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ORIGINAL ARTICLE

CLINICAL EXPERIENCE OF NEUROLOGICAL MITOCHONDRIAL DISEASES IN CHILDREN AND ADULTS: A SINGLE-CENTER STUDY

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ABSTRACT

The goal of the study was to retrospectively evaluate a cohort of children and adults with mitochondrial diseases (MDs) in a single-center experience. Neurological clinical examination, brain magnetic resonance imaging (MRI) and spectroscopy, muscle biopsy, metabolic and molecular-genetic analysis were evaluated in 26 children and 36 adult patients with MD in Slovenia from 2004 to 2018. Nijmegen MD criteria (MDC) were applied to all patients and the need for a muscle biopsy was estimated. Exome-sequencing was used in half of the patients. Twenty children (77.0%) and 12 adults (35.0%) scored a total of ≥8 on MDC, a result that is compatible with the diagnosis of definite MD. Yield of exome-sequencing was 7/22 (31.0%), but the method was not applied systematically in all patients from the beginning of diagnostics. Brain MRI morphological changes, which can be an imaging clue for the diagnosis of MD, were found in 17/24 children (71.0%). In 7/26 (29.0%) children, and in 20/30 (67.0%) adults, abnormal mitochondria were found on electron microscopy (EM) and ragged-red fibers were found in 16/30 (53.0%) adults. Respiratory chain enzymes (RCEs) and/or pyruvate dehydrogenase complex (PDHc) activities were abnormal in all the children and six adult cases. First, our data revealed that MDC was useful in the clinical diagnosis of MD, and second, until the use of NGS methods, extensive, laborious and invasive diagnostic procedures were performed to reach a final diagnosis. In patients with suspected MD, there is a need to prioritize molecular diagnosis with the more modern next-generation sequencing (NGS) method.

Keywords: Exome-sequencing; Magnetic resonance imaging (MRI); Mitochondrial disease (MD); Nijmegen mitochondrial disease criteria (MDC); Muscle biopsy.

INTRODUCTION

It is still a challange to diagnose a primary mitochondrial disease (MD) in a clinical setting. In general, MDs are characterized as a dysfunction of oxidative phosphorylation (OXPHOS) and/or enzymes involved in the mitochondrial role in the oxidation of sugars, amino acids and fats to water and carbon dioxide. Primary MD is a genetic and/or a biochemical defect of OXPHOS. Mitochondrial diseases are the most commonly inherited metabolic diseases in neurology, both in children and in adults. The minimum prevalence rate for mitochondrial DNA (mtDNA), mutations was one in 5000 (20 per 100,000) and for nuclear mutations was 2.9 per 100,000 adults in Northeast England [1]. Childhood MD prevalence has been estimated at 3.0-6.2 per 100,000 with a point prevalence in Sweden of mitochondrial encephalomyopathies in children of 4.8 per 100,000 [2]. The clinical, biochemical and genetic heterogeneity is shaping the right diagnostic approach, one which is changing rapidly with next-generation sequencing (NGS) methods [3].

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Mitochondria is now recognized as able to perform multiple essential functions involved in cellular homeostasis and cellular energetics [4]. Oxidative phosphorylation is a very sophisticated mitochondrial mechanism being able to produce chemical energy in a form of adenosine triphosphate (ATP). Glucose, glutamin and branched chain amino acids are the main micronutrients that are the substrates of the chain of reactions, where a pool of acetyl coenzyme A (acetyl-CoA) and Krebs cycle intermediates in a mitochondrial matrix is filled by three key-enzymes: pyruvate dehydrogenase complex, alpha-ketoglutarate dehydrogenase complex and branched chain alpha-keto acid dehydrogenase complex with a thiamine, flavin adenine dinucleotide (FAD) and Mg^{2+} as essential cofactors in all three enzymatic complexes. The Krebs cycle produces reduced equivalents in a form of $NADH_2$ and $FADH_2$, which push the respiratory chain enzymes to create a proton gradient that promotes ATP synthesis. Mitopathogenic mechanisms are recognized in a number of medical disciplines [5].

A muscle biopsy has been the gold standard used to set a diagnosis of primary MD in the pre genomic era. Nevertheless, the procedure has been invasive, costly, and usually requires general anesthesia in children. In order to evaluate the probability of underlying MD according to scoring of the clinical picture, Nijmegen MD Criteria (MDC) was developed [6,7].

Recent reanalysis of the MDC revealed that they are still useful in the clinical diagnosis of MD, both in children and adults, interpreting whole-exome results, as well as in deciding whether muscle biopsy needs to be performed [8]. In the last few years, NGS methods of leucocyte-derived DNA with yield of 40.0% have reversed the diagnostic approach from a “function to gene” to a “gene to function” approach and decreased the need for muscle biopsy [9]. Nevertheless, a muscle biopsy remains the method of choice for enabling diagnosis in selected clinical cases, where a confirmatory diagnostics is required.

