Three families with ‘de novo’ m.3243A > G mutation

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A R T I C L E   I N F O

Article history:
Received 8 February 2016
Received in revised form 28 April 2016
Accepted 28 April 2016
Available online 29 April 2016

Keywords:
Inheritance
m.3243A > G mutation
Mitochondrial myopathy, encephalopathy, lactate acidosis and stroke-like episodes (MELAS)
Maternally inherited diabetes and deafness (MIDD)
Genetic counselling

A B S T R A C T

The m.3243A > G mutation is the most prevalent, disease-causing mitochondrial DNA (mtDNA) mutation. In a national cohort study of 48 families harbouring the m.3243A > G mutation, we identified three families in which the mutation appeared to occur sporadically within these families. In this report we describe these three families. Based on detailed mtDNA analysis of three different tissues using two different quantitative pyrosequencing assays with sensitivity to a level of 1% mutated mtDNA, we conclude that the m.3243A > G mutation has arisen de novo in each of these families. The symptomatic carriers presented with a variety of symptoms frequently observed in patients harbouring the m.3243A > G mutation. A more severe phenotype is seen in the de novo families compared to recent cohort studies, which might be due to reporting bias. The observation that de novo m.3243A > G mutations exist is of relevance for both diagnostic investigations and genetic counselling. Firstly, even where there is no significant (maternal) family history in patients with stroke-like episodes, diabetes and deafness or other unexplained organ dysfunction, the m.3243A > G mutation should be screened as a possible cause of the disease. Second, analysis of maternally-related family members is highly recommended to provide reliable counselling for these families, given that the m.3243A > G mutation may have arisen de novo.

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1. Introduction

In recent years, a large body of data concerning the inheritance of mitochondrial disorders has been published [1–3]. Knowledge relating to the mode of inheritance has great importance for both the diagnosis and counselling of patients. Due to the involvement of the nuclear and mitochondrial genomes, mitochondrial diseases can be transmitted in a Mendelian manner, in the case of a nuclear aetiology, be maternally mitochondrial genomes, mitochondrial diseases can be transmitted in

Abbreviations: mtDNA, mitochondrial DNA; MELAS, mitochondrial myopathy, encephalopathy, lactate acidosis and stroke-like episodes; MIDD, maternally inherited diabetes and deafness; MERRF, myoclonic epilepsy with ragged-red fibres.

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1 Available online 29 April 2016

1.1. Genetics

Mitochondrial diseases are rare and can be transmitted in the form of mtDNA point mutations and large-scale mtDNA deletions. It is estimated that 1% of the population carry a mtDNA mutation [4]. Not all mtDNA mutations are harmful, but some can cause disease when present in the heteroplasmic state. Some mutations such as the m.3243A > G mutation are relatively common, occurring sporadically in the offspring of some families [5]. Our study focuses on the m.3243A > G MTTL1 gene mutation, first described by Goto, Nonaka and Horai [6] as a cause of the mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome but which also causes maternally-inherited diabetes and deafness (MIDD) [7]. Other clinical phenotypes include cardiac, ocular, gastrointestinal and renal involvement. The m.3243A > G mutation is the most prevalent, multi-system disease-causing mitochondrial DNA mutation, with a reported mutation prevalence of between 7.59 and 236 per 100,000 [8–10].

A considerable number of case series and small cohorts have been published describing the phenotypic expression of the m.3243A > G mutation [11–17]. In contrast to what is seen for other mtDNA mutations, the sporadic occurrence of the m.3243A > G mutation, with an expressed phenotype, is rare with only four de novo cases published to date [18–21] (see Table 1).

In our own cohort of Dutch m.3243A > G mutation carriers, we identified three families where the m.3243A > G mutation appears to have arisen de novo. The purpose of this paper is to document these families, describing the probands’ presentation in detail.
and highlighting the family members in whom the m.3243A > G mutation was not detected. Maternal inheritance in a pedigree is a trigger for clinicians to consider the presence of an mtDNA mutation but even if there is no significant family history in the maternal line, the m.3243A > G mutation should be considered in patients with symptoms consistent with the MIDD or MELAS syndromes.

