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Clinical and molecular characterization of mitochondrial DNA disorders in a group of Argentinian pediatric patients

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ABSTRACT

Objective: To describe the clinical and molecular features of a group of Argentinian pediatric patients with mitochondrial DNA (mtDNA) disorders, and to evaluate the results of the implementation of a classical approach for the molecular diagnosis of mitochondrial diseases.

Methods: Clinical data from 27 patients with confirmed mtDNA pathogenic variants were obtained from a database of 89 patients with suspected mitochondrial disease, registered from 2014 to 2020. Clinical data, biochemical analysis, neuroimaging findings, muscle biopsy and molecular studies were analyzed.

Results: Patients were 18 females and 9 males, with ages at onset ranging from 1 week to 14 years (median = 4 years). The clinical phenotypes were: mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome ($n = 11$), Leigh syndrome ($n = 5$), Kearns-Sayre syndrome ($n = 3$), Chronic Progressive External Ophthalmoplegia ($n = 2$), Leber hereditary optic neuropathy (n = 2), myoclonic epilepsy associated with ragged-red fibers ($n = 1$) and reversible infantile myopathy with cytochrome-C oxidase deficiency ($n = 3$). Most of the patients harbored pathogenic single nucleotide variants, mainly involving mt-tRNA genes, such as *MT-TL1, MT-TE* and *MT-TK.* Other point variants were found in complex I subunits, like *MT-ND6, MT-ND4, MT-ND5*; or in *MT-ATP6*. The m.13513G > A variant in *MT-ND5* and the m.9185 T > C variant in *MT-ATP6* were apparently *de novo*. The rest of the patients presented large scale-rearrangements, either the "common" deletion or a larger deletion.

Conclusions: This study highlights the clinical and genetic heterogeneity of pediatric mtDNA disorders. All the cases presented with classical phenotypes, being MELAS the most frequent. Applying classical molecular methods, it was possible to achieve a genetic diagnosis in 30% of the cases, suggesting that this is an effective first approach, especially for those centers from low-middle income countries, leaving NGS studies for those patients with inconclusive results.

1. Introduction

Mitochondrial diseases (MD) are a heterogeneous group of disorders caused by pathogenic variants in genes that primarily affect oxidative phosphorylation and ATP synthesis [1]. They are individually considered rare diseases, but collectively they have an estimated incidence of ~1/5000 births [2], and thus they are included among the most common forms of inherited metabolic disorders.

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Mitochondria are highly specialized organelles, present in all tissues (except mature erythrocytes), and are the major generators of cellular energy through the oxidative phosphorylation (OXPHOS) system [3]. The OXPHOS system consists of 85 subunits and is encoded at the genomic level by both the nuclear genome (nDNA) and mitochondrial DNA (mtDNA) [4]. Due to this dual genetic control, MD may show different modes of inheritance, such as mendelian pattern, mostly autosomal recessive, and maternal pattern related to mitochondrial DNA.

Mitochondrial DNA is a 16,569 base-pairs (bp) circular double-stranded molecule that encodes for 37 genes, including 13 polypeptides involved in the OXPHOS system, along with 2 ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs), necessary for their translation within the organelle. Each cell contains multiple copies of mtDNA, which can be genetically identical (homoplasmy) or present different amounts of different populations of mtDNA (heteroplasmy). Differences in mutational load surpassing the pathogenic threshold in some tissues but not in others may contribute to the heterogeneity of phenotypes that is observed in MD [5].

Pathogenic changes in mtDNA were first associated with human disease in 1988 [6,7]. Since then, more than 260 pathogenic variants and 120 large-scale rearrangements have been identified [8]. Pathogenic variants have been described affecting every mtDNA gene; remarkably, more than half of these variants are located in tRNA genes.

MD may affect any organ or tissue; however, those with higher energy demand are preferentially affected, such as central nervous system, heart, skeletal muscle, liver, kidney, and the eyes [9]. While some patients disclose an organ-specific disease, such as "pure" myopathy, cardiomyopathy or optic neuropathy, most patients show multisystemic involvement, either at presentation or during the course of the disease. Considerable clinical variability does exist, and many individuals do not fit neatly into one particular category [10]. However, several well recognizable syndromes have been described, *e.g.* mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS); or myoclonic epilepsy associated with ragged-red fibers (MERRF).

