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EFNS GUIDELINES/CME ARTICLE

EFNS guidelines on the molecular diagnosis of mitochondrial disorders

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Objectives: These European Federation of Neurological Sciences (EFNS) guidelines are designed to provide practical help for the general neurologist to make appropriate use of molecular genetics for diagnosing mitochondrial disorders (MIDs), which gain increasing attention and are more frequently diagnosed due to improved diagnostic tools.

Background: Since the publication of the first EFNS guidelines on the molecular diagnosis of inherited neurological diseases in 2001, rapid progress has been made in this field, necessitating the creation of an updated version.

Search strategy: To collect data about the molecular diagnosis of MIDs search for literature in various electronic databases, such as Cochrane library, MEDLINE, OMIM, GENETEST or Embase, were carried out and original papers, meta-analyses, review papers, and guideline recommendations were reviewed.

Results: The guidelines summarise the possibilities and limitations of molecular genetic diagnosis of MIDs and provide practical recommendations and diagnostic criteria in accordance with the EFNS Scientific Committee to guide the molecular diagnostic work-up of MIDs.

Recommendations: The proposed guidelines suggest an approach to the molecular diagnosis of MIDs in a manner accessible to general neurologists.

Objectives

These European Federation of Neurological Sciences (EFNS) guidelines on the molecular diagnosis of mitochondrial disorders (MIDs) are designed to summarise the possibilities and limitations of molecular genetic techniques and to provide diagnostic criteria for deciding, in which case a molecular diagnostic work-up is indicated.

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This is a Continuing Medical Education article, and can be found with corresponding questions on the Internet at http://www.efns.org/EFNSContinuing-Medical-Education-online.301.0.html. Certificates for correctly answering the questions will be issued by the EFNS.

Background

Since the publication of the first EFNS guidelines about the molecular diagnosis of inherited neurological diseases in 2001 [1,2], rapid progress has been made in this field, necessitating the creation of an updated version of

these guidelines, which follows the EFNS Scientific Committee recommendations for guideline papers [3].

Search strategy

To collect data about planning, conditions and performance of molecular diagnosis of MIDs, a literature search in various electronic databases, such as Cochrane library, MEDLINE, OMIM, GENETEST or Embase, was carried out and original papers, meta-analyses, review papers and guideline recommendations were reviewed.

Method for reaching consensus

Consensus about the recommendations was reached by a step-wise approach. First, task force members met at the EFNS congresses in 2007 and 2008 to discuss the preparations of the guidelines. In a second step, experts in the field of genetics of MIDs wrote a guideline proposal. In a third step, these recommendations were discussed in detail amongst all task force members until a final consensus had been reached.

Results and recommendations

Recommendations follow the criteria established by the EFNS [3], with some modifications to account for the specific nature of genetic tests. Since genetic testing is by definition the gold standard to diagnose a genetic disease, its diagnostic accuracy cannot be tested against another diagnostic method. Therefore, the level of recommendations will be based on the quality of available studies [3], which investigate the proportion of cases of a clinically defined group of patients that are explained by a specific molecular diagnostic test. As nearly all of these studies have a retrospective design and look for a specific mutation in a previously ascertained and clinically diagnosed cohort of patients, the highest achievable recommendation level will be B [3]. If only small case-series studying genotype-phenotype correlations are available, the level of recommendation will be C. If only case reports are available but experts still provided recommendations, the recommendation level is assessed as 'good practice point'.

Genetic background of mitochondrial disorders

Introduction

Primary MIDs comprise a wide range of phenotypes due to mutations in mitochondrial DNA (mtDNA) or nuclear DNA (nDNA) located genes resulting in respiratory chain (RC) or oxidative phosphorylation (OXPHOS) defects. MIDs are regarded as one of the most common groups of inherited metabolic disease. The prevalence of mtDNA point mutations that cause disease is estimated as 1/5000–10 000 and the frequency of mtDNA mutations amongst healthy subjects as 1/200 [4]. The high prevalence of MIDs urges the clinician to diagnose these disorders accurately, which is difficult in the light of highly variable and overlapping phenotypes, transmission patterns and molecular backgrounds. In the following chapters, we will shortly describe the genetic background of human MIDs and provide recommendations for a diagnostic algorithm for suspected MID.

Classification

The RC/OXPHOS pathway is conducted by two separated and partially autonomous genetic systems, the nuclear and mitochondrial genome. Identification of mutations in mtDNA or nDNA located genes provides the basis for the current classification of MIDs (Fig. 1). The first group of MIDs is due to sporadic or maternally transmitted mtDNA mutations and the second group due to sporadic or Mendelian nDNA mutations.