### 2. Methods

All patients participated in the m.3243A > G mutation cohort study at the Radboud Center for Mitochondrial Medicine [22]. 135 patients from 48 families were included in this study. The study was approved by the ethics committee of the Nijmegen–Arnhem region. Written informed consent according to the Helsinki agreement was obtained from all parents and patients ≥12 years.

Heteroplasmy levels were determined in urinary epithelial cells, blood and buccal cells of all participants using Pyrosequencing™ technology (Pyrosequencing, Uppsala, Sweden) as described before by Lowik, Hol, Steenbergen, Wetzels and van den Heuvel [23]. The pyrosequencing assay of the m.3243A mutation in the mtDNA (Genbank accession# NC_012920.1) has a precision of 1.5%, the precision relates to the correlation between independent measurements of the same sample. A sensitivity of 4.5% has been quoted for this technique, as determined by serial dilution of an m.3243A > G positive sample in a sample containing wild type mtDNA, and by determining the background signal +3× SD in a panel of 43 control samples.

The tissue samples of patient II-3 of family 3 were collected within 2 h post-mortem. Samples were snap frozen using liquid nitrogen and stored at −80 °C. DNA was extracted using a Genomic DNA Purification Kit (Genta, Mineapolis, USA), following the manufacturer’s procedures. In the families in which a de novo m.3243A > G mutation was suspected, heteroplasmy levels in urinary epithelial cells from first degree family members were measured using a second pyrosequencing assay with a sensitivity calculated at 1% as previously described by Alston et al. [24].

The data of this study were submitted to the MITOMAP database (www.mitomap.org).

#### 2.1. Family reports

**2.1.1. Family 1**

The proband (III-2, Fig. 1a) presented with aphasia and encephalopathy at the age of 34 years having experienced difficulty in speaking for a few months with a history of hearing impairment since age 5. During this episode of encephalopathy he spoke in short sentences and was unable to respond to complex commands. There were no abnormalities in the evaluation of cranial nerves, reflexes, motor skills or sensation. MRI imaging showed abnormal signal intensities in both cortical and subcortical areas of the left parieto-temporal lobe. MRI-spectroscopy demonstrated elevated levels of lactic acid in the affected areas. A muscle biopsy was performed showing diminished ATP production and decreased complex I activity (see Table 2). Unfortunately no histopathological data are available. m.3243A > G mutation screening was performed, revealing a high level of m.3243A > G mutation load (82%) in muscle, confirming the diagnosis of m.3243A > G related mitochondrial disease. Assessment of m.3243A > G mutation levels in leukocytes, urinary epithelial cells and buccal cells were 23%, 63% and 40%, respectively. During follow-up, the patient developed epilepsy at the age of 35 years and diastolic dysfunction on echocardiography. He was myopathic and unable to perform normal work. Lactate levels in blood were elevated to an average level of 4.0 mmol/L (reference 2.2 mmol/L). An extensive family study was performed. Family members with symptoms that could be consistent with the m.3243A > G mutation included a cousin (III-6) and her baby child (IV-6) who experienced severe congenital hearing impairment, and the proband’s mother (II-3) who reported non-insulin dependent diabetes and mild hearing impairment at the age of 71 years. A total of 16 family members were tested for the presence of the m.3243A > G mutation and the result was positive in mother, father and a third cousin (III-6).

**2.1.2. Family 2**

The proband (IV-1, Fig. 1b) presented at the age of almost 2 years, with neurological deterioration following a viral infection resulting in

