA analysis and mitochondrial respiratory chain (MRC) assays remain the corner stones for the diagnosis, but in several instances numerous other skills and disciplines must concur, not only for the complete elucidation of the etiology and pathogenesis of a disease entity, but also to formulate evidence-based predictions about the clinical course and prognostic outcome.

Truly enough, some patients present with syndromes that are strongly suggestive of a mitochondrial disease, but many others have clinical signs that overlap with those of other disorders. Children in particular usually show very generic clinical features, so that a host of laboratory and physiological studies must be planned, including neuroimaging examination, proton magnetic resonance spectroscopy (MRS) of brain and/or phosphorus MRS of muscle, neurophysiological assessment of the central and peripheral nervous systems, and biomarker search in body fluids. Morphological and biochemical hallmarks characterize many, albeit not all, of these syndromes. For instance, the "ragged red" transformation of muscle fibers (RRF) is a frequent morphological clue, especially in adult patients; RRFs consist of abnormal mitochondria that accumulate in discrete segments along the muscle fiber, especially near the sarcolemma, eventually causing the fiber to "crack". Muscle fibers that stain negative to the histochemical reaction to cytochrome *c* oxidase (COX, respiratory complex IV) are also frequently observed in mitochondrial encephalomyopathy, and usually coincide with RRFs. Nevertheless, typical "mitochondrial" findings may be absent in otherwise demonstrated mitochondrial disorders. This is the case for Leber's Hereditary Optic Neuropathy (LHON), for Neuropathy Ataxia and Retinits Pigmentosa (NARP), and it is also true in many paediatric cases, for instance, but not only, Leigh syndrome (LS). In these instances, biochemistry of the

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Table 1
Clinical, biochemical and genetic features of 33 patients with mtDNA mutations

Patient	Age at onset	Clinical presentation	Skeletal muscle morphology	Biochemical analysis	Affected gene	Mutation (aminoacid change)	Reference
1	10 mo	LS	Normal	Reduction CI	*ND1*	3688G>A (p.A128T)	Present work
2	7 yrs	Ataxia, PEO	Normal	Normal	*ND5*	13094T>C (p.V253A)	Present work
3	36 yrs	Myopathy	RRFs and COX− fibers	Reduction CIV	*COI*	6698delA (p.K265fs271X)	Present work
4	54 yrs	Myopathy	COX− fibers	Normal	*tRNATrp*	5567T>C	Present work
5	47 yrs	Ataxia, PEO, deafness	RRFs and COX− fibers	Reduction CI, CIII and CIV	*tRNAPhe*	642T>C	Present work
6	6 yrs	MELAS, retinal dystrophy	RRFs	Reduction CI	*ND1*	3481G>A(p.E59K)	[10]
7	10 mo	LS	Normal	Reduction CI	*ND1*	3697G>A (p.G131S)	Mutation already reported www.mitomap.org
8	10 mo	LS	Normal	Reduction CI	*ND3*	10158T>C (p.S34P)	[5]
9	15 mo	LS	Normal	Reduction CI	*ND3*	10191T>C (p.S45P)	[5]
10	21 yrs	EPC, optic atrophy	Normal	Reduction CI	*ND3*	10191T>C (p.S45P)	[10]
11	7 mo	LS	Normal	Reduction CI	*ND3*	10197G>A (p.A47T)	Mutation already reported www.mitomap.org
12	9 mo	LS	Normal	Reduction CI	*ND4*	11777C>A (p.R340S)	[5]
13	32 yrs	Ataxia, fragmentary hypnic myoclonus	RRFs	Reduction CI	*ND5*	13063G>A (p.V243I)	[10]
14	6 yrs	MELAS	RRFs	Reduction CI	*ND5*	13513G>A (p.D393N)	[48]
15	5 mo	LS	Normal	Reduction CI	*ND5*	13513G>A (p.D393N)	[5]
16	13 yrs	MELAS	Normal	Reduction CI	*ND5*	13514A>G (p.D393G)	[48]
17	17 yrs	MELAS	Normal	Reduction CI	*ND5*	13514A>G (p.D393G)	[48]
18	6 yrs	Optic atrophy, Leigh-like syndrome	Normal	Reduction CI	*ND5*	13514A>G (p.D393G)	[5]
19	2 mo	LS, sensory-neural deafness	Normal	Reduction CI	*ND6*	14600G>A (p.P25L)	[10]
20	Birth	LS	Normal	Reduction CI	*ND6*	14487T>C (p.M63V)	[5]
21	5 mo	LS, dilating cardiomyopathy	Normal	Reduction CI	*ND6*	14487T>C (p.M63V)	[10]
22	16 yrs	Optic atrophy, ataxia	Normal	Reduction CI	*ND6*	14487T>C (p.M63V)	[10]
23	4 yrs	LS	COX− fibers	Reduction CIV	*COIII*	9537insC (p.Q111fs113X)	[18]
24	10 yrs	Myopathy	RRFs	Reduction CI and CIII	*CYTB*	15800C>T (p.Q352X)	[49]
25	6 yrs	MELAS	RRFs and COX− fibers	Reduction CI, CIII and CIV	$tRNALeu^{UUR}$	3291T>C	[50]
26	51 yrs	Myopathy	RRFs and COX− fibers	Normal	$tRNALeu^{UUR}$	3291T>C	Mutation already reported www.mitomap.org
27	12 yrs	Deafness, cognitive impairment	RRFs	Reduction CI	$tRNALeu^{UUR}$	3291T>C	Mutation already reported www.mitomap.org
28	27 yrs	Spastic paraparesis	Normal	Normal	*tRNAIle*	4284G>A	[51]
29	16 yrs	Severe encephalopathy	Normal	Reduction CI and CIV	*tRNAIle*	4290T>C	[52]
30	10 yrs	Ptosis, myopathy, epilepsy	Myopathic changes	Reduction CI	*tRNACys*	5814T>C	Mutation already reported www.mitomap.org
31	55 yrs	PEO, myopathy	RRFs and COX− fibers	Normal	*tRNAAsn*	5698G>A	[53]
32	45 yrs	Motor Neuron Disease-like	COX− fibers	Reduction CIV	$tRNASer^{CUN}$	7472insC	[54]
33	48 yrs	PEO, myopathy	RRFs	Reduction CIII and CIV	$tRNALeu^{CUN}$	12315G>A	Mutation already reported www.mitomap.org

LS, Leigh syndrome; PEO, progressive external ophthalmoplegia; EPC, epilepsia partialis continua; RRF, ragged red fibers; COX−, COX-negative fibers; CI, complex I; CIII, complex III; CIV, complex IV.

MRC can help. The identification of single or multiple MRC defects in a muscle (sometimes liver) biopsy, in cultured fibroblasts or in both is often the first step that can orient towards the molecular definition of a mitochondrial disease.

When a mitochondrial disease is suspected, a crucial decision is how to investigate the patient in the most efficient, least invasive and least expensive way. In patients suspected of well-established conditions, such as LHON, MELAS, MERRF, chronic progressive external ophthalmoplegia (CPEO), Kearns–Sayre, Pearson and Leigh syndromes, direct genetic analysis is indicated for the detection of point mutations or large-scale rearrangements of mtDNA. DNA can be extracted by different sources, for instance from blood, oral swab, or urine sediment. In several cases, however, analysis of mtDNA extracted from a muscle biopsy is advisable or mandatory, particularly for mtDNA mutations that propend to accumulate in post-mitotic tissues (e.g. large-scale mtDNA rearrangements). Still other patients, who do not fall in any well-established clinical entity, do require the systematic screening of the entire mtDNA genome. This analysis must precede the eventual screening of a host of nuclear-disease genes involved in mitochondrial energy defects.

We here report the results of a complete mtDNA analysis carried out in a cohort of 240 patients suspected of having a mitochondrial disorder based on clinical or biochemical evaluation, not classified in any of the "typical" syndromes. A total of 33/240 patients (14%) did have pathogenic mutations, five of which were novel (Table 1).

Table 2
RFLP analysis

Patient	mtDNA mutation	Primers	Restriction Enzyme
1	3688 G→A	**3629Fw** 5′-CCTCTAGCCTAGCCGTTTACTCAATCCTCTGATCAGGGTGAGCATCAAACTCAAAC**GA**C	HgaI/cuts the wild-type sequence
	ND1	**3900Rc** 5′-GAAGGGGGTTCGGTTGGTCTCTG-3′	
2	13094T→C	**12906Fw** 5′-CCTACACTCCAACTCATGAGACCCA-3′	AluI/cuts the mutant sequence
	ND5	**13310Rc** 5′-TGCTAGGTGTGGTTGGTTGATGCCG -3′	
4	5567 T→C	**5517Fw** 5′-TTAGGTTAAATACAGACCAAGAGCCTTCAAAGCCCTCAGTAAGTTG**GT**A-3′	RsaI/cuts the mutant sequence
	$tRNA^{Trp}$	**5800Rc** 5′-TTGCAAATTCGAAGAAGCAGCTTC-3′	
5	642T→C	**477Fw** 5′-CCTCCCACTCCCATACTACT-3′	NdeI/cuts the wild-type sequence
	$tRNA^{Phe}$	**700Rc** 5′-TTGCATGTGTAATCTTACTAAGAGCTAATAGAAAGGCTAGGACCAAACCTATTTG**CA**T-3′	

Sequences of primers used to detect and quantify the mutations: single nucleotides in bold and underlined are modified in order to create the specific restriction site.

