Mutation loads in different tissues from six pathogenic mtDNA point mutations

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a r t i c l e i n f o

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a b s t r a c t

In this work, we studied the mtDNA mutations *m.3243A* N *G*, *m.3252A* N *G*, *m.15923A* N *G*, *m.13513G* N *A*, *m.8993T* N *G* and *m.9176T* N *C* in the blood, urine and buccal mucosa of a cohort of 27 subjects.
Urine cells had the highest mutation load for all of the mtDNA mutations studied. The mutation loads in the blood, urine and the buccal mucosa were significantly higher in the mitochondrial disorder group that manifested clinical signs than in the asymptomatic subjects. In conclusion, urine is a suitable biological sample for molecular diagnosis of mtDNA mutations and for the study of the attendant risk of recurrence in the offspring of asymptomatic mothers identified as non-carriers after mutation analysis in blood.

1. Introduction

Mitochondrial DNA (mtDNA) point mutations affect protein-coding or protein-synthesis machinery genes of the oxidative phosphorylation system (Scaglia and Wong, 2008; Zeviani and Di Donato, 2004; Zifa et al., 2007). These mutations cause a wide spectrum of clinical phenotypes with high transmission maternal inheritance risks (Monnot et al., 2011).

Pathogenic mtDNA point mutations usually co-exist with wild-type mtDNA in the same tissue, and the degree of heteroplasmy varies widely between individuals and also between tissues within the same subject (Chinnery et al., 1997). These facts seems to be the main factors responsible for the varied clinical expressions of mtDNA mutations, that include a diversity of clinical phenotypes involving different organs, severities and ages of the onset of disease (Chinnery et al., 1997; Ciafaloni et al., 1991; Holt et al., 1990; Macmillan et al., 1993; Matthews et al., 1994). Although muscle is the gold standard for the study of mtDNA mutation loads (muscle has the highest mutation load that remains invariable with time) ('t Hart et al., 1996), muscle biopsy is an invasive procedure that is difficult to perform routinely in clinical practice (Ma et al., 2009).

Previous reports have studied the *m.3243A* N *G* mutation loads in different accessible samples to identify a non-invasive and rapid diagnostic method that can identify patients with mitochondrial disorders (Chinnery et al., 1999; de Laat et al., 2012; Sue et al., 1998). These studies have shown that the *m.3243A* N *G* mutation load in urine is consistently higher than that in blood and the buccal mucosa (de Laat et al., 2012; McDonnell et al., 2004; Rahman et al., 2001; Shanske et al., 2004) and closely resembles the mutation load in muscle (de Laat et al., 2012; Frederiksen et al., 2006; McDonnell et al., 2004). The mutation load in urine decreases with age to a lesser extent than the mutation load in blood (Frederiksen et al., 2006; Rahman et al., 2001; 't Hart et al., 1996).

In this work, we present a family that harbored the *m.3243A* N *G* mutation, describe the clinical features of every member and examined the mutation loads in the blood, urine and buccal mucosa. Furthermore, we report the mutation loads of five different pathogenic mtDNA point mutations in the blood, urine and buccal mucosa in a series of cases and their relatives.

Abbreviations: DNA, mtDNA, mitochondrial; MELAS, mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; MD, mitochondrial disease.

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2. Material and methods

2.1. Patients

The index case for the *m.3243A* N *G* mutation was a woman (Fig. 1; case IV.6) who visited the Neurology Department of our Hospital. Due to her familial antecedents (one relative (Fig. 1; case III.1) was diagnosed with MELAS after necropsy), we studied her and 18 relatives including cases from previous generations. The other 6 index cases were Caucasian pediatric patients (2 boys and 4 girls) from 6 different families. We studied all of their mothers and, in 2 cases, also studied 2 siblings. The clinical and laboratory data are presented in Tables 1 and 2.

Regarding the clinical symptoms, all of the cases were scored and classified into 2 groups, asymptomatic patients ($n = 11$), and symptomatic patients ($n = 16$) (see Tables 1 and 2), based on the clinical signs and symptoms of the Morava's Mitochondrial Disease (MD) Criteria (Morava et al., 2006). The ages of all patients correspond to the times at which the biological samples were collected.

