

## REVIEW ARTICLE

# Moving towards clinical trials for mitochondrial diseases

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## Abstract

Primary mitochondrial diseases represent some of the most common and severe inherited metabolic disorders, affecting ~1 in 4,300 live births. The clinical and molecular diversity typified by mitochondrial diseases has contributed to the lack of licensed disease-modifying therapies available. Management for the majority of patients is primarily supportive. The failure of clinical trials in mitochondrial diseases partly relates to the inefficacy of the compounds studied. However, it is also likely to be a consequence of the significant challenges faced by clinicians and researchers when designing trials for these disorders, which have historically been hampered by a lack of natural history data, biomarkers and outcome measures to detect a treatment effect. Encouragingly, over the past decade there have been significant advances in therapy development for mitochondrial diseases, with many small molecules now transitioning from preclinical to early phase human interventional studies. In this review, we present the treatments and management strategies currently available to people with mitochondrial disease. We evaluate the challenges and potential solutions to trial design and highlight the emerging pharmacological and genetic strategies that are moving from the laboratory to clinical trials for this group of disorders.

## KEYWORDS

antioxidants, clinical trial, gene therapy, mitochondrial biogenesis, mitophagy, nucleosides, primary mitochondrial disease, treatment

## 1 | INTRODUCTION

Primary mitochondrial diseases are recognised as some of the most common and severe inherited metabolic disorders, affecting ~1 in 4,300 live births.<sup>1</sup> They are defined

as genetic diseases in which mutations primarily or secondarily lead to dysfunction of oxidative phosphorylation (OXPHOS) or other disturbances of mitochondrial structure and function, including perturbed mitochondrial ultrastructure.<sup>2</sup> Mitochondrial disorders are inherently heterogeneous at the genetic, mechanistic, and clinical level. Defects of nearly 400 genes across two genomes have been linked to primary mitochondrial diseases.<sup>3</sup>

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Patients may exhibit manifestations in almost any organ and tissue in the body, leading to enormous diagnostic challenges.

The complexity of mitochondrial disease contributes to a lack of therapeutic options for patients. With the exception of a handful of vitamin and cofactor biosynthesis and transporter defects, nearly all mitochondrial disorders currently lack disease-modifying curative therapies. Contributing factors to this current state include a poor understanding of disease pathomechanisms, inadequate disease models, the inaccessibility of the double-membraned mitochondrion, and the logistical challenges of conducting clinical trials for ultra-rare disorders.<sup>2</sup> However, the current outlook for mitochondrial disease therapeutics is encouraging, with dozens of therapies in development and reaching clinical trials. Disease modelling has also improved significantly, and preclinical studies have been performed in mouse models of multiple mitochondrial DNA (mtDNA) deletions, a mt-tRNA point mutation, mtDNA depletion syndrome (MDDS), epileptic encephalopathy, OXPHOS defects, coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) biosynthesis defects, and mitochondrial translation defects.<sup>4-9</sup> Table 1 summarises a selection of the active mitochondrial disease clinical trials currently listed in [clinicaltrials.gov](http://clinicaltrials.gov).

## 2 | TREATABLE DISORDERS

Although the vast majority of mitochondrial diseases lack licensed therapies, a subset are responsive to treatment; specifically, single-gene disorders of cofactor transport and metabolism. Vitamin and other organic cofactors, including thiamine, riboflavin, biotin and CoQ<sub>10</sub>, are required to catalyse the enzyme reactions necessary for OXPHOS and other mitochondrial pathways. In disorders of cofactor transport and metabolism, cofactor replacement at pharmacological doses has the potential to enhance downstream energy production. Deficiency of the SLC19A3 thiamine transporter is a relatively uncommon cause of Leigh syndrome, but prompt treatment with high dose biotin and thiamine is associated with an excellent clinical outcome.<sup>10</sup> Riboflavin (vitamin B2) is a precursor of two flavoenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) which act as cofactors for more than 90 human enzymes, many of which are localised in the mitochondrion. Mitochondrial disorders that have been reported to respond to riboflavin supplementation include deficiencies of ACAD9, AIFM1 and complex I subunits located near the FMN binding site such as NDUFV1 and NDUFV2, as well as deficiency of FAD synthase, the only known disorder of riboflavin metabolism.<sup>11,12</sup> Furthermore, the riboflavin transporter

### SYNOPSIS

Decades of work elucidating mitochondrial disease mechanisms have culminated in the commencement of several clinical trials of novel pharmacological agents that may herald the discovery of urgently needed disease-modifying therapies.

disorders (Brown-Vialetto-Van Laere syndrome) may mimic mitochondrial disease, including deficiencies of respiratory chain enzymes.<sup>13,14</sup> Ten disorders of the biosynthesis of CoQ<sub>10</sub>, a non-vitamin organic cofactor, are known and some cases may respond to supplementation with pharmacological doses of CoQ<sub>10</sub>.<sup>15</sup> Positive responses to treatment have been reported for defects of COQ2 and COQ8B (previously known as ADCK4).<sup>16,17</sup> However, there have been disappointing outcomes in other primary CoQ<sub>10</sub> deficiencies, particularly those affecting the brain and with prenatal onset such as deficiencies of COQ4 and COQ9.<sup>18,19</sup> The lack of clinical response to CoQ<sub>10</sub> supplementation in these patients has led to a search for new compounds to treat CoQ<sub>10</sub> biosynthesis disorders. One promising strategy still in preclinical development is to use alternative benzoquinone ring precursors, including 4-hydroxybenzoate and its analogues (such as 2,4-dihydroxybenzoic acid), *p*-coumarate, vanillic acid, resveratrol and kaempferol, to bypass the block in CoQ<sub>10</sub> biosynthesis.<sup>20</sup>

## 3 | SUPPORTIVE CARE

The current clinical management of mitochondrial disorders relies almost entirely on symptomatic treatment. Although vitamins and cofactors are frequently prescribed to patients with primary mitochondrial diseases, sometimes as a 'cocktail', there is no proven efficacy for these agents outside the disorders of vitamin/cofactor metabolism and transport described above.<sup>21</sup> Symptomatic therapies are specific to the manifestations of the patient. For example, seizures may be treated with anti-epileptic drugs or a ketogenic diet, cardiac dysfunction can be treated with drugs including  $\beta$ -blockers, renal insufficiency may require haemodialysis, hearing aids or cochlear implants may be needed for patients with hearing loss, and brow suspension surgery may be indicated to improve vision or for cosmesis in patients with severe ptosis. Organ transplantation is also an option, but should be considered carefully in cases of multi-system disease.<sup>22</sup>

**TABLE 1** Ongoing clinical trials targeting primary mitochondrial disease

Therapy	Clinical trial identifier	Phase	Mechanism of action	Disorder	Age range (years)	Primary outcomes measure(s)	Status
EPI-743	NCT01370447	II	Mitochondrial Redox Modulator	PMD	1+	Change in neuromuscular function, IAE, NPMDS	Active, not recruiting
Vatiquinone (PTC743, EPI-743)	NCT04378075	II	Mitochondrial Redox Modulator	PMD with Refractory Epilepsy	≤18	Change from baseline in number of observable motor seizures per 28 days, number of disease-related hospital days, number of participants with status epilepticus	Not yet recruiting
Vincerinone (EPI-743)	NCT02352896	II	Mitochondrial Redox Modulator	Leigh Syndrome	1–18	Long term effect on disease severity measured by NPMDS	Active, not recruiting
KH176 (Sonlicromanol)	NCT04165239	II	Mitochondrial Redox Modulator	MELAS, MIDD, MM, PMD	18+	Cognitive function: attention domain	Recruiting
Idebenone (Raxone)	NCT02774005	IV	Mitochondrial Redox Modulator	LHON	12+	Proportion of eyes with clinically relevant recovery of visual acuity from baseline	Active, not recruiting
Idebenone (Raxone)	NCT02771379	PASS	Mitochondrial Redox Modulator	LHON	Child, Adult, Older Adult	Long-term safety profile - IAE	Recruiting
Nicotinamide Riboside	NCT03432871	N/A	NAD Modulator Mitochondrial Biogenesis Enhancer	MELAS, MM, PEO, PMD	18-70	Bioavailability—pharmacokinetics. Safety—IAE, change in blood analytes, temperature, blood pressure, pulse. Mitochondrial biogenesis <sup>31</sup> P-MRS, respiratory chain enzyme analysis, mtDNA copy number	Recruiting
KL1333	NCT03888716	I	NAD Modulator Mitochondrial Biogenesis Enhancer	MELAS, MM, MRCD, PMD, HV	18-75	IAE, ECG, incidence of abnormal vital signs, incidence of abnormal physical examinations	Recruiting
REN001	NCT03862846	I	Mitochondrial Biogenesis Enhancer	MM	16+	IAE	Active, not recruiting
ABI-009 (Nab-sirolimus)	NCT03747328	II	Inhibition of Mitophagy	Leigh/ Leigh-like Syndrome	2–17	IAE, GMFM	Not yet recruiting
L-Citrulline	NCT03952234	I	Nitric Oxide Precursor	MELAS	18-65	Maximal tolerable dose, IAE	Not yet recruiting
Sodium Phenylbutyrate	NCT03734263	I/II	Inhibition of Pyruvate Dehydrogenase Kinase	PDHC Deficiency	0.25-18	Blood lactate levels	Recruiting