2. Materials and methods

2.1. Fibroblast and cybrid cell cultures

Fibroblast cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplied with 10% fetal calf serum (FCS) at 37 °C in a 5% CO_2 atmosphere [2]. Transmitochondrial cybrids were obtained by polyethylene glycol (PEG) fusion, followed by selection in a uridine-free medium, as described [3]. Cytoplasts derived from cytochalasin-treated patients' fibroblasts were fused with a mtDNA-less (ρ°) derivative of the human osteosarcoma 143B cell line. The absence of mtDNA in ρ° cell lines, and its presence in transmitochondrial cybrids, was confirmed by PCR analysis using pairs of primers to amplify the D-loop region, as described [4].

2.2. Morphological and biochemical analyses

Morphological analysis of skeletal muscle, and biochemical assays of the individual MRC complexes on muscle homogenate, digitonin-treated fibroblasts and cybrids were carried out as described [5,6]. Specific activities of each complex were normalised to that of citrate synthase (CS), an indicator of the number of mitochondria.

2.3. Sequence, restriction fragment length polymorphism analysis and single-fiber PCR analysis

Molecular analysis was performed on genomic DNA extracted either from muscle, fibroblasts or other tissues. According to a standardized protocol [5], the entire mtDNA was PCR-amplified into eight overlapping fragments using a set of coupled primers. Each of the eight fragments was then sequenced, with four different 'sense' primers, using a 3100 ABI Prism Automated Sequencer. The presence and the amount of the mutations were verified by restriction fragment length polymorphism (RFLP) analysis of PCR products. Primers and restriction enzymes used for each mutation are reported in Table 2. The single-fiber PCR analysis was performed according to Sciacco et al. [7].

2.4. Blue native electrophoresis and in-gel activity assays

The detection of the assembled MRC complexes in isolated mitochondria was performed using blue native gel electrophoresis (BNGE) as described [8]. After first dimensional (1D) or second dimensional (2D) electrophoresis, proteins were electroblotted onto nitrocellulose filters and sequentially immunostained with specific antibodies against complex I subunits NDUFA9 (39 kDa) and NDUFB6 (17 kDa), complex IV subunits I, II, IV, and against SDH subunits, (30 kDa or 70 kDa) (Molecular Probes, Invitrogen). Immuno-visualization was performed with the 'ECL Western-blotting detection analysis system' (GE Healthcare, Amersham).

The in-gel activity assay for complex I was performed on 1D-BNGE as described [9].

2.5. Statistics

The complex I values, normalised with CS activity, in 11 homoplasmic cybrids from patient 1 vs. 10 wt cybrids from his mother were compared using non-paired, two-tail Student's t test. The same was applied in the analysis of single muscle fibers from patients 4 and 5. Regression analysis was performed for complex I values vs. heteroplasmy percentages measured in cybrid clones from patients 2 ($n = 15$).

2.6. Patients

Since 1990 we have been collecting a total of 4200 DNA samples from patients with an initial suspect of mitochondrial disease. The latter was suggested by (a) Clinical evaluation, based on symptoms and signs of encephalomyopathy, with or without other clues including, for instance, elevated levels of lactate in body fluids, and/or CT/MRI findings typical of mitochondrial disorders such as MELAS, Leigh syndrome, Alpers' syndrome, etc.; (b) Morphological and/or biochemical analysis of skeletal muscle biopsy and/or skin fibroblasts, compatible with defective respiratory chain; (c) identification of pathogenic mutations associated with mitochondrial defects (e.g. the mutations responsible of MELAS, MERRF, LHON, NARP, Kearns–Sayre s., etc); (d) strong suggestion of maternal inheritance; (e) the above points combined.

Genetic diagnosis was reached in approximately 30% of the cases. Of the remaining cases, in 240 consecutive individuals the suspect of a mtDNA defect was strong enough as to justify systematic, sequence-based analysis of the entire mitochondrial genome. A pathogenic mutation in mtDNA was identified in 33/240 cases (Table 1). In five of these 33 positive patients, we found new mtDNA mutations; the corresponding clinical presentations are hereafter reported.

Patient 1 the only child of unrelated Italian parents, was born after 37 weeks of gestation by cesarean delivery because of maternal gestosis. The birth weight was 3440 g and the Apgar score was 9-10-10. Three days after birth, he presented with an episode of acute respiratory distress treated with N-CPAP for 24 h. Laboratory and instrumental findings at that time were normal except for a systolic murmur due to a patent foramen ovalis. At 10 months of age, the patient had an acute episode of metabolic failure, characterized by vomiting, pallor and profuse sweating followed by lethargy. Since then, his clinical conditions worsened progressively with psychomotor regression, impairment of upgaze ocular motility with divergent strabismus, recurrent episodes of vomiting and tonic seizures with absence, well controlled by barbituric treatment. A first MRI study, performed at 11 months, showed symmetric signal abnormalities in the basal ganglia and midbrain (not shown). At 24 months the MRI showed a progression of the symmetric lesions in the basal ganglia, and the presence of brain cortex and subcortical white matter alterations (Fig. 1). Proton magnetic resonance spectroscopy ([H]-MRS) showed a markedly elevated lactate peak in correspondence to the T2-weighted abnormal signals, with slight reduction of the NAA and choline peaks. Both PEVs and BAEPs were abnormal. Serum lactic acid ranged from 2.75 to 3.34 mmol/L (normal range < 1.33 mmol/L), but pyruvic acid levels were normal. The child, now 5 years old has a spastic tetraparesis, with dystonic postures triggered by external stimuli, especially at the neck and trunk; sub-continuous myoclonic jerks at the upper limbs partially controlled by antiepileptic polytherapy with carbamazepine, clobazam and barbiturates, severe growth and mental retardation. A muscle biopsy performed at 16 months of age was morphologically normal (not shown).

Patient 2 is an 11-year-old child with onset at 7 years with ataxic–spastic gait; she later developed CPEO, facial weakness with drooling and difficulty in swallowing, occasional seizures, severe growth and mental retardation. The clinical course has been downhill, with progressive worsening of the ataxia and PEO, and hearing loss. PEVs and BAEPs were both abnormal. At 10 years, the blood lactate was in the normal range, while it was elevated in the CSF. MRI examinations showed the presence of abnormal signals in the brainstem, cerebellar dentate nuclei, and basal ganglia, suggestive of a mitochondrial encephalopathy. An [H]-MRS of the brain showed the presence of a lactate peak in the abnormal areas. However, a skeletal muscle biopsy taken at 7 years of age was morphologically and biochemically normal (Table 3).

Patient 3 is a 38-year-old man complaining of muscle pain, weakness and exercise intolerance with no muscle wasting. He had one episode of myoglobinuria, with very high levels of serum creatine kinase (CK). An EMG showed modest myopathic abnormalities. A muscle biopsy showed the presence of numerous COX-negative fibers and RRFs (Fig. 2A–C).

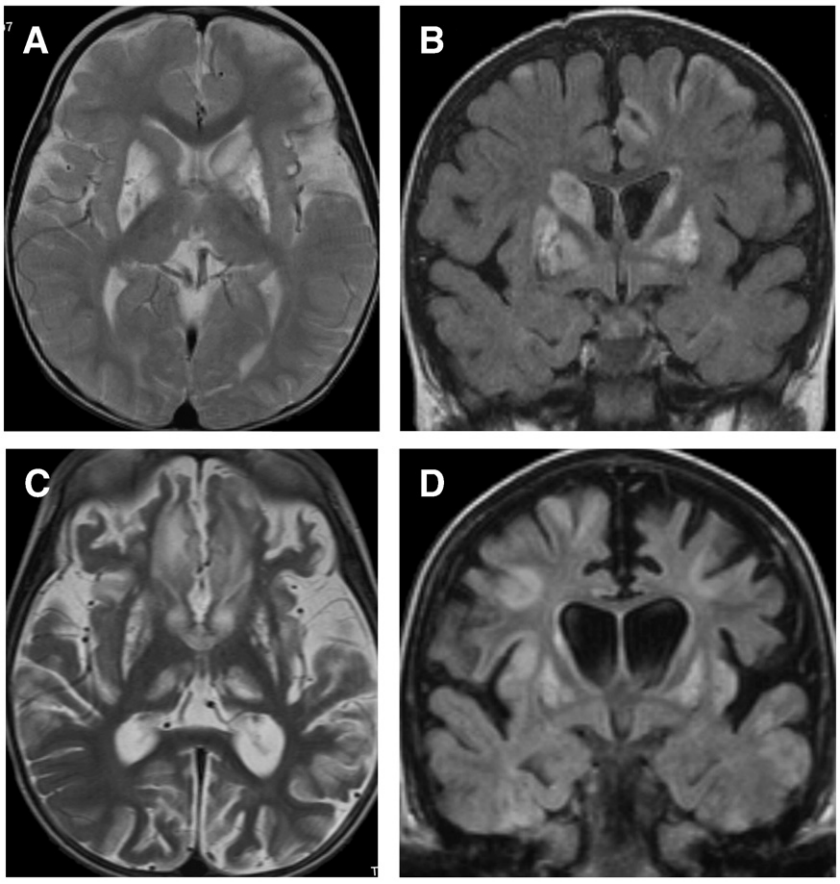

Fig. 1. Brain MRI of patient 1. (A, B) MRI performed at 2 years of age. (A) Axial T2-weighted and (B) coronal FLAIR images, showing lesions in the basal ganglia, that involve putamina, globi pallidi and caudate nuclei. In A, hyperintense lesions are present in the thalami. In B small bilateral signal abnormalities are present in the cortex. (C, D) MRI at 3.5 years of age. (C) T2-weighted and (D) coronal FLAIR images, showing diffuse and marked brain atrophy which involved also the basal ganglia.