Considering all index cases and their relatives, we studied a total of 33 subjects including 8 males and 25 females whose ages ranged from 2 to 78 years (average 33.3 years; SD = 19.2). Biological samples (blood, urine and buccal mucosa) were available for 27 cases.

2.2. Samples

Blood samples were obtained by venous puncture, urine samples were obtained from spontaneous urination from the first morning void (minimum sample volume: 25 mL) and buccal mucosa cells were obtained by brushing the cheek in the oral cavity with a sterile stick brush. In 7 cases, muscle biopsies was performed and processed as previously reported (Montero et al., 2008).

2.3. Histopathological investigations

Open muscle biopsies of the deltoid were performed in cases I, III and IV, and the quadriceps muscle was biopsied in case V and her sister. The specimens were either frozen in isopentane or cooled in liquid nitrogen for histochemical analysis. The specimens from case III and the sister of case V were fixed in glutaraldehyde for electron microscopy. Serial frozen sections were stained with standard techniques for hematoxylin and eosin, modified Gomori trichrome, nicotinamide adenine dinucleotidtetrazolium reductase, succinate dehydrogenase and cytochrome c oxidase. A standard technique was applied for electron microscopy.

2.4. Laboratory studies

Genomic DNA was extracted from the different biological samples with standard procedures. Mitochondrial DNA was amplified by polymerase chain reaction using specific oligonucleotides primers that corresponded to each studied mutation. The percentages of mutations were analyzed by last-cycle radioactive polymerase chain reaction and restriction fragment length polymorphisms after cutting the amplified fragment with the specific restriction enzyme. The digested products were electrophoresed in agarose gels.

2.5. Statistical analyses

A Kolmogorov–Smirnov test was applied to study the data distribution. Because the data followed a Gaussian distribution, parametric Student's *T* tests for paired data analyses were used to compare the mutation loads in the blood, urine and buccal mucosa from the cases that harbored the different studied mutations. Pearson tests were applied to examine the correlation of the mutation loads in different tissues with the ages of the patients. Levene and Student's *T* tests were applied to compare the mutation loads in the blood, urine, buccal mucosa and saliva between the cases with symptomatic mitochondrial disorder and the cases with no symptoms or signs. The calculations were performed with the SPSS 20.0 program. Statistical significance was considered at p b 0.05.

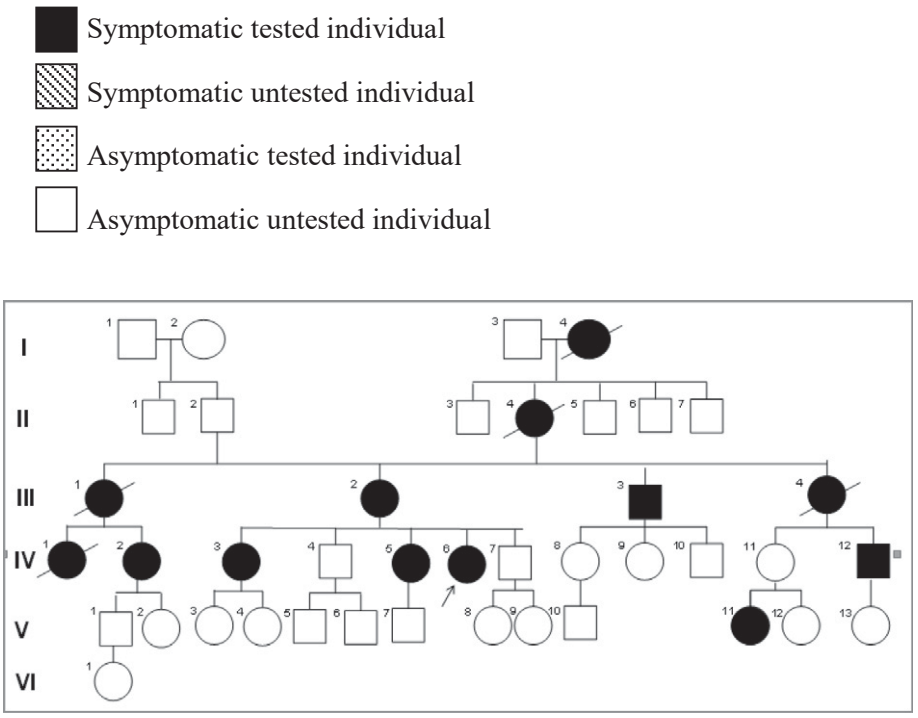

Fig. 1. Pedigree of *m.3243A* N *G* family. Symptomatic patients are in black. Arrow points index case.

