

Mitochondrial DNA Depletion and Deletions in Paediatric Patients with Neuromuscular Diseases: Novel Phenotypes

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Abstract Objective: To study the clinical manifestations and occurrence of mtDNA depletion and deletions in paediatric patients with neuromuscular diseases and to identify novel clinical phenotypes associated with mtDNA depletion or deletions.

Methods: Muscle DNA samples from patients presenting with undefined encephalomyopathies or myopathies were analysed for mtDNA content by quantitative real-time PCR and for deletions by long-range PCR. Direct sequencing of mtDNA maintenance genes and whole-exome sequencing were used to study the genetic aetiologies of the diseases. Clinical and laboratory findings were collected.

Results: Muscle samples were obtained from 104 paediatric patients with neuromuscular diseases. mtDNA depletion was found in three patients with severe early-onset encephalomyopathy or myopathy. Two of these patients presented with novel types of mitochondrial DNA depletion syndromes associated with increased serum creatine kinase (CK) and multiorgan disease without mutations in any of the known mtDNA maintenance genes; one patient had pathologic endoplasmic reticulum (ER) membranes in muscle. The third patient with mtDNA depletion was diagnosed with mersine-deficient muscular dystrophy caused by a homozygous mutation in the *LAMA2* gene. Two patients with an early-onset Kearns-Sayre/Pearson-like phenotype harboured a large-scale mtDNA deletion, minor multiple deletions and high mtDNA content.

Conclusions: Novel encephalomyopathic mtDNA depletion syndrome with structural alterations in muscle ER was identified. mtDNA depletion may also refer to

secondary mitochondrial changes related to muscular dystrophy. We suggest that a large-scale mtDNA deletion, minor multiple deletions and high mtDNA content associated with Kearns-Sayre/Pearson syndromes may be secondary changes caused by mutations in an unknown nuclear gene.

Abbreviations

CK	Creatine kinase
ER	Endoplasmic reticulum
mtDNA	Mitochondrial DNA
MDDS	Mitochondrial DNA depletion syndrome
nDNA	Nuclear DNA
PCIAA	Phenol-chloroform-isoamyl alcohol extraction
qRT-PCR	Real-time quantitative PCR
XL-PCR	Long-range PCR

Introduction

Mitochondrial DNA depletion syndromes (MDDS) are a clinically and genetically heterogeneous group of typically recessively inherited diseases with early or juvenile onset that are characterized by a severe reduction of mtDNA content (Alberio et al. 2007). Based on affected tissues and their mtDNA content, the clinical presentations of MDDS can be classified into three different forms: encephalomyopathic, myopathic and hepatocerebral. For clinical purposes, mtDNA depletion has been defined as mtDNA content of <0.30 relative to age-matched controls (Rahman and Poulton 2009). However, measuring intracellular mtDNA content is technically challenging and the amount of mtDNA is age- and tissue-related (Dimmock et al. 2010; Morten et al. 2007). Numerous pathogenic mutations have been found in the 12 nuclear genes responsible for encoding proteins vital to mtDNA maintenance (El-Hattab and Scaglia 2013; Suomalainen and Isohanni 2010).

Mitochondrial DNA (mtDNA) deletions are qualitative mitochondrial genome defects that come from the loss of mtDNA molecule fragments (Krishnan et al. 2008). They are most likely to be caused by defects that occur during the repair or replication of mtDNA (Krishnan et al. 2008; Yu-Wai-Man and Chinnery 2012). mtDNA deletions are associated with several clinical syndromes, but they also increase with age (Krishnan et al. 2007). Mutations in nuclear genes involved in mtDNA replication and maintenance, e.g., *POLG1* (NM_001126131.1) encoding the catalytic subunit of mitochondrial DNA polymerase γ , can cause accumulation of mtDNA point mutations and deletions throughout life (Chan and Copeland 2009). The most common single large-scale mtDNA deletion is 4,977

base pairs (bp) spanning between the genes for cytochrome B (*CytB*) and cytochrome c oxidase subunit II (*COXII*) (Krishnan et al. 2007; Remes et al. 2005).

The aim of this study was to investigate the occurrence of mtDNA depletions and deletions in muscle biopsy samples of paediatric patients with undefined encephalomyopathy or myopathy, in order to estimate the role of mtDNA rearrangements in pathogenesis of these diseases. Further objective was to identify novel clinical phenotypes associated with mtDNA depletion or deletions.

