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Review

Mitochondrial respiratory chain disorders in childhood: Insights into diagnosis and management in the new era of genomic medicine ☆

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ABSTRACT

Background: Mitochondrial respiratory chain disorders (MRCDs) are some of the most common metabolic disorders presenting in childhood, however because of it clinical heterogeneity, diagnosis is often challenging. Being a multisystemic disorder with variable and non-specific presentations, definitive diagnosis requires a combination of investigative approaches, and is often a laborious process.
Scope of review: In this review we provide a broad overview of the clinical presentations of MRCDs in childhood, evaluating the different diagnostic approaches and treatment options, and highlighting the recent research advances in this area.
Major conclusions: Extensive research over the years has significantly increased the frequency with which accurate diagnosis is being made, including the identification of new biomarkers and next generation sequencing (NGS) technologies. NGS has provided a breakthrough in unravelling the genetic basis of MRCDs, especially considering the complexity of mitochondrial genetics with its dual genetic contributions.
General significance: With an increased understanding of the pathophysiology of this group of disorders, clinical trials are now being established using a number of different therapeutic approaches, with the hope of changing the focus of treatment from being largely supportive to potentially having a positive effect on the natural history of the disorder.
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1. Introduction

The most important carrier of chemical energy in almost all living organisms is adenosine triphosphate (ATP). The useful energy in ATP is bound in highly energetic phosphor-anhydride bonds, which release free energy when hydrolyzed. This energy is used for diverse cellular functions such as synthesis of macromolecules, contraction of muscle cells, movement of individual cells from one location to another and the transport of molecules against a concentration gradient [1].

This range of fundamental cell functions is dependent on energy, and so it should not be surprising that impaired cellular energy production can affect any organ or tissue [2]. The vast majority of ATP is produced within mitochondria, intracellular organelles present in virtually all eukaryotic cells. The most important pathway for the synthesis of most cellular ATP is the mitochondrial respiratory chain (MRC), through a process of oxidative phosphorylation (OXPHOS) [3]. The MRC, which is composed of five enzyme complexes (complexes I–V) (refer to Fig. 1), and consists of approximately 90 subunits, is located in the inner mitochondrial membrane. Mitochondrial proteins are encoded by two distinct genetic systems, mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). Of the 90 subunits of the RC only 13 are encoded by mtDNA, which is a small 16.6 kb circle of double stranded DNA [4].

Complex I [NADH-coenzyme Q (CoQ) reductase] consists of 47 polypeptides, seven of which are encoded by mtDNA. Complex I carries reducing equivalents from NADH to CoQ. Complex II (succinate-CoQ reductase), which includes FAD-dependent succinate dehydrogenase and iron–sulphur proteins, is the only complex for which all four of its subunits are nuclear encoded, and carries reducing equivalents from $FADH_2$ to CoQ [4]. Complex III (reduced CoQ-cytochrome *c* reductase) consists of 11 subunits, of which only one is encoded by the mitochondrial genome. Complex III forms a homodimer and transfers electrons from CoQ to cytochrome *c* [5]. Complex IV [cytochrome *c* oxidase (COX)] consists of 13 protein subunits (three of which are encoded by mtDNA), two cytochromes (cytochromes *a* and *a*3) and two copper atoms. It is the terminal oxidase of the MRC, and catalyses the transfer of reducing equivalents from cytochrome *c* to molecular oxygen [4]. Complex V is composed of a membrane-bound subcomplex (F_0), a large extra membranous complex (F_1) that resides in the matrix

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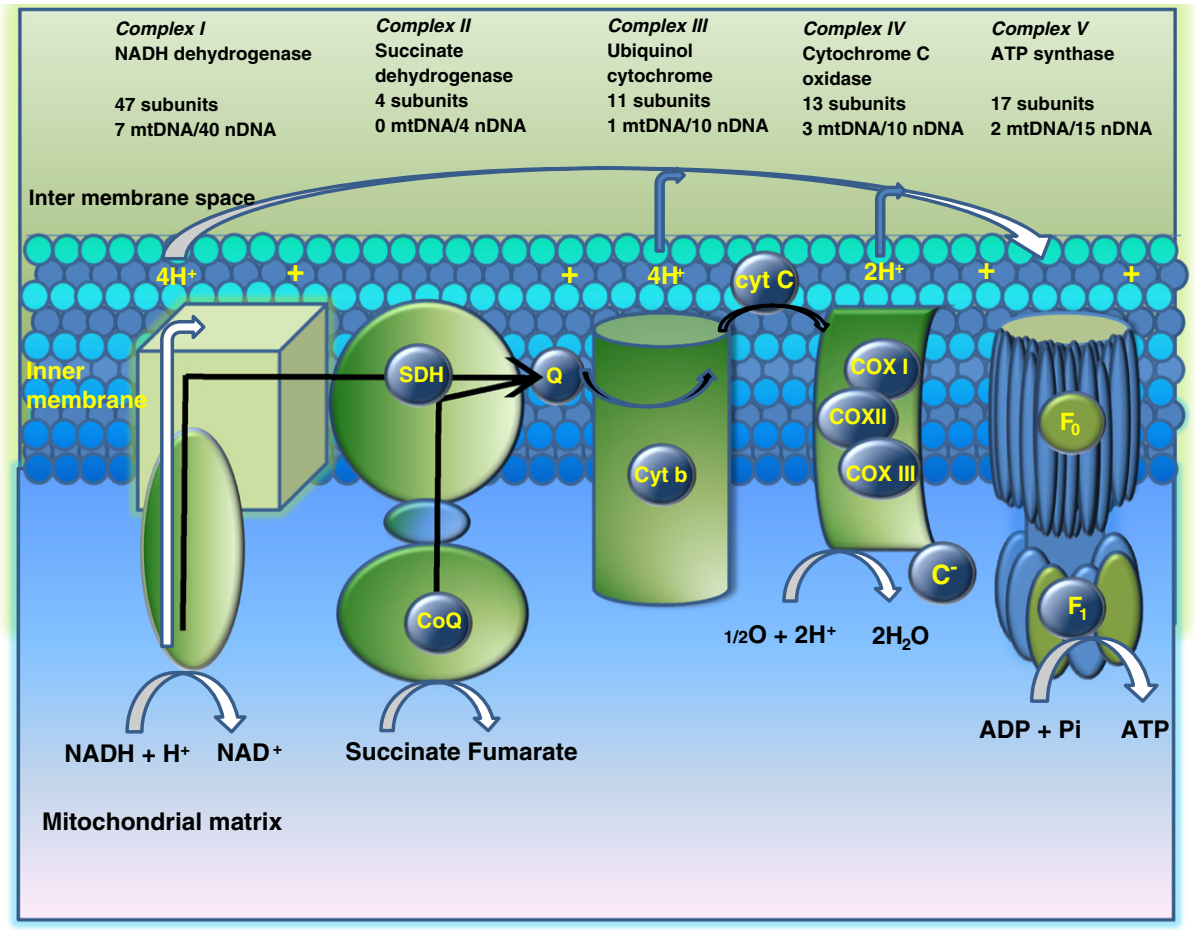

Fig. 1. The mitochondrial respiratory chain. The respiratory chain consists of five enzyme complexes (complexes I–V) and two intermediary substrates (coenzyme Q and cytochrome *c*). Complexes I and II are the two main entry points of reducing equivalents in the MRC. The NADH + H^+ and $FADH_2$ produced by intermediary metabolism are oxidised further by the MRC to establish an electrochemical proton gradient. Complex III forms a homodimer and transfers electrons from CoQ to cytochrome *c*. Electron transfer is then coupled to complex V, which uses the proton motive force created to generate ATP.

space, and a stalk connecting the two complexes. Protons from the intermembrane space are allowed to enter complex V through the (F_0) complex, leading to subunit rotation within the enzyme complex. The energy from this rotation is then used to generate ATP, which takes place in the (F_1) complex [6].