(Continues)

TABLE 1 (Continued)

Therapy	Clinical trial identifier	Phase	Mechanism of action	Disorder	Age range (years)	Primary outcomes measure(s)	Status
Dichloroacetate	NCT02616484	III	Inhibition of Pyruvate Dehydrogenase Kinase	PDHC Deficiency	0.5-17	Observer Reported Outcome (ObsRO) measure of health, IAE	Recruiting
Thymidine and Deoxycytidine	NCT03639701	I/II	Nucleosides	Myopathic Thymidine Kinase 2 Deficiency	All	Liver transaminase levels, lymphocyte count, creatinine, ECG, incidence of diarrhoea	Enrolling by invitation
EE-TP	NCT03866954	II	Erythrocyte Encapsulated ERT	MNGIE	12+	Safety—IAE, laboratory indices, vital signs. Pharmacodynamics—changes in plasma and urine thymidine and deoxyuridine levels. Efficacy—change in body mass index.	Not yet recruiting
CD34+ cells enriched with MNV-BLD	NCT03384420	I/II	Biological	PMD, PS	Child, Adult, Older Adult	IAE, IPMDS QoL questionnaire	Enrolling by invitation
scAAV2-P1ND4v2	NCT02161380	I	Gene Therapy	LHON	15+	IAE	Recruiting
GS010 (rAAV2/2-ND4)	NCT02064569	I/II	Gene Therapy	LHON	18+	IAE	Active, not recruiting
GS010 (rAAV2/2-ND4)	NCT03293524	III	Gene Therapy	LHON	15+	BCVA	Active, not recruiting
GS010 (rAAV2/2-ND4)	NCT03406104	III	Gene Therapy	LHON	15+	Long term follow up of gene therapy—IAE	Recruiting
rAAV2-ND4	NCT03153293	II/III	Gene Therapy	LHON	10-65	BCVA, computerised visual field	Active, not recruiting

Abbreviations: BCVA, best corrected visual acuity; CPET, cardiopulmonary exercise testing; ECG, electrocardiogram; ERT, enzyme replacement therapy; GMFM, gross motor function measure; HV, healthy volunteers; IAE: incidence of adverse events; IPMDS, international paediatric mitochondrial disease scale; LHON, Leber hereditary optic neuropathy; MDDS, mitochondrial DNA depletion syndrome; MELAS, mitochondrial encephalopathy lactic acidosis and stroke-like episodes; MIDD, maternally inherited diabetes and deafness; MM, mitochondrial myopathy; MNGIE, mitochondrial neurogastrointestinal encephalopathy; MRCD, mitochondrial respiratory chain deficiency; MRS, magnetic resonance spectroscopy; NPMDS, Newcastle paediatric mitochondrial disease scale; PASS, post-authorisation safety study; PDHC, pyruvate dehydrogenase complex; PEO, progressive external ophthalmoplegia; PS, Pearson syndrome; QoL, quality of life.

<sup>a</sup>Selection of clinical trials for primary mitochondrial disease (PMD) listed in <https://clinicaltrials.gov> accessed May 22, 2020.

#### 4 | BRIDGING THE TRANSLATIONAL GAP: THE LONG ROAD TO CLINICAL TRIALS

In 2012, a Cochrane Systematic Review of Treatments concluded that there was no clear evidence supporting the use of any intervention in mitochondrial disorders.<sup>21</sup> Twelve trials fulfilled the entry criteria to the review, which included randomised controlled trials (including cross-over studies). Since then, there have been significant advances in therapy development for mitochondrial

diseases, as outlined below. However, there remains no licensed disease-modifying therapies for patients. This might partly reflect a lack of efficacy of the interventions studied. However, other factors may have contributed to the failure of these trials. Trial design in mitochondrial diseases has historically been hampered by the lack of natural history data, given their relative rarity and heterogeneity, and validated biomarkers and outcome measures that correlate with disease progression. Progress is finally being made in these areas, as discussed in the following sections.

## 4.1 | Natural history studies

Before attempting to design a clinical trial, it is important to possess a good understanding of the natural history of the disease. This includes appreciation of the age of onset of symptoms, the major clinical features as they evolve and any variation in disease severity in the patient cohort, together with morbidity and mortality outcomes.<sup>23</sup> This has been undertaken on a large scale for several mitochondrial disorders to date.<sup>24–30</sup> The therapeutic time window defines a duration of time within which a putative treatment may achieve its intended benefit, that is, duration between establishing the diagnosis and the time-point after which disease severity is too great to achieve the desired outcome. It is important to determine whether there is any genotype-phenotype correlation that modifies this therapeutic window. Since mitochondrial disorders often have a long delay to diagnosis<sup>31</sup> many patients who are index cases within families will already be symptomatic by the time they are diagnosed. Neurological disease involvement is often severe and brain injury that accompanies metabolic decompensations such as stroke-like episodes often results in progressive neurodisability.<sup>32</sup> The utility of disease modifying treatments in patients with severe disease is therefore unclear. The situation for patients who are deemed to be at high risk of developing disease, for example siblings of affected patients who have been diagnosed pre-symptomatically, should be more straightforward, but this is not always the case. For some mitochondrial disorders, individuals with identical genotypes may present with vastly differing clinical features<sup>33</sup> and for patients with pathogenic variants in mtDNA, disease severity is dependent on the degree of mutation load in affected tissues.<sup>34</sup>

## 4.2 | Selection of optimal outcome measures

Selection of appropriate outcome measures is one of the most important factors in enabling a clinical trial to determine the efficacy of a novel therapy correctly. Optimal outcome measures should be robust, sensitive, specific, validated for mitochondrial disease and clinically meaningful. Development of such measures has been particularly challenging given the clinical variability of mitochondrial diseases (even within genetically homogeneous groups), their relapsing-remitting course and variable progression, which tends to occur over years to decades in adults, unlike most clinical trials that are conducted over much shorter time periods. Nevertheless, collaborative efforts are underway to overcome these

hurdles, including the development of large national and international ‘trial-ready’ patient cohorts,<sup>35–37</sup> and consensus recommendations on quality of life and clinical outcome scales and potential mitochondrial biomarkers to monitor efficacy.<sup>38–40</sup> Prospective natural history studies may help to identify appropriate biomarkers that could be used to assess the response to the trial drug. Presently however there is a lack of biomarkers that are sensitive enough to be used for this purpose in large patient cohorts.<sup>41,42</sup> Recently, there have been promising advances in the utility of multi-omic approaches to identify novel biomarkers, however these are yet to be validated in patients.<sup>42</sup>

## 4.3 | Clinical trial design

It is essential that patients who are to be enrolled into clinical trials seeking to treat primary mitochondrial diseases possess a genetic diagnosis which confirms their eligibility, since there are many other diseases that are not considered primary mitochondrial diseases which may be associated with secondary mitochondrial dysfunction. Traditional clinical trials typically have three main phases with escalating numbers of patients in each phase. Phase I studies, performed in small numbers of individuals (often healthy volunteers), test safety, pharmacokinetics and dosing whilst phase II trials begin to address effectiveness, with further assessment of safety. Phase III trials usually involve hundreds (or thousands for common disorders) of patients at multiple centres, and compare effectiveness of the drug under investigation to the standard of care, together with further safety studies and evaluation of side effects (Figure 1). The ideal clinical trial should be adequately powered, statistically valid, randomised, double-blinded, placebo controlled and include a large group of patients with the same genetic defect (similar mutation load if mtDNA), the same clinical presentation, the same biochemical findings and at a similar stage of disease progression. It is clearly impossible to achieve all of these aims in the context of mitochondrial disease, a collection of hundreds of ultra-rare disorders with variable and unpredictable progression. However, this does not mean that good quality trials cannot be performed for this group of patients, and care should be taken to design trials that will answer the issue being addressed.<sup>43</sup> One aspect of mitochondrial disease that has remained virtually unchanged is disease prognosis, which is still disappointing. More than 80% of disorders have an onset in childhood, and the central nervous system is the most frequently involved. Overall mortality is high, with close to 75% of mitochondrial disorders for which natural history data have been reported having a

life expectancy of under 10-years.<sup>23</sup> This implies that for a majority of disorders recruitment of children under the age of 12 years to a clinical trial is likely to be limited and that in order to achieve adequate statistical power for studies involving this age group, multicentre collaboration will be necessary. Moreover, alternative trial designs need to be explored, owing to small numbers of patients affected by ultra-rare disorders.<sup>43</sup> Another important initiative is to identify potential barriers to participating in clinical trials for patients affected by primary mitochondrial diseases.<sup>44</sup>

## 5 | PHARMACOLOGICAL STRATEGIES FOR MITOCHONDRIAL DISEASES

A pharmacological therapy can be defined as a chemical compound which elicits a mechanistic change to a cellular component. In the context of mitochondrial diseases, these can include vitamin and cofactor administration discussed above, but also comprises a variety of novel compounds, many of which are in clinical trials. A summary of mechanisms of action of drugs discussed in the following sections is depicted in Figure 2. Figure 3 illustrates the clinical trials landscape of emerging therapies for mitochondrial diseases.