Table 1
Case, age, sex, clinical signs and symptoms, and *m.3243A N G* $tRNA^{Leu}$ mutation load in blood, urine, and buccal mucosa.

Case	Age/sex M: male F: female	Clinical signs and symptoms	*m.3243A N G* mutation load in $tRNA^{Leu}$ (%)		
			Blood	Urine	Buccal mucosa
I.4	Exitus/F	Deafness and sudden death in young age.	Not done	Not done	Not done
II.4	Exitus (90y)/F	Migraine and deafness since 30 years old.	Not done	Not done	Not done
III.1	Exitus (54y)/F	Migraine, renal failure and arterial hypertension. MELAS diagnosed after necropsy (stroke like).	Not done	Not done	Not done
III.2	78y/F	Migraine, exercise intolerance, weakness, deafness, retinopathy, insulin resistance, coronary bypass.	Und.	16	b 5
III.3	76y/M	Migraine, deafness, arterial hypertension, diabetes, weakness, polyneuropathy, stroke-like episodes, wheelchair. Muscle biopsy: ragged red fibers.	Not done	Not done	Not done
III.4	Exitus (60y)/F	Migraine, weakness, deafness, ataxia, retinopathy, diabetes, arterial hypertension, psychiatric disorders, stroke-like episodes. Muscle biopsy: ragged red fibers.	Not done	Not done	Not done
IV.1	Exitus (39y)/F	Migraine, weakness, deafness, arterial hypertension, diabetes, ataxia, epilepsy, stroke-like episodes. MELAS diagnosed post-mortem.	Not done	Not done	Not done
IV.2	54y/F	Migraine, weakness, deafness, arterial hypertension.	13	4	13
IV.3	52y/F	Migraine.	b5	15	Und.
IV.4	51y/M	Asymptomatic.	b5	9	b 5
IV.5	49/F	Migraine, weakness, deafness, diabetes, depression, abdominal pain, myoclonus.	21	25	20
IV.6 index case	46y/F	Exercise intolerance, weakness, abnormal EMG, ptosis, migraine, deafness, seizures, depression, abdominal pain, hyperlactacidosis, increased CK.	b5	54	17
IV.7	44y/M	Asymptomatic.	b5	48	Und.
IV.11	39y/F	Asymptomatic.	18	23	5
IV.12	37y/M	Weakness, deafness, short stature, psychiatric disorders. EEG: photoparoxistic response.	28	65	32
V.1	29y/M	Asymptomatic.	b5	21	15
V.2	27y/F	Asymptomatic.	10	51	30
V.11	8y/F	Migraine, hyperlactacidosis. Brain MRI/spectroscopy: normal.	33	75	31
V.12	6y/F	Asymptomatic. Brain MRI/spectroscopy: normal.	47	b 5	32

Und.: undetectable, Y: years, EMG; electromiography, MRI: magnetic resonance imaging.

2.6. Ethical aspects

All samples from the patients were obtained in accordance with the 2000 revision of the Declaration of Helsinki of 1975. The study was approved by the local ethics committee review board; i.e., the "Comitè d'Ètica d'Investigació Clínica" from Sant Joan de Déu Foundation. All parents or guardians provided the written informed consent on behalf of the participants for participation in the study.

3. Results

3.1. Mutation loads in different samples

The pedigrees of the *m.3243A N G* family and those of the other families are shown in Figs. 1 and 2. Data regarding age, sex, clinical signs and symptoms and *m.3243A N G* mutation loads in the blood, urine and buccal mucosa of this family are shown in Table 1. We included the clinical signs and symptoms of some of the cases of generations I, II and III despite not having any samples from these cases because recurrent or familial diagnosis is one of the Morava's MD Criteria (Morava et al., 2006).