### Table 1

<table>
<thead>
<tr>
<th>Report: author (year)</th>
<th>Gender and age at onset</th>
<th>Probands clinical sign &amp; symptoms</th>
<th>Heteroplasmy levels</th>
<th>Number of tested maternal family members and tested tissues</th>
<th>Special characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yanamoto (1995)</td>
<td>m, 21 years</td>
<td>MELAS syndrome; SLE, mild deafness, weakness, MELAS/MERRF overlap syndrome; epilepsy, SLE, weakness, psychomotor delay</td>
<td>Muscle: 80% Blood: 36% Muscles: 70% Blood: 30% Muscle: 54% Blood: 56% Hair: 70% Buccal saliva: 64%</td>
<td>3; all studied in muscle.</td>
<td>Mother was the de novo patient experiencing MIDD. (muscle 79%, blood 10%)</td>
</tr>
<tr>
<td>Campos et al. (1996)</td>
<td>m, 2 years</td>
<td>MELAS syndrome; epilepsy, SLE, ataxia, blurred speech, paralytic ileus,</td>
<td>Muscle: 80% Blood: 36% Muscles: 70% Blood: 30% Muscle: 54% Blood: 56% Hair: 70% Buccal saliva: 64%</td>
<td>4; all studied in blood, 1 studied in muscle, 1 studied in hair 6; all studied in blood, hair and buccal saliva</td>
<td>Mother was the de novo patient experiencing mild deafness, (blood 11%, hair 27%, buccal saliva 32%); the younger brother was a dormant carrier. (blood 65%, hair 79%, buccal saliva 70%)</td>
</tr>
<tr>
<td>Ko et al. (2001)</td>
<td>m, 5 years</td>
<td></td>
<td>Muscle: 80% Blood: 36% Muscles: 70% Blood: 30% Muscle: 54% Blood: 56% Hair: 70% Buccal saliva: 64%</td>
<td>6; all studied in blood, UEC and buccal saliva</td>
<td></td>
</tr>
<tr>
<td>Maassen et al. (2002)</td>
<td>f, 8 years</td>
<td>MIDD; bilateral deafness, diabetes, hypertension, proteinuria</td>
<td>Muscle: 23% Buccal saliva: 38% UEC: 63%</td>
<td>6; all studied in blood, UEC and buccal saliva</td>
<td></td>
</tr>
<tr>
<td>Patient 1 (this study)</td>
<td>m, 34 years</td>
<td>MELAS syndrome; aphasia, encephalopathy, deafness, SLE, epilepsy, myopathy.</td>
<td>Muscle: 12% Blood: 16% UEC: 20% Buccal saliva:16%</td>
<td>3; all studied in blood, UEC and buccal saliva</td>
<td></td>
</tr>
<tr>
<td>Patient 2 (this study)</td>
<td>m, 1 year</td>
<td>Transient hypotonia, ataxia, ptosis and ophthalmoplegia, motor retardation. Improvement to normal at age 8. Foetal distress, transient tachypnoea of the neonate, transient left ventricular hypertrophy.</td>
<td>Muscle: 23% Buccal saliva: 40</td>
<td>4; all studied in blood and buccal saliva 16; all studied in blood, UEC and buccal saliva</td>
<td></td>
</tr>
<tr>
<td>Patient 3 (this study)</td>
<td>m, 1 day</td>
<td></td>
<td>Muscle: 23% Buccal saliva: 40</td>
<td>4; all studied in blood and buccal saliva 16; all studied in blood, UEC and buccal saliva</td>
<td></td>
</tr>
</tbody>
</table>

m = male, f = female, SLE = stroke-like episodes, UEC = urinary epithelial cells.
hypotonia, axial ataxia, ptosis and ophthalmoplegia. He had elevated lactate levels (5.5 mmol/L, reference 2.2 mmol/L) and abnormal signal intensities in the parieto-occipital region on MRI. His neurological symptoms improved after normalization and increase of his caloric intake. A muscle biopsy was performed, demonstrating diminished ATP production, decreased complex I activity but no histopathological abnormalities (Table 2). Genetic testing revealed the m.3243A > G mutation to be present at a level of 23% heteroplasmy in skeletal muscle. Motor skill development was somewhat slow, requiring physiotherapy but by the age of years he attended mainstream school, and had normal motor skills and strength. Heteroplasmy levels in urinary epithelial cells and buccal cells were 38% and 27%, respectively. Six family members were assessed; they were all in good health. Heteroplasmy analysis showed a low level (6% mutation load) of m.3243A > G mutation in the urinary epithelial cells from the mother (III-3), whereas the mutation was undetectable in her blood and buccal cells. The patient’s mother (III-3) underwent muscle biopsy which showed a slightly diminished ATP production, normal complex activities and no histopathological abnormalities suggestive of mitochondrial disease; heteroplasmy analysis showed a 5% m.3243A > G mutation load in the muscle (see Table 2).

