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Leading Edge
Review

Mitochondrial Diseases: Hope for the Future

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Mitochondrial diseases are clinically heterogeneous disorders caused by a wide spectrum of mutations in genes encoded by either the nuclear or the mitochondrial genome. Treatments for mitochondrial diseases are currently focused on symptomatic management rather than improving the biochemical defect caused by a particular mutation. This review focuses on the latest advances in the development of treatments for mitochondrial disease, both small molecules and gene therapies, as well as methods to prevent transmission of mitochondrial disease through the germline.

Mitochondrial diseases are a group of complex metabolic disorders that are defined by a genetic defect predominantly affecting mitochondrial oxidative phosphorylation (Gorman et al., 2016). These diseases are challenging both from a clinical and a treatment perspective since they can affect virtually any organ and may present at any age. They are further complicated by the involvement of both nuclear and mitochondrial genes coding for essential mitochondrial proteins. This review focuses on recent progress in treatment and prevention of mitochondrial diseases.

Challenges for Treatment of Mitochondrial Disease

Biochemical and Genetic Challenges

Mitochondrial diseases are among the most common genetically determined metabolic diseases (Gorman et al., 2015). Oxidative phosphorylation (OXPHOS), the main source of ATP generation in cells, occurs at the inner mitochondrial membrane. There are five complexes that are directly involved in OXPHOS, three of which pump protons into the intermembrane space (complexes I, III, and IV), and complex V uses the electrochemical gradient to generate ATP from ADP and Pi. However, OXPHOS depends on many other pathways to function efficiently and thus can be affected by a broad range of genetic defects.

Mitochondrial diseases are the only genetic conditions in humans that have involvement of two different genomes. mtDNA is a small circular genome that encodes 13 mitochondrial proteins (all components of the OXPHOS system), 22 mt-tRNAs, and 2 mt-rRNAs. However, the vast majority of mitochondrial proteins (>1,200) are nuclear encoded and transported into mitochondria. Not all nuclear mitochondrial proteins are involved in OXPHOS but many are involved in OXPHOS complex assembly, mtDNA replication, expression and repair, and other metabolic pathways.

mtDNA genetics is complex due to multiple copies of mtDNA being present in each cell. Mutations can be either homoplasmic with essentially all mtDNA affected or heteroplasmic with a mixture of mutated and wild-type mtDNA present (Taylor and Turnbull, 2005). Mutations in mtDNA are functionally recessive and thus even in the presence of mutated mtDNA, a biochemical phenotype is only observed if the levels of mutated mtDNA reach a critical threshold. There are many large-scale deletions and point mutations of mtDNA described (Lott et al., 2013). The threshold for biochemical deficiency varies for each mutation and cell type and may even be different for the same mutation in different patients. However, a pathogenic mutation is normally required to reach a high heteroplasmy (>70%) before a deleterious biochemical phenotype is expressed at a single-cell level. The clinical phenotype can also vary markedly in patients with the same mtDNA mutation at apparently similar levels of heteroplasmy. A good example of this is m.3243A > G disease where there is marked phenotypic variability and this is likely to reflect nuclear genetic factors influencing the expression of disease (Pickett et al., 2018).

Until recently, establishing a genetic diagnosis for many patients was challenging, especially those with nuclear gene mutations. However, with the advent of accessible next generation sequencing there has been an explosion in the discovery of new genetic defects affecting OXPHOS, with pathogenic mutations in over 300 genes identified to date (Frazier et al., 2019; Thompson et al., 2020). The identification of specific genetic defects is important as it not only gives insights into the underlying disease mechanism for that particular patient but also may highlight the potential importance of patient specific treatments not considered previously (Gerards et al., 2011; Hirano et al., 2012; Repp et al., 2018).

Determining the prevalence of mitochondrial disease has been challenging and subject to incomplete case ascertainment because of the varied clinical features, genotype-phenotype heterogeneity and intricacy of referral care pathways. Several studies have shown that nuclear genetic defects are the main cause of childhood mitochondrial disease whereas mtDNA mutations are more prevalent in adult cases. For childhood-onset mitochondrial disease (<16 years of age) estimates have varied from 5–15 cases per 100,000 of the population (Skladal et al., 2003; Ryan et al., 2006). For adults, the prevalence of mtDNA disease has been calculated at 9.6 cases per 100,000

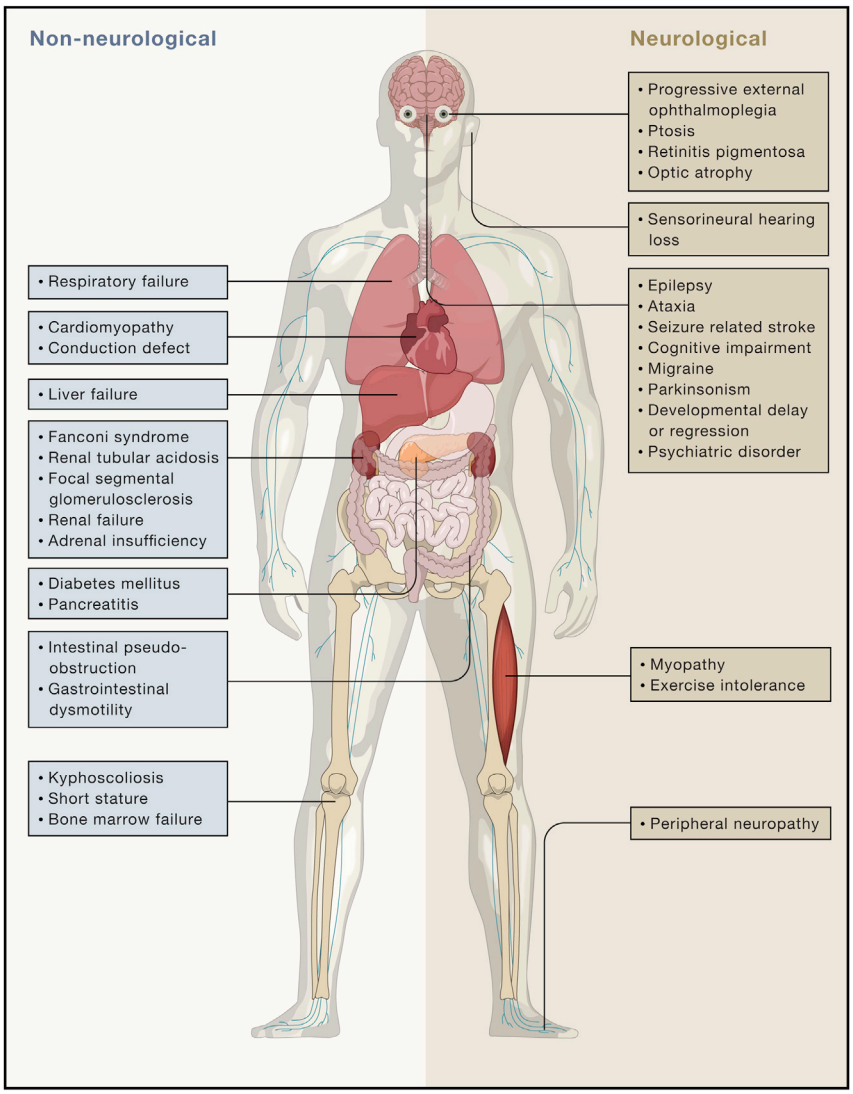

Figure 1. Clinical Features of Mitochondrial Disease

Mitochondrial dysfunction can cause a range of neurological and non-neurological symptoms. The spectrum of tissues involved varies between the mutation (mtDNA or nDNA), heteroplasmy, and age of onset and thus makes it difficult to predict disease progression.

with another 10.8 cases at risk because a first degree relative was affected. For nuclear genetic causes, the prevalence was 2.9 cases per 100,000 (Gorman et al., 2015).

Clinical Challenges

One of the major challenges in terms of treatment is the extremely varied phenotype-genotype relationship seen in patients with mitochondrial disease (Gorman et al., 2016) (Figure 1). This is reflected not only in the involvement of different organs, but also in the severity of disease. Children may be affected by a severe neurodegenerative condition called Leigh's syndrome (subacute necrotizing encephalomyelopathy), but even with this syndrome, the prognosis varies markedly depending on the underlying genetic defect (Lake et al., 2016). Adult onset disease is typically less severe and includes symptoms such as chronic progressive external ophthalmoplegia, deafness, and diabetes, but it may also manifest as relentlessly progressive seizures and stroke-like episodes culminating in a neurodegenerative dementia syndrome.

While this review will focus on the development of specific treatments, it is important to recognize that patients with mitochondrial disease require symptomatic treatments, which may improve quality of life and potentially increase life expectancy. These treatments are often organ-specific, for example use of anticonvulsants for mitochondrial epilepsy, insulin, or oral hypoglycemic for diabetes, or cardiac pacemakers and implantable electronic devices for patients with cardiac conduction disorders. There are several expert opinion guidelines available (https://www.newcastle-mitochondria.com/clinical-professional-home-page/clinical-publications/clinical-guidelines/) (Ng et al., 2019), and while the search for better therapies continues, it is crucial that patients access the best possible symptomatic treatment now.

Development of Small-Molecule Therapies

The search for small molecules is an essential component in the development of treatments for mitochondrial disease. Recently, there has been an increased interest in this area, with several biotech companies dedicated to the discovery or refinement of mitochondrial therapeutics. This has largely been fueled by recent regulatory and financial incentives aimed at invigorating investment in orphan drug discovery to treat such disorders (Field and Boat, 2010). Although mitochondrial disease is relatively rare when compared to other neurological disorders, modulation of mitochondrial function can also play a key role in decreasing the pathogenesis of diseases such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Huntingdon's disease, and Friedrich ataxia (Murphy and Hartley, 2018). Indeed, metabolic syndromes such as obesity, diabetes, and non-alcoholic fatty liver disease may also represent targets for mitochondrial modifiers. Although we discuss small molecules that are designed to improve primary mitochondrial disease, it is likely that their use will not be limited to these disorders, and should be considered in the wider context of syndromes with mitochondrial involvement.