Patient 4 is a 68-year-old woman. She was well until 54 years of age, when she started complaining fatigue, muscle weakness, joint pain, xerostomy and xerophthalmy, and low-grade fever. She also experienced episodes of dysphagia and fluctuating gait instability. Because of her clinical features and the laboratory findings of low-titer antinuclear antibodies, persistently elevated erythrocyte sedimentation rate (ESR), and high-titer PCR, she was suspected as having connectivitis and treated with high-dose corticosteroids. Since the anti-inflammatory therapy was of modest if any benefit, she was re-evaluated for her muscle weakness and gait instability. Myasthenia Gravis was excluded. Laboratory findings were all normal except serum creatinine (1.21 mg/L, n.v. 0.5–0.9), serum CK (195 U/L, n.v. 24–150), ESR (60 mm/h, n.v. 0–20). A muscle CT scan showed moderate adipose substitution of paravertebral muscles and bilateral atrophy of ileo-psoas muscles. A muscle biopsy of the left quadriceps revealed the presence of numerous COX-negative fibers with a few pre-RRF and lipid storage in some fibers (Fig. 2D–F).

Patient 5 is a 65-year-old woman with moderate mental retardation, who developed in her fifties a syndrome characterized by PEO, muscle weakness with proximal wasting, especially in the upper girdle and neck district, gait ataxia, peripheral neuropathy, urinary incontinence, hearing loss and neurosensory deafness. The deep tendon reflexes were absent with reduced vibratory sensation. She was dysmetric in both upper and lower limbs, and had a positive Romberg sign and gait ataxia. A muscle biopsy showed the presence of numerous COX-negative and RRFs (Fig. 2G–I). Analysis of the *POLG1* gene was negative. Family history is negative for neurodegenerative or neurometabolic disorders.

Table 3
Mitochondrial respiratory chain activities in patients 1–5

Patient	CI	CII	CIII	CIV	CV	CS
Muscle						
1	7.2	19.5	127	165	132	154
2	16.1	16.2	91	141	150	130
3	23.7	18.9	89	91	156	117
4	24.8	20.4	108	261	215	57
5	8.9	31.7	45	65	97	197
Controls' mean ± s.d.	18.5 ± 2.8	21.5 ± 3.3	128 ± 20	170 ± 26	155 ± 34	145 ± 33
Fibroblasts						
1	7.4	12.6	111	92	85	129
2	21.6	17.3	123	115	76	193
4	15	13.4	158	111	134	92
Controls' mean ± s.d.	18.4 ± 3.9	13.5 ± 2.5	108 ± 11	125 ± 19	98 ± 14	150 ± 25

Values are expressed as nmol/min mg of protein and normalised with citrate synthase (CS) activity. Fibroblasts from patient 3 and 5 were not available. CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V.

3. Results

3.1. Biochemical assays

Biochemical assay of MRC revealed the presence of specific and isolated complex I deficiency in both muscle (39%) and fibroblasts (40%) of patient 1, while an isolated defect of complex IV (53%) was detected in the muscle of patient 3. Patient 5 presented with multiple defects of the MRC complexes I, III and IV (Table 3). However, MRC activities were normal in both muscle and cultured fibroblasts of patients 2 and 4 (Table 3). The fibroblasts of patient 3 and 5 were not available.

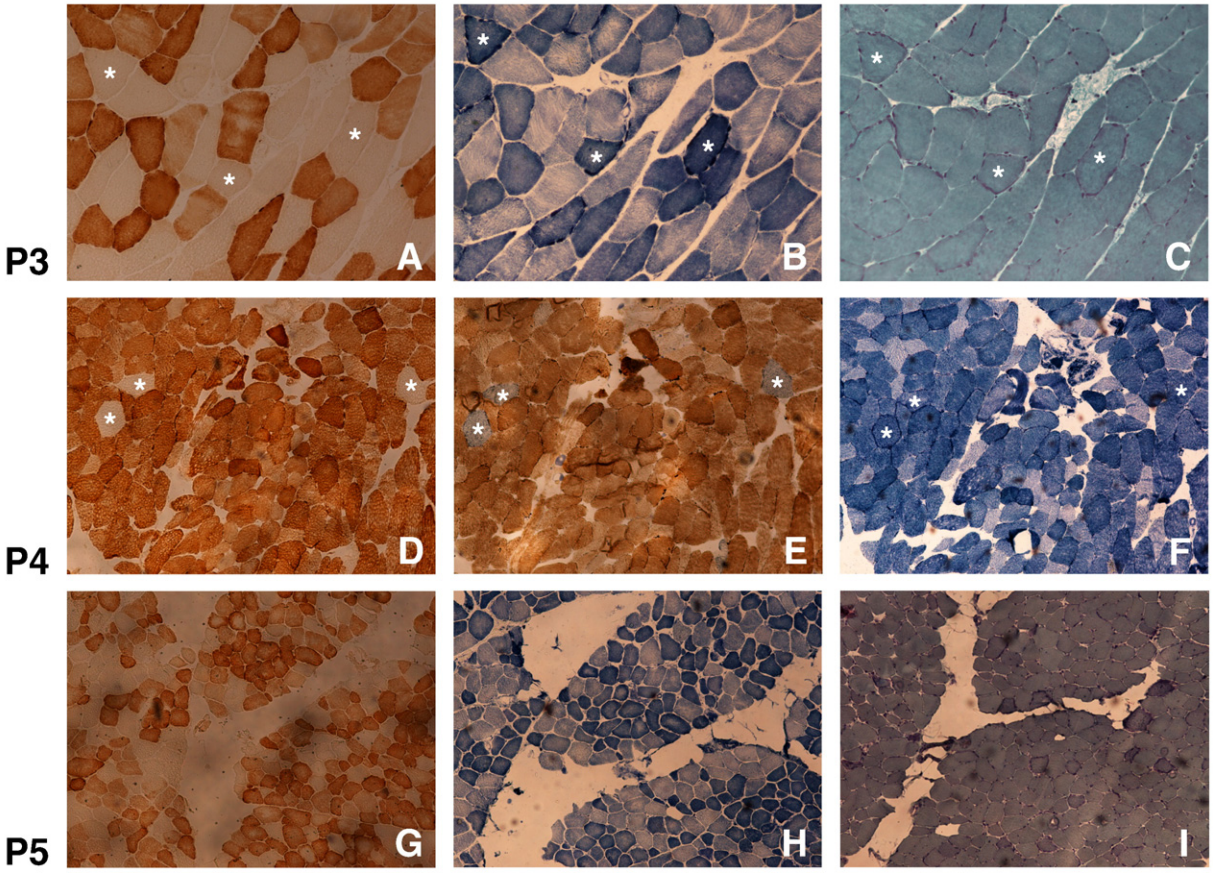

Fig. 2. Histochemical analysis in patient 3, 4 and 5 (respectively P3, P4, P5). P3: (A) Cytochrome *c* Oxidase (COX) stain; (B) NADH Dehydrogenase stain; (C) Modified Gomori trichrome stain. P4: (D) COX stain; (E) COX and Succinic Dehydrogenase (SDH) stain; (F) NADH Dehydrogenase stain. P5: (G) COX stain; (H) NADH Dehydrogenase stain; (I) Modified Gomori trichrome stain. Serial sections show COX-negative RRF (asterisks). COX activity is diffusely reduced in patient 3, numerous COX-negative RRF are present in patient 5, while only few COX-negative fibers are present in patient 4.

3.2. Sequence analysis of mtDNA

The sequence analysis of entire mtDNA revealed that patient 1 carried a homoplasmic 3688G>A transition in *ND1* gene, causing an alanine to threonine change in a highly conserved residue (p.A128T) of the protein (Fig. 3A). The mutation is homoplasmic in muscle, blood lymphocytes and fibroblasts, while his mother did not carry the mutation in blood lymphocytes and in fibroblasts (not shown).

Patient 2 showed the presence of the 13094T>C mutation in the *ND5* gene, that causes a valine to alanine change in a highly conserved residue (p.V253A) of the protein (Fig. 3B). The mutation was heteroplasmic in skeletal muscle (50%), lymphocytes (40%) and fibroblasts (30%) of the patient, while it was absent in the lymphocytes of her mother, her maternal aunt and two uncles (not shown).

Patient 3 had a single nucleotide deletion, 6698delA in the *COI* gene, causing a sequence frame shift of COX I subunit with the formation of a premature stop codon at aminoacid position 271 and the production of a truncated protein (p.K265fs271X) (Fig. 3C). The mutation was heteroplasmic in muscle (70%) and in urinary epithelial cells (15%), while it was absent in myoblasts and in maternal lymphocytes (not shown).

Patient 4 harboured a heteroplasmic 5567T>C mutation (Fig. 3D) in mt-tRNA^{Trp}, that destroys a base pair in the TΨC stem of the tRNA secondary structure. This mutation was heteroplasmic in muscle (50%), fibroblasts (30%) and urinary epithelial cells (40%) (not shown). A sister had no mutation in blood lymphocytes; no other relatives could be investigated.

Finally, patient 5 carried a 642T>C transition (Fig. 3E) in mt-tRNA^{Phe}, that destroys a base pair in the aminoacyl acceptor stem. The mutation was heteroplasmic in skeletal muscle (80%), whilst neither other tissues nor other family members were available for this patient (not shown).

None of these five mutations was found in a series of 300 consecutive control DNA samples.