Disorders due to mtDNA mutations
Mutations may be either present in all mtDNA copies (homoplasmy) or only part of the mtDNA copies (heteroplasmy, coexistence of wild-type and mutated mtDNA within a mitochondrion, cell or tissue). Only if mutated mtDNA copies accumulate above a critical threshold (threshold level), which depends on age and tissue, a mutation is phenotypically expressed. This is why heteroplasmic mtDNA mutations behave as 'recessive-like' traits. However, phenotypic expression may vary according to the intrinsic pathogenicity of a mutation, its tissue distribution, the variable aerobic energy-demand of different tissues or organs and the individual genetic background. Homoplasmic mtDNA mutations usually manifest as single-organ or even single cell-type-failure, like retinal ganglion cells in Leber's hereditary optic neuropathy (LHON), which may be due to primary or secondary LHON mutations. mtDNA mutations may be either classified as large-scale rearrangements or as point mutations.

mtDNA rearrangements Large-scale mtDNA rearrangements comprise single partial mtDNA-deletions and, more rarely, partial duplications, which both are heteroplasmic. Three main phenotypes are associated with single mtDNA deletions: *Kearns-Sayre-syndrome* (KSS), *sporadic progressive external ophthalmoplegia* (PEO) and *Pearson's syndrome* (Table 1).

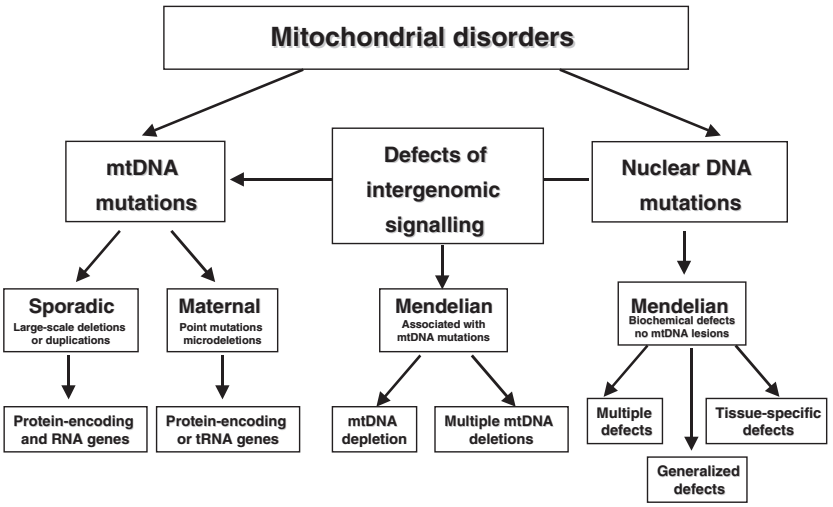

Figure 1 Classification of MIDs.

Kearns-Sayre-syndrome is a sporadic, severe MID characterised by the invariant triad of PEO, pigmentary retinopathy and onset <20 y. Frequent additional features include progressive cerebellar syndrome, dysphagia, myopathy, endocrine dysfunction (diabetes, short stature), atrio-ventricular-block, increased CSF-protein or lactacidosis. Muscle biopsy shows ragged-red fibres (RRF).

Sporadic PEO is characterised by bilateral ptosis and ophthalmoplegia and is frequently associated with muscle weakness and exercise-intolerance. Occasionally, patients present with additional signs, such as ataxia, cataract, retinitis pigmentosa, hearing loss or cardiomyopathy.

Pearson's syndrome is a rare sporadic disorder of early infancy, characterised by sideroblastic anaemia or pancytopenia and exocrine pancreatic insufficiency. Infants surviving into childhood develop features of KSS.

Mitochondrial disorders caused by mtDNA rearrangements occur in most cases sporadically, most probably during oogenesis in the patient's mother, rather during repair of damaged mtDNA than replication [5]. However, mother-to-child transmission of mtDNA duplications or deletions has been occasionally reported and may be responsible for a 5% recurrence-risk of these conditions. mtDNA rearrangements in KSS or PEO can be exclusively detected in muscle and in Pearson's syndrome exclusively in blood cells.

mtDNA point mutations Point mutations are usually maternally inherited and either affect protein-synthesis genes (tRNA, rRNA) or protein-encoding genes. Mutated protein-synthesis genes impair translation of all mtDNA-encoded polypeptides, thereby determining defects of multiple RC-complexes (RCCs). Mutated protein-encoding genes affect only the activity of the RCC, which houses the mutant protein. Despite the large number of pathogenic mtDNA mutations in humans (http://www.mitomap.org), only four of them occur frequently in various human populations: 3243A > G (MELAS), 8344A > G (MERRF), 8993T > G (NARP) and 11778A > G (LHON) (Table 1).

Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like-episodes (MELAS) syndrome is characterised by the presence of stroke-like-episodes (SLEs), lactacidosis or RRF. Additional manifestations include confusional state, dementia, psychosis, seizures, basal ganglia calcification, migraine-like headache, ataxia, optic atrophy, retinopathy, deafness, myopathy, diabetes, intestinal pseudo-obstruction or cardiomyopathy. In ~80% of the cases, MELAS-syndrome is caused by the heteroplasmic transition 3243A > G in the $tRNA^{Leu(UUR)}$ gene. The second most frequent mtDNA mutation is the transition 3271T > C. Other mtDNA genes mutated in MELAS-syndrome include the $tRNA^{Phe}$, $tRNA^{Val}$, $tRNA^{Lys}$, *COXII*, *COXIII*, *ND1*, *ND5*, *ND6* or *rRNA* genes. The genotype-phenotype correlation of the 3243A > G mutation is weak, as it also causes maternally-inherited PEO, KSS, maternally inherited diabetes and deafness (MIDD), Leigh syndrome (LS), cluster headache, isolated myopathy, cardiomyopathy, renal failure or pancreatitis. In single cases, polymerase-gamma (POLG1) mutations may cause MELAS.

