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The mitochondrial DNA A3243G mutation in Portugal: clinical and molecular studies in 5 families

Laura Vilarinho[a,b,*], Filippo M. Santorelli[b,h,j,k], Isabel Coelho[c], Lurdes Rodrigues[c], Maria Maia[d], Isabel Barata[e], Pedro Cabral[e], Alexandre Dionísio[f], Alfredo Costa[i,j], António Guimarães[g], Salvatore DiMauro[b]

[a]*Department of Clinical Biology, Pr. Pedro Nunes, Instituto de Genética Médica, 88 4050 Porto, Portugal*
[b]*Department of Neurology, College of Physicians and Surgeons, Columbia University, New York, NY, USA*
[c]*Department of Neurology, Hospital Senhora da Oliveira, Guimarães, Portugal*
[d]*Department of Neuropediatrics, Hospital S. Januário, Macau, Portugal*
[e]*Department of Neuropediatrics, Hospital D. Estefânia, Lisboa, Portugal*
[f]*Department of Neurology, Centro Hospitalar de Coimbra, Coimbra, Portugal*
[g]*Division of Neuropathology, Hospital de S. António, Porto, Portugal*
[h]*Instituto di Malattie Nervose e Mentali, La Sapienza University, Rome, Italy*
[i]*University Center for Adaptive Disorders and Headache (UCADH), Headache Center, University of Pavia, Pavia, Italy*
[j]*Laboratory of Molecular Neurobiology, Insitute of Neurology 'C. Mondino', University of Pavia, Pavia, Italy*
[k]*Neuromed IRCCS, Pozzilli (IS), Italy*

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Abstract

Out of 90 Portuguese patients with mitochondrial cytopathy, six harbored the A3243G mutation in the mtDNA $tRNA^{Leu(UUR)}$ gene ('MELAS mutation'). They had heterogeneous clinical features, including myopathy with stroke-like episodes, progressive external ophthalmoparesis, diabetes mellitus, and subacute encephalopathy. Histochemical and biochemical analyses of muscle biopsies showed abundant ragged-red fibers reacting positively with the cytochrome oxidase stain, and decreased respiratory chain enzyme activities. On average, the proportion of mutated mtDNA was 67% (20–88%) in tissues from patients and 21% (0–49%) in blood from 20 maternal relatives. The proportion of mutated mitochondrial genomes in muscle did not correlate with clinical presentation or duration of disease. This study, the first in Portuguese patients, confirms the frequent occurrence of the A3243G mutation in patients with mitochondrial diseases, and emphasises the usefulness of genetic testing in reaching a correct diagnosis.

Keywords: Mitochondrial DNA; A3243G; MELAS

1. Introduction

A wide range of clinical phenotypes have been classified under the common heading of Mitochondrial Encephalomyopathies (ME) [1]. Initially, the diagnosis was based on clinical features and on muscle biopsy abnormalities, including ragged-red fibres (RRF) with the modified Gomori trichrome stain, and scattered fibres failing to react with the cytochrome oxidase histochemical stain (COX-negative fibers). Later, systematic biochemical studies led to the identification of single or multiple defects in the mitochondrial respiratory chain complexes. However, it soon became evident that neither RRF nor biochemical data alone could provide a systematic classification of ME. Advances in molecular genetics have increased

*Tel.: +351-2-6070-306; fax: +351-2-6070-399.
E-mail address: laura.vilarinho@igmjm.pt (L. Vilarinho)

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our understanding of the genetic bases of these diseases, providing new tools for classification and diagnosis of putative patients. To date, over 50 point mutations in the mitochondrial genome (mtDNA) have been associated with a variety of maternally-inherited disorders, and we are starting to gleam molecular mechanisms that may be relevant to pathogenesis. However, the relationship between individual mutations and disease phenotypes is often puzzling: well-defined clinical entities can be genetically heterogeneous, while, conversely, a single mutation may be associated with different clinical syndromes. The best example of one mutation resulting in highly variable combinations of clinical features is the A3243G mutation, typically associated with MELAS. MELAS is characterised by the triad: (1) stroke-like episodes before age 40; (2) encephalopathy with seizures, dementia or both; and (3) lactic acidosis and/or ragged-red fibers (RRF) [2]. However, the spectrum of clinical presentations associated with the A3243G mutation [3] has expanded to include diabetes mellitus (DM) and deafness [4], PEO [5], or pure cardiopathy [6]. Conversely, the number of mutations associated with typical MELAS has also expanded: a survey of reports from different countries shows that 80–90% of MELAS patients harbor the A3243G mutation [7], while the remainder of MELAS patients have rarer mutations, including some in protein-encoding mtDNA genes [8–10].