Fig. 1. a–c Pedigrees of the three patients with a de novo mutation. Fathers are not indicated in the pedigrees. The patients with a detectable m.3243A > G mutation load are indicated as black squares and circles. The patients that were tested using pyrosequencing as earlier described by Lowik et al. (2005) are indicated in light grey shading; the patients that were additionally tested using the more sensitive pyrosequencing assay described by Alston et al. (2011) are indicated with darker grey shading. a: Family 1; the proband is indicated as individual III-2. Family members in whom the mutation was undetectable in urinary epithelial cells, leucocytes and buccal cells are; II-2, II-3, II-4, II-5, II-7, II-9, III-1, III-3, III-4, III-5, III-6, III-7, III-8, III-9, III-10, IV-6. No further family members were available to participate in the study. Family members II-3 and III-1, mother and sister of the proband respectively, were screened for m.3243 A > G heteroplasmy in urinary epithelial cells using the more sensitive pyrosequencing assay. b: Family 2; the proband is indicated as patient IV-1 however individual III-3 (the proband’s asymptomatic mother) is considered to be the de novo case following carrier testing. The m.3243A > G mutation was undetectable in urinary epithelial cells, leucocytes and buccal cells of relatives denoted II-1, II-3, II-5, III-4 and III-7. The other family members did not participate in the study. Heteroplasmy was tested in urinary epithelial cells from individual II-1, the proband’s mother, using the more sensitive pyrosequencing assay. c: Family 3; the proband is indicated as patient II-4. Tested family members in whom the mutation was undetectable in urinary epithelial cells, leucocytes and buccal cells are; I-1, II-1 and II-2. In all three family members, m.3243A > G mutation load in urinary epithelial cells was tested using the more sensitive pyrosequencing assay. Patient II-3 died at the age of 4 months. Post mortem muscle and liver biopsies were screened and no evidence of the m.3243A > G mutation was reported.
Five further asymptomatic maternally-related individuals including grandmother underwent genetic testing in urinary epithelial cells and did not harbour the m.3243A > G mutation (Fig. 1b). Patients III–1 and III–2 did not consent to genetic testing, their case history did not suggest any mitochondrial symptoms.

2.1.3. Family 3

The proband (II–4, Fig. 1c) was born at term with a birth weight of 4750 g (>97th centile). He was delivered by an emergency caesarean section due to foetal distress and required short time respiratory support because of transient tachypnea of the neonate. He had hypoglycaemia that was treated with glucose infusions and elevated lactate (pH 7.32, reference 7.34–7.45; lactic acid 4.2 mmol/L, reference <2.2 mmol/L). Left ventricular hypertrophy was seen on echocardiogram with no hemodynamic consequence. Muscle biopsy was performed and showed a decrease of complex I and complex IV activities, histopathological assays were normal (Table 2); mtDNA analysis revealed the m.3243A > G mutation at a heteroplasmic level of 12% in muscle. Following a rapid recovery, the patient was discharged from the hospital. At age five months, a second echocardiogram showed a normal heart with no signs of hypertrophy or cardiomyopathy. Quantitative mutation analysis of urinary epithelial cells, leucocytes and buccal cells showed m.3243A > G heteroplasmic levels of 16%, 20% and 16%, respectively. At six years of age the patient had no medical problems, in particular no cardiomyopathy, fatigue or muscle weakness. DNA samples from his mother and siblings showed no detectable levels of the m.3243A > G mutation in their more severely affected children.

An isolated incidence of an mtDNA mutation within a family can have one of three causes, as stated by Maassen et al. [21]: 1) other family members in the maternal lineage harbour the mutation, but below the detection limit, 2) there is no biological relationship between the proband and the other family members tested, 3) a de novo mutational event has occurred. We investigated the cause of the isolated m.3243A > G mutation in the three families described in this report using these three possibilities.

To investigate the possibility that the mutation is present below the sensitivity of the assay, we used two different pyrosequencing techniques as described previously [23,24]. The pyrosequencing assay described by Lowik et al. has a sensitivity of 4.5% whereas the assay described by Alston et al. has a higher sensitivity (1.0%). We tested three different tissues for each available family member, urinary epithelial cells, leucocytes and buccal cells. No muscle was available of the family members (except for patient II-3 of family 3), but previous studies have shown a good correlation between the m.3243A > G mutation load in urinary epithelial cells and muscle biopsy [25,26]. With sensitivities of 4.5% and 1.0% respectively for the two pyrosequencing assays, the probability of multiple false-negative results are highly unlikely, it is therefore unlikely that other family members in the maternal lineage also carry the mutation below the detection limit. The family members were also clinically assessed using the NMDAS for symptoms that could be related to the m.3243A > G mutation. From a total of 23 adult relatives, two had hearing problems (including patient III-6 from family 1), two had diabetes mellitus (one insulin dependent), three reported constipation, two relatives reported psychiatric complaints and two reported visual abnormalities. No relatives reported ataxia or myopathy. The results from the screening of non-invasive familial samples suggest that the m.3243A > G mutation is unlikely to be present below the detection threshold in our proposed de novo m.3243A > G families.