Myoclonus Epilepsy with Ragged-Red Fibres (MERRF) syndrome is a maternally inherited encephalomyopathy characterised by myoclonus, epilepsy, myopathy, cerebellar ataxia, deafness and dementia. Additional features include SLEs, basal ganglia atrophy, optic atrophy, pyramidal signs, polyneuropathy,

Table 1 Mitochondrial disorders

MID syndrome	MIM	Trait	Type of mutation	Mutated gene	Characteristics	Reference
mtDNA						
Chronic progressive external ophthalmoplegia (mtPEO)	252110, 220110, 252011	Spor	mtDNA deletion, duplication	*mtDNA*	Molecular genetic analysis should be performed in muscle	[16]
Kearns-Sayre syndrome (KSS)	530000	Spor	mtDNA deletion, duplication	*mtDNA*	Molecular genetic analysis should be performed in muscle	[17]
Pearson's bone marrow-pancreas syndrome (PS)	557000	Spor	mtDNA deletion, duplication	*mtDNA*	Surviving infants may later develop KSS	[18]
Mitochondrial encephalomyo pathy with lactacidosis and 'stroke-like episodes' (MELAS)	540000	Mat	np 3243, np 3271	*mt-tRNA^{Leu}*	Other point mutations have been described in rare cases	[19]
Myoclonus epilepsy with ragged red fibres (MERRF)	545000	Mat	np 8344	*mt-tRNA^{Lys}*	Other point mutations have been described in rare cases	[20]
Leber's hereditary optic neuropathy (LHON)	535000	Mat	np 11778, np 3460, np 14484	RCCI subunits	Other point mutations have been described in rare cases	[21]
Neurogenic weakness, ataxia and retinitis pigmentosa (NARP)	551500	Mat	np 8993	*ATPase6*	High percentage of this mutation may lead to MILS	[22]
Maternally inherited Leigh syndrome (MILS)	590050	Mat	np 8993	*ND1-6, COXIII, ATPase6, tRNAs*		[23]
nDNA						
Autosomal dominant PEO (adPEO)	157640, 609283, 609286, 610131	AD	4q34–35, 10q23–24, 15q25	*ANT1, PEO1, POLG1*	Multiple deletions of mtDNA	[24–27]
Autosomal recessive PEO (arPEO)		AR	15q25	*POLG1*	Multiple deletions of mtDNA	[28]
Myo-neuro-gastro-intestinal encephalomyopathy (MNGIE)	603041	AR	22q13-qter,	*TYMP*	Multiple deletions or depletion of mtDNA	[29]
Mitochondrial depletion syndrome (DPS)	609560	AR	15q25, 2p23–p21, 4q34–35, 8q23.1, 13q12.2–q13 2p11.2, 2p13, 16q22	*POLG1, MPV17, PEO1, RRM2B, SUCLA2, SUCLG1, DGUOK, TK2*	Reduction of the mtDNA copy number	[30,31]
Leigh syndrome (LS)	256000	AR	22q13, 17p13–p12 17 p12–p11.2, 10q24, 5p15, 9q34 and others	*SCO2, SCO1, COX10, COX15 NDUFS, NDUFV, SDH, SURF1, CoQ*	Genetically extremely heterogeneous	[32]
Dominant optic atrophy (ADOA)	165500	AD	3q28	*OPA1*	Mitochondrial biogenesis	[33]

AD, autosomal dominant; AR, autosomal recessive; X, X-chromosomal; mat, maternal transmission; spor, sporadic; Pm, point mutation; Del, deletion, Ins, insertion; MIM, Mendelian inheritance in men; Note that this compilation of MIDs is not complete. The selection of MIDs was chosen at random to provide the reader with an orientation. The speed at which progress is achieved in molecular genetics allows such tables become outdated quickly. In cases of doubt, it is recommended that current publications or specialised centres be consulted. For many, if not most of the diseases listed here, mutations in other currently unknown genes may also be responsible.

cardiomyopathy, heart block, pancytopenia or lipomatosis. The most common mtDNA mutation causing MERRF-syndrome is the $tRNA^{Lys}$ transition 8344A > G. Other mutations include the transitions 8356T > C or 8363G > A. Although the genotype–phenotype correlation of the 8344A > G mutation is tighter than that of other mtDNA mutations, it also causes phenotypes as different as LS, isolated myoclonus, familial lipomatosis or myopathy.