Data regarding age, sex, clinical signs and symptoms, metabolic/imaging studies, morphology of histopathology, locations of the mtDNA mutations, molecular mutations, and mutation loads in the muscle, blood, urine and buccal mucosa of the other 6 index cases and their relatives are presented in Table 2.

Across the entire cohort of patients, Student's *T* tests for paired data revealed significant differences (p b 0.01) in the percentages of the different mutations between the urine (average = 42%; SD = 34.3) and blood (average = 26%; SD = 29.0) and between the urine and buccal mucosa (average = 27%; SD = 30.8) but not between the blood and buccal mucosa. These differences were also observed when we compared the percentage of the tRNALeu, m.3243A N G mutation among the different tissues ($p = 0.033$ when comparing urine and blood and $p = 0.023$ for urine and buccal mucosa). Furthermore, the urinary mutation loads for the other mutations were in most cases, higher than those detected for the other samples (Table 2).

3.2. Mutation load and age

When the correlations of the mutation load across the entire cohort of patients with age were examined, the results revealed significant negative correlations in the urine (Pearson test: $r = -0.549$, $p = 0.003$), blood (Pearson test: $r = -0.646$, p b 0.001) and buccal mucosa (Pearson test: $r = -0.634$, $p = 0.001$). Strongly significant positive correlations were observed between the blood and buccal mucosa (Pearson test: $r = 0.966$, p b 0.001), between the urine and blood (Pearson test: $r = 0.784$, p b 0.001) and between the urine and buccal mucosa (Pearson test: $r = 0.840$, p b 0.001).

3.3. Mutation load in symptomatic patients and in asymptomatic relatives

According to Morava's MD Clinical Criteria, we compared the mutation loads in the blood, urine, buccal mucosa and saliva between symptomatic patients ($n = 16$; average age = 30 years; SD = 21.2) and the asymptomatic cases ($n = 11$; average age = 36 years; SD = 16.6). Student's *T*-tests revealed a significantly higher mutation loads in the symptomatic group in the blood (average 40%; SD = 32.3), urine (average 58%; SD = 34.3) and buccal mucosa (average 39%; SD = 34.2) when compared to the asymptomatic participants' for blood (average 13%; SD = 17.2), urine (average 23%; SD = 22.7) and buccal mucosa (average 9%; SD = 12.4) ($p = 0.027$, $p = 0.003$ and $p = 0.008$, respectively). No difference was observed when we compared the ages of these 2 groups of patients. The most relevant finding was that 12 patients who were classified as asymptomatic presented with different percentages of mtDNA mutation loads in the urine.

Table 2
Case, age (at the time of the urine, blood, oral mucosa collection), sex, clinical signs and symptoms, metabolic/imaging studies, morphology, location of mtDNA, molecular mutation and mutation load (%) in muscle, blood, urine, and buccal mucosa of index cases and their mothers and sibs.