Traditionally, initial presumptive diagnosis of MD relied upon clinical phenotype, metabolic changes, image studies and muscle pathology while genetic diagnosis was partially achieved through candidate gene studies. With the development of novel high throughput diagnostic techniques and the increased availability of next-generation sequencing (NGS), most diagnostic centers have changed their diagnostic strategy to customized targeted panels, clinical exome or whole-exome sequencing [11]. However, at present, NGS is not available as a first-approach technique for MD diagnosis in middle-income-countries. Therefore, traditional molecular studies remain the first choice, especially for those patients with syndromic phenotypes.

The aims of the present study are to describe the clinical and molecular features of a group of patients with mtDNA disorders diagnosed in a tertiary care pediatric public hospital of Argentina, and to evaluate the results of the implementation of a classical approach for the molecular diagnosis of MD.

2. Material and methods

2.1. Patients and clinical evaluation

Clinical data from 27 patients with confirmed mtDNA pathogenic variants were obtained from a database containing data from a total of 89 patients with suspected mitochondrial disease registered at the *Hospital de "Pediatría J. P. Garrahan"*, Argentina, from 2014 to 2020. Of them, DNA samples and clinical data corresponding to 6 patients had been received from other local institutions. Diagnosis of MD was based on stablished clinical and biochemical criteria [12]. Written informed consents for molecular analyses were obtained from the parents of the affected children using a form approved by the local Institutional Ethics Committee. Patients with MD related to nDNA variants or without certain molecular diagnosis were excluded from this report. Data collected from medical records included gender, age at onset of disease, current age, initial manifestations, and neurological and multisystemic signs and symptoms during the course of the disease. Clinical data were available from 25 patients.

2.2. Complementary studies

Biochemical tests evaluated were blood lactate, amino acids (increased alanine), creatine kinase (CK), urine organic acids (lactate and Krebs cycle intermediates); cerebral spinal fluid (CSF) lactate and amino acids (increased alanine). Neuroradiological studies included brain computerized tomography (CT) scan, brain magnetic resonance imaging (MRI) and spectroscopy (MRS), and lactate peak on spectroscopy. Muscle biopsy specimens were processed and stained according to standard protocols with modified Gomori trichrome, succinate dehydrogenase activity (SDH), cytochrome *c* oxidase activity (COX) and oil-red O stain for lipids, according to manufacturers' instructions. The following muscular pathological features were recorded: ragged red fibers (RRF), fibers with increased SDH staining (blue fibers), COX-negative fibers, COX reduction and SDH reduction or increase. Electron microscopy (EM) for the detection of abnormal mitochondria was performed when available.

2.3. Genetic analysis

DNA was obtained by standard methods. Samples analyzed were: peripheral blood in 25 cases, skeletal muscle in 4 cases, and buccal mucosa in 1 case. Mothers' samples were derived from peripheral blood in all cases ($n = 12$) and buccal mucosa in 1 case. A list of known mitochondrial DNA pathogenic variants in *MT-TL1, MT-ND1, MT-TI*, *MT-TK*, *MT-ATP6, MT-TF*, *MT-ND4* and *MT-ND6* were screened by Sanger sequencing of the specific PCR products on an ABI PRISM 3130 or 3500 Genetic Analyzers (Applied Biosystems) (Table 1, supplemental data). For other variants identified within the sequences analyzed, MITOMAP database was accessed as a gold standard reference for variant classification (MITOMAP: A Human Mitochondrial Genome Database. http://www.mitomap.org, 2019). Every variant was categorized as homoplasmic or heteroplasmic as Sanger sequencing is not a quantitative method. The real time allele refractory mutation system (ARMS) qPCR assay was performed to evaluate the heteroplasmy level of the m.13513A > G variant according to Brautbar et al. [13]. The so called "common" deletion in mtDNA (del4,977 bp or m.8470_13446del) was studied by long distance-PCR. Other deletions or duplications in mitochondrial DNA were detected in 2 patients by multiplex ligation-dependent Probe Amplification (MLPA) according to manufacturers' protocol using SALSA® MLPA® P125-Mitochondrial DNA kit, MRC Holland, and the breakpoints of the deletions were determined by long distance-PCR and Sanger sequencing. Heteroplasmy of deletions detected by MLPA was determined as = the mean ratio of normal copy regions - the mean ratio of the deleted regions [14].