Subjects and Methods

Patients and Controls

Skeletal muscle biopsy samples were taken as a part of the diagnostic protocol and collected from patients under 18 years with undefined encephalomyopathy or myopathy, who were examined at the Department of Paediatrics of Oulu University Hospital between 1990 and 2012 by the protocol including both clinical assessments and histological, biochemical and/or molecular genetic analyses (Uusimaa et al. 2000). All 104 patients were screened for the eight common *POLG1* mutations (p.T251I, p.A467T, p.N468N, p.G517V, p.P587L, p.R722H, p.W748S and p.Y955C) and the common MELAS m.3423A>G, MERRF m.8834A>G and NARP m.8993T>G mutations in mtDNA. The control muscle samples were collected by the Department of Paediatrics at Oulu University Hospital between the years 2008 and 2012. The samples were taken from patients with no signs of mitochondrial or other neurological diseases during surgical treatments for orthopaedic conditions where mitochondrial function has not been shown to play a major role. Muscle biopsies of 0.25–0.5 cm³ in diameter were performed during an operation requiring an incision through muscle tissue. The biopsies were only done if unnecessary trauma could be avoided. The collection of muscle tissue samples did not interfere with the result of the operation or the recovery of the patient.

The study protocol has been approved by the Ethics Committee of the Faculty of Medicine at the University of Oulu and the Ethics Committee of the Northern Ostrobothnia Hospital District and it is in compliance with the Helsinki Declaration. Written informed consent was given by the guardians of the subjects prior to the study.

DNA Extraction from Muscle Biopsy Samples and Fibroblasts

Total genomic DNA was extracted from skeletal muscle samples using standard phenol-chloroform-isoamyl alcohol

extraction (PCIAA) and ethanol precipitation or by using a commercially available Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). All extracted DNA samples were aliquoted into small fractions and stored in -80°C . DNA for whole-exome sequencing was extracted from patient fibroblasts by using a commercially available QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and ethanol precipitation. Fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) including 5 mM glucose, sodium bicarbonate and pyridoxine and supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin.

Real-Time Quantitative PCR of Muscle DNA Samples

Real-time quantitative PCR (qRT-PCR) was used to determine the amount of mtDNA relative to nuclear DNA in both patient and control muscle samples and was shown as an mtDNA/nDNA ratio. mtDNA was amplified using PCR primers targeted at the mitochondrial NADH dehydrogenase 1 (*ND1*) gene as described by He et al. (He et al. 2002). The values were normalized using the nuclear-encoded brain natriuretic peptide (*BNP*) gene as a single-copy nuclear gene. Amplification products were detected by sequence-specific 6FAM/TAMRA-labelled fluorogenic probes (Sigma-Genosys, Suffolk, UK). The sequences of the primers and probes and description of reaction conditions are available on request. The PCR programme was performed and amplification products were detected by an iCycler thermal cycler and an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). The mtDNA/nDNA ratio was calculated with the ΔCT method described by Pfaffl (Pfaffl 2001). mtDNA depletion was defined as an mtDNA ratio of <0.30 relative to median of age-matched controls (Rahman and Poulton 2009). Unfortunately, we were not able to obtain controls below 5 years of age. Thus, patients below 5 years were compared to median of 5-year-old controls.

Detection of Mitochondrial DNA Deletions in Muscle DNA Samples

Deletions of mitochondrial DNA were detected by long-range PCR (XL-PCR) amplification of mtDNA using Phusion DNA polymerase (New England Biolabs, Ipswich, MA, USA). The amplification reaction was carried out with light-strand primer starting from the nucleotide 10 (L10) and heavy-strand primer starting from the nucleotide 16496 (H16496). The sequences of the primers and probes and

description of reaction conditions are available on request. The PCR products, along with a GeneRuler λ mix ladder and 1 kb ladder (New England Biolabs, Ipswich, MA, USA), were electrophoresed on 0.7% agarose gel stained with a SYBR Safe DNA gel stain (Invitrogen, Eugene, OR, USA).

Molecular Genetic Studies on Patients with Mitochondrial DNA Depletion or Deletions

Based on the clinical phenotype and muscle histology findings, one patient (Patient 3) was analysed for laminin subunit alpha 2 (*LAMA2*) gene. All other patients with mtDNA depletion or deletions (Patients 1–2 and 4–5) were analysed for all the exons and intron-exon boundaries of the *POLG1*, *C10orf2* and thymidine kinase 2 (*TK2*) genes and the entire mtDNA coding region by direct sequencing. In addition, two patients (Patients 4–5) with a large single mtDNA deletion associated with Kearns-Sayre/Pearson-like phenotypes were analysed also for ADP/ATP translocase 1 (*ANT1*) gene.