Neurogenic weakness, Ataxia and Retinitis Pigmentosa (NARP) syndrome is a maternally inherited MID arising from the heteroplasmic 8993T > G transversion in the *ATPase6* gene. If the heteroplasmy rate is > 95%, the mutation manifests as *Maternally Inherited Leigh Syndrome (MILS)*. MILS patients present with dysmorphism, developmental delay, epilepsy, SLEs, dystonia, ophthalmoparesis, myopathy or polyneuropathy. NARP and MILS may coexist within the same family. RRF are absent on muscle biopsy (Table 1).

Leber's hereditary optic neuropathy (LHON) is characterised by bilateral, acute or subacute loss of vision due to retinal ganglia degeneration or demyelination or optic nerve atrophy. Occasionally, the heart is affected. The penetrance of LHON is ~40% in males and ~10% in females and onset is usually in the second or third decade. Although ~20 mtDNA mutations potentially cause LHON, three are most commonly found in all human populations: np11778 (*ND4*, RCCI), np3460 (*ND1*, RCCI) and np14484 (*ND6*, RCCI) and considered as high-risk (primary LHON mutations).

Several other mtDNA point mutations have been detected in single patients or pedigrees affected with a number of other syndromic and non-syndromic MIDs.

Disorders due to nDNA mutations

Mitochondrial disorders due to nDNA mutations include disorders due to: (i) mutated RC-subunits, (ii) mutated ancillary proteins, (iii) faulty intergenomic communication affecting mtDNA maintenance or expression, (iv) mutated biosynthetic enzymes for lipids or cofactors, (v) coenzyme Q-deficiency, (vi) defective mitochondrial trafficking or transport machinery, (vii) mutant proteins involved in mitochondrial biogenesis or (viii) defective apoptosis.

Mutations in RC-subunits Although 72 of 85 subunits of the RC/OXPHOS-system are nDNA-encoded, nDNA mutations have been identified only in a minority of MID-patients so far. Isolated RCCI-deficiency is the most common biochemical finding in RC/OXPHOS-disease, being present in about one-third of the cases. RCCI is composed of 45 proteins; seven of which are encoded by mtDNA and the remaining 38 by nDNA genes. Only 20% of children with RCCI-deficiency harbour mtDNA mutations in *ND* genes, suggesting that the vast majority of patients carries mutations in nDNA genes encoding for structural RCCI-subunits (*NDUFS1*, *NDUFS2*, *NDUFS3*, *NDUFS4*, *NDUFS6*, *NDUFS8*, *NDUFV1*, *NDUFV2*) or assembly factors. The majority of patients presents with variable non-syndromic MIDs with early-onset hypotonia, cardiomyopathy, ataxia, psychomotor delay or LS. Lactacidosis is only a rare finding. RCCI-deficiency may be also due to splice-site mutations in the *NDUFA11* or *NDUFA1* gene, respectively, phenotypically presenting as encephalo-cardiomyopathy with severe lactacidosis [6]. RCCII is composed of four nuclear encoded subunits. Mutations in *SDHA*, encoding for the 75 kDa flavoprotein subunit of RCCII, have been occasionally found in children with LS or late-onset neurodegenerative disease. An autosomal recessive (ar), non-lethal phenotype of severe psychomotor retardation, extrapyramidal signs, dystonia, athetosis and ataxia, mild axial hypotonia and dementia has been associated with a missense mutation in *UQCRQ*, one of 10 nDNA genes encoding RCCIII subunits. Mutations in nDNA-encoded COX-subunits are assumed to be incompatible with extra-uterine survival. However, recently, a mutation in one such subunits, COX6B1, was identified in a severe infantile encephalomyopathy [7].

Mutations in ancillary proteins Mitochondrial ancillary proteins are not part of RC complexes but involved in RCC formation, turnover and function. The most important gene of this group is *SURF1*, which encodes a COX assembly-factor. Mutations in *SURF1* manifest as LS and are associated with severe cytochrome-c-oxidase (COX)-deficiency. The SURF-1 protein is absent in all tissues of these individuals [8].

Leigh syndrome, also known as subacute necrotizing encephalomyopathy, is an early-onset, infantile progressive neurodegenerative disorder manifesting as severe psychomotor delay, cerebellar and pyramidal signs, seizures, dystonia, respiratory abnormalities, ophthalmoparesis, floppy infant, polyneuropathy, cardiomyopathy, dysphagia or recurrent vomiting. MRI shows focal symmetric lesions in the basal ganglia, thalamus, brainstem, cerebellum or posterior columns of the spinal cord. RRF are consistently absent, except for cases with MILS [9]. LS is a genetically heterogeneous entity. In some cases, it is attributable to mtDNA mutations (8993T > G NARP/MILS'), in others it arises from an AR defect. In still other cases, LS is X-linked or sporadic in case of the mutated E1-alpha-subunit of the pyruvate-dehydrogenase complex. All defects described to date in patients with LS affect the terminal oxidative metabolism and are likely to

