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Hydrogen sulfide supplementation as a potential treatment for primary mitochondrial diseases

Luke Slade [a,b], Colleen S. Deane [c], Nathaniel J. Szewczyk [d,e], Timothy Etheridge [f,*], Matthew Whiteman [a,*]

[a] University of Exeter Medical School, University of Exeter, St. Luke's Campus, Exeter EX1 2LU, UK
[b] Leibniz-Institut für Analytische Wissenschaften-ISAS-e.V, Dortmund, Germany
[c] Human Development & Health, Faculty of Medicine, University of Southampton, Southampton General Hospital, Southampton, UK
[d] Medical Research Council Versus Arthritis Centre for Musculoskeletal Ageing Research, Royal Derby Hospital, University of Nottingham, Derby DE22 3DT, United Kingdom
[e] Ohio Musculoskeletal and Neurologic Institute, Heritage College of Osteopathic Medicine, Ohio University, Athens, Ohio 45701, Greece
[f] Public Health and Sport Sciences, Faculty of Health and Life Sciences, University of Exeter, Exeter EX1 2LU, United Kingdom

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ABSTRACT

Primary mitochondrial diseases (PMD) are amongst the most common inborn errors of metabolism causing fatal outcomes within the first decade of life. With marked heterogeneity in both inheritance patterns and physiological manifestations, these conditions present distinct challenges for targeted drug therapy, where effective therapeutic countermeasures remain elusive within the clinic. Hydrogen sulfide (H_2S)-based therapeutics may offer a new option for patient treatment, having been proposed as a conserved mitochondrial substrate and post-translational regulator across species, displaying therapeutic effects in age-related mitochondrial dysfunction and neurodegenerative models of mitochondrial disease. H_2S can stimulate mitochondrial respiration at sites downstream of common PMD-defective subunits, augmenting energy production, mitochondrial function and reducing cell death. Here, we highlight the primary signalling mechanisms of H_2S in mitochondria relevant for PMD and outline key cytoprotective proteins/pathways amenable to post-translational restoration *via* H_2S-mediated persulfidation. The mechanisms proposed here, combined with the advent of potent mitochondria-targeted sulfide delivery molecules, could provide a framework for H_2S as a countermeasure for PMD disease progression.

Significance statement

There remain no effective therapeutic countermeasures for treating PMDs, a class of genetically inherited mitochondrial pathologies that are imminently fatal after birth. Hydrogen sulfide, with its primary roles in stimulating mitochondrial function, offers a promising avenue for targeted PMD therapies and addresses the urgent need for effective interventions in these life-threatening conditions.

1. Introduction

Primary mitochondrial diseases (PMD) are a class of heterogeneous, genetically inherited disorders typically resulting in death within the first decade of life [1]. These pathologies are amongst the most common inborn errors of metabolism with a prevalence rate of ~1:4000 [2]. To date, pathogenic variants for over 300 PMD causing genes have been discovered [3], with the patient phenotype presenting deficits in metabolically demanding tissues, culminating in neurodegeneration, neuromuscular dystrophy, cardiorespiratory failure and severe oxidative damage [4]. In turn, the financial healthcare cost of these pathologies is considerably higher than the average costs within the population [2]. The heterogeneous nature of both PMD mutation and physiological manifestations means therapeutic treatment for these diseases is extremely difficult, where effective therapeutic counter-measures remain elusive within the clinic. "*Indeed, there exists a huge unmet medical need for disease-modifying therapeutics of PMDs. As a regimen, vitamins and supplements remains the standard of care for most patients. Given the genotypic and phenotypic heterogeneity of PMD, significant research is*

* Correspondence to: University of Exeter Medical School, St. Luke's Campus, Magdalen Road, Exeter EX1 2LU, United Kingdom.
E-mail addresses: T.etheridge@exeter.ac.uk (T. Etheridge), M.whiteman@exeter.ac.uk (M. Whiteman).

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required to address the many forms of PMD therapeutically" – Philip Yeske, Science & Alliance Officer at the United Mitochondrial Disease Foundation.

Severe mitochondrial perturbation is a common driver of physiological decline in PMD patients, either from direct genetic mutation of mitochondrial coding genes, or genes encoding related pathways that subsequently disrupt mitochondrial function. Whilst direct modulation of PMD-causing genetic material is reserved for emerging gene-editing tools [5,6]· exploration of small molecules capable of improving mitochondrial health at clinically relevant doses to delay physiological degeneration is of critical demand. Thus, to address this clinical shortfall, we review key insights from hydrogen sulfide (H_2S) signalling that appear as favourable potential treatments for PMD.

H_2S is a chemically versatile signalling molecule produced endogenously across the animal kingdom [7]. Cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) are two key cytosolic H_2S-producing enzymes that catalyse the pyridoxal 5′-phosphate-dependent conversion of homocysteine to cystathionine and cystathionine to cysteine, respectively, producing H_2S [8]. Additionally, mammals possess the (primarily) mitochondrially-localised enzyme 3-mercaptopyruvate sulfurtransferase (3-MST) that, in combination with cysteine aminotransferase, produces H_2S via the reaction with cysteine [9]. These pathways are highly conserved and reflect the fundamental role of H_2S in pre-biotic energy provision at the origin of life (reviewed extensively previously [10,11]). The emergence of molecular oxygen as a primary energy source meant previously H_2S-dependent eukaryotes either became extinct, migrated to sulfide environments or adapted [12]. Importantly, these H_2S-metabolising pathways were not lost, and their molecular machinery remain physically embedded in mitochondria across phyla [10]. Today, a wealth of literature supports endogenous H_2S as a conserved mitochondrial substrate and post-translational regulator across species, essential for a multitude of physiological signalling events [11]. The cytoprotective effects of H_2S signalling are now generally agreed to act predominantly *via* protein *S*-persulfidation (sometimes referred [incorrectly] as *S*-sulfhydration) [13,14], a process whereby the addition of a sulfur atom onto the thiol (-SH) adduct of a cysteine residue can alter its structure, stability [15] and functional capacity post-translationally [16]. Importantly, such cytoprotective effects of *S*-persulfidation have been shown for key mitochondrial defence proteins [14] and subunits of the mitochondrial respiratory chain [17,18]. Thus, given the aberrant mitochondrial and protein dysfunction that occurs in PMDs, mechanisms of H_2S signalling represent a fundamentally under-explored potential therapeutic role for disease treatment and modification in PMD.

The emergence of potent mitochondrial-targeted sulfide delivery molecules (such as AP39 and related structures) and their evaluation in animal models of human disease has identified a large number of human conditions that could be potentially "druggable" with mitochondria-delivered sulfide. For example, existing data shows that biologically relevant doses (<300 nM; similar concentrations to circulating H_2S levels in humans [16]) of the parent compound are effective at ameliorating both ageing and disease-related mitochondrial dysfunction across *in vitro* and *in vivo* biological models [19–35]. Although detailed reviews on H_2S-mediated signalling [13,36] and mechanisms of PMD inheritance [1,37,38] are abundant, this review focuses precisely on the therapeutic potential H_2S holds as a countermeasure for PMD progression, with emphasis on the mitochondrial and post-translational regulatory capabilities of this molecule that appear favourable to PMD. Specifically, we will discuss key pathways/proteins with known H_2S-mediated modulation across various models, offering insights into their potential therapeutic roles in PMD, given the widespread pathological phenotypes these diseases share.

2. Primary mitochondrial diseases – inheritance and manifestations

Mitochondria are ubiquitous double-membraned organelles that form intricate networks within cells controlling aerobic respiration within the electron transport chain (ETC), producing the majority of cellular adenosine triphosphate (ATP). Along with their roles in regulating apoptosis, autophagy, and calcium homeostasis [39], its agreed their most important role (particularly in PMD) is the provision of biochemical energy. Both morphological alterations and fission/fusion events are tightly controlled by membrane-bound proteins, providing dynamic responses to energetic demands within the cell [40], and migration of mitochondria along microtubular cytoskeletal apparatus facilitates ATP provision in localised areas demanding of biochemical energy [41]. Unsurprisingly, tissues/organs of high metabolic activity and reliance on oxidative phosphorylation are primary culprits of perturbations in mitochondrial function such as the brain, cardiac and skeletal muscle, and genetically inherited mutations disrupting mitochondrial function can induce severe pathophysiological diseases in humans [42].

Of the approximate 80 proteins that comprise the ETC, mitochondrial DNA (mtDNA) encodes for a mere 13 of these structural genes, where evolution has handed much of the transcription and translation of ETC proteins to nuclear encoding DNA (nDNA) [43]. In fact, 99% of the mitochondrial proteome, thought to comprise ~1158 proteins, is encoded by nDNA [44]. The remaining genes under mtDNA control are thus crucial for mitochondrial respiration, encoding hydrophobic components essential for ETC functionality, where mutations in these genes are known to cause severe mitochondrial dysfunction and the onset of PMDs [45]. Mutations in nDNA are the largest contributors to PMD, given their greater involvement in coding for structural ETC proteins, requirements for proper subunit assembly and the reliance of mtDNA integrity and replication on nDNA [43]. Whilst the inheritance of mtDNA mutations occurs predominantly through the maternal lineage, mutation-inheritance for nDNA can occur via autosomal recessive (requiring inheritance of two mutated alleles for the pathology to present in offspring), autosomal dominant (requiring only one mutant allele), or X-linked recessive (mutations on the X chromosome induce pathology) patterns [38].