Manipulating Cell Content of Mitochondria

It has often been argued that increasing the level of mitochondrial mass in cells or animals with mitochondrial dysfunction could be beneficial (Wredenberg et al., 2002; Whitaker et al., 2016), but how can such an increase be achieved? First, mitochondrial biogenesis could be upregulated and second, mitochondrial turnover (mitophagy) could be reduced. Over the past fifteen years, master regulators of mitochondrial biogenesis have been found, with important players being identified and mechanisms leading to proliferation having been unearthed. In particular, the peroxisome proliferator-activated receptor (PPAR) family of isoforms of fatty acid-regulated nuclear receptors and the transcriptional co-activator of one of these isoforms (and many other nuclear receptors), PPAR-gamma coactivator 1-alpha or PGC1α, are now known to be key mediators of mitochondrial biogenesis, initiating expression of further factors central to mitochondrial function (Puigserver et al., 1998; Scarpulla, 2002). Consequently, bezafibrate, a panPPAR agonist used routinely for treating hyperlipidemia, has been piloted for use in patients with mitochondrial myopathy and used for treating models of mitochondrial dysfunction. Mouse models of cytochrome *c* oxidase (COX) deficiency with late onset multiple mtDNA deletions showed no evidence of induced mitochondrial biogenesis on exposure to bezafibrate (Viscomi et al., 2011; Yatsuga and Suomalainen, 2012). Interestingly, in a mouse model with a mutant mtDNA helicase (Deletor mouse), there was a marked delay of mtDNA deletion accumulation (Yatsuga and Suomalainen, 2012) and improvements in age-related skin and spleen phenotypes (Dillon et al., 2012a). The history of using bezafibrate to successfully treat models of mitochondrial disease has been muddied by the unfortunate retraction of a couple of key papers (Wenz et al., 2016a, 2016b; Noe et al., 2013). However, it is perhaps timely to reconsider its use.

Overexpression of PGC1α can induce mitochondrial proliferation and increase COX activity in the skeletal muscle of a *Surf1*$^{-/-}$ mouse model deficient in cytochrome *c* oxidase assembly (Viscomi et al., 2011). It has also been shown to improve skeletal muscle and heart function in the mtDNA mutator mouse model (Dillon et al., 2012b). The regulation of PGC1α itself is highly complex, with many forms of posttranslational modification, such as multiple phosphorylation and acetylation sites, ubiquitination, methylation, and others (Fernandez-Marcos and Auwerx, 2011), making it a difficult target for pharmaceutical intervention. Upstream activators of PGC1α, such as the AMP-activated protein kinase AMPK or the sirtuin SIRT1 are also central players in regulating mitochondrial biogenesis, being activated themselves either by the adenine nucleotide or NAD^+/NADH balance in the cytosol (Cantó et al., 2009). Activation of AMPK, for example, has been reported to stimulate skeletal muscle metabolism possibly through mitochondrial biogenesis (Winder et al., 2000; Jäger et al., 2007; Egan et al., 2011). Activators such as 5-aminoimidazole-4-carboxamide ribonucleotide (*AICAR*), which in turn can also activate SIRT1, could therefore be considered as a putative therapeutic strategy for mitochondrial disease (Corton et al., 1995; Golubitzky et al., 2011; Viscomi et al., 2011). Indeed, this compound is listed on the World Athletics Anti-doping Agency (WADA) prohibited list due to its potential performance enhancing effects. Metformin, a biguanine analog believed to act by weakly inhibiting complex I (El-Mir et al., 2000; Owen et al., 2000) or mitochondrial glycerophosphate dehydrogenase (Madiraju et al., 2014) has also been implicated as a potential activator of AMPK (Zhou et al., 2001), but there appears to be no clear consensus that metformin can promote mitochondrial biogenesis. Resveratrol, a natural plant polyphenol, has also been reported as an activator of AMPK and SIRT1 (Baur et al., 2006). A double-blind, randomized, placebo-controlled, cross-over trial of resveratrol in patients with mitochondrial myopathy (and skeletal muscle fatty acid oxidation disorders) will report in April 2020 (Table 1).

As part of the regulatory cascade that promotes mitochondrial biogenesis, the nuclear respiratory factors NRF1 and NRF2 are upregulated or translocated to the nucleus where they in turn regulate the expression of numerous genes. Additionally, these products are central to other cytoprotective functions (Piantadosi et al., 2008; Scarpulla, 2008; Dinkova-Kostova and Abramov, 2015). Small molecules that activate NRF2 by preventing its degradation have been identified, thereby potentially increasing mitochondrial biogenesis (for review, see Robledinos-Antón et al., 2019). A recent phase II randomized, double-blind, placebo-controlled interventional study on the efficacy of 12 weeks treatment with one such molecule, the synthetic triterpenoid omaveloxolone (RTA 408) failed to improve the primary outcome measure (peak exercise workload) or secondary outcome (6-min walk test) at week 12 in patients with mitochondrial myopathy (Table 1) (Madsen et al., 2020).

In summary, while the data supporting increased mitochondrial biogenesis as a way of potentially restoring mitochondrial function in patients are strong, to date there is limited evidence to support any small molecule as a therapeutic for mitochondrial proliferation. Exercise and endurance training are the most compelling methods for increasing mitochondrial mass, are safe across a range of systemic outcome measures and appear to cause no harm (Cejudo et al., 2005; Jeppesen et al., 2006). Hence, it is an area that may produce important new therapeutics in the near future.

Restoring NAD^+ Levels

A notable effect in cells harboring defective mitochondria is the dramatic decrease of NAD^+ and the reduction in NAD^+/NADH ratio (Gomes et al., 2013; Cerutti et al., 2014; Khan et al., 2014). NAD^+ is an essential substrate for the function of key proteins such as polyADP ribose polymerase (PARP), cyclic ADP ribose synthetases, and the family of sirtuin deacetylases that have many key pleiotropic functions in the cell (Katsyuba and Auwerx, 2017). To compensate for this decrease, pyruvate, which is produced by glycolysis and accumulates due to a decrease in use by the defective respiratory chain, is reduced to lactate with the concomitant oxidation of NADH by lactate dehydrogenase (Robinson, 2006) (Figure 2). Excess intracellular lactate is then transported out of the cell by the monocarboxylate carrier (Halestrap and Price, 1999), resulting in lactate acidemia, a common hallmark of mitochondrial disease. One activity of SIRT1 is the NAD^+-dependent deacetylation and activation of PGC1α and consequent mitochondrial biogenesis (Cantó et al., 2009). Trying to increase the cellular load of NAD^+ through supplementation with precursors for *de novo* biosynthesis or through manipulation of enzymes involved in its synthesis has become a key

Table 1. List of Recent and Current Clinical Trials

Title	Compound	MOA	Design	Participants	Sample Size	Trial Period (week)	Reference	Status
AIMM	acipimox; 5-carboxyl-2-methyl pyrazine 1-oxide	mitochondrial biogenesis	phase IIa/IIb adaptive design; single center	adults (≥16 years old) m.3243A > G or single large-scale mtDNA deletion, muscle involvement	n = 80 ≤ n ≤ 120;1:1 randomization	12 weeks	N/A	open to recruitment
SPIM-300; RePOWER	elamipretide; D-Arg-dimethylTyr-Lys-Phe-NH2	mitochondrially targeted tetrapeptide; ↓production of toxic ROS and stabilizes cardiolipin	phase II observational case-only (prospective); multicenter	16–80 years old; PMD with signs or symptoms suggestive of myopathy	n = 215	52 weeks	NCT03048617; linked to NCT02544217; MMPOWER	completed; March 2019
MMPOWER-3; SPIMM-301	elamipretide; D-Arg-dimethylTyr-Lys-Phe-NH2	mitochondrially targeted tetrapeptide; ↓production of toxic ROS and stabilizes cardiolipin	phase III; randomized, double-blind, parallel-group, placebo-controlled trial; followed by open label	adults (≥16–80 years old) PMD with signs or symptoms suggestive of myopathy	n = 218	24 weeks (part 1); up to 144 weeks (part 2)	NCT03323749	active; not recruiting
MITO-001	cysteamine bitartrate; RP103; (Procysbi)	anti-oxidant	phase II/III open-label dose escalation and long-term extension	pediatric (2–18 years old) phase II (including Leigh syndrome) childhood mitochondrial diseases	n = 36 (n = 30 completed; part 1) n = 22 (part 2)	24 weeks/ up to 2 years	NCT02023866	terminated (lack of efficacy)
Emergency use protocol for EPI-743 in acutely ill patients with inherited MRCD within 90 days of end-of-life care	vincerinone; EPI-743; quinone oxidation product of alpha tocotrienol	Structurally related to vitamin E	phase II; non-randomized, open label; dose escalation from 50 mg bid to 100 mg tid	>1 year old; genetically confirmed diagnosis of inherited mitochondrial respiratory chain disease	n = 87	13 months	NCT01370447 (previous trials linked NCT01721733 NCT01642056)	active; not recruiting estimated completion Dec 2021
EPI743-13-023: long-term safety and efficacy evaluation of EPI-743 in children with Leigh syndrome	vincerinone; EPI-743; quinone oxidation product of alpha tocotrienol		phase II; open-label	1–18 years old; genetically confirmed Leigh syndrome; completion of EPI743-12-002 protocol; initiation of treatment within 14 days (M12)	n = 31	up to 3 years	NCT02352896	active; not recruiting

