Mitochondrial disorders are a diverse group of debilitating conditions resulting from nuclear and mitochondrial DNA mutations that affect multiple organs, often including the central and peripheral nervous system. Despite major advances in our understanding of the molecular mechanisms, effective treatments have not been forthcoming. For over five decades patients have been treated with different vitamins, co-factors and nutritional supplements, but with no proven benefit. There is therefore a clear need for a new approach. Several new strategies have been proposed acting at the molecular or cellular level. Whilst many show promise in vitro, the clinical potential of some is questionable. Here we critically appraise the most promising preclinical developments, placing the greatest emphasis on diseases caused by mitochondrial DNA mutations. With new animal and cellular models, longitudinal deep phenotyping in large patient cohorts, and growing interest from the pharmaceutical industry, the field is poised to make a breakthrough.
premature death. Therefore, although they are rare diseases, with a minimum reported prevalence of 1 in 4300 (Schaef er et al., 2004), they have substantial impact on families and healthcare services.

Most mitochondrial disorders are ultimately thought to arise through a bioenergetic defect linked to a deficiency of ATP synthesis. ATP synthesis is the final step of respiration, which is carried out by five oxidative phosphorylation (OXPHOS) complexes situated on the inner mitochondrial membrane. Each complex has multiple protein subunits encoded by two distinct genomes: nuclear chromosomal DNA (nDNA), and the 16.5 kb mitochondrial genome (mtDNA).

Pathogenic mtDNA mutations often cause a subset of classical mitochondrial clinical syndromes (Chinnery and Hudson, 2013). However, an increased number of multi-system mitochondrial disorders are being described in the literature, many of which are yet to be fully characterized clinically and genetically. These include an emerging myriad of nuclear encoded mitochondrial disorders caused by mutations in some of the ~1500 nuclear genes thought to code for mitochondrial proteins (Chinnery and Hudson, 2013). In the past, the phenotypic and genetic diversity has made clinical diagnosis very challenging. However, with international diagnostic standards (Wolf and Smeitink, 2002), and the widespread availability of molecular genetic techniques, an accurate diagnosis is less challenging than before. Next generation sequencing is revolutionizing the diagnostic approach, with multi-gene panels, whole exome, and whole genome sequencing increasing the pace of diagnosis, and probably reducing the overall costs. As a consequence, more and more patients are being diagnosed with a mitochondrial disorder, placing even greater emphasis on developing treatments.

A recent systematic review identified over 1300 reports using a variety of approaches expected to bypass or enhance components of mitochondrial function. However, the vast majority of these reports are open-labelled case series with less than five subjects. Although ~30 randomized trials have been carried out to date, no treatment has shown a clear cut benefit on a clinically meaningful end-point (for reviews see Pfeffer et al., 2012; Kerr, 2013). It is therefore likely that components of the traditional ‘mitochondrial cocktail’ do not have a major therapeutic impact on most mitochondrial diseases. There is therefore a clear need for the field to ‘think outside the box’ when developing new treatments, harnessing the massive increase in our understanding of mitochondrial disease pathogenesis. After preclinical evaluation in cellular and animal models, new treatments showing promise should be studied in patients using a rigorous approach (Pfeffer et al., 2013). This review focuses on these new developments, with a particular emphasis on mtDNA diseases, which were previously thought to be intractable. Here we critically appraise each approach, and highlight areas where there is likely to be traction in the future. This is timely, because both small and large pharmaceutical companies are starting to see the potential market in developing treatments for these so-far untreatable disorders.

**Capitalizing on the unique properties of mitochondria**

Mitochondria have unusual characteristics that are potentially targetable for treatment at the molecular level. Mitochondria contain numerous copies of their genome, and most patients with mtDNA disease harbour both mutated and wild-type mtDNA (heteroplasmy) (Wallace, 1999). The proportion of mutated mtDNA needs to be in excess to cause a biochemical defect of the respiratory chain. This critical threshold varies from mutation to mutation, and also between tissues and organs. This partly explains the tissue selectivity and clinical heterogeneity of mitochondrial disorders (Macmillan et al., 1993). The overall amount of mtDNA within a cell is also tightly regulated in a tissue-specific manner, and some tissues (such as skeletal muscle) display a close correlation between mtDNA content and mitochondrial activity (Wang et al., 1999). This complexity raises the possibility of enhancing mitochondrial function by manipulating the ratio of mutant to wild-type mtDNA, or by increasing the amount of wild-type mtDNA. Targeting mutated mtDNA is an alternative approach, although this would need to be highly specific to avoid adverse effects on wild-type genomes. The delivery of wild-type nDNA (Di Meo et al., 2012; Bottani et al., 2014; Torres-Torrenteras et al., 2014) or mtDNA (Ellouze et al., 2008; Yu et al., 2012a,b) using viral vectors is another possibility, or perhaps the replacement of dysfunctional proteins via the cell nucleus, hitch-hiking on the mitochondrial import mechanism (Perales-Clemente et al., 2011). Moving away from the two genomes, small molecule screens may enhance function of the respiratory chain, stem cell therapies could correct enzyme defects due to nuclear gene defects (Hirano et al., 2006; Hill et al., 2009; Halter et al., 2011; Lenoci et al., 2011; Filosto et al., 2012; Sicurelli et al., 2012; Hussein, 2013), and treatments aimed at non-specifically preventing neurodegeneration may be the way forward. Some of these approaches capitalize on the ‘uniqueness’ of mitochondria, where other approaches build on knowledge acquired from rare and common neurological disorders. Having an open mind is critical at this stage—the first effective treatment may not come from an obvious place. This review considers each, structured into four sections (Fig. 1): manipulating DNA; new protein delivery; small molecule pharmaceuticals; and finally, stem cell approaches. Our discussion focuses on the likelihood of these treatments being used in the clinic for patients with mitochondrial disease, and does not discuss recent work aimed at preventing these disorders.
### Nucleic acid-based approaches

Several approaches, described below, have been developed to manipulate or replace mutated nDNA and mtDNA. As for all potential therapies, the multi-organ nature of mitochondrial disorders and difficulties of transferring therapies across cellular and mitochondrial membranes without causing toxic effects (Mingozzi and High, 2011), makes therapeutic targeting difficult. However, as explored further below, methods exist to overcome these difficulties including the use of viral vectors (Mingozzi and High, 2011), harnessing allotopic expression (nuclear expression of mtDNA-derived cybrid cells. Over time, the treatment resulted in repopulation with wild-type mtDNA, and normalization of cellular ATP content (Tanaka et al., 2002).  

