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Literature Review

Potential Therapies for Mitochondrial Diseases Caused by mtDNA Mutations

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Abstract: Mitochondrial diseases, caused by mutations in mitochondrial DNA (mtDNA), present significant genetic and clinical complexities that challenge accurate diagnosis and treatment. This review examines the critical molecular mechanisms underlying these disorders, particularly the role of heteroplasmy, where mixtures of mutated and normal mtDNA influence disease severity, and the nuclear-mitochondrial DNA interactions that compound diagnostic and therapeutic challenges. The threshold effect, wherein clinical symptoms manifest only when mutant mtDNA exceeds a critical proportion, exemplifies the complexity of genotype-phenotype correlations in these conditions. We analyze recent advancements in therapeutic strategies, including targeted small molecule treatments designed to enhance mitochondrial function and restore Nicotinamide Adenine Dinucleotide (NAD+) levels, which have demonstrated efficacy in preclinical models. Additionally, we explore emerging gene therapy approaches that aim to directly correct mtDNA mutations, representing innovative strategies with potential for definitive treatment. This review provides comprehensive insights into the interplay between genetic mechanisms and therapeutic innovations, emphasizing the critical need for translational research. By elucidating the molecular mechanisms driving mitochondrial diseases, we aim to inform development of targeted therapies and improve clinical outcomes for affected patients.

Keywords: Mitochondrial Diseases; mtDNA Mutations; Heteroplasmy; Gene Therapy; NAD+ Metabolism; Mitochondrial Function; Therapeutic Strategies; Nuclear-Mitochondrial Interactions

Introduction

Mitochondria are vital organelles enclosed by double membranes and found in every cell containing a nucleus in mammals. They play a key role in enabling aerobic respiration and are the main producers of Adenosine Triphosphate (ATP) through the process of oxidative phosphorylation (OXPHOS). The OXPHOS system consists of over 80 different proteins organized into five transmembrane complexes (Rossmann *et al*., 2021). Electrons produced through the oxidation of fats and carbohydrates are transported to oxygen via the first four complexes (CI–CIV) of the Respiratory Chain (RC), leading to the formation of water. This process generates

the energy required to move protons across the Inner Membrane (IMM), from the matrix into the intermembrane space (Ahmad *et al*., 2023). The resulting electrochemical gradient allows the fifth RC complex, ATP synthase, to add a phosphate group to ADP, resulting in the formation of ATP (Jonckheere *et al*., 2012).

Beyond their role in OXPHOS, mitochondria also regulate calcium concentrations in the cytosol and play a crucial role in the regulation of programmed cell death (apoptosis). They are a major source of Reactive Oxygen Species (ROS) within cells and facilitate several vital biochemical pathways, including the Tricarboxylic Acid (TCA) cycle and aspects of the urea cycle. Additionally, mitochondria are pivotal in the formation of iron–sulfur clusters, representing the only function preserved across all eukaryotic mitochondria (Nguyen *et al*., 2023; Moon, 2023).

In the past two decades, the role of mitochondrial dysfunction in contributing to various neuromuscular and neurodegenerative diseases has gained increasing recognition. Since mitochondria are regulated by genetic information from both mitochondrial and nuclear DNA, mutations in either can lead to deficiencies in the RC (Johri and Beal, 2012). This review will primarily focus on the role of primary mutations in mitochondrial DNA (mtDNA) in human diseases, while literature on defects in nuclear genes related to mitochondrial disorders is also available for consultation. The article will begin with an overview of the unique aspects of mitochondrial genetics, followed by an examination of the clinical syndromes that arise from mtDNA mutations. It will then explore the current understanding of how mitochondrial dysfunction leads to disease at the molecular level. Lastly, the review will highlight the latest progress in developing potential treatments for these conditions.

Mitochondrial DNA Mutations

As stated in the Introduction, a single cell contains many mitochondria, with each mitochondrion housing multiple copies of its genome. Additionally, the mitochondrial genome mutates at a rate approximately 100 times higher than that of the nuclear genome. This high mutation rate results in a diverse mix of mitochondrial DNA within the same cell and even within individual mitochondria, leading to a condition known as heteroplasmy (Diroma *et al*., 2020; Guo *et al*., 2023). During cell division, mitochondria are partially distributed to the daughter cells; however, this distribution is random and lacks the precision seen in the segregation of nuclear chromosomes during mitosis. Consequently, the mitochondrial DNA passed to daughter cells is similar but not identical (Mishra and Chan, 2014).

