HHS Public Access

Author manuscript

Mol Genet Metab. Author manuscript; available in PMC 2023 August 04.

Published in final edited form as:

Mol Genet Metab. 2022 ; 137(1-2): 40–48. doi:10.1016/j.ymgme.2022.07.003.

Mitochondrial DNA maintenance defects: potential therapeutic strategies

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Abstract

Mitochondrial DNA (mtDNA) replication depends on the mitochondrial import of hundreds of nuclear encoded proteins that control the mitochondrial genome maintenance and integrity. Defects in these processes result in an expanding group of disorders calledmtDNA maintenance defects that are characterized by mtDNA depletion and/or multiple mtDNA deletionswith variable phenotypic manifestations. As it applies formitochondrial disorders in general, current treatment options for mtDNA maintenance defects are limited. Lately, with the development of model organisms, improved understanding of the pathophysiology of these disorders, and a better knowledge of their natural history, the number of preclinical studies and existing and planned clinical trials has been increasing. In this review, we discuss recent preclinical studies and current and future clinical trials concerning potential therapeutic options for the different mtDNA maintenance defects.

Keywords

Mitochondria; mtDNA replication; mtDNA depletion; Preclinical studies; Nucleoside bypass therapy; Clinical trials

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1. Introduction

Mitochondria are ubiquitous organelles involved in several cellular functions. Although they are well known for energy production, they have other biological roles including apoptosis, cellular signalling, calcium homeostasis, and biosynthesis of several molecules [1].

Mitochondrial DNA (mtDNA) is a double-stranded circular DNA of 16.6 kilobases in length [2]. MtDNA contains 37 genes, 13 of which encode for subunits of the electron transport chain (ETC) complexes while the remaining encode for 22 tRNAs and two rRNAs. While mitochondria contain their own DNA, most mitochondrial proteins are encoded by nuclear genes [1]. In particular, all proteins essential to maintain mtDNA replication, expression and repair are nuclear encoded and imported into the mitochondria. Defects in these genes lead to mtDNA maintenance defects, an expanding and heterogeneous group of disorders characterized by multisystem disease along with mtDNA depletion and/or multiple mtDNA deletions in affected tissues. These disorders are inherited in Mendelian fashion with onset at any age and variable presentation [3,4].

Treatment of mitochondrial disorders in general, including mtDNA maintenance defects, has been challenging and limited to supportive and symptom specific therapies. Recently, the number of preclinical studies and clinical trials has been increasing with different approaches utilized including amelioration of oxidative stress, enhancement of mitochondrial biogenesis, modulation of mitophagy, nucleoside bypass therapy, mitochondrial augmentation therapy, stem cells and organ transplantation, and gene therapy (for detailed review see [5]).

In this review, we will focus on recent preclinical studies, and ongoing and prospective clinical trials concerning therapeutic options aimed at treating mtDNA maintenance defects.

2. MtDNA replication

MtDNA replication is a continuous process independent of the cell cycle. MtDNA is replicated and repaired by polymerase γ (polγ), encoded by *POLG* [6]. MtDNA contains a large non-coding D-loop, which acts as a promoter for the heavy and light strands transcription [7]. MtDNA replication is initiated at the origin of heavy-strand replication (OH) and then proceeds clockwise along the heavy chain until the origin of light-strand replication (OL) is exposed. Subsequently, transcription of the light chain starts and proceeds counter-clockwise until the entire molecule is replicated [8]. Besides polγ, other components of the mtDNA replisome include TWINKLE (the mitochondrial replicative DNA helicase) [9], mitochondrial topoisomerase I, which relieves tension and DNA supercoiling [10], and mitochondrial single-stranded DNA (ssDNA)-binding protein (mtSSB) which binds to ssDNA and prevents renaturation.

MtDNA replication requires constant supply of deoxyribonucleotide triphosphates (dNTPs). This is achieved by mitochondrial transport of dNTPs synthesized in the cytosol and through salvage pathway enzymes within the mitochondrial matrix [11]. The two principal enzymes in the salvage pathway are thymidine kinase 2 (TK2) [12] and deoxyguanosine kinase (DGUOK) [13] which catalyze the phosphorylation of pyrimidine deoxyribonucleosides

and purine deoxyribonucleosides respectively into deoxyribonucleotide monophosphates (dNMPs). The dNMPs are eventually converted into dNTPs through two phosphorylation reactions catalyzed by nucleotide monophosphate kinase (NMPK) and nucleotide diphosphate kinase (NDPK). In addition to the salvage pathway, dNTPs synthesized in the cytosol are imported to the mitochondria through specific transporters like adenine nucleotide translocator (ANT) and MPV17 [14,15]. Cytosolic enzymes like thymidine phosphorylase (TP) and ribonucleotide reductase (RNR) are essential to maintain balanced dNTPs pool for mtDNA replication and repair [16,17].

Finally, the dynamic nature of the mitochondria with constant fission and fusion is important to maintain the organization and integrity of mtDNA [18]. Through fission and fusion, the mitochondria maintain a well-balanced proteome and protein stoichiometry [19]. During fission, the mitochondria divide into multiple new mitochondria and this process is established through progressive constriction of the mitochondrial membranes through the action of dynamin-related protein 1 (DRP1) [18]. Mitochondrial fusion is the physical merge of two mitochondria through action of three GTPase; mitofusin 1 (MFN1), mitofusin 2 (MFN2), and OPA1 [20].