Whole-exome sequencing was performed in two patients with novel MDDS phenotypes with unknown genetic aetiology (Patients 1–2) and patients with single large-scale mtDNA deletions and minor multiple deletions (Patients 4–5). For whole-exome sequencing, total genomic DNA was extracted from skin fibroblasts. DNA samples of Patients 1–2 were analysed by the commercially available Agilent SureSelect in-solution target enrichment system (Agilent SureSelect Human All Exon V5, Agilent Technologies, Santa Clara, CA, USA) with mean sequencing coverage of $30\times$ using the Illumina HiSeq sequencing platform at the FIMM Technology Center, Helsinki, Finland, as described by Sulonen et al. (Sulonen et al. 2011). DNA samples of Patients 4–5 were analysed using Agilent SureSelect Human All Exon Kit V1 (Agilent Technologies) and sequenced on the Illumina GAIIx sequencing platform at the McGill University, Montreal, Canada, and Genome Quebec Innovation Center, Montreal, Canada. Bioinformatics of the whole-exome sequencing data (variant filtering and interpretation) on these four patients (Patients 1–2 and 4–5) was performed by Dr. Javad Nadaf, Dr. Somayyeh Fahiminiya and Prof. Jacek Majewski at the Department of Human Genetics, McGill University, and Genome Quebec Innovation Center.

Results

Characteristics of Patient and Control Cohorts

Muscle samples were obtained from 104 paediatric patients with encephalomyopathy or myopathy. The median age was 4.1 years (range of 1 month to 17 years). Control muscle samples were obtained from 14 subjects under 18 years with no suspicion of mitochondrial or other neurological diseases. The median age of controls was 12 years (range of 5–16 years). Muscle mtDNA content of the controls is presented in Fig. 1a.

Molecular Studies and Clinical Features of the Patients

Among the 104 paediatric patients with neuromuscular disorders, we identified three patients (Patients 1–3 in Table 1) with mtDNA depletion (Fig. 1b). Two of them presented with severe multiorgan phenotype of MDDS and increased serum creatine kinase (CK) levels. Muscle histology of Patient 2 disclosed myopathic muscle tissue with rounded fibres and endomysial collagen and electron micrograph (EM) showed disordered myofibrillar structure, pathologic ER membranes and accumulation of glycogen and extracellular collagen fibres (Fig. 2a, b). Molecular genetic studies including whole-exome sequencing did not reveal any mtDNA point mutations or mutations in any of the known mtDNA maintenance genes. Patient 3 presenting

with severe myopathy and mtDNA depletion was diagnosed with merosine-deficient muscular dystrophy (MDC1A) based on histological and clinical features. This patient was found to harbour a homozygous p.G1591X mutation creating a stop codon in the *LAMA2* gene.

Two patients (Patients 4 and 5; Table 2) harboured a major large-scale 5 kb deletion and minor multiple deletions (Fig. 3a, b). In addition, qRT-PCR of these two patients showed high mtDNA content (4.9- and 6.9-fold increases, respectively) relative to the median of age-matched controls (Fig. 1a, b). The clinical features of both patients included early-onset external ophthalmoplegia, sensorineural hearing impairment, progressive tremor, severe ataxia, migraine and dysarthria. Furthermore, Patient 5 presented with transient anaemia and granulocytopenia, pigmentary retinopathy and a trifascicular cardiac conduction block requiring a pacemaker in connection to Kearns-Sayre and Pearson syndromes. Muscle histology of both patients disclosed ragged-red fibres and COX-negative fibres as demonstrated in Fig. 2c. In addition, EM of muscle biopsy of Patient 5 revealed accumulation of mitochondria with pathologic internal structure and abnormal cristae (Fig. 2d). Respiratory chain activity measurement showed decreased activity of complexes I and I + III in muscle of Patient 5. Molecular genetic studies including whole-exome sequencing did not disclose any mtDNA point mutations or mutations in any of the known mtDNA maintenance genes.