The severity of symptomological manifestation of PMD from mtDNA or nDNA mutations can vary for many reasons; heteroplasmic levels of mutant mtDNA within a cell (known as the mtDNA 'bottleneck'), mutant allelic 'doses' from distinct inheritance patterns (i.e., recessive *vs* dominant) and tissue specificity-induced perturbations, reviewed extensively previously [37,46]. Precise disorders can vary dramatically, for example, common mtDNA mutation-induced disorders include chronic progressive external ophthalmoplegia, Kearns-Sayre syndrome, Leber hereditary optic neuropathy and mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes, whereas Leigh syndrome, a severe neurodegenerative PMD is caused predominantly by mutations in nDNA. Importantly however, the general patient phenotype is similar across mutation types and inheritance patterns, where muscle weakness, neurodegeneration, ataxia, cardiomyopathy and ophthalmologic abnormalities are widespread features of PMD [47]. Tissue/organ degeneration that underlies these phenotypic changes (i.e., in muscle, brain and heart) ensues largely due to mitochondrial insufficiency and energy provision. Crucially, pharmacological H_2S is known to augment mitochondrial function within each of these organs to restore function (see below), thus, an evaluation of common pharmacological donors and key mechanisms relevant for PMD follows.

3. Proposed naturally occurring H_2S-generating compounds

With the discovery of endogenous H_2S as a physiological signalling molecule [48] and accumulating evidence that intracellular levels of H_2S decline with ageing [14] and certain age-related, acute and chronic

diseases [49,50], early works began to explore naturally occurring H_2S-releasing compounds in biological research, for example, H_2S-producing thiosulfinates found in garlic and onions. Allicin, a sulfur-containing compound found in garlic is produced from the amino acid alliin via the enzyme alliinase when garlic is crushed. It is unstable in solution and quickly decomposes to diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS). It has been shown that H_2S production occurs from 100 μM administration of DADS and DATS (but not DAS) only *in vitro* in the presence of free thiols, and induces vascular smooth muscle cell relaxation of aortic rings from rodent endothelial cells [51]. However, their poor bioavailability (typically administered via intraperitoneal or intravenous injection, not orally) makes it uncertain whether such levels are achievable *in vivo,* known to be rapidly metabolised or degraded in blood with a half-life of 20 minutes from intravenous injection [52]. Furthermore, drug-garlic component interactions [53] likely limit their use in PMD where patients require a cocktail of therapeutics.

Further work investigated human umbilical endothelial cells with 246 μM of allicin to address treatment effects on lipopolysaccharide (LPS)-induced oxidative stress and inflammation [54]. The authors found that 246 μM of allicin improved cell viability, reduced apoptosis and offset increases in ROS production under LPS stress. Additionally, LPS significantly impaired mitochondrial ATP production, induced mitochondrial membrane potential collapse and increased mitochondrial cytochrome *c* release into the cytosol, all features attenuated by allicin treatments [54]. However, it wasn't clear how much of that effect was derived from a H_2S generation itself. Similarly, high concentrations of allium-derived thiosulfinates have been shown to upregulate gene expression of both heme oxygenase and NAD(P)H:quinone oxidoreductase 1 detoxifying enzymes in HepG2 cells after 6 hour exposure to 100 μM of DADS and DATS [55]. Similarly, cell viability was increased from DATS and DADS treatments ranging from 25 to 500 μM exposures, with no effect seen from DAS treatments. The main physiological signalling effects from garlic and onion-related compounds has been previously reviewed [56–59], and many examples exist for their anti-inflammatory, anti-cancerous and cytoprotective effects in mammalian cell cultures [60–63]. However, almost all reported anti-oxidant effects of allicin are from *in vitro* data, and *in vivo* data suggests that the anti-oxidative properties are attributed to the reaction of allicin with protein thiols as opposed to direct scavenging [64], and the concentrations achieved *in vivo* are unlikely to account for these observations. Although garlic-derived thiosulfinates superficially look to be immediately exploitable options for treating PMD (i.e., these are nutritionally available molecules), there is mounting evidence that garlic, or its decomposed bioactive forms (DATS, DADS) can elicit many adverse affects *in vivo*, precluding their uses as therapeutics. These include (but are not limited to): inducement of anaemia, growth retardation, liver injury, structural deformations in vital organs and stomach injury-induced death in rodent models from prolonged garlic treatments (reviewed extensively previously [64]). When combined with known drug interactions, limited bioavailability, poor half-life and rapid metabolism and/or degradation *in vivo*, the clinical prospects of these compounds are limited.

Another historical molecule with the potential for intracellular H_2S generation is thiosulfate ($S_2O_3^{2-}$), an oxidation product of hydrogen sulfide metabolism within mitochondria that can increase levels of H_2S upon exogenous administration [65], suggesting this molecule might be useful and act as an indirect source of mitochondrial H_2S. However, $S_2O_3^{2-}$ can be generated *in vivo* by several other mechanisms (reviewed in [66]). It has historically been used clinically for cyanide and heavy metal poisoning and calciphylaxis, but due to exceptionally poor bioavailability ($S_2O_3^{2-}$ is not systemically absorbed) requires intravenous use with dosing in gram quantities to achieve efficacy (e.g., 25 g three times daily) [67], increasing the likelihood of off-target effects and toxicity. However, studies using experimental animals have proved useful. For example, intravenous application of $S_2O_3^{2-}$ (10 mg/kg) has been shown to improve neurological function and survival in a mouse model of cerebral ischemia-reperfusion injury [68]. *S*-persulfidation of caspase-3 at Cys163 inhibited caspase-3 activity mediating cytoprotective effects, and $S_2O_3^{2-}$ levels (but not H_2S levels) associated with the protective effects of thiosulfate treatments. A drawback to many of these studies is the lack of suitable controls to remove or measure H_2S, clouding any interpretation of H_2S involvement in these phenomena. Further exampling the complexities surrounding $S_2O_3^{2-}$ as a "H_2S donor", Ichinose's group found that CSE *deficiency* (a primary H_2S-synthesising enzyme) attenuated survival rates in a chemical-induced liver injury mouse model, upregulating Nrf2 antioxidant defence genes and downregulating chemical-induced elevations in caspase 3, poly(ADP-ribose) polymerase (PARP-1) and inflammatory cytokine activities [69]. Although elsewhere others "could not detect any increase in SF7-AM [H_2S] or SSP4 [polysulfide] fluorescence in the presence of STS [15 mM] with or without human great saphenous vein vascular smooth muscle cells" [70]. Thus, as a putative "H_2S-releasing molecule", it is surprising that $S_2O_3^{2-}$ (administered intraperitoneally at 2 g/kg, 30-minutes before and 2-hours after chemical induction of liver failure) attenuated histological readouts of cell health, upregulated antioxidant defence proteins and reduced the expression of apoptosis-related proteins. These data using exceptionally high doses/concentrations suggest $S_2O_3^{2-}$ treatments signal predominantly *via* distinct mechanisms to that of increasing cellular H_2S levels, counterposing the cytoprotective effects seen from inhibitions of CSE levels.

Further, 1 mM concentrations of thiosulfate in a rat model of ischemia-reperfusion injury exhibited protective effects via a preservation of mitochondrial enzymatic activity in respiratory chain subunits, attenuation of apoptosis through caspase-3 activity and an increase in antioxidant defence through increases in SOD and catalase enzymatic activities [71]. Although it is not clear how much of these observations were due to H_2S generation from $S_2O_3^{2-}$, it has been suggested the beneficial effects from thiosulfate are reliant on the expression of endogenous H_2S synthesising enzymes, positing that any H_2S-related beneficial effects from thiosulfate treatments are from increases in endogenous H_2S-producing enzymes and not via thiosulfate. Similar effects have been seen with 1 mM thiosulfate pre-treatments under ischemia-reperfusion conditions in rats, showing significant improvements in the activities of ETC complex enzymes, preserved ultrastructure of mitochondria and attenuation of ATP production to control levels [72]. Separately, oral thiosulfate administration (400 mg/kg, once daily) for 28-days has also shown to be protective in a renal mitochondrial ischemia-reperfusion injury model in rats, maintaining mitochondrial function and increasing NADH hydrogenase activity [73]. These measures were, however, performed *in vitro*, and H_2S levels were not measured to be able to assess its potential contribution to these effects.

More recent work sought to establish H_2S/P-SSH changes in response to $S_2O_3^{2-}$ treatments in HUVEC cells [74]. Administration of 15 mM $S_2O_3^{2-}$ for 90-minutes caused a 50% increase in the fluorescence intensity of the semi-quantitative acetoxymethyl ester-containing probe SF_7-AM, and a 4-hour incubation with 15 mM $S_2O_3^{2-}$ seemed to increase PSSH levels within cells using the dimedone tag-switch method. Interestingly, the mRNA expression levels of key H_2S-regulating genes were elevated under 15 mM $S_2O_3^{2-}$ treatments: sulfite oxidase (SUOX), thiosulfate sulfurtransferase-like domain containing 1 (TSTD1), mercaptopyruvate sulfurtransferase (MPST), sulfide:quinone oxidoreductase (SQOR) and persulfide dioxygenase (ETHE1). Although not tested directly (by employing target-gene knockdown + $S_2O_3^{2-}$ co-treatment), these data suggest that $S_2O_3^{2-}$ -mediated increases in H_2S/PSSH might act via an upregulation of H_2S-producing gene expression rather than $S_2O_3^{2-}$ acting as a H_2S "donor". Crucially, the authors concluded that cell-protective effects from $S_2O_3^{2-}$ treatments result primarily *via* an inhibition of mitochondrial respiration and increased flux through glycolytic pathways, although at mM concentrations of $S_2O_3^{2-}$ purely osmotic effects cannot be entirely ruled out and control experiments to take this into

account or to account for "spent" $S_2O_3^{2-}$ or contaminants (which may include sulfide: e.g., patent JP7408753B2) are generally lacking. Whilst impairments in mitochondrial respiration might be a suitable treatment approach to attenuate endothelial-cell proliferation and migration, PMDs encompass severe mitochondrial dysfunction causal to disease onset and pathology progression. Thus, $S_2O_3^{2-}$ use, at the supra-physiological concentrations required to elicit cytoprotective effects in pre-clinical works, should be considered with caution when considering potential therapeutic options for PMD. Indeed, the therapeutic utility of $S_2O_3^{2-}$ is being explored with several patents relating to $S_2O_3^{2-}$ and oral formulation patents have been filed and either not yet grant (e.g., EP4212149A1) or have lapsed (e.g., WO2015056013A1). Others (e.g., CN112805238A) have not mentioned H_2S in the mechanism of action.