Damaged mtDNA is repaired through base excision repair (BER) and it is not clear if other mechanisms like mismatch repair (MMR) and DNA double-strand break (DSB) are involved in mtDNA repair. Alternatively, damaged mtDNA molecules could undergo degradation [21].

3. MtDNA maintenance disorders

MtDNA maintenance disorders represent an expanding group of diseases that result from defects in nuclear genes involved in mtDNA maintenance [1,3]. These genes code for proteins that are part of the mtDNA replisome (*POLG, POLG2, TWNK, TFAM, RNASEH1, MGME2, DNA2*), perform other functions critical for mtDNA replication like supply of nucleotides (*TK2, DGUOK, SUCLG1, SUCLA2, TYMP, ABAT, RRM2B, SLC25A4, AGK, MPV17*) or are involved in mitochondrial dynamics (*OPA1, FBXL4,MFN2*) [1]. As a result, mtDNA depletion and/or multiple deletions will develop in affected tissues with variable manifestations depending on the tissues involved [3]. The clinical presentation of mtDNA maintenance disorders is broad and the age of onset is variable as well. Common systems involved include central nervous system (CNS), liver, skeletal muscle, peripheral nerves, and gastrointestinal tract, either in combination like in mitochondrial neurogastrointestinal encephalopathy (MNGIE) or single organs could be involved as exemplified by adult onset progressive external ophthalmoplegia (PEO) [3].

Treatment of mtDNA maintenance defects is currently mostly limited to supportive and symptom specific therapies. Some therapeutic options, like nucleoside bypass therapy, seem promising and currently being explored in phase III studies. Animal models are available for several mtDNA maintenance defects and some of these models recapitulate human phenotypes making them good tools to study these diseases and evaluate therapeutic targets [22]. However, these model organisms may fail to recapitulate the observed phenotype in humans affected with these disorders. This factor could be explained in part by the different

genetic backgrounds of these animal models when compared to humans, and represents a challenge to the replication of the human phenotype in model organisms.

4. POLG-related disorders

Human polγ is a heterotrimer consisting of one catalytic subunit encoded by *POLG* and two accessory subunits encoded by *POLG2* [23]. Polγ is the DNA polymerase that replicates and repairs mtDNA [24]. Recently, PrimPol, which has primase and polymerase activity, has been shown to be involved in mtDNA repair, as it is required for reinitiation of replication after DNA damage [25].

Pathogenic variants in *POLG* cause a wide spectrum of phenotypes than range from adult onset progressive external ophthalmoplegia to Alpers-Huttenlocher syndrome (MIM #: 203700), a severe phenotype that is characterized by early onset encephalopathy with intractable seizures and liver failure [26]. Other phenotypes of *POLG*-related disorders include childhood myocerebrohepatopathy spectrum (MCHS), myoclonic epilepsy-myopathy-sensory ataxia (MEMSA); ataxia neuropathy spectrum (ANS), mitochondrial neurogastrointestinal encephalopathy (MNGIE), and sensory ataxic neuropathy and dysarthria (SANDO) [27].

Treatment of *POLG*-related disorders is supportive and directed to associated symptoms. Preclinical studies evaluated several agents on cell or animal models. Treatment of dopaminergic neurons differentiated from induced pluripotent stem cells (iPSCs) generated from two POLG deficiency patients with *N*-acetylcysteine amide reduced reactive oxygen species (ROS) levels and ameliorated the loss of mitochondrial membrane potential [28]. Chen et al. found that iPSC-derived astrocytes expressing *POLG* pathogenic variants exhibit downregulation of mitophagy-related genes, and this finding could be rescued with combined treatment of nicotinamide riboside and metformin [29]. Treated cells showed improvement of the mitophagy impairment via regulating SIRT1/AMPK/mTOR signalling pathway [29]. The effect of nicotinamide may be mediated by an increase of the NAD^+/NADH ratio and the activation of SIRT1, an NAD^+-dependent deacetylase that plays a role in autophagy flux [29,30]. These studies potentially suggest the suitability of SIRT1 activating compounds and NAD^+ boosting compounds as potential therapeutic strategies for mitochondrial disorders.

In the yeast *Saccharomyces cerevisiae* model for POLG deficiency with mutations in *MIP1*, the yeast homologue of *POLG*, increasing dNTPs pool (through overexpression of RNR1 or deletion of its inhibitor, SML1), resulted in reduction in the frequency of mutations [31]. In fibroblasts from patients with different POLG mutations, supplementation with deoxyribonucleosides plus erythro-9-(2-hydroxy-3-nonyl) adenine (inhibitor of deoxyadenosine degradation) led to increased mitochondrial dNTP pools and mtDNA repopulation in all cells, regardless of the phenotype [32]. Finally, in vivo transfer of hemopoietic stem cells harbouring healthy mitochondria (mitochondrial augmentation therapy) to a mouse model with *polg* missense mutation, resulted in sustained engraftment in these non-conditioned animals [33]. Based on these preliminary results and the impressive improvements reported in one patient with a mtDNA deletion syndrome (Kearns-Sayre

syndrome), mitochondrial augmentation therapy could represent a potential therapy that could be trialed in patients with mtDNA depletion syndromes [34].