3. Results

Using traditional molecular studies, pathogenic variants in mtDNA were detected in 27 patients from 22 families, out of 89 patients recorded in the hospital database of pediatric patients with suspected MD. The distribution of positive cases was: MELAS syndrome ($n = 11$; 10 families), Leigh syndrome (LS) ($n = 5$; 4 families), Kearns-Sayre syndrome (KSS) ($n = 3$; unrelated), chronic progressive external ophthalmoplegia (CPEO) ($n = 2$; unrelated), Leber hereditary optic neuropathy (LHON) (n = 2; 1 family), MERRF (n = 1) and reversible infantile myopathy with cytochrome-C oxidase deficiency (n = 3; 1 family). In total, 8 cases showed a family history consistent with MD. The patients were 18 females and 9 males, with ages at onset ranging

from 1 week to 14 years (median = 4 years). LS and reversible myopathy patients seemed to have the earliest onset compared to the other syndromes. In contrast, children with KSS, CPEO and LHON had a predominant older onset, and patients diagnosed with MELAS showed a wider range of ages from less than 1 year to 12 years.

3.1. MELAS syndrome (OMIM #540000)

Eleven patients had MELAS syndrome (9 females, 2 males). The median age at onset was 7.5 years (range = 0–14 years). The median age at last follow-up was 12.4 years (range = 5–20 years). Only one patient died at the age of 18. The clinical manifestations at onset of disease were stroke-like (n = 4), global developmental delay (n = 2), peripheral myopathy/exercise intolerance (n = 2), and endocrine dysfunction (n = 2). Clinical data at onset is not available in one case. All the patients presented cognitive impairment. Ten patients reported epilepsy and stroke-like episodes before the age of 14, and 7 of them presented more than 2 events. Other neurological manifestations observed during the course of the disease were: migraine (n = 4), pyramidal signs (n = 4), ophthalmoparesis (n = 2) and chronic ataxia (n = 1). The multisystemic manifestations most frequently observed were sensorineural hearing loss (n = 7) and constitutional symptoms (n = 7). Less frequent manifestations were diabetes (n = 2), endocrine disorders (n = 2), cardiac involvement (n = 1) and gastrointestinal dysfunction (n = 1). Increased serum level of lactic acid was present in all the patients. Brain MRI showed lesions consistent with stroke-like lesions (SLLs) at supratentorial level in all but one of the cases, and one patient also presented stroke-like images in both cerebellar hemispheres. Muscle biopsy was performed only in one patient who initially presented with peripheral myopathy, and it disclosed RRF and COX-negative fibers (Table 1).

The same pathogenic variant in the mitochondrial *MT-TL1* gene was detected in 10 patients, showing the typical m.3243A>G variant in a heteroplasmic state (Fig. 1. Supplemental data). Molecular studies were performed in 7 mothers, confirming the presence of the variant in all of them. Six of them showed the following manifestations: cognitive impairment (n = 1), diabetes (n = 1), sensorineural hearing loss (n = 1)

Table 1
Demographic, clinical manifestations, biochemical features, and neuroimaging and muscle biopsy findings. *One patient with LS died at 18 months of age. PEO: progressive external ophthalmoplegia; CSF: cerebrospinal fluid; KCI: Krebs Cycle Intermediates; MRS: magnetic resonance with spectroscopy; RRF: red ragged fibers; COXN: COX-negative fibers; COXD: COX-decreased; BRF: blue ragged fibers; SIM: subsarcolemmal increased mitochondria; EM: electron microscopy; (−): not done. NA: not available. For muscle biopsy and biochemical and neuroradiological features, the fraction of positive / cases studied is shown.

