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Strategies for fighting mitochondrial diseases

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Abstract. Viscomi C, Zeviani M (University of Padova, Padova; and Venetian Institute of Molecular Medicine, Padova, Italy). Strategies for fighting mitochondrial diseases (Review-Symposium). *J Intern Med* 2020; **287**: 665–684.

Mitochondrial diseases are extremely heterogeneous genetic conditions characterized by faulty oxidative phosphorylation (OXPHOS). OXPHOS deficiency can be the result of mutation in mtDNA genes, encoding either proteins (13 subunits of the mitochondrial complexes I, III, IV and V) or the tRNA and rRNA components of the in situ mtDNA translation. The remaining mitochondrial disease genes are in the nucleus, encoding proteins with a huge variety of functions, from structural subunits of the mitochondrial complexes, to factors involved in their formation and regulation, components of the mtDNA replication and expression machinery, biosynthetic enzymes for the biosynthesis or incorporation of prosthetic groups, components of the mitochondrial quality control and proteostasis, enzymes involved in the clearance of toxic compounds, factors involved in the formation of the lipid milieu, etc. These different functions represent potential targets for 'general' therapeutic interventions, as they may be adapted to a number of different mitochondrial conditions. This is in contrast with 'tailored', personalized therapeutic approaches, such as gene therapy, cell therapy and organ replacement, that can be useful only for individual conditions. This review will present the most recent concepts emerged from preclinical work and the attempts to translate them into the clinics. The common notion that mitochondrial disorders have no cure is currently challenged by a massive effort of scientists and clinicians, and we do expect that thanks to this intensive investigation work and tangible results for the development of strategies amenable to the treatment of patients with these tremendously difficult conditions are not so far away.

Keywords: AAV, antioxidants, autophagy, experimental therapy, mitochondrial biogenesis, mitochondrial disease.

Experimental therapeutic strategies

In the last 30 years, remarkable progress has been made towards a substantial elucidation of the essential mechanisms causing mitochondrial disorders, as well as the pathways controlling biogenesis of and signalling from mitochondria. The increasing knowledge mechanistic aspects of mitochondrial disease have allowed to propose numerous therapeutic approaches aimed at combating these conditions that have been tested in suitable cells and animals. These include strategies that could in principle be used to treat several diseases (which we previously called 'generalist' [1]) as well as treatments specific for a single disease ('disease-tailored' strategies) (Table 1). Table 2 summarizes the ongoing clinical trials cited in the following sections.

The following discussion will cover the state of the art of both preclinical and clinical therapies for mitochondrial diseases. The approaches based on mitochondrial replacement therapy, that is those aiming at replacing mitochondria carrying mtDNA mutations with healthy mtDNA molecules in early-stage embryos or in maternal oocytes, for which numerous excellent reviews have been recently published (see for instance [2]), will not be covered here.

The development of mitochondrial medicine

Mitochondria are complex organelles, characterized by the presence of an outer and an inner membrane (OMM and IMM), the latter presenting serial invaginations denominated mitochondrial cristae, where respiratory chain (RC) complexes

Table 1 *Therapeutic strategies*

General (i.e. fighting common mechanisms)	Specific (e.g. AAV gene therapy)
↙ Less specific	↙ Tailored for specific diseases
↙ Off-target effects	↙ Potentially highly effective
↙ Wide applicability	↙ Limited to a few conditions
↙ Cost effective	↙ Expensive
Achieved by • Activating mitochondrial and MRC biogenesis • Acting on mitodynamics>autophagy>apoptosis • Correcting mtDNA mutations post-transcriptionally	Achieved by • Delivering proteins involved in detoxification • Gene replacement in specific tissues • Stabilizing mutant proteins

(complexes I to IV) and the mitochondrial ATP synthase (complex V, cV) are localized. Mitochondria oxidatively extract the energy stored in nutrients, converting it into two main different forms, heat and ATP, the energy currency of the cell, through respiration, which uses molecular oxygen as a sink on which electrons oxidatively stripped off nutrients converge, to form water. This process is carried out by the first four complexes of the respiratory chain (RC). The electron flow carried out by redox reactions in the different RC complexes, and ultimately reducing molecular oxygen into water, is coupled in the electron flow carried out by cI, cIII and cIV (or cytochrome c oxidase) by the translocation of protons from the inner mitochondrial compartment, called the mitochondrial matrix, MM, through the IMM, into the intermembrane space (IMM). This asymmetric distribution of positively charged protons creates a membrane potential (ΔP) composed of a chemical (ΔpH) and an electrostatic ($\Delta\psi$) components, that have the characteristic of a capacitor, and provide the chemiosmotic and electronmotive forces that are exploited to eventually phosphorylate ADP + Pi into ATP, operated by complex, or mitochondrial ATP synthase. The entire process is known as oxidative phosphorylation (OXPHOS). In summary, respiration, that is oxidation of reduced shuttle molecules by oxygen, is carried out by four multiheteromeric RC complexes, cI-cIV, that transfer electrons from NADH and $FADH_2$, reduced by extraction of reducing equivalent from nutrients by intermediate metabolism, mainly sugars and fatty acids, to molecular oxygen. The electron flow is coupled with the translocation of protons across the IMM operated by complexes I, III and IV. This generates an electrochemical gradient, that stores energy similar to a capacitor, which is then exploited by complex V (or ATP synthase) to synthesize ATP from ADP and Pi [3].

Mitochondria contain a double-stranded circular DNA molecule (mtDNA) of 16.5 Kb in mammals. Mammalian mtDNA, present in multiple copies in each mitochondrion, encodes 13 subunits of the RC complexes I, III, IV and also ATP synthase (complex V), 22 tRNAs, and 2 rRNA genes, which constitute the RNA apparatus necessary for the *in situ* translation of the 13 mtDNA-encoded protein subunits. The four subunits of complex II are all encoded from nucleus-encoded genes. In normal conditions, the vast majority of mtDNA molecules are all identical to each other (a condition called homoplasmy), although low levels of mutant mtDNA molecules are detected in all the subjects by ultradeep sequencing techniques, and may increase in specific areas of certain tissues such as the brain during ageing [4]. Contrariwise, pathogenic mtDNA mutations often coexist in substantial amounts with wild-type mtDNA molecules (a condition called heteroplasmy) [5].

The approximately 1500 proteins forming the rest of the mitochondrial proteome are encoded by nuclear genes. These protein products are translated in the cytosol and finally targeted to and imported into the organelles by an active process largely operated by the respiration-dependent membrane potential (ΔP). These proteins participate in a large number of essential biological pathways, including mtDNA replication, transcription and translation, formation assembly and functional and structural regulation of the respiratory chain complexes, fission-fusion of the

Table 2 *Drug: injection of scAAV2-P1ND4v2 1.18x10e9 vg (Low), |drug: injection of scAAV2-P1ND4v2 5.81 X10e9 vg (Med),|drug: injection of scAAV2-P1ND4v2 2.4 X10e10vg (High) and |drug: injection of scAAV2-P1ND4v2 1.0 X10e11vg (Higher)*