Recently, ferroptosis, a form of iron- and lipid-dependent regulated cell death, has been implicated as a possible pathomechanism in the epilepsy associated with mitochondrial disorders [35]. Ferroptosis is associated with glutathione depletion and the production of lipid peroxides [36]. Targeting this mechanism has been the focus of recent studies [35]. In fibroblasts and B-lymphocytes from patients with mitochondrial disease-associated epilepsy (including *POLG* related disorders), the administration of EPI-743 (now called PTC-743), a para-benzoquinone analog that exerts its antioxidant effects through repletion of reduced intracellular glutathione, prevented ferroptosis [35]. A randomized trial to assess the efficacy and safety of PTC-743 for the treatment of mitochondrial disease subjects with refractory epilepsy (including *POLG* related disorders), is active and recruiting (https://clinicaltrials.gov/ct2/show/NCT04378075). This same compound had been previously used in a randomized, double-blind, placebo-controlled trial in children with Leigh syndrome. Recruited subjects had first participated in a 6-month placebo-controlled phase (NCT01721733), followed by an extension phase (NCT02352896) to assess the long-term safety and impact on disease morbidity of this investigational compound. The comparison of the scores for subjects in the active treatment group and the placebo group of their respective changes from baseline to six months in the Newcastle Pediatric Mitochondrial Disease Scale (NPMDS) Sections 1–3 was chosen as a primary outcome measure. The primary outcome measure was not met. The adequacy of this outcome measure in this trial may have been complicated by the extreme clinical, biochemical, and molecular heterogeneity of the recruited subjects. Analysis of extension phase data revealed a progressive decline in hospitalizations and serious adverse events from the first six months of EPI-743 treatment to months 19 to 24 [37]. In addition to the heterogeneity of the population of research subjects, the NMPDS may not have been the ideal scale to use as it was not primarily designed to capture data on hospitalizations or serious adverse events.

Elamipretide, a cardiolipin stabilizing agent [38], was trialed in a phase 3 study in subjects with primary mitochondrial myopathy (MMPOWER-3), but the study was terminated as it did not meet its primary endpoints [5]. Although all participants had primary mitochondrial myopathy, their clinical and molecular heterogeneity, and the multisystemic nature of their mitochondrial disorders likely represented an insurmountable challenge in this trial. These features made it unlikely that a universal primary outcome measure would have applied to all primary mitochondrial myopathies caused by mtDNA and nuclear defects. Based on the findings of this trial and the potential effects of this compound on a subpopulation of patients with genetic disorders affecting the mtDNA replisome, there is an active and recruiting study to evaluate the efficacy and safety of elamipretide in subjects with primary mitochondrial disease resulting from nuclear DNA mutations (NuPower) (https://clinicaltrials.gov/ct2/show/NCT05162768).

5. Thymidine kinase 2 (TK2)-related mitochondrial disorders

Thymidine kinase 2 (TK2) is a mitochondrial matrix enzyme, which phosphorylates the nucleosides deoxycytidine (dC) and deoxythymidine (dT) to generate deoxycytidine

monophosphate (dCMP) and deoxythymidine monophosphate (dTMP) and in the first step of the salvage pathway [39]. In 2001, pathogenic variants in TK2, which codes for the TK2 enzyme, were first identified in four children with severe myopathy and mtDNA depletion syndrome 2 (myopathic type); MIM #:609560) [40]. Subsequently, more cases were identified and the phenotype expanded with three forms being recognized; infantile-onset, childhood onset, and adult onset [41]. Infantile-onset myopathy is observed in around 40% of cases and is characterized by early mortality and severe mtDNA depletion in muscle. The childhood-onset form is also observed in 40% of cases and is characterized by longer survival (post-onset survival at least 13 years). The late-onset form is less common (20% of cases) and presents in the form of mild weakness with slow progression and multiple mtDNA deletions [41].

In a study conducted in the *Tk2* H126N knock-in mouse model, oral treatment with dCMP+dTMP was associated with an increase in mtDNA levels, improvement in mitochondrial respiratory chain enzymes, and a three-fold increase in prolongation of survival [42]. In addition, it was noted that dCMP and dTMP were rapidly catabolized to dC and dT suggesting that nucleosides rather than nucleotides are the major active therapeutic agents. Based on this observation, another study was conducted using the same murine model in which two therapies were trialed; dT + dC alone and the deaminase inhibitor, tetrahydrouridine (THU) administrated with dTMP+dCMP orally. While treatment with nucleosides achieved similar results to those observed with nucleotides treatment; THU administration was associated with a decreased life span. This effect was attributed to the dNTPs pool imbalance in the brain with lowering of deoxythymidine triphosphate (dTTP) levels and more severe mtDNA depletion in brain mitochondria when THU is co-administered with dCMP+dTMP [43]. Nevertheless, treated mice with either nucleotides or nucleosides showed mtDNA depletion in brain as early as postnatal day 13 and later in other tissues [42]. A stronger response was observed in small intestine and this finding was attributed to the higher bioavailability of dC and dT with oral administration [42]. Parenteral dC+ dT was then administrated to the same mouse model and although it was more efficient compared to oral treatment at raising levels of nucleosides in blood and liver; it did not improve the response in brain [44]. This result was attributed to age related down-regulation of thymidine kinase-1 (TK1), which together with deoxycytidine kinase (dCK), phosphorylate dC to dCMP and dT to dTMP and are thought to serve as the initial step in the mechanism of action of dC + dT therapy [44]. In fact, dT + dC treatment in *Tk2* knockout mouse model also resulted in similar survival, suggesting that therapy would be beneficial for any *Tk2* mutation through the recruitment of alternative cytosolic salvage pathways for dNTPs synthesis [45]. The age related down-regulation of TK1 in human muscle was found to be minimal only compared to mice and this suggests that TK2 deficient myopathy is likely to respond to dC + dT therapy, since most patients present mainly with a myopathic phenotype [44].