In this paper, we describe the clinical characteristics and the results of imaging and laboratory investigations [muscle histology, histochemistry, electron microscopy (EM), respiratory chain enzymes (RCEs), pyruvate dehydrogenase complex (PDHc) activities, and/or molecular genetics] in a cohort of Slovenian neurological mitochondrial patients, of whom 26 are children and 36 are adults. Use of MDC, new NGS methods and repositioning of a muscle biopsy in the genomic era is emphasized. The study outlines the clinical experience of neurological mitochondrial diseases in Slovenia, a European country of 2 million people. It is important to improve early recognition and noninvasive diagnostics of MDs due to its devastating character and current unsatisfactory treatment options.

MATERIALS AND METHODS

Clinical data (*i.e.,* clinical, metabolic, electrophysiological, muscle biopsy and brain imaging data) of the children and adult patients with MDs have been summarized and analyzed. Data have been collected retrospectively analyzing the medical records of patients from 2000-2018, performing neurological examination and taking medical histories of all patients by the first author.

Cohort Analysis. The cohort of children with MDs comprised 26 patients, who manifested significantly decreased activity of RCEs and/or PDHc in the skeletal muscle with less than 50.0% enzyme activity [10]. Metabolic evaluations (serum lactate and pyruvate, blood gas analysis, amino acids in plasma, organic acids in urine, and acyl-carnitine profile in plasma) were performed in all children; cerebral spinal fluid (CSF) analysis with lactate measurements was assessed in eight children, electromyography was performed in 14 children, and brain magnetic resonance imaging (MRI) was performed in 24 children. Activities of RCEs and PDHc as well as molecular analysis for the thymidine kinase 2 *TK2* gene and synthesis of cytochrome C oxidase 2 (*SCO2*) gene mutations were investigated as described previously [11,12]. Additional molecular analysis of mtDNA by polymerase chain reaction (PCR) and Southern blotting techniques in patients with RCEs deficiencies was investigated and included testing for large scale deletions/duplications and for point mutations related to the classical MDs, including mitochondrial encephalopathy lactic acidosis stroke-like episode (MELAS) syndrome mt.3242A>G and mt.3271T>C; myoclonic epilepsy and ragged-red fiber (MERRF) syndrome, mt.8344A>G and mt.8356T>C; neuropathy ataxia retinitis pigmentosa (NARP) syndrome mt.8993T>G and mt.8993T>C; Leigh syndrome mt.9176T>C, and in cardiomyopathy mt.8363G>A. Molecular analysis in patients with PDHc deficiency included testing for the E1α subunit gene *PDHA* and *PDX* (E3 binding protein subunit) gene mutations. The clinical exome sequencing of leucocytes-derived DNA has been performed in six children.

The cohort of adults with MDs comprised 36 patients who had a clinical picture of a classical mitochondrial syndrome, significantly reduced OXPHOS activity in the skeletal muscle with less than 50.0% enzyme activity [10] and/or a positive mutational analysis in a mtDNA or nuclear mitochondrial genes. The age range of patients was 23 to 79 years old. Most of the patients presented with a neuromuscular anomaly, and therefore, electromyography was performed in 31 patients and a muscle biopsy in 30 patients. Thirty-two patients presented as having a multisystem disease, but brain MRI was performed in only eight patients. Respiratory chain enzyme activities in a muscle

were investigated in 14 patients, and additional sequencing of the mitochondrial genome was performed in 28 patients (mtDNA was isolated from the muscle specimen in 13 patients and from a buccal swab in 15 patients). Large scale deletion/duplication analysis of the mitochondrial genome isolated from the muscle specimen was done in 13 patients by the Southern blotting method. Finally, with the adult group, the clinical exome sequencing of leucocytes-derived DNA was performed in 19 patients.

Clinical examination and tests of all patients, children and adults, were scored according to the Nijmegen MDC [6-8]. The mean clinical score, metabolic and brain imaging score, and morphology score, were estimated and the probability of an MD diagnosis was determined. With the addition of the results from RCEs deficiencies and molecular genetic testing, Twenty of 26 children and 12/36 adults were scored as being definite MD carriers. The follow-up period ranged from 15 months to 18 years in children, and 1 to 15 years in adults.

Clinical-Exome Sequencing (CES). Clinical-exome sequencing was performed using target capture from Trusight One (Illumina Inc., San Diego, CA, USA) targeting 12 Mb of exonic coding sequences in 4.813 Mendelian disease-associated genes. Sequencing was performed on HiSeq 2500 in 2 × 100 reads paired-end sequencing mode (Illumina Inc.). Sequencing data was processed using an in-house analysis pipeline, based on the Burrows-Wheeler aligner (BWA) (https://sourceforge.net) pipeline. Briefly, after the alignment of reads to hg19 human reference assembly using the BWA, duplicate sequences were removed using MarkDuplicates (Picard)-GATK (https://gatk.broad insti tute. org/), followed by base quality score recalibration, variant calling, variant quality score recalibration and variant filtering using elements of the GATK software [13].