We have previously described the clinical and molecular features of 29 Portuguese patients harboring mtDNA deletions [11]. To evaluate the incidence of the A3243G mutation in Portugal and its relationship to clinical phenotype, we studied 90 patients with mitochondrial encephalopathy. Here, we present the clinical, morphological and molecular genetic features in the six patients who harbored the 'MELAS mutation'.

2. Patients and methods

2.1. Patients

We studied 398 Portuguese patients who had clinical presentations or laboratory features (hyperlactacidemia) suggestive of mtDNA-associated disorders. Among these, 90 subjects had clinical and morphological evidence of mitochondrial cytopathy. Three out of 90 patients had the typical clinical features of MELAS, but we have detected the A3243G mutation in six patients (3 men and 3 women). Detailed clinical histories are given only for these cases.

2.1.1. Patient 1

(Family A). A 12-year-old-boy (A.II-3 in Fig. 1) was the second child born to healthy, unrelated parents. Pregnancy and delivery were uneventful. Birth weight was 3020 g (25th percentile). Early developmental milestones were normal, but at 6 years of age, the patient presented anorexia, exercise intolerance, nausea with 'cerebral' vomiting, and migraine with aura (MA). Migraine spells were usually accompanied by visual manifestations (fortification spectra and blurred vision) and did not last longer than one hour. At age 8, sensorineural hearing loss was diagnosed. At age 12, he was hospitalised because of unilateral palpebral and foot edema. Physical examination showed short stature (122 cm<5th percentile) lumbar scoliosis, and generalised muscle atrophy. Echocardiography revealed hypertrophy of the interventricular septum and of the left ventricle, consistent with idiopathic hypertrophic cardiomyopathy. Neurological examination was normal although the patient complained of tiring easily. Brain magnetic resonance imaging (MRI) showed diffuse cortical atrophy. Blood levels of venous lactate (2× normal), creatine kinase (CK) (2–3× normal), lactic dehydrogenase (LDH) (3× normal), and aspartate transaminase (4× normal) were markedly elevated, while the values of thyroxine and growth hormones were reduced to 50% of normal values. He started treatment with growth hormone (GH) (0,6 U/kg/week) and biotin, but three months later he was hospitalised because of a transient stroke episode with aphasia, and right homonymous hemianopsia.

His mother was asymptomatic but had had two miscarriages, and another child, who had died at 6.5 months of age of unspecified 'respiratory complications'. Maternal family history was otherwise negative.

2.1.2. Patient 2

(Family B). This patient has already been described [6]. Briefly, a 6-year-old boy (B.III-7 in Fig. 1), born after an uneventful pregnancy to non-consanguineous parents, was hospitalised because of vomiting, 'malaise', and muscle pain. He had developed normally until age 3, when asthenia, anorexia, and poor physical growth were first noted. He had had recurrent episodes of postprandial vomiting without any other symptoms, which had worsened over a two-year period. On admission, he was in physical distress but alert, and complained of general fatigue. General examination revealed short stature (height 110 cm and weight 20 kg) and a cardiac systolic bruit; echocardiography showed a dilated left ventricle. Holter electrocardiogram showed some episodes of sinusal arrhythmia at night. Neurological examination, brain CT scan, and electromyography were normal. Metabolic investigations showed increased levels of CPK (3×normal), LDH (5× normal), and lactic acid (6.2 mM/l when fasted, 4.8 mM/l when fed, normal <2.5 mM/l) with increased lactate/pyruvate ratio.

Family history was significant for the 36-year-old mother, who suffered from an affective disorder and had attempted suicide in the past. The proband's 10-year-old