In a recent study, the *Tk2* knock-in mice were treated with adeno-associated virus (AAV) mediated gene therapy containing human *TK2*. Treatment was associated with delayed disease onset and increased lifespan. Subsequently, these mice received dC and dT supplementation which result in significant improvement in mtDNA copy number, animal growth, and lifespan [46].

Based on the promising results from animal studies evaluating nucleoside bypass therapy, deoxynucleosides were administered to 16 TK2-deficient patients on a compassionate basis. Five of the patients presented with the infantile form and all of them survived for two years compared to 27.3% survival in historical controls. The remaining 11 patients presented with childhood and adult-onset disease. In all of them, clinical measures of motor and respiratory function stabilized or even improved [47]. A retrospective observational study of six patients treated with dC and dT over 30 months also showed that treatment could stabilize lung function [48].

Three active clinical trials are currently listed in clinicaltrials.gov to evaluate safety and efficacy of nucleoside bypass therapy in subjects with TK2 deficiency (NCT03845712, NCT04581733, and NCT03639701) (Table 1).

6. Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE)

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (MIM#:131222) is a rare, autosomal recessive disease caused by pathogenic variants in *TYMP* gene, which codes for thymidine phosphorylase (TP). TP catalyzes the conversion of dT and deoxyuridine (dU) into their respective bases. MNGIE is a multisystemic disease with a mean age of onset around 17.9 years. It is characterized by gastrointestinal (GI) involvement with abdominal pain, vomiting, intestinal pseudoobstruction, weight loss, and hepatopathy, and neurological involvement with ophthalmoparesis, polyneuropathy, and leukoencephalopathy. It is a progressive disease with mean age of death between 35 and 37 years. Death is attributed mainly to GI and liver complications and cachexia [49].

Gene therapy for MNGIE was attempted in several preclinical studies, using either a lentiviral vector targeted to hematopoietic stem cells or an AAV vector targeted to liver. This therapeutic approach restored liver TP activity and normalized nucleoside levels in treated mice models of MNGIE [50–54]. Vectors with an alpha-1-antitrypsin (AAT) promoter showed the best efficacy [50]. In an attempt to evaluate the effects of gene therapy on biochemical and clinical profile, the Tymp/Upp1 double knock-out(dKO) mice were treated with both dT and dU and later received AAV mediated gene therapy. This strategy resulted in increased TP activity in liver and lowering of the systemic nucleoside levels in the exposed mice. Moreover, AAV treatment prevented the development of enlarged ventricles and motor impairment in the exposed mice [55]. Recently, liver-directed gene editing approach was successful in achieving long term, normalization of plasma metabolite levels [56].

As dT and dU are freely diffusible across cell membranes; lowering of plasma levels of both metabolites could be achieved by either direct removal of the metabolites or replacement of the deficient enzyme. One of the approaches to remove the toxic metabolites is through dialysis, but the effect is short term only with metabolite levels returning to pretreatment concentration at 19 h post-dialysis [57]. Therefore, continuous dialysis methods are required. A 16-year-old girl with MNGIE was treated with continuous ambulatory peritoneal dialysis (CAPD) for three years and she showed clinical improvement in terms of weight gain, resumption of menstruation, and resolution of vomiting [58]. In another report, a

29-year-old MNGIE patient had one year of extensive hemodialysis. The treatment provided only transient response in terms of decreasing the accumulated metabolites and it did not reduce CSF levels of these toxic metabolites [59]. A 22-year-old female patient received CAPD and after one year, she showed a marked improvement in GI and neurological symptoms. Fifteen months after the initiation of CAPD, dT and dU levels increased again with reappearance of symptoms. Bone marrow transplant was performed but the patient died two months later from severe cytomegalovirus pneumonia [60]. The authors attributed the decline in response to CAPD to peritoneal sclerosis.