Rank	Title	Status	Study Results	Conditions	Interventions	Locations	URL
1	Efficacy & Safety Study of Bilateral IVT Injection of GS010 in LHON Subjects Due to the ND4 Mutation for up to 1 Year	Active, not recruiting	No Results Available	Leber Hereditary Optic Neuropathy	Genetic: GS010|Drug: Placebo	Doheny Eye Center UCLA Pasadena, Pasadena, California, United States|University of Colorado Health Eye Center, Aurora, Colorado, United States|Emory Healthcare - The Emory Clinic, Atlanta, Georgia, United States|Massachusetts Eye and Ear Infirmary, Boston, Massachusetts, United States|Icahn School of Medicine at Mount Sinai, New York, New York, United States|Wills Eye Institute - Ocular Oncology Service, Philadelphia, Pennsylvania, United States|Vanderbilt Eye Institute, Nashville, Tennessee, United States|Universitair Ziekenhuis Gent, Gent, Belgium|CHNO Les Quinze Vingts, Paris, France|IRCCS Istituto delle Scienze Neurologiche di Bologna UOC Clinica Neurologica, Bologna, Italy|Hospital Universitario Ramon y Cajal, Madrid, Spain|Taipei Veterans General Hospital, Taipei, Taiwan|Moorfields Eye Hospital, London, Greater London, United Kingdom	https://ClinicalTrials.gov/show/NCT03293524
2	Safety Study of an Adeno-associated Virus Vector for Gene Therapy of Leber's Hereditary Optic Neuropathy	Recruiting	No Results Available	Leber's Hereditary Optic Neuropathy	Drug: injection of scAAV2-P1ND4v2 1.18x10e9 vg (Low),| Drug: injection of scAAV2-P1ND4v2 5.81 X10e9 vg (Med)|Drug: injection of scAAV2-P1ND4v2 2.4 X10e10vg (High)|Drug: injection of scAAV2-P1ND4v2 1.0 X10e11vg (Higher)	Bascom Palmer Eye Institute, University of Miami, Miami, Florida, United States	https://ClinicalTrials.gov/show/NCT02161380
3	EAP_GS010_single Patient	Available	No Results Available	Leber Hereditary Optic Neuropathy (Optic, Atrophy, Hereditary, Leber)	Genetic: GS010		https://ClinicalTrials.gov/show/NCT03672968

Table 2 (*Continued*)

Rank	Title	Status	Study Results	Conditions	Interventions	Locations	URL
4	REALITY LHON Registry	Recruiting	No Results Available	Leber Hereditary Optic Neuropathy	Other: Patient-reported outcomes (PROs)	Doheny Eye Center UCLA Pasadena, Pasadena, California, United States|Emory University Hospital, Atlanta, Georgia, United States|Massachusetts Eye and Ear Infirmary, Boston, Massachusetts, United States|Wills Eye Institute, Philadelphia, Pennsylvania, United States|Alkek Eye Center, Houston, Texas, United States|CHU d'Angers, Angers, France|CHNO Les Quinze Vingts, Paris, France|Ospedale Bellaria, Bologna, Italy|Ospedale San Raffaele, Milano, Italy|Institut Catala de Retina, Barcelona, Spain|Moorfields Eye Hospital, London, Greater London, United Kingdom	https://ClinicalTrials.gov/show/NCT03295071
5	RESCUE and REVERSE Long-term Follow-up	Recruiting	No Results Available	Leber Hereditary Optic Neuropathy (Optic, Atrophy, Hereditary, Leber)	Genetic: GS010|Other: Sham Intravitreal Injection	Doheny Eye Center UCLA, Pasadena, California, United States|Emory University Hospital, Atlanta, Georgia, United States|Wills Eye Institute, Philadelphia, Pennsylvania, United States|CHNO Les Quinze Vingts, Paris, France|LMU Klinikum der Universität München/ Friedrich-Baur-Institut, Munich, Germany|Ospedale Bellaria, Bologna, Italy|Moorfields Eye Hospital, London, Greater London, United Kingdom	https://ClinicalTrials.gov/show/NCT03406104
6	Efficacy Study of GS010 for the Treatment of Vision Loss up to 6 Months From Onset in LHON Due to the ND4 Mutation	Completed	Has Results	Optic, Atrophy, Hereditary, Leber	Biological: GS010|Device: Sham Intravitreal Injection	Doheny Eye Center, University of California, Los Angeles, Los Angeles, California, United States|Department of Ophthalmology, Emory University School of Medicine, Atlanta, Georgia, United States|Neuro Ophthalmologic Associates, Wills Eye Hospital, Thomas Jefferson University, Philadelphia, Pennsylvania, United States|Centre National Hospitalier d'Ophtalmologie des Quinze-VingtCentre National Hospitalier d'Ophtalmologie des Quinze-Vingt, Paris, France|Department of Neurology, University of Munich, Friedrich-Baur-Institute, Munich, Germany|IRCCS Istituto delle Scienze Neurologiche di Bologna, UOC Clinica Neurologica, Ospedale Bellaria, Bologna, Italy|Moorfields Eye Hospital NHS Foundation Trust, London, United Kingdom	https://ClinicalTrials.gov/show/NCT02652767

Table 2 (*Continued*)

Rank	Title	Status	Study Results	Conditions	Interventions	Locations	URL
7	Efficacy Study of GS010 for Treatment of Vision Loss From 7 Months to 1 Year From Onset in LHON Due to the ND4 Mutation (REVERSE)	Completed	Has Results	Optic, Atrophy, Hereditary, Leber	Biological: GS010| Device: Sham Intravitreal Injection	Doheny Eye Center, University of California, Los Angeles, Los Angeles, California, United States| Department of Ophthalmology, Emory University School of Medicine, Atlanta, Georgia, United States| Neuro Ophthalmologic Associates, Wills Eye Hospital, Thomas Jefferson University, Philadelphia, Pennsylvania, United States|Centre National Hospitalier d'Ophtalmologie des Quinze-Vingt, Paris, France|Department of Neurology, University of Munich, Friedrich-Baur-Institute, Munich, Germany|IRCCS Istituto delle Scienze Neurologiche di Bologna, UOC Clinica Neurologica, Ospedale Bellaria, Bologna, Italy|Moorfields Eye Hospital NHS Foundation Trust, London, United Kingdom	https://ClinicalTrials.gov/show/NCT02652780

mitochondrial network, signalling and execution mechanisms (e.g. ROS production and apoptosis), scavenging of toxic compounds, and many other relevant metabolic functions, as diverse as fatty acid oxidation, biosynthesis of pyrimidines, haeme, Fe-S clusters, etc [3].

Primary mitochondrial diseases are due to pathogenic mutations in either mitochondrial or nuclear genomes (Table 3). Mutations in mtDNA include homo- or heteroplasmic point mutations and large-scale rearrangements, which are always heteroplasmic since the deleted mtDNA portion invariably contains one or more tRNA species essential for mtDNA translation. Heteroplasmic point mutations lead to rather well-established syndromes, such as mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) [6], myoclonic epilepsy with ragged red fibres (MERRF) [7], neurogenic weakness, ataxia and retinitis pigmentosa (NARP) [8], and Leigh syndrome (LS). The main condition associated with homoplasmic mtDNA mutations is Leber's hereditary optic neuropathy (LHON) [9]. Rearrangements (single deletions or duplications) of mtDNA are responsible for sporadic progressive external ophthalmoplegia (PEO) [10], Kearns–Sayre syndrome (KSS) [10] and Pearson's syndrome [11].

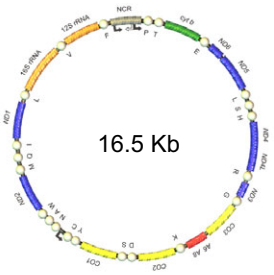
Nuclear mutations affect a large number of genes directly or indirectly related to the respiratory chain, encoding (i) structural subunits of the respiratory chain complexes; (ii) factors involved in the assembly of the respiratory complexes, in the biosynthesis of their prosthetic groups, or in their structural and functional regulation; (iii) proteins involved in mtDNA replication and maintenance of its integrity and abundance in different tissues; (iv) components of the mtDNA transcription and translation machineries; (v) proteins involved in execution pathways, such as autophagy/mitophagy, quality control of proteostasis, mitochondrial dynamics and apoptotic signals stemming from abnormal mitochondria; and (vi) a miscellaneous category of genes however participating in OXPHOS homeostasis [12].