Variant Analysis. Variants were stored and annotated using volunteer tools (vtools) and ANNOVAR (http:// anno var.openbioinformatics.org) [14,15]. The refseq gene models were used for transcript positioning of variants, and annotations from the dbSNP (single nucleotide database), version 138 were used for SNP annotation. We used in-house background population variant frequency estimates based on compilation of 2000 Slovenian exomes. Frequency information for worldwide populations was based on the data from GnomAD project (https:// gnomad.broad institute.com). Pre computed pathogenicity predictions in the dbNSFP version 2 database were used for evaluation of pathogenicity for missense variants [16]. Additionally, we used SNPeff predictors for additional annotation of variant effect [17]. Evolutionary conservation rates of the variant sites was based on genomic evolutionary rate profiling (GERP++) rejected substation (RS) scores [18]. We also used ClinVar (https://www.ncbi. nlm.nih.gov/clinvar/) as the source for known variants, associated with human disease [19].

Variant Filtration Strategy. Variants were filtered according to autosomal dominant, autosomal recessive and X-linked models of inheritance. We filtered out general variation attaining frequency above 0.01% in any of the surveyed populations for the dominant model and filtered out variants exceeding 0.1% for the autosomal recessive and X-linked models. All the variants passing these filters were subsequently inspected at aligned read level with the aim of avoiding false call due to misalignment or low-depth of coverage. Genes, previously associated with MDs were investigated in a targeted manner.

Magnetic Resonance Imaging Analysis. Of twenty children with a diagnosis of a definite MD (either RCEs deficiency or PDHc deficiency), additional brain MRI and magnetic resonance spectroscopy (MRS), under general anesthesia, were performed in 10 children. Progression of brain morphological MRI changes and metabolic changes in basal ganglia and white matter by MRS were also evaluated. Magnetic resonance imaging investigations were performed on 3T (Siemens AG, Munich, Germany) and 1.5T GE MR machine. Standard MR sequences were performed for structural brain imaging: 3DT1 sequence (TE/ TR = 6 ms/30 ms), T2 FSE sequence (TE/TR = 98.16 ms/4000 ms), fluid attenuated inversion recovery (FLAIR) sequence (TE/TR = 133 ms/8777 ms) and diffusion-weighted imaging (DWI) sequence (TE/TR = 134.8 ms/10000 ms). On the 3T MR machine, we also performed multi-voxel MR spectroscopy at TE 35 ms and TE 124 ms (TE/TR = 35 ms/1500 ms and TE/TR = 35 ms/1500 ms), and on the 1.5T MR machine we performed single-voxel MR spectroscopy at TE 35 ms and TE 144 ms (TE/TR = 35 ms/1500 ms and TE/TR = 144 ms/1500 ms) in the region of basal ganglia and deep white matter of the brain.

RESULTS

The main clinical charasteristics of the patients in the childrens' cohort is listed in Table 1, and those of patients in an adult cohort is listed in Table 2. The MDC were applied to all children and adults in the study. Clinical comparison between both cohorts of patients (*i.e.,* children and adults), was also performed and the data are presented in Table 3.

In a cohort of children, RCEs and/or PDHc deficiencies were found in all subjects. Twelve children had a PDHc deficiency. Molecular genetic analysis of the *PDHA* and *PDX* genes did not reveal a mutation in any child. Five children had Leigh syndrome, 10 children had RCE deficiencies, and four children had combined PDHc and RCE deficiencies. Diagnostic evaluation in these children

Table 1. Clinical characteristics in our cohort of children with mitochondrial diseases.

Clinical Characteristics	*n* (%)
Number of patients	26
males	12 (46.0)
females	14 (54.0)
Age:	
range	0.5-18.5
average±SD	7.3±5.1
Age at diagnosis of MD:	
neonatal presentation	10 (39.0)
>2 years	11 (42.0)
<2 years	5 (19.0)
First clinical symptoms:	
severe central hypotonia	8 (31.0)
psychomotor regression	9 (35.0)
exercise intolerance	5 (19.0)
seizures	4 (15.0
Nijmegen MD clinical criteria:	
unlikely	0 (0.0)
possible	1 (4.0)
probable	5 (19.0)
definite MD	20 (77.0)
Clinical mitochondrial diagnosis:	
Leigh or Leigh-like syndrome	5 (19.0)
unspecific encephalomyopathy	16 (62.0)
myopathy	5 (19.0)

MD: mitochondrial disease; Nijmegen MDC: mitochondrial disease criteria.