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Table 1. *Continued*

Title	Compound	MOA	Design	Participants	Sample Size	Trial Period (week)	Reference	Status
MOTOR	omaveloxolone; RTA-408	potent Nrf2 activator; inhibits NF-κB; induces anti-oxidant and anti-inflammatory phenotype	phase II	adult (18–75 years old) RCD (all forms-nuclear and mitochondrial)	n = 52 (53)	12 weeks/up to 84 weeks	NCT02255422	completed (2018; no results reported)
KHENERGY study	KH176	potent intracellular reduction-oxidation-modulating compound	phase II; double-blind, randomized, placebo-controlled, two-way cross-over	≥18 years old m.3243A > G	n = 20	28 days	NCT02909400; linked to NCT02544217	completed (2018; primary outcome not achieved; positive effect on alertness and mood)
KHENERGIZE study	KH176	potent intracellular reduction-oxidation-modulating compound	phase IIb; double-blind, randomized, multicenter, placebo-controlled, 3-way cross-over	≥18 years old m.3243A > G	n = 27	112 days	NCT04165239	recruiting
Phase Ia/Ib, SAD and MAD study of KL1333 in healthy subjects and patients with PMD	KL1333	NAD^+ modulator	phase Ia/Ib; dose block-randomized, double-blind, placebo-controlled, single-dose, dose-escalation	adults m.3243A > G-related mitochondrial disease	n = 8 patients; n = 24 healthy subjects	15 days	NCT03888716 (linked to NCT03056209)	recruiting
The effect of arginine and citrulline supplementation on endothelial dysfunction in PMD	arginine and citrulline	NO precursors	phase I; randomized, cross-over, open label	pediatric (3–18 years old) mitochondrial disease with multi-organ disease (molecular/ respiratory chain assay abnormalities diagnosis)	n = 10 patients and 10 controls; n = 9 (actual enrolment)	2 week Rx/2 week washout/ 2 week Rx	NCT02809170	active; not recruiting; due to complete (Dec 2019)

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Table 1. ***Continued***

Title	Compound	MOA	Design	Participants	Sample Size	Trial Period (week)	Reference	Status
Trial of coenzyme Q10 in mitochondrial disease	Q10	natural electron carrier	phase III; 10 mg/kg daily up to 400 mg; randomized, cross-over, triple blinded	pediatric (1–17 years old) mitochondrial disease with multi-organ disease (molecular/ respiratory chain assay abnormalities diagnosis)	n = 24	12 months	NCT00432744	completed
Niacin supplementation in healthy controls and mitochondrial myopathy patients (NiaMIT)	niacin	NAD^+ precursor, ↑PGC1α-dependent mitochondrial biogenesis pathway	phase II; 750–1000 mg/day; non-randomized; open label	Single or multiple mtDNA and pure mitochondrial myopathy	n = 15	10 months	NCT03973203	completed
The role of nicotinamide riboside in mitochondrial biogenesis	nicotinamide riboside; vitamin B	NAD^+ precursor, ↑PGC1α-dependent mitochondrial biogenesis pathway	N/A- dietary supplement; open label	adult (18–70 years) mitochondrial disease due to SD or m.3243A > G	n = 5; 10 mg/kg (part 1); n = 10; 10 mg/kg/BD (part 2)	part 1: 24 h (single dose); part 2: 4 weeks)	NCT03432871	recruiting (due to complete Dec 2019)
Resveratrol supplementation in patients with mitochondrial myopathies and skeletal muscle fatty acid oxidation disorders	resveratrol; 1,000 mg/day	increase mitochondrial biogenesis	N/A; dietary supplement; double-blind, randomized, placebo-controlled, cross-over study	Adult (≥18 and ≤ 80 years old) mitochondrial disease/VLCAD and CPTII	n = 20 (MM:10; FAO:10)	Rx:8 weeks/ WO:4 weeks/ Rx:8 weeks) 20 weeks	NCT03728777	recruiting
A phase 2a, open-label study to evaluate the safety, tolerability, and clinical activity of abi-009 (nab-sirolimus) in patients with genetically confirmed Leigh or Leigh-like syndrome	nabsiralimus (ABI-009; rapamycin)	nanoparticle albumin bound sirolimus (rapamycin)	phase IIa; open-label	pediatric: age 2–17 years genetically confirmed Leigh or Leigh-like syndrome	n = 32	24 weeks	NCT03747328	not yet recruiting
An open-label study to evaluate the safety and tolerability of 12 weeks treatment with oral ren001 in patients with pmm; optional extension of treatment	REN001	PPARδ agonist	phase I; open-label, single-dose phase I clinical Study	adults (≥16 years old); mtDNA-related mitochondrial myopathy	n = 24	48 weeks	NCT03862846	recruiting
A feasibility study of bezafibrate in MM	bezafibrate	increase mitochondrial biogenesis	open label	adults (18–64 years old)	n = 6	12 weeks	NCT02398201	completed; results awaited

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Table 1. *Continued*

Title	Compound	MOA	Design	Participants	Sample Size	Trial Period (week)	Reference	Status
A study to evaluate the safety and therapeutic effects of transplantation of mnv-bm-bld in pediatric patients with Pearson Syndrome	$CD34^+$ cells enriched with MNV-BLD	biological; mitochondria augmentation therapy; $CD34^+$ cells enriched with MNV-BLD (blood-derived mitochondria)	phase I/II, open label, single dose clinical study	pediatric patients with Pearson syndrome	n = 7	primary outcome = 1 year; secondary outcome = 2 years	NCT03384420	recruitment by invitation
TEETPIM – Trial of erythrocyte encapsulated thymidine phosphorylase in mitochondrial neurogastrointestinal encephalomyopathy	replacing functional thymidine phosphorylase (ERT)	this treatment uses the patients' own red blood cells in which thymidine phosphorylase is encapsulated to produce EE-TP (the study drug)	phase II; multi-center, multiple dose, open label; sequential assignment	adults ($\geq$18 years old); MNGIE	n = 20	24 patient-months exposure to treatment	NCT03866954	not yet recruiting
MNGIE allogeneic hematopoietic stem cell transplant safety study (MASS)	allogeneic stem cells	stem cell transplant to introduce normal thymidine phosphorylase and rescue mtDNA homeostasis	phase 1 open label	patients with MNGIE	n = 12	24 months	NCT02427178	recruiting
Treatment of TK2 deficiency with thymidine and deoxycytidine	nucleoside precursors	rebalancing of precursor pools to restore mtDNA levels	phase 1/2 open label, single group assignment	patients with TK2 deficiency	n = 20	<60 months	NCT03639701	recruiting by invitation
Bone marrow-derived stem cell ophthalmology treatment study II		autologous bone marrow derived stem cells (BMSC) for treatment of retinal and optic nerve damage or disease	N/A; open label, non-randomized, parallel assignment	adults ($\geq$18 years old)	n = 500; to include LHON	12 months	NCT03011541	recruiting
A prospective, randomized, double-masked, vehicle controlled, phase 2 clinical study to evaluate the safety, tolerability, and efficacy of elamipretide (MTP-131) topical ophthalmic solution in subjects with LHON	elamipretide (MTP-131)	binds cardiolipin	phase II; prospective, randomized, double-masked, vehicle controlled	adults $\geq$18 and $\leq$ 50 years old; m.11778G > A	n = 12	up to week 56	NCT02693119	active, not recruiting

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Table 1. *Continued*

Title	Compound	MOA	Design	Participants	Sample Size	Trial Period (week)	Reference	Status
Phase-1, dose finding and safety study on L-citrulline treatment of nitric oxide deficiency in MELAS	L-citrulline	amino acid; precursor of arginine (NO deficiency)	phase I; open label, single group assignment	18–65 years old; m.3243A > G mutation	n = 24	4 weeks	NCT03952234	not yet recruiting
External natural history controlled, open-label intervention study to assess efficacy & safety of treatment with Raxone in LHON	idebenone	electron carrier and antioxidant	phase IV; open label, single group assignment	≥12 years old; confirmation of either m.11778G > A, m.3460G > A or m.14484G > C LHON mtDNA mutations	n = 250	12 months	NCT02774005	active not recruiting
Post authorization safety study with Raxone in LHON patients (PAROS)	idebenone		prospective cohort study	child, adult, older adult; patient prescribed Raxone for the treatment of LHON	n = 250	up to 5 years	NCT02771379	recruiting
Study to assess efficacy, safety, and tolerability of Idebenone in the treatment of Leber's hereditary optic neuropathy (RHODOS)	idebenone		phase II, interventional	≥14 years old; confirmation of either m.11778G > A, m.3460G > A or m.14484T > C LHON mtDNA	n = 85	3 years	NCT00747487	completed
Safety and efficacy study of gene therapy for the treatment of LHON	rAAV2-ND4	allotopic expression of mtDNA encoded protein ND4	phase II/III; open label, single group assignment	10–65 years old; M.11778G > A mutation	n = 159	12 months	NCT03153293	active, not recruiting
Long-term follow-up of ND4 LHON subjects treated with GS010 ocular gene therapy in the RESCUE or REVERSE phase III clinical trials	GS010		observational	15 years and older; subjects who were treated with GS010 in either of the 2 phase III trials—RESCUE and REVERSE	n = 74	up to 5 years	NCT03406104	recruiting
Mitochondrial oxidative stress and vascular health in chronic kidney disease	MitoQ	mitochondrially targeted antioxidant	interventional		n = 24	4 weeks	NCT02364648	recruiting