CASE	Age/Sex M: male F: female	Clinical signs and symptoms	Metabolic and imaging studies	Age at the time of biopsy, histopathology and electron microscopy	mtDNA mutations	Mutation load (%)			
						Muscle	Blood	Urine	Buccal mucosa
Case VII	9y/M	Migraine, recurrent stroke-like episodes, epilepsy (seizures, myoclonus), ptosis, exercise intolerance, myopathy (abnormal EMG), hypothyroidism, adrenal insufficiency, cognitive delay. MELAS. Exitus at 11 years old.	Elevated lactate and alanine in blood/CSF. MRI: Stroke-like. MRS: elevated lactate.	9y RRF, SDH (+) and COX (−) fibers.	$tRNA^{Leu}$, m.3243A N G	83	60	98	71
Mother Case VII	40y/F	Depression, insulin dependent diabetes mellitus, breast carcinoma.	Not done.	Not done	$tRNA^{Leu}$, m.3243A N G	Not done	16	52	12
Case VIII	13y/F	Migraine, recurrent stroke-like episodes, photoparoxistic response, epilepsy (seizures, myoclonus), exercise intolerance, myopathy (abnormal EMG), gastro-intestinal symptoms, hearing loss, decreased vision, cognitive delay. MELAS. Exitus at 18 years old.	Elevated lactate and alanine in blood/CSF MRI: Stroke-like, MRS: elevated lactate	Not done	$tRNA^{Leu}$, m.3252A N G	Not done	42	78	48
Brother Case VIII	7y/M	Asymptomatic.	Not done	Not done	$tRNA^{Leu}$, m.3252A N G	Not done	Undetectable	Undetectable	Undetectable
Mother Case VIII	46y/F	Migraine, depression, hypothyroidism, hearing loss, weakness.	Not done	Not done	$tRNA^{Leu}$, m.3252A N G	Not done	Undetectable	10	5
Case IX	19y/F	Exercise intolerance, myopathy (abnormal EMG), gastro-intestinal symptoms, myoclonic epilepsy (seizures, myoclonus), neurosensorial deafness, pigmentary retinopathy, ataxia, and cognitive delay. MERRF.	Elevated lactate and alanine in blood/CSF. MRI: Basal Ganglia calcification.	13 y RRF, subsarcolemmal aggregates of mitochondria, SDH (+) and COX (−) fibers EM: subsarcolemmal aggregates of enlarged mitochondria	$tRNA^{Thr}$, m.15923A N G	78	10	26	18
Mother Case IX	53y/F	Asymptomatic	Not done	Not done	$tRNA^{Thr}$, m.15923A N G	Not done	Not available	26	Not available
Case X	18y/F	Ataxia, exercise intolerance, muscle weakness, axonal neuropathy, pyramidal signs, pigmentary retinopathy, failure to growth, cognitive delay. NARP.	Elevated lactate and alanine. In blood/CSF. MRI: Leigh syndrome.	18 y RRF. Subsarcolemmal aggregates of mitochondria, SDH (+) and COX (−) fibers.	ND5, m.13513G N A	60	48	93	Not done
Mother Case X	48y/F	Asymptomatic	Not done	Not done	ND5, m.13513G N A	Not done	Undetectable	Undetectable	Undetectable
Case XI	17y/F	Ataxia, muscle weakness, dysarthria, axonal neuropathy, pigmentary retinopathy, myoclonus, seizures, cognitive delay. NARP.	Elevated lactate and alanine in blood/CSF. MRI: T2 hyperintensity on cerebellum and atrophy of folies.	2 y Normal	ATPase6, m.8993T N G	90	82	91	95
Sister Case XI	20y/F	Mild pigmentary retinopathy	Mild elevated blood lactate and alanine. MRI: normal.	9 y Subsarcolemmal aggregates of mitochondria with trichromic stain. EM: subsarcolemmal aggregates of enlarged mitochondria	ATPase6, m.8993T N G	83	88	90	85
Mother Case XI	49y/F	Asymptomatic	Not done	Not done	ATPase6, m.8993T N G	Not done	5	5	5
Case XII	2y/M	Psychomotor delay, exercise intolerance, ataxia, ptosis, axonal neuropathy, failure to growth, impaired visual evoked potentials, pigmentary retinopathy. NARP.	Elevated lactate and alanine in blood/CSF. MRI: Leigh syndrome. MRS: elevated lactate.	Not done	ATPase6, m.9176T N C	Not done	95	99	99
Mother Case XII	30y/F	Asymptomatic.	Not done	Not done	ATPase6, m.9176T N C	Not done	45	70	Not available

Y: years, RRF: ragged red fibers, SDH: succinate dehydrogenase, COX: cytochrome c oxidase, EM: electron microscopy, MRS: magnetic resonance with spectroscopy; MRI: magnetic resonance imaging, CSF: cerebrospinal fluid.