The oxidation process is fuelled by pyruvate generated from the glycolysis of carbohydrates and fatty acids released from triglycerides. They are imported into the mitochondrial matrix, allowing pyruvate to be metabolised by the pyruvate dehydrogenase complex, and fatty acids to be catabolised by the β-oxidation pathway, both leading to the generation of acetyl CoA. The citric acid cycle then utilises these acetyl groups to produce substrates like NADH and $FADH_2$ for OXPHOS. In addition, acetyl CoA may be used to generate ketone bodies, which can be utilised as an alternate energy source by the brain and striated muscle. The electrons released from NADH and $FADH_2$ are then shuttled through the various complexes of the MRC, resulting in the production of ATP and water. The metabolism of one molecule of glucose in the mitochondria produces 36 molecules of ATP, whereas glycolysis in the cytoplasm produces only 2 molecules of ATP. This means that organs with a high-energy demand are vulnerable if mitochondrial energy production is compromised. Indeed, disorders affecting energy metabolism are often multisystemic, particularly affecting high energy-demanding tissues like skeletal muscle and brain, and together are collectively often referred to as the mitochondrial encephalomyopathies [7,8].

1.1. Prevalence of mitochondrial respiratory chain disorders

The first reported mitochondrial respiratory chain disorder (MRCD) was in a woman with severe hypermetabolism in 1962 [8], and subsequently the range of clinical conditions attributed to this group of disorders has expanded greatly. This group of disorders is now reported to be one of the most common groups of inborn errors of metabolism, with an estimated frequency of one in 5000 live births [9], and in paediatric neurology they are regarded as the most frequent cause of metabolic abnormality [10]. This is most likely an underestimate of the actual prevalence of these disorders as the symptoms are nonspecific and can be mistaken for other diseases [10]. Most MRCDs presenting in childhood have an autosomal recessive pattern of inheritance and therefore have a higher incidence in ethnic groups where consanguinity is seen [11], although all forms of inheritance have been reported. On the other hand, most diagnosed MRCDs first presenting in adulthood are due to an underlying primary mtDNA defect [11,12].

2. Clinical and molecular features of MRCDs

2.1. Clinical presentation in childhood

A defective OXPHOS system should be suspected in a patient who presents with a combination of unexplained neuromuscular and non-neuromuscular symptoms [2]. Childhood manifestations of MRCDs are

often progressive and more often fatal as opposed to adult onset disease. The clinical spectrum in the paediatric population is quite broad, with the first symptoms potentially developing as early as the neonatal period [4] (refer to Table 1). Apart from the neuromuscular system many other organ systems may be affected e.g., the heart, eyes, ears, kidneys, endocrine glands, liver, bone marrow, and gastrointestinal tract [13]. Childhood clinical presentations include lethargy, hypotonia, developmental delay, failure to thrive, seizures, cardiomyopathy, hearing or visual impairment, movement disorders, and lactic acidosis [14]. Definitive diagnosis is more of a challenge when only one symptom or clinical abnormality is present, whilst an MRCD should be considered a strong possibility when two or more apparently unrelated symptoms manifest [15]. In a study by Scaglia and colleagues, 71% of a total of 113 paediatric patients with mitochondrial disease (diagnosis based on clinical, pathologic, enzymatic, molecular and metabolic parameters) were found to have significant functional defects in RC enzymology. The most frequently identified functional MRC defects were complex I (32%) combined complexes I, III and IV (26%), followed by complex III (16%) and complex II (7%). Forty percent of the patients presented with cardiac disease, and those with cardiac disease as a component of the initial diagnosis had a survival rate of 18% at 16 years, as opposed to those with neuromuscular features but no cardiomyopathy, who had a higher survival rate of 95% [16].

2.2. Clinical and molecular spectrum of RC disorders

The mitochondrion is unique in that it requires the contribution of two physically separate genomes for construction, maintenance and function of the OXPHOS system. For four of the five enzyme complexes of the MRC to be functional, nuclear and mitochondrial genes coding for protein subunits of the RC must be expressed in a co-ordinated manner, some of which show tissue-specific expression [3]. In addition, there are a number of essential proteins, encoded by other nuclear genes that are involved in regulation of mtDNA transcription and translation or in RC subunit assembly or stability. Therefore, normal MRC function necessitates the coordinated expression of well over 200 different gene loci from two very different genomes. Molecular defects in nuclear or mitochondrial encoded genes have been directly implicated in patients with MRCDs [3,9].

2.3. Mitochondrial disease associated with primary mtDNA mutations

mtDNA mutations are maternally inherited or may occur spontaneously. Heteroplasmy, where both mutant and normal copies of the mtDNA genome can co-exist within a tissue or organ, is a distinctive feature of mitochondrial disease genetics. The mutation load needed to cause mitochondrial dysfunction varies from tissue to tissue depending on the energy demands of the tissue; a phenomenon called the threshold effect [17]. Some pathogenic mtDNA mutations are homoplasmic (i.e. 100% mutated mtDNA), and depending on the context may be more likely to be fatal. The link between pathogenic mutations in mtDNA and defects in the MRC was first reported in 1988. These initially described mutations included large-scale deletions in patients with mitochondrial myopathy [18], and a family with Leber hereditary optic neuropathy (LHON), affected members who had a point mutation in the mtDNA gene encoding subunit 4 of complex I (MT-ND4) [19]. There are now more than 300 point mutations, deletions and duplications reported in mtDNA [20]. Important clinical syndromes mainly related to point mutations or large-scale deletions of mtDNA are described below (refer to Table 2).

2.3.1. mtDNA deletions

Most deletions of mtDNA are single, sporadic events occurring early in embryonic development, and, depending on the segregation of mutated mtDNA and subsequent clonal proliferation, will present with different pictures including Kearns–Sayre syndrome (KSS), progressive external ophthalmoplegia (PEO) and Pearson syndrome (PS) [21].

KSS is characterised by ophthalmoplegia, ptosis, pigmentary retinopathy, ataxia and cardiac conduction block. Interestingly, there is a crossover of symptoms in some instances of PS, whereby if the patient survives childhood, features of KSS begin to develop [22]. These changes are explained by a progressive loss of the deleted mtDNA in rapidly dividing tissue (bone marrow), and accumulation of the same deleted mtDNA molecule in postmitotic tissue (muscle) [23].

PEO is defined by the onset of disease usually in adulthood with ptosis, external ophthalmoplegia, and slowly progressive skeletal muscle weakness, or with a phenotype presenting in childhood that is more heterogeneous and can be more severe, including infantile cardiomyopathy [24]. Another disorder associated with both multiple deletions in mtDNA and mtDNA depletion is mitochondrial neuro-gastro-intestinal encephalomyopathy (MNGIE). Clinically this disorder is characterised by onset in adolescence or early adulthood of ptosis, progressive external ophthalmoplegia, gastrointestinal dysmotility with pseudo-obstruction, peripheral neuropathy, myopathy, leukoencephalopathy and lactic acidosis. Analysis of skeletal muscle shows the presence of ragged-red fibres (due to the accumulation of abnormal mitochondria in the subsarcolemmal region of muscle fibres) and reduced respiratory chain enzyme activities of complexes I, III and IV, with normal or

Table 1
Clinical manifestations of MRCDs in childhood.
Adapted from [16].