There are different approaches to replace the deficient enzyme, one of which is platelets transfusion given that platelets are rich in TP [61]. In two patients with MNGIE, platelets infusion restored transiently circulating TP and reduced plasma dT and dU levels [61]. Another approach is allogeneic hemopoietic stem cell transplantations (aHSCT). In a retrospective study, data from 24 patients with MNGIE who underwent ASCT between 2005 and 2011 were reviewed. The median age was 25 years (range 10–41 years) and the follow-up period ranged from 27 months to 8.5 years. Nine patients were alive at last follow-up with a median follow-up of 1430 days. In the remaining 16 patients, deaths were attributed to transplant in nine and to the primary disease in six patients. TP activity normalized in all survivors. Seven patients showed improvement of body mass index, GI manifestations, and peripheral neuropathy. It was suggested that survival was associated with two factors; human leukocyte antigen match (10/10 versus <10/10) and disease characteristics (liver disease, history of gastrointestinal pseudo-obstruction or both) [62]. In a recent single center experience, six patients with MNGIE who had aHSCT were reported. Survival was 66%, including one patient who was transplanted at ten years of age (diagnosed based on positive family history) with only mild symptoms at the time of aHSCT. He remained clinically stable at 15 years posttransplant. The authors concluded that aHSCT could be a curative therapeutic option for carefully selected, young and pre-symptomatic or mildly affected patients. Another conclusion of the study was that aHSCT should not be pursued in patients with advanced MNGIE disease as severe GI symptoms were mostly irreversible leading to a poor outcome [63]. In line with this, one study evaluated the effects of aHSCT on small intestinal pathology of MNGIE patients and knockout mice models of MNGIE and showed that treatment may be insufficient to correct gastrointestinal pathology completely, especially at later stages of the disease [64]. Two active clinical trials are enrolling patients with MNGIE to evaluate engraftment success after aHSCT (Table 1).

Liver is another source for TP enzyme [65]. Orthotopic liver transplantation (OLT) in three patients with MNGIE normalized the metabolites level but clinical improvement was limited to some neurological features and one patient died 32 months after transplant with GI bleeding and anemia [66]. In a subsequent study of four patients, including one child, OLT again resulted in decline in metabolites levels and stabilization in clinical status was noted [67].

A potentially successful and emerging therapeutic approach for MNGIE is exemplified by enzyme replacement therapy with erythrocyte encapsulated thymidine phosphorylase (EE-TP). EE-TP is formulated by the *ex vivo* encapsulation of *Escherichia coli* TP within the patient's autologous erythrocytes. In a proof of concept study, EE-TP was administered

to an adult patient with MNGIE and it resulted in the decrease in the urinary excretion of dT and dU at three days post infusion to 6% and 13%, respectively and a decrease in plasma concentrations of these metabolites in parallel [68]. On a compassionate basis, three adult patients with MNGIE (ages 25, 26, and 28 years) were treated with intravenous escalating doses of EE-TP. Treatment was well tolerated and resulted in reductions in dT and dU. Clinical improvement in terms of weight gain and improved disease score was observed in two patients who were treated for 28 and 79 months respectively. The first patient who was treated for 28 months developed rapid progression of disease following an illness and eventually died. The third patient received only four cycles of treatment that was terminated as the patient was not able to travel to receive the tretament [69,70]. A phase two, multi-centre trial is planned to study EE-TP in patients with MNGIE. The primary outcomes are to determine the safety, tolerability, pharmacodynamics, and efficacy of multiple doses of EE-TP [71] (https://clinicaltrials.gov/ct2/show/NCT03866954).

7. Mitochondrial deoxyguanosine kinase deficiency

Mitochondrial deoxyguanosine kinase (DGUOK), encoded by *DGUOK*, mediates the phosphorylation of purine deoxyribonucleosides (deoxyadenosine and deoxyguanosine) into the corresponding nucleotides (deoxyadenosine monophosphate (dAMP) and deoxyguanosine monophosphate (dGMP)) in the salvage pathway. Majority of patients with DGUOK deficiency present in the neonatal period with liver disease associated with neurologic manifestations and most die within the first year of life due to liver failure (MIM #:251880) [72].

In vitro study of liver organoids and hepatocytes developed from iPSCs of *DGUOK*-related mitochondrial DNA depletion syndrome patients showed increased sensitivity to iron overload-induced ferroptosis. This finding could be recused by the glutathione precursor, *N*-acetylcysteine (NAC) [73]. In another study, nicotinamide adenine dinucleotide (NAD) was found to improve mitochondrial function in DGUOK-deficient hepatocyte-like cells by activating the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) [74]. *In vitro* studies of fibroblasts from a DGUOK deficiency patient demonstrated that, although mtDNA synthesis is cell cycle independent, pathogenic variants in *DGUOK* compromise cell cycle independent mtDNA synthesis and mtDNA synthesis can still occur during S phase via cytosolic supply of dNTPs [75]. This phenomenon explains why quiescent tissues, including liver, muscle and brain, are particularly affected in this disorder. It was demonstrated that mtDNA depletion can be prevented by dGMP and dAMP *in vitro* supplementation in fibroblasts [75,76]. Using patients' myotubes, similar results were also obtained after in vitro supplementation with dAMP/dGMP in terms of significant increase of mtDNA copy number [77,78]. In mutant dguok −/− knockout adult zebrafish, supplementation of both purine nucleosides resulted in a significant increase in liver mtDNA content [79]. In a recent study, a novel ProTide nucleotide of dGMP, CERC-913, was trialed in a primary hepatocyte culture model of DGUOK deficiency and it restored mtDNA content in a dose-dependent fashion [80]. However, nucleoside bypass therapy with purine nucleosides may only provide a potential benefit in those patients with DGUOK deficiency who primarily have liver disease and not in those with a hepatocerebral phenotype.