#### Mitochondrially-targeted nucleases

Restriction endonuclease approaches were developed to recognize specific DNA sequences, produce double-stranded DNA breaks, and thereby initiate target molecule degradation. Unlike other gene therapy approaches, restriction endonucleases are not contingent on stable vector integration into the nuclear genome. In theory, a single treatment could induce a stable modulation of mtDNA heteroplasmy, correcting the biochemical defect within diseased cells (Minczuk et al., 2008; Bacman et al., 2013; Gammage et al., 2014). Restriction endonucleases recognize a broad repertoire of mtDNA sequences (‘restriction sites’) and some pathogenic mtDNA mutations add restriction sites that restriction endonucleases specifically target. The m.8993T>G mtDNA mutation causes Leigh syndrome or neuropathy ataxia retinitis pigmentosa, and mitochondrially-targeted restriction endonuclease Smal has been shown to selectively eliminate m.8993T>G from patient-derived cybrid cells. Over time, the treatment resulted in repopulation with wild-type mtDNA, and normalization of cellular ATP content (Tanaka et al., 2002).

Unfortunately, a key limitation of restriction endonucleases is that very few human pathogenic mutations create restriction sites amenable to this form of targeted destruction. This can in part be overcome by restriction endonucleases custom designed to bind specific DNA sequences.

<table>
<thead>
<tr>
<th>Examples</th>
<th>Challenges</th>
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<tr>
<td>Zinc Finger Nucleases</td>
<td>Lack of mutation-defined restriction sites for restriction endonucleases to target</td>
</tr>
<tr>
<td>Transcription Activator Like Effector Nucleases</td>
<td>Specificity of restriction endonuclease targeting</td>
</tr>
<tr>
<td>Human non-cognate mitochondrial leucyl tRNA synthetase</td>
<td>Efficiency of targeting recombinant proteins into cells and mitochondria</td>
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<tr>
<td>Carboxy-terminal domain of human mitochondrial leucyl tRNA synthetase</td>
<td>Solubility</td>
</tr>
<tr>
<td>AAV-ETH4 (nuclear gene)</td>
<td>Efficiency of delivery</td>
</tr>
<tr>
<td>AAV-ND4 (mitochondrial gene)</td>
<td>Cross-reactivity</td>
</tr>
</tbody>
</table>

#### Systemic protein delivery

**Cellular and mitochondrial protein delivery**

- Transfusion of platelets or erythrocyte-encapsulated thymidine phosphorylase
- Mitochondrially-targeted transcription factor A

Efficiency of targeting recombinant proteins into cells and mitochondria. This can in part be overcome by restriction endonucleases that recognition sites amenable to this form of targeted destruction. This can in part be overcome by restriction endonucleases custom designed to bind specific DNA sequences.

#### New protein delivery

**Exogenous stem cell therapy for nuclear gene mutations**

- Bone marrow and stem cell transplantation
- Bupivicaine injections
- Exercise

- Avilability of stem cell transplants
- Sustaining effect
- Side effects of myeloablative protocol
- Finte capacity of repair
- Limited to patients with isolated mitochondrial myopathies

#### Small molecule pharmaceuticals

**Manipulating mitochondrial and nuclear DNA**

- Bezaflurate
- AICAR
- Resveratrol
- PAPR inhibitors
- Rapamycin
- Cyclosporin A

- Hepatomegaly and abnormal lipid metabolism in animal models
- Potential hepatic side effects
- Mild gastrointestinal side effects
- Mild side effects including fatigue, nausea vomiting and exercise
- Hyperlipidaemia, poor wound healing and immunosuppression
- Immunosuppression

#### Stem cell approaches

**Manipulating mtDNA with peptide nucleic acids**

- Zinc Finger Nucleases
- Transcription Activator Like Effector Nucleases

- Solubility
- Efficiency of delivery

**Manipulating tRNA enzymes**

- Carboxy-terminal domain of human mitochondrial leucyl tRNA synthetase

- Cross-reactivity
- Efficiency of delivery

**Gene transfer using adeno-associated viral vectors**

- AAV-ETH4 (nuclear gene)
- AAV-ND4 (mitochondrial gene)

- Adeno-associated viruses cannot carry constructs > 5kb
- Misexpression of the imported gene
- Efficient delivery to desired cell population
- Obtaining adequate titres

**Mitochondrially-targeted restriction endonucleases**

- Very few human pathogenic mutations create restriction sites amenable to this form of targeted destruction.
- Restriction endonucleases custom designed to bind specific DNA sequences.