As a consequence, mitochondrial diseases are extremely heterogeneous clinically, biochemically and genetically. This is a severe obstacle that hampers the possibility to collect homogeneous and sufficiently numerous cohorts of patients, in order to provide unequivocal evidence about the efficacy (or inefficacy) of a given treatment.

Table 3 *Genetic classification of OXPHOS mitochondrial diseases*

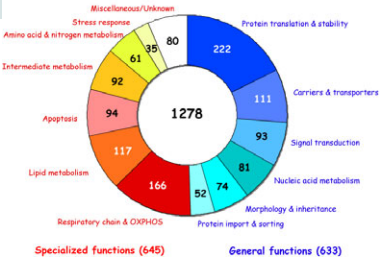
Defects of Mitochondrial DNA

- Protein synthesis genes (rRNAs, tRNAs)
- Protein-encoding OXPHOS subunit genes
- Large deletions



Nuclear DNA mutations

- Protein-encoding OXPHOS subunit genes
- OXPHOS assembly factors
- Factors affecting mtDNA maintenance
- Mitochondrial protein synthesis genes
- Biosynthetic enzymes for lipids or cofactors
- Genes involved in mitochondrial dynamics
- Genes involved in mitochondrial biogenesis
- Genes involved in mitochondrial detoxification
- Others



Generalist strategies

Activation of mitochondrial biogenesis

Given the pivotal role of mitochondria in energy conversion processes, a rather obvious consequence of mitochondrial dysfunction is the reduced synthesis of ATP. Interventions aimed at increasing mitochondrial content are thus expected to promote some improvement in mitochondrial diseases by increasing the number of ATP-synthesizing units. Peroxisome proliferator-activated receptor-gamma 1 (PGC1) α is a transcriptional coactivator of a number of transcription factors, including the nuclear respiratory factors (NRF1 and NRF2), and the peroxisomal proliferator activator receptors (PPAR) α, β and γ. NRF1 and NRF2 control the transcripts levels of OXPHOS-related genes, whereas PPARs those of genes related to fatty acid oxidation. PGC1α is post-translationally activated by phosphorylation carried out by the AMP-dependent kinase (AMPK) or by deacetylation operated by the nuclear deacetylase Sirtuin 1 (Sirt1) (Figure 1). Both AMPK and Sirt1 activities can be pharmacologically modulated and exploited to activate PGC1α [13]. AMPK is activated by AMP or analogue compounds, signalling a symmetric reduction of ATP supply, whereas Sirt1 is activated by its own substrate NAD^+.

We and others used either the AMP analogue AICAR to stimulate AMPK or the NAD^+ precursor nicotinamide riboside (NR) to activate Sirt1 [14-16]. In both cases, amelioration of the clinical phenotype, particularly motor endurance and coordination, was observed in recombinant mouse models of mitochondrial disease. Alternative approaches to increase Sirt1 activity, based on the inhibition of a major NAD^+ consumer, poly(ADP-ribosyl)-polymerase 1 (PARP1), effectively improved the clinical phenotype in the same mouse models. Also, the pan-PPAR agonist bezafibrate has been proposed to activate PGC1α but the results are more controversial [16-18].

Other compounds have been used to stimulate mitochondrial biogenesis in cell and animal models of mitochondrial disfunction, including resveratrol ([19,20]) and retinoic acid ([21]), again with controversial results.

Translation into the clinic

Three clinical trials are currently ongoing using bezafibrate (NCT02398201); nicotinamide riboside (NCT03432871) and niacin, another precursor of NAD^+ (NCT03973203). For none of these, the outcome has been disclosed, although those with

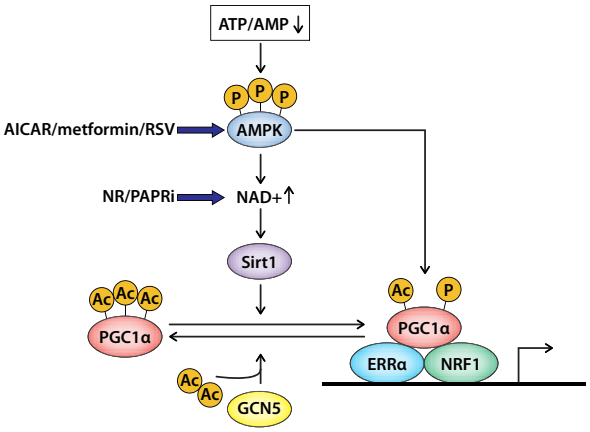


Fig. 1 *PGC1α-dependent mitochondrial biogenesis pathway*

bezafibrate and niacin have now been completed and the results are expected soon.

Targeting mTORC1

mTOR (mechanistic target of rapamycin) is a cytosolic Ser/Thr kinase belonging to the family of PI3K-related kinases (PIKK) [22]. It interacts with several proteins forming two different complexes: mTORC1 is formed by three main components, that is mTOR, Raptor (regulatory protein associated with mTOR) and mLST8 (mammalian lethal with Sec13 protein 8), whereas mTORC2 contains Rictor (rapamycin-insensitive companion of mTOR) instead of Raptor. These two complexes regulate different cellular processes. mTORC1 plays essential roles in a vast number of cellular metabolic (mainly anabolic) pathways, including activation of protein translation, immune response, nucleotide and lipid synthesis, and glucose metabolism, and, in parallel, inhibition of catabolic pathways, such as autophagy and lysosomal biogenesis. mTORC1 is activated by availability of amino acids and other nutrients, and it essentially modulates cell metabolism towards activation of anabolic pathways. In contrast, mTORC2 controls cell proliferation and survival as well as cytoskeleton remodelling. In 2013, Johnson and colleagues published the results of a study showing that strong and prolonged inhibition of mTORC1 by rapamycin inhibited cytosolic translation and markedly prolonged the lifespan of a mouse with early neurodegeneration similar to Leigh disease, the *Ndufs4*$^{-/-}$