Manifestation	Clinical presentations	% of MRCD for each manifestation
Neurological signs	Muscle weakness, hypotonia, peripheral neuropathy, ataxia, spasticity, stroke-like episodes, migraine headaches, tremor, chorea, and ballismus, dystonia, seizures, myoclonus	45%
Intellectual dysfunction/psychiatric disturbances		20%
Hepatic signs	Liver failure	10%
Cardiological features	Arrhythmia, cardiomyopathy, cardiac murmur, or sudden death	
Haematological abnormalities	Pancytopenia; sideroblastic anaemia	10%
Renal dysfunction	Proximal tubulopathy, nephritic syndrome, tubulo-interstitial nephritis, nonspecific renal failure	5%
Growth failure		20%
Endocrine abnormalities	Hypoparathyroidism, hypothyroidism, diabetes insipidus, diabetes mellitus, hypogonadism, ketotic hypoglycaemia, adrenocorticotropin hormone deficiency	
Dermatological findings	Multiple lipomatosis, scaly pruritic erythema, reticular pigmentation, hypertrichosis, eczema, or vitiligo	
Ophthalmologic abnormalities	Retinitis pigmentosa, ophthalmoplegia, strabismus	
Hearing	Sensorineural deafness	7–26%
Gastrointestinal abnormalities	Anorexia, frequent emesis, abdominal pain, diarrhoea, and constipation, pseudo-obstruction	

Table 2
Mitochondrial disorders caused by primary mutations in mitochondrial DNA.
Adapted from [27].

Mitochondrial DNA disorder	mtDNA genotype	Gene(s)	Status	Inheritance
KSS	A single large deletion	Multiple deleted genes	Heteroplasmic	Usually sporadic
CPEO	A single large deletion	Multiple deleted genes	Heteroplasmic	Usually sporadic
PS	A single large deletion	Multiple deleted genes	Heteroplasmic	Usually sporadic
MELAS	m.3243A>G; m.3271T>C; other individual mutations	*TRNL1* *ND1* and *ND5*	Heteroplasmic	Maternal
MERRF	m.8344A>G; m.8356T>C	*TRNK*	Heteroplasmic	Maternal
NARP or MILS	m.8993T>G	*ATP6*	Heteroplasmic (Leigh disease if homoplasmic)	Maternal
Later onset Leigh syndrome	m.8993T>C	*ATP6*	Heteroplasmic	Maternal
LHON	m.3460G>A m.11778G>A m.14484T>C	*ND1* *ND4* *ND6*	Usually homoplasmic	Maternal
Fatal, infantile encephalopathy; Leigh/Leigh-like syndrome	m.10158T>C; m.10191T>C	*ND3*	Hetero- or homoplasmic	Sporadic

ATP6, ATPase 6; CPEO, chronic progressive external ophthalmoplegia; CYB, cytochrome *b*; LHON, Leber hereditary optic neuropathy; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy and ragged-red fibres; MILS, maternally-inherited Leigh syndrome; ND1,3–6, NADH dehydrogenase subunits 1, 3–6; NARP, neurogenic weakness, ataxia and retinitis pigmentosa.

increased complex II and citrate synthase (the latter two often indicative of a proliferation of mitochondrial numbers), in association with multiple mtDNA deletions and mtDNA depletion [25].

2.3.2. mtDNA point mutations

MRCDs have been associated with point mutations in protein coding regions of mtDNA (Table 2) [26]). Mutations in these protein-coding genes have mainly been described in association with a number of clinical pictures, including LHON, NARP (neuropathy, ataxia, retinitis pigmentosa) and MILS (maternally inherited Leigh syndrome).

LHON (MIM 535000) is a maternally inherited disease characterised by acute or subacute blindness, which presents in early adulthood, usually more frequently in males, with central vision loss due to bilateral optic nerve atrophy [23]. The three common mtDNA mutations encoding complex I subunits causing LHON are typically homoplasmic [27]. NARP (MIM 551500) usually affects young adults and is characterised by retinitis pigmentosa, proximal neurogenic muscle weakness, intellectual disability, seizures, ataxia, sensory neuropathy and dementia [23,28]. MILS is a severe, progressive infantile encephalopathy with characteristic symmetrical lesions in the basal ganglia and the brainstem [29]. The onset is usually in the first year of life, and although clinical features can vary, they reflect the damage to the brainstem or basal ganglia, and are characterised by developmental delay, hypotonia, dystonia, respiratory abnormalities, ataxia, optic atrophy, as well as renal involvement [23,30]. For some of these disorders the primary defect may be in a nuclear encoded gene, and often patients show a similar phenotype to those with mtDNA defects. Moreover some nuclear encoded mutations can result in secondary abnormalities of the mitochondrial genome (mtDNA depletion, deletions or duplications) [26].

Other MRCDs associated with point mutations in tRNA genes encoded by the mtDNA include MELAS (Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) and MERFF (Myoclonic Epilepsy with Ragged Red Fibres). MELAS is a heterogeneous disorder defined by seizures, episodic vomiting and repeated cerebral insults resembling strokes at a young age, elevated lactate in plasma and CSF (particularly during acute episodes), migraine headaches and either focal or generalised epilepsy, and ultimately dementia [23]. Ragged-red fibres can be found in skeletal muscle biopsies of MELAS patients, but unlike the MERRF mutations, show patchy cytochrome *c* oxidase staining on immunohistochemistry [31]. The most common mtDNA tRNA point mutation associated with this syndrome is the m.3243A>G transition in the gene coding for the mitochondrial leucine tRNA ($tRNA^{Leu\ (UUR)}$) [32]. The m.3243A>G mutation may also cause a distinct phenotype of deafness and diabetes [33].

MERRF is a severe neuromuscular disorder that often has its onset in childhood, and is characterised by epilepsy (myoclonic epilepsy, generalised seizures, or focal seizures), cerebellar ataxia, intention tremor, myopathy, proximal renal tubule dysfunction, cardiomyopathy, peripheral neuropathy, optic atrophy, deafness and ultimately dementia. Muscle biopsies show ragged red fibres [34].

2.4. Mitochondrial disease associated with nuclear gene mutations

Numerous clinical disorders have been associated with mutations in mtDNA, however, defects in nuclear encoded genes are becoming increasingly recognised, and are likely to account for the vast majority of patients with a defect, at least in paediatric patients. This group includes both nuclear mutations in structural subunits of RC enzymes and nuclear genes that encode proteins involved in assembly and maintenance of the mitochondrial genome. Also included in this grouping are the MRCDs in which defective oxidative phosphorylation is due to mutations in nuclear genes encoding non-RC proteins involved in mitochondrial biogenesis, which directly or indirectly result in reduced mitochondrial energy metabolism and disease (see Table 3) [35].

2.5. Nuclear mutations in structural subunits of RC enzymes and proteins involved in respiratory chain assembly and maintenance

Complex I deficiency is a common deficiency, either as an isolated defect or in combination with other RC enzyme defects. Thirty-eight of the 45 subunits of Complex I are nuclear encoded and mutations in these subunits have been identified as leading to complex I deficiency, with Leigh syndrome (LS) being the most common manifestation. Complex II deficiency appears to be rare among the RC disorders, as very few cases with an isolated complex II deficiency have had mutations identified in any structural subunits of the enzyme, all of which are nuclear-encoded. A number of patients have been identified with mutations in the succinate dehydrogenase component, with mutations in succinate dehydrogenase complex subunit A (SDHA) being associated with LS, epilepsy, optic atrophy, ataxia, myopathy with exercise intolerance, cardiomyopathy, and leukoencephalopathy [36,37]. Other individuals with mutations in complex II structural subunits may develop inherited paragangliomas [38]. Complex III deficiency is another rare group of disorders. Mutations in the nuclear genes encoding CIII subunits or CIII assembly factors can cause a wide-range of tissue-specific defects in affected patients, including: encephalopathy with renal involvement or lethal infantile hepatic failure (*BCS1L*, GRACILE syndrome) [39,40], severe psychomotor retardation and extrapyramidal signs, dystonia, athetosis and ataxia (*UQCRQ*) [41], mitochondrial encephalopathy (*BCS1L*, *TTC19*, *CYTB*) [42], *pili torti* and sensorineural hearing loss, known as Björnstad syndrome (*BCS1L*) [43], optic neuropathy, and

Table 3
Nuclear genes causing isolated RC complex defects.
Adapted from [17].