OLT is another therapeutic option that have been attempted in several patients in literature. One study reported 14 patients who underwent OLT in infancy. Eight patients died during early follow-up, while five survived and no follow up was available for the last patient. The one-year survival rate was 64% with 36% survived for >5 years. Those who survived had good quality of life with a stable neurological condition [81]. Two Japanese patients with DGUOK deficiency died less than one year after OLT [82]. Liver transplant was performed in a 9-month-old boy who had liver failure. He survived for a follow up period of six years post-OLT with stable neurological status [83]. In two infants, OLT was performed at the age of six and seven months. One died two months after OLT due to procedure-related complications whereas the other survived for three years of follow-up, with good liver function, mild hypotonia, and age appropriate development [84].

8. MPV17-related mitochondrial disorders

*MPV*17 encodes the MPV17 protein which is a mitochondrial inner membrane protein that has been shown to be essential in maintaining adequate nucleotides inside the mitochondria. The function of MPV17 is not completely understood, but it has been suggested that MPV17 may be involved in importing nucleotides into the mitochondria [85,86]. Electrophysiological studies showed that MPV17 is a non-selective channel regulated by different factors [87]. In a recent study, Sperl et al. used Nuclear Magnetic Resonance (NMR) and showed that MPV17 protein has six membrane-embedded α- helices that form disulfide-stabilized oligomers that may transport nucleotides into the mitochondrial matrix [88].

Biallelic pathogenic variants in *MPV17* are associated with two phenotypes: encephalohepatopathic (hepatocerebral) (MIM#256810) disease and neuromyopathic (neuropathic) disease. The vast majority of affected individuals present with the hepatocerebral phenotype which is characterized by an early-onset (neonatal or infantile) liver dysfunction that typically progresses to liver failure leading to death in infancy or early childhood. It is associated with mtDNA depletion, particularly in the liver. The neuromyopathic disease is characterized by later onset (childhood to adulthood) myopathy and neuropathy, and associated with multiple mtDNA deletions in muscles [89].

Current management options are only symptomatic and include physiotherapy, nutritional support, and prevention of hypoglycemia with frequent feeds and uncooked cornstarch. Although OLT remains the only treatment option for liver failure, it is controversial because of the multisystem involvement in this disorder [90].

In an *Mpv17*$^{-/-}$ mouse model, AAV-mediated expression of human *MPV17* in mouse liver restored mtDNA content and oxidative phosphorylation [91]. Through a screen of a large number of molecules in a yeast model with *SYM1* mutation, the ortholog of the human *MPV17*, several molecules were identified that could increase the dTTP pool and therefore improve the mtDNA stability. Some of the identified molecules affect ergosterol levels in the yeast and therefore could work through increasing the permeability of mitochondrial membranes [92]. These molecules were also effective in other yeast models with mutations

in *MIP1* and *RNR2*, which are the orthologs of the human genes *POLG* and *RRM2B* respectively [92].

As *MPV17* deficiency results in decreased nucleotides supply in mitochondria, increasing the availability of nucleotide pools and enhancing the salvage pathway could be a potential therapeutic target, although no reports of such approach have been published yet. There is an ongoing phase 2 clinical trial evaluating supplementation of dC and dT in children with mtDNA depletion syndromes associated with pathogenic variants in the following genes; *POLG, C10orf2, TYMP, RRM2B, MPV17, SUCLA2, SUCLG1, FBXL4* (https://clinicaltrials.gov/ct2/show/NCT04802707). The study is still recruiting subjects.

9. Disorders of mitochondrial dynamics

OPA1 is essential for mitochondrial fusion which occurs in a two-step process: fusion of the mitochondrial outer membrane (MOM) and fusion of the mitochondrial inner membrane (MIM). Fusion of the MOM is mediated by mitofusin 1(MFN1) and mitofusin 2 (MFN2) [93]. Fusion of the MIM is mediated by OPA1 which is a dynamin-like GTPase that is anchored to the MIM by an N-terminal transmembrane domain protein [94]. Mitochondrial fusion enables the exchange of intramitochondrial material between mitochondria [95].

Monoallelic pathogenic variants in *OPA1* are associated with *OPA1*-related autosomal dominant optic atrophy (MIM#:165500) whereas biallelic pathogenic variants are associated with Behr syndrome (MIM#:210000) and *OPA1*-related cardioencephalomyopathy (MIM#:616896). OPA1-related autosomal dominant optic atrophy is the most common cause of hereditary optic neuropathy and has reduced penetrance. Affected individuals usually present between four and six years of age with gradual decline of vision acuity and bilateral optic atrophy. Vision loss is occasionally asymmetric. It is also associated with visual field defects and color vision defects. Additional extra-ophthalmologic abnormalities can be observed in a minority of affected individuals including sensorineural hearing impairment, proximal myopathy, exercise intolerance, myalgia, muscle weakness, ataxia, axonal sensorimotor neuropathy, ptosis, and ophthalmoplegia. Multiple mtDNA deletions can be observed in skeletal muscle tissue [96].

Behr syndrome presents during infancy or early childhood with optic atrophy, developmental delay, and spinocerebellar degeneration resulting in ataxia, pyramidal signs, and sensory axonal neuropathy. Other variable features include gastrointestinal involvement (constipation, vomiting, and dysphagia), developmental delay, nystagmus, deafness, and spasticity. Neuroimaging can show cerebellar vermian atrophy, with periventricular white matter changes in some affected children [97–99].