Thus, $S_2O_3^{2-}$ represents a very interesting therapeutic tool clearly displaying efficacy across a range of *in vitro* and *in vivo* pre-clinical models. As an already established drug used for cyanide poisoning and occasionally "off-label" for calciphylaxis with a known safety profile, $S_2O_3^{2-}$ could be promising, and its utility for safe and effective use in treating PMDs demands further work. Although efficacy for $S_2O_3^{2-}$ is clear, it is the authors opinion that to date, the "H_2S-specific" effects of $S_2O_3^{2-}$ are currently poorly defined and ambiguous (even within the same group, e.g., [70,74]) and with such high concentrations/doses required (e.g. $\geq$15 mM cell culture, $\geq$ 2 g/kg *i.v., in vivo* in experimental animals, or 25 g *i.v., t.i.d.* in humans) "off-target", non-H_2S or effects on trans-sulfuration genes/enzymes cannot at present be ruled out.

Despite paving the way in exploring natural options for exogenous H_2S molecules, garlic-derived H_2S-producing thiosulfinates (such as DAS and DATS) have many concerns: a high reactivity, poor pharmacogenetics, very rapid metabolism/degradation, interaction with other drugs and off-target effects, limiting their likelihood in being safe, beneficial clinical options for PMD. However, formulation studies could circumvent some, but not all, of these limitations in the future (e.g., EP4212149A1). Similarly, the exceedingly high doses required for positive effects from $S_2O_3^{2-}$ treatments (e.g., in humans, requiring 25 g intravenously, three times daily) could prove problematic, given that biological effects of H_2S are dose-responsive, and high levels of H_2S are cytotoxic [75]. Such untargeted approaches of H_2S release are unlikely to become clinical tools for restoring mitochondrial H_2S levels, and compounds with more appropriate pharmacological properties are required, e.g., potency, efficacy, bioavailability, stability and target tissue penetration.

4. Pharmacological H_2S molecules

Extensive efforts have gone into designing pharmacological approaches to restore diminishing H_2S in the form of delivery molecules as potential restorative therapies. Some examples of widely used H_2S donors can be categorised based on their chemical structure, source composition and release dynamics in Table 1. Traditionally, inorganic compounds such as sodium sulfide (Na_2S) and sodium hydrosulfide (NaSH) are regularly used in biological studies, providing a water-soluble system to investigate the effects of H_2S generation. However, these molecules generate H_2S in physiological media almost instantly *via* non-targeted, pH-dependent dissociation, producing an extremely high local concentration of H_2S that poorly mimic endogenous enzymatic H_2S kinetics in biological systems [75]. With the physiological effects of H_2S known to act predominantly within mitochondria, providing cytoprotection at low-moderate (i.e., sub micromolar) concentrations [75], these early molecules are incompatible with clinical requirements.

As a result, the slow-release donor GYY4137 (morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate) was designed to provide a sustained, low-level H_2S release over longer durations (proposedly, up to 7 days) [76]. Although well-established as an experimental H_2S donor tool in biological research, this compound still suffers from an untargeted H_2S-releasing approach and requires high micromolar concentrations for therapeutic effects to become detectable (see Table 1). Alternative related structures to GYY4137 have been synthesised and evaluated previously, such as AP67 and AP72 with modified phosphorodithioate cores [77]. These two compounds showed increased H_2S generation *vs* GYY4137 from equimolar dosing in human synoviocytes, an effect that seemed to largely explain the cellular response from treatments [77]. Importantly, physiological effects have been seen from 1 nM treatments of both AP67 and AP72, where K_{ATP} and prostaglandin-dependent dilation of bovine ciliary artery restored vasorelaxation in apoE$^{-/-}$ atherosclerotic mice [78]. Later, the H_2S compound FW1256 was synthesised providing a donor tool comparable to GYY1437, but with generally faster rates of hydrolysis and H_2S generation [79]. Initially, the compound was used to inhibit cancer cell proliferation, proving superior to GYY4137 due to its faster H_2S release rate [80]. Later, its effects were explored in a *Caenorhabditis elegans* (*C. elegans*) model of ageing and showed increased lifespan and healthspan when animals were grown in the presence of 250–500 μM throughout the life-course [81]. Once more, an exceedingly high compound concentration was required for positive health effects and mitochondrial improvements with FW1256, negating the likelihood of its success as a clinical H_2S-modulating therapy.

Importantly, we have seen successful implementation of H_2S-related therapies used in clinical settings. For example, the H_2S-coupled anti-inflammatory donors – specifically, ATB-346 and SG1002 - were first extensively tested in animal models. Although initially designed as a treatment for arthritis, ATB-346, typically in the range of 50–200 mg/kg, has shown restorative effects in settings of inflammation [82], cancer progression [83], brain and spinal injury [84,85] and cognitive impairment [86] across animal and human cell models. Additionally, the H_2S pro-drug SG1002 is known to significantly attenuate cardiac dysfunction in animal models, primarily via the modulation of adenosine monophosphate-activated protein kinase [87], reduced endoplasmic reticulum stress [88] and upregulation of cytoprotective proteins such as SOD1, catalase and CBS [89]. From here, both ATB-346 and SG1002 have progressed into phase I and II clinical trials, with positive results in settings of arthritis and cardiovascular dysfunction, respectively, and minimal adverse effects and good tolerability with >400 mg daily treatments [116]. Similarly, the pH-dependent H_2S donor ammonium tetrathiomolybdate (ATTM) showed attenuative effects in rat models of cerebral ischemia-reperfusion injury, with improvements in heart and brain function [119]. This compound has also progressed into clinical trials as a result, where it is being explored for its roles in anti-cancer treatments. Lastly, Zofenopril – an already approved drug for treating cardiovascular disease – was found to release H_2S upon hydrolysis of the compound, underlying many of its positive effects on inflammation, angiogenesis and anti-apoptotic activity [120]. This drug continues to be tested in clinical cardiac settings (https://clinicaltrials.gov), but its application should be considered in wider pathologies. Currently available data on these clinical compounds is largely exclusive to specific pathologies (i.e, arthritis, cardiovascular dysfunction, cancer), where treatments exploiting these clinical tools in PMD could be a valuable next step in the clinic. Although these studies are currently lacking, these data do however, support the effectiveness of a 'bench to bedside' pipeline for establishing effective clinical H_2S molecules.

5. Mitochondria-targeted H_2S as a preferred strategy for H_2S delivery

Mitochondria display functional improvements in the presence of nanomolar/low micromolar concentrations of H_2S in lower organisms *in vivo* [20,35,75,90], therefore, the high micromolar compound doses required with non-targeted sulfide salts likely reflect a surplus requirement to allow smaller amounts to diffuse from cytosolic compartments into mitochondria. Knowing this, our group previously designed the first mitochondria-targeted H_2S molecule, AP39, exploiting a triphenylphosphonium (TPP^+) cation linker coupled to an established sulfide generating molecule, anethole dithiolethione (ADT-OH) [121–123].

Table 1
Primary pre-clinical and clinically tested pharmacological H_2S delivery molecules. Above, the most commonly utilised delivery molecules in pre-clinical research. Details of targeting mechanisms, H_2S release, effective (parent) compound concentration range and models of study. Below, a summary of clinically tested H_2S compounds that have shown positive effects in human clinical trials.