impair ATP-production. Other COX assembly-factors include SCO1, SCO2, COX10 and COX15. These factors are associated with COX-deficient LS or other multisystem fatal infantile disorders, in which encephalopathy is accompanied by cardiomyopathy (SCO2, COX15), nephropathy (COX10) or hepatopathy (SCO1). Mutations in a RCCIII assembly protein, known as BCS1L, have been associated with Leigh-like syndromes as well as with lethal infantile Growth Retardation, Aminoaciduria, Cholestasis, Iron overload, Lactacidosis and Early death (GRACILE)-syndrome. Only in two RCCV assembly genes, pathogenic mutations have been identified so far. A mutation in the *ATP12* gene was associated with congenital lactacidosis and fatal infantile multisystem disease, involving brain, liver, heart and muscle. *TMEM70* mutations cause isolated ATP-synthase deficiency and neonatal encephalo-cardiomyopathy. LS may be also due to mutations in the RCCI assembly factors *NDUFA12L*, *NDUFAF1* or *C6ORF66*. *NDUFA12L* mutations manifest phenotypically as leucencephalopathy.

NDUFAF1 and *C6ORF66 m*utations manifest as severe cardio-encephalo-myopathy. Mutated *C20ORF7* was made responsible for lethal, congenital encephalopathy due to a RCCI defect.

Defects of intergenomic signalling Maintenance of mtDNA requires the concerted activity of several nuclear-encoded factors that participate in its replication as part of the mitochondrial replisome or by supplying dNTPs to mitochondria. Autosomal disorders classified as defects of nuclear-mitochondrial intergenomic communication may be associated with accumulation of multiple, large-scale mtDNA deletions in muscle or brain (mtDNA breakage syndromes) or with severe reduction of the mtDNA copy number (mtDNA depletion syndromes). A third group is due to mutations in genes encoding for proteins involved in the protein synthesis machinery.

mtDNA breakage syndromes

- *Autosomal dominant PEO (AdPEO) and arPEO*. Most adPEO families carry heterozygous mutations in *ANT1, POLG1* or *twinkle* genes. *ANT1* encodes the muscle-heart-specific mitochondrial adenine nucleotide translocator, *twinkle* encodes a mtDNA helicase and *POLG1* encodes the catalytic subunit of the mtDNA-specific polymerase-gamma. *POLG1* is particularly relevant because *POLG1* mutations are the most common cause of Mendelian PEO (see: http://dir-apps.niehs.nih.gov/polg/index.cfm) and specific mutations or a combination of mutations can be associated with a wide phenotypic spectrum, including adPEO or arPEO, sensory ataxic neuropathy, dysarthria, ophthalmoplegia (SANDO), spino-cerebellar ataxia and epilepsy with or without ophthalmoplegia (SCAE), or recessive, infantile Alpers-Huttenlocher-syndrome (AHS), characterised by myopathy, hepatopathy, epilepsy, migraine, intractable seizures and mental retardation (hepatic poliodystrophy). Whilst *POLG1*-associated PEO is characterised by accumulation of multiple mtDNA deletions, the latter syndromes are associated with tissue-specific mtDNA depletion. The phenotypic heterogeneity is explained by the complexity of the enzyme, which is composed of an exonuclease domain with predominantly proof-reading functions and a polymerase domain that primarily mediates mtDNA replication. Only a single heterozygous dominant mutation has been identified in *POLG2*, which encodes the accessory subunit of POLG.
- *Myo-neuro-gastro-intestinal encephalomyopathy (MNGIE)*. MNGIE is an AR multisystem disorder of young adults characterised by PEO, peripheral neuropathy, leucencephalopathy and severe gastrointestinal dysmotility leading to kachexia and early death. The gene responsible for MNGIE encodes the thymidine phosphorylase (*TYMP*), which promotes the phosphorylation of thymidine into thymine and deoxyribose-phosphate. TYMP-defects result in systemic accumulation of thymidine and deoxyuridine, which leads to deoxynucleotide pool imbalance and mtDNA instability, manifesting as point mutations, multiple mtDNA deletions or partial mtDNA depletion in the skeletal muscle. In addition, a MNGIE-like phenotype has been associated with POLG1 mutations [10].

mtDNA depletion syndromes. Mitochondrial DNA depletion syndromes (DPSs) are early-onset, age-specific syndromes and are phenotypically quite heterogeneous. Southern-blot analysis or quantitative PCR is diagnostic, demonstrating severe reduction of mtDNA in affected tissues (up to 98% in most severe forms). So far, DPSs have been linked to mutations in nine genes (*POLG1*, *PEO1 (twinkle)*, *thymidine-kinase* (*TK2*), *DGUOK*, *SUCLA2*, *SUCLG1*, *MPV17*, *RRM2B*, *TYMP*). Three main clinical presentations are differentiated: a myopathic, encephalo-myopathic and hepato-cerebral form. Consistent with these phenotypes, mtDNA depletion affects either a specific tissue (most commonly muscle, liver or brain) or multiple organs including heart, brain or kidney.

- *Myopathic DPS*. Myopathic DPS is due to mutations in the *TK2* or *RRM2B* genes. *TK2* mutations are responsible for approximately 20% of the myopathic DPS cases. Recently, mutations in p53-dependent ribonucleotide reductase (*RRM2B*) have been reported in children with mtDNA depletion in muscle, manifesting as developmental delay, microcephaly and proximal tubulopathy.