Table 2. Clinical characteristic in our cohort of adult mitochondrial patients.

Clinical Characteristics	*n* (%)
Number of patients:	36
males	21 (60.0)
females	15 (40.0)
Age:	
range	23-79
average±SD	58±13
Age at diagnosis of MD:	
<18 years	16 (46.0)
18-45 years	9 (26.0)
>45 years	10 (28.0)
First clinical symptoms:	
ptosis	14 (40.0)
exercise intolerance	15 (43.0)
developmental delay	2 (5.0)
stroke	1 (3.0)
vision deterioration	1 (3.0)
rapidly progressive dementia	1 (3.0)
epilepsy	1 (3.0)
Nijmegen MD clinical criteria:	
unlikely	0 (0.0)
possible	8 (23.0)
probable	15 (43.0)
definite MD	12 (34.0)
Clinical mitochondrial diagnosis:	
CPEO	8 (23.0)
CPEO plus	8 (23.0)
MELAS	2 (5.0)
MERRF	3 (9.0)
MNGIE	1 (3.0)
mitochondrial myopathy	7 (20.0)
unspecific encephalomyopathy	5 (14.0)
adult Leigh syndrome	1 (3.0)

CPEO: chronic progressive external ophthalmoplegia; MELAS: mitrochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; MERRF: myoclonic epilepsy with ragged-red fibers; MNGIE: mitochondrial neugastrointestinal encephalopathy.

is presented in Table 4, and results of molecular genetic testing are available in Table 5.

In a cohort of adults the main clinical presentation of MD was chronic progressive external ophthalmoplegioa (CPEO) or CPEO+, where a clinical diagnosis with musculus levator palpebrae or musculus orbicularis oculi biopsy and EM in 16 patients. Classical mitochondrial syndromes (CPEO, CPEO plus, MELAS, MERRF, MNGIE) were found in 22 patients (Table 2). Twelve adults were found to carry a diagnosis of definite MD, and 15 adults were found to carry a probable MD. A muscle biopsy with histochemical analysis has been performed in 30 patients, but RCEs were measured in only 14 patients. Diagnostic evaluation of these adult patients is presented in Table 6, and results of molecular genetic testing are available in Table 5.

Twenty children were scored according to MDC as a definite MD, five children as a probable MD and one

Table 3. Comparison of mitochondrial diseases between children and adults in our cohort of patients.

Parameters	Children (<18 years) *n* = 26	Adults *n* = 36
Main clinical signs	encephalomyopaathy	neuromuscular
Serum lactate measurements	increased in 80.0%	increased in 14.0%
Changes in brain imaging	common	rare
Muscle histology	less informative	more informative
Genetic analysis	nuclear defects	mtDNA defects
Metabolic decompensation	more common	almost never
MD syndrome suspected	rarely	common
Multisystemic signs	common	common

mtDNA: mitochondrial DNA; MD: mitochondrial disease.

Table 4. Diagnostic investigations in our cohort of children with mitochondrial diseases.

Diagnostic Investigations	***n* (%)**
Number of patients	26
Laboratory:	
increased lactate serum (>2.2 mmol/L)	20 (77.0)
increased pyruvate serum (>80.0 nmol/L)	14 (54.0)
increased lactate in CSF (>1.8 mmol/L)	5 (19.0)
abnormal amino acids in plasma	1 (4.0)
abnormal organic acids in urine	7 (27.0)
abnormal acyl-carnitine profile	0 (0.0)
Electrophysiology:	
EMG:	14
myopathic	3 (22.0)
neuropathic	2 (14.0)
normal	9 (64.0)
EEG:	16
focal	4 (25.0)
multifocal	3 (19.0)
hypsarrhythmia	1 (6.0)
3T MRI imaging:	24
Leigh or Leigh-like syndrome	5 (21.0)
cerebral atrophy	7 (29.0)
dysmielination	8 (33.0)
neuronal migration disorder	6 (25.0)
cerebellar hypoplasia	2 (8.0)
ischemic lesion	1 (4.0)
normal	4 (16.0)
Muscle biopsy:	26
COX negative fibers	7 (27.0)
ragged-red fibers	0 (0.0)
abnormal mitochondria on EM	7 (27.0)
normal results	1 (4.0)
OXPHOS and PDHc biochemical	26
measurements:	12 (46.0)
PDHc deficiency	6 (23.0)
single OXPHOS deficiency	4 (15.0)
combined OXPHOS deficiency	4 (15)
combine PDHc and OXPHOS deficiency	

CSF: cerebralspinal fluid; EMG: electromyography; EEG: encephalomyography; COX: cytochrome oxidase; EM: electron microscopy; OXPHOS: oxidative phosphoryla-tion; PDHc: pyruvate deydrogenase complex.