RC deficiency	Genes	Clinical presentations
Complex I	*C20orf7*, *FOXRED1*, *NDUFA1*, *NDUFA2*, *NDUFA7*, *NDUFA8*, *NDUFA10*, *NDUFA11*, *NDUFA13*, *NDUFAF1*, *NDUFAF2*, *NDUFAF3*, *NDUFAF4* (*HRPAP20*), *NDUFB6*, *NDUFS1*, *NDUFS2*, *NDUFS3*, *NDUFS4*, *NDUFS5*, *NDUFS6*, *NDUFS7*, *NDUFS8*, *NDUFV1*, *NDUFV3*, *NUBPL*, *ACAD9*	Leigh syndrome, cardiomyopathy, hepatopathy, myopathy, encephalomyopathy, tubulopathy, hypotonia, Parkinson disease, and leukoencephalopathy
Complex II	*SDHA*, *SDHAF1 SDHB*, *SDHC*, *SDHD*, *SDH5*, (*SDHAF2*)	Leigh syndrome, epilepsy, optic atrophy, ataxia, myopathy with exercise intolerance, cardiomyopathy, and leukoencephalopathy Hereditary paraganglioma and pheochromocytoma syndromes
Complex III	*BCS1L*, *TTC19*, *UQCRB*, *UQCRQ*, *CYC1*	Epilepsy, severe psychomotor retardation, movement disorder, ataxia, hypotonia, and global developmental delay, lactic acidosis, hypoglycemia, hyperglycemia, magnetic resonance imaging abnormalities involving the deep grey nuclei, hepatopathy, renal tubulopathy, *BCS1L*: GRACILE and Bjornstad syndromes
Complex IV	*COX10*, *COX15*, *COX4I1*, *COX4I2*, *COX6B1*, *COX7A1*, *FASTKD2*, *LRPPRC*, *SCO1*, *SCO2*, *SURF1*, *TACO1*	Leigh syndrome; encephalocardiomyopathy; hypotonia; lactic acidosis
Complex V	*ATPAF2* (*ATP12*), *ATP5E*, *TMEM70*	Hypertrophic cardiomyopathy, epilepsy
Coenzyme Q10	*PDSS1*, *PDSS2*, *CoQ2*, *CoQ3*, *CoQ6*, *CoQ7*, *CoQ9*, *ADCK3*	Encephalomyopathy, seizures, and ataxia; infantile encephalopathy, cardiomyopathy, and renal failure; cerebellar syndrome with ataxia and atrophy; Leigh syndrome; isolated myopathy; and steroid-resistant nephrotic syndrome

transient episodes of hypoglycaemia or hyperglycaemia and lactic acidosis (*UQCRB*, *UQCRC2*, *CYC1*) [44–46].

Mutations in genes encoding proteins involved in the function and assembly of Complex IV have been identified in patients with LS and COX deficiency. LS associated with isolated COX deficiency (usually identified in all tissues) is largely caused due to mutations in *SURF1*, with close to 75% of COX-deficient Leigh patients having *SURF1* mutations [47]. Clinically, patients with LS caused by *SURF1* mutations have a severe, early onset encephalopathy with characteristic symmetric lesions in the basal ganglia and brainstem, and most die as a result of central ventilatory failure [48]. Other genes involved in complex IV deficiency are *COX10* (tubulopathy and leukodystrophy), *COX15* (hypertrophic cardiomyopathy) [49], *SCO1* (neonatal-onset hepatic failure), and *SCO2* (cardioencephalomyopathy). Mutations in *LRPPRC*, involved in the translation or stability of the mRNA and *TACO1*, which encodes a translational activator of the mtDNA encoded *COX1* subunit encoded by mtDNA, result in LS [47].

Only a few nuclear mutations involving complex V have been reported. Mutations in *ATP12* which encodes a protein required for assembly of the alpha and beta subunits of complex V, resulted in dysmorphic features, neurological involvement and 3-methylglutaconic aciduria. Another gene causing isolated complex V deficiency is *TMEM70*, a gene encoding a transmembrane mitochondrial protein. Mutations in this gene can result in encephalo-cardiomyopathy, infantile onset cataracts, dysmorphism and 3-methylglutaconic aciduria [50].

2.6. Mutations in nuclear genes resulting in mtDNA depletion syndromes (MDDS)

A number of nuclear gene products are also responsible for maintenance of mitochondrial integrity, mtDNA biogenesis and maintenance of dNTP pools. Mutations in these nuclear genes result in mtDNA depletion syndromes (MDDS), which are usually autosomal recessive disorders, however an autosomal dominant inheritance pattern has been observed with mutations in *POLG* and *C10orf2* [51,52]. At present, mutations in at least nine genes (*TYMP*, *POLG*, *DGUOK*, *TK2*, *SUCLA2*, *MPV17*, *SUCLG1*, *RRM2B*, and *C10orf2*) may result in mtDNA depletion with somewhat tissue-specific involvement [53–57]. Features associated with mtDNA depletion include progressive muscle weakness or liver failure, or multisystem involvement with lactic acidosis. In one report, the affected individuals in three consanguineous kindreds first presented with MDDS in the neonatal period and died during the first year of life [58]. However, in general with MDDS, the age at onset and clinical presentations are very variable [57]. Consistent with this clinical heterogeneity, the tissues most often affected in this syndrome include either muscle or liver, however, other affected tissues include brain, heart and kidneys [58].

2.7. Mutations in genes involved in mitochondrial protein synthesis

There are a number of nuclear genes that encode proteins which are involved in regulation of mtDNA transcription, or the translation and maintenance of the assembly and stability of the MRC subunits. These include genes which encode ribosomal proteins, aminoacyl-tRNA synthetases, tRNA modification enzymes, rRNA base-modification enzymes, and elongation and termination factors [47]. Mitochondrial protein synthesis is affected by mutations in these genes, and the patients usually present with multiple RC deficiencies and a range of clinical phenotypes (see Table 4).

Table 4
Nuclear genes with mutations affecting mtDNA protein synthesis.
Adapted from [114,46].

Genes	Clinical presentation
AARS2	Infantile cardiomyopathy
DARS2	Leukoencephalopathy with brain stem and spinal cord involvement, lactate elevation
EARS2	Leukoencephalopathy with thalamus and brain stem involvement, lactate elevation
FARS2	Mitochondrial encephalopathy
HARS2	Ovarian dysgenesis, sensorineural hearing loss
MARS2	Spastic ataxia with leukoencephalopathy
RARS2	Pontocerebellar hypoplasia
SARS2	Hyperuricemia, pulmonary hypertension, renal failure in infancy, alkalosis
YARS2	Myopathy, lactic acidosis and sideroblastic anaemia (MLASA)
MRPL3	Psychomotor retardation
MRPS16	Early-onset encephalopathy
MRPS22	Early-onset encephalopathy, microcephaly
PUS1	Myopathy, lactic acidosis and sideroblastic anaemia (MLASA), exercise intolerance
EFTu	Infantile encephalopathy
EFG1	Hepatoencephalopathy
TSFM	Hypertrophic cardiomyopathy, encephalopathy
TRMU	Hepatic failure
TUFM	Leukoencephalopathy

3. Diagnosis of MRCDs

Diagnosis of MRCDs is challenging because of the considerable clinical variability. While some individuals identified with MRCD display a cluster of clinical features that fall into a defined clinical syndrome, many individuals do not. To reach an accurate and reliable diagnosis it is essential to utilise a combination of clinical, laboratory, pathological, biochemical, and genetic investigations [59].