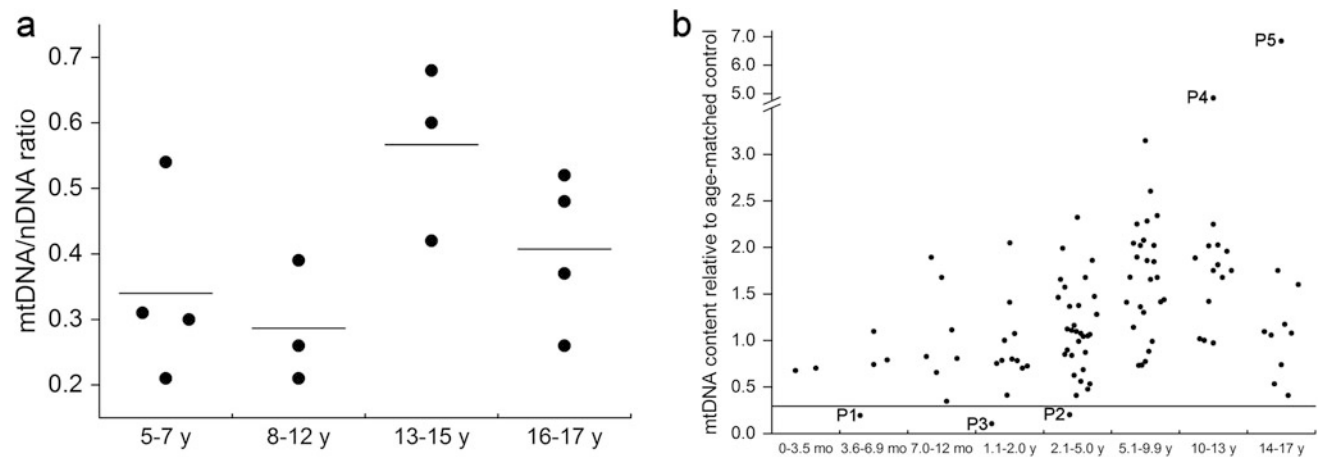


Fig. 1 mtDNA content determined using quantitative real-time PCR. (a) Muscle mitochondrial DNA content of the control samples. The results are presented as mitochondrial DNA/nuclear DNA ratio. Each dot represents one sample. (b) Muscle mitochondrial DNA content of paediatric patients with encephalomyopathies and myopathies ($N = 104$) compared to median muscle mtDNA content of the age-matched control samples. mtDNA depletion was determined as mtDNA content < 0.30 of age-matched controls. The cutoff point at

0.30 is marked with a horizontal line. Three patients with mtDNA depletion were identified (Patients 1–3). Two samples present with marked increase in mtDNA content relative to median of age-matched controls: a single sample in the age group 10–13 years (Patient 4) presents with a 4.9-fold increase and another sample in the age group 14–17 years (Patient 5) show a 6.9-fold increase in mtDNA content relative to age-matched controls. *mo* months, *y* years

Table 1 Clinical features of paediatric patients with neuromuscular diseases associated with mtDNA depletion (Patients 1–3)

Patient	Age at onset	Age at biopsy	Deletion status	mtDNA/mtDNA ratio (\pm S.D.)	mtDNA relative to control	Enc	Myo	Hep	FTT	Epi	Ata	Others	Laboratory findings	OXPHOS activity	Muscle histology	Genetic aetiology
P1	Newborn	5 mo	No	0.06 \pm 0.05	0.19	+	-	-	+	+	-	Spasticity, microcephaly EEG, burst suppression; MRI, white matter necrosis, basal ganglia and thalamic lesions	L: + CFSL: + P: + CK: + UAA: +	N	Atr, F	Unknown
P2	Newborn	3 y 5 mo	No	0.06 \pm 0.02	0.2	+	+	-	+	-	-	Optic atrophy, pigmentary retinopathy, nystagmus, SNHI, death at age 4 y 8 mo	L: N P: N CK: +	N.A.	Gly, ER, Fib,	Unknown
P3	9 mo	1 y 10 mo	No	0.03 \pm 0.01	0.1	-	+	-	-	-	-	Merosine-deficient muscular dystrophy (MDC1A), severe muscular hypotonia ENMG: myopathy Brain MRI: leukodystrophy	CK: +	N.A.	Atr, F, Fib	LAMA2 p. G1591X +/- +

Abbreviations for clinical features: *Enc* encephalopathy, *Myo* myopathy, *Hep* hepatopathy, *FTT* failure to thrive, *Epi* epilepsy, *Ata* ataxia, *EEG* electroencephalography, *MRI* magnetic resonance imaging, *SNHI* sensorineural hearing impairment