### 5.1 | Antioxidant approaches

Under physiological conditions, reactive oxygen species (ROS) are important signalling molecules, with wide-ranging effects on cell migration, viability, and differentiation.<sup>2</sup> However, the pathological over-production of ROS leads to a perturbed mitochondrial network, increased phospholipid peroxidation, activation of an inflammatory response, and hyper-activation of apoptosis.<sup>45,46</sup> A number of antioxidant agents have been developed, with the aim of ameliorating the deleterious effects of ROS on membrane integrity, DNA damage, and activation of apoptotic pathways. A cautionary note when considering antioxidant therapy is that prolonged exposure to excessive antioxidant doses may potentially have deleterious consequences. One study reported worse survival of *cox15* knock-out mice treated with N-acetylcysteine.<sup>47</sup>

Idebenone was among the first artificial mitochondrial antioxidants to be developed and was reported to improve respiration in rat brain mitochondria and counteract lipid peroxidation and cerebral vascular lesions.<sup>48</sup> Idebenone is a synthetic analogue of CoQ<sub>10</sub> with a shorter chain, granting greater solubility for improved pharmacokinetics and an ability to cross the blood brain

barrier, making it attractive for the treatment of mitochondrial diseases affecting the central nervous system. In clinical trials, idebenone demonstrated mild improvement of neurological phenotype in Friedreich ataxia and was found to mildly improve visual acuity and colour contrast sensitivity in Leber hereditary optic neuropathy (LHON).<sup>49</sup> It is currently approved for treating LHON in Europe but is not FDA approved. A clinical trial of idebenone in MELAS (NCT00887562) has been completed, with a primary outcome measure of mean change in cerebral lactate concentration measured by magnetic resonance spectroscopy (MRS), but the results have not been published. Another CoQ<sub>10</sub> analogue EPI-743, claimed to have 1000 to 10 000-fold greater potency than both CoQ<sub>10</sub> and idebenone, has been investigated in open-label trials in Leigh syndrome, with inconclusive results.<sup>50,51</sup> These CoQ<sub>10</sub> analogues appear to function by restoring redox balance and counteracting ROS, notably by increasing glutathione production.<sup>50,51</sup>

Sonlicromanol (previously known as KH176) is another ROS-redox modulator which has been shown to decrease cellular ROS levels and protect fibroblast cell lines, derived from patients harbouring mutations in nuclear encoded complex I subunits, against redox perturbation by targeting the thioredoxin/peroxiredoxin system.<sup>52</sup> Long-term Sonlicromanol treatment of *Ndufs4*<sup>-/-</sup> mice, a mammalian model for Leigh syndrome, retained brain microstructural coherence in the external capsule and normalised lipid peroxidation in this area and the cerebral cortex.<sup>53</sup> It also significantly improved rotarod and gait performance and decreased retinal ganglion cell degeneration in the mice. A recent double-blind, randomised, placebo-controlled, two-way crossover phase IIa study (the KHENERGY study) of Sonlicromanol in 18 adults with the m.3243A > G *MT-TL1* mutation showed that the drug was well-tolerated with no treatment-emergent adverse events.<sup>54</sup> Although no significant improvement in gait parameters or other outcome measures was obtained, a positive effect on alertness and mood was detected. A phase IIb study of Sonlicromanol in adult m.3243A > G disease is currently recruiting (KHENERGYZE, NCT04165239).

### 5.2 | Harnessing mitochondrial biogenesis

Mitochondrial biogenesis refers to an increase in mitochondrial mass. It is activated by a large number of physiological stimuli including fasting, cold exposure and exercise, with the ultimate aim of matching energy supply and demand. Pathways related to mitochondrial biogenesis centre on the Peroxisome proliferator-activated

receptor gamma coactivator 1-alpha (PGC1 $\alpha$ ), a transcriptional coactivator that interacts and boosts activity of several metabolism-related transcription factors.<sup>55</sup> PGC1 $\alpha$  is a target for Sirtuin1 (Sirt1), a nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-dependent protein deacetylase. Irrespective of the underlying molecular mechanism, the downstream consequence of impaired OXPHOS is reduced synthesis of ATP. Thus, pharmacological stimulation of mitochondrial biogenesis via manipulation of the PGC1 $\alpha$  axis represents a potential therapeutic strategy in primary mitochondrial diseases, and several small molecules have been identified that exert their effect through this pathway. Examples include bezafibrate,<sup>56</sup> glitazones,<sup>57</sup> resveratrol,<sup>58</sup> omaveloxolone,<sup>59</sup> aminoimidazole-4-carboxamide ribonucleotide (AICAR),<sup>60</sup> NAD<sup>+</sup> modulators (eg, tryptophan, nicotinic acid, nicotinamide, nicotinamide riboside, KL1333), poly(ADP-ribose) polymerase 1 (PARP1) inhibitors,<sup>61,62</sup> REN001, and decanoic acid.<sup>63</sup> Although there is promise from animal and patient-derived models for some of these compounds,<sup>61-63</sup> others have generated data that have not been reliably reproducible or suggested toxicity in mice.<sup>60,64,65</sup>

Three human clinical trials investigating agents purported to increase mitochondrial biogenesis have been completed recently. An open-label observational study of bezafibrate in six patients with mitochondrial myopathy caused by the m.3243A>G mutation showed improvement in cytochrome *c* oxidase (COX)-deficient muscle fibres and cardiac function, with no clinically significant adverse events.<sup>66</sup> Although liver function was unaffected, fibroblast growth factor 21 (FGF21) and growth and differentiation factor 15 (GDF15) levels increased and fatty acid and amino acid metabolism was altered. The authors concluded that although bezafibrate might have short-term benefits, concerns about the longer-term consequences of metabolic reprogramming potentially restrict its application in mitochondrial diseases. A double-blind, randomised, placebo-controlled, cross-over study of resveratrol supplementation in patients with mitochondrial myopathies and skeletal muscle fatty acid oxidation disorders (NCT03728777) has been completed recently but the results have not been reported yet.

A phase II randomised double-blinded placebo controlled clinical trial was recently completed to assess safety and efficacy of omaveloxolone in 53 patients with mitochondrial myopathy (NCT02255422). Omaveloxolone induces mitochondrial biogenesis by activating NRF2. Patients on the treatment arm received the drug in six dose escalations. The primary and secondary outcome measures included peak cycling exercise workload and distance travelled in the six-minute walk test (6MWT). Results indicated that omaveloxolone did not