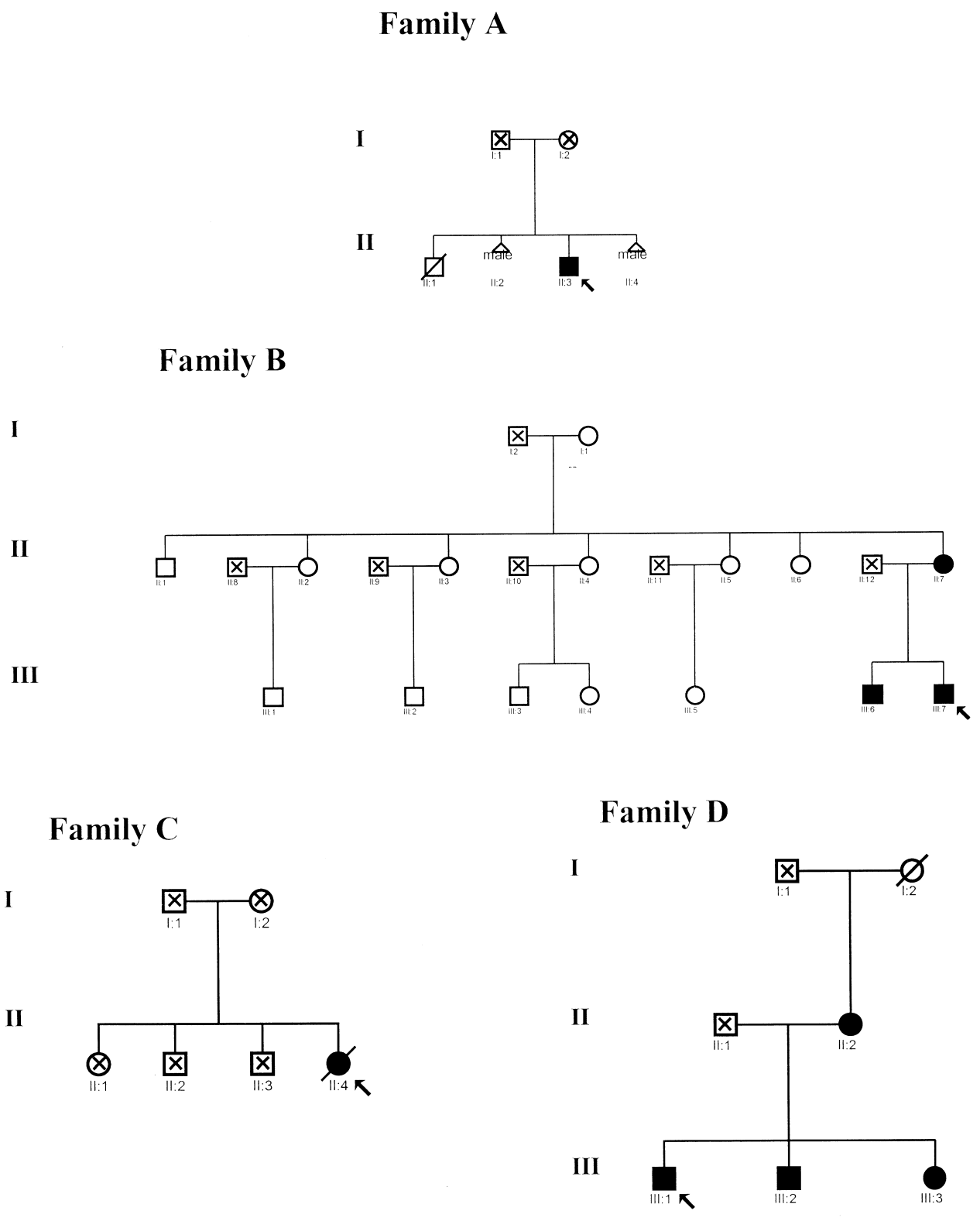

Fig. 1. Five Portuguese pedigrees harboring the mitochondrial DNA A3243G mutation. Dark symbols indicate subjects with detectable levels of mutated genomes in muscle or blood. Arrows show the probands.

brother was asymptomatic but mild hyperlactacidemia was found in both mother and brother (2.8 mM/l and 2.7 mM/l, respectively).

2.1.3. Patient 3

(Family C). A 52-year-old woman, the fourth child of unrelated parents, presented with progressive external