Abbreviations for laboratory findings and OXPHOS activity: *CK* creatine kinase, *L* blood lactate, *CSFL* cerebrospinal fluid lactate, *P* blood pyruvate, *N* normal, + elevated, - decreased, *UAA* + in patient P1 generalized aminoaciduria, *OXPHOS* respiratory chain, *N.A.* not available

Abbreviations for histology: *N* normal, *Atr* atrophy, *F* fat excess, *Gly* increased glycogen, *ER* pathologic endoplasmic reticulum membranes, *Fib* accumulation of extracellular collagen fibres, *N.A.* not available

Abbreviations and footnotes: *y* years, *mo* months

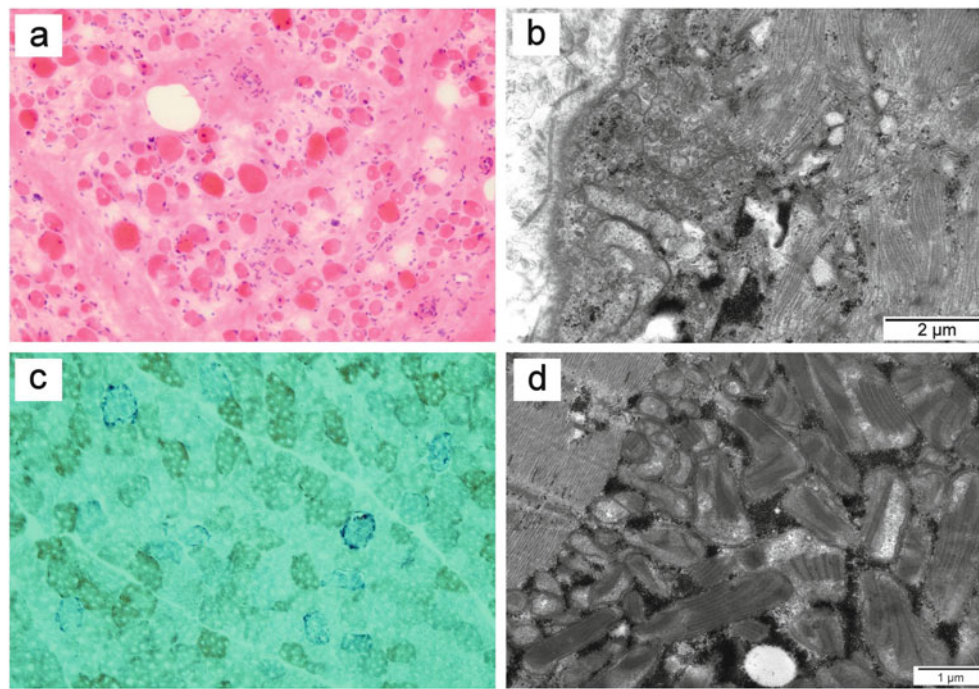


Fig. 2 Histological findings in skeletal muscle biopsies associated with mtDNA depletion or deletions. **(a)** Muscle histology of Patient 2 disclosed myopathic muscle tissue with rounded fibres and endomysial collagen. HE staining. **(b)** Electron micrograph of the muscle of Patient 2 showing disordered myofibrillar structure, pathologic endoplasmic reticulum membranes, accumulation of

glycogen and extracellular collagen fibres. **(c)** Muscle histology of Patient 5 demonstrated COX-negative fibres. SDH-COX staining. **(d)** Electron micrograph of the muscle of Patient 5 displayed accumulation of mitochondria with pathologic internal structure and abnormal cristae

Discussion

We investigated mtDNA depletion and deletion in relation to paediatric neuromuscular diseases. As it is not ethically justifiable to collect muscle biopsies from healthy children with a sole intention to obtain control samples, the control samples had to be collected from patients who underwent surgical operation. This obviously had an effect on the selection process. Thus, we were unfortunately lacking control muscle samples from healthy children younger than 5 years old, but as presented by Dimmock et al. (Dimmock et al. 2010), muscle mtDNA content is relatively stable between 0 and 6 years of age. Therefore, mtDNA ratio of patients under 5 years was determined relative to median of 5-year-old controls. In all, we identified 4 patients with a typical presentation of a mitochondrial disorder in association with mtDNA depletion or deletions. Furthermore, mtDNA depletion was found as a secondary finding in hereditary muscular dystrophy.