What causes such a high mutation rate in mitochondria? The enzyme responsible for copying mitochondrial DNA, DNA Polymerase Gamma (POLG), is produced by a nuclear gene. The POLG protein consists of a catalytic domain, which carries out the polymerase function, and an exonuclease domain, which identifies and corrects errors in the DNA sequence during replication. Recent research indicates that an imbalance in nucleotides within mitochondria could lead to reduced accuracy of POLG, resulting in an increase in mutations in mitochondrial DNA (Rahman and Copeland, 2019; Lee *et al*., 2009).

Based on the gathered data, it was discovered that the levels of mitochondrial deoxynucleotide Triphosphates (dNTPs) varied greatly, with dGTP being the most predominant nucleotide in mitochondria across most tissues. The research subjects tissues collected from both young and old rats were tested for levels of mitochondrial free dNTPs, which are essential components for new DNA strands during the replication process (Gandhi and Samuels, 2011; López *et al*., 2009). However, no significant differences were observed in the nucleotide levels between tissues from young and old rats. For instance, in both heart and skeletal muscles, dGTP accounted for 85 to 91% of the mitochondrial nucleotide pool, while dTTP made up only 0.5%. In the brain, dGTP constituted 62% of the mitochondrial nucleotide pool, in contrast to dTTP, which comprised just 4%. In the liver, the composition was 37 dGTP, 51 dCTP, 9 dATP, and 3% dTTP. These findings highlight the significant abundance of dGTP in the mitochondria compared to its levels in the rest of the cell; for example, in whole rat embryos, dGTP represents only 10% of the free nucleotide pool (Ylikallio *et al*., 2010; Song *et al*., 2005).

The theory suggests that altering the balance of dNTP levels might lead to increased errors by mitochondrial DNA polymerase during replication. To investigate this theory, a series of experiments were conducted to assess whether the accuracy of POLG, the mitochondrial DNA polymerase, was affected under conditions of unequal dNTP levels (Dalla *et al*., 2016). Human POLG, in both its proofreading (Exo+) and non-proofreading (Exo-) forms, was utilized to fill a DNA gap in the lacZ gene, using either balanced dNTP levels or dNTP concentrations that resemble those found in mitochondria. The integrity of the lacZ gene repair was then evaluated by introducing the repaired gene into bacterial cells and observing the color of the colonies formed (Park *et al*., 2023). Blue colonies indicated that the lacZ gene was correctly repaired, representing the wild-type sequence, while light blue or white colonies indicated the presence of mutations in the repaired lacZ gene segment (Gupta *et al*., 2013).

The DNA sequences of individual lacZ clones were analyzed to identify the specific mutations present. When

Exo- POLG was supplied with dNTP pools that replicate those found in the mitochondria of different muscle types, including Interfibrillar Heart Muscle (IFM), Subsarcolemmal heart Muscle (SSM), and Skeletal Muscle (SM), there was an observed increase in lacZ mutation frequencies by two to six times (Stumpf and Copeland, 2011). A significant finding from the DNA sequence analysis of the lacZ templates was a 30-fold increase in the occurrence of T to C substitutions. Additionally, when Exo + POLG was exposed to the same dNTP pools, there was a notable four- to eight-fold increase in T to C substitutions. These findings indicate that elevated levels of dGTP adversely affect the accuracy of DNA replication by both Exo- and Exo + POLG. Thus, the imbalance in the mitochondrial dNTP pool is suggested to be a significant factor contributing to the high rate of mitochondrial DNA mutations (Mertz *et al*., 2015; Schmidt *et al*., 2017).

Definite Features of Mitochondrial Disease

The appearance of clinical symptoms, the range of physical manifestations, and the inconsistent expression of mitochondrial diseases are influenced by several factors, including the threshold effect, the distribution of mitochondria during cell division, the selective growth of certain cell populations, and a genetic bottleneck (Bakare *et al*., 2021; Wallace and Chalkia, 2013).