	MELAS	LS	KSS	CPEO	LHON	MERRF	Reversible myopathy	Total MD
Patients								
Number of patients (%)	11 (41%)	5 (18%)	3 (11%)	2 (7%)	2 (7%)	1 (4%)	3 (11%)	27
Age								
Age at onset (median)	7.5y	1 y	NA	9y	6.5y	6y	2 m.	4y
Age at last follow-up (median)	12.4y	9 y	20.3y	20y	18y	10y	NA	13.7y
Neurological manifestations								
Cognitive decline	11	3	1	–	–	1	–	16
Epilepsy	10	1	–	–	–	1	–	12
Stroke-like	10	–	–	–	–	–	–	10
Pyramidal	4	4	–	–	–	–	–	8
PEO	2	–	3	2	–	–	–	7
Ataxia	1	2	1	–	–	1	–	6
Global development delay	2	2	–	–	–	–	–	4
Myopathy/Exercise intolerance	2	–	–	1	–	–	3	4
Migraine	4	–	–	–	–	–	–	4
Acute encephalopathy	–	1	–	–	–	–	1	2
Extrapyramidal	–	3	–	–	–	–	–	3
Myoclonus	1	–	–	–	–	1	–	2
Neuropathy	–	–	1	–	–	–	–	1
Multisystemic manifestations								
Constitutional symptoms	7	2	3	–	–	1	1	14
Hearing loss	7	1	2	1	–	1	–	12
Ophthalmologic	1	1	2	–	–	1	–	5
Cardiac	1	1	2	–	–	–	–	4
Endocrine	2	–	–	–	–	–	–	2
Diabetes Mellitus	2	–	–	–	–	–	–	2
Gastrointestinal/liver	1	–	–	–	–	–	–	1
Renal	1	–	–	–	–	–	–	1
Biochemical Features								
Plasma lactic acid	11/11	2/5	3/3	1/2	0/2	1/1	2/2	20/25
CSF lactic acid	2/2	2/2	–	–	–	–	–	4/4
Aminoacids (alanine)	3/3	1/4	0/1	–	–	0/1	0/1	4/10
Urine organic acids (lactic acid/KCI)	4/4	4/5	2/2	1/2	0/2	1/1	1/1	13/17
Neuroradiological features								
Stroke like	10/11	–	–	–	–	–	–	10/11
Basal ganglia	2/11	5/5	–	–	–	–	1/2	8/17
Lactic acid MRS	2/3	3/3	–	–	–	0/1	–	5/7
Brainstem	–	3/5	1/2	–	–	–	–	4/5
Thalamic	1/11	–	1/2	–	–	1/1	–	3/14
Cerebellum	1/11	2/5	–	–	–	–	–	3/16
Infratentorial stroke like	1/11	–	–	–	–	–	–	1/11
Optic nerve/chiasma	–	–	–	–	1/2	–	–	1/2
Muscle biopsy								
RRF	1/1	–	2/2	1/2	–	1/1	1/1	5/7
COXN/COXD	1/1	–	2/2	2/2	–	1/1	–	6/6
BRF	–	–	–	–	–	–	1/1	1/1
SIM	–	1/3	–	–	–	–	1/1	2/4
EM (abnormal mitochondria)	–	–	–	–	–	–	1/1	1/1

or short stature ($n = 3$). The eleventh patient harbored the variant m.13513G > A in the *MT-ND5* gene in heteroplasmic state, which was not detectable in peripheral blood DNA from her mother by sanger sequencing. The mutant load of the patient addressed by real time PCR ARMS was approximately 20%, while it was not detectable in the mother's blood.

3.2. Leigh syndrome (OMIM #256000)

Five patients presented with Leigh syndrome due to variants in mtDNA. The median age at onset of disease was 1 year (range = 0–4 years) and the median age at last follow-up was 9 years. They presented with global developmental delay ($n = 2$), gait disturbances related to spastic hemiparesis (n = 2) and acute encephalopathy with loss of acquired skills ($n = 1$). Neurological manifestations during the course of the disease were: cognitive impairment ($n = 3$), spasticity ($n = 4$), dystonia (n = 3), epilepsy (n = 1), chronic ataxia (n = 1) and episodes of intermittent ataxia (n = 1). The registered multisystemic manifestations were constitutional signs (n = 2), hypertrophic cardiomyopathy (n = 1), retinitis pigmentosa (n = 1), sensorineural hearing loss (n = 1). Lactate was increased in plasma of 2/5 and in CFS of 2/2 patients. Neuroimaging revealed bilateral lesions in the basal ganglia in all LS cases, being associated to brainstem lesions in 3 and to cerebellar involvement in 2 of them. Spectroscopy was performed in 3 children and showed lactate peak in all of them. Muscle biopsy was performed in 3 patients, showing subsarcolemmal mitochondrial aggregates in one case.