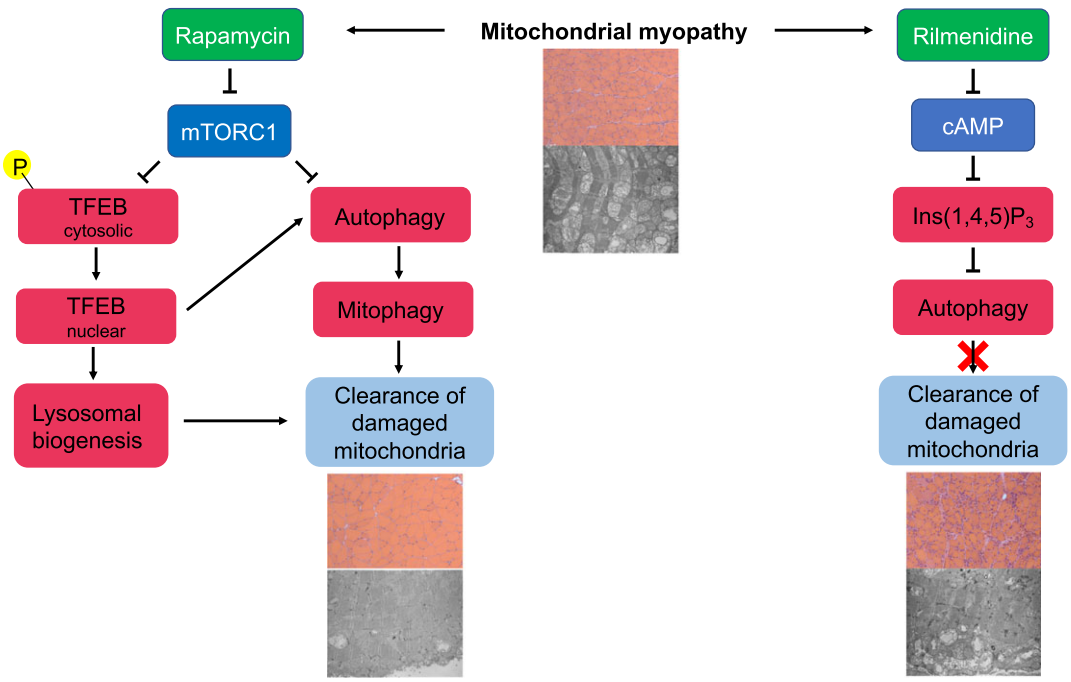


Fig. 2 *Differential effects of rapamycin, which increases both autophagy and lysosomal biogenesis and rilmenidine, which increases only autophagy, on mitochondrial myopathies*

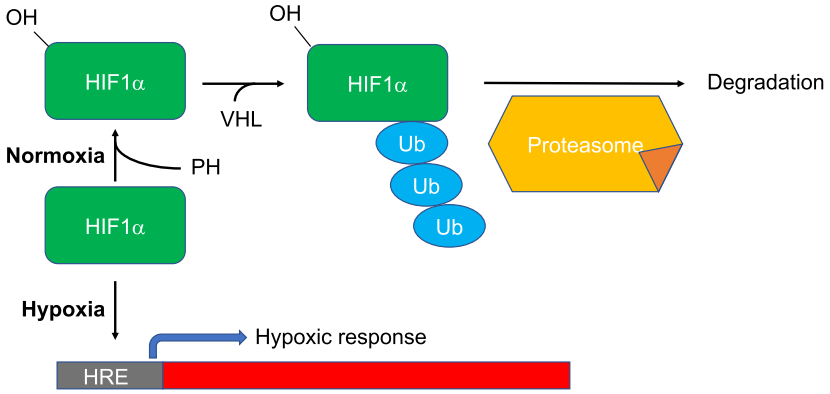


Fig. 3 *Regulation of hypoxic response. VHL, Von Hippel–Lindau Factor; PH, prolyl hydroxylases; Ub, ubiquitin; and HRE, hypoxia response element*

mouse, which lacks the 18 kDa accessory cI subunit NDUFS4 [23].

A rather large number of studies on different in vivo and cellular models confirmed this result, including (i) a muscle-specific Cox15 knockout mouse [24], (ii) a *ND2*-deficient *Drosophila* model of LS [25], (iii) iPSCs-derived neurons carrying a mutation in the MT-ATP6 gene [26], (iv) the CoQ-deficient mouse *B6.Pdss2*$^{kd/kd}$, (v) the gas-1(fc21) nematodes [27], carrying a homozygous mutation in the complex I *NDUFS2* subunit homologue, (vi) the Deletor mouse and (vi) the *Tk2* knock-in mouse model (*Tk2*^{H126N}) [28]. However, the mechanism by which rapamycin has beneficial effects in mitochondrial disease models is highly debated. Several mechanisms have been hypothesized, including translation inhibition, activation of FGF21 and GDF21 and of the mitochondrial unfolded response, metabolic remodelling, including the one carbon metabolism, and activation of autophagy. The latter is indeed increased by rapamycin-exposed cIV-deficient COX15 muscle KO mice, but the concomitant activation of lysosomal biogenesis by increased nuclear migration of the TFEB transcription factor seems essential for the effectiveness of rapamycin, contrary to the no effect of other autophagy inhibitors such as rilmenidine (Figure 2). TFEB is phosphorylated by mTORC1, and this impedes it to migrate to the nucleus and activate its transcription programme. Thus, inhibition of mTORC1 by rapamycin is likely to promote TFEB nuclear localization and perform a synergistic action on both autophagy and lysosomal clearance of defective mitochondria [28].

Notably, rapamycin was ineffective in a *Coq9*^{R239X} knock-in mouse, possibly due to the lack of neuroinflammation induced by microglia, the limited capacity to trigger autophagy in this model due to lysosomal impairment. Interestingly, rapamycin upregulated the transcription of several metabolic pathways, including lipid, amino acid and nucleotide metabolism in liver and, to a lesser extent, in brain. These changes were reflected in parallel changes in the corresponding metabolites. However, they failed to improve the phenotype of the *Coq9*^{R239X} mice, likely because of the absence of a functional CoQ-junction [29,30].

Translation into the clinic

Following the preclinical evidence, four MELAS patients were treated with everolimus, a rapamycin analogue, following kidney transplant [31]. Primary fibroblasts derived from these patients showed improved mitochondrial morphology, membrane potential and replicative capacity. Blood markers showed reduced oxidative damage but no decrease of heteroplasmy levels.

In another study, two children, one affected by MELAS and one by Leigh disease, have been treated with everolimus [32].

The Leigh syndrome patient was a girl carrying a homozygous missense mutation in NDUFS4 and started treatment (2-4 gr/day) at 23 months of age. Six months after starting the treatment, improvement by brain MRI consisted of reduction of the bilateral signal hyperintensity in thalami and

brainstem. After 19 months of treatment, she was walking independently with a slightly ataxic gait, she spoke in sentences, and she no longer required tracheostomy and gastrostomy. MRI did not improve further, but the clinical scales continued to improve up to 47 months. In contrast, the MELAS patient was a boy, who started the treatment at 69 months, did not show any improvement in the brain MRI and continued to deteriorate until he passed away at 79 months of age.

An open-label phase 2a study to evaluate the safety, tolerability and clinical activity of ABI-009 (the rapalogue Nab-sirolimus) in Patients with genetically confirmed Leigh or Leigh-like Syndrome is currently ongoing but not yet recruiting (NCT03747328).

Overall, the available data support the idea that rapamycin may be effective in several mitochondrial disorders. Both mouse and human studies suggest that the specific gene affected or gender-specific effects should be carefully evaluated. It is currently unknown whether the immunosuppressive effect of mTORC1 inhibitors can be detrimental for mitochondrial patients in the long term.

Hypoxia

A CRISPR/Cas9 screening identified the Von Hippel–Lindau (VHL) factor as the most effective suppressor of antimycin induced mitochondrial dysfunction [33], opening the possibility to use this pathway to treat mitochondrial diseases. VHL is a ubiquitin ligase that recognizes and targets for degradation the hydroxylated forms of the hypoxia-induced transcription factors (HIFs) during the hypoxic response [34] (Figure 3). The same team followed up the original paper in order to explore this concept in vivo and to dissect the mechanism by which hypoxia acts. In a first study, chronic normobaric hypoxic conditions (11% O_2) starting at 30 days were shown to arrest the clinical progression of the *Ndufs4*$^{-/-}$ mice, with the median lifespan increasing from 58 to 270 days. This effect was accompanied by marked rescue of the neuropathological lesions in olfactory bulbs, cerebellum and brainstem of *Ndufs4*$^{-/-}$ mice. Notably, hyperoxia (55% O_2) had the opposite effect and worsened all the parameters mentioned above [33]. Less drastic hypoxic conditions (17% O_2) or alternation of hypoxia and normoxia had no beneficial effects on the phenotype of *Ndufs4*$^{-/-}$ mice [33]. Finally, return to normoxic conditions rapidly reversed the beneficial effects described above, whilst switching to hypoxia after the onset of the symptoms reversed the brain lesions [35]. Very recently, the same research group showed that this effect is mediated by the alleviation of brain hyperoxia, a consequence of reduced respiration, rather than by activation of the hypoxic genetic program. Accordingly, the authors showed that other interventions aimed at reducing oxygen partial pressure in the brain, namely exposure to nonlethal concentration of carbon monoxide and severe anaemia, have similar beneficial effect [36]. These interesting findings, however, do not fully address whether ROS may play a role in the development of the disease and partly contradict the observation that the genetic activation of the hypoxic response protected against respiration defects [37].