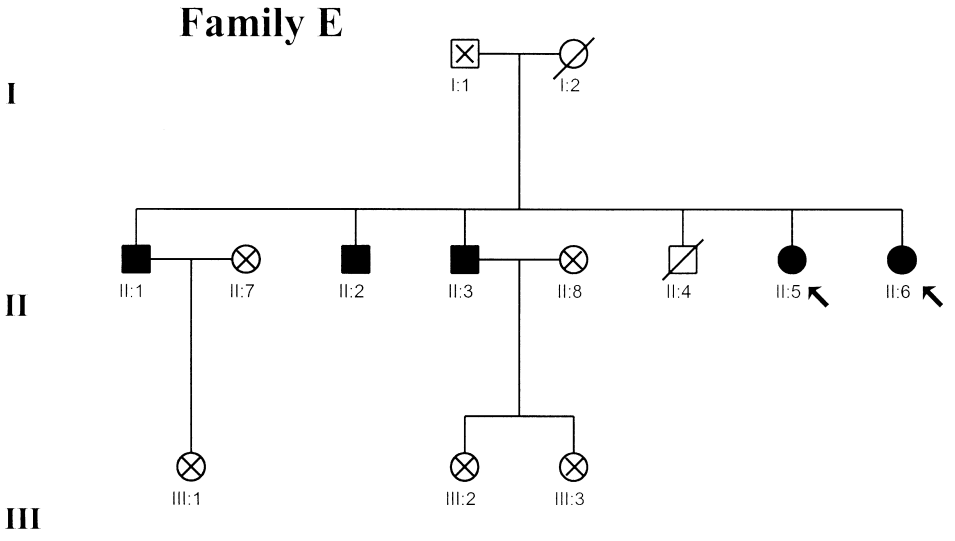

Fig. 1. (*continued*)

ophthalmoparesis (PEO), diplopia, proximal muscle weakness, mild mental retardation, sensorineural hearing loss, migraine (MA), and pigmentary retinopathy. Physical examination also showed short stature (148 cm). She had been diagnosed with non-insulin-dependent diabetes mellitus (NIDDM) in her early 30s. At age 47, she had been hospitalised twice because of strokelike episodes. At the time of admission, a brain CT scan showed mild diffuse cerebral and cerebellar atrophy. At age 50, she suffered a left MCA ischemic stroke followed, some months later, by a subarachnoid haemorrhage. Family history was remarkable for a sister and the mother, who were short, and a brother who had been diagnosed with NIDDM. Another brother was healthy. None of the patient′s maternal relatives consented to be studied. Our patient died suddenly after a severe migraine attack followed by a stroke and coma. Autopsy was not performed.

2.1.4. Patient 4

(Family D). This patient has already been described in detail elsewhere [12]. Briefly, a 10-year-old Asian boy (D.III-1 in Fig. 1), the first child of healthy and unrelated parents had normal development until age 5 years, when he had a left-sided convulsion with transient hemiparesis, followed by recurrent focal or complex partial seizures. Repeated seizures with poor performance in school and gait difficulties were the main symptoms at age 7 years. He gradually improved, but during febrile illnesses his neurological symptoms worsened. On neurological examination, he was short and presented extrapyramidal tract signs and gait ataxia. Brain CT scan showed symmetrical and bilateral hypodensities of the lenticular nuclei (Fig. 2). EEG showed repetitive spike-wave complexes in the right temporal area. Lactic acid levels were elevated in blood (8.90 mM/l) and CSF (6.08 mM/l). Because of the subacute neurological illness and the striking CT findings, a diagnosis of Leigh syndrome (LS) was considered.

2.1.5. Patients 5 and 6

(Family E). Patient 5 (E.II-5 in Fig. 1) weighted 2400 g (25th percentile) at birth. Her milestones were delayed: she sat at 7 months and walked unaided at 15 months.

She was hirsute and had dysmorphic features, including coarse facies, short neck, and lumbar kyphosis. Psychomotor development was normal until age 7, when she first suffered from myoclonic seizures. At that time, because of her peculiar facies and habitus, a mucopolysaccharidosis was considered but ruled out by appropriate testing. Around age 13, she had a stroke-like episode with transient left hemiparesis. She progressively developed mental retardation, ataxia, and deafness. Hypotonia and pyramidal tract signs were observed during a neurological examination. At age 16, she developed seizures. Brain CT scan showed cerebral and cerebellar atrophy and basal ganglia calcifications. Her cortical functions deteriorated pro-

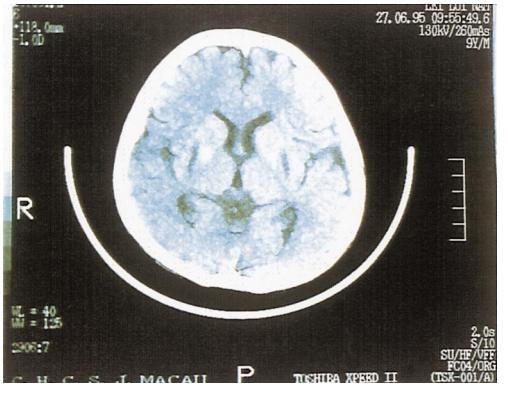

Fig. 2. Brain CT scan in patient D.III-1 showed symmetrical and bilateral hypodensities of the lenticular nuclei.

gressively. Repeated episodes of prostration and nocturnal vomiting also occurred. She died of cardiopulmonary arrest at age 22. Autopsy was not performed.