• *Encephalomyopathic DPS*. Encephalomyopathic DPS is due to mutations in the *SUCLA2* or *SUCLG1* genes. *SUCLA2* encodes for the β-subunit of the adenosine diphosphate-forming succinyl-CoA-ligase and manifests phenotypically as Leigh-like syndrome with muscle hypotonia, lactacidosis, dystonia and moderate methyl-malonic-aciduria. Mutations in the *SUCLG1* gene, encoding for the alpha-subunit of GDP-forming succinyl-CoA-ligase, manifest as fatal infantile lactacidosis, dysmorphism and methyl-malonic-aciduria with muscle and liver mtDNA depletion.

• *Hepato-cerebral DPS*. Hepato-cerebral DPS is due to mutations in the *PEO1*, *POLG1*, *DGUOK* or *MPV17* genes [11]. Phenotypes of *POLG1* mutations have been described above. Mutations in *PEO1* may cause an AR Alpers-like phenotype. *MPV17* encodes for a protein of unknown function located at the inner mitochondrial membrane. *MPV17* mutations manifest as hepatic failure, hypoglycemia, muscle hypotonia, ataxia, dystonia or polyneuropathy. Hepato-cerebral DPS from *MPV17* mutations is allelic to Navajo neurohepatopathy, characterised by hepatopathy, polyneuropathy, corneal anaesthesia and scarring, acral mutilation, leucencephalopathy, failure to thrive, recurrent metabolic acidosis and intercurrent infections. Mutations in the *TYMP*-gene may not only cause multiple mtDNA deletions but also mtDNA depletion, manifesting clinically as MNGIE.

Defective mitochondrial protein synthesis machinery Autochthonous translation of mtDNA proteins is carried out by an apparatus composed of tRNAs and rRNAs, synthesised *in situ* from mtDNA genes and numerous proteins encoded by nDNA genes, including 77 mito-ribosomal proteins, several tRNA maturation enzymes (e.g. pseudouridylate synthases), the amino-acyl-tRNA synthetases and translation initiation, elongation and termination factors. Mutations in the *PUS1* gene, encoding for the catalytic domain of the pseudouridylate synthase-1, cause AR mitochondrial myopathy and sideroblastic anaemia (MLASA). Mutations in *DARS2*, the gene encoding mitochondrial aspartyl-tRNA synthetase, manifest as leucencephalopathy with brainstem and spinal cord involvement and lactacidosis (LBSL)-syndrome. Lactacidosis in LBSL is due to RC-defects in tissues other than muscle or skin. Missplicing mutations in the *RARS2* gene, encoding for the mitochondrial arginine-tRNA-synthetase, cause AR pontocerebellar hypoplasia (PCH) with prenatal-onset, cerebellar/pontine atrophy/hypoplasia, microcephaly, neocortical atrophy and severe psychomotor impairment. Impaired RNA processing is speculated to preferentially affect the brain because of a tissue-specific vulnerability of the splicing machinery.

Defects of the mitochondrial lipid milieu Barth-syndrome is an X-linked recessive disorder characterised by mitochondrial myopathy, hypertrophic or dilated cardiomyopathy, left ventricular hypertrabeculation/non-compaction, growth retardation, leukopenia and methyl-glutaconic aciduria. Barth-syndrome is caused by mutations in the *G4.5* gene, encoding for one of the taffazins. Tafazzins are homologous to phospholipid-acyl-transferases, which are involved in the biosynthesis of cardiolipin, a phospholipid species present in the inner mitochondrial membrane. Cardiolipin is required for structural stabilisation and functional modulation of RCCV.

CoQ-defects Coenzyme Q_{10} (CoQ), a lipid-soluble component of virtually all cell membranes, transports electrons from RCCI and RCCII to RCCIII and is essential for stabilising RCCIII. Primary CoQ-deficiency causes AR MIDs, like: encephalomyopathy with recurrent myoglobinuria, brain involvement and RRF; a severe infantile multisystem MID; cerebellar ataxia; or LS. So far, mutations in *COQ2*, *COQ8 ADCK3*, *CABC1*, *PDSS1*, *PDSS2,* genes encoding for enzymes of the CoQ-biosynthesis have been reported in primary CoQ-deficiency [12]. Secondary CoQ-deficiency is due to mutations in genes not directly related to ubiquinone biosynthesis, such as *APTX (*cerebellar ataxia*), ETFDH (*pure myopathy*) or BRAF* (cardio-facio-cutaneous syndrome). However, in the majority of CoQ-deficiencies, the causative molecular genetic defect remains elusive. Most cases with primary or secondary CoQ-deficiency respond favourably to CoQ-supplementation.