3.3. Biochemical studies on cybrids

To establish the pathogenic role of the two novel *ND* gene mutations, we generated transmitochondrial cybrids obtained from fibroblast cell lines. Since in patient 1 the mutation was homoplasmic, we compared cybrids derived from this patient with cybrids derived from his homoplasmic wild-type mother. The mean activity of complex I, normalised to that of CS, was 4.0 ± 1.5 in the homoplasmic mutant clones vs. 17.0 ± 4.0 in the maternal homoplasmic wild-type clones (Student's *t*-test $p = 8.17E-09$) (Fig. 4A). For patient 2, we analysed a series of 15 cybrids with different percentages of heteroplasmy. As shown in Fig. 4B, the complex I/CS activity ratio was highly correlated with the percentage of the mutation. These data clearly demonstrate that the mutations in *ND1* and *ND5* genes are indeed responsible for the complex I deficiency detected in patients 1 and 2 respectively.

3.4. BNGE analysis of complex I and in gel activity

In order to understand whether the novel *ND1* and *ND5* gene mutations interfere with the assembly and amount of complex I, we performed 1D- and 2D-BNGE on protein derived from fibroblasts of patient 1, and from two cybrid clones harbouring different percentages

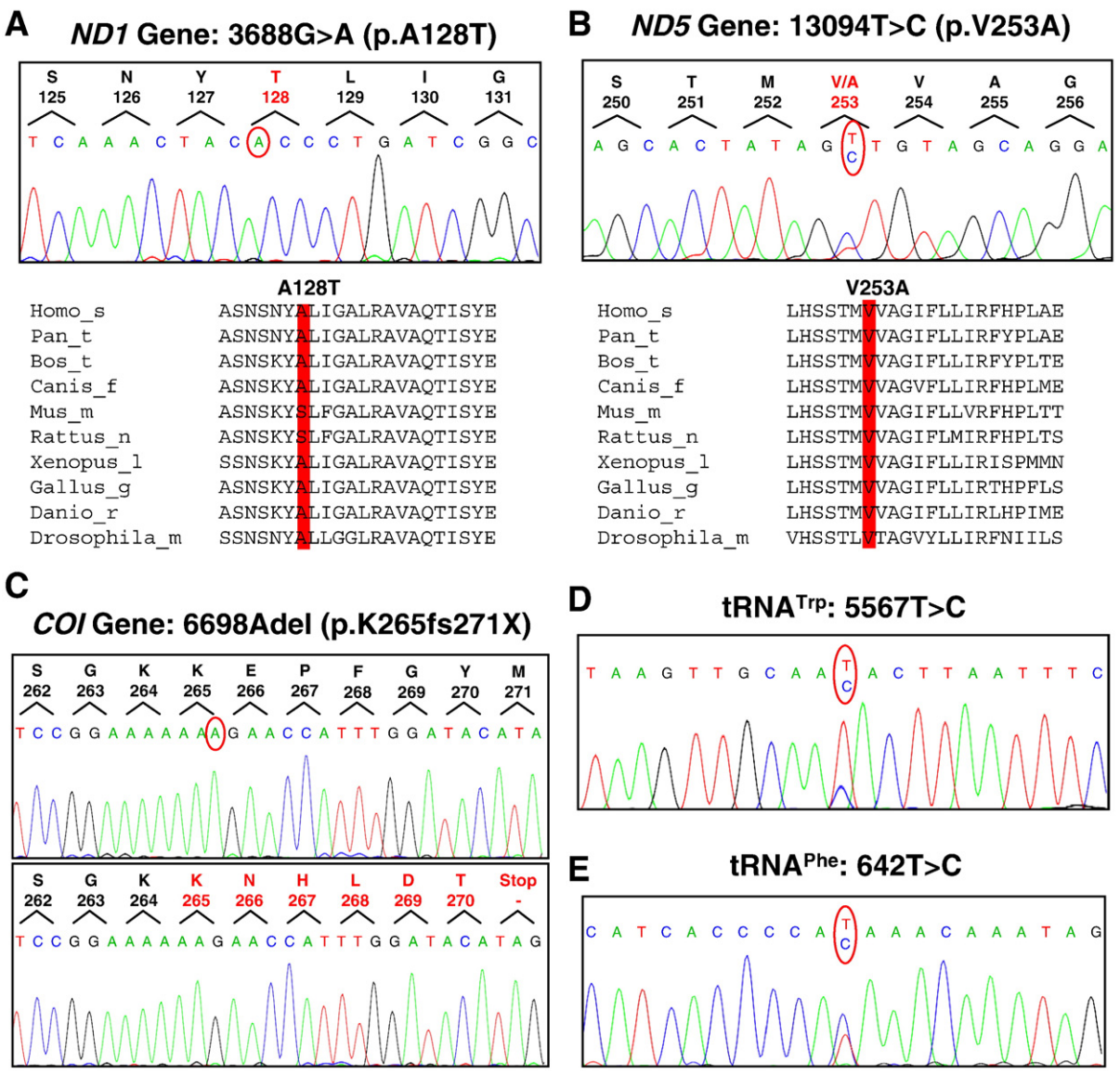

Fig. 3. Mutation analysis in patients 1–5. (A) Sequence analysis of mtDNA in patient 1; top panel: electropherogram of the ND1 gene showing the 3688G>A transition (p.A128T substitution); bottom panel: multiple alignment of the mutation-containing ND1 region in different species: a red box indicates the mutated aminoacid. (B) Sequence analysis of mtDNA in patient 2; top panel: sequence of the *ND5* gene region with the 13094T>C transition (p.V253A substitution); bottom panel: multiple alignment of the mutation-containing ND5 region in different species; a red box indicates the mutated aminoacid. (C) Sequence analysis of muscle mtDNA in patient 3 after subcloning in suitable vector; *top panel*: wild-type *COI* sequence; *bottom panel*: mutant *COI* sequence with a 6698A deletion (p.K265fs271X). (D) Electropherogram of the mtDNA $tRNA^{Trp}$ from patient 4, showing the 5567T>C transition. (E) Sequence analysis of mtDNA $tRNA^{Phe}$ from patient 5, showing the 642T>C transition.

of mutation, derived from patient 2: homoplasmic wild-type (Clone #13), and 80% heteroplasmic mutant (Clone #6).

Complex I was immunodetected by using antibodies against the subunit NDUFB6 (17 kDa), that is part of the membrane arm of complex I, and the 39 kDa subunit NDUFA9, which is located in the peripheral arm, but very close to the membrane arm.

Western-blot (WB) analysis on 1D-BNGE in fibroblasts of patient 1 demonstrated a strong reduction in the amount of cross reacting material (CRM) corresponding to the *bona fide* fully assembled complex I, using both NDUFB6 and NDUFA9 antibodies, as compared to control fibroblasts (Fig. 5A). As for patient 2, using the NDUFB6 antibody, cybrid Clone #6 (80% mutant) showed, in addition to fully assembled Complex I, the accumulation of a lower molecular weight band, which was absent in cybrid Clone #13 (100% wt). The anomalous band was not evident using the NDUFA9 antibody (Fig. 5B). Furthermore, by complex I-specific in-gel activity a clear reduction of complex I activity was detected in both fibroblasts from patient 1 and patient 2 (Fig. 5C). Abnormal assembly and function of CI-containing supercomplexes due to the ND1 or ND5 mutations cannot be excluded and could indeed lead to the OXPHOS impairment of these conditions; future work is warranted to demonstrate it.

The assembly of complex I was further investigated by WB analysis on 2D-BNGE. In fibroblasts from patient 1, the antibody against subunit NDUFB6 (17 kDa) showed the presence of fully assembled complex I, although its amount was strongly reduced as compared to the control (Fig. 6A). Since identical amounts of the very same samples were used in 1D- and 2D-BNGE, normalization of the protein loading was performed only in the first dimension, by using the antibody against the 70 kDa SDH subunit (see Fig. 5A).

In mutant cybrid Clone #6 of patient 2, NDUFA9 (39 kDa) cross-reacting material (CRM) was present only in a position corresponding to fully assembled complex I. However, NDUFB6 CRM was also found in low molecular weight (MW) complexes in the 600–270 kDa range (Fig. 6B), representing partially assembled complex I species. Normalization

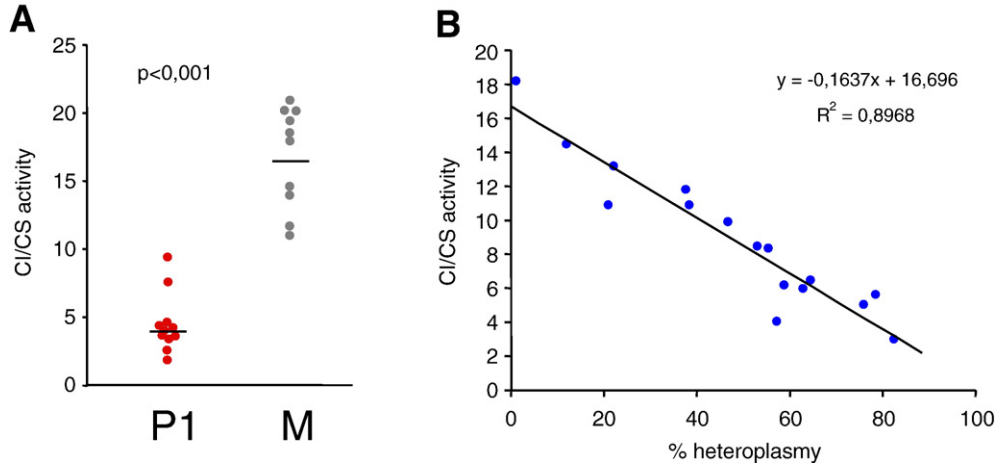

Fig. 4. Genotype-to-phenotype correlation in patient 1 (A) and patient 2 (B) transmitochondrial cybrids. (A) Complex I activity in homoplasmic cybrids of patient 1 (P1, $n=11$) vs. homoplasmic wt clones from his mother (M, $n=10$). Student's t-test $p=8.17E-09$. (B) Complex I activity vs. percentage of heteroplasmy in cybrids ($n=15$). $R2=$ squared correlation coefficient by linear regression analysis.

for protein loading was assessed by analysing assembly levels of complex II, using an anti-SDH antibody on the same filters. Complex II specific CRM was similar in cybrid Clone #6 and Clone #13 from patient 2, thus indicating a specific complex I abnormality (Fig. 6B).