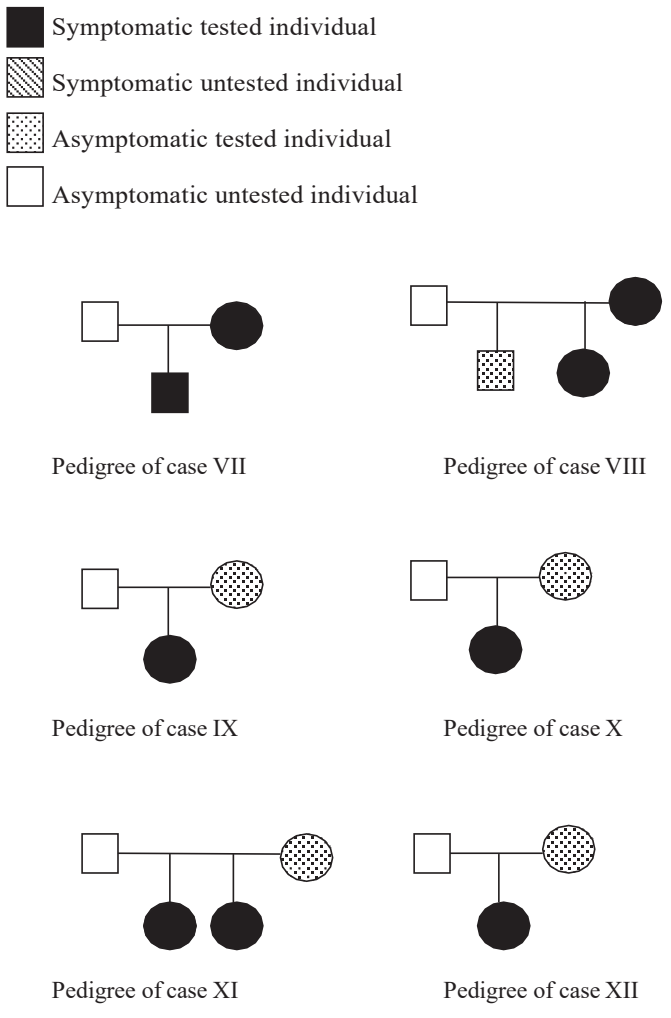

Fig. 2. Pedigrees of cases VII (tRNA^{Leu}, m.3243A N G), VIII (tRNA^{Leu}, m.3252A N G), IX (tRNA^{Thr}, m.15923A N G), X (ND5, m.13513G N A), XI (ATPase6, m.8993T N G) and XII (ATPase6, m.9176T N C).

4. Discussion

Due to the phenomenon of heteroplasmy, several reports have studied the *m.3243A N G* mutation loads in different accessible samples to identify a non-invasive and rapid diagnostic method that can assess mutation load in the muscle (Chinnery et al., 1999; de Laat et al., 2012; Sue et al., 1998). In 2004, Shanske and McDonnell reported for the first time that the *m.3243A N G* mutation load in urinary sediment is higher than that in other tissues (i.e., blood, buccal mucosa, fibroblasts and hair follicles) (McDonnell et al., 2004; Shanske et al., 2004). Later, it was demonstrated that not only is the *m.3243A N G* mutation load in urine consistently higher than those in blood (de Laat et al., 2012; Frederiksen et al., 2006; Ma et al., 2009; McDonnell et al., 2004; Shanske et al., 2004) and buccal mucosa (Frederiksen et al., 2006; Shanske et al., 2004), but it is also correlated with the mutation load present in skeletal muscle (Frederiksen et al., 2006; Ma et al., 2009; Shanske et al., 2004). In our *m.3243A N G* family, we found similar results; the *m.3243A N G* mutation load in the urine was 2-fold higher than those in the blood and buccal mucosa. Furthermore, 6 cases (III.2, IV.3, IV.4, IV.6, IV.7, and V.1; Table 1) harbored nearly undetectable (b5%) blood *m.3243A N G* mutation loads. These individuals, in base of the low percentage of the mutation in blood may have no risk to develop the disease. However, the mutation load found in urine would predict the possibility of developing the pathologic phenotype. In relation to the risk of recurrence of the mutation in their offsprings, it has already been shown several times that mothers with very low mutation load and asymptomatic, may have children with very high percentage of the mutation and symptomatic; there is a great tendency to become homoplasmic. In any case, all these individuals should be considered as carriers (Table 1). Similar results have been observed regarding the mutation loads of other mtDNA point mutations affecting tRNA protein-synthesis machinery genes. Previous descriptions of isolated patients (Emmanuele et al., 2011; Mayr et al., 2006; Nishigaki et al., 2003; O'Callaghan et al., 2012; Tay et al., 2005) and our results regarding *m.3252A N G* in tRNA^{Leu} and *m.15923A N G* in tRNA^{Thr} (Table 2) suggest that urine has the highest mutation load for tRNA protein-synthesis machinery genes among the accessible tissues.