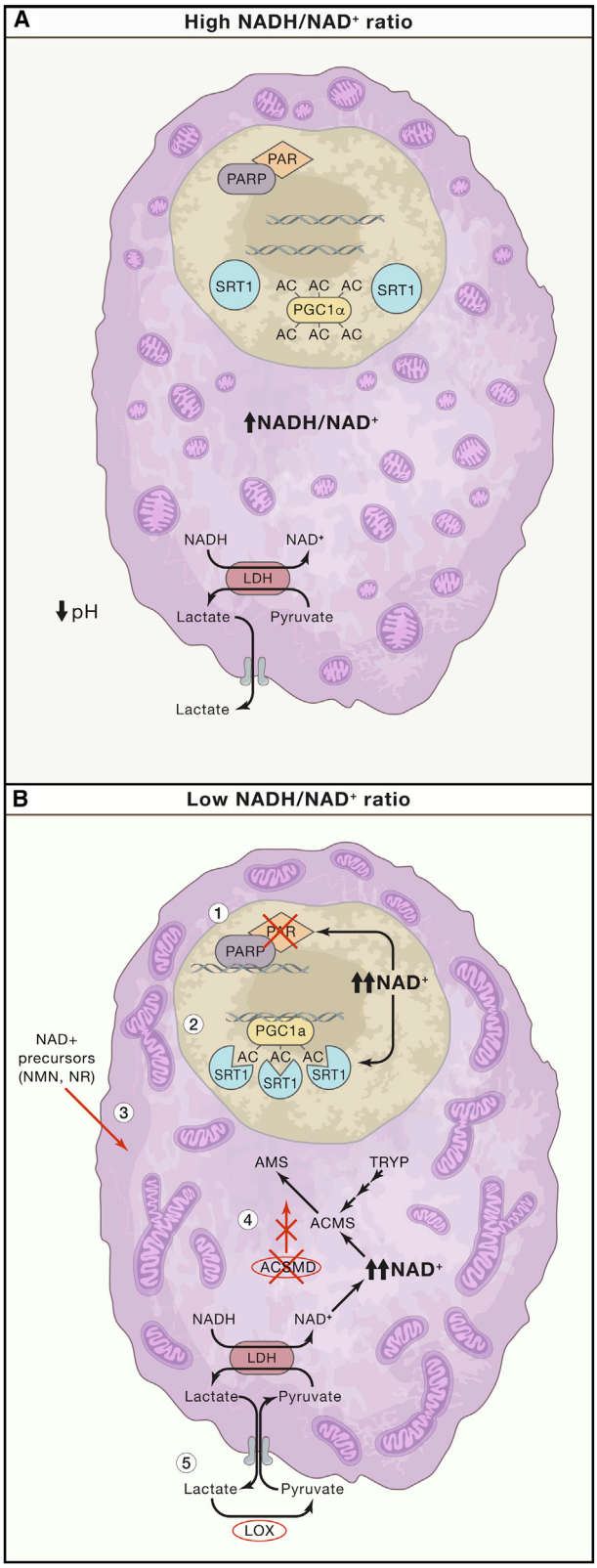


Figure 2. NAD+-Mediated Rescue of Mitochondrial Function

(A) An increase in the NADH/NAD+ ratio due to mitochondrial dysfunction leads to a cascade of detrimental effects. Low NAD+ concentration decreases the amount of sirtuin mediated PGC1α deacetylation, inhibiting mitochondrial biogenesis. The need to increase NAD+ levels, combined with a decreased ability to process pyruvate via OXPHOS, leads to the conversion of pyruvate to lactate by the NADH-linked lactate dehydrogenase. Although this reaction provides NAD+, it also decreases pH via production of lactic acid.

(B) Several therapies have been proposed to correct these issues. (1) The use of PARP inhibitors can prevent the polyADP ribosylation (PAR) of PARP, a major NAD+ consumer in the nucleus. This has the secondary effect of increasing the NAD+ concentration available for use by sirtuins (2), deacetylating PGC1α, and increasing mitochondrial biogenesis. (3) Direct addition of NAD+ precursors such as NMN or NR, or (4) by inhibiting ACMSD to prevent the conversion of ACMS to AMS, also increases NAD+ levels. (5) Extracellular conversion of lactate to pyruvate by LOXCAT has the synergistic effect of increasing extracellular pH and increasing cellular pyruvate concentration, providing more substrate for LDH to generate NAD+. PGC1α, PPAR-gamma coactivator 1-alpha; SRT, sirtuin; PARP, polyADP ribose polymerase; PAR, PARP inhibitor; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; ACMS, 2-amino-3-caroxymuconate 6-semialdehyde; ACMSD, 2-amino-3-caroxymuconate 6-semialdehyde decarboxylase; AMS, 2-aminomuconate 6-semialdehyde; TRYP, tryptophan; LDH, lactate dehydrogenase; LOX, lactate oxidase/catalase fusion protein.

strategy, not only for the treatment of mitochondrial disease but for many diverse medical conditions including the aging process itself (Cantó et al., 2015) (Figure 2). Nicotinamide riboside (NR) has been successfully used to treat the Deletor mouse model of mitochondrial disease, resulting in increased mitochondrial biogenesis in skeletal muscle and the restoration of normal mitochondrial morphology, albeit with extremely high doses (Khan et al., 2014). NR has also been used to improve motor performance in skeletal muscle and increases OXPHOS-related gene expression in a Sco^{KOKI} mouse model characterized by impaired COX function, with a similar improvement seen by pan-PARP inhibition (Cerutti et al., 2014). Nicotinamide mononucleotide (NMN) ameliorated lactic acidosis in *Ndufs4* KO mice, increasing lifespan and substantially increasing both NAD+ and NADH levels in skeletal muscle, although no modulation of levels in brain tissue was noted (Lee et al., 2019). Acipimox, a niacin derivative previously used to treat dyslipidemia, has been shown to boost NAD+ levels in murine C2C12 myotubes and increase mitochondrial respiration in human skeletal muscle, *ex vivo* (van de Weijer et al., 2015). A clinical trial on patients with mitochondrial disease has been established to determine if acipimox can relieve the extensive muscle symptoms associated with this disorder (Table 1). A recently completed trial of niacin supplementation in patients with pure mitochondrial myopathy caused by mtDNA deletions is due to report results (Table 1).

There has been a recent and very interesting approach to the NAD+ booster concept. One of the four metabolic pathways for the *de novo* synthesis of NAD+ includes the breakdown of L-tryptophan via the kynurenine pathway, generating 2-amino-3-carboxymuconate 6-semialdehyde (ACMS). This can be enzymatically decarboxylated by the enzyme ACMS decarboxylase (ACMSD). In the absence of this activity, quinolinic acid is non-enzymatically generated by spontaneous cyclization of ACMS and further converted to NAD+ (Katsyuba et al., 2018). Auwerx and colleagues have been able to show that by using an orally available synthetic inhibitor of ACMSD, NAD+ levels are indeed elevated in kidney, liver, and brain of mice (Katsyuba et al., 2018) (Figure 2B). With the caveat that

this particular NAD^+ synthesis pathway is only present in certain tissue-types, treating patients with ACMSD inhibitors may be a viable therapy, in particular, for the subset of disorders whose patients present with mainly liver-specific defects.

Another elegant approach to manipulating the NAD^+/NADH ratios has been to reoxidize extracellular lactate back to pyruvate, which is then transported back into the cell by the monocarboxylate carrier, allowing re-reduction of the pyruvate by lactate dehydrogenase to re-establish the NAD^+ poise. Patgiri et al. (2020) have had some success with this approach by using a bacterial lactate oxidase fusion protein injected into the circulation of mice with a drug induced mitochondrial dysfunction.

Inducing Mitochondrial Turnover

Rapamycin, a macrolide first isolated from the soil bacterium *Streptomyces hygroscopicus* as an anti-inflammatory and anti-proliferative compound, has shown promise in ameliorating many aspects of mitochondrial dysfunction. Its target is a component of the mammalian target of rapamycin (mTOR) complex, mTORC1, which is a key regulator of cellular homeostasis and has been linked to activation of the mitochondrial stress response in mitochondrial myopathy (Khan et al., 2017). Rapamycin has been shown to delay symptoms and extend life expectancy in the $Ndufs4^{-/-}$ mouse model of Leigh's disease (Johnson et al., 2013). Strikingly, while the effect was profound, the molecular rescue mechanism was unclear. Using a skeletal muscle-specific complex IV assembly-deficient model, $COX15^{sm/sm}$, Civiletto et al. (2018a) have now shown that rapamycin may be having its effects through increasing autophagic flux coupled with an upregulation of lysosome biogenesis, all thought to be a consequence of mTORC1 inhibition. In Deletor mice, treatment with rapamycin rebalances one carbon metabolism and serine synthesis and silences the mitochondrial integrated stress response (Khan et al., 2017). Increasing autophagic flux is believed to rid the cell selectively of mitochondria that have become dysfunctional as a consequence of the complex assembly defect, leading to restored mitochondrial morphology. However, OXPHOS can never be fully restored, due to the COX assembly defect. Interestingly, rapamycin treatment also promotes metabolic reprogramming, suggesting other methods to mimic this reprogramming event may have therapeutic potential. The partial rescue of mitochondrial function due to rapamycin treatment in these models is exciting and it has been used in the clinic for many years. However, there are substantial side effects not merely around immunosuppression that may limit its use for treating mitochondrial disease in humans (Johnson and Kaeberlein, 2016).

Could Increasing Mitophagy Prove Beneficial?

Current understanding is that the PINK1:PARKIN axis can selectively orchestrate turnover of dysfunctional mitochondrial fragments (Narendra et al., 2008; Twig et al., 2008). It has therefore been argued that for heteroplasmic mtDNA disease, it may be possible to selectively remove defective mitochondria that have accumulated high levels of pathogenic mtDNA, reducing heteroplasmy (Suen et al., 2010). In a heteroplasmic model of *C. elegans*, loss of Parkin resulted in increased heteroplasmy of the mtDNA deleted molecule compared to wild-type mtDNA (Valenci et al., 2015). In flight muscles of insects artificially manipulated to produce heteroplasmy, overexpression of either Pink1 or Parkin resulted in the selective loss of deleted mtDNA compared to wild-type mtDNA (Kandul et al., 2016) and in human cells generated by cell fusion and harboring a *MTCO1* pathogenic mtDNA mutation, Parkin overexpression dramatically reduced the levels of heteroplasmy and rescued mitochondrial dysfunction (Suen et al., 2010). Pink1-mediated mitophagy requires the addition of ubiquinated molecules on the mitochondrial outer membrane for the binding of autophagic receptors leading to degradation. Inhibition of a series of deubiquitinating enzymes has also been suggested as a way to promote mitophagy (Desai et al., 2018) that may also lead to reduced heteroplasmy. Although the importance of the PINK1:PARKIN axis is established in cell lines the relevance to mammalian tissues is less clear, with *in vivo* imaging studies in mice showing no role for PINK1 in mitochondrial turnover (McWilliams et al., 2018).