3.1. Initial investigations

Patients suspected of a having a MRCD based on clinical features undergo a series of preliminary investigations, usually beginning with biochemistry and imaging.

3.1.1. Biochemistry

Biochemical tests are routinely done as the first line of diagnosis however in some cases, they may return normal results even in the presence of MRCD [60]. Initial investigations usually involve measuring the levels of lactate, pyruvate and ketone bodies in blood, and their relative molar ratios, especially lactate: pyruvate ratios, which give an indication of the oxidation–reduction status of the cytoplasm and mitochondria, and whether a potential MRCD exists [2]. Disturbance at any step in the intra-mitochondrial oxidation of pyruvate at the level of the pyruvate dehydrogenase complex (PDHC), the tricarboxylic acid cycle (TCA), or the electron transport chain (ETC) will cause an overproduction of pyruvate to compensate for a lack of ATP [61]. Excess pyruvate can then be transaminated to alanine or reduced to lactate, however, an elevated lactate concentration (>2.5 mM), with an increased lactate: pyruvate ratio (>20:1) and an elevated 3-hydroxybutyrate:acetoacetate ratio (>2) are highly suggestive of a MRCD, whereas a defect of PDHC usually results in low lactate: pyruvate ratios (<10) [62]. In some instances, patients with mitochondrial encephalopathy should have lactate measured in cerebrospinal fluid (CSF), as blood biochemistry may show normal levels of lactate and pyruvate, whereas the CSF will more often have increased lactate values (and an increased lactate:pyruvate ratio) [61]. In some instances measurement of basal lactate or pyruvate concentrations may be inconclusive. Therefore, if a strong suspicion of a defect exists, provocative tests may then be carried out, using glucose loading test to unmask an exaggerated lactate build-up. As multiple organ involvement is a key feature of the clinical presentation of MRCDs, all possible target organs and tissues should be screened irrespective of the presenting symptom [2,4]. The cytokine FGF-21 is a relatively new potential biomarker for mitochondrial disorders. It is involved in lipid metabolism, and is induced on starvation [63]. A study by Suomalainen et al. showed that the levels of FGF-21 were high in patients with muscle-specific mitochondrial disorders, with no increase in individuals with non MRCD muscle disease or in healthy controls. Compared to other conventional biomarkers, FGF-21 is more specific and sensitive in identifying MRCDs, and may reduce the need for muscle biopsies [63,64].

3.1.2. Imaging

Patients with a mitochondrial encephalopathy may have characteristic CT and MRI patterns, pointing to a MRCD. Patients with an encephalopathy often show cerebral and cerebellar atrophy, for example in LS, and may also show bilateral signal hypersensitivities in the basal ganglia and brainstem. MELAS patients typically display stroke-like lesions in the posterior regions of the cerebral cortex, and especially the occipital region, and may also show basal ganglia calcification [28]. KSS patients characteristically have diffuse signal abnormalities in the central white matter and also show basal ganglia calcification. Leukoencephalopathy patients with NUBPL and complex I deficiency have a very typical and unique MRI pattern, which makes diagnosis of these cases much faster [65]. Brain proton magnetic resonance spectroscopy (MRS) has proved to be a useful and non-invasive test for patients with MRCDs, being able to detect an abnormal lactate peak, even in cases when the blood and CSF lactate levels were normal [66].

3.1.3. Other investigations

In the right clinical context, specific investigations may help point to specific mtDNA mutations. For instance, LS associated with a Wolf–Parkinson–White cardiac arrhythmia suggests the possibility of the m.13513G>A mutation [67].

3.2. Subsequent investigations

The next phase of investigation often involves biopsy samples for further analyses including histochemistry, electron microscopy and functional assays. The biopsied material may be analysed directly or used to establish cell cultures as a source of material for subsequent assays including molecular investigations. It is at this stage where most patients, particularly children, are diagnosed with an MRCD, usually through a functional measure.

Analysis of muscle biopsies is regarded as one of the more important and reliable means for identifying a MRCD, as many but not all, mitochondrial cytopathies express a defect in this tissue [68].

However, deciding which tissue to investigate should usually be determined by which tissue clinically expresses the disease, and so for a patient with muscle weakness, a skeletal muscle biopsy is the most obvious choice. When a patient presents with a liver disease or a cardiomyopathy, then an open biopsy of the liver or an endomyocardial biopsy respectively should be considered [4]. Where the disease manifests primarily in tissues such as brain, retina, endocrine organs, or smooth muscle, which cannot be readily biopsied, careful and extensive analysis of multiple peripheral tissues such as liver, skeletal muscle, skin fibroblasts and lymphocytes should be carried out [2]. In addition, regardless of the actual primary tissue investigated, it is essential to also obtain a skin fibroblast biopsy to establish a cultured skin fibroblast line for further diagnostic studies, in particular where prenatal diagnosis may be contemplated, and also for molecular-genetic analysis [2,68]. Enzyme activities may be normal in an organ that does not express the disease clinically, as observed in the case of myopathy, lactic acidosis and sideroblastic anaemia (MLASA), which is a muscle specific MRCD [69]. However, in some biochemical and functional assays a defect may not be identified in fibroblasts, as they express a defect in only 50% of the cases where a defect is demonstrable in skeletal muscle and liver [70].

3.2.1. Histology

Analysis of a muscle biopsy using a variety of histochemical stains is a powerful tool in the diagnosis of mitochondrial myopathies. Frozen sections can be stained with the modified Gomori trichrome stain to reveal ragged-red fibres, a hallmark of mitochondrial dysfunction caused by the proliferation of mitochondria. Staining with SDH, a more sensitive marker for mitochondrial proliferation produces ragged-blue fibres on muscle sections [2,71]. Mitochondrial proliferation can also affect the smooth muscle of intramuscular blood vessels, with detection made easier using the SDH stain, and is characteristically seen in MELAS [72,73]. COX shows reduced staining where there is a mtDNA mutation affecting protein synthesis or a mutation in the mtDNA COX genes. On the other hand, SDH staining does not show any abnormalities in disorders caused by primary mtDNA mutations, as complex II is encoded only by nuclear genes. Therefore, the combination of COX-negative fibres seen in association with SDH ragged-red blue, is suggestive of a mtDNA mutation affecting protein synthesis. On the other hand, COX-positive staining with SDH ragged-blue fibres is suggestive of a mtDNA mutation in a protein-encoding gene (but not COX) [3].

3.3. Electron microscopy

Electron microscopy (EM) can reveal subsarcolemmal accumulation and ultrastructural changes of mitochondria, which may include enlarged pleiomorphic mitochondria and paracrystalline inclusions, which can be helpful in pointing to an underlying mitochondrial defect. However, EM would need to be used in combination with other diagnostic modalities, as the abnormalities identified with EM are not specific to MRCD [73,74].