**Figure 1** Overview of novel therapeutic approaches for the treatment of mitochondrial disorders. AICAR = 5-aminoimidazole-4-carboxamide ribonucleotide; PAPR = poly adenosine diphosphate-ribose polymerase receptor; TP = thymidine phosphorylase.
A zinc finger nuclease (ZFN) consists of tandem repeat zinc fingers, each binding approximately three DNA bases, combined with a FokI endonuclease domain functioning as the DNA cleavage module (Klug, 2010). Specificity is achieved through different combinations of zinc fingers. ZFNs have also been shown to selectively eliminate the m.8993T>G mutation in a cybrid model (Minczuk et al., 2008). Early ZFNs caused significant cytotoxicity, homodimerization (Ramalingam et al., 2011) and off-target binding. This would be detrimental in vivo, thereby limiting their clinical use (Radecke et al., 2010), but are being addressed by improved design (Gammage et al., 2014). The architecture of dimeric ZFNs ensures that the active cleavage reagent is only assembled at desired restriction sites, overcoming targeting difficulties and concerns about constitutive nuclease activity (Gammage et al., 2014). Customized ZFNs targeted to mitochondria cause shifts in heteroplasmy through the selective degradation of mtDNA containing the m.8993T>G point mutation, and the large scale (4977 bp) mtDNA ‘common deletion’, which is the most common cause of chronic progressive external ophthalmoplegia, Kearns-Sayre syndrome, and Pearson marrow pancreas syndrome (Gammage et al., 2014). This work opens up the opportunity to develop a library of bespoke ZFNs against more common pathogenic mtDNA mutations, but the shifts in heteroplasmy have been limited to date. Longer-term studies, particularly using animal models, will hopefully show that ZFNs can improve biochemical function in vivo—but this will be technically demanding, not least because of the challenges delivering these agents at an appropriate concentration to affected tissues.

When combined with a non-specific nuclease (e.g. FokI), TALEs (transcription activator-like effectors) become target-specific DNA nucleases (TALENs) (Christian et al., 2010). TALENs are potentially much more potent than ZFNs, but their larger size limits their use with adeno-associated viral vectors (AAVs) (Ellis et al., 2013), TALEs contain repetitive

**Figure 2.** Endonucleases. Endonucleases are used to target specific sequences in mtDNA causing double-strand breaks and degradation of mtDNA. For example the endonuclease ZFN has been shown to reduce mutation load in a cybrid model of Leigh and NARP syndrome, which are caused by the mtDNA mutation m.8933T>G within the ATP6 domain. ZFN binds specifically to the mutant form of the mtDNA and the FOK1 endonuclease domain cleaves the DNA molecule, which is then degraded.
domain modules, each of which bind individual nucleotides (Moscou and Bogdanove, 2009), thus different domain module combinations can be used to ensure specificity.

A recent report described the use of mitochondrially localized TALENs targeted for mtDNA species with the ‘common deletion’ (m.8483_13459del4977), and a separate TALEN that recognizes a point mutation (m.14459G>A in the MT-ND6 gene) in an osteosarcoma cybrid cell model (Bacman et al., 2013). The TALEN markedly reduced the level of deleted mtDNA species allowing compensatory mitochondrial biogenesis to preferentially restore wild-type mtDNA (Bacman et al., 2013). In studies with the point mutation, relative specificity was demonstrated for the mutant over the wild-type sequence (Bacman et al., 2013). Interestingly the heteroplasmy shift persisted after the TALENs were no longer detectable, suggesting long-lasting effects after a single treatment (Bacman et al., 2013). However, the impact of the rapid reduction in mtDNA copy number following TALEN treatment raises serious concerns about their potential toxicity, not least because several fatal mitochondrial diseases are caused by mtDNA depletion. However, the co-induction of mitochondrial biogenesis could circumvent this issue, but will need careful evaluation in animal models before any clinical studies.

The most recent addition to this group of endonucleases is Cas9 nuclease. Unlike the ZFN and TALE proteins that target the FokI endonuclease, Cas9 is targeted using a much smaller short RNA sequence CRISPR (Hsu et al., 2014). This RNA sequence can more easily be altered to change target site than for TALEs and distinct RNA sequences can be used target Cas9 to induce multiple double-stranded DNA breaks (Hsu et al., 2014; Cox et al., 2015). Furthermore this system is not hindered by context-specific binding reported for ZFNs and TALENs (Hsu et al., 2014; Cox et al., 2015). Although there has been controversy surrounding this approach, recently Cas9/CRISPR has been demonstrated to restore the weight loss phenotype of a mouse model of fatal disease hereditary tyrosinaemia type 1 (Yin et al., 2014).

The potential use of restriction endonucleases to prevent germline transmission has also been investigated. MitoTALENs have been demonstrated to reduce levels of human mutated mtDNA responsible for Leber hereditary optic neuropathy (LHON) and NARP (neurogenic muscle weakness, ataxia, and retinitis pigmentosa) in oocytes (Reddy et al., 2015).

**Peptide nucleic acids**

Sequence-specific peptide nucleic acids selectively bind mutant mtDNA and induce direct mtDNA strand degradation (Mukherjee et al., 2008). Although conjugation with
Mitochondrial targeting peptides promotes importation and successful targeting into human cells in culture (Chinnery et al., 1999), this initially failed to modulate heteroplasmy in patient-derived cell lines (Kyriakouli et al., 2008). In part this is due to a failure to transmit the peptide nucleic acids across the mitochondrial membrane, so techniques of traversing the cell membrane are being explored, such as ‘cell membrane crossing oligomers’ (Kyriakouli et al., 2008), which possess greater polarity, and RNA vectors (Comte et al., 2013). Although these examples show promise, evidence for therapeutic benefit remains sparse despite nearly two decades of research. Targeted delivery will be a common problem, and potential toxic effects need to be excluded in long-term animal studies. Again, limited availability of animal models of human mtDNA diseases has hindered progress.