Threshold Effect

Some detrimental mutations in mtDNA are present in all copies of the genome, known as homoplasmic mutations, while most are heteroplasmic, meaning they occur in only a fraction of the mtDNA copies. In cases of heteroplasmy, the severity and onset of disease symptoms are influenced by the ratio of wild-type to mutant mtDNA (Taylor and Turnbull, 2005). A certain critical level of mutated mtDNA is required before biochemical abnormalities and tissue dysfunction manifest. This critical threshold varies depending on the specific mutation and the tissue involved, with tissues that rely heavily on OXPHOS having a lower threshold compared to those that can utilize anaerobic glycolysis. Commonly, this threshold falls between 60% and 90% mutant mtDNA relative to wild-type (Thangaraj *et al*., 2015; Chinnery, 2000). While this threshold concept helps in understanding the variability in disease presentation among patients, a direct and precise link between the proportion of mtDNA mutations and clinical severity is not consistently observed.

Distribution of Mitochondria During Cell Division

During the process of mitosis, mitochondria are distributed randomly. In cells with heteroplasmy, this can lead to changes in the ratio of mutant mtDNA in the resulting daughter cells. If the level of mutant mtDNA surpasses the disease-causing threshold specific to that tissue, symptoms may manifest. Conversely, mutant mtDNA can completely disappear, especially in tissues that undergo rapid division. For instance, there has been an observation of a yearly decrease of 1% in the levels of the m.3243A>G mutation in blood (Nissanka and Moraes, 2020; Burr and Chinnery, 2024).

Selective growth of certain cell populations

Clonal expansion describes the process by which mutations in mtDNA are selectively amplified to a significant extent within tissues that do not undergo cell division. This phenomenon is believed to occur due to random genetic fluctuations, influenced by the less stringent replication mechanisms of the mitochondrial genome (Lawless *et al*., 2020; Kowald and Kirkwood, 2018).

Genetic Bottleneck

The observation that mammalian heteroplasmic mtDNA genotypes can rapidly segregate across generations, leading to homoplasmy in some descendants, has prompted the proposal of an mtDNA bottleneck during development. The specific mechanism behind this genetic bottleneck is currently a subject of intense debate (Wallace and Chalkia, 2013; Wai *et al*., 2008). A widely accepted theory suggests that the bottleneck occurs during embryonic development due to a significant decrease in the number of mtDNA copies in the germ line. Interestingly, there is also a theory suggesting that the bottleneck does not involve a reduction in germ line mtDNA copy number but instead arises from the selective replication of certain mtDNA subgroups during oogenesis (Cao *et al*., 2009). Recently, another perspective has emerged, proposing that the genetic bottleneck might occur during postnatal folliculogenesis rather than during embryonic oogenesis. This theory is based on observations of variations in mtDNA heteroplasmy and copy number in single germ cells throughout oogenesis, indicating the replication of a subset of mitochondrial genomes. Further research is needed to definitively determine the nature of the mtDNA bottleneck (Johnston, 2019).

Symptoms

Common symptoms of mitochondrial diseases can vary depending on the specific type and the location of the affected cells. These symptoms can differ in severity, ranging from mild to severe, and may include (Hanaford and Johnson, 2022):

- Poor growth

- Muscle weakness, muscle pain, or low muscle tone
- Vision and/or hearing loss
- Developmental delays or cognitive issues
- Diarrhea or constipation
- Unexplained vomiting
- Acid reflux and/or difficulty swallowing
- Seizures
- Migraines
- Respiratory problems
- Fainting

Signs of mitochondrial diseases may be evident from birth or may emerge at any point in life. It is not uncommon for medical professionals to identify symptoms that impact multiple organs or organ systems simultaneously. The manifestations of the same condition can vary widely among individuals, including within the same family (Aldossary *et al*., 2022).