Her 31-year-old sister, Patient 6 (E.II-6 in Fig. 1), was short (147 cm) and mildly retarded. At age 28, she was diagnosed with NIDDM and hearing impairment. Two brothers (E.II-1 and II-3 in Fig. 1) were asymptomatic, and another brother (E.II-2) had undefined psychiatric problems. A fourth brother (E.II-4) died at age 25 with a diagnosis of right parietal glioblastoma; he had had hearing loss, optic atrophy, and myoclonic jerks since childhood. The mother was diminutive and thin, and appeared prematurely aged; for the past few years, she had suffered from a progressive affective disorder. She refused clinical or molecular investigations.

2.2. Methods

Muscle biopsy specimens from all six patients were analysed for abnormal mitochondrial function using standard morphological and biochemical methodologies, including modified Gomori-trichrome, cytochrome c oxidase (COX), and succinate dehydrogenase (SDH) stains [13], and spectrophotometric measurement of respiratory chain complexes and citrate synthase (CS) [14]. Total DNA was extracted from biopsied muscle or peripheral leukocytes using reported methodologies [15]. Screening for the A3243G mutation in muscle or blood from patients and their relatives, and quantitation of mutant mtDNAs were performed employing described PCR-RFLP methodology [16].

3. Results and discussion

Fig. 1 shows the pedigrees of the six patients who tested positive for the A3243G mutation. Clinical features, morphological and biochemical findings, and abundance of mutated mitochondrial genomes are summarised in Table 1.

Clinically, our patients showed great variability. Age at onset varied from late childhood to early adulthood. Cardiomyopathy and NIDDM were observed in three individuals and hearing loss in four. Interestingly, the first symptom in patient 1 was MA 'plus'. Later, he developed myopathy, seizures and, ultimately, a transient stroke with concomitant lactic acidosis, thus satisfying the clinical criteria for MELAS. In most cases, non−neuromuscular clinical features such as cardiomyopathy, diabetes, and short stature dominated the clinical picture. This stresses the need for thorough clinical and laboratory investigation, including mtDNA analysis, whenever, patients present with multiorgan involvement, especially if there is evidence of maternal inheritance. Morphologically, all six patients had RRF (13±10% of all examined fibers), documented either with the modified Gomori trichrome

Table 1
Clinical, biochemical, morphological, and molecular genetic features in six Portugese patients carrying the A3243G mutation

Patients	1	2	3	4	5	6
Clinical phenotype	MELAS	CM	PEO-DM	LS	MELAS	MELAS
Sex	M	M	F	M	F	F
Age at onset (years)	6	3	Adolescence	5	7	20
Age of diagnosis	12	6	52	10	20	28
Exercise intolerance	+	+	−	+	+	−
Muscle weakness	+	−	+	−	+	−
PEO	−	−	+	−	−	−
Strokes	+	+	+	−	+	−
Seizures	+	−	−	+	+	−
Nausea, vomiting	+	+	−	+	+	−
Hearing loss	+	−	+	−	+	+
Cardiomyopathy	Hypertrophic	Dilated	−	−	Hypertrophic	−
Short stature	+	+	+	+	+	+
Ataxia	+	−	−	+	+	−
Diabetes mellitus	+	−	+	−	−	+
Learning disability	+	−	+	+	+	+
Other manifestations	Anorexia; migraine; scoliosis	Anorexia	Diplopia; migraine; retinopathy	Rigidity alkinesia	Microcephaly; hirsutisme; dysmorphic features	−
RRFs in muscle (%)	25	20	2	3	10	20
Residual respiratory chain complex activity (%)	I (10); III (35); IV (15)	I (20); III (22); IV (12)	Normal	I (9); IV (11);	nd	nd
Mutated mtDNA (%)	78 (m)	88 (m); 68 (b)	65 (m)	82 (m); 54 (b)	79 (m);	67 (m); 20 (b)

stain or with the SDH stain. In addition, excessive accumulation of lipid droplets, and minimal-to-severe atrophy were found in some cases. The biopsy of patient 1 also showed perivascular inflammatory infiltrates. Histochemistry showed that most RRF had COX activity while only a few fibres were COX-negative. Electron microscopy showed elongated, balloon-like mitochondria in all patients and paracristalline inclusions in patients 3 and 5.