**Manipulating tRNAs**

The large array of pathogenic mtDNA mutations affecting tRNA genes prompted the exploration of tRNA-targeted therapies (Yarham et al., 2011). Of particular interest are tRNA synthetases that catalyse the addition of specific amino acid molecules to cognate tRNA molecules during protein translation. Early work demonstrated that the overexpression of cognate aminoacyl mt-tRNA synthetase stabilized mt-tRNA (mitochondrial tRNA) molecules (Rorbach et al., 2008). More recently overexpression of human non-cognate mitochondrial leucyl tRNA synthetase and its small carboxy-terminal domain were shown to partially rescue the biochemical dysfunction secondary to mt-tRNA defects (Hornig-Do et al., 2014; Perli et al., 2014).

**Gene transfer using adeno-associated viral vectors**

Harnessing viral vectors to transfer wild-type genes into the cell nucleus dates back to the late 1990’s. Although initial excitement was tempered by the death of a patient in an early clinical trial (Raper et al., 2003), much progress has been made in developing new, less immunogenic vectors such as AAV (Wang et al., 2012) (Fig. 3).

**Gene therapy for nuclear-mitochondrial disorders**

Ethylmalonic encephalopathy classically has heterogeneous phenotypes with neurological, gastrointestinal, metabolic and psychiatric sequelae (Tiranti et al., 2004) and results from mutations in the *ETHE1* gene (Tiranti et al., 2004). *ETHE1* encodes a ubiquitous mitochondrial sulphur dioxygenase that detoxifies hydrogen sulphide (Tiranti et al., 2004). AAV-mediated hepatic *ETHE1* gene expression restores sulphur dioxygenase activity in a murine model of ethylmalonic encephalopathy, correcting biochemical abnormalities within liver, muscle, and brain; and increasing survival from a few weeks to more than 6 months (Di Meo et al., 2012). However, targeting human skeletal muscle and other large tissues is extremely challenging, given currently achievable viral titres. Targeting the relatively impenetrable blood–brain barrier presents another hurdle in the treatment of nuclear-encoded mitochondrial diseases. However, there are exceptions where the approach has real potential for therapeutic benefit in the near future.

Mitochondrial neurogastrointestinal encephalopathy (MNGIE) is a rare defect of the thymidine phosphorylase encoding gene *TYMP* (Nishigaki et al., 2003). Mutations in *TYMP* cause high circulating levels of thymidine, which in turn lead to mtDNA depletion and the formation of secondary mtDNA deletions and point mutations. Treating a single organ, such as the liver, has been proposed to generate enough enzyme to ‘detoxify’ the systemic circulation and thereby prevent disease progression (Torres-Torronteras et al., 2014). In keeping with this idea, the permanent and dose-dependent reduction of systemic toxic metabolite levels in a murine model of MNGIE has been demonstrated with the construct, AAV2/8-hcTYMP, which possesses a liver-specific promoter (Torres-Torronteras et al., 2014). Interestingly, recent evidence that the liver can synthesize potentially therapeutic thymidine phosphorylase levels has prompted the suggestion that liver transplantation would be an effective therapy for MNGIE (Boschetti et al., 2014).

The Harlequin mouse model of mitochondrial complex 1 deficiency results from a pro-viral X-linked gene insertion causing severely reduced apoptosis-inducing factor 1 (AIF1) expression. These mice exhibit retinal and optic nerve respiratory chain complex 1 deficiency causing glial and microglial cell activation, retinal ganglion loss and optic atrophy. The intra-vitreal administration of an AAV2/2_AIF1 vector has been shown to counteract these changes (Bouaita et al., 2012).

Finally, mutations in the *MPV17* gene result in a hepatocerebral mtDNA depletion syndrome that is also potentially amenable to gene therapy (Bottani et al., 2014). *MPV17* knockout (KO) mice exhibit hepatic mtDNA depletion and liver failure when subjected to a ketogenic diet (Bottani et al., 2014). This phenotype is rescued by AAV viral vector expression of human *MPV17* cDNA with a hepatic specific promoter (Bottani et al., 2014). However, it is unclear whether the tissue-specific expression of the transgene will correct the cerebral disorder. This is critical because the CNS features of the disorder are fatal.

**Gene therapy for mitochondrial DNA disorders**

Delivering gene therapy into mitochondria presents an even greater challenge. Many cells vulnerable to mitochondrial disease contain thousands of mitochondria, and the mitochondrial membrane is relatively impermeable. There is little evidence that AAVs penetrate into the mitochondrial matrix, so achieving the high titres required to intercalate...
with multiple copies of mtDNA seems almost insurmountable at present. An alternative approach is to harness well-established techniques for nuclear gene therapy to ‘allotopically express’ mtDNA encoded proteins. If carefully engineered, and with a targeting peptide presequence, this has the potential to deliver wild-type proteins to the mitochondrial membrane.

After early in vitro work expressing the subunits of the mitochondrial ATPase (Bokori-Brown and Holt, 2006), several laboratories have focused on the allotropic expression of complex I subunits with a view to treating the most common mtDNA disorder: LHON. The mtDNA m.11778G > A mutation affects nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 4 (ND4 of complex I, and accounts for ~50% of LHON patients). Not surprisingly, this mutation has been the focus for most of the studies to date. In vitro and preclinical work (Guy et al., 2002; Qi et al., 2007; Ellouze et al., 2008; Yu et al., 2012a,b) provide some evidence base for this approach, and preparations for early phase clinical (Lam et al., 2010; Bin, 2015; Vignal, 2015) studies are well under way in the USA, China and Europe.