In vitro/in vivo H_2S donor tools	Targeting mechanism	H_2S release characteristics	Effective range	Pre-clinical preservation/effects
AP39	Mitochondria-targeted (TPP^+)	Release *via* ADT-OH	100 pM – 300 nM	PMD [19], DMD [20, 90], AD [21], CA [27], hyperglycaemia [26], ischemia-reperfusion [91], hypoxia [24], photoaging [92], burn injury [23], inflammation [32], irradiation [92]
AP123	Mitochondria-targeted (TPP^+)	Release *via* HTB	30 – 300 nM	Hyperglycaemia [26] , photoaging [92]
RT01	Mitochondria-targeted (TPP^+)	Release via RT02	30 – 300 nM	Gastric mucosal injury [93], hyperglycaemia-induced mitochondrial dysfunction [94], cell senescence [95]
AP67	Modified Phosphinodithioate	Slow release - hydrolysis	1 nM – 10 μM	Vasorelaxation in atherosclerosis [78]
AP72	Modified Phosphinodithioate	Slow release - hydrolysis	1 nM – 10 μM	Vasorelaxation in atherosclerosis [78]
MitoPerSulf	Mitochondria-targeted (TPP^+)	Fast release via thiol interaction	10 μg/kg	Ischemia-reperfusion [96]
NaSH	Non-targeted	Fast release – pH dependent	10 – 500 μM	AD [97], diabetes [98, 99], apoptosis [100, 101], hyperglycaemia [102], endothelial dysfunction [103], ischemia-reperfusion [104], mitochondrial biogenesis [105] neural injury [106],
Na_2S	Non-targeted	Fast release – pH dependent	10 – 500 μM	IR [91, 107], cardiac dysfunction [108], myocardial injury [109]
GYY4137	Phosphinodithioate	Slow release - hydrolysis	30 – 500 μM	DMD [20, 90], ischemia-reperfusion [110], myocardial infarction [111], ipsilateral epididymis injury [112], lung injury [113], apoptosis [114], atherosclerosis [115]
FW1256	Non-targeted	Slow release - hydrolysis	250 – 500 μM	Ageing [81], inflammation [80]

Risk of H_2S-induced toxicity

Clinically-tested H_2S molecules	Targeting mechanism	Properties/ mechanisms	Clinical preservation
ATB-436	Cyclooxygenase inhibitor	H_2S-releasing NSAID molecule	Phase II clinical trial – Osteoarthritis [116]
SG1002	Non-targeted	Thiol activated H_2S release	Phase I clinical trial – Heart failure [117]
ATTM	Non-targeted	Thiol-dependant and pH controlled H_2S release	Phase II clinical trial – Breast cancer [118] (in trials for mechanisms separate to H_2S generation)
Zofenopril	Non-targeted	Sulfhydryl containing angiotensin-converting enzyme inhibitor	Phase IV clinical trial – Hypertension [116] (in trials for mechanisms separate to H_2S generation)

Abbreviations: ADT-OH: Anethole dithiolethione, **HTB**: 4-hydroxythiobenzamide, **TPP^+:** tetraphenylphosphonium, **RT02:** H_2S-releasing moiety for the parent compound, RT01, **NaSH:** sodium hydrosulfide, **Na_2S:** sodium sulfide, **PMD**: primary mitochondrial disease, **DMD**: Duchenne muscular dystrophy, **AD**: Alzheimer's disease, **CA**: cardiac arrest, **NSAID:** Non-steroidal anti-inflammatory drug. In the effective range column, green indicates low, physiological doses of the compound required to elicit therapeutic effects, whereas orange > red indicate doses approaching/beyond endogenous H_2S levels. Note: the clinically tested molecules ATTM, Zofenopril are in trials for mechanisms separate to H_2S release [93–104,106–115,117,118].

With a strong negative electrochemical charge within mitochondria, TPP^+ drives the accumulation of AP39 into mitochondria in large amounts due to strong potentiometric differences [124]. As a result, this sulfide delivery molecule is currently the most potent tool for pharmacological H_2S administration, with an efficacious range of picomolar to low nanomolar across *in vitro* and *in vivo* models [19–22,26,27,35,90] – an approximate 1000 - 10,000 fold lower compound dose than salt-based H_2S molecules (see Table 1).

With a growing interest surrounding the development of mitochondria-targeted sulfide delivery molecules (including the advent of enzyme-mediated and thiol promoted H_2S-releasing donors [125]), the potential future for target-driven H_2S molecules effective at physiologically relevant doses is ever growing. Therefore, we will discuss the available literature surrounding H_2S-mediated physiological signalling, focusing on key targets and mechanisms that appear favourable in the potential treatment of PMD. Despite the majority of biological H_2S research underpinned by clinically (and chemically) unfavourable molecules (such as Na_2S, NaSH and GYY4137), these experiments nevertheless provide key insights into potential signalling mechanisms of H_2S that hold strong therapeutic potential if these can be retained using sulfide delivery molecules with improved drug-like properties.

6. H_2S-mediated improvements in mitochondrial structure, integrity and function

The loss of well-organised mitochondrial networks and structural integrity is a common feature in PMD [126], which is unsurprising, given that the majority pathogenic genetic variants encode for fundamental mitochondrial processes [127]. Importantly, administration of pharmacological H_2S has been shown to rescue mitochondrial structure and membrane integrity in diverse models. For example, a rodent model of myocardial ischemia-reperfusion injury showed that murine mitochondria isolated from the heart treated with 50 μg/kg of Na_2S upon reperfusion displayed a 2.2-fold increase in mitochondrial respiration rate to vehicle controls [91]. In addition, these Na_2S treated cardiomyocytes retained well-organised mitochondrial structure and cristae formation compared to disorganisation and swelling seen in vehicle controls, supported by reduced caspase-3 activity and TUNEL positive nuclei [91], both indicative of reduced apoptosis-induced cell death – a common defective system in PMD [128]. Similarly, neonatal rat cardiomyocytes pre-treated with 50 μM of NaSH prior to angiotensin II insult (to evoke cardiomyocyte hypertrophy) displayed improvements in mitochondrial ultrastructure, reduced mitochondrial swelling and improved cristae number in a rodent model of myocardial hypertrophy *via* SIRT3-dependant mechanisms [129]. This was also demonstrated with the slow-release H_2S donor GYY4137 in a lung ischemia-reperfusion injury in diabetic rats [130]. Together, these data highlight the ability of pharmacological H_2S to restore mitochondrial architecture across diverse disease states and, particularly, in settings that display phenotypic similarities to PMD pathologies, suggesting the possibility that mitochondrial H_2S could be efficacious in PMD.

The occurrence of neurodegenerative phenotypes in PMD are exceedingly common and typically result in death within the first few years of human life [131]. Previous data show beneficial effects of mitochondrial sulfide-delivery molecules in brain *in* vivo. For example, Ikeda and colleagues used a cardiac arrest model of neurological decline in mice, administering 10, 100 and 1000 nmol/kg of mitochondria-targeted AP39 either prior to or immediately after arrest. Treatments of 100 and 1000 nmol/kg prior to, and 10 nmol/kg (7 μg/kg) immediately after arrest improved neurological performance *via* preserved mitochondrial integrity, reduced oxidative stress and attenuation of mitochondrial permeability transition pore opening [27]. The authors showed no change in levels of CSE, CBS or 3-MST in the brain, but both sulfide and thiosulfate levels were increased, indicative of increased mitochondrial H_2S generation from AP39 independent from endogenous H_2S pathways. Additionally, post-arrest survival rates were increased by up to 69% with AP39 treatments, an important finding for PMD therapy. Similarly, others showed that a 50 nmol/kg pre-treatment of AP39 for 7 days in rats prior to ischemia onset improved brain tolerance to cerebral artery occlusion (a model of stroke) by reducing pro-inflammatory markers Il-1β, Il-6 and TNFα in the brain [30]. The same group more recently revealed that 100 nmol/kg given intravenously shortly after (i.e., a post *vs* pre-stroke treatment as previously reported) reperfusion form cerebral artery occlusion, significantly improved neurological deficits and infarct volume, largely via a reduction in inflammatory cytokines, reduced activation of cell death machinery (PARP1) and improvements in blood-brain barrier integrity [132]. The implications of these data are crucial for PMDs such as Leigh syndrome, an aggressive (and highly common[133]) neurodegenerative PMD that, in light of these works, could potentially be treated via pharmacological mitochondrial H_2S.

More specifically, H_2S-mediated protection of mitochondrial integrity within pre-clinical models of heritable disease have recently been noted. For example, administration of 100 μM GYY4137 and 100 nM AP39 preserved the proportion of well-networked mitochondria in severely fragmented muscle-mitochondria in a *C. elegans* model of Duchenne muscular dystrophy [20], an X-linked heritable disease associated with severe mitochondrial perturbation. Importantly, AP39 was efficacious at a 1,000-fold lower dose than GYY4137, highlighting the potent therapeutic potential of mitochondrial H_2S molecules. More recently, treatment with 100 nM of AP39 improved survivability and healthspan *via* preserved mitochondrial membrane potential and increased ATP content in a *C. elegans* model of Leigh syndrome [19], important findings for higher translational work into mitochondrial H_2S treatment of Leigh syndrome.

The ability of pharmacological H_2S to modulate diverse aspects of mitochondrial morphology in both diseases associated with mitochondrial dysfunction and disease states where mitochondrial dysfunction underlies the very cause of disease progression, highlights the therapeutic potential of this diatomic molecule in PMD. Abnormalities in mitochondrial structure are inextricably linked to its function, where compounds able to directly preserve its integrity serve as potential mitochondrial treatment tools for PMD either as standalone therapies, or in combination with current treatment options.

7. Regulation of mitochondrial dynamics by H_2S

7.1. Fission/ fusion

In order to adapt to energetic fluctuations within the cell, mitochondria undergo frequent fusion and fission events to improve biochemical energy provision, alongside regulated death of architecturally perturbed mitochondria and elimination of damaged mitochondrial DNA (mtDNA). Mitochondrial fusion, the process in which two distinctly separate mitochondria merge both their outer and inner membranes, is predominantly controlled by the dynamin-like GTPase's mitofusin 1/2 (MFN1/2) in the outer mitochondrial membrane, and optic atrophy 1 (OPA1) in the inner membrane [134]. Fission events are regulated by the GTPase dynamin-related protein 1 (DRP1), which binds to the outer mitochondrial membrane translocating from the cytosol to induce mitophagic processes [134]. Abrogated mitochondrial dynamics are a common feature in neurodegenerative forms of PMD [135], with wider implications into age- related causes of neurodegeneration such as Alzheimer's, Huntington's, Parkinson's and Amyotrophic lateral sclerosis. Specifically, mutations in DRP1 have been reported in a neonate female, displaying abnormal brain development, optic atrophy and hypoplasia [136], with more recent reports for inborn DRP1 mutations causing severe PMD pathology [137–139]. MFN1/2 and OPA1 mutations are also known to lead to axonal degeneration, peripheral neuropathy and muscle atrophy, as seen in Charcot-Marie-tooth disease type 2 A, reviewed extensively previously [134].