The bacterial natural product urolithin A, a mitophagy activator, has been trialed on individuals aged 61 to 85 years and shown after 28 days to induce a molecular signature in skeletal muscle similar to regular exercise (Andreux et al., 2019). It has previously been shown to enhance mitochondrial function and improve muscle strength in mice (Ryu et al., 2016).

Metabolic Reprograming

Elegant high throughput chemical and CRISPR screens have been used to determine whether any small molecules could resolve a complex I defect in cultured cells associated with Leigh's syndrome (Barrow et al., 2016). Both screens showed benefit from loss of function of the bromo-domain containing protein BRD4, resulting either in PGC1α upregulation or prevention of cell death in galactose, a carbon source that forces cells to utilize OXPHOS. BRD4 was shown to occupy promoter sites for a variety of nuclear-encoded mitochondrial genes and by using inhibitors of BRD4 binding, such as I-BET-525762A, expression could be rescued. Similar to rapamycin (Civiletto et al., 2018a), the complex I deficiency was not resolved, but metabolic reprogramming was apparent, consistent with glutamine becoming a key metabolic substrate, effectively bypassing complex I and feeding into the respiratory chain at complex II via TCA cycle product, $FADH_2$. Additionally, in a similar response to rapamycin treatment, autophagic flux and lysosome biogenesis were also increased by BRD4 ablation, although there is no suggestion that this leads to increased mitochondrial turnover (Barrow et al., 2016).

Chemical-genetic screens investigating individual OXPHOS complexes have highlighted surprising therapeutic targets. Under conditions of OXPHOS complex inhibition, which may have been expected to worsen the phenotype, genome-wide CRISPR screening revealed that metabolic remodeling promoted cell survival. Inhibition of complex V by oligomycin was alleviated either by knock out of complex I genes or chemical inhibition of complex I. The authors attributed this to an increase in glycolysis and reductive carboxylation that was facilitated by redox rebalancing (To et al., 2019).

Hypoxia

Perhaps one of the more surprising recent observations from screens looking to identify putative mitochondrial therapeutics has been the discovery of gene products involved in oxygen sensing, with Jain et al. (2016) showing that chronic, low level

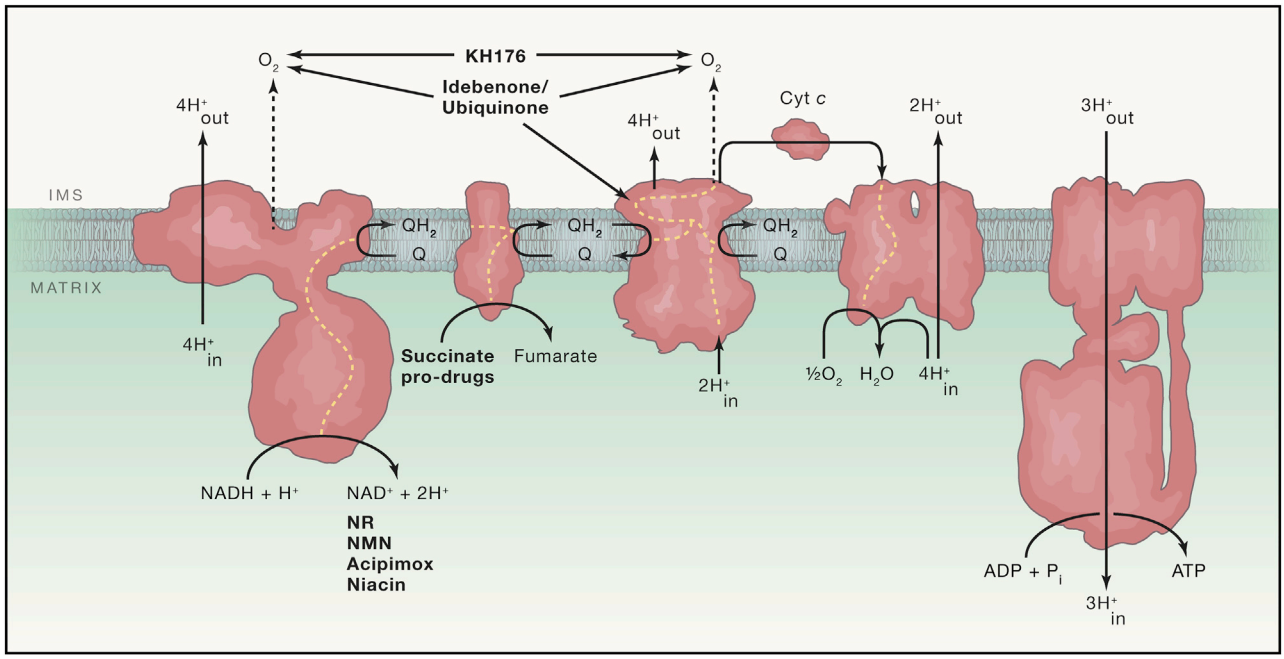


Figure 3. Mitochondrially Localized Small-Molecule Therapies

There are several compounds which have been recently, or are currently, involved in clinical trials. These compounds enable mitochondria to compensate for deficient OXPHOS, in particular complex I-related deficiencies. Provision of NAD^+ to restore cellular REDOX is currently under investigation, with both NR and Acipimox in clinical trials. In mitochondria with complex I deficiency it can also be beneficial to bypass complex I altogether and provide electrons directly to complex III (idebenone/ubiquinone) or by increasing available substrate for complex II (succinate pro-drugs) (Ehinger et al., 2016). These drugs, along with KH176, also have the potential to scavenge reactive oxygen species produced by deficient mitochondria. NR, nicotinamide riboside: NMN, nicotinamide mononucleotide.

hypoxia (equivalent of an altitude of 4,500 m) slowed disease progression in the *Ndufs4* knockout, Leigh's syndrome mouse model. Remarkably, there was no evidence of brain lesions, a hallmark of Leigh's syndrome, in knockout mice after 250 days in 11% O_2, whereas the normoxic group typically had visible lesions after 55 days. In mice allowed to develop brain lesions prior to initiation of chronic hypoxia, 70% were alive after 210 days, compared to the median lifespan of 58 days in normoxic-treated knockout animals (Ferrari et al., 2017). While these data are encouraging, the use of hypoxia as a treatment in patients is still some way off, in particular because intermittent hypoxia is not sufficient to rescue the phenotype. Although Jain et al. (2019) have demonstrated other methods to lower the partial pressure of O_2 in the brain, to the best of our knowledge, these have not yet been safely and effectively trialed in patients.

Modulating the Production of Reactive Oxygen Species and Oxidative Stress

Mitochondria are a major source of reactive oxygen species such as superoxide, because electrons at complex I and III of the respiratory chain are often offloaded to molecular oxygen (Murphy, 2009). Superoxide can act to nucleate the formation of numerous highly chemically active intermediates that can damage protein, nucleic acids, or lipids. Reactive oxygen species scavengers have been considered for some time as therapeutics to try and protect mitochondria from damage (Murphy and Smith, 2007). Clinical trials have been undertaken with mitochondrial antioxidants such as KH176, a derivative of the water-soluble form of vitamin E, and with mitochondrially targeted reactive oxygen species scavengers such as the ubiquinone derivative MitoQ (Figure 3; Table 1). Clearly, any such approach has to be carefully controlled, as mitochondrially produced reactive oxygen species play an important role in intracellular signaling. Recently, KHENERGY, a phase 2 clinical trial in patients with m.3243A > G mitochondrial disorders treated with KH176 for 28 days, reported no serious treatment-emergent adverse events. Although it did not meet its stated primary outcomes, KH176 demonstrated a positive effect on alertness and mood (Table 1). A follow-on (phase IIB) trial (KHENERGIZE) to confirm the potential effects of KH176 (Sonlicromanol) in patients with m.3243A > Grelated mitochondrial disease is now underway.

Another antioxidant, which already has the US Food and Drug Administration (FDA) approval for treatment of cystinosis, is cysteamine bitartrate (RP103). Believed to promote the synthesis of the major intracellular antioxidant glutathione (Besouw et al., 2013), RP103 has been trialed for a mixed cohort of pediatric patients with mitochondrial disease but the trail was terminated after 3 years due to lack of efficacy. Interest in RP103 has recently been re-evaluated in pre-clinical studies, leading to the suggestion that effective dosage may have to be very carefully titrated. Although these results are of interest, no effect on the cellular glutathione levels could be measured (Table 1).

Elamipretide (Bendavia, MTP131, or SS31) is a modified and cell-permeant cationic tetrapeptide whose exact function

remains unclear. It locates to mitochondria and binds cardiolipin (Birk et al., 2013). Its protective function is thought to be due to the protection of cardiolipin, which under certain conditions can become tightly bound by cytochrome *c*, leading to unfolding and repurposing of cytochrome *c* as a peroxidase, resulting in oxidative damage of cardiolipin (Szeto, 2014). Elamipretide, therefore, may dually protect cardiolipin, an essential lipid in the inner mitochondrial membrane and cytochrome *c*, safeguarding normal bioenergetics in the mitochondrion. More recent experiments *ex vivo* in failing human hearts have led to the suggestion that elamipretide may promote supercomplex formation (Chatfield et al., 2019). Numerous clinical trials testing the efficacy of elamipretide in patients suffering from a variety of clinical problems have been performed or are ongoing. In particular, it has recently been announced that a phase III trial with over 200 patients suffering from mitochondrial myopathy (MMPOWER-3) did not meet its primary endpoints assessing changes in the 6-min walk test and Primary Mitochondrial Myopathy Symptom Assessment (PMMSA) total fatigue score (Table 1) (Prnewswire, 2019).