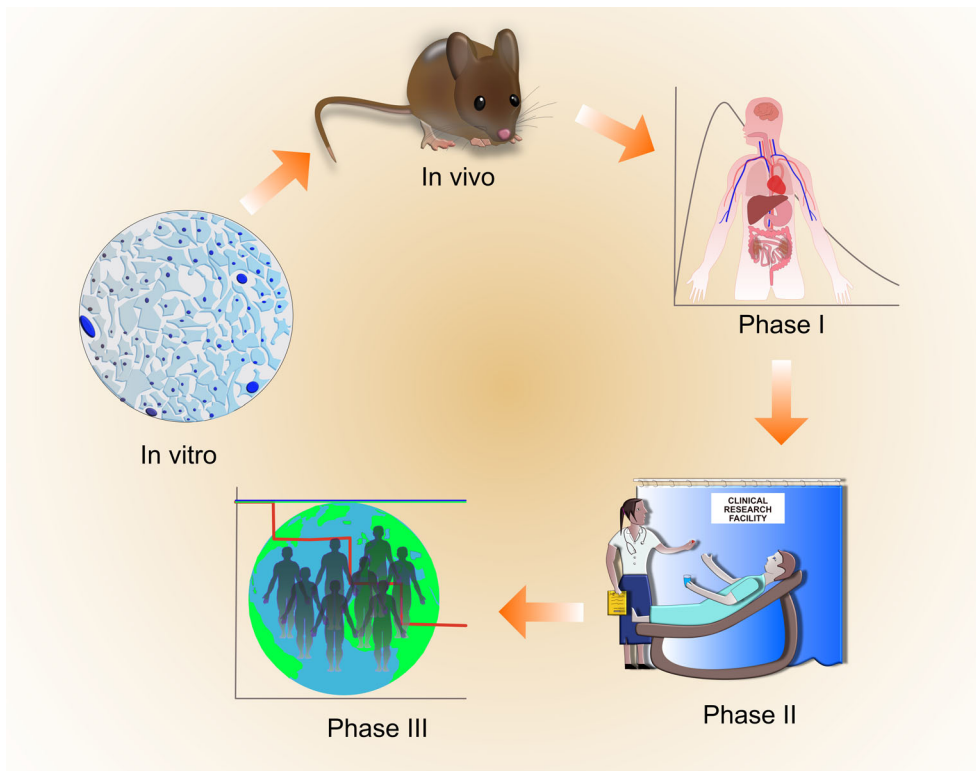
meet its primary and secondary endpoints although it was well tolerated in the majority of subjects.<sup>67</sup>

An open label phase Ib study evaluating the safety and tolerability of REN001 (NCT03862846), a peroxisome proliferator-activated receptor delta (PPAR $\delta$ ) agonist, in patients with primary mitochondrial myopathy, is currently underway, while a phase Ia/Ib trial of KL1333 (NCT03888716), a modulator of cellular NAD<sup>+</sup> levels, is being conducted in people with primary mitochondrial diseases.

Another group of therapies currently undergoing clinical trials involved the restoration of redox balance in mitochondria as a means of stimulating mitochondrial biogenesis. Disrupted redox in the form of the NADH/NAD<sup>+</sup> ratio has wide-ranging cellular effects, including epigenetic changes via the action of NAD<sup>+</sup>-dependent sirtuins and intramitochondrial changes in ROS production and calcium signalling. Supplementation with the vitamin B3 derivative nicotinamide riboside, an NAD<sup>+</sup> precursor, stimulated mitochondrial biogenesis and improved mitochondrial disease phenotypes in two mouse models.<sup>61,62</sup> An open label study of niacin (nicotinic acid) has recently been conducted in patients with mitochondrial myopathy, with post-treatment increased NAD<sup>+</sup> levels detected in blood and muscle tissue, alongside enhanced mitochondrial biogenesis.<sup>68</sup> Finally, an open label study of nicotinamide riboside in mitochondrial biogenesis is currently recruiting patients (NCT03432871).

### 5.3 | Stabilisation of cardiolipin

Elamipretide (also known as SS-31, MTP-131, and Bendavia) is a mitochondrially-targeted Szeto-Schiller tetrapeptide that is reported to decrease the production of ROS. Although the mechanism of action is not entirely clear, Elamipretide is thought to exert its effects by stabilising cardiolipin and thus improving the efficiency of the mitochondrial respiratory chain. Elamipretide recently completed a phase III clinical trial (MMPOWER-3, NCT03323749), following a phase II study that demonstrated a clinically meaningful but not statistically significant increase in exercise performance (6MWT) after 4 weeks of daily subcutaneous treatment in patients with primary mitochondrial myopathy.<sup>69</sup> Safety results showed that treatment with Elamipretide was well tolerated, with most adverse events being mild to moderate in severity. Unfortunately, a press release from the manufacturer in December 2019 confirmed that the phase III study did not meet its primary endpoints assessing changes in the 6MWT and Primary Mitochondrial Myopathy Symptom Assessment (PMMSA) Total Fatigue



**FIGURE 1 Translational pipeline.** Candidate drugs are first investigated in vitro for example, in patient cell lines before in vivo toxicity and efficacy studies in appropriate animal models of disease are undertaken. Clinical trials include phase I studies, in which the candidate therapy is administered to patients or healthy volunteers to assess safety and tolerability, as well as drug pharmacokinetics. Phase II studies assess safety and efficacy of the drug in a small number of patients. Phase III studies assess safety and efficacy of the drug in a larger number of patients with defined outcome measures

Score.<sup>70</sup> Elamipretide has also recently completed a phase II randomised controlled blinded crossover study with open-label follow-up in 12 patients with Barth syndrome (TAZPOWER). The trial failed to meet its primary endpoint of improving 6MWT and patient-reported fatigue scores during the first 12 weeks of the study which were blinded. However, there were statistically significant improvements in both outcomes at 36 weeks' follow up in the open-label extension. In addition, during open-label follow-up echocardiographic assessments indicated an improvement in cardiac function as determined by increased mean stroke volume and left ventricular end-diastolic volume.<sup>71</sup>

## 5.4 | Targeting mitophagy

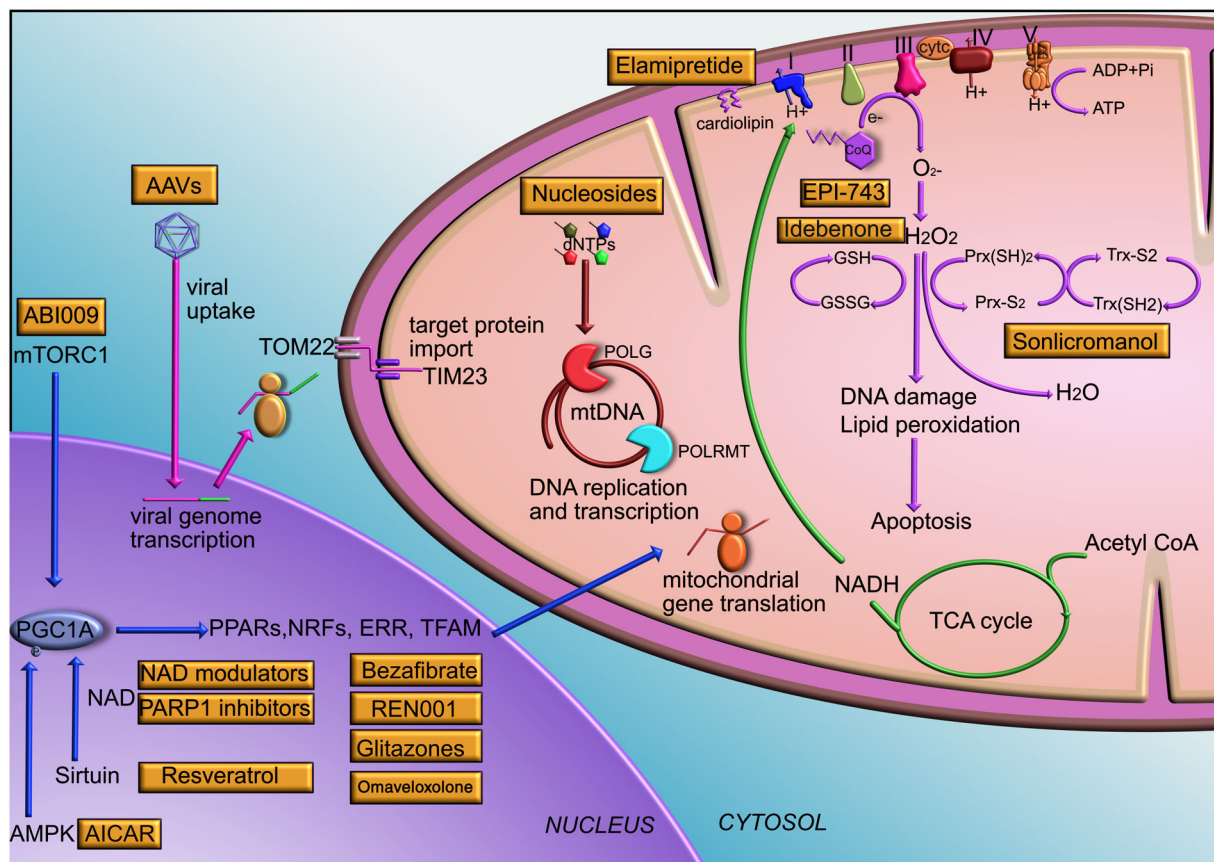
Mitophagy is the physiological maintenance of normal mitochondrial function by selective elimination of damaged mitochondria.<sup>72</sup> Mitophagy is regulated by numerous cellular pathways, one of which includes mTOR (mechanistic inhibition of rapamycin).<sup>73</sup> Rapamycin inhibits mTOR and was shown to prolong lifespan of the *Ndufs4*<sup>-/-</sup> Leigh syndrome mouse model.<sup>74</sup> It has subsequently been shown to benefit a large number of cell-based and in vivo mitochondrial disease models,<sup>75-80</sup> although the underlying mechanism is unclear. However, there was no evidence of benefit from rapamycin in a mouse model of CoQ<sub>10</sub> deficiency,<sup>81</sup> suggesting that it

may not be a universal mitochondrial panacea. Observational studies in humans have yielded conflicting results. Four renal transplant patients with m.3243A>G disease were reported to have clinical improvement after their immunosuppression was changed from calcineurin inhibitors to rapamycin or everolimus<sup>82</sup> whilst one child with Leigh syndrome apparently improved and another with MELAS deteriorated after commencing everolimus therapy.<sup>83</sup> Formal clinical trials are needed, and an open-label phase IIa Study to Evaluate the Safety, Tolerability, and Clinical Activity of ABI-009 (the rapamycin derivative, Nanoparticle albumin-bound Sirolimus) in patients with genetically confirmed Leigh or Leigh-like Syndrome is currently ongoing but not yet recruiting (NCT03747328).