Interestingly, pharmacological H_2S has shown significant

preservation of mitochondrial dynamics in the face of compromised fission/fusion events. Specifically, 400–600 μM of NaSH exposure inhibited mitochondrial fission in SH-SY5Y neuroblastoma cells, increasing the proportion of elongated mitochondria and overall ATP production [140]. Additionally, a mouse model of hyperhomocysteinemia displayed severe impairments in mitochondrial number, with an upregulation of DRP1 expression and impaired MFN1 expression in response. Treatment with 30 μM NaSH increased mitochondrial number predominantly *via* a preservation of DRP1/MFN1 expression ratios [141]. Further, cardiomyocyte senescence in rats was inhibited with 100 μM/kg NaSH treatment by increasing the blunted stress-induced decline in DRP1 expression, with concordant decreases in stress-induced MFN2 upregulation [142]. Recently, potential persulfide/polysulfide-mediated regulation of DRP1 has been suggested. By revealing roles for cysteinyl-tRNA synthases (*CARSs*) as principal persulfide synthases *in vivo,* the authors demonstrate that polysulfidation and de-polysulfidation events can reversibly regulate DRP1 activity [143], with *CARS2* inhibition increasing DRP1 expression. This loss of *CARS2* expression also drastically altered mitochondrial architecture towards fragmented morphologies.

7.2. *Improving the mitochondrial pool – roles for mitochondrial biogenesis and mitophagy*

With mitochondrial dysfunction and reduced oxidative capacity primary features of PMD, strategies to increase healthy mitochondrial content within a given tissue would seem complementary to improving respiratory capacity. Exercise and therapeutic interventions to modulate mitochondrial biogenesis are ongoing area of research in PMD [144, 145]. However, enhancing mitochondrial replication/number through a genetically compromised framework (i.e., PMD) could simply increase the number of dysfunctional mitochondria within the cell, particularly if the PMD of concern is caused by mutations in mtDNA. Importantly, rates of mitophagy are decreased in Methylmalonic acidemia models of PMD [146] (and wider [147]), supporting the notion that dysfunctional mitochondria are not effectively eliminated, and simply adding to this through mitochondrial biogenesis may prove pernicious to cell environments by expanding the number of ROS-producing mitochondria. Despite this, others have shown that overexpression of the mitochondrial biogenesis regulator, peroxisome proliferator-activated receptor-γ coactivator (PGC1-α), augments respiratory capacity in primary cells obtained from PMD patients [148], suggesting that increased mitochondrial number (despite aberrant genetic frameworks in both nuclear and mitochondrial DNA for mitochondrial production) can improve

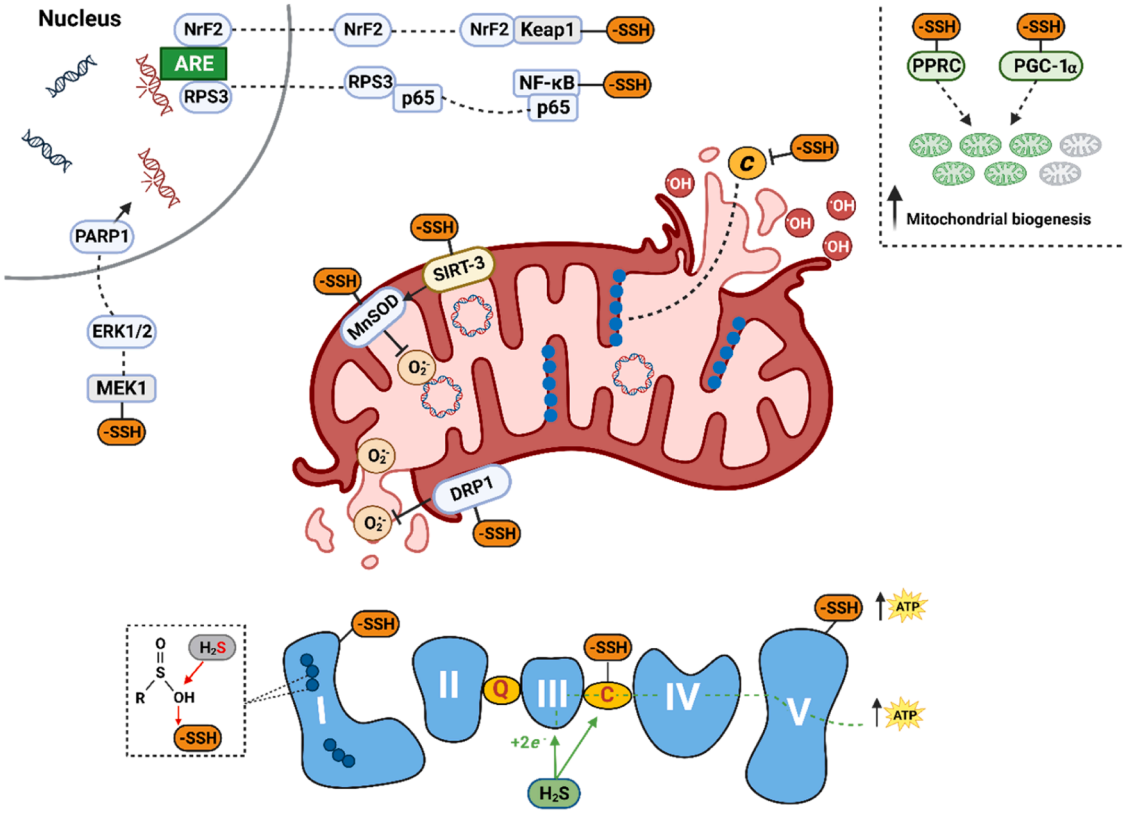

Fig. 1. Therapeutic effects of H_2S and protein *S*-persulfidation within mitochondria. Orange '-SSH' annotations denote direct *S*-persulfidation interactions of key cytoprotective proteins. *S*-persulfidation of kelch-like ECH-associated protein 1 (Keap-1) uncouples bound nuclear factor erythroid 2–related factor 2 (NrF2) allowing its translocation into the nucleus to upregulate anti-oxidant response elements (ARE). Similarly, *S*-persulfidation of the p65 subunit of nuclear factor kappa B (NF-κB) permits its binding to ribosomal protein S3 (RSP3) which translocates to the nucleus to upregulate ARE. Poly [ADP-ribose] polymerase 1 (PARP1) is a nuclear bound DNA repair enzyme that, through *S*-persulfidation of the mitogen-activated protein kinase signalling cascade MEK1/ ERK1/2, can be translocated to the nucleus to promote DNA repair. *S*-persulfidation of the peroxisome proliferator-activated receptor-γ coactivator-related proteins PPRC and PGC1-α can increase enzymatic activity and increase overall mitochondrial content. Direct *S*-persulfidation-induced mitochondrial protection includes *S*-persulfidation of cytochrome *c,* increasing its activity and inhibiting its cytosolic release; Mitochondrial superoxide dismutase 2 (MnSOD), increasing protein activity and reducing circulating mitochondrial ROS and subsequent mtDNA attack; Dynamin-related protein 1 (DRP1), dynamically regulating DRP1 and mitofusin (MFN1/2) expression ratios and ATP synthase, augmenting ATP production independent of chemiosmotic gradients. *S*-persulfidation of i.e, complex I cysteine residues (lower left) is a propositional mechanism, given the persulfidation of multiple mitochondrial cysteines and the known oxidation of complex I cysteines [127]. Additionally, H_2S donates two electrons to the ETC at the level of complex III, increasing chemiosmotic proton pumping and ATP production downstream of complexes I and II. *This figure was made using BioRender.com.*

PMD pathogenesis in cells.

Interestingly, H_2S treatments have displayed direct modulation of proteins involved in both mitophagic and biogenesis pathways. For example, 30 μM of NaSH increased H_2S levels (as detected by the methylene blue assay) and induced *S*-persulfidation (detected using the biotin-switch assay) of PGC1-α and the proliferator-activated receptor-γ coactivator-related protein (PPRC), mediating an increase in mitochondrial biogenesis and mitochondrial content in primary hepatocytes isolated from mice [105]. *In vitro* gene-silencing of both PGC1-α and PPRC abrogated the NaSH-mediated mitochondrial biogenesis effects and, although not assessed directly by the authors, could be a result of reduced PGC1-α and PPRC protein levels for NaSH-mediated *S*-persulfidation of these targets. Importantly, the mitochondria-targeting molecules AP39 and AP123 induced similar effects on mitochondrial biogenesis through PGC1-α upregulation in an *in vivo* mouse model of photoaging, however, with as little as 100 nM - 1 μM topical treatments (i.e, >1,000-fold lower compound dose) [92].