Mitochondrial transport machinery defects An example of such a defect is the X-linked deafness-dystonia syndrome (DDS), also known as Mohr-Tranebjaerg syndrome, which is due to loss-of-function mutations in the *DDP1/TIMM8A* gene. The gene product is involved in the transport and sorting of proteins to the inner mitochondrial membrane [13]. RC functions and ATP-synthesis are intact. Patients present with childhood-onset progressive deafness, dystonia, spasticity, mental deterioration and blindness. Another example is X-linked sideroblastic anaemia with ataxia (XLSA/A), which is caused by partial inactivating mutations in the *ABCB7* gene, encoding for the ATP-binding cassette-transporter, responsible for the iron transport from mitochondrion to cytoplasm [14]. Mutations in the mitochondrial phosphate carrier SLC25A3, which transports inorganic phosphate into the mitochondrial matrix, causes congenital lactacidosis, hypertrophic cardiomyopathy and muscle hypotonia. Biochemically, muscular ATP synthesis is impaired.

Defects in mitochondrial biogenesis Mitochondria are dynamic organelles, which constantly fuse and divide. The equilibrium between fusion and fission controls the morphology of mitochondria, which either appear as corpuscles or elongated tubules depending on the prevailing process. Mitochondrial fusion requires the concerted action of three GTPases: mitofusin-1, mitofusin-2 and OPA1. For mitochondrial fission, another GTPase called dynamin-like protein-1 (DLP1) is required. Mutations in 'fusion' genes result in fragmentation of mitochondria due to ongoing fission but absent fusion. Disruption of 'fission' proteins results in tubulised, 'spaghetti-like' mitochondria, excessively long and interconnected. Heterozygous mutations in *MFN2* cause Charcot-Marie-Tooth neuropathy-2A, whereas *OPA1* mutations cause AD optic atrophy, the most common inherited optic atrophy. In some cases, optic atrophy may be associated with myopathy, PEO, hearing loss, ataxia and accumulation of multiple mtDNA deletions in skeletal muscle. *DLP1* mutations may cause severe infantile encephalopathy and since DLP1 is also expressed in peroxisomes, both elongated mitochondria and peroxisomes coexist.

Defects in apoptosis Mutated proteins involved in mitochondrial apoptosis have been recently shown to cause MIDs. In a patient with developmental delay, asymmetric cerebral atrophy, epilepsy, hemiplegia and myopathy with COX-deficiency a mutation in the *FASTKD2* gene, encoding for a protein localised in the mitochondrial inner compartment, were causative. Preliminary data indicate that this protein plays a role in mitochondrial apoptosis. In staurosporine-induced-apoptosis experiments, decreased nuclear fragmentation was detected in treated mutant fibroblasts.

Summary of recommendations for the genetic diagnosis of MIDs

Diagnostic work-up for suspected MID is a stepwise procedure. The first step comprises a comprehensive individual and family history and clinical investigations by specialists in neurology, ophthalmology, otology, endocrinology, cardiology, gastroenterology, nephrology, haematology or dermatology. Important instrumental procedures include chemical investigations of the serum, CSF and urine, electrophysiological investigations, functional and imaging studies of the cerebrum, and muscle biopsy [15]. Based on their results the probability for the presence of a MID can be assessed according to the Nijmegen, Bernier' or Walker diagnostic criteria.

In a second step, clinicians need to decide whether an individual phenotype conforms to any of the syndromic MIDs or represents a non-syndromic MID and need to determine if the phenotype occurred sporadically or followed a Mendelian or maternal trait of inheritance.

Genetic testing is the third step and depends on step 1 and 2. If the phenotype suggests syndromic MID due to mtDNA point mutations (MELAS, MERRF, NARP, LHON) DNA-microarrays using allele-specific oligonucleotide hybridisation, real-time-PCR or single-gene-sequencing are indicated. If the phenotype suggests syndromic MID due to mtDNA deletion (mtPEO, KSS, Pearson's syndrome) mtDNA analysis starts with RFLP or Southern-blot from appropriate tissues (Fig. 2). mtDNA deletions with low heteroplasmy rate may be detected only by long-range PCR. If neither a single deletion nor multiple deletions are found, mtDNA sequencing is recommended (level B).

If RFLP or Southern-blot of muscle mtDNA detects multiple mtDNA deletions (breakage syndromes), sequencing of *POLG1* (adPEO, arPEO, SANDO, SCAE, AHS), *POLG2*, *PEO1* (adPEO), *ANT1* (adPEO), *TYMP* (MNGIE) or *OPA1* (ADOAD) genes should follow (level B). Sequencing should start with the *POLG1* gene, since it is the most likely to carry a mutation. Sequencing of *TYMP* should be performed only if serum thymidine is elevated. If the phenotype suggests LS appropriate nDNA genes encoding for RCC subunits or assembly factors (*SURF1, ATP12, SCO1*, *SCO2*, *COX10*, *COX15 NDUFS*, *NDUFV*, *SDH*) need to be sequenced. Sequencing of appropriate nDNA genes is also required if the phenotype suggests GRACILE (*TMEM70*), IOSCA (*PEO1*), MLASA (*PUS1*), Barth syndrome (*TAZ/G4.5*), DDS/Mohr-Tranebjaerg syndrome (*DDP1*), MIRAS (*POLG1*) or CMT2A (*mitofusin-2*) (level B).