Two patients with mtDNA depletion (Patients 1–2) presented with novel types of mitochondrial DNA depletion syndromes manifesting with multiorgan disease. The clinical features of these two patients with the severe MDDS associated with increased CK levels suggested mutations in the *TK2* gene (Lesko et al. 2010), but

sequencing of *TK2* gene disclosed no mutations. Patient 1 manifested with a severe progressive metabolic encephalomyopathy and the brain MRI of Patient 1 disclosed white matter degeneration as well as basal ganglia and thalamic lesions resembling Leigh syndrome. Similar encephalopathic phenotypes have been described in MDDS caused by several mtDNA maintenance genes including in *SUCLA2* and *SUCLG1* (Ostergaard et al. 2007a, b; Carrozzo et al. 2007; Elpeleg et al. 2005), *DGUOK* (Mandel et al. 2001; Dimmock et al. 2008) and *RRM2B* (Bornstein et al. 2008; Acham-Roschitz et al. 2009; Kollberg et al. 2009). Patient 1 was also found with generalized aminoaciduria that has previously been described in MDDS patients (Uusimaa et al. 2014). Patient 2 presented with a novel early-onset and fatal MDDS phenotype including encephalopathy, optic atrophy, pigmentary retinopathy, sensorineural hearing impairment and pathologic ER membranes in histologically disordered muscle with accumulation of glycogen and extracellular collagen fibres (Fig. 2b). A patient with mtDNA depletion and similar clinical features has previously been described to harbour a missense mutation in *MFN2* gene encoding mitofusin 2, a protein essential in mitochondrial network dynamics (Renaldo et al. 2012). As ER and mitochondria are functionally related (Kommann

Table 2 Clinical features of patients with Kearns-Sayre/Pearson syndrome associated with a single large-scale deletion and minor multiple mtDNA deletions and high mtDNA content (Patients 4–5)

Patient	Age at onset	Age at biopsy	Deletion status	mtDNA/nDNA ratio (\pm S.D.)	mtDNA relative to control	CPEO	Pt	Mig	SNHI	Myo	Ata	Others	Laboratory findings	Muscle histology	Genetic aetiology
P4	5 y	12 y	Single 11 kb band + M	1.89 \pm 0.25	4.9	+	-	+	+	-	+	Progressive tremor Brain CT: subcortical and cerebellar hypodensity Brain MRI: T2-weighted lesions in globus pallidus and thalamic and frontal subcortical regions	L: + CFSL: + P: N CK: N	C-, RRF, F, Fib	Unknown
P5	1 y	16 y	Single 11 kb band + M	2.81 \pm 0.89	6.9	+	+	+	+	+	+	Transient anaemia and granulocytopenia, pigmentary retinopathy, growth retardation, progressive cognitive impairment, trifascicular conduction block Ultrasound: echogenic liver and kidneys Brain MRI: symmetric T2-weighted lesions in inferior colliculi and right thalamic and frontal subcortical regions	LE: + OXPHOS: CI + III and CIII-	C-, RRF, Mito, Cr	Unknown

Abbreviations for mtDNA deletion status: *M* multiple deletions

Abbreviations for clinical features: *CPEO* chronic external ophthalmoplegia, *Pt* bilateral ptosis, *Mig* migraine, *SNHI* sensorineural hearing impairment, *Myo* myopathy, *Ata* ataxia, *CT* computed tomography, *MR* magnetic resonance imaging

Abbreviations for laboratory findings: *L* blood lactate, *CSFL* cerebrospinal fluid lactate, *P* blood pyruvate, *CK* creatine kinase, *LE* liver enzymes, *OXPHOS* C respiratory chain complex activity, *N* normal, + elevated, - decreased, *N.A.* not available

Abbreviations for histology: *C-* COX-negative fibres, *RRF* ragged-red fibres, *F* fat excess, *Fib* fibrosis, *Mito* pathologic inner mitochondrial structures, *Cr* abnormal cristae in mitochondria, *N.A.* not available