Idebenone, an analog of ubiquinone (CoQ_{10}) with a shorter side chain and greater bioavailability, has recently been licensed to treat adolescent and adult patients with Leber's hereditary optic neuropathy (LHON). Idebenone has the potential to act as an electron carrier in the respiratory chain (Figure 3) and as an antioxidant against membrane damage caused by lipid peroxidation. The best evidence to assess Idebenone came from the RHODOS trial, a double blind, phase II randomized control trial (Klopstock et al., 2011, 2013). This study showed no significant difference between idebenone and placebo for the primary outcomes, but subgroup analysis suggested there may be some beneficial effect of idebenone in stabilizing or improving visual acuity in subgroups with discordant visual acuity (Table 1).

Restoring mtDNA Homeostasis

Neurogastrointestinal encephalomyopathy (MNGIE) is a rare mitochondrial disease where mutated thymidine phosphorylase (TYP) causes an imbalance in deoxynucleosides and subsequent profound effects on mtDNA composition. In the absence of therapeutics, safety studies are being assessed on patients for enzyme replacement therapy and allogeneic stem cell transplants (Bax et al., 2019) (Table 1).

Defects in a second enzyme involved in nucleoside metabolism, thymidine kinase 2 (TK2), are also known to have a profound effect on mtDNA levels in various tissues. Following promising results in mice (Garone et al., 2014), a compassionate use program has perhaps provided the greatest hope for success, to date, in these patients (Domínguez-González et al., 2019). The findings of this retrospective study indicated a favorable side effect profile and clinical efficacy of nucleoside therapies (Domínguez-González et al., 2019), paving the way for a phase 2 prospective, open-label continuation treatment study of the safety and efficacy of oral deoxypyrimidine (MT1621) in TK2-deficient patients over 36 months (Table 1). Deoxynucleoside therapy for other mtDNA maintenance disorders is also being considered, but each disease should be carefully investigated (Saada, 2019). However, the lack of mtDNA depletion in cell culture models of these disorders highlights the importance of the development of relevant animal models for the field.

Overall, there have been several interesting new approaches in the potential development of new drugs to treat mitochondrial disease. Encouragingly, these approaches involve several different pathways, and it seems that although some drugs could potentially treat only specific mitochondrial disease, others could have a more generic benefit. However with the exception of idebenone, (and perhaps emerging evidence of nucleoside therapies in TK2-deficient patients), there is no positive clinical trial evidence for any beneficial effects of small-molecule treatments, to date.

Manipulating the Mitochondrial Genome

The multi-copy nature of the mitochondrial genome adds an extra challenge to the diagnosis and the prediction of progression of mtDNA disease. However, the fact that many disease-causing mtDNA mutations are heteroplasmic actually provides a route to curing these disorders. The presence of wild-type mtDNA copies in the cell provides a pool of molecules from which unaffected mtDNA molecules can replicate, decreasing heteroplasmy and curing the cellular biochemical defect, assuming mutated copies can be selectively removed or inhibited from replicating. The ability to manipulate heteroplasmy in cells has been an area of interest for the last 20 years, but until recently, a viable method has proven elusive.

There are several barriers to manipulating mtDNA, the first and most difficult to overcome is crossing the inner mitochondrial membrane to access the mtDNA-containing mitochondrial matrix. In order to maintain an electrochemical gradient, mitochondria have evolved specialized transporters so molecules can cross the membrane without dissipating the membrane potential. This impermeability severely limits the ability to accumulate foreign molecules within the mitochondrial matrix and requires any therapeutic to be able to directly cross the membrane or to utilize an existing transporter to enter the matrix. The second barrier is the requirement of the therapy to selectively target mutated molecules of mtDNA and cause their degradation, allowing wild-type mtDNA copy number to propagate with time. Anti-sense peptide nucleic acids (Chinnery et al., 1999; Taylor et al., 1997, 2001), endonucleases (Alexeyev et al., 2008; Srivastava and Moraes, 2001; Tanaka et al., 2002), zinc-finger nucleases (ZFNs), transcription activator-like effector nuclease (TALENS), and Crispr-Cas9 all have the ability to selectively target single nucleotide mutations and cause their degradation. Unfortunately, the evidence for RNA import into mitochondria is limited, which precludes the use of Crispr-Cas9 due to its requirement for single-guide RNA (sgRNA) guides to facilitate the gene editing, as reviewed by Gammage et al. (2018a).

Current promising technologies are ZFNs and TALENs, proteins that recognize and cleave DNA in a sequence-specific manner. Modification of their N termini to express the COXVIII-MTS drives efficient localization to mitochondria, although ZFNs also require the presence of a nuclear exclusion sequence to ensure there are no off-target effects in genomic DNA (Minczuk et al., 2008). In both instances, a dual system of two ZFNs or TALENS is utilized, each fused to a FokI nuclease. The activity of this nuclease relies on dimerization of two FokI molecules, therefore requiring the close proximity of two ZFNs or TALENS. Typically, the systems consist of one ZFN/TALEN

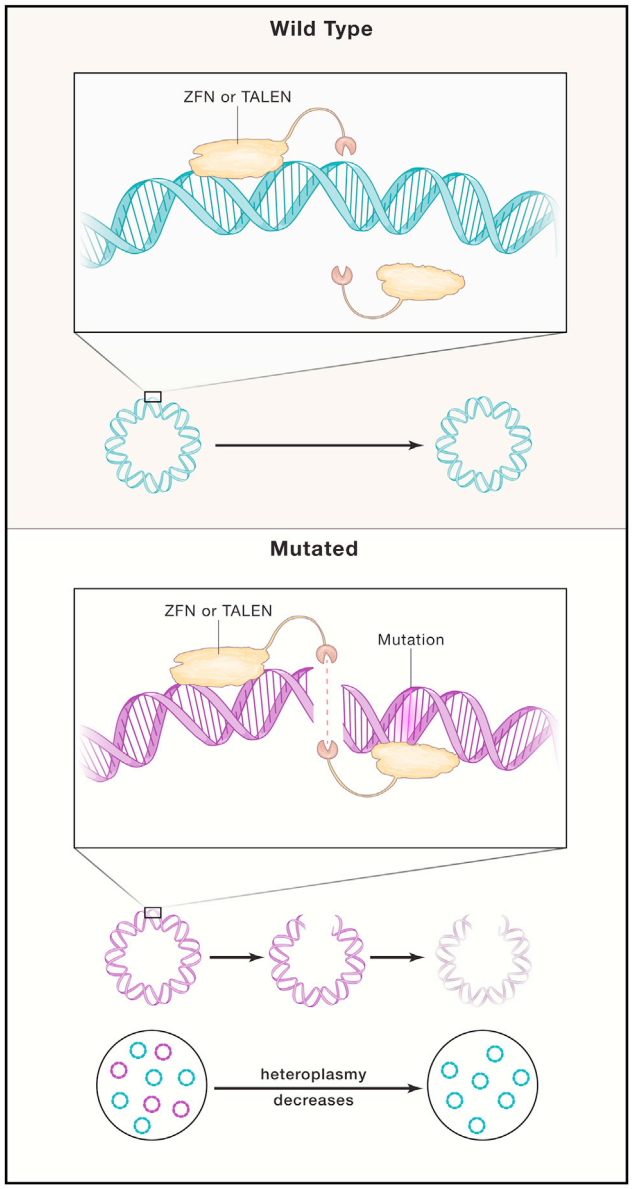


Figure 4. Direct Manipulation of mtDNA

Heteroplasmic mtDNA disease can be corrected using either ZFNs or TALENs to recognize mutated regions of the genome and introduce a double strand break. Top: the FokI nuclease requires dimerization of two molecules, therefore a ZFN or TALEN that binds a region adjacent to the mutation site is required, even though both wild-type and mutated mtDNA molecules will be bound by this ZFN/TALEN. However, as the second ZFN/TALEN will only bind mutated molecules, only mutated mtDNA is cut. Bottom: ZFN/TALEN target sites (red dots) are bound and cut in mutated genomes. Wild-type genomes only contain one target site, preventing the binding of both ZFNs/TALENs, thus wild-type mtDNA is not cut. Heteroplasmy changes as cut genomes are degraded, followed by replication of wild-type genomes, decreases the heteroplasmy to sub-pathogenic levels. ZFN, zinc-finger nucleases; TALEN, transcription activator-like effector nuclease.

that recognizes a wild-type sequence adjacent to the mutated region, with the second ZFN/TALEN designed to bind the mutation and several bases adjacent to it. Therefore, only those mtDNA molecules harboring the mutation in question will have both iterations of the ZFN/TALEN bound and therefore dimerized FokI nucleases (Figure 4).

Bacman et al. (2013) have pioneered the use of TALENs to eliminate mutant mtDNA genomes, with initial work focusing on the selective degradation of mtDNA heteroplasmic for either the m.8483_13459del4977 "common deletion" or the m.14459G > A point mutation. In both instances, there was a significant decrease in the mutant level of heteroplasmy. Subsequent refinement of the technique has shown that it can also cause selective elimination of m.8344A > G and m.13513G > A mtDNA respectively in cell lines in culture (Hashimoto et al., 2015; Pereira et al., 2018) along with germline editing (Reddy et al., 2015) and manipulation of m.3243A > G molecules in iPSC (Yang et al., 2018). Recently, manipulation of heteroplasmy in a mouse model with a heteroplasmic pathogenic variant in mt-tRNA^{Ala} has been demonstrated. Using AAV9 adenoviruses to deliver TALENs designed to selectively degrade the mtDNA with the m.5024C > T mutation, the authors demonstrated a significant decrease in mutant load in both skeletal muscle and cardiac tissue (Bacman et al., 2018).