Bond Between Nuclear DNA and mtDNA in Mitochondrial Disease

Mitochondrial disorders can arise from mutations and variations in both mtDNA and nuclear DNA (nDNA). The majority of mitochondrial proteins, around 1,500, including those involved in oxidative phosphorylation (OXPHOS), the TIM/TOM complexes, nucleoid proteins, matrix proteins, and channel proteins, as well as those that regulate processes like mitophagy, mitogenesis, and mitochondrial dynamics (fusion and fission), are encoded by nuclear genes (Ryzhkova *et al*., 2018; Slone and Huang, 2020). Mutations in nuclear DNA can lead to instability in the mitochondrial genome, manifesting as large deletions or point mutations in mtDNA. For instance, the presence of nuclear genome polymorphisms rs6493454 and rs7182946 at the TRPM1 locus on chromosome 15, in combination with the mitochondrial genome mutation m.4917A>G in the MT-ND2 gene, has been linked to an increased risk of developing age-related macular degeneration (AMD) (Heuer, 2023). A similar increase in risk was noted when the mtDNA mutation m.12771G>A in the MT-ND5 gene occurred alongside nuclear DNA polymorphisms rs4932478, rs4932480, rs11459118, rs875390, rs875391, rs2351006, rs144871045, and rs2070780 at the ABHD2/RLBP1 loci on chromosome 15. Recent studies have demonstrated that the risk of deafness is heightened by the combination of the nuclear modifier allele A10S in the TRMU gene with the mitochondrial genome mutation m.1555A>G in the MT-RNR1 gene (Persad *et al*., 2017).

It is important to highlight that there are currently very few studies exploring the impact of combined mutations in the mitochondrial and nuclear genomes on the incidence and progression of diseases.

Difficulties in the Treatment of Mitochondrial Diseases

Mitochondrial disorders rank among the most prevalent genetically inherited metabolic conditions. The primary mechanism for ATP production in cells, oxidative phosphorylation (OXPHOS), occurs on the inner membrane of mitochondria. Five key complexes are directly involved in OXPHOS, with three of them (complexes I, III, and IV) transporting protons into the space between membranes, while complex V utilizes the resulting electrochemical gradient to convert ADP and inorganic Phosphate (Pi) into ATP. Nonetheless, OXPHOS relies on numerous additional pathways for optimal function, making it susceptible to a wide array of genetic abnormalities (Zhao *et al*., 2022; Clemente-Suárez *et al*., 2023).

Mitochondrial disorders represent a unique category of human genetic diseases that involve two distinct genomes. Mitochondrial DNA (mtDNA), a compact circular genome, is responsible for producing 13 proteins integral to the OXPHOS system, along with 22 mt-tRNAs and 2 mt-rRNAs (Rossmann *et al*., 2021; Smits *et al*., 2010). Nevertheless, over 1,200 mitochondrial proteins are encoded by nuclear DNA and subsequently imported into the mitochondria. While not every nuclear-encoded mitochondrial protein plays a role in OXPHOS, a significant number contribute to the assembly of OXPHOS complexes, replication, expression, and repair of mtDNA, as well as various other metabolic processes (Walker and Moraes, 2022).

The genetics of mtDNA is intricate because each cell contains multiple copies of mtDNA. Mutations in mtDNA can be either homoplasmic, affecting nearly all mtDNA copies, or heteroplasmic, where a mix of mutated and normal mtDNA exists. These mutations are functionally recessive, meaning that a biochemical phenotype becomes apparent only when the mutated mtDNA exceeds a certain threshold (Filograna *et al*., 2021). Numerous large-scale deletions and point mutations in mtDNA have been identified. The threshold at which a biochemical deficiency occurs varies depending on the specific mutation and the type of cell, and can even differ for the same mutation among different individuals. Typically, a pathogenic mutation must reach a high level of heteroplasmy (over 70%) before a harmful biochemical phenotype manifests at the level of a single cell (Bernardino *et al*., 2021; Alston *et al*., 2017). The clinical manifestations can vary significantly among patients with the same mtDNA mutation, even when levels of heteroplasmy are similar. An example of this variability is seen in the disease caused by the m.3243A > G mutation, which shows significant phenotypic variability, likely due to the influence of nuclear genetic factors on

disease expression (Ryytty and Hämäläinen, 2023; Li *et al*., 2022).

Until recently, pinpointing a genetic diagnosis for many patients, particularly those with mutations in nuclear genes, proved difficult. However, the emergence of readily available next-generation sequencing has led to a surge in identifying novel genetic abnormalities impacting OXPHOS, with pathogenic mutations in more than 300 genes discovered so far (Ng and Turnbull, 2016; Stenton and Prokisch**,** 2020). Recognizing specific genetic anomalies is crucial because it sheds light on the disease mechanisms affecting individual patients and could reveal the importance of tailored treatments that might not have been previously considered (Palmieri *et al*., 2023).