The allotropic expression of a synthetic ND4 gene is reported to increase ATP synthesis in a human cell harbouring the m.11778G > A mutation (Guy et al., 2002). More recently, in murine LHON models (Qi et al., 2007; Ellouze et al., 2008; Yu et al., 2012a,b), intraocular injection of human nuclear ND4 gene constructs expressed by AAV has been shown to be safe (Ellouze et al., 2008; Yu et al., 2012a); non-mutagenic to host mtDNA (Yu et al., 2013); prevent retinal ganglion cells loss; and improve optic atrophy and vision (Ellouze et al., 2008; Yu et al., 2012a). However, as explored further below, this contradicts the longstanding evidence of interspecies protein incompatibility (McKenzie et al., 2003; Perales-Clemente et al., 2011). Furthermore, although the allotypically-expressed proteins appear to be imported, it is not clear whether the new peptides integrate within the respiratory chain (Figueroa-Martinez et al., 2011). Indeed, one study demonstrated that mitochondrially-encoded NADH (reduced nicotinamide adenine dinucleotide) dehydrogenase (with an additional mitochondrial transduction domain) was expressed in the nucleus and partially rescued the phenotype of cells with a homoplasmic knockout mutation for mt-Nd6 (Perales-Clemente et al., 2011). However, the expressed protein seemed to lie largely outside the outer mitochondrial membrane (Perales-Clemente et al., 2011). Despite these concerns, groups in Miami (Lam et al., 2010) and China (Bin, 2015) are currently recruiting for a trial to examine intraocular AAV-ND4 for LHON. A further safety trial is currently recruiting in France (Vignal, 2015).

**Expression of non-mammalian genes**

There is interest in using AAV expressing mature yeast complexes (not troubled by issues of integration) to bypass mutations in multi-subunit mammalian equivalents. This ‘transkingdom approach’ has been shown to preserve retinal ganglion cells, improve optic nerve function and improve vision in a rotenone-induced LHON rodent model (Marella et al., 2010; Chadderton et al., 2013). Lentiviral vectors expressing a yeast alternative oxidase have also been demonstrated to rescue COX (cytochrome c oxidase) deficiency in a mouse model (El-Khoury et al., 2013).

Despite the emerging evidence supporting transkingdom protein expression, it remains a contentious issue. The co-evolution of nDNA and mtDNA has led to species-specific genome compatibility (Kenyon and Moraes, 1997). Incompatibilities between nDNA and mtDNA explain why Human-Gorilla xenomitochondrial hybrids have a respiratory chain deficiency (Barrientos et al., 1998), and incompatibles of interspecies mtDNA gene products (McKenzie et al., 2003; Perales-Clemente et al., 2011), suggest that caution is needed with this approach, which is very much at the early preclinical phase.

**Improving mitochondrial DNA copy number in mitochondrial DNA depletion syndromes**

In vitro and preclinical evidence support the therapeutic potential of increasing deoxyribonucleotides to treat autosomal recessive mtDNA depletion syndromes. One approach involves deoxyribonucleoside supplementation, which increased mtDNA levels (copy number) in primary cell cultures from patients with deoxyguanosine kinase deficiency due to DGUOK mutations (Bulst et al., 2009; Camara et al., 2014). A similar effect has also been seen in an in vitro model of MNGIE (Camara et al., 2014). Another strategy involves inhibition of deoxyribonucleoside catabolism, which has also been shown to improve mtDNA copy number in vitro (Camara et al., 2014). More recently, deoxyribonucleoside supplementation in a knock-in mouse model of thymidine kinase 2 (TK2) deficiency increased mtDNA copy number, improved mitochondrial respiratory chain function, and prolonged life span (Garone et al., 2014).

**New protein delivery**

**Systemic protein delivery**

The identification of a specific protein dysfunction or deficiency resulting in mitochondrial disease has led to the possibility of protein replacement or the removal of accumulated toxic metabolites.

In MNGIE, haemodialysis has been used to remove toxins (Spinazzola et al., 2002) and the transfusion of platelets (Lara et al., 2006) or erythrocyte-encapsulated thymidine phosphorylase (EE-TP) has been explored as a means of delivering exogenous thymidine phosphorylase (Table 2) (Moran et al., 2008; Bax et al., 2013; Hussein, 2013). At present there is no convincing evidence of
<table>
<thead>
<tr>
<th>Reference</th>
<th>Age/Gender</th>
<th>Intervention</th>
<th>Outcome measures</th>
<th>Outcome</th>
<th>Reported adverse events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hussein 2013</td>
<td>Patient 1: M/24</td>
<td>HLA fully matched BMT</td>
<td>TP activity</td>
<td>TP activity initially rose from 1 to 180 nmol/h/mg/protein but then dropped to 10 nmol/h/mg/protein 10 months post-transplantation. Normalized.</td>
<td>GVHD</td>
</tr>
<tr>
<td>Filosto et al., 2012</td>
<td>Patient 1: F/34</td>
<td>HLA matched HSCT</td>
<td>TP activity</td>
<td>Patient 1 and 2: TP activity rose from 0 nmol/h/mg/protein to within normal range.</td>
<td>Patient 1: Pyrexia of unknown origin, hyperglycaemia, pancreatitis, CMV reactivation, GVHD and complications of immunodeficiency. Patient 2: mild GVHD. Posterior reversible encephalopathy syndrome and C. difficile diarrhoea, followed by septic shock and ARDS.</td>
</tr>
<tr>
<td>Sicurelli et al., 2012</td>
<td>Patient 1: F/23</td>
<td>HLA matched HSCT</td>
<td>TP activity</td>
<td>TP activity rose from 0 nmol/h/mg/protein to within normal range.</td>
<td>Worsening of sensory neuropathy.</td>
</tr>
<tr>
<td>Hirano et al., 2006</td>
<td>Patient 1: F/21</td>
<td>HLA matched placental cord blood transplant</td>
<td>TP activity</td>
<td>Blood lactate decreased from 2.3 to 1.5 mmol/l.</td>
<td>Patient 1: no symptomatic improvement was documented. Died 86 days post-transplantation from disease progression.</td>
</tr>
<tr>
<td>Patient 2: F/30</td>
<td>HLA matched related SCT</td>
<td>dThd and dUrd levels</td>
<td>Blood lactate</td>
<td>Patient 1 and 2: dThd and dUrd levels were reduced in both patients and to within normal range for patient 2 after 2 months.</td>
<td></td>
</tr>
<tr>
<td>Lenoci et al., 2011 (abstract only)</td>
<td>Patient 1: F/21</td>
<td>HLA matched related HSCT</td>
<td>TP activity</td>
<td>TP activity normalized 55 days post-transplantation.</td>
<td>None reported.</td>
</tr>
<tr>
<td>Hill et al., 2009</td>
<td>Patient 1: F/41</td>
<td>HLA matched related peripheral blood SCT</td>
<td>TP activity</td>
<td>TP activity normalized by day 25 post-transplantation and was still normal 14 months post-transplantation. Improvement in oral intake and increase in weight. Neurological performance status, repeat EMG and cerebral MRI scan were unchanged.</td>
<td>Idiopathic thrombocytopenic purpura and gastrointestinal haemorrhage.</td>
</tr>
</tbody>
</table>