Similarly to TALENs, the mt-tRNA^{Ala} mouse was the only suitable model for ZFN *in vivo* proof of concept work. Using AAV9.45 serotypes to deliver the ZFNs to the animals, the authors detected a significant decrease in heteroplasmy of cardiac tissue when compared to the level of heteroplasmy in DNA extracted from an ear-notch biopsy (Gammage 2018b). Unfortunately, the mouse model does not have an easily assessed disease phenotype so it is not possible to ascertain if the heteroplasmy fall was physiologically relevant (Kauppila et al., 2016); however, the data from this study and the similar work by Bacman et al. (2013) shows significant promise for treating patients with mitochondrial disease. As these therapies move into the clinic, it is important to consider that CNS involvement is a common phenotype associated with mtDNA disease, and therefore innovative delivery strategies to improve efficacy at the target site is essential. Previously, delivery of mitoTALEN AAV was performed using intramuscular, intravenous, and intraperitoneal injections, and in all cases, there was no evidence of AAV delivery into the brain, despite the use of AAV9 serotypes reported to cross the BBB (Hudry and Vandenberghe, 2019). However, there are several clinical trials currently underway that include cerebral targets, which provide further validity to the potential use of these therapies in mitochondrial disease patients (Hudry and Vandenberghe, 2019).

Delivery of wild-type copies of mutated mtDNA genes is another potential route of gene therapy for mitochondrial disease, but is complicated by the lack of recombination in mammalian mitochondria. Pathogenic mutations in any of the 13 mtDNA-encoded peptides can cause dysfunctional OXPHOS, with the relatively common m.8993T > G and m.11778G > A mutations causing maternally inherited Leigh's syndrome/NARP and Leber's hereditary optic neuropathy, respectively. The affected polypeptides encoded by these genes, *MTATP6* (m.8993T > G) and *MTND4* (m.11778G > A), have both been investigated as possible candidates for allotopic expression. Although there is evidence to suggest that cytosolically expressed versions of these proteins conjugated to a mitochondrial localizing sequence can be imported into

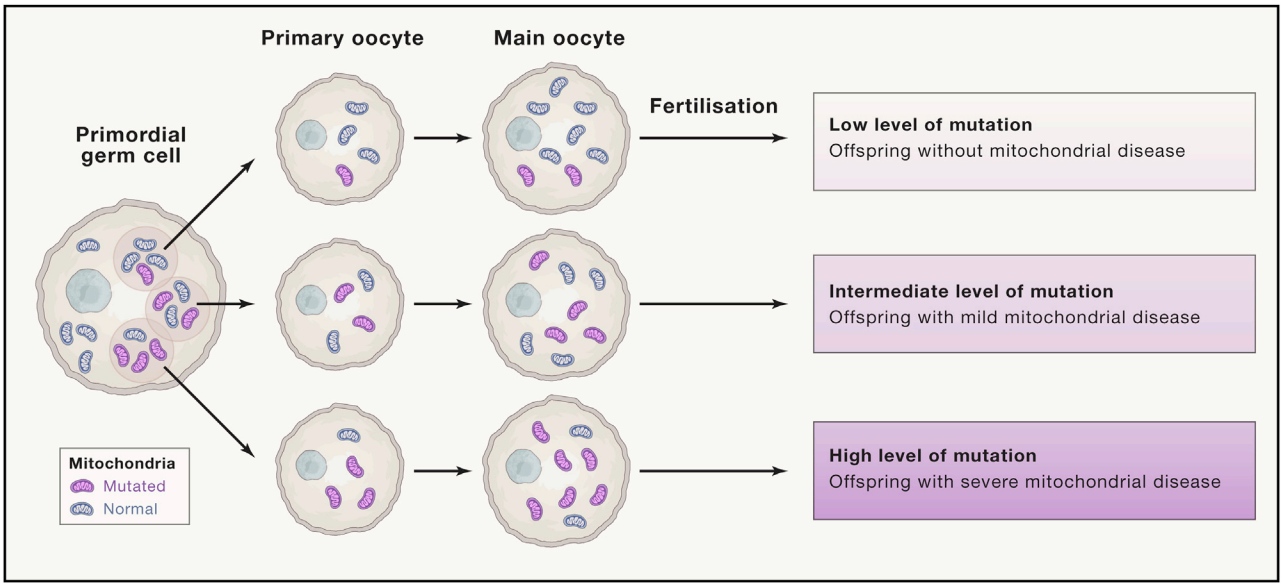


Figure 5. The Mitochondrial Genetic Bottleneck

This bottleneck occurs owing to a profound dilution of mtDNA followed potentially by selective replication of mtDNA genomes and asymmetric segregation of the mitochondria, therefore creating oocytes with heteroplasmy levels different to the germ cell. Fertilization of the mature oocytes can lead to the development of offspring with a wide range of mtDNA heteroplasmy.

mitochondria (allotopic expression) (Ellouze et al., 2008), conflicting evidence suggests that rescue of a mutated mtDNA gene can be due simply to reversion to the wild-type mtDNA sequence (Perales-Clemente et al., 2011). However, following numerous positive claims for allotopic expression in cells, trials have attempted to use allotopically expressed *MTND4* to treat LHON via intraocular AAV delivery of the gene (Table 1). By injecting only one eye and using the other eye as a control for progression of the visual symptoms, the authors of these studies suggest there is an improvement in vision in injected eyes (Guy et al., 2017). However, the efficacy of the treatment is not the same in all patients, indeed, in some, the uninjected eye performs better during follow up (Yang et al., 2016), and recent phase III trials failed to satisfy a primary endpoint (Fightaging, 2019). Although allotopic expression as a potential for treating mtDNA encoded mutations is an interesting concept, it is difficult to interpret these studies.

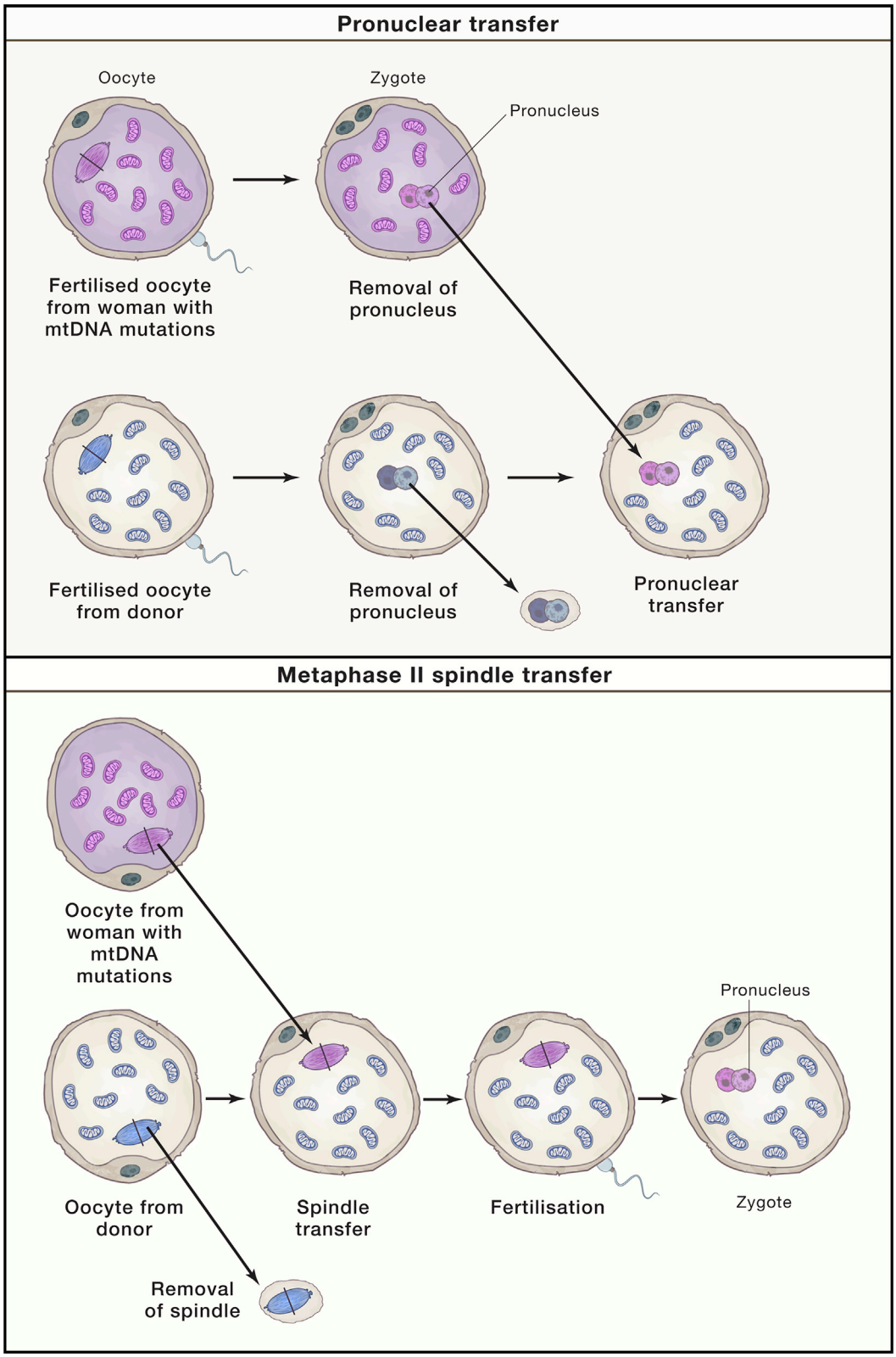
Overall, the recent results with manipulating the mutated mtDNA show great promise, especially the more recent studies using a heteroplasmic mouse model. However, there are still many hurdles to overcome before gene therapy will be possible for patients with mitochondrial disease, and this highlights the important of developing methods to prevent transmission of these diseases.

Prevention of Transmission of Mitochondrial Diseases

Mitochondrial diseases are often relentlessly progressive and, until safe curative strategies are developed, families with mitochondrial disease will understandably look for options to prevent their transmission. This may mean different options for those with nuclear genetic defects compared to those with mtDNA defects, and it highlights why the recent advances in genetics have been so important in determining the primary genetic defect in most patients with mitochondrial disease. For those families with nuclear defects the options will be similar to any other nuclear genetic disease. This will include counselling, prenatal testing (chorionic villus biopsy [CVS] and amniocentesis), and preimplantation genetic diagnosis (PGD) (Gorman et al., 2018). In the future, correction of the genetic defect at the germ cell or embryo stage may be an option, but at present, the safety, efficacy, and ethics of using these techniques at such stages needs to be established.