Estimating the frequency of mitochondrial disease has been challenging due to its diverse clinical manifestations, variability in how genetic changes manifest as symptoms, and the complexity of the healthcare referral process. Research indicates that nuclear genetic abnormalities are primarily responsible for mitochondrial diseases in children, while mutations in mtDNA are more common in adults (Gorman *et al*., 2015; Saneto, 2020). Prevalence rates for mitochondrial disease in children under 16 years old range from 5 to 15 cases per 100,000 people. In adults, the prevalence of mtDNA-related disease is about 9.6 per 100,000, with an additional 10.8 per 100,000 individuals at risk due to having a first-degree relative with the condition. For diseases caused by nuclear genetic issues, the prevalence is lower, at 2.9 per 100,000 (Riquin *et al*., 2020).

One of the principal challenges in treating mitochondrial disease stems from the complex relationship between a patient's genetic makeup and the manifestation of the disease, which varies widely. This variability is evident in the range of organs affected and the severity of the disease (Alston *et al*., 2017; Aldossary *et al*., 2022). For instance, children can suffer from a severe neurodegenerative disorder known as Leigh's syndrome (or subacute necrotizing encephalomyelopathy), yet the outlook for those with this syndrome can differ greatly based on the specific genetic mutation they have. In adults, the disease often presents less severely, with symptoms that may include chronic progressive external ophthalmoplegia, hearing loss, and diabetes. However, some adults may experience severe, progressive symptoms such as seizures and stroke-like episodes that lead to a dementia syndrome (Saneto and Ruhoy, 2014).

MtDNA Mutations and Therapy of Cytopathies

The molecular and cellular processes underlying the origin and progression of mitochondrial cytopathies remain inadequately understood and require further research. Consequently, the management of mitochondrial disorders is currently limited to symptomatic care, supplementation with cofactors, precursors to nitric oxide, and physical activity (Almannai and El-Hattab, 2021; Bottani *et al*., 2020).

Mutations in the mitochondrial genome can serve as a basis for developing models aimed at exploring the molecular and cellular mechanisms underlying cytopathies. These models have been created to study conditions such as MELAS, LHON, LS, and MERRF (Burr and Chinnery, 2024; Khotina *et al*., 2023).

Moreover, examining how mutations in the mitochondrial genome contribute to cytopathies facilitates the implementation of tailored medical treatments for patients. This approach involves selecting specific medications that target the mutations present in an individual's mtDNA. For instance, researchers are developing targeted treatments for MELAS syndrome in patients with the m.3260A>G mutation in the MT-TL1 gene (Lax *et al*., 2011; Hirano *et al*., 2018). This mutation is thought to cause mitochondrial dysfunction and a decrease in cellular energy production. Treatments for MELAS have included a ketogenic diet and magnesium supplementation, both of which have shown potential in enhancing the function of the respiratory chain complexes.

In Germany, a team attempted to treat a patient with the m.11778G>A mutation, associated with Leber's Hereditary Optic Neuropathy (LHON) and multiple sclerosis. The patient underwent immunosuppressive therapy with mitoxantrone and experienced an improvement in condition after 12 months (Koňaříková *et al*., 2020; Argudo *et al*., 2022).

The latest approaches to treating mitochondrial cytopathies involve the development of gene therapy. Several strategies within gene therapy can be employed, including shifting or reducing mtDNA levels to decrease heteroplasmy, introducing normal mtDNA polypeptides directly into the mitochondria, and modifying mtDNA itself. Additionally, there is growing interest in employing donor mitochondria during fertilization as a preventative measure against mitochondrial disorders inherited from the mother (Di Donfrancesco *et al*., 2022; Soldatov *et al*., 2022). Advanced studies have demonstrated that the integration of donor mitochondria with the recipient cell's nucleus does not disrupt normal transcriptomic and energetic functions. Furthermore, the targeted treatment of mitochondrial diseases may also be facilitated through the transfer of mtDNA from one cell to another via nanotubes (Liu *et al*., 2022).