## 5.5 | Enzyme bypass studies

Single-peptide enzymes present in yeast and lower eukaryotes have been harnessed to bypass specific mitochondrial respiratory chain enzyme complexes. Examples include NADH reductase (Ndi1) and alternative oxidase (AOX) which have been expressed in cellular and *Drosophila* models to bypass complex I and complex III and IV defects, respectively.<sup>84-87</sup> AOX has also been expressed in two mouse models of mitochondrial disease, with apparently beneficial effects in *Bcs1l*<sup>p.S78G</sup> knock-in mice but exacerbation of mitochondrial myopathy secondary to aberrant redox signalling in the skeletal muscle-



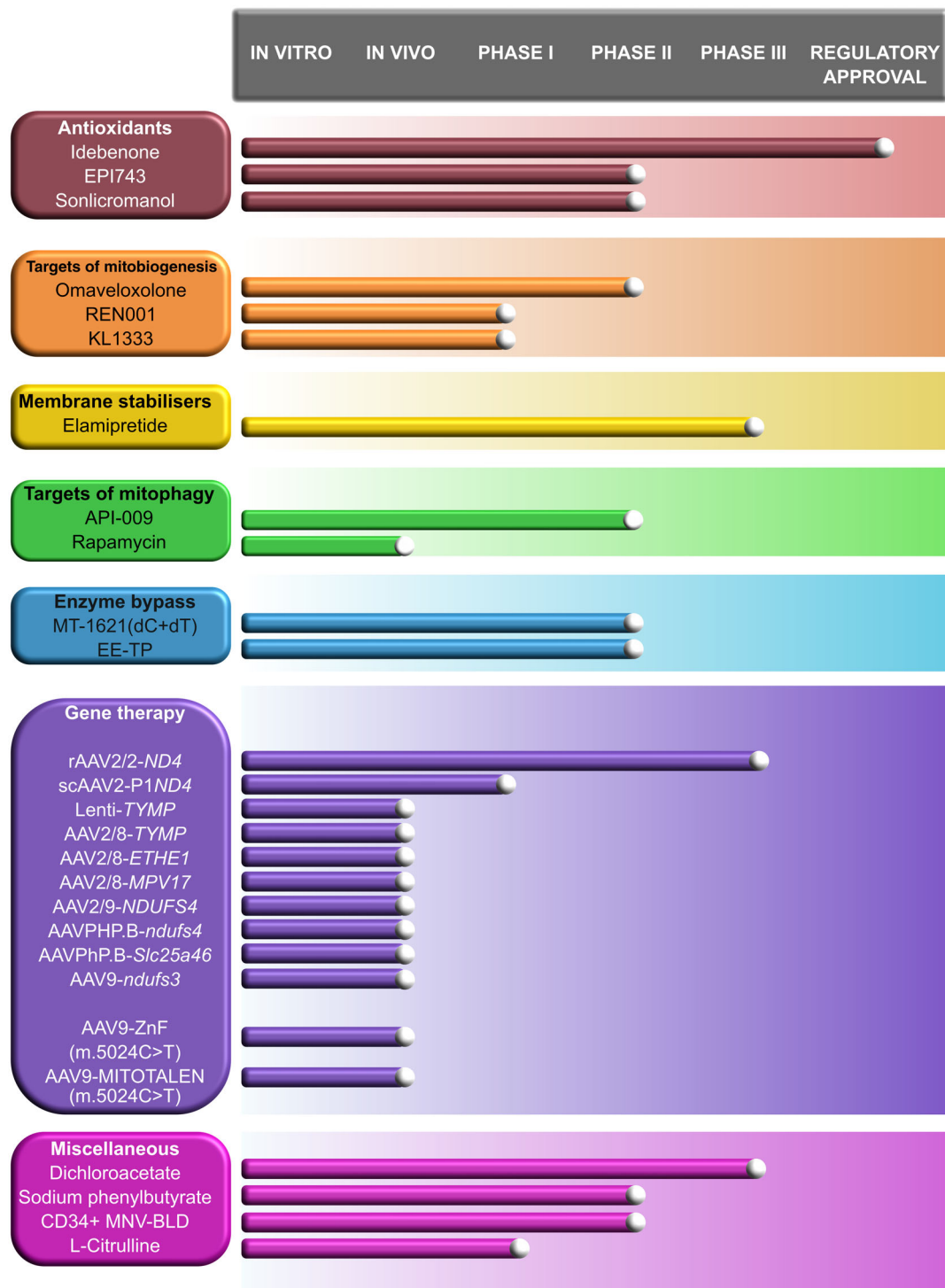


**FIGURE 2 Mechanisms of action of emerging therapies.** Drugs affecting mitochondrial biogenesis act on the PGC1 $\alpha$  pathway. PGC1 $\alpha$  is a master transcriptional coactivator of several transcription factors including PPAR $\alpha,\delta,\gamma$ , NRF1,2, ERR and TFAM. PGC1 $\alpha$  is activated by phosphorylation by AMPK and deacetylation by NAD<sup>+</sup>-dependent sirtuin, and is also controlled by mTOR. Drugs acting on these pathways include AICAR which activates AMPK, resveratrol which activates sirtuin, NAD<sup>+</sup> modulators and PARP1 inhibitors which increase NAD<sup>+</sup> levels, rapamycin and ABI009 which act on mTORC1, bezafibrate which activates PPAR $\alpha$ , REN001 which activates PPAR $\delta$ , glitazones which activate PPAR $\gamma$  and omeveloxolone which activates NRF2. Gene therapy vectors for example, AAVs transduce target cells by first being endocytosed at the plasma membrane. The viral genome is released in the nucleus where it forms an episome and is transcribed by target cell transcriptional machinery. mRNAs are translated in the cytosol. The nascent protein contains a mitochondrial targeting sequence which enables entry into mitochondria by interacting with the TOM22/TIM23 complex. Nucleoside based trial drugs are currently only applicable to one subtype of MDDS, namely thymidine kinase 2 deficiency. Several candidate therapies act on pathways related to the production of ROS, such as superoxide and hydrogen peroxide. Their intermediates have important cellular signalling functions, but also contribute to disease pathophysiology and cell death in mitochondrial disease. Levels of ROS are controlled by the glutathione and peroxidoredoxin/thioredoxin pathways. EPI743 and idebenone are both CoQ analogues which are thought to affect glutathione levels and Sonlicromanol acts on the peroxidoredoxin/thioredoxin pathway. Key: AAV, adeno-associated virus; cytc, cytochrome c; CoQ, coenzyme Q; AMPK, AMP activated protein kinase; GSH, glutathione (reduced); GSSG, glutathione (oxidised); ERR, oestrogen related receptor; MDDS, mitochondrial DNA depletion syndrome; mRNA, messenger RNA; mTORC1, mechanistic target of rapamycin complex 1; NAD, nicotinamide adenine dinucleotide; NRF, nuclear respiratory factor; PARP1, poly(ADP-ribose) polymerase 1; PGC1 $\alpha$ , peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPAR, peroxisome proliferator-activated receptor; POLG, polymerase gamma; Prx, peroxidoredoxin; ROS, reactive oxygen species; TCA, tricarboxylic acid; TFAM, transcription factor A, mitochondrial; TIM, translocase of inner membrane; TOM, translocase of outer membrane; Trx, thioredoxin

specific *Cox15* knockout mouse crossed with an AOX-transgenic mouse.<sup>47,88</sup>

More recently, an engineered enzyme LOXCAT, fusing a bacterial lactate oxidase (LOX) and catalase (CAT), was developed with the aim of decreasing reductive stress by oxidising extracellular lactate to pyruvate.<sup>89</sup> Applying LOXCAT to cell lines with chemically-induced and

disease-relevant genetic knockout models of mitochondrial respiratory chain dysfunction, in addition to human mutant fibroblasts, reduced extracellular lactate:pyruvate, normalised intracellular NADH:NAD<sup>+</sup>, increased ATP production by glycolysis and improved cell proliferation. Similar findings were observed in wild-type and metformin-treated mice following LOXCAT tail vein injections.