A series of case reports of stem cell therapies in eight patients with MNGIE showed variable results. Overall thymidine phosphorylase activity tended to increase post-transplantation and some improvements in gastrointestinal and neurological symptoms were reported. ARDS = acute respiratory distress syndrome; BMT = bone marrow transplant; CMV = cytomegalovirus; EMG = electromyogram; F = female; GVHD = graft versus host disease; HLA = human leukocyte antigen; HSCT = haematopoietic stem cell transplantation; M = male; MRC = Medical Research Council; SCT = stem cell transplant; TP = thymidine phosphorylase; dTHd = thymidine; dURD = deoxythymidine.
### Table 2 New protein delivery to treat MNGIE

<table>
<thead>
<tr>
<th>Reference</th>
<th>Age/gender</th>
<th>Intervention</th>
<th>Outcome measures</th>
<th>Outcome</th>
<th>Reported adverse events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax et al., 2013</td>
<td>M/28</td>
<td>Encapsulated TP</td>
<td>Plasma dThd and dUrd levels between cycles</td>
<td>Thd and dUrd levels reduced to 8.1 and 12.6 mmol/l from 20.5 and 30.6 mmol/l, respectively after 27 cycles. Urinary dThd and dUrd levels reduced to 192-282 and 0.0-184 mmol/l from 421 and 324 mmol/l, respectively from cycle 21.</td>
<td>Mild transient reaction to infusion with coughing and head and neck erythema.</td>
</tr>
<tr>
<td>Moran et al., 2008</td>
<td>F/21</td>
<td>Encapsulated TP</td>
<td>Urinary dThd and dUrd levels</td>
<td>3 days post-infusion plasma dThd and dUrd levels reduced (but not to within normal range) but then began to rise.</td>
<td>None reported.</td>
</tr>
</tbody>
</table>

Systemic protein delivery: thymidine phosphorylase (TP) encapsulated in erythrocytes. Thymidine phosphorylase can be encapsulated in autologous blood erythrocytes by reversible hypo-osmotic dialysis, these erythrocytes can then be used for infusion to increase the functional thymidine phosphorylase levels of the recipient. Results from two case reports of infusion of thymidine phosphorylase encapsulated in erythrocytes for patients with MNGIE demonstrated initial reductions in toxic product levels post transplantation and some reported clinical improvement. MRC = Medical Research Council; dTHd = thymidine; dURD = deoxythymidine.
the complexity of the diverse genotype–phenotype relationship still complicates drug discovery (Macmillan et al., 1993; Wallace, 1999; Wang et al., 1999; Koopman et al., 2012), this could be circumvented by screening a panel of drugs on specific patient cell lines, leading to a personalized or precision medicine approach (Koopman et al., 2012), which can be optimized for testing in animals models then clinical studies (Koopman et al., 2012). So called ‘N-of-one’ clinical trials provide one way of moving forward in patients, proving the drugs have a measurable effect in a short time period.

**Induction of mitochondrial biogenesis**

**Bezafibrate**

Several studies have focussed on peroxisome proliferator-activated receptor (PPAR) gamma co-activator 1 alpha (PGC-1α, encoded by PPARGC1A) as a potential treatment for mitochondrial disease. PGC-1α co-ordinates the vast majority transcriptional mitochondrial biogenesis regulation in accordance with metabolic demand (Scarpulla et al., 2012). The array of novel pharmaceuticals discussed below either modulate PGC-1α mtDNA expression (e.g. PPARα) and protein expression or target downstream pathways. Bezafibrate is pharmacological ligand for the transcriptional co-factor PGC-1α. AICAR activates AMP-activated protein kinase (AMPK) and is thought to modulate increased mitochondrial biogenesis through PGC-1α. The natural polyphenol resveratrol activates sirtuin 1 (SIRT1). Sirtuins are part of a group of oxidizing NAD-dependent protein deacetylases. Upon activation, for example, by PGC-1α or transcription factor A, mitochondrial (TFAM) they promote mitochondrial respiratory chain activities and the transcription of genes modulating mitochondrial biogenesis and function. Nicotinamide riboside can be used to supplement NAD+ levels. PARP1 functions as a NAD+ consuming enzyme. Thus in turn inhibition of PARP1 has been demonstrated to increase NAD+ bioavailability and SIRT1 activity (not shown above) promoting oxidative phosphorylation. Rapamycin inhibits mTOR, which in turn releases mTOR inhibition of autophagy. Cyclosporin A inhibits the mitochondrial permeability transition pore (MPTP). Opening of the mitochondrial permeability transition pore is thought to deplete pyridine nucleotides thus impairing mitochondrial oxidative respiration.