OPA1-related cardioencephalomyopathy presents in early infancy with developmental delay, muscle weakness and wasting, poor feeding, failure to thrive, hypertrophic cardiomyopathy, hypertonia, opisthotonic posturing, optic atrophy, sensorineural hearing impairment, lactic acidemia, and mtDNA depletion in muscle tissue [100].

Management for *OPA1*-related mitochondrial diseases is symptomatic. However, several therapeutic approaches have been experimented. Small ribonucleoprotein particle U1 (U1

snRNP) can be a promising approach for some splice-site variants which account for 30% of *OPA1* pathogenic variants [101]. Jüschke and colleagues have studied the *OPA1* splice site variant c.1065+5G>A which was identified in individuals with optic atrophy and found to cause exon 10 skipping reducing the OPA1 protein expression significantly [101]. Engineered U1 splice factor targeted to bind to intron 10 was able to silence the effect of the variant in skipping exon 10 and increase the expression level of normal transcripts in patient-derived fibroblasts. This proof-of-concept study indicated the feasibility of splice variant correction as a treatment option for *OPA1*-related optic atrophy [101].

Another approach utilized in treatment of patients with dominant optic atrophy (DOA), including those with *OPA1* pathogenic variants, is autologous bone marrow derived stem cells (BMSC). Six patients with DOA (three documented to have *OPA1* pathogenic variants) were treated with BMSC and five of them experienced statistically significant visual improvements with average improvement of 29.5% in LogMAR [102]. This open label, non-randomized study is still ongoing and recruiting (https://clinicaltrials.gov/ct2/show/NCT03011541).

Gene therapy has also been tried in animal models. Intravitreal injections of an adeno-associated virus carrying the human OPA1 cDNA in genetically-modified mouse model resulted in wild-type OPA1 expression that was able to alleviate the OPA1-induced retinal ganglion cell degeneration [103]. Gene therapy has been also explored using lipoplexes which are non-viral vectors based on cationic lipids used to deliver DNA into cells [104]. Utilizing this lipofection methodology, Muñoz-Úbeda and colleagues demonstrated a transgene expression of the Opa1 mitochondrial transmembrane protein in mice. The expression was sufficient in magnitude supporting the notion that lipoplexes can be valuable therapeutic agents in the field of gene delivery [104].

Pathogenic variants in *MFN2* are associated with Charcot Marie Tooth Disease 2A (CMT2A), an axonal peripheral sensorimotor neuropathy that is inherited in an autosomal dominant manner in most cases [105]. It can cause also a multisystemic disease [106]. It was shown that the open conformation form of MFN2 protein favours mitochondrial fusion whereas the closed conformation is fusion incompetent [107]. Rocha et al. showed that the closed conformation was stabilized by alignment of specific amino acid residues in the heptad repeat (HR) 1 and 2 domains. The authors then identified small molecule mimics of the peptide-peptide interface that disrupted this interaction and therefore activated MFN2 and promoted mitochondrial fusion. These “mitofusin agonists” reversed mitochondrial fragmentation, dysmotility and hypopolarization in cultured mouse neurons expressing mutants Mfn2. They also normalized axonal mitochondrial trafficking within sciatic nerves of Mfn2 mutant mice [108]. A 4-hydroxy cyclohexyl analogue was recently discovered to be a good candidate as “mitofusion agonist” for preclinical studies [109]. Another therapeutic option trialed in mice models is histone deacetylase 6 (HDAC6) inhibition. Treating $Mfn2^{R94Q}$ mutant mice with a highly selective HDAC6 inhibitor, SW-100, restored α-tubulin acetylation and motor and sensory performance [110]. In an adult patient with CMT2A and optic atrophy, high dose of CoQ10 (200 mg/day) for eight months resulted in partial resolution of visual impairment [111].

10. Conclusions

MtDNA depletion syndromes constitute an expanding group of disorders with novel genes responsible for disorders of mtDNA maintenance being discovered constantly. Potential therapeutic strategies for patients with mtDNA depletion syndromes have increased exponentially in the past few years. This review aims to illustrate potential therapeutic approaches based on a large number of preclinical data recently collected from research performed in model organisms and cellular studies. Moreover, this review also discusses current and planned clinical trials for patients affected with these disorders. Nucleoside bypass therapy for TK2 deficiency and enzyme replacement therapy for MNGIE have the potential of providing a breakthrough for a group of rare disorders for which treatment remains supportive. Genetic based therapeutic strategies will likely deliver the most auspicious outcome, even if now these approaches are currently focused on early preclinical studies. An increasing understanding of the molecular and biochemical mechanisms involved in the maintenance of mtDNA copy number, a better knowledge of the natural history of these disorders, and the ongoing development of model organisms will lead to the development of ground-breaking therapies that could be trialed for patients affected with these disorders, ultimately leading to their application in the clinical setting. However, it must be taken into consideration that animal studies may not always recapitulate the clinical phenotype observed in humans affected with these disorders due to insurmountable genetic differences between humans and animals.