Possible Treatments

Development of Small-Molecule Therapies

The pursuit of small molecules plays a crucial role in developing treatments for mitochondrial diseases. Recently, there has been growing interest in this field, with numerous biotech firms focusing on finding or enhancing mitochondrial therapies. This surge in activity is largely due to new regulatory and financial incentives that encourage investment in the discovery of orphan drugs for these conditions (Meng and Wu, 2023). Although mitochondrial diseases are relatively uncommon compared to other neurological conditions, targeting mitochondrial function could significantly slow the progression of several diseases, including Parkinson's disease, Alzheimer's disease, Amyotrophic Lateral Sclerosis (ALS), Huntington's disease, and Friedrich's ataxia (Johri and Beal, 2012). Metabolic conditions such as obesity, diabetes, and non-alcoholic fatty liver disease may also benefit from mitochondrial-targeted treatments. While our discussion centers on small molecules aimed at treating primary mitochondrial diseases, their potential applications extend beyond these conditions and should be viewed within the broader context of disorders involving mitochondrial dysfunction (Legaki *et al*., 2022; Zheng *et al*., 2023).

Wielding Cell Content of Mitochondria

The idea that enhancing mitochondrial mass in cells or animals with mitochondrial dysfunction could be beneficial has been frequently discussed. The question is: how can such an enhancement be realized? Two strategies emerge: first, by boosting mitochondrial biogenesis, and second, by decreasing mitochondrial turnover, also known as mitophagy. Over the last fifteen years, significant progress has been made in identifying the main regulators of mitochondrial biogenesis (Daussin *et al*., 2021). Key among these are the Peroxisome Proliferator-Activated Receptor (PPAR) family, a group of fatty acid-regulated nuclear receptors, and PPAR-gamma coactivator 1-alpha (PGC1α), which is a transcriptional co-activator for one of the PPAR isoforms and several other nuclear receptors. These factors have been recognized as crucial drivers of mitochondrial biogenesis, triggering the expression of additional factors essential for mitochondrial function. As a result, bezafibrate, a drug commonly used to treat high blood lipid levels and a pan-PPAR agonist, has been explored as a treatment for mitochondrial myopathy and in models of mitochondrial dysfunction (Christofides *et al*., 2021; Tyagi etr al., 2011). However, in mouse models of Cytochrome C Oxidase (COX) deficiency characterized by late-onset multiple mtDNA deletions, bezafibrate did not induce mitochondrial biogenesis. Curiously, in a mouse model with a mutant mtDNA helicase (known as the Deletor mouse), there was a significant delay in the accumulation of mtDNA deletions and improvements in age-related skin and spleen conditions. Despite the mixed outcomes and the retraction of several key studies, the potential of bezafibrate in treating mitochondrial diseases warrants further consideration (Yatsuga and Suomalainen, 2012; Viscomi *et al*., 2011).

Elevated levels of PGC1α have been found to trigger an increase in mitochondrial numbers and enhance COX activity within the skeletal muscles of Surf1−/− mice, which lack proper assembly of cytochrome c oxidase. Additionally, this overexpression has been observed to bolster the function of skeletal muscles and the heart in the mtDNA mutator mouse model (Pulliam *et al*., 2014). The control mechanisms governing PGC1α are notably intricate, involving various post-translational modifications such as phosphorylation and acetylation at multiple sites, as well as ubiquitination and methylation. These complexities render PGC1α a challenging target for drug development. Key to the regulation of PGC1α are upstream activators such as AMP-Activated Protein Kinase (AMPK) and sirtuin 1 (SIRT1), which themselves are regulated by the balance of adenine nucleotides or NAD+/NADH in the cytosol (Abu Shelbayeh *et al*., 2023; Luo *et al*., 2019).

For instance, activating AMPK has been linked to enhanced metabolism in skeletal muscles, potentially through the promotion of mitochondrial biogenesis. Catalysts like 5-Aminoimidazole-4-Carboxamide Ribonucleotide (AICAR), which can also activate SIRT1, are being explored as potential treatments for mitochondrial diseases. However, AICAR is banned by the World Anti-Doping Agency (WADA) due to its possible performance-enhancing properties (Hinkle *et al*., 2022; Moore *et al*., 2020). Metformin, another compound thought to activate AMPK by slightly inhibiting complex I or mitochondrial glycerophosphate dehydrogenase, has been considered, but lacks unanimous support for its role in mitochondrial biogenesis. Resveratrol, a polyphenol found in plants, has been identified as an activator of both AMPK and SIRT1. Its effectiveness is still being evaluated and tested (Hur and Lee**,** 2015; Wang *et al*., 2019).