**FIGURE 3** Progress in clinical trial development for mitochondrial disorders including trials that have been completed and those that are currently recruiting

## 5.6 | Hypoxia

Von Hippel-Lindau (VHL) factor was identified as an effective suppressor of mitochondrial dysfunction through a CRISPR/Cas9 genome-wide screen to search for repressors of antimycin-induced complex III

deficiency.<sup>90</sup> The VHL ubiquitin ligase appears to exert its effects via activation of the hypoxic response pathway, negatively regulating hypoxia-induced transcription factors (HIFs) during the hypoxic response. In-vivo studies demonstrated an increased lifespan of *Ndufs4*<sup>-/-</sup> mice when exposed to chronic normobaric hypoxic (11% O<sub>2</sub>)

conditions. Normoxic conditions reversed these beneficial effects, while hyperoxia was detrimental.<sup>90</sup> Although these data are all preclinical, a speculative translational implication of these studies is that hyperoxia should be avoided in the mitochondrial patient in the ICU.<sup>91,92</sup>

## 5.7 | Nucleoside supplementation

Since mitochondrial dNTP pool imbalances contribute to the pathogenesis of MDDS, nucleoside precursor supplementation has been proposed as a method to correct these dNTP pool imbalances. In mice, substrate enhancement therapy by dT and dC administration in *Tk2*<sup>-/-</sup> mice improved mtDNA copy number and life span in the encephalomyopathic animals.<sup>93</sup> Subsequently, 38 patients with TK2 deficiency were treated at multiple centres with oral pyrimidine nucleosides dC and dT on a compassionate-use basis (NCT03701568). A retrospective analysis of these patients compared their outcomes to 68 published untreated cases and revealed a significant improvement in survival of treated patients compared to natural history data of untreated patients.<sup>94</sup> No deaths were reported in treated patients. Ninety-four percent of patients who received treatment were found to either have improved (especially in gross motor assessments) or remained stable (especially in respiratory and feeding assessments) regardless of the age of disease onset. The most common adverse effect was diarrhoea, followed by derangements in liver function and urolithiasis. A prospective open-label phase II clinical trial of MT-1621, a GMP grade combination of dC and dT, is now underway (NCT03845712). The primary aim is to assess safety of the drug treatment, with a secondary aim to assess efficacy including motor function, respiratory status and effects on growth and nutrition.

Nucleoside therapy has been proposed for other forms of MDDS. However, a major concern is that supplementation may potentially induce further dNTP imbalances causing unexpected and unpredictable side effects. Furthermore, there is evidence that mitochondria play a role in the toxicity of other nucleoside-based therapies. For example, anti-retroviral reverse transcriptase inhibitors such as 3'-azido-2',3'-dideoxythymidine (AZT) elicit deleterious effects on TK2 and DGUOK, leading to mitochondrial disease-like side effects in patients.<sup>95</sup> AZT and other nucleoside analogues used for the treatment of acquired immunodeficiency syndrome (AIDS) have also been found to be preferentially incorporated by POLG, resulting in gross mitochondrial genome instability.<sup>96</sup> In fact POLG is the most sensitive DNA polymerase to nucleoside analogue inhibition after the retroviral reverse transcriptase.<sup>97</sup> As a result, a secondary mitochondrial myopathy is an observed side effect of using these drugs in patients with

AIDS.<sup>98</sup> Thus, further studies of the effects of nucleoside therapy need to be conducted in different disease models of MDDS before considering clinical applicability.

## 5.8 | Enzyme replacement therapy: Erythrocyte encapsulated thymidine phosphorylase

Mitochondrial Neurogastrointestinal Encephalopathy (MNGIE) is caused by bi-allelic *TYMP* mutations resulting in a harmful accumulation of thymidine. In this disorder, enzyme replacement in the form of allogeneic haematopoietic stem cell transplantation (HSCT) has been shown to be effective in restoring thymidine phosphorylase activity and reducing thymidine levels to normal circulating levels in patients. However, a study of 24 patients who received HSCT demonstrated poor survival outcomes with deaths reported in >60% of cases.<sup>99</sup> It is clear that a safer treatment is needed.

An alternative approach that is being investigated is delivery of enzyme replacement via erythrocyte encapsulation. In this strategy, patient erythrocytes are removed from circulation and treated ex vivo with recombinant thymidine phosphorylase using a red cell loader device to enable encapsulation of the enzyme within the erythrocytes. These cells are then infused back into the patient. A phase II open-label trial in adults (NCT036866954) aims to investigate safety and efficacy of multi-dose erythrocyte encapsulated thymidine phosphorylase (EE-TP), utilising three dose levels to determine the minimum dose required to achieve metabolic correction on an individual patient basis.<sup>100</sup> The primary endpoint for this study is the mean change in baseline body mass index after 2 years of treatment. Secondary endpoints include evaluation of adverse events, laboratory blood tests, ECGs, clinical observations, and physical examination.

## 6 | GENETIC STRATEGIES FOR MITOCHONDRIAL DISEASES

In addition to novel small molecule therapies for mitochondrial disorders, a number of genetic therapies are also in development. Gene therapy seeks to correct the underlying defect of a genetic disease by delivering a normal copy of the mutated gene to affected individuals, and represents a potential cure, as it would rescue the molecular defect. The case for applying in vivo gene therapy to mitochondrial disorders is compelling. Solid organ transplantation for example, in liver (DGUOK deficiency, *TYMP* deficiency),<sup>101,102</sup> kidney (*COQ2* deficiency)<sup>103</sup> and heart (Kearns-Sayre syndrome)<sup>104</sup> has been used

with variable success, but crucially does not correct disease in other affected organs such as the brain.<sup>22</sup> Gene therapy has the potential to target multiple organs simultaneously, including the central nervous system, making it an attractive treatment strategy.

Different gene therapy approaches are being investigated for mitochondrial disorders, and can be grouped broadly into non-viral and viral-based methods. Non-viral approaches include physical methods of cell membrane bombardment (hydrodynamic injection of DNA, biolistic methods),<sup>105,106</sup> chemical methods (micelles of cationic surfactants, rhodamine nanoparticles, liposomes),<sup>107,108</sup> and harnessing endogenous import machinery by means of mitochondrial targeting signal peptide (MTS)-mediated translocation via the TOM22/TIM23 complexes.<sup>109</sup> Many of the non-viral methods are at preliminary in vitro stages and are limited by poor transfection efficiency, weak specificity to mitochondria or failure due to cytotoxicity.<sup>110</sup>

The most promising gene therapy vectors presently are adeno-associated viral vectors (AAV) which belong to the parvovirus family but do not cause disease in humans. Naturally occurring AAVs occur in several subtypes each reflecting a unique pattern of tissue tropism (targeting).<sup>111</sup> Recombinant AAV9 vectors represent an improvement on previous AAV vectors because they cross the blood brain barrier to transduce neurons well and provide a sustained effect after a single neonatal intravenous administration.<sup>112</sup> They also retain the ability to target visceral organs, have a low risk of insertion into the host genome, low immunogenicity and low toxicity.<sup>113-115</sup> AAV9 has already been applied to rodent models of various neurological and metabolic disorders including Hunter syndrome, Sanfilippo disease, Gaucher disease, GM1 gangliosidosis, Pompe disease and spinal muscular atrophy (SMA) with excellent efficacy.<sup>116-121</sup> Currently several AAV gene therapies are in clinical trials for a range of neurometabolic disorders including LHON (NCT02161380), Hunter syndrome (NCT03041324), GM1 gangliosidosis (NCT03952637), Pompe disease (NCT04174105), CLN2 disease (NCT00151216), Hurler syndrome (NCT02702115), AADC deficiency (NCT02852213) and Sanfilippo disease (NCT03612869). Currently, two gene therapies hold FDA approval, for the treatment of spinal muscular atrophy and retinal dystrophy.<sup>122,123</sup>