Separately, others note impaired mitophagy in diabetic rodent cardiomyocytes through loss of Parkin activity, which was restored by 80 μmol/kg (approximately 4500 mg/kg) of NaSH administration by intraperitoneal injection every 2 days for twelve weeks [149]. Specifically, *S*-persulfidation levels of the deubiquitinating enzyme USP8 were decreased in these mice, where NaSH exposures increased USP8 *S*-persulfidation and restored USP8/Parkin interactions facilitating mitophagy and the clearance of dysfunctional mitochondria. Together, these data suggest a multi-purpose role of H_2S in improving both mitochondrial quality and content, where H_2S treatments can simultaneously upregulate biogenesis and mitophagic pathways post-translationally in order to restore healthy mitochondrial environments.

8. Protein persulfidation and the prevention of cysteine over-oxidation

S-persulfidation is now well-established to be a primary mode of signalling by H_2S [13,14,16]. The post-translational addition of a sulfur group onto the cysteine thiol residues within proteins, can conformationally alter the structure and enzymatic activity of a protein, displaying a multitude of cytoprotective effects [13,16]. Additionally, the pK_a of persulfides is much lower than that of corresponding thiols in physiological conditions, making them a more favourable candidate for direct interactions with oxidative molecules, such as (at least) hydrogen peroxide (H_2O_2) [16] and peroxynitrite ($ONOO^-$) [14]. Whilst the reaction between H_2S and free thiols are chemically unfavourable [13], a strong affinity exists between H_2S and existing sulfenylated protein species, producing a persulfide in its place and cycling the sulfenylated cysteine back to a free thiol [150]. This interaction undoubtedly underlies a large proportion of the cytoprotective effects of H_2S-mediated signalling across phyla [14].

In PMD, elevated levels of reactive oxygen species (ROS) are a common feature [151], and are known to be drivers of disease pathology in some cases. Whilst ROS can provide essential messenger roles provoking adaptive protein responses in the face of cellular stress, superfluous levels can force the progression of mildly oxidised to irreversibly, over-oxidised proteins [14]. As such, protein cysteine residues are susceptible to a range of post-translational modifications, such as glutathionylation, nitrosation, prenylation, acylation, sulfenylation, sulfinylation and sulfonylation [152]. Although protein oxidation has been implicated in five of the most prevalent neurological PMD's [151], thiol modifications and their therapeutic targeting remain a heavily understudied topic in PMD.

Cysteine residues that have undergone protein sulfenylation (-SOH - the first step of H_2O_2-mediated thiol oxidation, see Fig. 2) will progress to irreversible sulfinylated ($-SO_2H$) and sulfonylated ($-SO_3H$) states in the presence of excessive ROS. However, the addition of reactive persulfide molecules onto -SOH (forming $-SSO_2H$) products allows for a protein 'rescue loop', whereby these products can be reduced back to free thiol states in the presence of sufficient reductants (such as the thioredoxin reductase pathway), limiting oxidative progression and protecting from loss-of protein functionality [50]. This concept has recently been extensively detailed [14]. The authors demonstrated cyclic increases in *S*-persulfidation levels in response to H_2O_2-mediated increases in protein sulfenylation by exploiting the redox sensitive protein, DJ-1 (PARK7). The addition of 100 μM of bolus H_2O_2 induced elevated *S*-persulfidation within minutes to (presumably) combat rising protein -SOH and $-SO_3H$. In cells lacking the primary H_2S producing enzyme CSE, *S*-persulfidation levels were reduced at baseline, and although levels of -SOH also continued to drop, this was met with progressive increases in protein sulfinylation and sulfonylation, confirming an inability of H_2S to trap sulfenylated adducts and limit over-oxidation.

Levels of *S*-persulfidation are known to decline with ageing across species [14], and certain neurodegenerative diseases display impairments in H_2S/persulfide production [49,153,154]. Additionally, pharmacological H_2S treatments can protect against ageing-related neurodegenerative disorders, in large part, by protein-persulfidation

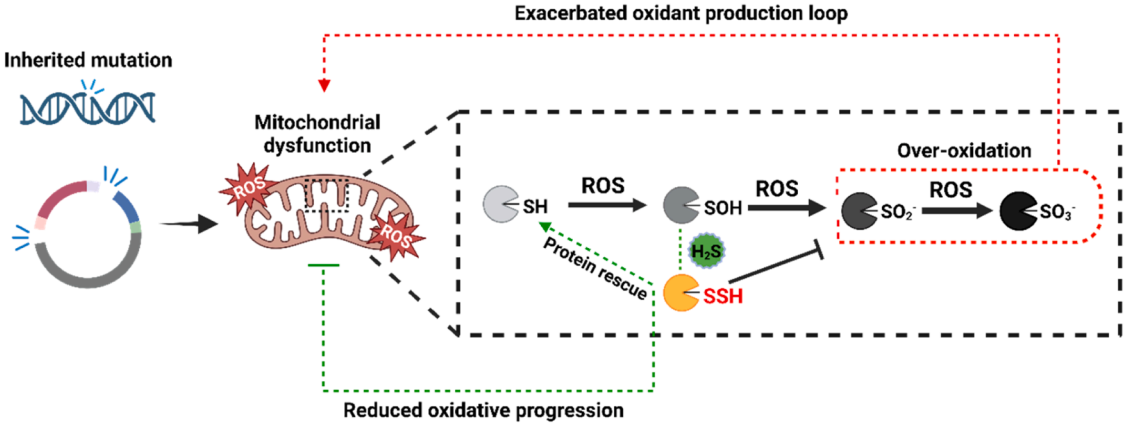

Fig. 2. The protective effects of persulfidation against protein oxidation in PMD. Inherited mutations in genes encoding for mitochondrial proteins cause ETC dysfunction, increase ROS production, induce membrane degeneration and ultimately cause cell death. Proteins within the mitochondrial environment harbour reactive cysteine residues susceptible to oxidative attack by superfluous ROS. Subsequently, the free thiol (-SH) adduct of these cysteines undergo conformational change *via* post-translational ROS signalling to sulfenylated (-SOH), sulfinylated ($-SO_2^-$) and sulfonylated ($-SO_3^-$) species in a progressive manner in situations of persistent ROS exposure. Typically, only -SOH is regarded as a reversible modification, with $-SO_2^-$ and $-SO_3^-$ deemed over-oxidised and non-functional proteins. In the presence of sufficient H_2S levels, -SOH adducts can undergo reversible modification back to free thiol states, avoiding further cysteine oxidation and restoring protein function. In turn, further oxidative stress through positive feedback with mitochondria are reduced [14]. This figure was made using BioRender.com.

protective mechanisms [49,153,155,156]. Therefore, exploring the effects of H_2S treatments in modulating PMD-mediated protein oxidation seems of critical importance, and may hold significant therapeutic potential given the strong oxidative environments these diseases present. Administration of pharmacological H_2S could offset or delay the time to irrecoverable protein oxidation in PMD, therefore, retaining/augmenting protein function of key health-regulating pathways. Specifically, mitochondria are the primary site of pathology in PMD and, also, produce some of the largest sources of endogenous ROS production. Thus, pharmacological H_2S could be a therapeutic strategy to protect from (excessively) oxidised mitochondrial environments seen in PMD, potentially augmenting constituents of, and whole systems apparatus involved in mitochondrial function.

9. Mitochondrial function and *S*-persulfidation of key proteins within the electron transport chain

9.1. Site-specific mitochondrial electron transport chain reduction

The PMD patient phenotype displays severely compromised function of metabolically demanding organs and tissues, for example, skeletal muscle, brain and neurons [157]. Unsurprisingly, mitochondrial respiration and subsequent ATP production is perturbed in the majority of PMD pathologies, underlying the metabolic deficit in the patient [157]. Mitochondrial electron donation and site-specific post-translational regulation of precise ETC proteins offers an interesting therapeutic modality for H_2S in PMD. Namely, the severe neurological disorder Leigh syndrome is amongst the most common forms of PMD, arising predominantly from mutations in nuclear DNA that encode mitochondrial complex I subunits [158]. This impaired complex I function affects the downstream chemiosmotic reduction of mitochondrial subunits, reducing electron flow, destabilising mitochondrial membrane potential as well as destabilisation of supercomplex formations with downstream respiratory subunits [159]. Importantly, H_2S can enter the ETC at the level of complex III, contributing two electrons to the ETC *via* sulfide: quinone oxidoreductase (SQOR) oxidation [75]. This contribution to mitochondrial respiration can increase electron flow and ATP production in cases of impaired ETC activity [75]. However, more importantly, the site of reduction is of significant utility for upstream PMD mutations such as complex I in Leigh syndrome and complex II deficient pathologies, potentially able to bypass the defective subunit and restore ETC function. In fact, a complex I-deficient *C. elegans* model of Leigh syndrome displayed improved survivability, healthspan and increased ATP production with (100 nM) AP39 administration throughout development, suggesting H_2S can act downstream of complex I to restore mitochondrial function [19]. Further isolated models of this proposition have been shown previously (albeit, with non-targeted salt-based compounds such as Na_2S), utilising complex I and II inhibitors with H_2S co-treatments, showing a restoration of mitochondrial function [160,161].