### Table 2

<table>
<thead>
<tr>
<th>Patient</th>
<th>Heteroplasm %</th>
<th>ATP (%)</th>
<th>Complex I (%)</th>
<th>Complex IV (%)</th>
<th>%b</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family 1</td>
<td>82%</td>
<td>5.8 (42.1–81.2)</td>
<td>13.8%</td>
<td>30 (100–401)</td>
<td>30%</td>
<td>809 (810–3120)</td>
</tr>
<tr>
<td>Family 2</td>
<td>23%</td>
<td>24.4 (42.1–81.2)</td>
<td>60.0%</td>
<td>49 (70–251)</td>
<td>70%</td>
<td>1269 (810–3120)</td>
</tr>
<tr>
<td>Family 3</td>
<td>5%</td>
<td>13.8 (15.4–30.2)</td>
<td>90.0%</td>
<td>53 (47–154)</td>
<td>113%</td>
<td>1172 (470–1842)</td>
</tr>
<tr>
<td>Overall</td>
<td>12%</td>
<td>10.4 (42.1–81.2)</td>
<td>24.7%</td>
<td>52 (70–251)</td>
<td>74.2%</td>
<td>759 (810–3120)</td>
</tr>
</tbody>
</table>

a ATP metabolism (nmol/h.mt.tUCS).
b percentage of lower limit of normal range.
c Complex activity (mt/U.CS); na = not available.
In order to investigate the second possibility of non-kinship as the cause of isolated m.3243A > G occurrence, we obtained confirmation of kinship from the mothers within the families. Paternity testing was not performed given that paternity is not relevant in establishing the inheritance of a mitochondrial DNA mutation. Having excluded kinship and sensitivity issues, we conclude that there is most likely a de novo appearance of the m.3243A > G mutation in our three families.

The m.3243A > G mutation has previously been detected in patients with different haplotypes [8,27], indicating that de novo appearance of the m.3243A > G mutation has occurred frequently in the past. Although the mechanism behind de novo appearance of the m.3243A > G mutation is unknown, it is speculated that the mutational event is likely to have occurred during oogenesis (in the mother’s embryonic development) or during early embryonic development of the proband [19]. These hypotheses are supported by our results given that each proband harbours the mutation in tissues that originate from each of the three primary germ cell layers.

This report detailing a further three cases harbouring a de novo m.3243A > G mutation highlights the importance of screening for the MELAS subgroup of mitochondrial encephalomyopathies, Nature 348 (1990) 651–653.

Conflict of interest
Paul de Laat received research support from the Stichting Energy4All. Prof. Smeitink is the founder and CEO of Khondrion and is funded by the Netherlands Organization for Scientific Research and by ongoing Marie-Curie and Eurostars grants and grants of Stichting Energy4All and the Mitochondrial Medicine Foundation, all not related to the current study.

Mirian C.H. Janssen, Charlotte L Alston, Robert W Taylor and Richard J.T. Rodenburg declare that they have no conflict of interest.

This study was not industry sponsored.

Informed consent
All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients, or their legal guardians for being included in the study.

Details of the contributions of individual authors
Paul de Laat: manuscript preparation, patient collection, sample collection, analyses, study design.
Mirian C.H. Janssen: manuscript preparation, study design, patient collection.
Charlotte L Alston: heteroplasmy percentage determination, manuscript correction.
Robert W Taylor: heteroplasmy percentage determination, manuscript correction.
Richard J.T. Rodenburg: heteroplasmy percentage determination, manuscript correction.
Jan A.M. Smeitink: manuscript correction, study design.

Transparency document
The Transparency document associated with this article can be found in the online version.

Acknowledgements
Part of this work was supported by the Stichting Energy4All. Jan Smeitink is CEO of Khondrion.

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