## 6.1 | Gene therapy for nuclear genes: Preclinical studies

AAV-mediated gene therapy in mitochondrial disease has been tested mostly in mouse models, and overall, the

results in mice have been promising.<sup>124-128</sup> Gene therapy has been applied to a mouse model of MNGIE. Pyrimidine metabolism in mice is different to humans. Thus, in order to model disease in mice, a double knockout of both enzymes thymidine phosphorylase and uridine phosphorylase is required. These knockout animals (*Tymp*<sup>-/-</sup>/*Upp1*<sup>-/-</sup>) recapitulate the elevation of thymidine and deoxyuridine seen in MNGIE although they do not demonstrate liver or gastrointestinal disease. Both lentiviral and AAV-based approaches have been trialled. For the lentiviral approach, haematopoietic progenitors were treated ex vivo with a lentivirus containing the *hTYMP* cDNA driven by the human phosphoglycerokinase promoter. A transduction efficiency of up to 28% was demonstrated using flow cytometry. Cells were then infused into partially myeloablated *Tymp*<sup>-/-</sup>/*Upp1*<sup>-/-</sup> animals. After a period of 4-weeks, there was a supranormal level of thymidine phosphorylase activity and normalisation of thymidine and deoxyuridine levels in peripheral blood as compared to sham treated animals.<sup>129</sup> An alternative AAV-based approach has been used to reconstitute thymidine phosphorylase activity by targeting mouse hepatocytes in vivo.<sup>130</sup> In this study, an AAV2/8 recombinant vector was produced containing the *hTYMP* cDNA sequence driven by the liver specific thyroxine-binding globulin promoter (TBG). Adult *Tymp*<sup>-/-</sup>/*Upp1*<sup>-/-</sup> mice were treated with single intravenous injections of the AAV at doses ranging from  $2 \times 10^{11}$  to  $2 \times 10^{12}$  vector genomes (vg)/kg. Long-term follow up of animals receiving the highest dose over 22 months revealed a reduction in the circulating thymidine and deoxyuridine levels which was sustained to the end of the period of follow-up. There was also a resolution of liver intramitochondrial deoxyribonucleoside imbalances (elevated dTTP and low dCTP) that are seen in the knock-out animals. However, in all dosage groups in long term follow-up there was a reduction in transgene copy number and a consequent reduction in thymidine phosphorylase, but notably at the highest dose, enzyme activity was still supranormal. Overall, these studies demonstrate effective hepatic transduction with consequent clearance of thymidine.

An AAV2/8 recombinant vector has been used to ameliorate the ethylmalonic encephalopathy phenotype of *Eth1* knock-out mice.<sup>126</sup> This knock-out model recapitulated the biochemical and clinical features of ethylmalonic encephalopathy including reduced sulphur dioxygenase (SDO) activity in liver, COX deficiency in skeletal muscle and brain and reduced survival to 4 weeks' age. The transgene cassette used was AAV2/8-TBG-*hETHE1* and route of administration was intracardiac. High doses of AAV vector ( $4 \times 10^{13}$  vg/kg) were able to restore SDO activity, which correlated with lower

levels of plasma thiosulphate levels, lower ethylmalonic acid levels and longer survival. Prolonged survival correlated with a higher transgene copy number in liver. COX activities also improved in skeletal muscle and brain after AAV treatment. Treated animals showed improved weight gain, normal motor activity and improved survival to beyond 6 months.

The *Mpv17*<sup>-/-</sup> mouse model of MDDS does not demonstrate liver disease at baseline despite the presence of liver mtDNA depletion, unless animals are maintained on a high-fat (ketogenic) diet which induces weight loss, liver cirrhosis and hepatic failure. AAV2/8-based gene therapy using the *hMPV17* cDNA sequence under control of the TBG promoter was used to treat *Mpv17*<sup>-/-</sup> mice.<sup>124</sup> Doses of  $4 \times 10^{12}$  and  $4 \times 10^{13}$  vg/kg were administered by intravenous (retro-orbital) delivery to 2-month old knock-out animals which were then maintained on a ketogenic diet for a further 2 months. Liver transaminase levels normalised in peripheral blood in the treated animals, and mtDNA copy number and liver OXPHOS activity were restored to wild-type levels. Weight of the animals improved and liver cirrhosis was prevented, based on histological analysis.

AAV-based gene therapy strategies have been trialled in a *Ndufs4*<sup>-/-</sup> mouse model of Complex I deficient Leigh syndrome. This mouse model demonstrated encephalopathy from P40, together with weight loss, abnormal gait and lethargy, with death at P50.<sup>126</sup> Initially, AAV2/9-CMV-*hNDUFS4* was administered intravenously to neonatal mice and presymptomatic mice (P21) at a dose of 1 to  $2 \times 10^{12}$ vg/mouse. Correction of complex I activity was best seen in the heart and skeletal muscle, with poor brain transduction and consequent persistence of complex I deficiency in the brain. Intravenous delivery was therefore unable to alter the survival of knock-out animals. Intracerebroventricular administration of AAV2/9-CMV-*hNDUFS4* did not improve survival despite improving brain transduction. A combined intravenous/ intracerebroventricular approach achieved better overall brain and visceral organ transduction. Complex I dysfunction was rescued completely in skeletal muscle and heart but only partially in brain. Consequently, there was a modest improvement in survival of the animals to just over 80 days. Further analysis demonstrated that transduction in brain was limited to glial cells, not neurons, and was poor in the basal ganglia.

An alternative approach has been used to treat *Ndufs4*<sup>-/-</sup> mice utilising the PHP.B capsid to improve brain transduction via intravenous delivery.<sup>131</sup> In biodistribution studies, AAV-PHP.B was shown to transduce both neurons and glia effectively throughout the brain of *Ndufs4*<sup>-/-</sup> mice after single intravenous administration, in addition to transducing the visceral organs. AAV-PHP.

B containing the *CBA-Ndufs4* cassette was injected into 1 month old knock-out animals at a dose of  $10^{12}$  vg/mouse. This improved the lifespan of knock-outs from 0% survival at 75 days to 50% survival at 250 days' follow up. From the perspective of neurological function, improvements in paw clasping, grip strength, locomotor activity, and a significant reduction in seizures were observed in treated animals. In addition, restoration of *Ndufs4* protein expression in the brain and visceral organs, complex I activity and supercomplex formation was demonstrated. Histologically, there was a reduction in neuroinflammatory markers and gliosis in the cerebellum, olfactory bulb and vestibular nuclei. This work demonstrated that systemically administered gene therapy might be an effective method of treating mitochondrial diseases that involve both brain and visceral organs. Furthermore, adequate multisystemic targeting is dependent on selecting a combination of a ubiquitously active promoter and an appropriate capsid that enables efficient viral uptake across different tissues.

AAV-PHP.B gene therapy was also able to ameliorate the disease phenotype of a mouse model of a mitochondrial dynamics defect in *Slc25a46* deficiency.<sup>128</sup> Clinically, this defect causes hereditary sensory and motor neuropathy. Mice with the defect demonstrate ataxia, feeding difficulty and premature death. Intravenous neonatal administration of AAV-PHP.B CMV-*Slc25a46-eGFP* administered at doses of  $1 \times 10^{11}$  and  $2 \times 10^{11}$  vg/g was able to transduce affected tissues including the cortex, cerebellum, sciatic nerve, restore *Slc25a46* protein in affected tissues, reduce neuroinflammation and neuronal loss in the cerebellum and optic nerve. Furthermore, gene therapy improved the body weight, coordination and lifespan of the animals in a dose-dependent manner. This approach needs further refinement before clinical translation since it has been shown that AAV-PHP.B has a more restricted intravenous biodistribution in non-human primates than it does in mice.<sup>132</sup>

AAV9 gene therapy has been applied recently to a skeletal muscle specific conditional *Ndufs3* knock-out mouse model.<sup>133</sup> Untreated knock-out animals develop weight loss from 3 months and die prematurely. They also exhibit myopathic features including exercise intolerance and lactic acidosis, reduced complex I activity and compensatory increases in complex II and IV activities in skeletal muscle. AAV9 vectors containing the mouse *Ndufs3* cDNA driven by the CMV promoter were administered to *Ndufs3*<sup>-/-</sup> mice by retro-orbital injection at a dose of 1.25 to  $1.66 \times 10^{15}$  vg/kg at a pre-symptomatic age of 15 to 18 days as well as to symptomatic mice aged 2 months. In both cases, the mice showed improvements in body weight, motor coordination, muscle strength and increased survival. Laboratory investigations showed a

restoration of Ndufs3 protein expression which persisted at 15 months of follow-up, with normal complex I activity and skeletal muscle histochemical appearances. These data suggest that AAV9-based gene therapy can reverse the pathophysiological changes in post-symptomatic animals with myopathy due to complex I deficiency.