9.2. Post-translational complex I interactions – a key defective protein in aggressive PMDs

The ability of H_2S to persulfidate free thiols within proteins and augment protein activity represents an attractive method to either i) augment enzymatic activity within defective, mutated proteins and/or ii) modulate the activity of proteins downstream of mutation sites. For example, the large (~1MDa) complex I protein within the ETC contains at least 45 subunits, rich in reactive cysteine residues [162]. Inherited mutations in approximately 8 of these nuclear encoded subunits are known to cause Leigh syndrome [163], where the *S*-persulfidation of an available cysteine residue within either the defective subunit, or a neighbouring subunit(s), could prevent the loss of functionality and improve overall complex I function. Interestingly, oxidation of 34 from 130 complex I cysteines have been previously reported in a Parkinsonian mouse model [162], highlighting the ability of small reactive species to modulate complex I cysteine residues. More so, protein *S*-persulfidation of cysteine adducts are dependant on existing thiol oxidation [14,164], where direct reaction of H_2S with free thiols is chemically unfavourable, however, displays a strong affinity with oxidised modifications. In support, mapping of the mitochondrial cysteinome has identified over 1500 cysteine residues from ~450 proteins [165], further highlighting the rich environment for H_2S-mediated *S*-persulfidation of a multitude of mitochondrial proteins. With over 1000 sulfenylated species identified across a multitude of proteins [166], and the strong affinity of H_2S to react with sulfenylated protein adducts, protein *S*-persulfidation and its protein activity-enhancing/protecting effects could provide widespread protection within PMD environments. More importantly, the *in vivo* efficacy of, and modulation in mitochondrial dynamics from mitochondrial sulfide-delivery molecules (i.e., AP39 and AP123; detailed above) provides an option for targeting H_2S to cysteine-rich mitochondrial environments using physiologically relevant compound doses.

9.3. S-persulfidation of downstream ETC proteins

Persulfide-mediated modulation of proteins downstream of complex I (i.e., complexes II, III, IV and V) could be beneficial for pathologies such as Leigh syndrome, but also a wider patient reach. For example, *S*-persulfidation of cytochrome *c* oxidase has been reported to inhibit caspase 9-induced apoptosis *via* a downregulation of caspase 9 activity [18], suggesting an improvement in mitochondrial membrane integrity and cell survival. The authors also showed that pharmacological H_2S was able to reduce cytochrome *c* and contribute to mitochondrial respiration, confirmed by increased oxygen consumption in colonic HT29 and hepatic HepG2 cells treated with a complex III inhibitor (antimycin A) and 20 μM Na_2S. Indirectly, this suggests a potential mechanism for restoring mitochondrial function in PMD's defective upstream of cytochrome *c* (i.e., harbouring deleterious mutations in complex I, II or III), whereby H_2S-mediated *S*-persulfidation of cytochrome *c* could restore diminished electron flow, inhibit apoptosis signals and restore mitochondrial respiration downstream of these subunits (see Fig. 3). Importantly, the authors demonstrate AP39 was able to induce significant cytochrome *c* *S*-persulfidation in HeLa cells with a clinically meaningful exposure of 200 nM [18].

Lastly, in order for mitochondria to produce sufficient biochemical energy, the ATP synthase/complex V subunit harnesses the electrochemical energy produced from proton pumping *via* complexes I-IV to drive rotor turning and synthesis of ATP. Diverse PMD phenotypes with inherited ATP synthase mutations have been detailed previously [167], where ATP deficits of up to 90% can be seen, culminating in severe neurological and neuromuscular pathologies. Interestingly, Módis et al. demonstrated Na_2S-induced *S*-persulfidation of the α subunit (ATP5A1) of ATP synthase at cysteines Cys244 and Cys294 in HepG2 and HEK293 cells which increased ATP synthase activity and ATP production [17]. This finding represents a key therapeutic modality for H_2S treatments in PMD: firstly, if inherited mutations within the ATP synthase subunit do not compromise the structure of these two highly conserved cysteine residues, H_2S-mediated *S*-persulfidation of these sites could augment protein activity and subsequent ATP production in PMD patients with direct complex V mutations. Secondly, as eluded earlier, PMDs with mutation sites that precede complex V may benefit from this downstream effect, bypassing the defective subunits to restore mitochondrial function. Similarly, impaired function of complexes I, III and IV can all effect sequential proton pumping, thus, decrease the electrochemical energy required to drive ATP synthase rotors. Direct modulation of complex V activity would, therefore, be an attractive modality for augmenting mitochondrial function in PMD.

10. Upregulation of key cytoprotective pathways

Previous evidence has implicated H_2S as a direct oxidant scavenger

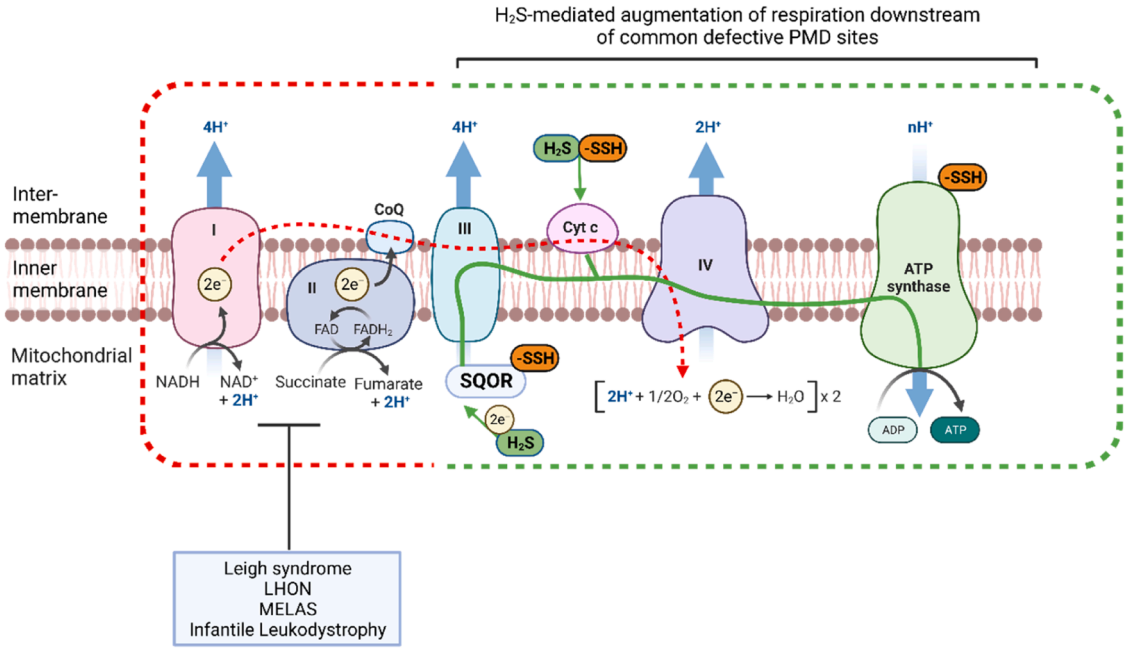

Fig. 3. H_2S-mediated restoration of mitochondrial function downstream of common defective mutation sites that impair proton-motive forces. Genetically inherited mutations in mitochondrial subunits upstream of complex III disturb normal electron flow and resultant proton pumping for ATP generation via ATP synthase. Pharmacological supplementation of H_2S reduces mitochondria at the level of complex III (via sulfide:quinone oxidoreductase; SQOR) and complex IV (via cytochrome C; Cyt c), re-establishing electron flow through the ETC and increasing ATP production. Thus, PMDs upstream of complex III could benefit from a downstream restoration of energy production that bypasses the dysfunctional subunits. Additionally, PMDs with mutations in complexes III, IV and V themselves could also benefit from the *S*-persulfidation of the terminal complex V subunit that is known to increase ATP production [16] and likely, protein stability [14,15]. *This figure was made using BioRender.com.*

and anti-inflammatory. However, given the low concentrations of H_2S within biological systems compared to thiols (several orders of magnitude lower in some cases), the ability to outcompete thiols as oxidant scavengers is unfavourable, unless present in extremely high concentrations [13]. Unsurprisingly, those reports for direct scavenging frequently utilise supra-physiological bolus administrations (~100–500 μM) of fast release H_2S donors (i.e., NaSH, Na_2S) [168]. Therefore, it is likely that pharmacological H_2S treatments act *via* indirect mechanisms to reduce pernicious oxidative stress as opposed to direct quenching of radical molecules. In fact, recent evidence supports pharmacological H_2S-mediated upregulation of endogenous anti-oxidative machinery that, in turn, acts to reduce oxidative environments [14]. Similarly, our understanding into the post-translational effects of H_2S-mediated protein interactions (*via S*-persulfidation) is developing, where growing evidence suggests intracellular H_2S can interact with oxidised proteins adducts (i.e – sulfenylated and sulfinylated terminal cysteines within proteins) to inhibit oxidation progression [14,50,164,169]. Moreover, these mechanisms might provide insight into why antioxidants (i.e., idebenone, MitoQ, *N*-acetylcysteine) prove largely ineffective in PMD clinical trials.

Importantly, many of the pathways with known targeting by pharmacological H_2S are perturbed in PMD and can be drivers of disease progression. Here, we will detail the primary cytoprotective pathways with known *S*-persulfidation regulation that could aid in delaying PMD degeneration.

10.1. Keap1/ Nrf2

Nuclear factor-erythroid factor 2-related factor 2 (Nrf2) is a transcription factor responsible for upregulating crucial cell protective genes in cases of oxidative stress. It exists in the cytoplasm bound to Kelch-like-ECH-associated protein 1 (Keap1) which controls its fate through ubiquitination [170]. The presence of reactive oxidants can disrupt cysteines 273 and 288 on Keap1, allowing the dissociation of Nrf2 into the nucleus to bind to and upregulate the expression of anti-oxidant response element (ARE) genes [171]. Importantly, Nrf2 is also responsible for regulating glutathione (GSH) production through glutathione reductase, a central governor for cycling post-translational modifications of cysteines from sulfenic acids back to free thiols and preventing over-oxidation [172]. Crucially, Nrf2 function is compromised in multiple PMD's, including Leigh syndrome [173], Friedreich ataxia [174–178] and inherited Parkinson's disease [179], highlighting the importance of restoring Nrf2 function to offset oxidant-induced disease progression.