3.5. Studies on COX

To verify the presence and amount of COX subunits in muscle of patient 3, we performed WB analysis of COX subunits I, II and IV in mitochondrial protein extracts separated by denaturing SDS-PAGE. As predicted by the *COI* mutation load detected in skeletal muscle (approximately 70%), the CRM amount for COX I was markedly reduced in the patient's sample compared to a control sample. The reduction is estimated to be approximately 20% by densitometric analysis and using the intensity of the band detected with SDH antibody for normalisation (Fig. 7A). In the same way we evaluated the partial reduction for COX II and COX IV, 41% and 45% respectively (Fig. 7B). Unfortunately, muscle was no longer available for further studies.

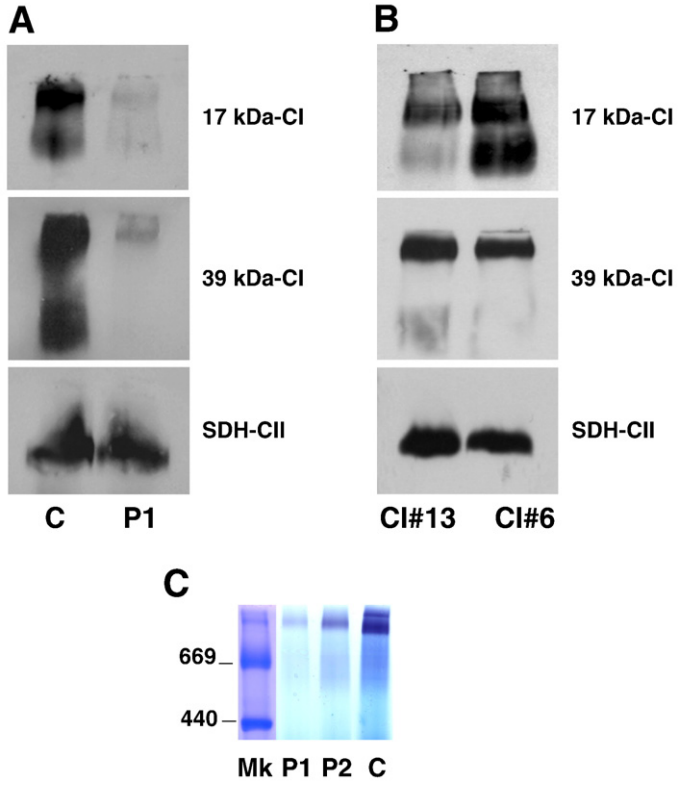

Fig. 5. Complex I assembly state and in-gel activity assays in patient 1 and patient 2. (A) 1D-BNGE WB analysis of mitochondria from fibroblasts of patient 1 (P1) and control (C). (B) 1D-BNGE WB analysis of patient 2 cybrids. Clone #13 is wild type, while clone #6 is 80% heteroplasmic for the 13094T>C mutation in *ND5*. Complex I was immunovisualized using antibodies against the NDUFB6 (17 kDa) and the NDUFA9 (39 kDa) subunits. Antibodies against the 70 kDa (A) or the 30 kDa (B) subunit of complex II were used for normalisation. (C) In-gel activity of complex I on one-dimension blue-native gel electrophoresis (1D-BNGE) of fibroblast extracts from a control (C), patient 1 (P1), and patient 2 (P2). Both patients show a less intense complex I-specific band compared to control. Molecular weight (Mk) are in kDa.

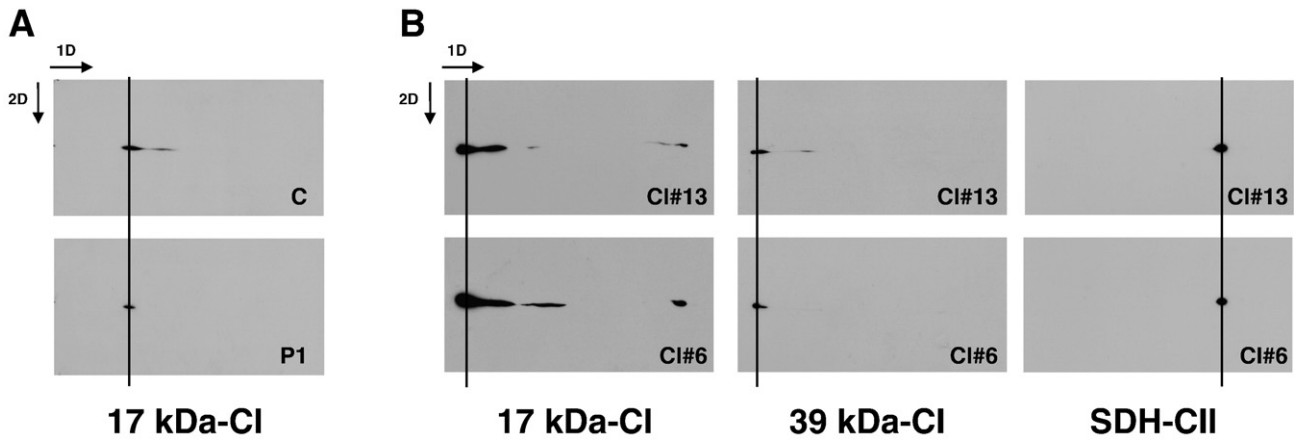

Fig. 6. Immunodetection of complex I assembly intermediates in patient 1 and patient 2. (A) 2D-BNGE WB of patient 1 (P1) and control (C) fibroblasts. The amount of loaded samples is the same as in the 1D-BNGE WB analysis of Fig. 5. Patient 1 shows decrease in the steady-state levels of complex I holoenzyme detected by an anti-NDUFB6 (17 kDa) antibody. (B) 2D-BNGE WB of patient 2 cybrids. Samples and antibodies are the same as in the experiment shown in Fig. 5. Similar to results in Fig. 5, clone #6 shows an additional sub-assembly intermediate in the 600 kDa range detected by the anti-NDUFB6 (17 kDa) antibody. To normalize for the amount of loaded proteins, the same filter was hybridized with an anti SDH (30 kDa) antibody. Arrows indicate the direction of the electrophoretic runs in the first (1D) and second (2D) dimensions.

3.6. Single muscle fiber analysis

To establish the pathogenic role of the two novel mt-tRNA mutations found in patients 4 and 5, we performed single-fiber PCR analysis in muscle sections stained for COX.

Results obtained in the $tRNA^{Trp}$ mutant muscle sample (patient 4) show a significantly higher mean mutant load in COX-negative muscle fibers (95%, $n=4$) than in COX-positive fibers (29%, $n=10$) (Student's t test $p=2.75E-07$) (Fig. 8A). Likewise, the mean mutant load in COX-negative muscle fibers of patient 5, carrying the $tRNA^{Phe}$ mutation, was higher (97%, $n=8$) than in COX-positive fibers (40%, $n=7$) (Student's t test $p=2.89E-04$) (Fig. 8B).

These data strongly suggest a pathogenic role of both mutations.

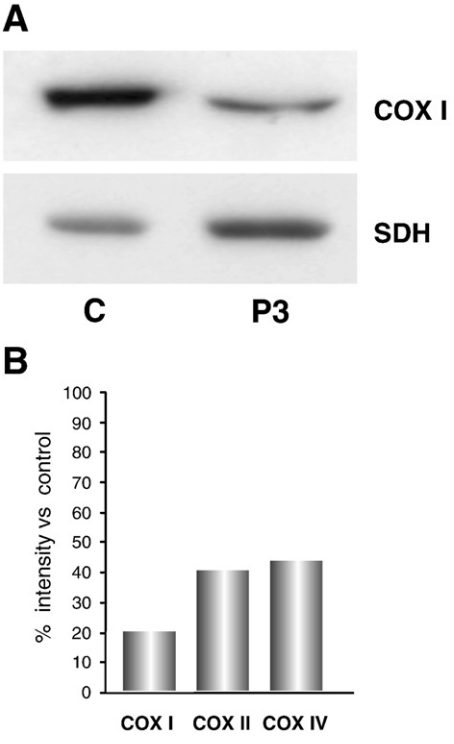

Fig. 7. Western blot analysis in patient 3. (A) Western blot analysis in patient 3 (P3) and control (C) skeletal muscle. Loaded samples correspond to 40 μg of total protein. Levels of the COX I, COX II and COX IV proteins were quantified by WB immunodetection, using specific antibodies against the corresponding polypeptides. SDH (30 kDa) immunodetection was used for normalisation. (B) Graphic representation of densitometric analysis of COX I, II, and IV of patient 3 vs. control samples.