3.4. Biochemical analyses

Biochemical analysis involving measurement of functional defects in the MRC probably remains the most useful and common way of confirming pathogenicity. This can be done either through spectrophotometric enzyme assays or polarographic studies, each providing considerable value to the diagnosis of MRCD. Samples that can be analysed include isolated mitochondria or tissue homogenates prepared from biopsied tissues (skeletal muscle, liver, heart) and/or cultured cells (fibroblasts, lymphoblasts and chorionic villus sampling cells [70,75]. Unlike histochemical studies, biochemical studies of the RC enzymes enable quantitation of the defect, thus providing a measure of the severity of the functional defect and/or the number of enzymes affected [11].

Functional evaluation of a MRCD using polarography is useful for obtaining an overall estimation of OXPHOS activity, whereas spectrophotometric assays measure the activity of individual RC complexes [76]. Polarographic studies measure oxygen consumption of enriched mitochondrial fractions in the presence of various oxidative substrates. A sufficient sample (400 μg to 500 μg of protein) can be obtained from small muscle biopsies (100 mg to 200 mg [77]), lymphocytes and also detergent-permeabilized cultured cells (including lymphoblasts and fibroblasts), thus making polarography feasible in infants and children. Polarography can also be used to evaluate other mitochondrial metabolic disorders, including PDH deficiency and TCA enzyme defects, as well as defects of carriers, shuttles and substrates like cytochrome *c* [62]. However, a disadvantage of using polarographic assays is that in most patients with mitochondrial disorders, there is an overpopulation of mitochondria which may result in an increased electron transport chain activity, masking the deficiency [76]. Another limitation of these techniques is that prepared tissue has to be handled carefully, to ensure that mitochondria are kept intact, and therefore investigations can only be carried out using fresh tissue or cellular material [70].

Spectophotometric assays measure the individual activity of the respiratory chain enzyme complexes by assaying the oxidation/reduction of various substrates or substrate analogues [75]. Unlike other functional studies, enzyme assays can be performed using fresh or frozen material, and the sample material can be from isolated mitochondrial fractions or whole-cell or tissue homogenates [62], with the exception being complex V where assays on frozen tissues fail to give reliable results [78]. However, samples need to be frozen immediately after collection and stored at −80 °C to minimise deterioration of enzyme activity which can result in a false positive result [76]. If stored in this way, activities are stable for over a decade [62,70]. Since MRCD usually result from defects in either isolated or combined RC complexes, analysis of the activity of the complexes is an important diagnostic step in the assessment of a patient suspected to have a MRCD [75].

Although a biochemical defect does not always correlate with the clinical phenotype, certain RC enzyme profiles may provide useful diagnostic clues as to the underlying genetic defect responsible for the MRCD. Isolated defects of complex I, complex III or complex IV activity suggest a mutation in either an mtDNA or nuclear DNA encoded subunit of the defective respiratory chain complex. Another possibility may be that the defect is in a nuclear gene, which encodes an ancillary or assembly protein, involved in the formation or maintenance of the MRC complex [61]. On the other hand, combined defects of complexes I, III and IV, but not complex II suggest a mutation in a mitochondrially encoded tRNA or rRNA gene, affecting mitochondrial protein synthesis, or in a nuclear encoded gene affecting mtDNA replication or maintenance, resulting in mtDNA depletion or multiple mtDNA deletions [61], or mutations in nuclear genes encoding proteins involved in mitochondrial protein synthesis and/or maintenance.

Though spectrophometric assays are robust and reliable there are many factors that complicate enzymatic diagnosis. Some defects can cause a proliferation in mitochondrial numbers, seen histochemically in skeletal muscle as ragged-red fibres, usually as a mechanism to compensate for a decrease in ATP production. A MRCD can also be influenced by secondary factors, which can alter mitochondrial numbers and RC activity, particularly in skeletal muscle. These include inactivity with age, fitness, immobility and variation in fibre-type composition [70]. However, this variation in mitochondrial numbers can also result in increased or decreased RC enzyme activity, possibly masking or "creating" a RC defect. To adjust and normalise for this under- or overcompensation, MRC enzyme activities are often described as a ratio relative to citrate synthase, a nuclear-encoded enzyme marker of mitochondrial abundance [61,70]. Another issue is that it is very difficult to define a reference range for RC enzyme analysis, as it tends to be very broad for some analytes in certain tissues, and there is often overlap between controls and patients [76]. This may be explained by the observation that, unlike other inborn errors of metabolism in which the mutant enzyme may have virtually no activity, MRC enzyme defects often have significant residual enzyme activity [79,80].

Another factor which complicates diagnosis using spectrophometric assays, is the tissue-specific manifestation of RC enzyme defects, whereby a defect may be expressed in one tissue (e.g. skeletal muscle) but not in other tissues (e.g. fibroblasts or liver). Therefore a normal result in RC enzyme activities in one tissue does not exclude the possibility of a defect in others. The choice of tissue is usually based on one which can be obtained in the least invasive manner such as blood or skin fibroblasts [9].

Blue native gel electrophoresis is another useful diagnostic tool, which is a separation method with high resolution, allowing analysis of OXPHOS complexes in their native form i.e. monomeric (complexes I, II, IV, and V) or dimeric forms (complex III). This method can be used to analyse the size, assembly, relative abundance, and enzyme activity of the individual complexes [81,82].

3.5. Somatic cell investigations

The development of the mtDNA-less ρ^0 cell line, and subsequent in vitro somatic cell studies involving production and functional evaluation of transmitochondrial cybrids, have been critical in determining the pathogenicity of mtDNA mutations. This system ensures that the nuclear genetic complement is held constant, such that any changes detected in the functioning of the OXPHOS can be linked to the introduced mtDNA [83]. The primary application of the transmitochondrial cybrid technique has been to characterise the impact of a mtDNA mutation and associated defects on mitochondrial function, including mtDNA transcription, translation, RC activity and ATP synthesis. Transmitochondrial cybrids have also been used to garner evidence as to the likely genetic origin of a MRCD [84]. Various types of mtDNA mutations have been investigated using this technique, including tRNA point mutations associated with the common MELAS mutation m.3243A>G, and the m.8344A>G mutation with MERRF. Cybrid studies showed that tRNA mutations impaired both protein synthesis and MRC enzyme activity at mutant loads greater than 90% [85,86].

3.6. DNA based testing

With the very broad variability in the clinical presentations of MRCDs, biological issues such as tissue specificity, and technical issues relating to functional analysis of the MRC, accurate diagnostic screening of MCRDs still remains one of the most difficult challenges in the field of genetic metabolic disorders [87]. Genetic screening in combination with

data from clinical phenotype, family history, histochemistry, electron microscopy and biochemical functional assays provides a more definitive diagnosis, leading to the development of scoring systems in adults [88] and children [79,89] for the classification of the likelihood of having a MRC. In some instances the family history will point to the likely genetic origin, nuclear or mitochondrial, of the MRCD, whilst the clinical and neuroradiological picture, histology and biochemical studies may suggest the possible underlying specific genetic defect. For example, a child with LS and isolated complex IV deficiency affecting all tissues is very likely to have mutations in the *SURF1* gene [90]. Initial mutation detection in mtDNA may involve direct sequencing or PCR and restriction fragment length polymorphism (RFLP) analysis to screen for specific point mutations associated with several "classic" syndromes, including MELAS (m.3243A<G), MERRF (m.8344A<G), NARP/MILS (m.8993T<G or m.8993T<C) and LHON (m.11778G<A). Disorders caused by mtDNA rearrangements (single large deletions, large duplications, and multiple large deletions) can be detected by Southern-blot analysis, long-range PCR or real-time PCR protocols, which amplify across the major arc of the mitochondrial genome, the site of most mtDNA deletions [23,91].