Biochemically, the activities of all respiratory chain complexes containing mtDNA-encoded subunits were decreased in 3 patients. Specifically, the average residual activity of complex I was 13±6% and that of complex IV was 12±2%, after correction for CS values. No biochemical abnormalities were found in patient 3.

Genetic analysis detected the A3243G mutation in tissues from all 6 patients. The percentage of mutant mtDNAs was 76±9% (range 65–88%) in muscle and 47±25 (20–68%) in blood from the patients. We also looked for the A3243G mutation in tissues from three oligosymptomatic and 17 asymptomatic maternal relatives. A total of 26 subjects were studied: blood alone in 20 individuals, muscle alone in 3, and both muscle and blood in 3. Heteroplasmy, that is, co-existence of normal and mutant mtDNA, was observed in 14 subjects. Twelve symptom-free individuals did not harbor detectable mutated mtDNAs in blood but did not undergo a muscle biopsy. Abnormal mtDNA species were less abundant (0–49%) in blood from 20 maternal relatives. However, when the overall clinical phenotype was roughly scored for severity, there was poor correlation between severity of clinical involvement and abundance of mutant mtDNA in muscle from patients. Morphological changes and biochemical impairment also did not seem to correlate with percentage of the mutation in blood. On the contrary, the abundance of mutant mtDNAs in blood was correlated with severity of the phenotypic presentation ($r=0.89$; $P=0.02$ and with earlier age at onset ($r=0.93$; $P=0.006$).

Point mutations in the mitochondrial genome have been associated with a number of neurological disorders. Some are well-defined clinical syndromes, including myoclonus epilepsy with ragged-red fibres (MERRF) and MELAS. However, other clinical phenotypes are less distinctive and mitochondrial studies are often included in the workup of complex neurological syndromes of uncertain etiology. To determine the incidence of recognised point mutations, we have investigated 90 consecutive Portuguese patients with varied clinical phenotypes and suspected mtDNA mutations. The A3243G base substitution, which is present in the majority of cases of MELAS syndrome, was detected in six cases, only three of whom had typical MELAS symptoms. Studies of three large series have concluded that roughly 83% MELAS patients harbor the A3243G mutation [16–18]. Our experience, in a smaller group, is consistent with those findings.

The range of phenotypic expression of the A3243G mutation is baffling, and has been confirmed in our study. Only half of our A3243G cases had typical MELAS syndrome, whereas the remaining three patients presented ocular myopathy and diabetes mellitus, severe dilated cardiomyopathy, and a LS-like disorder. As far as LS is concerned, the majority of maternally inherited LS (MILS) are due to the T8993G point mutation in the mtDNA ATP6 gene. Other mtDNA mutations are much rarer. Case 4 appears to be the first MILS patient harboring the A3243G mutation [12], although a similar Asian patient was briefly described [19]. Although we could not study this patient′s brain, it is possible that the earlier and more aggressive neurological disorder was associated with very high percentages of mutant genomes in the CNS. Indeed, a review of the neuropathogical features of mtDNA-associated encephalopathies suggests that the LS-type pattern might represent the extreme expression of defective cerebral energy metabolism, irrespective of the primary molecular etiology [20].

Our data show that the proportion of mutant mtDNA in blood was significantly greater in symptomatic than asymptomatic subjects, and was inversely correlated with age in both groups. This has not been observed in patients with the A8344G ‘MERRF’ mutation. The proportion of mutant mtDNA A3243G was always greater in muscle than in blood but the absence of the mutation in blood does not exclude the possibility of its presence in muscle. Thus, peripheral leukocytes is a useful but not absolutely foolproof diagnostic tool. Indeed, the proportions of mutant mtDNA in blood in affected and unaffected cases overlapped, limiting the use of the blood for prognostic purposes.

The most important observation provided by our data is the unpredictable phenotypic-genotype correlation. Nonetheless, when there is an unusual and unexplained association of symptoms, with involvement of unrelated organs, and evidence of maternal inheritance for at least some of the patients symptoms, we should consider molecular investigations. There are still incomplete data to explain the wide clinical spectrum of mtDNA-associated disorders. Tissue-to-tissue, or even organelle-to-organelle heteroplasmy, seems an important but not exclusive factor. Additional genetic mechanisms, including specific mitochondrial or nuclear genetic backgrounds, could interact with still undefined tissue-specific modulating elements or epigenetic factors. Together, these might determine clinical presentation. Recent data indicate that the relationship between the nuclear and the mitochondrial genomes in mammalians is, indeed, more enigmatic that previously thought [21,22].

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