Within the regulatory network that encourages the growth of mitochondria, the nuclear respiratory factors NRF1 and NRF2 play crucial roles. They are either upregulated or translocated to the nucleus, where they influence the expression of a wide array of genes. Moreover, these factors are integral to various protective mechanisms within cells. Small molecules that can activate NRF2 by inhibiting its degradation, potentially enhancing mitochondrial production, have already been discovered (Hu *et al*., 2022; Dinkova-Kostova and Abramov, 2015). A recent phase II study, which was randomized, double-blind, and placebo-controlled, tested

the effectiveness of a 12-week treatment with a specific molecule, the synthetic triterpenoid omaveloxolone (RTA 408), in patients with mitochondrial myopathy. Unfortunately, no improvements were found in the primary outcome measure (peak exercise workload) or in the secondary outcome (6-minute walk test) after 12 weeks (Madsen *et al*., 2020).

In conclusion, the evidence strongly suggests that enhancing mitochondrial biogenesis could improve mitochondrial function in patients. However, there is currently scant proof that any specific small molecule can effectively serve as a treatment for boosting mitochondrial numbers. The most convincing strategies for augmenting mitochondrial volume are exercise and endurance training, which have been shown to be safe based on various systemic health indicators and seem to pose no risk. Therefore, this field holds promise for yielding significant new treatments in the foreseeable future (Uittenbogaard and Chiaramello, 2014; Meng and Wu, 2023).

Recovering NAD + Levels

Cells with defective mitochondria experience a significant drop in NAD + levels and a decrease in the NAD + /NADH ratio. NAD + is crucial for the activity of important proteins such as poly (ADP-ribose) polymerase (PARP), cyclic ADP ribose synthetases, and sirtuin deacetylases, which play multiple vital roles within the cell. To offset the reduced NAD+ levels, pyruvate, produced through glycolysis and accumulating due to diminished consumption by the faulty respiratory chain, is converted to lactate (Hopp *et al*., 2019; Zapata-Pérez *et al*., 2021). This conversion involves the oxidation of NADH by lactate dehydrogenase. The resulting excess lactate is then expelled from the cell via the monocarboxylate transporter, leading to lactic acidosis, a typical sign of mitochondrial disease. For instance, SIRT1 activates PGC1α through NAD+-dependent deacetylation, promoting mitochondrial biogenesis. Enhancing cellular NAD+ levels, either by supplementing with precursors for its synthesis or by modifying enzymes involved in its production, has emerged as a critical approach for treating not only mitochondrial diseases but also a variety of other conditions, including aging (Kane, 2014).

Nicotinamide Riboside (NR) has shown promise in treating mitochondrial disease in the Deletor mouse model, increasing mitochondrial biogenesis in skeletal muscle and normalizing mitochondrial shape, although very high doses were required. NR has also been found to enhance motor function and upregulate OXPHOS-related gene expression in a ScoKO/KI mouse model with compromised COX function, a benefit similarly observed with pan-PARP inhibition (Lapatto *et al*., 2023). Nicotinamide Mononucleotide (NMN) improved lactic acidosis and extended lifespan in Ndufs4 KO mice, significantly raising NAD+ and NADH levels in skeletal muscle, though it did not affect brain tissue levels. Acipimox, a niacin derivative previously used for dyslipidemia, has been shown to increase NAD + levels in murine C2C12 myotubes and boost mitochondrial respiration in human skeletal muscle ex vivo. A clinical trial is underway to explore whether acipimox can alleviate the severe muscle symptoms in patients with mitochondrial disease (Lee *et al*., 2019; Wang *et al*., 2022).

A novel and intriguing method has been developed for enhancing NAD + levels. Among the pathways for synthesizing NAD+ from scratch, one involves breaking down L-tryptophan through the kynurenine pathway. This process produces 2-Amino-3-Carboxymuconate 6-Semialdehyde (ACMS), which can undergo decarboxylation by the enzyme ACMS Decarboxylase (ACMSD). Without this enzyme's action, ACMS spontaneously converts to quinolinic acid, which can then be transformed into NAD + (Castro-Portuguez and Sutphin, 2020; Houtkooper *et al*., 2010). It has already been established that administering a synthetic ACMSD inhibitor orally successfully raises NAD + concentrations in the kidney, liver, and brain of mice. However, the effectiveness of this strategy is limited to tissues where this specific pathway for NAD + synthesis exists. Consequently, the use of ACMSD inhibitors could potentially be an effective treatment, especially for diseases characterized by liver-specific issues (Katsyuba *et al*., 2018; Okabe *et al*., 2022).