**Figure 4** Schematic representation of pharmaceutical modulators of mitochondrial biogenesis. There are multiple signalling pathways involved in mitochondrial biogenesis. PGC-1α (encoded by PPARGC1A), which is a co-activator for a family of transcriptional factors known as PPARs, co-ordinates via a cascade of nuclear encoded proteins the vast majority transcriptional mitochondrial biogenesis. Novel pharmacological therapies aim to modulate PGC-1α mtDNA expression (e.g. PPARα) and protein expression or target downstream pathways. Bezafibrate is pharmacological ligand for the transcriptional co-factor PGC-1α. AICAR activates AMP-activated protein kinase (AMPK) and is thought to modulate increased mitochondrial biogenesis through PGC-1α. The natural polyphenol resveratrol activates sirtuin 1 (SIRT1). Sirtuins are part of a group of oxidizing NAD-dependent protein deacetylases. Upon activation, for example, by PGC-1α or transcription factor A, mitochondrial (TFAM) they promote mitochondrial respiratory chain activities and the transcription of genes modulating mitochondrial biogenesis and function. Nicotinamide riboside can be used to supplement NAD+ levels. PARP1 functions as a NAD+ consuming enzyme. Thus in turn inhibition of PARP1 has been demonstrated to increase NAD+ bioavailability and SIRT1 activity (not shown above) promoting oxidative phosphorylation. Rapamycin inhibits mTOR, which in turn releases mTOR inhibition of autophagy. Cyclosporin A inhibits the mitochondrial permeability transition pore (MPTP). Opening of the mitochondrial permeability transition pore is thought to deplete pyridine nucleotides thus impairing mitochondrial oxidative respiration.
Suomalainen, 2012). Bezafibrate induced hepatomegaly and abnormal lipid metabolism is reported in Surf1 knockout (Viscomi et al., 2011) and Deleter mice (Yatsuga and Suomalainen, 2012). Importantly this does not appear to occur at comparable doses in humans.

**AICAR**

5-Aminooimidazole-4-carboxamide ribonucleotide (AICAR) is an adenosine monophosphate (AMP) analogue (Merrill et al., 1997) causing AMPK to initiate catabolic mechanisms to produce ATP and inhibit anabolic processes (Canto and Auwerx, 2009), promote PPARGC1A gene transcription (Terada et al., 2002) and PGC-1α activity by direct phosphorylation (Jager et al., 2007). AICAR has been shown to increase mitochondrial biogenesis and ATP production alongside decreasing reactive oxygen species human complex I deficient fibroblasts (Golubitzky et al., 2011). AICAR also causes partial correction of COX defects in mice (Viscomi et al., 2011). AICAR is reported to have only mild gastrointestinal side effects (Patel et al., 2011).

**Resveratrol**

Resveratrol is a natural polyphenol, known to increase NAD+ (oxidized nicotinamide adenine dinucleotide) levels, which activate the protein deacetylase SIRT1 (Kanabus et al., 2014). SIRT1 activates PGC-1α by deacetylation sparking a chain of signalling pathways promoting mitochondrial biogenesis and function (Canto and Auwerx, 2009; Kanabus et al., 2014).

Resveratrol activation of SIRT1 has been demonstrated to restore normal function in human fibroblasts with inborn errors of mitochondrial fatty acid β-oxidation (Bastin et al., 2011). Such preclinical data have prompted investigation of resveratrol for diseases with mitochondrial pathogenesis such as Friedreich’s ataxia (Delatycki, 2012; Yiu et al., 2013).

A recently completed open-label clinical pilot study investigating resveratrol in Friedreich’s ataxia has presented preliminary data (full publication is awaited) showing improvement in disease severity and oxidative stress markers (Delatycki, 2012; Yiu et al., 2013). Resveratrol is reported to have only mild gastrointestinal side effects (Patel et al., 2011).

**Modulating NAD+ bioavailability**

The balance between NAD+ and NADH is crucial to the process of oxidative phosphorylation. With increased NAD+ bioavailability enhancing oxidative phosphorylation. Indeed NAD+ supplementation with nicotinamide riboside has produced promising biochemical and clinical improvements in two mitochondrial myopathy murine models; a nuclear gene (Sc02) knockout/knockin mouse (Cerutti et al., 2014), and the Deleter mouse possessing a nuclear gene mutation resulting in mtDNA deletions (Khan et al., 2014).

**PARP inhibitors**

Inhibition of the NAD+-consuming enzyme poly ADP (adenosine diphosphate-ribose) polymerase 1 (PARP1) increases NAD+ bioavailability, SIRT1 activity and subsequently oxidative metabolism (Bai et al., 2011). There is convincing evidence from murine models of myopathy (Cerutti et al., 2014) and mitochondrial encephalopathy (Felic et al., 2014), of the potential benefit of PARP inhibition in human mitochondrial disease. Toxicity is also reported to be low (Tutt et al., 2010).

**Inhibiting cytosolic translation and autophagy**

During starvation states damaged cellular components are degraded to maintain cellular activity and viability (Kim et al., 2011). This process termed autophagy is inhibited by mammalian target of rapamycin (mTOR) (Kim et al., 2011).

Administration of the mTOR inhibitor rapamycin in a murine model of Leigh syndrome delayed the onset of symptoms, reduced neuroinflammation and prevented brain lesions, leading to an increased life span (Johnson et al., 2013). Although the safety profile and clinical use of rapamycin is well documented, the associated immunosuppressive effects, reduced wound healing and hyperlipidaemia may limit its use for mitochondrial disease (Johnson et al., 2013). In keeping with this, recent evidence in patient cell lines also showed benefit from the partial inhibition of intramitochondrial translation using cycloheximide, and the inhibition of autophagy by lithium chloride (Peng et al., 2015).