Screening for less frequent or unknown mutations can be undertaken using a number of molecular methods including: direct sequencing, RFLP, denaturing high performance liquid chromatography (dHPLC) analysis, Southern-blot, and chip arrays [61,92,93]. Although considered the gold standard for the identification of mutations, direct sequencing of mtDNA should be interpreted with caution, as it does not detect large deletions, and, importantly because of the low sensitivity of sequencing in detecting low level heteroplasmic (<20%) mtDNA mutations [9,93,94]. In addition, care should be taken when annotating a variant as pathogenic because of the high frequency of single nucleotide polymorphisms which may not be of pathogenic significance [59]. Sanger sequencing is still used as the first line of diagnosis to identify nuclear encoded mutations in genes known to cause MRCDs. However, because of the clinical and genetic heterogeneity of MRCDs, there is still a large proportion of individuals with a MRCD where the underlying genetic cause is unknown, and selection of candidate genes and sequential Sanger sequencing can be time consuming and expensive.

3.7. Next generation sequencing (NGS) for the diagnosis of MRCDs

NGS has revolutionised genomic science by facilitating gene screening spanning the entire genome in a cost effective and efficient manner. NGS has allowed the discovery of more than 50 new genetic disease genes in recent years, and this technology is now being applied more often for the clinical diagnosis of Mendelian disorders. It has proven to be useful for screening not only well characterised genes, but also for novel MRC disease gene identification [93,95].

Whole genome sequencing, though proven to be an efficient strategy to identify genetic variants leading to rare or common diseases, is still too expensive an approach to be used routinely [96]. Whole exome sequencing (WES) on the other hand is a much more cost effective alternative, and also a reasonable approach, as coding exons harbour most functional variations [97]. However WES sequencing has its own disadvantages, including the fact that it will not detect potentially pathogenic deep intronic mutations or functional variants in the 5′ UTR and 3′ UTR regulatory regions of genes. Moreover, most current bioinformatics resources are poor at characterising insertion-deletions and accurate analysis of homopolymeric runs. Finally, current capture methods are imperfect, so some exons will not be well represented, and if the fold coverage per exon is not sufficiently high WES will also fail to detect whole exon deletions [98,99]. These limitations can make selection and identification of the candidate disease gene more challenging [87]. Targeted sequencing of the "MitoExome" which involves sequencing the entire mitochondrial genome and all nuclear genes encoding mitochondrial proteins (based on the Mitocarta dataset) has been a successful approach to identify known and novel disease causing genes [95].

Several groups have undertaken large cohort studies and demonstrated the diagnostic utility of the NGS techniques. Calvo and colleagues sequenced the MitoExome of 42 unrelated patients who had no genetic diagnosis for mitochondrial disorders, but who had clinical and biochemical evidence characteristic of an infantile OXPHOS disorder. These studies resulted in the identification of known reported mutations in ten patients, and 13 novel recessive mutations. *NDUFB3* and *AGK* were among the novel disease genes identified whose pathogenicity was confirmed [95]. Vasta et al. also performed a similar study using targeted next generation sequencing in 26 patients with either known or suspected MRCD. In this study, the panel for targeted sequencing was expanded to include 908 nuclear genes, including genes which could cause secondary inhibition of the MRC. The interesting finding of this study was though several of the patients had multiple RC complex deficiencies none of the identified mutations affected the subunits or assembly of the RC complex enzymes. They identified two probable novel candidate genes *SLC7A13* and *MTO1* which still need functional validation [87]. Lieber and colleagues performed a large cohort study, with 102 patients screened, some of whom already had a molecular diagnosis. The approach was efficient in accurately recovering 94% of the previously identified molecular diagnoses; however the outcome was relatively low for the patients with unknown genetic diagnosis. Only five out 84 (6%) patients had a new molecular diagnosis, which included the genes *NDUFV1*, *POLG2*, *DPYD*, *KARS* and *WFS1* [100]. The discrepancy in the detection rate between these studies is most likely a reflection of the rigour with which a clinical diagnosis of a MRCD was initially established, highlighting the key role which current non-molecular diagnostic methods still play. NGS has been proven to be efficient approach which can be applied to clinical diagnostics, however care should be taken to clearly validate and confirm the functional pathogenicity of novel candidate disease genes [93].

3.8. Approaches to mitochondrial therapy

The current focus with regard to treatment and management of mitochondrial disorders is mainly on providing support to ameliorate symptoms, improving quality of life, physical therapy, and management of the complications that arise secondary to the disorder itself [101]. The approaches to treatment are varied and include metabolic manipulation using nutriceuticals, altering the balance between mutated and normal mtDNA, enzyme replacement therapy, regulation of specific gene activators and gene therapy. However, as therapeutic benefits are limited, prenatal diagnosis to prevent recurrences is an important option. The performance of clinical trials and deriving conclusions from them can be challenging, because in some cases even though the subjects selected for the trials have the same genotype, they may show variability in their phenotypic presentation and evolution [102]. Another factor to be considered is that some symptoms like stroke-like episodes in MELAS patients may spontaneously resolve without specific therapy [103] and this could affect the outcome measures of clinical trials.

3.9. Metabolic manipulation

A defect in mitochondrial functioning may result in increased free radical production. These free radicals potentially have damaging effects by increasing oxidative stress, which has been implicated as a contributing factor to disease progression in patients with MRCDs [104]. Metabolic manipulation involves modifying the nutritional composition of the diet through supplementation with vitamins and co-factors which could have an antioxidant effect. A number of antioxidants, including vitamin C, vitamin E, beta-carotene and zinc in different combinations have been trialled with some appearing to have beneficial effects in patients with MRCDs such as LHON [104,105]. There have been several clinical studies evaluating the effect of coenzyme Q10. Though they have been shown to have some benefit, none have demonstrated a benefit of clinical relevance. However, coenzyme Q10 has gained popularity

in the treatment of mitochondrial disorders for its ability to reduce free radicals, and also because it has a well-documented safety record even when administered at high doses [106].

MRCDs are often characterised by increased lactate levels, which is due to the shift towards the anaerobic pathways to generate energy. Creatine, an endogenously synthesised compound, functions as an alternate energy source, and has been shown to be of benefit in some neurodegenerative disorders, with a demonstrated neuroprotective effect in animal models. However, there are conflicting reports on its beneficial effect, and it has been suggested that is more likely to be effective as a combination therapy along with other vitamins and cofactors [104]. L-arginine is known to dilate blood vessels, increasing blood flow, through the generation of nitric oxide. A recent clinical trial with L-arginine was shown to have beneficial effects in MELAS patients by decreasing the frequency and the severity of acute stroke-like episodes [106–108]. There is however a need for a well-controlled long term clinical trial of L-arginine to evaluate it benefits and assures its safety during long term use [102]. Citrulline supplementation, which increases arginine levels, has been suggested to have superior therapeutic benefits compared to arginine [109].

For some individuals, the ketogenic diet has proved to be effective, particularly in reducing the frequency of intractable seizures, and has been considered to be safe even for children with mitochondrial disorders [110]. It has been suggested that the ketogenic diet may play a role in inducing mitochondrial biogenesis, thereby slowing disease progression, or by the virtue of the ketones generated being used as an alternate energy source for brain and muscle [111].

3.10. Altering the balance between mutated and normal mtDNA

For MRCDs caused by a primary mtDNA mutation, reducing the mutant mtDNA load may help in recovering mitochondrial function. The ketogenic diet has been shown to reduce the proportion of mtDNA deletions in cybrid cell lines [112]. This can also be achieved genetically. Another strategy is by targeting the novel restriction sites introduced by some mutations, for example a *SmaI* site in the *MTATP6* gene caused by the m.8993T>G mutation that causes MILS. In recent years new technologies such as zinc fingers and transcription activator like (TAL) effectors, which can be modified to selectively bind specific DNA sites, are other approaches which could be used to target mutated mtDNA for degradation [113].