Antioxidants

Reactive oxygen species (ROS) are normal by-products of respiration generated at several sites in the matrix and intermembrane space, including complex I FMN moiety, complex III ubiquinone-binding sites, glycerol 3-phosphate dehydrogenase, the electron transferring flavoprotein:Q oxidoreductase (ETFQOR) involved in the terminal phases of oxidation of both fatty acids and branched-chain amino acids, and pyruvate and 2-oxoglutarate dehydrogenases [38].

ROS, for instance the superoxide anion (O_2^{2-}) and most of all the hydroxyl radical ($^{\bullet}OH$), are highly reactive molecules, whose production is increased in the presence of respiratory chain dysfunction, ageing [39] or specific OXPHOS defects [40], and may damage key cell components, including proteins, lipids and nucleic acids.

Cells have evolved highly efficient antioxidant defences to prevent oxidative damage. For instance, O_2^{2-} is rapidly converted into the much less harmful and more stable hydrogen peroxide (H_2O_2) by the mitochondrial manganese superoxide dismutase (SOD2) [41]. H_2O_2 is then converted to water by mitochondrial glutathione peroxidases (GPX) or peroxiredoxins (PRX) or can diffuse to the cytosol, where it has important signalling functions by oxidizing protein thiol residues. In turn, cytosolic GPXs and PRXs and peroxisomal catalase tightly regulate H_2O_2 levels by converting it into water [41].

Based on these observations, antioxidants have been proposed in the therapy of mitochondrial

diseases, and several compounds with antioxidant properties are or have been used in the clinical practice as well as in experimental setups.

KH176 is a compound found by systematic screening of a cell library that works both as an antioxidant and as a redox modulator by activating the thioredoxin/peroxiredoxin system [42]. In the *Ndufs4*$^{-/-}$ mouse, KH176 administration improved rotarod performance and gait abnormalities with retention of the microstructural organization in some areas of the brain; KH176 treatment was neither able to reduce or prevent the severe brain pathology, nor had it tangible effects on the onset or severity of the disease, as measured by phenotypic scoring or lifespan [43]. However, recent data showed a slight, but significant, increase in the lifespan and amelioration of some gait and motor parameters in KH176-treated Ndufs4-/- mice [44].

N-acetyl cysteine (NAC) and vitamin E were able to rescue the phenotype of gas-1(fc21) in mutant nematodes, carrying a mutation in the orthologue of complex I 49 KDa subunit (NDUFS2) [44]. CoQ, lipoate, orotate and vitamin C partially prolonged the lifespan of the same model [44]. These results were possibly due to a reduction of oxidative stress without correction of the underlying mitochondrial defect [44]. In another series of experiment, probucol rescued zebrafish developmental delay induced by rotenone inhibition of complex I and oligomycin-dependent complex V inhibition, but failed to rescue complex IV inhibition by azide [45]. The reasons for the different outcome with the complex IV inhibitor are not known.

Finally, NAC and ascorbate were shown to decrease ROS production in fibroblasts with a mutation in COX4-1 subunit [46].

Translation into the clinic

In spite of these controversial data, antioxidants remain central in clinical practice, and several antioxidant-based clinical trials have been completed or are ongoing.

In a double-blind, randomized, placebo-controlled, phase 1 study (NCT02544217). KH176 was shown to be well tolerated up to single doses of 800 mg and multiple doses of 400 mg b.i.d. and had a pharmacokinetic profile supportive for a twice daily dosage. Only at high doses, KH176 caused clinically relevant QTc prolongation [46]. A phase 2a clinical trial (KHENERGY, NTC0290400) investigated tolerability, safety, pharmacokinetics, pharmacodynamics, and efficacy of twice daily oral 100 mg KH176 for 28 days in 18 adult m.3243A>G patients without cardiovascular involvement. No significant improvements in gait parameters or other motor outcome measures were observed in this 28 days study. Beneficial effects on mood and alertness as measured by the BDI, HADS and TAP, were observed [46], supporting a phase 2b study , which is currently ongoing (KHENERGYZE, NCT04165239: see https://clinicaltrials.gov/ct2/show/NCT04165239).

Idebenone is a compound structurally related to CoQ and is the first drug approved by EMA (European Medicines Agency) for the therapy of Leber's Hereditary Optic Neuropathy (LHON), with an indication for the treatment in patients with acute, subacute or dynamic clinical course, whereas it was not recommended for the treatment for chronic patients [49].

Several idebenone-based clinical trials have been carried out, including (i) NCT00887562 on MELAS, in which the primary end-point did not reach statistical significance, and (ii) NCT00747487 on LHON, in which the primary end-point did not reach statistical significance, whilst the secondary outcomes significantly differed in a subgroup of patients with discordant visual acuity at baseline.

RTA408 (omaveloxone) is an oleanane triterpenoid compound activating nuclear factor erythroid-derived 2-related factor 2 (Nrf2), a master regulator of the antioxidant response, which is suppressed in Friedreich's ataxia. A randomized, double-blind phase 2 study on FRDA patients (NCT02255435) provided evidence of good tolerability of the compound administered at 160mg/day over three months, with minor adverse effects and improved neurological abilities assessed by modified Friedreich's Ataxia Rating Scale [50]. A second trial (NCT02255422) with the same compound on mitochondrial myopathy has been completed but the results are not yet available.

EPI-743 is an antioxidant compound, which has been widely tested in several clinical trials on different diseases. However, either the trial has been suspended or the results not published. These include the following: (i) NCT01370447 on mitochondrial respiratory chain diseases, active not recruiting; (ii) NCT01642056 on mitochondrial disorders; (iii) NCT02104336 on Pearson's

syndrome, discontinued; (iv) NCT02352896 on Leigh disease, completed with no published results; and (v) NCT01962363 on Friedreich's ataxia, completed with no published results.

Finally, two clinical trials were carried out with thioctic (lipoic) acid (NCT00004770) and curcumin (NCT00528151 on LHON patients with 11778 mutation). In both cases, the studies have been completed, but the results are not available.

From the above discussion, the necessity emerges to improve the quality of both preclinical data supporting the clinical studies, which, with the exceptions of KH176 and idebenone, are almost completely lacking, and the need for a more systematic and accessible policy to report the results.

Finally, it has become increasingly clear in recent years that ROS have also crucial signalling functions in a number of pathways [51], and there is evidence that interfering with these can have harmful consequences for the cell, as outlined in the next session.