Table 5. Results of molecular genetic analysis in both our cohorts of mitochondrial patients.

Molecular Genetic Analysis	***n* (%)**
Number of children analyzed	26 (100.0)
Molecular genetic methods:	
nuclear DNA muscle (Sanger)	3 (12.0)
nuclear and mtDNA blood (CES, PCR, Sanger	23 (88.0)
Results:	
TK2 p.Ala181Val, OMIM #609560	3
SCO2 p.Glu140Lys, OMIM #604377	1
MTFMT p.Ser209Leu, OMIM #614947	1
SCL16A2 p.Gly327Arg, OMIM #300523	2
CHAT p.Thr354Met, p.Ser694Cys, OMIM #254210	1
CES normal	3/6
E1α PDHc mutation analysis negative	12/12
common mtDNA mutation analysis negative	23/26
Number of adults analyzed	27 (77.0)
Molecular genetic methods:	
mtDNA muscle (PCR, Southern blotting)	13 (48.0)
mtDNA buccal swab (NGS)	15 (56.0)
nuclear DNA blood (CES)	19 (70.0)
Results:	
mtDNA point mutation:	4 (15.0)
mt.12213G>A	1
mt.8344A>G, OMIM #545000	3
mtDNA large deletion	4 (15.0)
mtDNA VUS	2 (7.0)
nuclear defects	3 (11.0)
C10orf1 p.Arg374Gln, OMIM #609286	1
SLC25A4 p.Ala123Asp, OMIM #615418	1
SURF1 p.Ser282fs, OMIM #256000	1
nuclear DNA VUS	3 (11.0)

CES: clinical-exome sequencing; PCR: polymerase chain reaction; Sanger: Sanger sequencing; PDHc: pyruvate dehydrogenase complex; VUS: variant of unknown significance.

child as a possible MD. The mean clinical score (I) was 3.7 (range 2-4), with a maximum score of 4 [including multi-system involvement taken alone, as part of the clinical score, had a mean score of 1.0 (range 0-2)]. The metabolic and brain imaging score (II) was 3.0 (range 0-4), and the histology score (III) was 1.8 (range 0-4). Before muscle biopsy, the mean MDC score (I+II) was 6.6 (±1.4 SD), and the final score (I+II+III) was 8.4 (±1.8 SD). Twenty children (77.0%) scored a total (I+II+III) of ≥8, a result that is comparable with the diagnosis of a definite MD.

Twelve adults were scored according to MDC as a definite MD, 15 adults as a probable, and eight adults as a possible MD. The mean clinical score (I) was 3.5 (range 2-4), with a maximum score of 4 [including multisystem involvement, which taken alone, had a mean score of 1.4 (range 0-3)]. The metabolic and brain imaging score (II) was 0.4 (range 0-3), and the histology score (III) was 2.6 (range 0-4). Before muscle biopsy, the mean MDC score (I+II) was 3.8 (±1.2), and the final score (I+II+III) was 6.5 (± 2.0). Twelve adults (35.0%) scored a total (I+II+III) of ≥8, a result that is comparable with the diagnosis of a definite MD. If we also include in this group of patients the patients with CPEO who were not genetically confirmed, the number of definite MD patients would be increased to 22 (63.0%).

Of 24 children evaluated with brain MRI imaging, five children (20%) had MRI signs of Leigh syndrome. Morphological brain MRI changes, which are not classified in MDC, but might be in accordance with the diagnosis of an MD, have been found in the following: eight (33.0%) children with delayed myelination, seven (29.0%) children with cerebral atrophy, two (8.0%) children with cerebellum changes, and in six (25.0%) children in whom neuronal migration disorders were found. In summary, brain MRI morphological changes, which can be an imaging clue for the diagnosis of MD, were found in 17/24 (71.0%) children.

Table 6. Diagnostic investigations in our cohort of adult mitochondrial patients.

Diagnostic Investigations in Our Cohort	***n* (%)**
Laboratory:	
increased lactate in serum	5 (14.0)
positive ischemic test	0
amino acids in plasma/organic acids in urine	0
CSF analysis	0
Electrophysiology:	
EMG:	31
myopathic	19 (61.0)
neuropathic	2 (6.0)
normal	10 (33.0)
EEG:	16
normal patterns	6 (38.0)
abnormal (slow waves or epileptic	10 (62.0)
discharges)	5
VEPs: abnormal	2
BERA: normal	
Brain imaging:	8
unspecific abnormalities on CT	3 (38.0)
unspecific abnormalities on MRI	3 (38.0)
stroke-like episodes on MRI	2 (24.0)
Muscle biopsy:	30
COX negative fibers	10 (33.0)
ragged-red fibers	16 (53.0)
blue ragged fibers	3 (10.0)
abnormal mitochondria on EM	20 (67.0)
normal results	4 (13.0)
OXPHOS biochemical measurements:	14
normal enzyme activities	8 (58.0)
single deficiency	3 (21.0)
combined deficiency	3 (21.0)

CSF: cerebralspinal fluid; EMG: electromyography; EEG: encephalomyography; VEPs: visual-evoked potentials; BERA: brainstem-evoked response audiometry; CT: computed tomography; MRI: magnetic resonance imaging; COX: cytochrome oxidase; EM: electron microscopy; OXPHOS: oxidative phosphorylation.