Gene therapy using viral vectors holds much promise based on preclinical data, but with the exception of LHON (see below), we are yet to see clinical translation in other mitochondrial disorders. Some of the challenges associated with clinical translation include ensuring sustained gene expression (ie, avoidance of transgene silencing) in target tissues and restrictions as to which patients could benefit from AAV treatments based on the presence of neutralising antibodies to AAV capsids in individual patients.<sup>134</sup> In addition AAVs have a limited packaging capacity of up to ~4.7Kb thereby limiting its use as a strategy for disorders where the transgene to be delivered is too large.<sup>135</sup> Gene therapy for rare diseases is becoming a form of personalised medicine for which the number of patients who could benefit from a specific therapy is likely to be small, and therefore the cost of the therapy, once commercialised, is likely to be high.<sup>136</sup> Nevertheless it is encouraging to see that some of the more recent preclinical studies have been able to demonstrate effective reversal of disease pathology following post-symptomatic gene transfer.

## 6.2 | Curing the mitochondrial genome 1: Selectively destroying mutant mtDNA using zinc finger nucleases and mitoTALENS

Genome editing in the mitochondrial genome is more difficult, owing to problems accessing the double membraned-mitochondrion and importing nucleic acids, which makes CRISPR-Cas9 gene editing impossible using currently available methods. Another element of complexity for mitochondrial disorders caused by mtDNA mutations is the high copy number of the mtDNA molecule, with hundreds or even thousands of copies per cell, depending on the cell type. An individual patient's tissue may possess both normal mtDNA molecules as well as mtDNA molecules containing deleterious mutations, a situation known as heteroplasmy. This gives rise to the concept of mtDNA heteroplasmy thresholds where a critical proportion of mutated mtDNA molecules may need to be present before the overall mitochondrial function of a tissue is deficient.

Despite these challenges, genetic strategies for therapeutic mtDNA manipulation are being developed. The existence of heteroplasmy introduces a potential

opportunity for treatment by selectively destroying mtDNA molecules which possess the mutation, using nucleases, thereby shifting heteroplasmy in favour of wild-type mtDNA.<sup>137</sup> Currently the most promising strategies to achieve shifts in heteroplasmy utilise zinc finger nucleases and mitochondrial transcription activator-like effector nucleases (mitoTALENS). In proof-of-principle experiments, zinc finger nucleases have been engineered that selectively destroy mtDNA molecules harbouring specific mtDNA mutations.<sup>138,139</sup> Subsequently, combined approaches for delivery of zinc finger nucleases to cells using AAV9 vectors have been undertaken in a mouse mitochondrial cardiomyopathy model caused by a m.5024C>T mutation in the mitochondrial tRNA for alanine (mt-tRNA<sup>Ala</sup>). These studies demonstrated a dose-dependent improvement in mt-tRNA<sup>Ala</sup> expression in the mouse heart.<sup>140</sup> Another nuclease-based approach is the use of mitoTALENS which can be engineered to recognise specific DNA sequences in order to induce double-stranded breaks for DNA degradation. MitoTALENS delivered to in vitro cellular models of mitochondrial disease selectively eliminated the common ~5 kb mtDNA deletion, thus shifting heteroplasmy in these patient-derived cybrid lines.<sup>141</sup> More recently, an AAV9-based approach has been used to deliver a mitoTALEN specific to the m.5024C>T mutation driven by a CMV promoter to treat the mt-tRNA<sup>Ala</sup> cardiomyopathy mouse model. This approach was successful in transducing cardiac and skeletal muscle and was able to restore mt-tRNA<sup>Ala</sup> levels in skeletal muscle.<sup>142</sup>

The clinical translatability of these approaches remains unclear since cells treated in this way become mtDNA depleted first before endogenous wild-type mtDNA repopulates. Furthermore, mistargeting of the zinc finger nucleases to the nucleus has been reported.<sup>138</sup> It also remains to be determined whether shifts in heteroplasmy are sustained over time.

## 6.3 | Curing the mitochondrial genome 2: Allotopic expression of mitochondrial proteins

When considering gene therapy for disorders caused by mutations in the 13 protein-coding mtDNA genes, another challenge is ensuring intra-mitochondrial expression of the transgene's protein product. One approach, rather than delivering the transgene itself across the mitochondrial membranes, is to express the mitochondrial gene allotopically within the nucleus, and designing the transgene cassette to contain a mitochondrial targeting sequence (MTS) that enables the newly translated polypeptide to enter the mitochondrion through

endogenous import mechanisms.<sup>143</sup> This approach has reached clinical trials in patients with LHON.<sup>144,145</sup> The most prevalent cause of LHON is a mutation in the *MT-ND4* gene that encodes a subunit of complex I. Ganglion cells within the retina are most affected in LHON, typically resulting in subacute loss of vision. In one phase I/II study (NCT02064569) which recruited patients in France, rAAV2/2-*ND4* (including a MTS derived from the COX10 complex IV assembly factor) was administered intravitreally unilaterally to an affected eye in four dose escalations from  $9 \times 10^9$  to  $9 \times 10^{10}$  vg/eye and followed up over a period of almost 5 years.<sup>144</sup> Adverse events following treatment included anterior chamber inflammation, vitritis and elevated intraocular pressure. In most cases these were managed with appropriate topical anti-inflammatory agents. Since the gene therapy was delivered unilaterally each patient had a fellow (control) eye for comparison. Best corrected visual acuity improved in the treated eye in 43% of individuals with better outcomes seen in patients with a shorter disease course and a better baseline visual acuity. An alternative strategy being trialled uses a different MTS, derived from the P1 isoform of ATP synthase subunit c, and a recombinant self-complementary AAV2 scaffold. An open-label phase I clinical trial (NCT02161380) of the scAAV2-P1ND4v2 vector with three dose escalations is ongoing. Data for the low and medium doses are available.<sup>145</sup> Low ( $5 \times 10^9$ ) and medium ( $2.46 \times 10^{10}$  vg/eye) doses of scAAV2-P1ND4v2 were administered unilaterally to 14 patients with either acute or chronic bilateral visual loss. Comparison was made between mean visual acuity in both injected and fellow eyes compared with baseline. Overall, both injected and fellow eyes demonstrated improvements in visual acuity over follow up, but the improvement seen in injected eyes was greater than that seen in fellow eyes. This effect was more evident in the acute visual loss group than the chronic visual loss group. In those for whom improvement was seen, a rapid treatment effect was noted within 1 month of gene therapy administration, with ongoing improvements in visual acuity over 18 months of follow-up. The only adverse event noted was anterior uveitis which was mild in all cases and did not require treatment. A high dose escalation to  $10^{11}$  vg/eye is currently recruiting.

## 7 | CONCLUSION

This review has discussed pharmacological and genetic therapies for mitochondrial disease, spanning the spectrum from treatments still at a preclinical phase of development to those that have reached phase III clinical trials. Traditionally pharmacological therapies for

mitochondrial disease have taken a generic approach, targeting mitochondrial biogenesis, lipid membranes, ROS, and mitophagy. However, for some diseases specific pharmacological approaches are underway, for example nucleoside replacement for TK2 deficiency and enzyme replacement for MNGIE. Genetic therapies are likely to be the most promising approaches ultimately, although most of these are currently at preclinical stages of development. However, as there are at least 350 known mitochondrial diseases, each with its own genetic cause, in future decisions will need to be made as to which are good candidates for gene therapy. Factors to be considered will include disease prevalence (or more precisely the number of patients who could benefit from treatment), the presence of a clinically relevant animal model, and the penetrance of disease as determined by genotype-phenotype correlation from the natural history.

The significant remaining challenges for trial design should not be underestimated. Several mitochondrial disease trials are actively recruiting but these are mainly early phase (I/II) trials targeting adults with mitochondrial myopathy. There remains a dearth of clinical trials specifically targeting paediatric mitochondrial diseases. Although there are still no curative therapies for the vast majority of individuals affected by primary mitochondrial diseases, it should be remembered that supportive therapies might be lifesaving or life preserving. The last 5 years have seen dramatic changes in the field of mitochondrial medicine, with increased diagnostic power achieved through next generation sequencing approaches. It is hoped that the next 5 years will finally bring licensed disease-modifying medicines for people affected by mitochondrial disease.

## COMPETING INTERESTS

S.R. is an Investigator on the EE-TP clinical trial (NCT03866954). R.D.S.P. is Chief Investigator for the clinical trial assessing KL1333 (NeuroVive Pharmaceutical AB, NCT03888716) and Principal Investigator for the REN001-101 (REN001, Reneo Pharmaceuticals Inc., NCT03862846) and MMPOWER-3 (Elamipretide, Stealth BioTherapeutics Inc., NCT03323749) clinical trials. R.D.S.P. has received honoraria from Stealth BioTherapeutics and Reneo Pharmaceuticals for S.A.B. meetings. N.K. and J.R. declare no competing interests.

## AUTHOR CONTRIBUTIONS

Drafting manuscript: all authors; Figures: N.K., Table: J.R., S.R.; Review and editing of manuscript: all authors.

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