Bypass of electron transfer chain defects

Alternative oxidase (AOX) and NADH reductase (Ndi1) are single-peptide enzymes present in yeast, plants and lower eukaryotes as alternative components of the respiratory chain. These xenoproteins have been expressed in cellular and Drosophila models to bypass the block of the RC due to defects in specific complexes. The rationale for using these nonproton pumping enzymes is the restoration of the electron flow, thus decreasing the accumulation of potentially harmful reduced intermediates and ROS production and increasing ATP production by allowing proton pumping at the nonaffected RC complexes. Ndi1, which in the yeast *Saccharomyces cerevisiae* replaces complex I, by transferring electrons from NADH to CoQ, has been expressed to bypass cI defects in cells and flies, but not yet in mammalian systems [52,53]. AOX, which in various organisms transfers electrons from CoQ to molecular oxygen, without pumping protons, may then bypass cIII and cIV defects [54,55]. A transgenic mouse moderately expressing *AOX* has been produced and did not show any obvious clinical abnormality [56]. Two recent studies explored the possibility to bypass cIII or cIV defects in mouse models of mitochondrial disease, leading to starkly different results. AOX was able to markedly prolong the lifespan of the cIII-defective *Bcs1l*$^{p.S78G}$ knock-in mouse model of GRACILE syndrome, preventing renal tubular atrophy and cerebral astrogliosis, but not liver disease. In addition, ultrastructure of cardiac mitochondria, respiration rate and transcriptome and metabolomic signatures were normalized by AOX, with no effect on ROS production [57].

In contrast, when crossed to skeletal muscle-specific *Cox15* knockout, AOX led to an unexpected worsening of the myopathic phenotype, which was associated with markedly reduced ROS production via RET, possibly impairing a compensatory mechanism controlling mitochondrial biogenesis and satellite cell differentiation [58].

Several aspects remain to be investigated in order to clarify the possibility of using AOX to bypass cIII and IV defects. The different outcomes may be due to different disease mechanisms underlying the specific defects, or to the different organs involved or to the specific *Ciona intestinalis* AOX used in these studies. Numerous AOX from different organisms have been studied in detail and have different kinetic and structural profiles that should be more (or less) favourable in the potential therapy of mitochondrial dysfunction.

Targeting mitochondrial dynamics and shape

Mitochondria are highly dynamic organelles. Their shape and mass are finely tuned by the activity of pro-fusion proteins, such as mitofusin 1 (MFN1), MFN2 (acting in the OMM) and optic atrophy protein 1 (OPA1, acting in the IMM)), as well as pro-fission proteins, such as dynamin-related protein 1 (DRP1) and mitochondrial fission 1 protein (FIS1) [59,60]. Mutations in these genes lead to disease in humans. Heterozygous dominant mutations in *OPA1* are associated with autosomal dominant optic atrophy [61], whereas dominant mutations in *MFN2* cause Charcot–Marie–Tooth disease type 2A [62].

Deletion of *Mfn1* and *Mfn2* in the skeletal muscle of the *POLG*^{D257A} *mutator* mouse leads to striking worsening of the clinical conditions, due to accumulation of mtDNA mutations. These results suggest that mtDNA integrity is protected by the physiological balance between mitochondrial fission and fusion, possibly promoting the continuous mixing and complementation of different mtDNA pools [63].

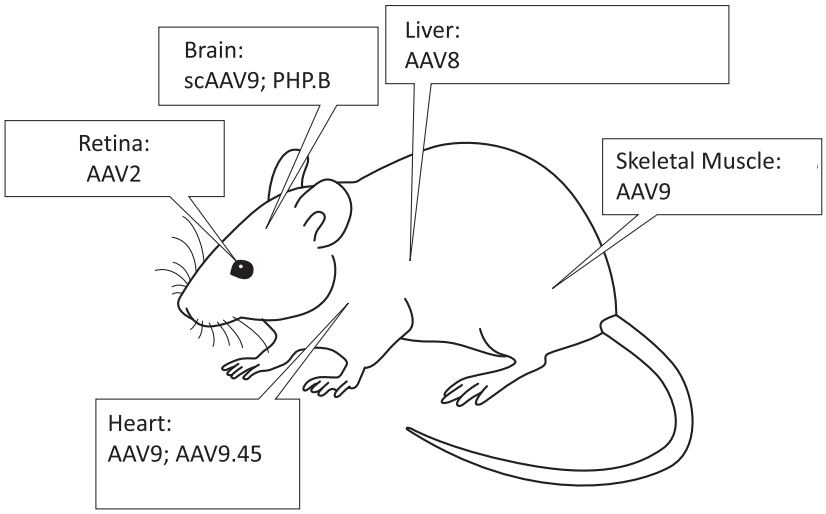


Fig. 4 *AAV serotypes used in mouse models of mitochondrial diseases*

In 2013, Cogliati et al. demonstrated that moderate overexpression of Opa1 increased the efficiency of the respiratory chain by regulating the structural and functional organization of the respiratory complexes into supercomplexes [64].

We reported that moderate overexpression of *Opa1* was beneficial in models of mitochondrial encephalopathy and myopathy by stabilizing the defective complexes and supercomplexes [65]. More recent data produced in our laboratory suggest that Opa1 overexpression can also prevent kidney focal glomerulosclerosis observed in *Mpv17* KO mice (Luna-Sanchez et al, manuscript in preparation, 2019). In addition, moderate Opa1 overexpression proved to be beneficial in several other conditions characterized by altered mitochondrial morphology, including denervation-induced muscle atrophy, liver damage and ischaemic brain damage [66].

Alternative strategies aimed at modifying mitochondria fission/fusion have been suggested. By using a computational approach, Rocha and colleagues were able to identify a small molecule acting as an allosteric activator of Mfn2, thus promoting fusion, and showed this compound effectively ameliorates mitochondrial abnormalities in cell lines carrying various mutations in MFN2 and normalizes axonal mitochondrial trafficking in sciatic nerves of MFN2 Thr105Met mice, a model of CMT2A [67]. Future work to assess their efficacy in models of OXPHOS deficiencies is warranted.

Translation into the clinic

Several clinical trials are currently ongoing on primary mitochondrial disease, primary mitochondrial myopathy and LHON using elamipretide, a Szeto and Schiller tripeptide able to enter cells and accumulate in mitochondria, possibly by binding cardiolipin. Cardiolipin is a phospholipid of the inner mitochondrial membrane where it plays a key role in regulating RC activities and structure and shaping mitochondrial cristae [68]. Although elamipretide seems to correct mitochondrial ultrastructure in cells with altered mitochondrial morphology, its mechanism is still poorly understood and no evidence that it may be effective in primary mitochondrial dysfunction has been provided. Only for one of the clinical trials on elamipretide (NCT02367014), the results have recently been published, being aimed at providing evidence for safety and initial efficacy assessment on mitochondrial myopathy patients [69]. Elamipretide showed substantially favourable safety profile and improved the 6-minute walking test in the treated group compared with the placebo group.

Dietary manipulations

Dietary measures have been attempted on cellular and in vivo models, as well as on mitochondrial patients.

Ketogenic high-fat, low-carbohydrate diet (KD) was used to stimulate mitochondrial beta-oxidation,

thus increasing the production of ketones, which can be used by several tissues as an alternative energy source. Ketone bodies are a source of acetyl-CoA, thus entering the TCA cycle and increasing the biosynthesis of succinate, which feeds the respiratory chain via complex II. In addition, high ketone levels have been shown to increase OXPHOS genes expression via a starvation-like response, which involves the same players controlling mitochondrial biogenesis, such as SIRT1, AMPK and PGC-1α [70]. It is worth mentioning here that succinate synthesis can be stimulated by using compounds such as triheptanoin, an anaplerosis-stimulating compound causing the rapid increase of plasma C4- and C5-ketone bodies. These are precursors of propionyl-CoA that is then converted into succinyl-CoA.

In cybrids carrying a heteroplasmic mtDNA deletion, KD reduced the mutation load [71], and in the *deletor* mouse, a model carrying a mutant copy of the helicase Twinkle, it slowed the progression of the myopathic phenotype [72]. In contrast, KD worsened the mitochondrial defect in vivo in the *Mpv17*$^{-/-}$ mouse model [73].