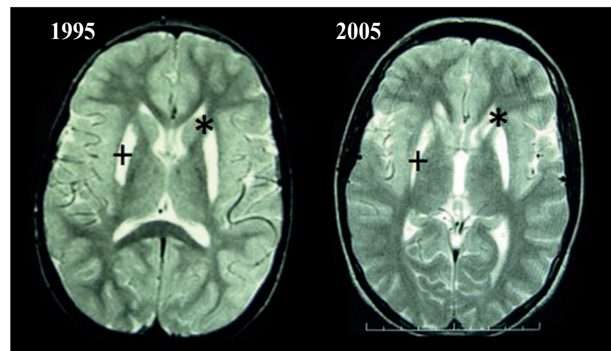

Figure 1. Progression of Leigh syndrome in an 18-year-old girl with a pathogenic homozygous mutation c.626C>T (p.Ser209Leu) on the *MTFMT* gene (OMIM #614947). *: progression of nucleus caudatus atrophy and T2-weighted hyperintensities; +: T2-weighted hyperintensities in putamen bilaterally.

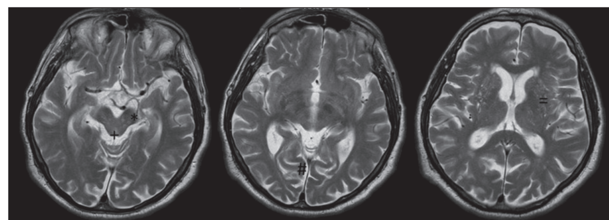

Figure 2. Brain MRI morphological changes in an adult with a pathogenic homozygous mutation p.Ser282fs on the *SURF1* gene, which is usually presented in childhood as Leigh syndrome (OMIM #256000). *: round T2-weighted hyperintensities in cerebral peduncles; +: T2-weighted periaqueductal hyperintensities; #: significant cerebral atrophy in occipital lobes. =: dilatations of Virchow-Robin spaces in basal ganglia bilaterally.

Variability of results of the MRS spectrum was large in children with MD. No statistically significant difference at relaxation time TE 35 ms was found in N-acetyl aspartate, myoinositol and cholin ratios between basal ganglia and white matter between children with MD and children in a control group. Lactate peak was found in one child with Leigh syndrome and complex I plus IV deficiency at 1.33 ppm. However, statistically significant metabolic differences were found between basal ganglia and white matter in a healthy control group, leading us to conclude that the MRS spectrum was well performed.

Follow-up of this group of children with brain MRI has been important to show the "normalization" of myelination in these children after several years of active disease, to evaluate the progression of morphological changes in Leigh syndrome, and revealed new neuronal migration disorders previously not found on brain MRI images (Table 7). Figure 1 demonstrates the progression of Leigh syndrome in an 18-year-old girl with a pathogenic homozygous mutation c.626C>T (p.Ser209Leu) on the *MTFMT* gene (OMIM #614947).

We concluded this phase of the study with a comparison of the clinical characteristics of MD in children and adults (Table 3). Mitochondrial diseases in children have revealed a chronic course with metabolic decompensation with increased serum lactate and often negative genetic analysis. However, MDs in adults have also presented a slow but chronic progression over the years and this has been more often related to mitochondrial genome rearrangements with normal serum lactate. Nuclear mitochondrial gene mutations can have a different clinical presentation depending on whether patients are children or adults. Figure 2 demonstrates brain MRI changes in an adult with a pathogenic homozygous mutation p.Ser282fs on the *SURF1* gene, a pathogenic variant that usually presents in childhood as Leigh syndrome (OMIM #256000).

Finally, we analyzed positive results of molecular genetic analysis methods for different groups of patients

Table 7. Brain magnetic resonance imaging morphological changes in children with mitochondrial encephalomyopathies at follow-up.