**Inhibiting opening of the mitochondrial permeability transition pore**

Opening of the mitochondrial permeability transition pore (PTP) is thought to deplete pyridine nucleotides thus impairing mitochondrial oxidative respiration. Cyclosporin A inhibits the mitochondrial PTP and has been shown to induce muscle regeneration, improve mitochondrial function, and suppress muscle apoptosis (Merlini et al., 2008) in both a murine model of collagen VI-deficiency myopathy and five patients with the same disorder. These findings lend support to the use of cyclosporin A in other mitochondrial disorders such as LHON, where a clinical trial is currently recruiting patients (Milea, 2014). However, like rapamycin, this widely used immunosuppressant may have a non-acceptable side effect profile in chronic use.

**Stem cell approaches**

Substantial preclinical, and increasing clinical evidence exists to support the use of stem cell therapies in several neurological disorders where mitochondrial dysfunction plays a role (e.g. Parkinson’s disease) (Lunn et al., 2011). The proposed therapeutic mechanisms of stem cells are...
Exogenous stem cell therapy for nuclear DNA mutations

A number of case reports and small case series, discussed below, have described the effects of allogenic stem cell treatment in patients with MNGIE (Table 2) (Hirano et al., 2006; Hill et al., 2009; Lenoci et al., 2011; Filosto et al., 2012; Sicurelli et al., 2012; Hussein, 2013). The rationale behind the treatment is based on work where haemoperitoneal dialysis or platelet transfusions reduced circulating levels of toxic thymidine, presumably by introducing cells with normal thymidine phosphorylase activity (Moran et al., 2008; Bax et al., 2013; Hussein, 2013). Restoring thymidine phosphorylase activity in the bone marrow has been shown to reduce toxic thymidine levels post-transplantation, although the results were highly variable (Hirano et al., 2006; Hill et al., 2009; Halter et al., 2011; Lenoci et al., 2011; Filosto et al., 2012; Sicurelli et al., 2012; Hussein, 2013), and there is no convincing evidence that objective clinical benefit was actually achieved.

Interestingly, a recent retrospective analysis of all the 24 patients with MNGIE known to undergo a haematopoietic stem cell transplantation between 2005 and 2011 reported that in the nine survivors, thymidine phosphorylase activity rose from undetectable to normal levels (Halter et al., 2015). Two trials at Columbia University, USA are currently recruiting; one to map the natural history study of MNGIE (Hirano, 2015a) and the other to assess the safety of stem cell transplant for MNGIE in a proposed 12 patients (Hirano, 2015b). These should help inform future clinical trial design and begin to address safety concerns highlighted by a consensus report published by Halter et al. (2011).

Endogenous stem cells for mitochondrial DNA mutations

There has been ongoing interest in the phenomenon of ‘gene shifting’ as a treatment for mitochondrial myopathy (Clark et al., 1997; Spendiff et al., 2013). In patients with
heteroplasmic mtDNA mutations, the muscle satellite cells (myogenic stem cells) typically contain less mutated mtDNA than mature post-mitotic skeletal muscle fibres. Pharmacological methods and resistance exercise to induce satellite cell proliferation and fusion with mature muscle to deliver wild-type mtDNA into mature muscle (Spendiff et al., 2013) have been demonstrated to reduce mtDNA load in mature muscle (Clark et al., 1997) and improve biochemistry. However, there is currently no evidence of clinical benefit (Andrews et al., 1999). Recent work using somatic cell nuclear transfer adds weight to this approach, showing the correction of the metabolic disturbance with minimal disruption of nuclear-mitochondrial communication using induced pluripotent stem cells, albeit in vitro (Ma et al., 2015).

A series of collaborative trials for patients with mtDNA mutations (comprehensively reviewed) (Kerr, 2013) demonstrated that endurance training increased work capacity and mitochondrial enzyme activity, but did not reduce heteroplasmy. Thus the benefits in this context were unlikely to be attributable to gene shifting and may simply reflect increased oxygen delivery through the development of capillary networks. Moreover, there may be a finite capacity for repair following induction of satellite cell proliferation by these methods; raising concerns about long-term sequelae. Finally, any potential benefits are most likely help the minority of patients with an isolated mitochondrial myopathy. Despite these concerns, there is currently an ongoing crossover trial investigating exercise versus inactivity in a cohort of patients with mitochondrial myopathy (Haller, 2012). This will hopefully provide a definitive answer to the role of exercise training in this context.

**Conclusion**

The past 5 years have seen several new approaches developing through our understanding of the molecular pathogenesis of mitochondrial diseases. For mtDNA disorders, the early clinical studies attempting to harness gene-shifting some 15 years ago have not really progressed beyond early open labelled studies—probably because the likely clinical impact is limited using current approaches. Although intriguing, other molecular approaches directed against mtDNA disease are very much at the preclinical stage, and will require substantial development to improve efficacy and ensure there is no substantial risk of toxicity before human trials. From a clinical perspective, nuclear-genetic enzyme defects show the greatest promise. Stem cell therapy is already being used in specific contexts, and its efficacy and safety being evaluated, and gene therapy trials in mouse models show clear benefits. Unfortunately, each one of these rare genetic diseases may require their own proprietary approach, and the impact needs to be evaluated long-term. Small molecules are attractive because they have the potential to provide a more generic solution applicable across the mitochondrial disease spectrum, and a greater understanding of cell signalling pathways opens up several unexpected disease targets. For some of these drugs, clinical evaluation is imminent, particularly for those being repurposed or repositioned drugs such as bezafibrate. It is critical at this stage that laboratory and clinical scientists work closely with patient organizations to ensure that the ultimate aims of therapy will actually tackle issues that are important to patients. Given limited resources, this will ensure that new treatments improve quality of life—a prerequisite if these treatments are going to be adopted by healthcare systems worldwide.

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**Literature search strategy**


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Emerging therapies for mitochondrial disorders