Abbreviations and footnotes: *y* years, *mo* months

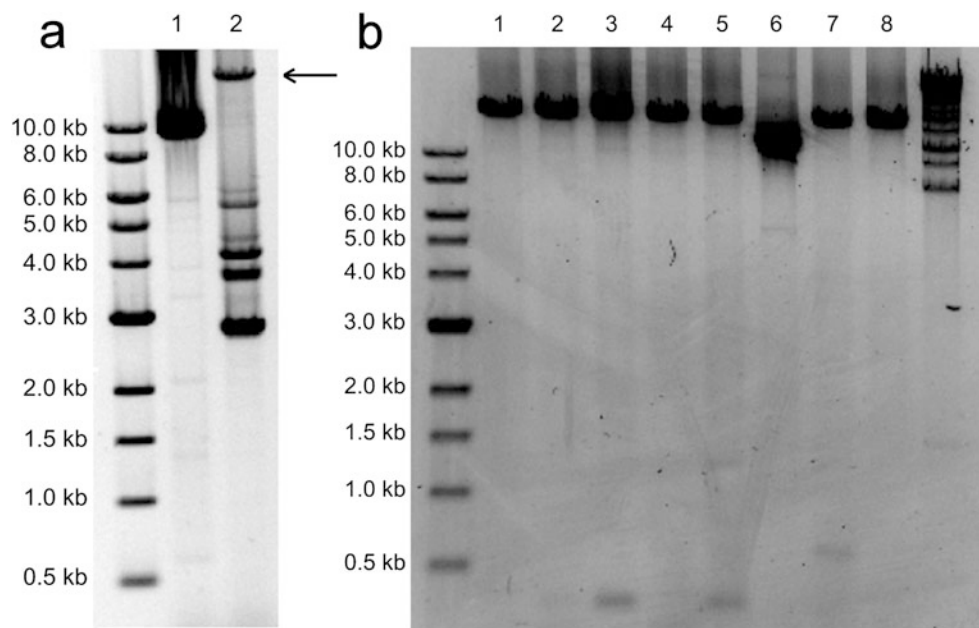


Fig. 3 Long-range PCR of muscle DNA. (a) Lane 1, patient 4 presenting with Kearns-Sayre/Pearson-like syndrome; lane 2, a positive control for mtDNA deletions. Intact 16.6 kb mtDNA is marked with a *black arrow*. (b) Lanes 1 and 8, 5-year-old healthy control; lanes 2–5, patient samples with unspecific deletion bands;

lane 6, Patient 5 presenting with a single large-scale deletion with no intact 16.6 kb mtDNA band. In addition, smaller deletion bands are visible signifying multiple mtDNA deletions; lane 7, a patient sample with unspecific 0.5 kb band

2013), the muscle histology of Patient 2 suggests involvement of ER in the pathogenesis of the disease.

Two patients (Patients 4 and 5) harboured a large-scale mtDNA deletion in addition to minor deletions in muscle DNA (Fig. 3). Although XL-PCR is not a quantitative method for mtDNA deletion analysis, we were not able to detect intact 16.6 kb mtDNA in an XL-PCR assay of these patients suggesting a very high heteroplasmy rate of the mutated mtDNA molecule. Most interestingly, we found markedly increased muscle mtDNA content with the qRT-PCR analysis in both cases referring to overamplification of mtDNA as a compensation mechanism for large-scale mtDNA deletion. These patients developed ophthalmoplegia at a young age along with other neurological symptoms including sensorineural hearing loss, muscle hypotonia and ataxia. Patient 5 also presented with pigmentary retinopathy and cardiac conduction block associated with KSS (#OMIM 530000), as well as transient anaemia and granulocytopenia suggesting Pearson syndrome (OMIM #557000). In the previous literature, we found only one similar case of mtDNA overamplification associated with mtDNA deletions. This case was a female patient presenting with KSS (Wong et al. 2003) including progressive external ophthalmoplegia, ptosis and pigmentary retinopathy; the atypical onset of the disease happened at 30 years of age. She harboured a 3,078 bp mtDNA deletion with a 92% proportion of mutant mtDNA and partial mtDNA duplication. Ragged-red fibres were seen in muscle histol-

ogy of this patient, but respiratory chain activity was normal. Quantitative analysis showed a ninefold increase in muscle mtDNA content, thus suggesting compensatory amplification of mtDNA due to a high deletion mutant load.

KSS is commonly associated with 4,977 bp mtDNA deletion (Remes et al. 2005). It has also been suggested that mtDNA duplications can be a typical feature of early-onset KSS (Poulton 1992; Odoardi et al. 2003). Consideration of the role of mtDNA duplications in KSS raises a question whether the high mtDNA content in qRT-PCR is actually an artefact caused by duplicated target gene in mtDNA molecules. In this work we did not analyse the DNA samples for duplications, but previous studies (Wong et al. 2003; Odoardi et al. 2003) suggest that the *ND1* gene, the target gene for qRT-PCR in our study, is not situated in common locations of either mtDNA deletions or duplications. This may indicate that the high mtDNA content found in our two KSS patients actually refers to a high copy number in the muscle suggesting compensatory overamplification of mtDNA, which is also suggested in the previous study (Wong et al. 2003). In addition, muscle histology of Patient 5 showed mitochondrial inclusion bodies, which could be related to high mtDNA content observed in quantitative analysis.