Regarding mtDNA point mutations that affect protein-coding genes, *m.13513G N A* in the ND5 subunit of complex I have been related to MELAS, Leigh and LHON-MELAS overlapping syndromes (Pulkes et al., 1999; Santorelli et al., 1997; Shanske et al., 2008). Case X is the first description of *m.13513G N A* associated with a NARP phenotype (Table 2). The urine mutation load in case X was higher than the mutation loads in the blood and even in the muscle (Table 2). Recently, a study of *m.13513G N A* mutation loads demonstrated that the mutation load in skeletal muscle was not much higher than that in blood in 12 of 13 patients screened and that the mutation load in the urinary sediment was higher in only a single sample (Shanske et al., 2008). The mutation loads in the asymptomatic mother of case X were undetectable in the blood, urine and buccal mucosa (Table 2), which suggests a de novo origin. A previous study of the *m.13513G N A* mutation observed no clinical involvement of the maternal relatives in 7 of 11 patients. Furthermore, in 2 of the 4 mothers studied, the mutation loads were detectable in the blood, urine and buccal mucosa, and the highest load was present in the urine as in case X (Shanske et al., 2008).

Regarding mutations in ATPase6, in case XI and her relatives (Table 2), the *m.8993T N G* mutation loads were similar in the blood, urine and buccal mucosa as has previously been described (White et al., 1999). It is important to note that the sister of case XI had high mutation loads in all samples and remained nearly asymptomatic at the age of 20 years. She only presented with a mild retinopathy and exhibited normal muscle histopathology. In contrast, case XI exhibited similar high mutation loads in all tissues and had a complete NARP phenotype and a pathological muscle biopsy (Table 2). This finding is striking in the context of previous reports, which have suggested that this *m.8993T N G* mutation probably exhibits the strongest correlation between mutant load and disease severity among the different mtDNA mutations (White et al., 1999). However, high m.8993T N G mutation percentage is not always associated with typical features of Leigh and NARP syndromes (Tsao et al., 2001), since these authors reported five individuals from a family harboring the m.8993T N G mutation who presented percentages over 90%, but none of them suffer retinitis pigmentosa and only one was diagnosed with Leigh syndrome.

The *m.9176T N C* mutation has previously been associated with familial bilateral striatal necrosis (Thyagarajan et al., 1995), Leigh syndrome (Campos et al., 1997) and Hereditary spastic paraplegia-like disorder (Verny et al., 2011). Our case XII is the first time that this mutation has been associated with a NARP phenotype (Table 2). Only one previous report has examined the *m.9176T N C* mutation loads in accessible tissues (Jacobs et al., 2005) but did not examine the urine. Thus, the present work is the first to report that the *m.9176T N C* mutation loads in the urine were higher than those in the blood and buccal mucosa in case XII and his mother.

It is interesting to note that the variability of the mutation heteroplasmy rate in different tissues is not the same for different mutations. For example, it is well known that the m.8993T N G mutation levels in the gene for the p.MT-ATP6 polypeptide are very similar in different tissues (Dahl et al., 2000). On the other hand, the load of pathologic mutations in tRNAs (m.3243A N G, m. 3252A N G, and m.15923A N G) shows large differences in different tissues. However, this difference between tissues is also found for the m.13513G N A

transition in the gene for the p.MT-ND5 polypeptide. Thus, this matter deserves further investigation.

It is interesting to note that when we statistically analyzed all of the mutations, the mutation loads in the blood, urine and buccal mucosa exhibited significant negative correlations with age. Our results and those from other groups were obtained from cross-sectional studies. Since the correlation between age and tissue heteroplasmy levels could therefore be biased (Frederiksen et al., 2006), long-term follow up studies are needed to determine whether there is an age associated decrease of mutation loads in mitotic tissues. Finally, mutations were significantly higher in the symptomatic group of patients than in the asymptomatic patients according to Morava's Criteria.

In conclusion, our study supports the hypothesis that urine is a reliable sample for the non-invasive molecular diagnosis of mtDNA mutation in protein-coding or protein-synthesis genes. Furthermore, urine could be the sample of choice for the study of the attendant risk of recurrence of the disease in non-carrier blood and asymptomatic mothers and their relatives.

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