A sophisticated method for adjusting the NAD + /NADH balance involves converting extracellular lactate back into pyruvate. This pyruvate is then transported back into the cell via the monocarboxylate transporter. Once inside, it undergoes reduction by lactate dehydrogenase to regenerate NAD + levels. It has been reported that utilizing a bacterial lactate oxidase fusion protein introduced into the circulation of mice with drug-induced mitochondrial dysfunction showed some positive results (Patgiri *et al*., 2020; Prochownik and Wang, 2021).

Impelling Mitochondrial Turnover

Rapamycin, originally derived from the soil bacterium Streptomyces hygroscopicus, exhibits a blend of anti-inflammatory and anti-proliferative properties, demonstrating potential in improving various symptoms of mitochondrial dysfunction. It primarily targets the mTORC1 component of the mammalian target of rapamycin (mTOR) complex, which plays a crucial role in maintaining cellular balance and is associated with the activation of the mitochondrial stress response in cases of mitochondrial myopathy. It has been shown that rapamycin is effective in delaying the onset of symptoms and increasing lifespan in the Ndufs4−/− mouse model of

Leigh's disease (Liu *et al*., 2019; Arriola Apelo and Lamming, 2016).

It's worth noting that the exact molecular mechanisms behind these significant effects are not fully understood. Recent investigations using a model with a specific defect in complex IV assembly in skeletal muscle, COX15sm/sm, indicate that rapamycin's benefits may stem from enhanced autophagic flux and increased lysosome biogenesis, likely due to the inhibition of mTORC1. In Deletor mice, rapamycin treatment has been shown to adjust one-carbon metabolism and serine synthesis pathways, as well as deactivate the mitochondrial integrated stress response (Roos *et al*., 2019). This increase in autophagic flux appears to selectively remove dysfunctional mitochondria caused by the assembly defect, leading to improved mitochondrial structure. Nonetheless, due to the COX assembly defect, OXPHOS cannot be fully restored.

Curiously, rapamycin also induces metabolic changes, suggesting that other strategies to replicate this metabolic shift could offer new therapeutic avenues. The partial improvement in mitochondrial function observed with rapamycin treatment in these models is promising, especially considering its long history of clinical use. However, its significant side effects, particularly concerning immunosuppression, may restrict its application for mitochondrial diseases in humans (Haeussler *et al*., 2020).

Conclusion

Mitochondrial diseases represent a complex challenge due to their multifactorial nature, encompassing genetic, biochemical, and clinical dimensions. While this complexity continues to complicate diagnosis and treatment, recent advancements in the field offer hope for improved outcomes. For instance, clinical trials of elamipretide have demonstrated potential in enhancing mitochondrial function in patients with primary mitochondrial myopathy, representing a significant step forward for small-molecule therapies. Additionally, gene therapy approaches, particularly those using AAV vectors to deliver corrective genes to mitochondria, are beginning to show promise in preclinical studies.

Key challenges remain, including the difficulty of accurate genetic diagnosis owing to the heterogeneous nature of mitochondrial disorders and the variability in clinical presentation among affected individuals. Ongoing efforts aim to develop more precise diagnostic tools and biomarkers that can aid in tailoring treatments to individual patient profiles.

Looking ahead, the future of mitochondrial disease therapies lies not only in refining existing strategies but also in fostering collaborative research initiatives that harness advances in genomics, personalized medicine, and biotechnology. A clear vision for the future involves prioritizing research into novel therapeutic targets and integrating multidisciplinary approaches that can lead to transformative treatments. Collaborations between research institutions, industry stakeholders, and regulatory bodies will be essential to navigate the challenges facing implementation and to accelerate the development of effective therapies.

In summary, while significant hurdles remain, the continued focus on specific therapeutic strategies, coupled with an emphasis on addressing identifiable challenges, positions the field for meaningful progress in the coming years.

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Authors Contributions

Anastasia V. Poznyak and Nikolay A. Orekhov: Conceptualization writing review and editing.
Vasily N. Sukhorukov and Tatiana Ivanovna Kovyanova: Methodology writing review and editing.
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Ethics

Authors should address any ethical issues that may arise after the publication of this manuscript.

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