4. Discussion

Among the 33/240 consecutive patients (14%) presenting with known, novel, private or uncommon mtDNA mutations (Table 1), 28 were reported or found to have an already described mutation: 17/28 patients (61%) presented with mutations in *ND* coding genes (see Table 1 for the references), 9/28 (32%) had mutations in *tRNA* genes (references in Table 1), while the remaining 7% had mutations in other protein-coding genes.

Of the five patients carrying novel mtDNA mutations, those with mutations in *ND1* and *ND5* genes were children, while the remaining three, with mutations in *COI*, mt-$tRNA^{Trp}$ and mt-$tRNA^{Phe}$, were adults. Interestingly, no evidence of transmission by descent was obtained in the three mutations for which the mothers of the patients could be investigated, although the absence of mtDNA mutations in blood lymphocytes does not exclude the possibility that other tissues, notably oocytes, could carry them. Patient 1 carried a novel homoplasmic mutation in *ND1* that was associated with a strong reduction of complex I activity in both muscle and fibroblasts. Patient 2 carried a heteroplasmic mutation in *ND5*, which was associated with a milder biochemical phenotype. In both cases, results on cybrids clearly demonstrated the pathogenic role of the mutations.

By studying specific mutations affecting mtDNA-ND subunits, several progresses have been made in the comprehension of their role in the assembly/stability of the complex. For instance, ND1, ND4 and ND6 are essential for the assembly of CI, since their mutations are associated with markedly reduced levels of fully assembled holoenzyme [10,11]. Mutations in ND2 are also associated with altered assembly demonstrated by accumulation of CI intermediates [12,13]. Some mutations in ND3 and ND5 did affect the enzymatic activity of CI but not its assembly status [10,14].

In our patients, the reduction in complex I activity on *ND1* mutant fibroblasts was paralleled by an akin decrease in the steady-state levels of the complex I holoenzyme content, as shown by both 1D- and 2D-BNGE WB analysis, while in *ND5* mutant cybrids the amount of complex I was comparable to that of wild-type cybrids (Figs. 5 and 6). However, the *ND5* mutation was associated with accumulation of low MW protein species that were detected by an antibody against the membrane-arm NDUFB6 subunit, but not by an antibody against the peripheral-arm NDUFA9 subunit. These results are in agreement with

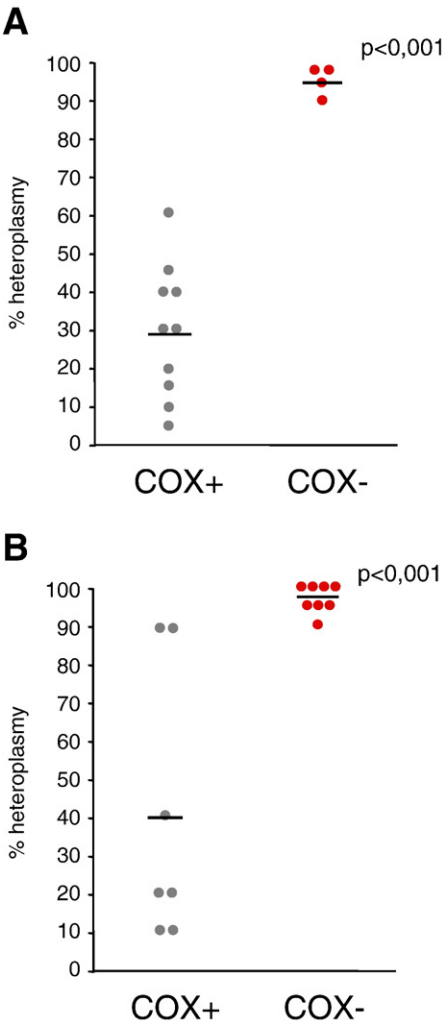

Fig. 8. Single-fiber PCR analysis from skeletal muscle of patient 4 and patient 5. (A) Correlation between the percentage of heteroplasmic mutation in COX-positive (COX+, $n=10$) and COX-negative (COX−, $n=4$) single fibers from patient 4 (Student's t-test $p=2.75E-07$). (B) Correlation between the percentage of heteroplasmic mutation in COX-positive (COX+, $n=7$) and COX-negative (COX−, $n=8$) single fibers from patient 5 (Student's t test $p=2.89E-04$). Bars show the means.

our previous study [10] and with the observation that, since ND5 is the last ND subunit to be incorporated into the membrane arm [15], it is likely not essential for the assembly of the other mtDNA-ND subunits into the complex. However, 2D-BNGE analysis demonstrated that the loss of ND5 is associated with instability of the membrane arm, as also reported by Chomyn [16]. Taken together, the results published by others, as well as previous data from our own laboratory and the findings reported in the present work, suggest that, irrespective of the particular ND subunit affected, some mutations may compromise catalysis but not assembly, while others, probably the majority, do affect both. This is not surprising considering that all ND subunits are essential for function and form the main protein backbone of the membrane arm of Complex I.

Patient 3 carries a heteroplasmic deletion of a single nucleotide in the *COI* gene that creates a premature stop codon and predicts the synthesis of a truncated protein lacking the 243/513 aminoacid residues on the carboxy-terminus. In contrast with ND mutation, just a handful of mutations have been reported in mtDNA *CO* genes have been reported as pathogenic (www.mitomap.org) by affecting the assembly/stability of Complex IV [17,18]. In particular, the following *COI* mutations are known: (i) two missense mutations in patients with idiopathic sideroblastic anemia [19]; (ii) an out-of-frame microdeletion in a patient with motor neuron disease [20]; (iii) a stop codon mutation in a patient with multisystem disorder [17]; (iv) a missense mutation in a girl with epilepsia partialis continua [21]; (v) a missense mutation in a patient with cardioencephalopathy [22]; and finally (vi) two different nonsense mutations in two patients virtually identical to our own, that is, affected by a myopathy with exercise intolerance, muscle pain, myoglobinuria and high levels of CK [23,24]. Additional features in common among the three patients were (i) the presence of numerous COX-negative fibers and RRFs in the muscle biopsy (Fig. 2); (ii) an isolated reduction of complex IV activity in the muscle; (iii) absent or very low mutation load in extra-muscular tissues; (iv) no mutation found in maternal relatives. Notably, the patient reported by Kollberg et al. [24] carried a mutation predicting a premature stop codon at position 269, which is only two codons upstream from the mutant stop codon of our patient 3. Similar to our patient, WB analysis in this patient showed reduced levels of COX subunits I, II and IV (Fig. 7). COX I is the first subunit to be inserted in the inner mitochondrial membrane during COX assembly, followed by incorporation of COX IV and COX II. Our results and those by Kollberg et al. indicate that a reduction in the amount of intact COX I is associated with a parallel reduction in the steady state levels of the other early-assembly subunits, suggesting that all three subunits are part of an assembled COX, while mutant, truncated COX I species is unable to drive the incorporation of the other subunits into the nascent COX holoenzyme, thus inducing their degradation. Unfortunately, lack of material prevented us to further investigate this point by performing BNGE on muscle homogenate. Furthermore the *COI* mutation failed to be detected in myoblasts of both our patient 3 and the patient reported by Kollberg et al. [24], despite the high mutant load found in mature skeletal muscle. Lack of mutation in satellite muscle cells has previously been reported also with another COX point mutation [25] and with several tRNA [26,27] and cytochrome *b* mutations [28]. Mutation heteroplasmy, tissue-specificity, and, possibly, vigorous induction of mitochondriogenesis, can also explain why a potentially fatal mutation was associated with isolated, adult onset, relatively benign myopathy. The absence of the mutation in satellite cells has potential therapeutic implications, making this patient eligible for treatment based on exercise-training protocols [29,30].

Mitochondrial human disorders are caused by dysfunction of the OXPHOS system and many of them are associated with altered assembly of one or more of the OXPHOS system components. The study of the assembly defects in patients has been useful to unravel and/or complete the understanding of the processes by which these large multimeric complexes are formed and to classify patients on the basis of an altered assembly pattern. In addition, the respiratory chain is organized, at least in part, as supra-molecular structures in which the individual complexes of the respiratory chain interact with each other, giving rise to the so-called respirasomes [31]. In mammalian mitochondria, almost all CI is assembled into supercomplexes containing monomeric CI, dimeric CIII and up to four COX complexes ($I_1III_2IV_{0-4}$). Analysis of supercomplex assembly in pathological conditions, for example in the patients reported in this paper, would add more information on the functional relevance of these supercomplexes and on the role that mtDNA encoded subunits play in this process.

Numerous mutations in mitochondrial *tRNA* genes have been described in association with diverse clinical phenotypes. Some *tRNA* genes, such as the $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$ genes are more frequently affected than others [32].

In particular, mutations in $tRNA^{Trp}$ gene have been found only in 5 patients affected by a wide range of different clinical presentations including: MILS [33], spinocerebellar ataxia with neurosensorial deafness [34], late-onset mitochondrial myopathy [35,36], gastrointestinal pseudo-obstructions [37], and dementia with chorea [38]. We identified a novel mtDNA point mutation, a 5567T>C in the $tRNA^{Trp}$ gene, in a 68 year-old woman presenting with fatigability, mild

bilateral ptosis, and COX-negative and RRFs. In this patient the MRC activities in muscle and fibroblasts were normal, possibly because the mutation load in both tissues were not particularly high (50% and 30% respectively); nevertheless, single-fiber PCR analysis on COX-positive vs. COX-negative fibers clearly demonstrated the pathogenic role of 5567T>C.