In parallel with these developments, novel clinical rating scales, and patient-reported outcome measures will have to be developed to capture the efficacy of these interventions. Furthermore, new biomarkers for mitochondrial disease will have to be ascertained that could be used as reliable endpoints. Moreover, there is an urgent need to conduct multinational clinical trials to better evaluate novel therapies for these rare mitochondrial syndromes. In order to do so, harmonization of a group of solidly validated, and clinically relevant primary outcome measures for these studies will have to be achieved.

Funding

Fernando Scaglia has received research support from NIH (5U54-NS078059-10); Mervar Foundation, Courage for a Cure Foundation, Stealth BioTherapeutics, PTC Therapeutics (Edison Pharma), Astellas Pharma, and Zogenix. He is on the Board of the Mitochondrial Medicine Society; and is an investigator for the North American Mitochondrial Disease Consortia (NAMDC) and the Frontiers of Congenital Disorders of Glycosylation Consortia (FCDGC).

May Ali has received research support from Courage for a Cure Foundation, Stealth BioTherapeutics, and Astellas Pharma.

Mahshid S. Azamian has received research support from Zogenix.

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Mol Genet Metab. Author manuscript; available in PMC 2023 August 04.

Table 1

Summary of recent and ongoing clinical trials concerning therapeutic options in mitochondrial DNA maintenance defects.

Ongoing clinical trial	Condition	Therapeutic approach	Primary outcome	Status
Efficacy and safety of vatiquinone for treating mitochondrial disease in participants with refractory epilepsy (MIT-E) (NCT04378075) (phase2/3)	Inherited mitochondrial disease with associated epilepsy phenotype (including *POLG*-related disorders)	Preventing ferroptosis through glutathione repletion	Seizure frequency (percent change from baseline to week 24)	Recruiting
Efficacy and safety of elamipretide in subjects with primary mitochondrial disease resulting from nuclear DNA mutations (nPMD) (NuPower) NCT05162768) (Phase 3)	Primary mitochondrial disorders due to pathogenic variants in mitochondrial replisome-related genes (*POLG1, POLG2 TWINKLE, TYMP, DGUOK, TK2, RRM2B, RNASEH1, SSBP, MGME1, DNA2, SLC25A4, SUCLG1, SUCLA2, MPV17*)	Cardiolipin stabilization	6-MWT	Recruiting
Continuation treatment with combination pyrimidine nucleosides in patients with TK2(continuation) (NCT03845712) (Phase 2)	*TK2*-related disorders	Nucleoside bypass therapy	Safety (AEs, Laboratory markers, EKG)	Active, not recruiting
Efficacy and Safety of MT1621 in TK2 deficiency (Treatment naïve) (NCT04581733) (phase 3)	*TK2*-related disorders	Nucleoside bypass therapy	Proportion of subjects acquiring a motor milestone	Not yet recuring
Treatment of TK2 deficiency with thymidine and deoxycytidine (NCT03639701) (Phase 1/2)	*TK2*-related disorders	Nucleoside bypass therapy	Safety (AEs, Laboratory markers, EKG)	Enrolling by invitation
Deoxynucleosides Pyrimidines as Treatment for mtDNA depletion syndrome (NCT04802707) (Phase 2)	MtDNA depletion syndrome due to pathogenic variant(s) in one of the following genes: *POLG, C10orf2, RRM2B, MPV17, SUCLA2, SUCLG1, FBXL4*	Nucleoside bypass therapy	Rate of Responder *versus* Non-Responder Status at 26 weeks	Recruiting
MNGIE allogeneic hematopoietic stem cell transplant safetystudy (MASS) (NCT02427178) (Phase 1)	MNGIE	aHSCT	Engraftment success	Recruiting
MT2013–31: aHSCT for metabolic disorders and severe osteopetrosis (NCT02171104) (Phase 2)	Metabolic disorders and severe osteopetrosis (including MNGIE)	aHSCT	Engraftment success	Recruiting
Trial of erythrocyte encapsulated thymidine phosphorylase in mitochondrial neurogastrointestinal encephalomyopathy (TEETPIM) (NCT03866954) (Phase 2)	MNGIE	EE-TP	-Safety (AEs, Laboratory markers, vital signs, use of concomitant medication, EKG) -Pharmacodynamic - Efficacy (change in BMI)	Not yet recruiting
Stem Cell Ophthalmology Treatment Study II (SCOTS2) (NCT03011541)	Retinal and optic nerve damage or disease (including *OPA1*-related disorders)	BMSC	Visual acuity	Recruiting
A study to evaluate ASP0367 in participants with primary mitochondrial myopathy (Mountainside) (NCT04641962) (Phase 2/3)	Primary mitochondrial myopathy due to pathogenic nuclear or mitochondrial genome variants demonstrated to cause primary mitochondrial disease	PPARδ modulator	-Pharmacokinetics - Safety (AEs, Laboratory markers, vital signs, EKG) - 6-MWT	Recruiting

AEs: adverse events; aHSCT: allogeneic hematopoietic stemcell transplant; BMI: bodymass index; BMSC: Bonemarrowmesenchymal stem cell; EE-TP: erythrocyte encapsulated Thymidine Phosphorylase; EKG: electrocardiogram; MNGIE: Mitochondrial neurogastrointestinal encephalopathy; TK2: Thymidine Kinase 2; 6-MWT: 6 min' walk test.