If an individual presents with a non-syndromic phenotype, biochemical investigations of the most affected tissues (muscle, liver, brain, skin) should clarify if a single or multiple RCCs are defective (level B). If a single autosomally inherited defect is present, sequencing of appropriate structural subunits or assembly-factors of RCCI, III, IV or V, is necessary (level B). If biochemical investigations in muscle tissue suggest CoQ-deficiency, sequencing of genes involved in the coenzyme-Q biosynthesis (most frequently *ETFDH*) should be carried out (level B). If the single RRC defect is maternally inherited, one should proceed with mtDNA sequencing of appropriate mtDNA genes (level B).

If multiple autosomally inherited biochemical defects are found, a Southern-blot or quantitative PCR should clarify if there is mtDNA depletion or not. If these investigations detect mtDNA depletion, sequencing of the *TK2* or *RRM2B* genes is indicated if the skeletal

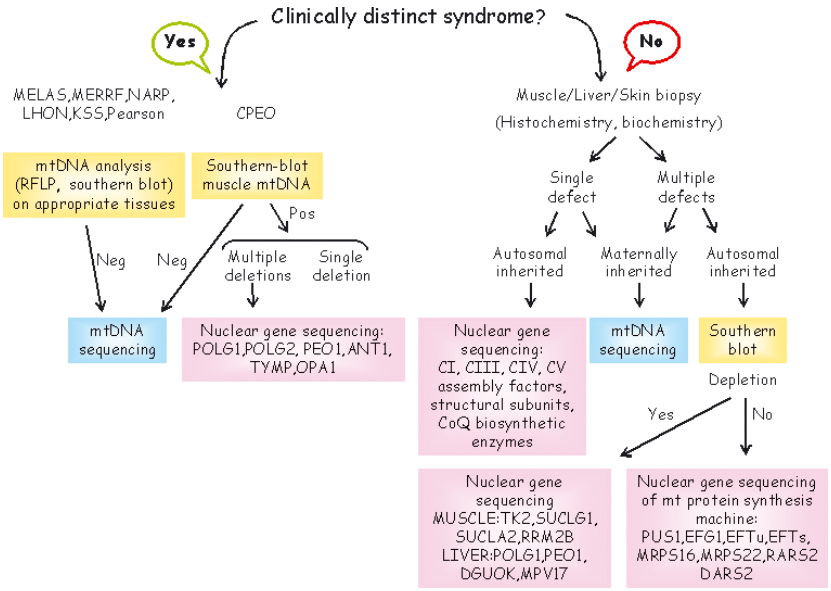

Figure 2 Algorithm for the genetic analysis of MIDs.

muscle is the predominantly affected organ. Elevated creatine-kinase may help to prioritise testing for *TK2* or *RRM2B*. If muscle and cerebrum are the most affected organs, sequencing of the *SUCLG1, SUCLA2* genes or if the liver is the predominantly affected organ sequencing of the *POLG1*, *PEO1*, *DGUOK* or *MPV17* genes is recommended (level B, Fig. 2).

If Southern-blot fails to detect mtDNA depletion, sequencing of genes involved in the mitochondrial protein synthesis machinery (*PUS1*, *EFT*, *EFG1*, *EFTs*, *MRPS16*, *MRPS22*, *RARS2*, *DARS2*) is recommended (level B, Fig. 2). Corresponding proteins of the *MRPS22*, *EFT* and *EFTs* genes are involved in the initiation and elongation of peptides during protein synthesis and are responsible for the group of elongation-factor-disorders. In case of PCH and severe psychomotor impairment, the *RARS2* gene should be sequenced for splice-site mutations. Sequencing of the *FASTKD2* gene is required in cases with developmental delay, asymmetric cerebral atrophy, epilepsy, hemiplegia and myopathy with COX-deficiency after exclusion of other causes of COX-deficiency.

Tissues most frequently chosen are blood (MELAS, MERRF, NARP, LHON, Pearson's syndrome) or muscle (KSS). Availability of muscle tissue for mtDNA-analysis is mandatory in patients with phenotypes associated with large-scale mtDNA rearrangements, which can be easily missed in leukocytes except for Pearson syndrome. Alternative tissues for DNA-analysis are cells from the urine sediment, buccal mucosa, or hair follicles. mtDNA point mutations can be also detected in blood samples. However, in maternally inherited phenotypes associated with heteroplasmic point mutations, the percentage of mutant mtDNA is often significantly higher in muscle than other tissues.

Conclusions

This second part of the EFNS guidelines should not only familiarise the clinician with mitochondrial genetics but also enable her/him to implement this knowledge into daily practice, when investigating the genotype of a suspected MID. The guidelines should allow her/him to collect essential and useful information for the geneticist and confront the neurologist with the limitations of the methods and incompleteness of currently available insight despite the rapid developments in the understanding of mitochondrial genetics. Because of the rapid developments in this field, there will be a need to regularly update these guidelines, provide information about laboratories offering genetic testing, and provide algorithms for cost-effective diagnostic work-up. To stimulate and support ongoing or future basic or therapeutic trials for human MIDs, Europe-wide networks and databases are required.

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