Pathogenic variants were identified in the *MT-ATP6* gene in 3 cases (m.8993 T > G, m.8993 T > C and m.9185 T > C), and in the *MT-ND6* gene in 2 siblings (m.14459G > A). The patient with the variant m.8993 T > G was the most severe clinically affected within this group and died due to an infectious intercurrence when he was 1 year old, and his mother reported a previous son who had died at 7 months without a molecular diagnosis. Molecular studies were carried out in 3 mothers, confirming the presence of m.8993 T > G and m.8993 T > C variants. The variant m.9185 T > C was not detected on maternal blood and buccal mucosa DNA, suggesting that it arose *de novo*. The only symptom detected was migraine in one mother carrying the variant m.8993 T > C.

3.3. Kearns Sayre syndrome (OMIM #530000)

Three patients (2 females, 1 male) presented with KSS. The age at onset was available in only 1 patient (10 years old) and the current median age of patients is 20 years (range = 14–21 years). All three cases started with ophthalmoparesis although they had shown a previous history of failure to thrive. The observed neurological manifestations were peripheral myopathy ($n = 1$), neuropathy (n = 1), cerebellar ataxia (n = 1) and cognitive impairment (n = 1). Multisystemic manifestations were pigmentary retinopathy ($n = 2$), sensorineural hearing loss (n = 2) and cardiac conduction disturbances (n = 2). All the patients showed increased plasmatic lactic acid levels. Brain MRI, available in 2 cases, showed thalamic lesions and brainstem involvement in 1 case, being normal in the other patient. Muscle biopsy was performed in 2 cases, both showing RRF and COX-negative fibers.

In all 3 cases, the large-scale deletion of 4799 bp, known as "common" deletion, was identified in mtDNA from skeletal muscle (n = 2) and peripheral blood leukocytes ($n = 1$). As patients' mothers were asymptomatic and mtDNA deletions are usually sporadic they were not screened.

3.4. Chronic progressive external ophthalmoplegia (CPEO) (OMIM #530000)

Two girls presented with external progressive ophthalmoparesis as an initial sign at 8 and 11 years of age and the current ages are 20 and 21. Both evolved towards complete ophthalmoplegia and 1 of them also developed associated proximal myopathy and dysphagia. The only systemic manifestation recorded was mild sensorineural hearing loss. Increased serum lactic acid was recorded in an isolated determination ($n = 1$). Both girls presented increased CK levels. Brain MRI was normal in both patients. The muscle biopsies showed RRF (n = 1) and COX-deficient fibers ($n = 2$).

Mitochondrial DNA large deletions were identified on skeletal muscle DNA in both patients. In one patient the deletion m.7996_15056del was present with 78% of heteroplasmy and the other patient had a deletion m.7488_14414del with a 40% of heteroplasmy [15] (Table 2), but was detectable by long-PCR only in the peripheral blood DNA of the first patient. The mothers were asymptomatic and for this reason they were not studied.

3.5. Leber hereditary optic neuropathy (OMIM #535000)

Two siblings had LHON (1 female, 1 male). They started with bilateral painless subacute visual loss when they were 4 and 9 years old, being their current ages 15 and 21, respectively. At present, visual acuity has deteriorated to the level of counting fingers in both of them. The girl had shown learning difficulties and scoliosis before visual symptoms. No biochemical abnormalities were found, and brain MRI disclosed bilateral optic nerve and chiasma atrophy in one of them but was normal in the other one. In both patients, the homoplasmic m.11778 G > A variant in *MT-ND4* was detected. There were no other maternal family members clinically affected, and the mother could not be still tested.