For mtDNA disease, the challenges are very different. MtDNA is almost invariably maternally inherited and there is a genetic bottleneck during development (Stewart and Chinnery, 2015). The bottleneck means that for women with heteroplasmic mutations, there can be considerable difference in the level of mutated mtDNA in different oocytes, and thus the result of any pregnancy is uncertain (Figure 5). Thus, for women with mtDNA mutations, there are a variety of different options available that vary depending on the mtDNA mutation carried and, in those with heteroplasmic mtDNA mutations, the level of mutation they harbor. This highlights the importance both of making a genetic diagnosis and of expert genetic counselling.

The options available for women after counselling include voluntary childlessness, adoption, and oocyte donation. In recent years, other options have become available including prenatal testing and preimplantation genetic diagnosis (PGD) (Gorman et al., 2018). Prenatal testing is only suitable for women who have a low risk of transmission of mtDNA disease and who would consider a termination. Either prenatal testing using chorionic villus biopsy or amniocentesis have a small risk to the viability of the pregnancy, which also needs to be considered by the couple. PGD is an option available in several countries and is suitable for



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some women with heteroplasmic mtDNA mutations. PGD involves *in vitro* fertilization (IVF) and egg collection with development of embryos to eight-cell or blastocyst stage. Certain mtDNA mutations (e.g., m8993T > G) often have widely varying levels in oocytes and thus are particularly suitable to PGD (Steffann et al., 2007). An embryo with the lowest level of mtDNA mutation is then selected and transferred, providing the quality of the embryo is sufficiently high (Hellebrekers et al., 2012). It is also important to be aware that only a limited number of embryos will be available with mutation levels that are unlikely to result in severe mitochondrial disease, and PGD is only successful in approximately one-third of cycles due to inherent problems associated with IVF (Steffann et al., 2018; Poulton et al., 2019).

For some women with mtDNA mutations, PGD is not suitable, and there were no reproductive options available for them to prevent transmission of mtDNA mutations at high levels of heteroplasmy to their offspring. In recent years, there have been considerable advances in developing techniques to prevent the transmission of mtDNA mutations, called mitochondrial replacement (MRT) or mitochondrial donation. The principle of these techniques is to move the nuclear DNA from a mother with pathogenic mtDNA mutations into an oocyte (metaphase II spindle transfer) or zygote (pronuclear transfer) of a donor woman (Greenfield et al., 2017) (Figure 6). The children born after this technique will have the nuclear DNA of both parents but the mitochondrial DNA of the donor woman. There have been several reports showing the feasibility of these techniques in human oocytes and that good quality embryos can be produced (Tachibana et al., 2013; Hyslop et al., 2016). Indeed, in the United Kingdom, after a change in the law and several independent scientific reviews, a current clinical trial will assess the outcome of mitochondrial donation on the first 75 children born.

Although mitochondrial donation is potentially a major advance, both the advice and counselling, and indeed the options available for women with mtDNA mutations remain challenging. Mitochondrial donation is only available as part of a highly regulated process in the United Kingdom and, in some countries, may never be allowed for ethical reasons. Although there have been several studies in rodents (McGrath and Solter, 1983), and one in primates (Tachibana et al., 2009), indicating the apparent safety and efficacy of the technique, there have been limited studies in humans. Some studies have suggested there may be health consequences associated with mitochondrial replacement therapies (Latorre-Pellicer et al., 2016) but these studies have been done using inbred strains of mice and thus may have very different effects compared to the situation in humans where the population is highly genetically mixed. Due to the lack of regulation of IVF techniques in many countries, mitochondrial replacement therapy is being used in unregulated environments and in some cases without appropriate ethical approval. Although there have been some reports of successful pregnancies, these reports have made only limited information available (Zhang et al., 2017). In addition, MRT is also being tried in some countries to treat infertility with little evidence to suggest that this would be beneficial. This highlights the importance of the United Kingdom study of the safety and efficacy. In addition, there is the evidence that MRT may not be 100% effective. In ~20% of stem cell lines derived from embryos after mitochondrial donation *in vitro*, there is reversion to the genotype of the mother (Hyslop et al., 2016). The reason and *in vivo* significance of this reversion is unknown.

For those women considering the possibility of mitochondrial donation, there are other challenges. In the United Kingdom, the body regulating fertility treatments, the Human Fertilization and Embryology Authority (HFEA), have made a number of specific criteria before a license for mitochondrial donation is permitted (https://www.hfea.gov.uk/8807.html). Two major conditions are the severity of the mitochondrial disease, and PGD is not suitable. In terms of disease severity, in the United Kingdom, PGD is only allowed for serious disease, and similar criteria are now used for mitochondrial donation. The suitability of PGD is difficult to determine because, for many mtDNA mutations, there is uncertainty about the threshold level of the mtDNA mutation before clinical manifestations emerge. Recent studies in patients harboring the m.3243A > G mutation have highlighted the importance of collecting genetic data to allow mathematical modeling to predict the likelihood of the success of PGD (Pickett et al., 2019). An additional issue for mitochondrial donation is the availability of suitable donors who are prepared to provide oocytes, which in itself requires both hormonal treatment and oocyte collection (Poulton et al., 2019).

Thus, for all patients with mitochondrial disease, accurate and specific genetic counselling is vital and should be made available for all patients. An in-depth discussion of the risks and potential benefits of all possible options will allow parents to decide on the most appropriate course of action for their family. Finally, it is also important that these discussions, especially for women who would consider one of the assisted reproduction options, should be as early as possible. With the age of mothers having their first child rising in many developed countries, there has to be a realization that there is an age-related decline in both oocyte quality and number that may negatively impact options.

Conclusions and Future Perspectives

To date, treatment strategies are predominantly symptom-based, focusing primarily on restorative (such as Q10 supplementation in primary genetic defects of coenzyme Q10 synthesis) or preventative strategies during episodes of acute metabolic decompensation due to physiological stressors (such as dehydration, fever, surgery, sepsis) (Avula et al., 2014). Although there are more than 50 clinical trials currently listed that purport to interrogate medicinal products targeting primary mitochondrial diseases, the evidence for most pharmacological strategies still remains largely anecdotal (Garone and Viscomi, 2018). Presently, only one drug, idebenone, has

Figure 6. Mitochondrial Replacement Techniques

Pronuclear replacement (top) involves the fertilization of both the mother and donor oocytes and transfer of the male and female pronuclei from the mother's oocyte into an enucleated donor oocyte. Metaphase II spindle transfer (bottom) involves the removal of the spindle from the donor oocyte and the replacement of the spindle from the mother's oocyte followed by fertilization.

provided sufficient scientific evidence for FDA/European Medicines Agency (EMA) approval for mitochondrial disease, and this use is only for the acute visual loss in LHON (Klopstock et al., 2011; Rudolph et al., 2013).

However, there is great excitement in the mitochondrial field regarding future treatment options. There has been remarkable progress in mitochondrial disease over the past decade, both in terms of our basic understanding of mitochondrial biology and our ability to identify the genetic defect in the vast majority of patients. The unmet clinical need for treatment of patients with mitochondrial disease has stimulated both academic and commercial interest in developing new treatments, as has the awareness of mitochondrial involvement in more common diseases (Murphy and Hartley, 2018). Small molecule and genetic screens using either patient or genetically modified cell lines has identified new targets and some of these are unexpected. For example, who would have predicted that relative hypoxia would potentially be valuable in the treatment of OXPHOS disorders? The development of new drugs is a lengthy process but the possibility of using repurposed drugs that have a positive effect on mitochondrial function is something that is likely to have a more immediate effect on patients with mitochondrial disease.

While the concept of using some form of gene therapy to correct heteroplasmic mtDNA disorders has been considered for over 25 years, this strategy is now much closer to the clinic as shown by the recent change in the level of heteroplasmy in the mt-tRNA^{Ala} mouse model using two different technologies (Bacman et al., 2018; Gammage et al., 2018b). While, again, there are many hurdles ahead, this is encouraging and advances in gene therapy in other areas may well allow this correction of the genetic defect in the future. This, in combination, with innovative new IVF techniques to prevent the transmission of mtDNA must give new hope to families who have seen many family members affected.

Finally, crucially important in the development of new treatments is the ability to conduct meaningful clinical trials. This previous lack of clinical trial data for mitochondrial therapeutics is, in part, due to the rare nature of primary mitochondrial disorders, which impacts successful clinical trial conduct, design, and funding (Augustine, Adams and Mink, 2013). The historical scarcity of scientific evidence about the natural history (disease symptoms, heterogeneity, and progression) and the underlying disease mechanisms, in addition to the lack of clinically and regulatorily meaningful, standardized measures to assess outcomes, have collectively been recognized as critical disadvantages in informing clinical trial design in the field (Newman et al., 2015; Koene et al., 2018; Parikh et al., 2019).

The lack of study power (impacted by limited sample sizes and invariably small treatment effect size), variable endpoint selection (single primary, multiple, or composite), dichotomy between statistical significance and clinically meaningful results (Schober et al., 2018), and the lack of established biomarkers to substitute for a clinical efficacy endpoint (“surrogate endpoint”) have also impacted the ability to perform high quality clinical trials. Transformative approaches to mitigate several of these disadvantages are now being implemented in a current trial (AIMM; see Table 1).

We believe that major breakthroughs in the development of treatments for mitochondrial disease will occur during the next decade. This will be achieved by further advances in gene therapy and the development of screening assays to discover new small molecules to improve mitochondrial function. Together with devising innovative approaches to clinical trial design including the development of new wearable and immersive technologies and the creation of virtual controls, these advances will herald a truly new era in innovative, personalized medicine for patients with mitochondrial disease.

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