A total of seven different mutations in the $tRNA^{Phe}$ have been reported, associated with phenotypes as diverse as tubulointerstitial nephritis and stroke [39], mitochondrial myopathy [40–42], acute rhabdomyolysis [43], exercise intolerance and deafness [44], MELAS [45], and MERRF [46]. Here we report an eighth point mutation in $tRNA^{Phe}$ in a 65-year old woman presenting with a phenotype characterized by mental retardation, ataxia, bilateral neurosensory deafness and peripheral neuropathy. The muscle biopsy presented numerous RRFs and COX-negative fibers. The mutation fulfills accepted criteria for pathogenicity, since it occurs at a highly conserved position within the *$tRNA^{Phe}$* gene, suggesting selective constraint of the wild-type nucleotide at this position. Accordingly, the mutation disrupts a conserved Watson–Crick T–A base pairing within the aminoacyl acceptor stem of the tRNA, thus potentially impairing the stability of the acceptor stem hairpin structure.

From a clinical and diagnostic standpoint, this work demonstrates that sequence analysis of the entire mtDNA is mandatory in all cases with a suspect of mitochondrial aetiology, even if not all the clinical and diagnostic criteria are fulfilled. This recommendation is further corroborated by the recent evidence that mutations of mtDNA are far more frequent in the general population than previously suspected, and that mutation load and reduced penetrance plays a crucial role in the clinical manifestation not only of homoplasmic mutations such as those associated with LHON, but also of several, perhaps most, of the heteroplasmic mtDNA mutations as well [47]. It is also noteworthy to observe that most of the mutations in our cohort hit structural genes belonging to complex I, while mutations in genes coding for other structural subunits are much rare, even after normalisation by the amount of mtDNA sequence encoding for each complex. This suggests that the selective constraints vary widely among the different MRC complexes. By the same token, we also found a high percentage of mutations (32%) in *tRNA* coding genes, while no mutations in ribosomal RNA (rRNA) genes were identified. This observation suggests either reduced selective stringency for ND- and tRNA-encoding gene variations, or that mutations in other *mtDNA* genes or region are indeed so deleterious to cause embryonic lethality.

Acknowledgements

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References

[1] A.M. Schaefer, R. McFarland, E.L. Blakely, L. He, R.G. Whittaker, R.W. Taylor, P.F. Chinnery, D.M. Turnbull, Prevalence of mitochondrial DNA disease in adults, Ann. Neurol. 63 (2008) 35–39.
[2] M. Munaro, V. Tiranti, D. Sandonà, E. Lamantea, G. Uziel, R. Bisson, M. Zeviani, A single cell complementation class is common to several cases of cytochrome *C* oxidase defective Leigh's syndrome, Hum. Mol. Genet. 6 (1997) 221–228.
[3] M. King, G. Attardi, Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation, Science 246 (1989) 500–503.
[4] C. Mariotti, V. Tiranti, F. Carrara, B. Dallapiccola, S. Di Donato, M. Zeviani, Defective respiratory capacity and mitochondrial protein synthesis in transformant cybrids harboring the tRNA-Leu(URR) mutation associated with maternally inherited myopathy and cardiomyopathy, J. Clin. Invest. 93 (1994) 1102–1107.
[5] M. Bugiani, F. Invernizzi, S. Alberio, E. Briem, E. Lamantea, F. Carrara, I. Moroni, L. Farina, M. Spada, M.A. Donati, G. Uziel, M. Zeviani, Clinical and molecular findings in children with complex I deficiency, Biochim. Biophys. Acta 1659 (2004) 136–147.
[6] V. Tiranti, M. Munaro, D. Sandonà, E. Lamantea, M. Rimoldi, S. DiDonato, R. Bisson, M. Zeviani, Nuclear DNA origin of cytochrome *c* oxidase deficiency in Leigh's syndrome: genetic evidence based on patient's derived rho° transformants, Hum. Mol. Genet. 4 (1995) 2017–2023.
[7] M. Sciacco, E. Bonilla, E.A. Schon, S. DiMauro, C.T. Moraes, Distribution of wild-type and common deletion forms of mtDNA on normal and respiration-deficient muscle fibers from patients with mitochondrial myopathy, Hum. Mol. Genet. 3 (1994) 13–19.
[8] L.G. Nijtmans, N.S. Henderson, I.J. Holt, Blue Native electrophoresis to study mitochondrial and other protein complexes, Methods 26 (2002) 327–334.
[9] E. Zerbetto, L. Vergani, F. Dabbeni-Sala, Quantification of muscle mitochondrial oxidative phosphorylation enzymes via histochemical staining of blue native polyacrylamide gels, Electrophoresis 18 (1997) 2059–2064.
[10] E. Malfatti, M. Bugiani, F. Invernizzi, C.F. De Souza, L. Farina, F. Carrara, E. Lamantea, C. Antozzi, P. Confalonieri, M.T. Sanseverino, R. Giugliani, G. Uziel, M. Zeviani, Novel mutations of *ND* genes in complex I deficiency associated with mitochondrial encephalopathy, Brain 130 (2007) 1894–1904.
[11] D.M. Kirby, R. McFarland, A. Ohtake, C. Dunning, M.T. Ryan, C. Wilson, D. Ketteridge, D.M. Turnbull, D.R. Thorburn, R.W. Taylor, Mutations of the mitochondrial *ND1* gene as a cause of MELAS, J. Med. Genet. 41 (2004) 784–789.
[12] H. Antonicka, I. Ogilvie, T. Taivassalo, R.P. Anitori, R.G. Haller, J. Vissing, N.G. Kennaway, E.A. Shoubridge, Identification and characterization of a common set of complex I assembly intermediates in mitochondria from patients with complex I deficiency, J. Biol. Chem. 278 (2003) 43081–43088.
[13] C. Ugalde, R. Hinttala, S. Timal, R. Smeets, R.J. Rodenburg, J. Uusimaa, L.P. van Heuvel, L.G. Nijtmans, K. Majamaa, J.A. Smeitink, Mutated ND2 impairs mitochondrial complex I assembly and leads to Leigh syndrome, Mol. Genet. Metab. 90 (2007) 10–14.
[14] R. McFarland, D.M. Kirby, K.J. Fowler, A. Ohtake, M.T. Ryan, D.J. Amor, J.M. Fletcher, J.W. Dixon, F.A. Collins, D.M. Turnbull, R.W. Taylor, D.R. Thorburn, De novo mutations in the mitochondrial ND3 gene as a cause of infantile mitochondrial encephalopathy and complex I deficiency, Ann. Neurol. 55 (2004) 58–64.
[15] I. Bourges, C. Ramus, B. Mousson de Camaret, R. Beugnot, C. Remacle, P. Cardol, G. Hofhaus, J.P. Issartel, Structural organization of mitochondrial human complex I: role of the ND4 and ND5 mitochondria-encoded subunits and interaction with prohibitin, Biochem. J. 383 (2004) 491–499.
[16] A. Chomyn, Mitochondrial genetic control of assembly and function of complex I in mammalian cells, J. Bioenerg. Biomembr. 33 (2001) 251–257.
[17] C. Bruno, A. Martinuzzi, Y. Tang, A.L. Andreu, F. Pallotti, E. Bonilla, S. Shanske, J. Fu, C.M. Sue, C. Angelini, S. DiMauro, G. Manfredi, A stop-codon mutation in the human mtDNA cytochrome *c* oxidase I gene disrupts the functional structure of complex IV, Am. J. Hum. Genet. 65 (1999) 611–620.
[18] V. Tiranti, P. Corona, M. Greco, J.W. Taanman, F. Carrara, E. Lamantea, L. Nijtmans, G. Uziel, M. Zeviani, A novel frameshift mutation of the mtDNA *COIII* gene leads to impaired assembly of cytochrome *c* oxidase in a patient affected by Leigh-like syndrome, Hum. Mol. Genet. 9 (2000) 2733–2742.
[19] N. Gattermann, S. Retzlaff, Y.L. Wang, G. Hofhaus, J. Heinisch, C. Aul, W. Schneider, Heteroplasmic point mutations of mitochondrial DNA affecting subunit I of cytochrome *c* oxidase in two patients with acquired idiopathic sideroblastic anemia, Blood 90 (1997) 4961–4972.
[20] G.P. Comi, A. Bordoni, S. Salani, L. Franceschina, M. Sciacco, A. Prelle, F. Fortunato, M. Zeviani, L. Napoli, N. Bresolin, M. Moggio, C.D. Ausenda, J.W. Taanman, G. Scarlato, Cytochrome *c* oxidase subunit I microdeletion in a patient with motor neuron disease, Ann. Neurol. 43 (1998) 110–116.
[21] D.A. Varlamov, A.P. Kudin, S. Vielhaber, R. Schroder, R. Sassen, A. Becker, D. Kunz, K. Haug, J. Rebstock, A. Heils, C.E. Elger, W.S. Kunz, Metabolic consequences of a novel missense mutation of the mtDNA *CO I* gene, Hum. Mol. Genet. 11 (2002) 1797–1805.
[22] S. Lucioli, K. Hoffmeier, R. Carrozzo, A. Tessa, B. Ludwig, F.M. Santorelli, Introducing a novel human mtDNA mutation into the *Paracoccus denitrificans COX I* gene explains functional deficits in a patient, Neurogenetics 7 (2006) 51–57.
[23] C.L. Karadimas, P. Greenstein, C.M. Sue, J.T. Joseph, K. Tanji, R.G. Haller, T. Taivassalo, M.M. Davidson, S. Shanske, E. Bonilla, S. DiMauro, Recurrent myoglobinuria due to a nonsense mutation in the *COX I* gene of mitochondrial DNA, Neurology 55 (2000) 644–649.
[24] G. Kollberg, A.R. Moslemi, C. Lindberg, E. Holme, A. Oldfors, Mitochondrial myopathy and rhabdomyolysis associated with a novel nonsense mutation in the gene encoding cytochrome *c* oxidase subunit I, J. Neuropathol. Exp. Neurol. 64 (2005) 123–128.
[25] M.G. Hanna, I.P. Nelson, S. Rahman, R.J. Lane, J. Land, S. Heales, M.J. Cooper, A.H. Schapira, J.A. Morgan-Hughes, N.W. Wood, Cytochrome *c* oxidase deficiency associated with the first stop-codon point mutation in human mtDNA, Am. J. Hum. Genet. 63 (1998) 29–36.
[26] K. Fu, R. Hartlen, T. Johns, A. Genge, G. Karpati, E.A. Shoubridge, A novel heteroplasmic tRNAleu(CUN) mtDNA point mutation in a sporadic patient with mitochondrial encephalomyopathy segregates rapidly in skeletal muscle and suggests an approach to therapy, Hum. Mol. Genet. 5 (1996) 1835–1840.
[27] K. Weber, J.N. Wilson, L. Taylor, E. Brierley, M.A. Johnson, D.M. Turnbull, L.A. Bindoff, A new mtDNA mutation showing accumulation with time and restriction to skeletal muscle, Am. J. Hum. Genet. 60 (1997) 373–380.
[28] A.L. Andreu, M.G. Hanna, H. Reichmann, C. Bruno, A.S. Penn, K. Tanji, F. Pallotti, S. Iwata, E. Bonilla, B. Lach, J. Morgan-Hughes, S. DiMauro, Exercise intolerance due