3.11. Enzyme replacement

MNGIE is caused by loss of activity of the enzyme thymidine phosphorylase (TPase) due to mutations in the *TYMP* gene. This results in increased levels of thymidine and deoxyuridine, which in turn result in mtDNA depletion, and/or deletions. Preliminary evidence suggests that restoration of TPase activity, either by gene therapy in a mouse model of MNGIE [114] or by allogeneic hematopoietic stem cell transplantation (ASHSCT) in patients [113] may be of therapeutic efficacy. To date, more than 10 patients have undergone ASHSCT, and though it improves some of the clinical features, the risks associated with ASHSCT are high. A number of the MNGIE patients who underwent ASHSCT were reported to have died, with the mortality rate increased in individuals in who the disease was more advanced. Therefore, the ASHSCT procedure may be more effective with less complications in the early stages of disease [115].

3.12. Regulation of specific gene activators

A number of therapies are aimed at increasing mitochondrial biogenesis. Peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α) is a transcriptional co-activator and has an established role in mitochondrial biogenesis. PGC-1α can be upregulated by increasing exercise [116], and also by drugs such as bezafibrate and resveratrol [117]. COX10 deficient mice transgenically expressing PGC-1α or those treated with resveratrol showed an increase in the number of mitochondria, and improvements in motor function and aerobic capacity, as well as a delayed onset of myopathy and prolonged lifespan [118].

3.13. Exercise as a therapy

Patients with mitochondrial myopathy are usually characterised by exercise intolerance. However, endurance training has been shown to improve their oxidative capacity. A study by T.D. Jeppesen and colleagues showed that training not only increased VO_{2max} in the patients, but some individuals even showed an increase in wild type mtDNA and a decrease in the muscle mtDNA mutation load [119]. The decreased mtDNA mutation load could be explained by the fact that eccentric exercise leads to activation of satellite cells (precursors to skeletal muscle cells) do not harbour mtDNA mutations. This activation in turn leads to regeneration of the muscle fibres without mutant mtDNA, which then potentially decreases the mutational burden, a process called mtDNA shifting [120].

3.14. Emerging therapies

As indicated above, CoQ10 is a popular therapeutic. An analogue of CoQ10 idebenone, with better bioavailability was developed, and its administration has led to improvement in patients with MELAS, LHON and Friedreich ataxia. Another synthetic analogue of CoQ10, EP1-743 was then developed which is reported to be much more potent and to have a favourable safety profile. In an open label study which included near terminal patients with MRCDs (*POLG*, LS, MELAS, and other mtDNA deletion related disorders) and Friedreich ataxia, twelve out of the fourteen patients survived beyond the expected 90 days. They also showed improvement in their Newcastle Paediatric Mitochondrial Disease Scale score (NPMDS) (NPMDS allows evaluation of the progression of mitochondrial disease) and brain SPECT scans (scans which can assess blood flow and activity in the brain) [121]. Similar results were seen in another study conducted by Martinelli et al., where all treated patients with LS treated showed significant improvement in their NPMDS score [122]. Based on these two preliminary studies and the apparently very good safety profile, EPI-743 has progressed to phase 2 trials in children with LS (http://clinicaltrials.gov/ct2/show/NCT01642056).

3.14.1. Gene therapy

There have been a few attempts in recent years to use gene therapy as tool to treat MRCDs, with the main challenge being the import of the normal copy of the gene of interest into mitochondria [123]. One of the approaches has been to use allotopic expression where the mutant mitochondrial gene is recoded to the universal (i.e. nuclear) genetic code and protein synthesised in the nuclear cytosolic compartment. This is followed by import of the protein into the mitochondria using a mitochondrial targeting sequence. In this case the gene was MT-ND6 (mutation in this gene leads to complex I deficiency). This resulted in restoration of the assembly and activity of complex I, however the allotopically expressed protein was not fully internalised into the mitochondria [124]. Another example of gene therapy using allotopic expression has been with the gene MT-TL1 which codes for mt-$tRNA^{Leu(UUR)}$. The m.3243A>G mutation in this gene is the main cause of MELAS syndrome. This study was done in human transmitochondrial MELAS cybrids, where a recombinant tRNA with a mitochondrial-targeting signal was able to rescue oxidative defects and levels of the RC complexes containing mtDNA encoded subunits [125]. Other approaches have been to use alternatives of the OXPHOS complexes, such as AOX (cyanide insensitive alternative oxidases) from sea squirt as an alternative to the defective human COX, and yeast soluble NADH oxidase as an alternative to the defective human complex I. Allotopic expression in both the above cases have been shown to rescue the

OXPHOS deficiencies [126]. All these studies are still in their early stages, and the generation of animal models to study the efficacy of these gene therapy techniques would give a clearer understanding of their translation into the clinical setting.

3.15. Challenges with mitochondrial disorders

MRCDs are some of the most clinically and genetically heterogeneous group of disorders. The broad spectrum of the clinical features, affecting physical abilities and/or cognitive function, may be static in some in some individuals but progressive in others. Currently diagnosis remains challenging and usually relies on a combination of biochemical, radiological, histological and functional tests. There is a need to develop more robust diagnostic markers like FGF-21, which will improve the accuracy and specificity with which diagnosis can be made.

Identification of pathogenic mutations by DNA sequencing may provide a more definitive diagnosis, however factors such as complex inheritance patterns (Mendelian versus maternal inheritance), heteroplasmy and tissue specificity all contribute to increased complexity of diagnosis. The advances in next generation sequencing technologies will increase the efficiency and accuracy with which diagnosis can be made [127]. Performing biopsies (both under general and local anaesthesia) in patients with MRCDs is associated with a number of risks which include seizures, strokes, respiratory difficulties and in some cases coma and death [128,129], hence NGS may obviate the need for invasive biopsies in at least some cases. Over the years, the management of MRCDs has improved, with a number of new trials currently underway. For the same reasons that make diagnosis difficult, evaluation of the effectiveness of treatments has also been a challenge, complicated by the lack of objective outcome measures. The number of mouse models for mitochondrial diseases is steadily growing, however there is still a great need for more diverse mouse models to better understand the pathophysiology of this complex group of disorders, and for effective preclinical therapeutic trials to be developed.

Therefore there is an urgent need for further research involving the development and validation of new therapeutic agents, the development of definitive objective endpoints to measure functional changes, so that more robust clinical trials can be established, with the primary goal being to improve the range of effective therapies for the MRCDs [130].

4. Conclusion

Since the first report of mitochondrial abnormality causing disease in humans [8], with the advances in technology and extensive research over the years, the diagnosis and management of MRCDs has become better defined, more accurate and less invasive. The approach to definitive diagnosis will still require integration of all levels of investigations. However, with the development of new biomarkers like FGF-21, modern approaches like MRS lactate and NGS techniques will make the diagnostic process less lengthy. Though the approaches to treatment of MRCDs are still largely aimed at lessening the secondary complications associated with the disorders rather than halting disease progression, a genetic diagnosis is invaluable for genetic counselling, allowing also an option for prenatal diagnosis for subsequent pregnancies.

Extensive research in the areas cell biology, molecular biology and biochemical approaches to investigate the underlying mechanisms of MRCDs will allow the development of more targeted therapies. Gene therapy, though still in its early stages, with no trials in multicellular organisms to date, still holds promise of a new possible approach to treatment of this rather complex and challenging group of disorders.

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