A high-fat diet (HFD) had protective effects on fibroblasts with cI deficiency and delayed the neurological symptoms of the Harlequin mouse, which carries a homozygous mutation in *AIFM1*, encoding the mitochondrial apoptosis-inducing factor, and is characterized by cI deficiency [74].

Translation into the clinic

A modified Atkins KD was administered to five patients with mitochondrial myopathy and progressive external ophthalmoplegia with single or multiple deletions [75]. All of them developed rhabdomyolysis, with severe damage to muscle fibres, determining the termination of the study after only two weeks. Notably, two years later, the same patients showed an increase in muscle strength, suggesting that the damage induced by the diet had stimulated muscle repair by satellite cells, which, for unknown reasons, are virtually free from mtDNA deleted molecules.

Low glucose medium was shown to reduce the accumulation of cI-subassemblies and to increase respiration along with mitochondrial content [76], but the mechanistic details are not known.

Accordingly, triheptanoin treatment markedly improved the cardiomyopathy in VLCAD-deficient patients and the myopathy of CPT2 deficient patients [77,78].

Endurance training

Endurance training is meant to activate the core components of the mitochondrial biogenesis system, including PGC-1α and AMPK. On this ground, it was shown to delay ageing in mice [79,80], and to be beneficial in the mtDNA *mutator* mice, where it rescued the progeroid phenotype associated with this mouse model [79]. Intriguingly, the beneficial effects were not limited to skeletal muscle but also involved other organs, including the brain. However, other people have not been able to replicate this result.

Translation into the clinic

Exercise seems to be beneficial in some mitochondrial disease patients [81,82]. Aerobic endurance training increased mitochondrial mass through the stimulation of mitochondrial biogenesis, which may lead to increased mitochondrial enzyme activities and muscle strength.

In another trial on patients with mitochondrial DNA mutations, endurance training was beneficial and safe [80,81].

Overall, the data suggest that combination of progressive endurance with resistance exercise may be beneficial in mitochondrial patients [85].

Disease-tailored strategies

Supplementation of nucleotides

MtDNA instability is related to defects either in the machinery controlling mtDNA replication or in the enzymes involved in the de novo or salvage pathway for nucleotides biosynthesis [86], which cause imbalanced nucleotide pools. Clinically, the diseases related to mtDNA instability present as a spectrum of heterogeneous disorders, including severe infantile hepatocerebral, encephalopathy or myopathy disorders, childhood onset myopathy or adult onset PEO.

The supplementation of the missing or insufficient dNTP can bypass the biosynthetic block and restore the deoxynucleotides triphosphate (dNTP) pools.

Oral molecular bypass therapy with deoxypyrimidine monophosphates (dCMP and dTMP) or substrate enhancement therapy with deoxypyrimidine nucleosides (dC and dT) increased the levels of mtDNA copy number, as well as the mitochondrial RC activities in the thymidine kinase *Tk2* ^{H126N} mice. Thymidine kinase is the mitochondrial enzyme that phosphorylates pyrimidine deoxynucleosides into deoxynucleotides that can then be used for mtDNA synthesis. This treatment resulted in a dose-dependent prolongation of the lifespan [87,88]. However, the efficacy of this therapy in the long term has recently been questioned by using Tk2 KO mice, because of the progressively reduced bioavailability of dThd and dCtd after early postnatal life [89].

Similar approaches were used for other disease models. Deoxyguanosine improved mtDNA depletion in human fibroblasts with mutations in *DGUOK*, the gene encoding the mitochondrial deoxyguanosine kinase, which phosphorylates purines to the corresponding nucleotides in the mitochondrial nucleotides salvage pathway [90]. Supplementation with pyrimidine and purine nucleosides corrected ethidium bromide-induced mtDNA depletion in human fibroblasts carrying mutations in *RRM2B,* the P53-dependent subunit of riboside reductase, the enzyme converting ribonucleosides into deoxyribonucleosides. However, the corresponding monophosphate nucleotides did not correct mtDNA depletion in *RRM2B* deficient human myoblasts [91,92]. Finally, deoxycytidine or tetrahydrouridine corrected mtDNA depletion in a *Tymp/Upp1* double knockout mouse model of MNGIE disease. Similarly, deoxycytidine and tetrahydrouridine were also able to prevent mtDNA depletion in a cell model of the same syndrome [92].

Translation into the clinic

The results of an open-label study in which deoxynucleoside monophosphates and deoxynucleoside were administered for compassionate use to 16 TK2-deficient patients were recently reported. Children with severe disease presented a marked amelioration of the clinical conditions compared with the ominous natural history of the disease, including prolonged survival and improvement of motor abilities. In patients with later onset of the disease, clinical outcome measures were improved as well. Out of 8, 3 nonambulatory patients recovered the ability to walk. Out of 5, 4 patients with enteric nutrition discontinued the use of feeding tube. Out of 9, 1 patient who required mechanical ventilation became able to breathe independently. Diarrhoea was the most common side effect but did not require discontinuation of the therapy [93].

Supplementation of CoQ

Primary coenzyme Q deficiency, due to mutations in the genes encoding the enzymes of the CoQ biosynthetic pathway [94], is a striking example of the huge variability of mitochondrial diseases. The clinical outcomes include encephalomyopathy, multisystem disease, cerebellar ataxia, isolated myopathy and nephrotic syndrome [95].

Some 4-hydroxybenzoate B (HB) analogues have been proposed as potential bypass molecules with higher bioavailability than CoQ. These water-soluble CoQ head precursors would bypass enzymatic steps disrupted by mutations in *COQ* genes, but their efficacy may differ depending on the stability of the CoQ biosynthetic complex. Recent examples are vanillic acid (VA) and 3,4-dihydroxybenzoate (3,4-dHB), which are able to bypass *COQ6* and *COQ9* mutations [96], or 2,4-dHB for *COQ7* defects [97]).

Translation into the clinic

CoQ supplementation at high doses is effective in treating both primary and secondary CoQ deficiencies [98]. However, for unknown reasons only 20% of the patients responded to CoQ_{10} supplementation [99]. Obstacles to tissutal CoQ_{10} delivery may be caused by its high molecular weight and high hydrophobicity, but at high doses, dietary supplementation increases CoQ_{10} levels in all tissues, including heart and brain, especially with certain formulations [10,101]. In addition, studies in cellular models suggest that the slow pharmacokinetics of CoQ_{10} can be a reason for the variable responses observed in patients, but additional investigation is needed to clarify this issue.

AAV-based gene therapy

Gene therapy consists in the expression of genes either as wild-type form of a missing or mutated gene or of other therapeutic genes (e.g. alternative oxidases and reductases) by using appropriate cellular or viral vectors. Adeno-associated viral vectors (AAVs) are currently at the forefront of human gene therapy because of the favourable

safety profile and the availability of several tissue-specific serotypes (Figure 4). However, the limited cloning capacity and the intrinsic difficulty in achieving therapeutic expression levels in several tissues limit their applicability to disease due to mutations in small genes and possibly involving a single organ.

In the context of mitochondrial diseases, AAVs have been used to deliver therapeutic genes in several mouse models of mitochondrial disorders, including the models for ADOA [12], MNGIE [13], EE [14] and Leigh syndrome [15].