3.6. MERRF syndrome (OMIM #545000)

One male patient was diagnosed with myoclonic epilepsy with ragged red fibers. He started with generalized tonic-clonic seizures when he was 6 years old, adding later-on epileptic and non-epileptic myoclonus. During the evolution of his disease he developed cerebellar ataxia, optic atrophy and neurosensorial hearing loss. Lactic acid was only increased in one isolated plasma determination and it was normal in CSF. Brain MRI showed transient bilateral subthalamic lesions, and MRS was normal. Muscle biopsy showed ragged red and COX-negative fibers. Molecular study revealed the pathogenic heteroplasmic variant m.8344

Table 2
Molecular diagnosis in the patients and their mothers. LSR = Large scale rearrangement; MMS = mother molecular study (positive / cases studied); ND = not determined. *This patient has been included in a previous publication by Mayorga *et. al* [16].

Syndrome	Gene	DNA/protein variant	State	MMS
MELAS (11)	*MT-TL1*	m.3243A > G (10)	Heteroplasmic	7/7
	MT-ND5	m.13513G > A,(1) p. Asp393Asn	Heteroplasmic	0/1
Leigh syndrome (5)	*MT-ATP6*	m.8993 T > G (1*) p. Leu156Arg	Homoplasmic	1/1
			Homoplasmic	1/1
		m.8993 T > C (1) p.Leu156Pro	Heteroplasmic	0/1
		m.9185 T > C (1) p. Leu220Pro		
	MT-ND6	m.14459G > A (2) p. Ala72Val	Homoplasmic	ND
SKS (3)	*LSR*	"common" deletion (3) m.8470_13446del	Heteroplasmic	ND
CPEO (2)	*LSR*	m.7996_15056del (1) m.7488_14414del (1)	Heteroplasmic	ND
LHON (2)	*MT-ND4*	m.11778 G > A (2) p. Arg340His	Homoplasmic	ND
MERRF (1)	*MT-TK*	m.8344 A > G	Heteroplasmic	1/1
Reversible Myopathy (3)	*MT-TE*	m.14674 T > C (3)	Homoplasmic	1/1

A > G in the *MT-TK* gene both in the patient and in his asymptomatic mother. His older brother had died at the age of 5 due to a neurological disorder without known etiology.

3.7. Reversible myopathy with COX-negative fibers (OMIM #500009)

Two siblings and their maternal cousin presented with myopathy of variable severity. The index case was a girl who started at 7 days of age with repetitive vomiting and failure to thrive, and then developed profound hypotonia and generalized muscle weakness requiring tracheostomy. At six-month-old she suffered a cardiopulmonary arrest developing a hypoxic encephalopathy. At 2 years old she died during an infectious disease. Laboratory studies showed increased plasmatic levels of lactic acid and CK, and the brain CT scan presented bilateral basal ganglia calcifications. Her muscle biopsy showed vacuolar changes, fibers with increased Gomori staining, and increased oxidative activity with SDH. The electron microscopy detected fibers with increased mitochondrial accumulations, dense inclusions and alteration of the crest networks associated with lipid-like vacuoles. The youngest brother presented with acute generalized muscle weakness, and hypotonia associated to swallowing difficulties at 4 months of age. He showed increased CK and lactic acidosis during acute stage and improved spontaneously after a 3-month period. Brain CT scan did not show any remarkable finding. The cousin presented with muscle weakness and hypotonia associated to respiratory involvement. This girl showed a progressive clinical improvement. The homoplasmic pathogenic variant m.14674 T > C in *MT-TE* gene was found in all 3 cases.

4. Discussion

In the present study we report the clinical and molecular characterization of mitochondrial DNA disorders in a group of pediatric patients from Argentina. The use of classical molecular methods to analyze common mtDNA abnormalities has led to a genetic diagnosis in 27 of 89 pediatric patients with suspected MD. This represents 30% of the analyzed cases, a diagnostic yield similar to previously reported pediatric series, in which testing for common mtDNA defects has shown positive results in a range from 5 to 25% of the cases [17].

The median age at disease onset was 4 years, similar to previous data from MD patients harboring pathogenic variants in the mtDNA [18]. Less than 30% of our patients were younger than 18 months at onset of symptoms, mostly patients with LS, MELAS and reversible myopathy. Even though several authors have reported that more than 50% of MD cases start before 18 months [19], it is important to consider that this percentage is calculated taking together nDNA and mtDNA variants.