to mutations in the cytochrome *b* gene of mitochondrial DNA, N. Engl. J. Med. 341 (1999) 1037–1044.
[29] K.M. Clark, L.A. Bindoff, R.N. Lightowlers, R.M. Andrews, P.G. Griffiths, M.A. Johnson, E.J. Brierley, D.M. Turnbull, Reversal of a mitochondrial DNA defect in human skeletal muscle, Nat. Genet. 16 (1997) 222–224.
[30] T. Taivassalo, K. Fu, T. Johns, D. Arnold, G. Karpati, E.A. Shoubridge, Gene shifting: a novel therapy for mitochondrial myopathy, Hum. Mol. Genet. 8 (1999) 1047–1052.
[31] J. Murray, B. Zhang, S.W. Taylor, D. Oglesbee, E. Fahy, M.F. Marusich, S.S. Ghosh, R.A. Capaldi, The subunit composition of the human NADH dehydrogenase obtained by rapid one-step immunopurification, J. Biol. Chem. 278 (2003) 13619–13622.
[32] E. Zifa, S. Giannouli, P. Theotokis, C. Stamatis, Z. Mamuris, C. Stathopoulos, Mitochondrial tRNA mutations: clinical and functional perturbations, RNA Biol. 4 (2007) 38–66.
[33] F.M. Santorelli, K. Tanji, M. Sano, S. Shanske, M. El-Shahawi, P. Kranz-Eble, S. DiMauro, D.C. De Vivo, Maternally inherited encephalopathy associated with a single-base insertion in the mitochondrial *tRNATrp* gene, Ann. Neurol. 42 (1997) 256–260.
[34] G. Silvestri, T. Mongini, F. Odoardi, A. Modoni, G. DeRosa, C. Doriguzzi, L. Palmucci, P. Tonali, S. Servidei, A new mtDNA mutation associated with a progressive encephalopathy and cytochrome *c* oxidase deficiency, Neurology 54 (2000) 1693–1696.
[35] G. Silvestri, M. Rana, A. DiMuzio, A. Uncini, P. Tonali, S. Servidei, A late onset mitochondrial myopathy is associated with a novel mitochondrial DNA (mtDNA) point mutation in the *tRNATrp* gene, Neuromuscul. Disord. 8 (1998) 291–295.
[36] R. Anitori, K. Manning, F. Quan, R.G. Weleber, N.R. Buist, E.A. Shoubridge, N.G. Kennaway, Contrasting phenotypes in three patients with novel mutations in mitochondrial *tRNA* genes, Mol. Genet. Metab. 84 (2005) 176–188.
[37] K. Maniura-Weber, R.W. Taylor, M.A. Johnson, Z. Chrzanowska-Lightowlers, A.A. Morris, C.P. Charlton, D.M. Turnbull, L.A. Bindoff, A novel point mutation in the mitochondrial *tRNA(Trp)* gene produces a neurogastrointestinal syndrome, Eur. J. Hum. Genet. 12 (2004) 509–512.
[38] I. Nelson, M.G. Hanna, N. Alsanjari, F. Scaravilli, J.A. Morgan-Hughes, A.E. Harding, A new mitochondrial DNA mutation associated with progressive dementia and chorea: a clinical, pathological, and molecular genetic study, Ann. Neurol. 37 (1995) 400–403.
[39] C.Y. Tzen, J.D. Tsai, T.Y. Wu, B.F. Chen, M.L. Chen, S.P. Lin, S.C. Chen, Tubulointerstitial nephritis associated with a novel mitochondrial point mutation, Kidney Int. 59 (2001) 846–854.
[40] A.R. Moslemi, C. Lindberg, J. Toft, E. Holme, G. Kollberg, A. Oldfors, A novel mutation in the mitochondrial *tRNA(Phe)* gene associated with mitochondrial myopathy, Neuromuscul. Disord. 14 (2004) 46–50.
[41] S. Kleinle, V. Schneider, P. Moosmann, S. Brandner, S. Krahenbuhl, S. Liechti-Gallati, A novel mitochondrial tRNA(Phe) mutation inhibiting anticodon stem formation associated with a muscle disease, Biochem. Biophys. Res. Commun. 247 (1998) 112–115.
[42] N. Darin, G. Kollberg, A.R. Moslemi, M. Tulinius, E. Holme, M.A. Grönlund, S. Andersson, A. Oldfors, Mitochondrial myopathy with exercise intolerance and retinal dystrophy in a sporadic patient with a G583A mutation in the mt *tRNA (Phe)* gene, Neuromuscul. Disord. 16 (2006) 504–506.
[43] P.F. Chinnery, M.A. Johnson, R.W. Taylor, R.N. Lightowlers, D.M. Turnbull, A novel mitochondrial tRNA phenylalanine mutation presenting with acute rhabdomyolysis, Ann. Neurol. 41 (1997) 408–410.
[44] M. Deschauer, H. Swalwell, M. Strauss, S. Zierz, R.W. Taylor, Novel mitochondrial transfer *RNA(Phe)* gene mutation associated with late-onset neuromuscular disease, Arch. Neurol. 63 (2006) 902–905.
[45] M.G. Hanna, I.P. Nelson, J.A. Morgan-Hughes, N.W. Wood, MELAS: a new disease associated mitochondrial DNA mutation and evidence for further genetic heterogeneity, J. Neurol. Neurosurg. Psychiatry 65 (1998) 512–517.
[46] M. Mancuso, M. Filosto, V.K. Mootha, A. Rocchi, S. Pistolesi, L. Murri, S. DiMauro, G. Siciliano, A novel mitochondrial tRNAPhe mutation causes MERRF syndrome, Neurology 62 (2004) 2119–2121.
[47] H.R. Elliott, D.C. Samuels, J.A. Eden, C.L. Relton, P.F. Chinnery, Pathogenic mitochondrial DNA mutations are common in the general population, Am. J. Hum. Genet. 83 (2008) 254–260.
[48] P. Corona, C. Antozzi, F. Carrara, L. D'Incerti, E. Lamantea, V. Tiranti, M. Zeviani, A novel mtDNA mutation in the ND5 subunit of complex I in two MELAS patients, Ann. Neurol. 49 (2001) 106–110.
[49] E. Lamantea, F. Carrara, C. Mariotti, L. Morandi, V. Tiranti, M. Zeviani, A novel nonsense mutation (Q352X) in the mitochondrial cytochrome *b* gene associated with a combined deficiency of complexes I and III, Neuromuscul. Disord. 12 (2002) 49–52.
[50] G. Uziel, F. Carrara, T. Granata, E. Lamantea, M. Mora, M. Zeviani, Neuromuscular syndrome associated with the 3291T → C mutation of mitochondrial DNA: a second case, Neuromuscul. Disord. 10 (2000) 415–418.
[51] P. Corona, E. Lamantea, M. Greco, F. Carrara, A. Agostino, D. Guidetti, M.T. Dotti, C. Mariotti, M. Zeviani, Novel heteroplasmic mtDNA mutation in a family with heterogeneous clinical presentations, Ann. Neurol. 51 (2002) 118–122.
[52] A. Limongelli, J. Schaefer, S. Jackson, F. Invernizzi, Y. Kirino, T. Suzuki, H. Reichmann, M. Zeviani, Variable penetrance of a familial progressive necrotising encephalopathy due to a novel tRNA(Ile) homoplasmic mutation in the mitochondrial genome, J. Med. Genet. 41 (2004) 342–349.
[53] A. Spinazzola, F. Carrara, M. Mora, M. Zeviani, Mitochondrial myopathy and ophthalmoplegia in a sporadic patient with the 5698G → A mitochondrial DNA mutation, Neuromuscul. Disord. 14 (2004) 815–817.
[54] V. Fetoni, E. Briem, F. Carrara, M. Mora, M. Zeviani, Monomelic amyotrophy associated with the 7472insC mutation in the mtDNA *tRNASer(UCN)* gene, Neuromuscul. Disord. 14 (2004) 723–726.