In general, in 40% of cases of mtDNA-related clinical syndromes, the genetic aetiology remains unsolved even after whole-exome sequencing (Calvo et al. 2012). In this study, with whole-exome sequencing, we did not identify

pathogenic mutations in any of the known mtDNA maintenance genes (El-Hattab and Scaglia 2013; Suomalainen and Isohanni 2010) to disclose the genetic aetiology of the disease in Patients 1 and 2, but further studies are ongoing to evaluate the role of the candidate genes identified by whole-exome sequencing (unpublished data provided by Dr. Javad Nadaf, Dr. Somayyeh Fahiminiya and Prof. Jacek Majewski, Department of Human Genetics, McGill University and Genome Quebec Innovation Center, Montreal, Canada) associated with these novel MDDS phenotypes. Most single mtDNA deletions are thought to be sporadic and thus not genetically transmitted (Chinnery et al. 2004), but multiple mtDNA deletions can be inherited as an autosomal dominant or recessive trait (Zeviani et al. 1990; Nishino et al. 1999). Our two patients with a KSS/Pearson-like phenotype presented with minor multiple deletions in addition to a major large-scale mtDNA deletion, which led us to suggest a genetic origin of the mtDNA arrangements leading to these clinical phenotypes. Whole-exome sequencing was performed in Patients 4–5, but no mutations in the known mtDNA maintenance genes were found. These circumstances could refer to mutations in a yet unknown nuclear gene responsible for mtDNA maintenance.

Patient 3, presenting with severe mtDNA depletion (mtDNA content only 0.10 compared to the age-matched controls) and severe myopathy with muscle fibrosis and atrophy, was diagnosed with merosine-deficient muscular dystrophy (MDC1A) based on further histological and clinical examinations. Molecular studies on this patient disclosed homozygous p.G1591X mutation in the *LAMA2* gene. Secondary mtDNA rearrangements have been previously found in neuromuscular disorders that are not primary mitochondrial diseases (Katsetos et al. 2013), but so far only a few studies concerning mtDNA rearrangements in the pathogenesis of muscle dystrophies have been performed. mtDNA deletions have been described in myotonic dystrophy (Sahashi et al. 1992) and oculopharyngeal muscular dystrophy (Lezza et al. 1997; Muqit et al. 2008). Another study on congenital myotonic dystrophy associated mtDNA depletion to a minor degree with the disease, but no evidence of mtDNA rearrangements in the pathogenesis was found and therefore mild mtDNA depletion was suggested to be secondary to the disease (Poulton et al. 1995).

In conclusion, we describe two novel early-onset phenotypes associated with mtDNA depletion, one of them presenting with pathologic endoplasmic reticulum membranes. In addition, we suggest that Kearns-Sayre and Pearson syndrome can present with multiple deletions and may be caused by mutations in an unknown mtDNA maintenance gene. Furthermore, mtDNA depletion can be a secondary finding in hereditary muscular dystrophy.

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Synopsis

Novel phenotypes associated with mtDNA depletion and deletions without mutations in the known mtDNA maintenance genes.

Compliance with Ethics Guidelines

Conflict of Interest

Tuomas Komulainen, Milla-Riikka Hautakangas, Reetta Hinttala, Salla Pakanen, Vesa Vähäsarja, Petri Lehenkari, Päivi Olsen, Päivi Vieira, Outi Saarenpää-Heikkilä, Johanna Palmio, Hannu Tuominen, Pietari Kinnunen, Kari Majamaa, Heikki Rantala and Johanna Uusimaa declare that they have no conflict of interest.

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 guardian for being included in the study.

Authors' Contributions

Tuomas Komulainen is responsible for the study design, gathering, analysing and interpreting the data and drafting the manuscript. Reetta Hinttala and Johanna Uusimaa are responsible for the study design, gathering, analysing and interpreting the data and revising the manuscript for important intellectual content. Milla-Riikka Hautakangas, Reetta Hinttala, Salla Pakanen, Vesa Vähäsarja, Petri Lehenkari, Päivi Olsen, Päivi Vieira, Outi Saarenpää-Heikkilä, Johanna Palmio, Hannu Tuominen, Pietari Kin-

nunen, Kari Majamaa and Heikki Rantala are responsible for gathering, analysing and interpreting the data and revising the manuscript for important intellectual content.

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