The clinical spectrum observed in this series of patients was broad, from cases with symptoms limited to a specific system (LHON or CPEO patients), to others with severe multisystemic impairment (LS and MELAS patients). All of them presented with a classic mitochondrial syndrome, which is in accordance with the fact that all children included presented a pathogenic variant in the mtDNA. MELAS syndrome was the most frequent phenotype, representing 41% of the cases, and LS only accounted for 18%, in contrast to literature that described LS as being the most common phenotype in children. This is probably due to the fact that only those LS patients harboring mtDNA variants were included in this study. Most of the LS patients are reported to be associated with nDNA variants [20], being mtDNA variants found in only 10–20% of cases [21]. Although all the patients presented with classical phenotypes, some cases were atypical, such as the family with reversible COX-deficient myopathy, whose index case presented a severe phenotype and early death, similar to previous reported cases [22], while the rest of the family members showed a typical clinical phenotype. The other unusual presentation was the LS patient associated to the variant m.8993 T > C. Even though this variant is quite frequent, this patient presented with episodic ataxia and lactic acidosis, which are not that common in children but is similar to the presentation shown by an adult patient, reported before [23].

Neurological manifestations were present in all the patients, either at presentation or during disease evolution, similar to previous studies that described neurological symptoms as the most frequent finding in MD [19,24,25]. Particularly, development delay or regression, epilepsy, and neuromuscular involvement were observed in almost half of the patients. Failure to thrive (51%) and hearing loss (44%) were the most frequent systemic manifestations observed. It has been documented that the presence of pathogenic variants in the mitochondrial tRNA genes is associated with hearing loss [26,27], being the heteroplasmic m.3243A > G variant the most frequent genetic defect causing syndromic hearing loss [27]. This fact probably explains the high percentage of patients with hearing loss detected in this series. Otherwise, ophthalmologic (18%), cardiac (14%) and gastrointestinal (4%) involvement were lower than previously reported [19].

The most common biochemical features found in these patients were elevated lactate levels in plasma and urine, and intermediates of Krebs cycle, which were present in around 70% of the cases. Hyperlactatemia is the most frequent biochemical abnormality present in around 70% of the patients with MD [19], being a helpful diagnostic biomarker. However, many patients showed increased lactate levels only during metabolic crisis or after exercise. On the other hand, the specificity is low since a wide variety of genetic and environmental conditions can lead to increased lactate values [28,29]. Curiously, lactic acid was elevated only in 40% of the LS patients, but those who had normal plasma levels presented increased values on CSF or urine. CSF lactic acid was measured in four cases, and it was increased in all, suggesting that CSF lactate values may therefore be a more reliable diagnostic marker than venous lactate in patients with neurological symptoms. Plasma amino acids profile was abnormal in 44% of the cases, being high levels of alanine the most frequent finding.

Typical neuroradiological lesions described in MD were found in 72% of the cases, being the most common findings stroke-like images and basal ganglia lesions. Interestingly, the MELAS patient with the m.13513 G > A variant showed an atypical pattern with SLLs involving cerebellar hemispheres. SLLs in MELAS are usually found in the occipital, temporal and parietal lobes [30], but are rarely observed in the cerebellum and they are known as “non-classic SLLs” [31–34]. Lactate peak on brain MRS was present in 4 out of 6 patients. Three LS patients showed lactate peak even in the absence of systemic lactic acidosis, as it was described before [35].

Muscle biopsy was performed in 37% of the patients, and the most frequent histopathological finding was COX reduction/COX negative fibers as it was reported previously [18], followed by the presence of RRF. Electron microscopy was completed only in one patient with reversible myopathy, showing a distinguished pattern characterized by increased and abnormal mitochondria with lipids and vacuolar changes, similar to what has been previously described for this syndrome [36].

Regarding molecular findings, we found that 81% of the cases presented with single point variants, and almost 65% of them involved mt-tRNA genes. It is well known that point variants within mt-tRNA genes represent the most common group of mtDNA variants to cause human mitochondrial disease [37]. The remaining 19% of the patients presented with large scale-rearrangements, a higher percentage compared with other authors who found that approximately 10% of patients with mtDNA defects presented with large scale deletions [38].