Case	Enzyme Deficiency	Age of First MRI (diagnostic)	First Brain MRI Results	Age of Last MRI (follow-up in years)	Last Brain MRI Results
1	PDHc	–	No data	11	Normal
2	PDHc	–	No data	17	Normal
3	complex IV	6 months	Cerebral atrophy F-T; delayed myelination; nucleus caudatus atrophy; callosal hypogenesis	5	Progression of nucleus caudatus atrophy; dysmyelination; cerbral atrophy F-T; callosal hypogenesis
4	complex II+PDHc	1 year	Delayed myelination; callosal hypoplasia; bilateral polymicrogyria of operculum	3	Normal myelination; callosal hypoplasia; bilateral polymicrogyria of operculum
5	PDHc	1 year	Cerebral atrophy F-T; delayed myelination; bilateral GM heterotopia along LV	4	Less cerebral atrophy; normal mye-lination; bilateral GM heterotopia along LV
6	PDHc	–	Lost data	7	Mild cerebral atrophy F-T; callosal hypoplasia; atrophy of cerebellar padunculi (wide 4th ventricle)
7	complex I+IV	4 years	Leigh syndrome	18	Leigh syndrome-progression; nucleus caudatus atrophy; bilateral high T2 signal in putamen and mesencephalon
8	PDHc	–	Lost data	7	Mild cerebral atrophy
9	complex IV+PDHc	6 months	Mild cerebral atrophy F-P	7	Mild cerebral atrophy F-P

PHDc: pyruvate dehydrogenase complex; F-T: frontotemporal region; F-P: frontoparietal region; GM: brain grey matter; LV: lateral ventricle.

Table 8. Yield of different diagnostic approaches used in Nijmegen mitochondrial disease criteria and positive molecular genetic analysis results in different groups of patients (children and adults) according to the Nijmegen mitochondrial disease criteria.

Nijmegen MDC	Adults		Children	
	Average	Range	Average	Range
Clinical signs and symptoms (I)	2.9	1-4	3.7	2-4
Multisystem disease:	1.0	0-2	1.0	0-2
metabolic/imaging studies (II)	0.4	0-2	3	0-4
morphology: muscle biopsy (III)	2.6	0-4	1.8	0-4
I + II	3.2	±1.3	6.6	±1.4
I + II + III	6.0	±1.9	8.4	±1.8
Molecular genetic analysis (number of cases):	unlikely	possible	probable	definite
WES blood	0	1	1	8
mtDNA muscle	0	0	0	8
mtDNA buccal swab	0	0	0	1

Nijmegen MDC: Nijmegen mitochondrial disease criteria; WES: whole-exome sequencing; mtDNA: mitochondrial DNA.

scored by Nijmegen MDC (Table 8). Positive molecular genetic results were in the great majority (17/19) in accordance with definite diagnosis of MD. Results also showed that NGS methods are beneficial to discriminate molecular mimicry and determining a diagnosis in cases of possible and probable groups of patients with MD (Tables 5 and 8). Despite a limited number of patients with mtDNA clinical syndromes, buccal swabs for mtDNA isolation are a promising noninvasive option instead of muscle biopsies (Table 8).

DISCUSSION

Slovenia is a small European high-income country with 2.1 million inhabitants and an estimated prevalence of MD of three per 100,000 inhabitants, a finding that is in accordance with other population studies of MD prevalence [2]. Determining a diagnosis of MD is a complex algorithm due to its extremely heterogeneous character, genetic diversity and complex laboratory diagnostic methods. With the majority of our mitochondrial patients we

still have to use a laborious approach with muscle biopsies in the end. Obviously, in the genomic era, the new diagnostic algorithms will improve the diagnostic approach, and influence the development of new standards for early diagnosis and possible treatment [3,9,20].

Our study has mostly been done on unspecific encephalomyopathies in children and neuromuscular patients in adults. Thirty-one percent of cases were defined by CES in both our cohorts. Clinical-exome sequencing has been used when a pathology in the mitochondrial genome was not suspected or was excluded. In almost all of the children in the study, a diagnosis of MD was first suspected, based on the results of metabolic deviations in plasma, urine or CSF or abnormal brain MRI. In adults, an MD diagnosis has still been defined by muscle histology findings. Twenty of 30 adult patients had abnormal mitochondria on EM and 16/30 adult patients presented ragged-red fibers. Therefore, clinical knowledge, metabolic investigations, brain imaging and muscle biopsies have still been valuable in our clinical scenario until 2018. Promising potential of whole-exome sequencing (WES) as a first diagnostic option in finding mutations in genes related to mitochondrial dys-functions will probably reverse the classical diagnostic approach [21,22].