Although MNGIE and EE are very different diseases, they are both characterized by accumulation of high levels of toxic compounds, pyrimidine nucleotides for the first, hydrogen sulphide for the latter [16]. Hepatotropic AAV8 vectors were used in the mouse models of both diseases to re-express the missing gene in the liver, the main filtering organ in the body, leading to scavenging of the toxic compound from the bloodstream and peripheral organs. This approach triggered the idea that a similar effect could be achieved in EE [17] and MNGIE [18] patients by using liver transplant, thus bypassing all the regulations needed to implement a clinical trial with AAVs. It should also be noted that pre-existing liver disease, as occasionally observed in MNGIE [18] and other diseases, may prevent the use of AAVs, since cellular damage may interfere with viral entry into the cells. Notably, liver transplant has also been used as a therapy for other mitochondrial diseases with hepatopathy, such as Alpers' disease, at early stages [19,110], although the neurological derangement was not prevented.

We recently showed that the phenotype of *Ndufs4*$^{-/-}$ mice could be partially rescued by human *NDUFS4* only when the AAV9 vector was simultaneously administered systemically and intracranially. This highlights the potential, but also the challenges of targeting several organs in multisystem disorders, and the brain in particular, which is protected by the blood–brain barrier (BBB) [15]. In fact, the AAV9 serotype did not efficiently cross the BBB and mainly targeted glial cells when injected intracranially in newborn mice. Interestingly, new engineered serotypes, such as PHP.B, raised great hopes because of their extremely high efficiency in crossing the BBB [111]. Unfortunately, PHP.B is of limited usefulness for translation into clinics because of its liver toxicity observed in nonhuman primates and its reliance for crossing the BBB on the presence of Ly6a, a glycosylphosphatidylinositol (GPI) protein, which is absent in primates [112]. However, preliminary results from our laboratory on the use of PHP.B on *Ndufs4*$^{-/-}$ mice confirm the potential benefits of brain-tailored AAV-based gene therapy for mitochondrial, and potentially for other, diseases (Viscomi et al, unpublished) and indicate that future efforts should be put in developing new tools for this goal.

AAV can also be used to deliver other therapeutic genes to the affected organs. The most important example on this matter is the use of molecular scissors (i.e. dimeric endonucleases such as *Fok*I) to selectively destroy mutated mtDNA, preserving wild-type molecules. Both mitochondrial targeted (mt)TALENs and mtZFNs were shown to be able to reduce the heteroplasmic load in several cellular models with mutations in mtDNA [113-115], and in vivo in the same mouse models carrying a heteroplasmic mutation in the MT-tRNA-Ala gene [116,117].

It is worth noting here that the CRISPR/Cas9 system, which has great potential for the manipulation of the nuclear genome, cannot be applied to mtDNA due the impossibility of importing RNAs (such as the guided RNAs which are integral components of the Cas9 nucleoprotein) into mammalian mitochondria [118].

Translation into the clinic

There are several AAV-based clinical trials registered for LHON disease and one has been completed using allotopic expression of ND4, that is expression of mtDNA-encoded protein from the nucleus. Although there is weak evidence that allotopically expressed mtDNA-encoded subunits can enter into mitochondria and can be inserted into respiratory complexes, Guy et al. reported amelioration of visual acuity in the injected eyes [119]. An alternative explanation to this clinical effect could be the presence of a secondary mutation in mtDNA and a spontaneous recovery that is often observed especially in the m.11778G> A mutation [120].

Another study using a single dose ($5x10^9$ vg/0.05 mL) of rAAV2-ND4 on 9 patients (NTC01267422) [121] reported no adverse effects. In 6/9 patients, visual acuity improved, and visual field was enlarged nine months after treatment, whilst other parameters were unchanged.

An open-label phase I/II clinical trial (NCT02064569) investigated both safety and preliminary efficacy of a rAAV2/2-*ND4* in 4 dose-escalation cohorts (9×10^9, 3×10^{10}, 9×10^{10}, 1.8×10^{11} vector genomes/eye). The treatment proved to be overall safe with only mild to moderate adverse effects, such as increase in ocular pressure, ocular pain and vitritis. A clinically relevant improvement in the best corrected visual acuity (i.e. the best vision achievable with the help of correction) was observed in the treated eyes in 6/14 patients. In parallel, a between-eye difference in the change of visual acuity from the baseline was observed in a subset of patients with disease duration of less than 2 years. A phase 3 study is currently ongoing on patients with vision loss for less than 6 months and between 7 months and 1 year (NCT03406104). So far the results of this study have been only disclosed on the sponsor website (https://www.businesswire.com/news/home/20180619006555/en/GenSight-Biologics-Key-Opinion-Leaders-Highlight-GS010), who reported a preservation of the retinal ganglion cell macular volume and nerve thickness and an improvement in contrast sensitivity of the treated vs. sham-treated eyes. No difference was observed in high-contrast visual acuity. According to the sponsor, the intervention was more effective on young patients with vision loss duration of less than 9 months. These results are promising but await confirmation through a peer-reviewed publication.

In spite of the difficulties related to the extremely high production costs and regulatory requirements, new stamina for the AAV-based gene therapy has been triggered by the positive results of a clinical trial on spinal muscular atrophy using AAV9, showing remarkable amelioration of the treated patients compared with the natural history of the disease. It remains anyway challenging to address the safety concerns and comply with all the regulations as every single vector has to be treated as a new therapeutic agent.

Conclusions

Mitochondrial diseases raise very peculiar challenges to the development of effective therapies. A first challenge is the extreme heterogeneity of these diseases, partly due to the huge number of genes, each involved in specific diseases, partly to the intrinsic variability of mitochondrial genetics, in which the same mutation in mtDNA can lead to different phenotypes, even within the same family. A second problem is that mitochondrial diseases often present as multisystem disorders affecting several organs and correcting the defect in all of them is still challenging and may imply several complementary approaches.

Third, the brain, which is often affected in mitochondrial disorders, is protected by the blood–brain barrier, which prevents most of the drugs from reaching this organ. This is particularly important for the development of AAV-based gene therapy approaches. As new serotypes become available, new hopes and, as we saw, new challenges arise.

Finally, a defect in ATP production is often the primary consequence of mitochondrial dysfunction, but, in many cases, this is accompanied by other consequences. Experimental work over the last 10 years has shown that several of these consequences can be at least partially corrected by pharmacological interventions, but it is difficult to anticipate a single intervention able to rescue all of them.

We think that a radical cure for these diseases will probably come only from gene therapy. The results obtained on other diseases, such as spinal-muscle atrophy [121], are very encouraging from this point of view. Mitochondrial gene therapy poses additional problems, including the fact that (i) mitochondria are surrounded by a double membrane, (ii) mitochondrial diseases are often multisystemic, making it difficult to reach the high titres required to target a majority of cells in all the affected organs, (iii) mammalian mitochondrial DNA has no recombination systems, preventing the use of homologous recombination-based approaches, (iv) mitochondria cannot import nucleic acids, preventing the use of CRISPR/Cas9-based techniques, and (v) last but not least, mtDNA is polyplasmic, that is present in multiple copies (up to 200000 in mature oocytes) instead of the diploid or haploid organization of nuclear genes, which implies an extremely high number of genomes to be targeted in each cell to produce a tangible effect. In spite of these problems, the recent development of approaches to shift mtDNA heteroplasmy levels using ZFNs or TALENs opened great therapeutic opportunities. However, a regulatory simplification is needed in order to move towards the clinical application. Under the current regulations by EMA and/or FDA, each single ZFN or TALEN, as well as each AAV-targeted therapeutic nuclear gene, is

considered as a new drug and would require a number of safety and toxicological assessments, with extremely high costs.

A final cure for mitochondrial diseases has still to come, but the exciting progresses in the field during the last decade give the realistic hope that a solution will be available in the near future.

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Conflict of interest statement

No conflicts of interest to declare.

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Authors contribution

CV and MZ co-authored this manuscript.

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