The most frequent pathogenic variant was the A-to-G transition at position 3243 in the *MT-TL1* gene which encodes for the tRNALeu (UUR), all related to MELAS syndrome, but with a heterogeneous clinical presentation and severity, which could be related to different levels of heteroplasmy. The load of this pathogenic variant is, at least in part, responsible for the diverse clinical manifestations of many mitochondrial disorders caused by mtDNA variants [8,39]. On the other hand, variants in different mtDNA genes can present with the same clinical phenotypes, as the case of the MELAS with the m.13513G > A variant in *MT-ND5* gene.

The second most frequent mtDNA pathogenic variants were those found in *MT-ATP6* gene, in 3 patients with LS with different clinical manifestations and severity. Variants in this gene account for approximately 10% of LS cases [40], being the most frequent the m.8993 T > G y m.8993 T > C variants [41]. The two LS cases with those variants showed a phenotype/genotype correlation that was in agreement with previous reports that described m.8993 T > C as a less severe variant than m.8993 T > G [42]. In contrast, the missense m.9185 T > C pathogenic variant detected in one patient has been less frequently reported, accounting for approximately 9% of *MT-ATP6* pathogenic variants according to recent publications [43], and has been generally associated with a late-onset maternally inherited LS [44,45]. However, Takada et al. published a case report of a 3-month-old girl with an apparently *de novo* m.9185 T > C homoplasmic variant, whose clinical manifestations and imaging were similar to those observed in our patient [46]. Two siblings with LS had a m.14459G > A missense pathogenic variant in the *MT-ND6* gene. Even though this variant was first associated with LHON [47,48], several cases of LS have been described harboring this variant in homoplasmy with no signs of optic atrophy [49,50].

Single large-scale deletions of mtDNA were observed in 5 patients from our cohort. The 3 patients with KSS harbored the so-called "common" deletion, while the 2 girls with CPEO showed larger deletions. In this small group of patients, we did not find a relationship between the course and severity of the disease and the size of the deletion, in line with the early work of Rotig et al. [51]. Several reviews exposed that the clinical heterogeneity associated with mtDNA deletions relates to a wide clinical spectrum and not only depends on the size of the deletion and the genes that are lost, but also relies on the levels of heteroplasmy in different tissues and the changes of these levels over time [52,53].

Maternal segregation of genetic variants was studied in 12 cases, and we found that 90% of these cases were inherited. Two cases were apparently *de novo*, the MELAS patient harboring the m.13513G > A variant, in concordance with Kirby et al. in 2003 [54], and the variant m.9185 T > C in one LS patient, as it was described by Takara et al. All the mothers with the m.3243G > A mtDNA variant in heteroplasmic state presented a wide spectrum of symptoms. In contrast, the mothers harboring any of the other single nucleotide variants were asymptomatic.

In this series, the identification of a classical phenotype, in most of the cases, eased the molecular diagnosis through one or two simple genetic tests, showing that a carefully clinical characterization is important to guide the molecular studies, especially in syndromic cases. A reasonable diagnostic algorithm, particularly for those centers from low- or middle-income countries, should include the study of "common" point mtDNA variants and large deletions as a first step. As heteroplasmic variants can have a variable level among tissues and blood levels might decrease over time, it may be necessary in some cases test other affected tissues. For those patients with negative results, next-generation sequencing (NGS) methodologies may improve the reliability and sensitivity of mtDNA genome analyses for low level heteroplasmic point variants and deletions.

5. Conclusions

This study highlights the clinical and genetic heterogeneity of pediatric mtDNA disorders. All the cases presented with classical phenotypes, being MELAS the most frequent. Applying classical molecular methods, it was possible to achieve a genetic diagnosis in 30% of the cases, suggesting that this is an effective first approach, especially for those centers of low-middle income countries, leaving NGS studies for those patients with inconclusive results.

Conflicts of interest

All authors declare that they have no conflicts of interest.

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Author statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in Molecular Genetics and Metabolism Reports.

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Appendix A. Supplementary data

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