Our clinical and diagnostic patient data were put together according to MDC scoring. It has previously been demonstrated that a high MDC scores increases the likelihood of finding a genetic diagnosis by WES [23,24]. Witters *et al.* [8] found that the combined metabolic findings and imaging sub scores were higher in mitochondrial patients who were diagnosed when Sanger sequencing was used as a genetic analysis tool, and where a clinician-directed genetic analysis to a target gene was based on a clinical phenotype. The MDC scores are still found very useful in the clinical diagnosis of MDs. The MDC scores are helpful in interpreting exome-sequencing results or deciding on the need to perform a muscle biopsy [8]. The importance of MDC scoring and recognizing the patient's clinical phenotype is in accordance with what we found in our clinical data. Moreover, in our cohort, MDC score was used to conduct muscle biopsy not molecular genetic studies. Twenty of 26 children and 22/36 adults in our cohort reached a score of a definite MD. In all children, a diagnosis was confirmed by RCEs and PDHc activities measurements and when a high MDC score was reached before a muscle biopsy. Molecular genetic diagnosis was confirmed in 5/26 children. In adults a diagnosis of MD was confirmed in six patients with measurements of RCE activities, and reached a confirmation in 11 patients by molecular genetic analysis. Sixteen of 36 patients had CPEO syndrome, which is still challenging to diagnose at the genetic level [25]. The MDC score was found to be a useful tool for determining whether a muscle biopsy was used in both cohorts of patients, as well as for affirming the certainty of a diagnosis. In future, the MDC score might be also useful in using WES results to reach a diagnosis of a definite MD before performing a muscle biopsy, especially in children.

Next-generation sequencing methods are becomig the first diagnostic tool in the genomic era. Whole-genome sequencing (WGS) of blood DNA is also going to contribute as the first-line method for diagnosing a primary MD [3]. Whole-genome sequencing has overtaken WES as the preferred NGS method for DNA sequencing [26-28]. Whole-genome sequencing is better for detecting copy number variations (CNVs), single nucleotide variants, insertions/deletions, and in principle, microRNA (miRNA) variants. However, even with WES, results are not going to be so straightforward. Four scenarios of molecular results are possible for diagnoses derived from the WES approach [9]. At the very least, a skin biopsy and fibroblast-related studies will be needed to evaluate variances of unknown significance (VUS) or new candidate genes. For now, the results of CES in our cohort of patients, gave us a promising yield (positive in 7/22 or 31.0% cases) compared to a yield of 39.0-55.0% in centers abroad [23,29]. Next-generation sequencing methods can also discriminate molecular mimicry or confirm a molecular diagnosis, even in cases of probable or possible groups of MD (Table 8). Buccal swabs for mtDNA isolation are showing us a potential as a noninvasive approach to diagnose mtDNA pathogenic variations, but we had a limited number of suspected mtDNA syndrome cases (Table 8).

Large mitochondrial centers are searching for a geno-type-phenotype correlation in neuroimaging of MDs [30-32]. Mitochondrial diseases are not only commonly described as multi-systemic diseases, but also demonstrate neuroradiological changes in several functional systems of the central nervous system. Pathognomonic MRI changes for MDs can be found in the cerebral cortex, white matter, basal ganglia, cerebellum and brainstem [31]. The same is true in neuroimaging of MDs, as the meaning of pleitropy is known in genetics. For example, it is true that one pathognomonic MRI characteristic can be a result of many genes related to mitochondrial dysfunction. A genetic cause of Leigh sydnrome can also be found in 75 genes [33]. Nevertheless, through our study, we can demonstrate that in 71.0% of children with MD we have found brain MRI changes that can be a clue to a diagnosis of MD. Therefore, we can suggest that MRI is still the diagnostic tool of choice in selected cases that serves to provide at least one piece of the puzzle, particularly if we have a genetic result of VUS or negative results by WES. Magnetic resonance imaging

also demonstrates that MD has the dynamic nature of a chronic disease (Table 7) (Figure 1). Some of the brain MRI characteristics present as a degree of cortical atrophy, dysmyelination and/or basal ganglia hyperintensities can change over a period of several years of follow-up (Table 7) (Figure 1).

One of the diagnostic problems in MDs is diagnostic mimicry. Clinical presentation of MD can present as a metabolic, neurodegenerative, neuromuscular or neuroin-flammatory diseases [34,35]. When we suspect a primary MD, another molecular cause can be found and mitochondrial dysfunction is only secondary [36]. This is demonstrated by the example of two clinical cases with pathogenic variants in a gene for a congenital myasthenic syndrome and Allan-Herndon-Dudley syndrome of triiodo-thyronine resistance that have been found by CES. In these cases, even muscle morphology and OXPHOS measurements of a skeletal muscle specimen and brain MRI have been misleading. We emphasize how important WES is in the differentiation of rare neurogenetic diseases mimicking as an MD.

In conclusion, MDs have remained a very heterogeneous group of neurometabolic diseases on a clinical, biochemical and genetic level of diagnostics. The goal of being noninvasive and cost-efficient is guiding us to choose a new way of the "gene-to-function" approach, instead of the old way as the "function-to-gene" approach. Solving the diagnostic puzzle(s) in either way is still a challenging task. For clinical purposes, the MDC has been a valuable tool in the diagnosis of MDs in children and adults. Obviously, the NGS methods are likely to contribute to end many diagnostic odysseys.

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