With accessible cysteine residues on Keap1, H_2S is now a known post-translational regulator of Nrf2. Administration of NaSH in CSE knockout mice showed *S*-persulfidation at cysteine-151, allowing dissociation of Nrf2 from Keap1 and nuclear translocation to upregulate ARE genes [180]. *S*-persulfidation of cysteine-151 on Keap1 has also been demonstrated separately in liver injury [181], diabetic-induced atherosclerosis [182] and microplastic induced inflammation [183] conferring protection through Nrf2 upregulation and accumulation. Similarly, both AP39 and AP123 (from 20 to 500 nM treatments) were recently shown to attenuate UV-induced photoaging partly by an upregulation and accumulation of Nrf2 within the nucleus, both in human dermal fibroblasts and in an *in vivo* topical treatment model in mice [92]. With Nrf2 function impaired in PMD, pharmacological H_2S could provide an excellent drug candidate for post-translational augmentation of NrF2 function, with positive downstream effects on other crucial antioxidant systems (e.g., GSH for regulating thiol cycling).

10.2. SOD2/MnSOD

Superoxide dismutase 2 (SOD2/ MnSOD) is a mitochondria-localised protein responsible for regulating levels of superoxide ($O_2^{\cdot-}$), capable of converting $O_2^{\cdot-}$ to H_2O_2 and oxygen (O_2). With the vast majority of mitochondrial ROS produced by complexes I and III [184], macromolecular damage to mtDNA can occur if oxidant levels go unchecked,

whereby mtDNA reside in close proximity to these ETC subunits. In fact, reductions in SOD2 expression and/or activity have been noted in cases of human PMD. For example, fibroblasts with pathogenic mutations in complex I of the ETC from Leigh syndrome patients displayed decreased SOD1 and SOD2 activity, subjecting cells to increased oxidative stress [185]. In accordance, reports of a patient suffering from 3-methylglutaconic aciduria displayed decreased levels of SOD2, increasing both cytosolic and mitochondrial levels of ROS, with H_2O_2 production to almost double that of control cells [186].

Utilising human recombinant MnSOD, Zivanovic et al. demonstrated that short-term exposure to H_2O_2 was sufficient to impair MnSOD activity *in vitro* [14]. Interestingly, co-incubation with pharmacological H_2S induced *S*-persulfidation at the redox-sensitive cysteine C193, preventing H_2O_2-induced MnSOD inactivation. *S*-persulfidation at this site rendered MnSOD more resilient to nitration/oxidation by peroxynitrite and H_2O_2, respectively – a common feature amongst PMD pathologies [187]. In support, H_2S-induced *S*-persulfidation of mitochondrially localised SIRT-3 can induce deacetylation of MnSOD [188], a process known to control MnSOD's enzymatic activity *via* alterations in lysine acetylation [189]. Additionally, mouse skin pre-treated with 0.3–1 μM/cm^2 of AP39 and AP123 prior to irradiation stress showed significantly upregulated MnSOD protein levels, implicating H_2S-mediated upregulation of MnSOD in an *in vivo* model of severe oxidative stress. Together, these data suggest H_2S could provide post-translational regulation of impaired MnSOD activity in PMD patients suffering from extreme mitochondrial ROS production. Indeed, if the required cysteine residue for H_2S-mediated MnSOD *S*-persulfidation is present in patient tissues, direct upregulation of enzymatic activity could be possible. Opposingly, structural degradation of the required site, thus, impaired persulfidative ability from H_2S, could in theory be bypassed by SIRT-3 persulfidation that in turn, could augment MnSOD activity.

10.3. PARP1

PARP-1 is an NAD^+-dependant DNA damage repair gene that regulates first-response detection and facilitation of repair pathways (reviewed extensively previously[190]). Despite significant debate, this once thought nuclear DNA-specific gene has been identified in mitochondrial extracts [191], whilst others suggest PARP-1-mediated repair of mtDNA is regulated from the nucleus, increasing the expression of nuclear genes that encode for mtDNA repair factors [192]. Irrespectively, as distinctly genetically based disorders, PMD unsurprisingly comes hand-in-hand with significant damage to both nuclear and mitochondrial DNA. Whilst PARP1 activity is unlikely to repair underlying disease-causing genetic configuration, it may offer opportunistic avenues to ameliorate genetic damage caused by the disease phenotype (i.e., excessive oxidative damage to nuclear and/or mitochondrial DNA).

PARP1 activation is regulated by multiple kinases, including the MEK/ERK signalling cascade [193], a MAPK-related signalling pathway involved in cell proliferation and survival [194]. Interestingly, *S*-persulfidation of cysteine 341 within MEK induces phosphorylated ERK1/2 translocation into the nucleus in human umbilical endothelial cells with 10 μM of NaSH exposure, where it binds directly with PARP1 to recruit DNA damage repair processes and protects from cell death [195]. Also, levels of NAD^+ are frequently reduced in situations of mitochondrial dysfunction e.g., mitochondrial disease [196] and ageing [197]. Therefore, as a key substrate for PARP1 function, post-translational modification *via* *S*-persulfidation of the MEK/ERK/PARP1 pathway could bypass compromised NAD^+ levels and maintain PARP1 activity in settings of PMD.

10.4. Parkin

Mutations in Parkin are the most common cause of recessive early-onset Parkinson's disease [198], however, its wider roles in targeted protein ubiquitination and mitophagy render its function crucial across multiple PMD's. Both proteasomal-linked ubiquitination and mitophagy are dysregulated in PMD disorders [199–201], proposing potential targets for therapeutic regulation of Parkin and affected pathways.

Decisive work of Snyder and colleagues demonstrated H_2S-mediated *S*-persulfidation of cysteines C59, C95 and C182 within Parkin caused significant increases in Parkin activity [154]. To add, *S*-persulfidation levels in brain matter of Parkinson's patients were diminished, where elevations in cysteine nitrosation (indicative of nitrosative stress) occurred. Interestingly, the site of Parkin nitrosation occurred at the same cysteine residues as *S*-persulfidation, suggesting nitrosation is pathogenic and *S*-persulfidation protects against thiol oxidation [14]. Additionally, *S*-persulfidation of Parkin could prove therapeutic outside of recessive Parkinson's disease, where increased Parkin activity would aid in covalent labelling of defective proteins with ubiquitin for subsequent degradation of damaged cellular material.

10.5. NF-κB

Nuclear factor kappa B (NF-κB) is a key cytoprotective transcription factor that regulates cell survival and cytokine production in situations of stress [202]. held in the cytoplasm by suppression factors that when phosphorylated, subjects them to degradation and allows NF-κB mobilisation to the nucleus [203]. NF-κB interacts with ribosomal protein S3 (RPS3; a subunit of NF-κB) for nuclear translocation and regulation of anti-apoptotic genes. The NF-κB pathway is a key signalling event to protect in settings of inflammation [202], a common molecular phenotype across multiple PMD's driving disease progression [204–206]. Recently, the expression of NF-κB-regulating proteins were increased in a mouse model of Leigh syndrome [207], suggesting its mobilisation is a protective response to pernicious cellular alterations in Leigh syndrome.

H_2S treatments, including mitochondria-targeted approaches [32, 208] have frequently been linked to anti-inflammatory effects, with limited mechanistic understanding [209]. Whilst some of these data may be misleading - given the widespread adoption for bolus administration of impure sulfide salts (see above) - post-translational regulation of the NF-κB pathway may partly underlie the role of pharmacological H_2S in inflammation. Sen and colleagues showed that endogenous H_2S levels tripled in response to inflammation-inducing TNF-α exposure in peritoneal macrophage cells [210]. In turn, cysteine 38 of the p65 subunit of NF-κB was endogenously persulfidated, inducing its binding to RPS3 and repressing apoptotic progression. Conversely, CSE knockout mice showed ablated H_2S responses to TNF-α and displayed elevated nitrosation of NF-κBs p65 subunit, further expanding the requirement of H_2S/persulfidation in protecting reactive cysteine residues (thus protein function) in key anti-inflammatory/anti-apoptotic proteins.

11. Protection against an oxidised epitranscriptome

Beyond H_2S's ability to protect/regulate protein function through protein *S*-persulfidation, the process of RNA thiolation (the addition of a sulfur molecule onto nucleotides) is seemingly overlooked. Whilst extensive literature surrounds DNA oxidative damage in settings of PMD, little attention is given to oxidative modifications of RNA molecules, despite RNA accounting for 80–90% of the cells total nucleic acid [211]. Since DNA is double-stranded with complementary nucleotide bases protected by hydrogen bonds, their structure is relatively stable, whereas single-stranded RNA is more unstable and subject to degradation [211]. Whilst mRNA is short-lived and degraded almost immediately after translation, tRNA and rRNA are stable and exist for a long time. Importantly, where protein translation is an extremely energetically expensive task for the cell, dynamic regulation of translational flux (in both cytosolic and mitochondrial compartments) is crucial to maintaining homeostasis. Critically, protein translation is impaired in many cases of PMD [212], and could be a result of over-oxidised RNA molecules that hinders translational fidelity (see Fig. 4).

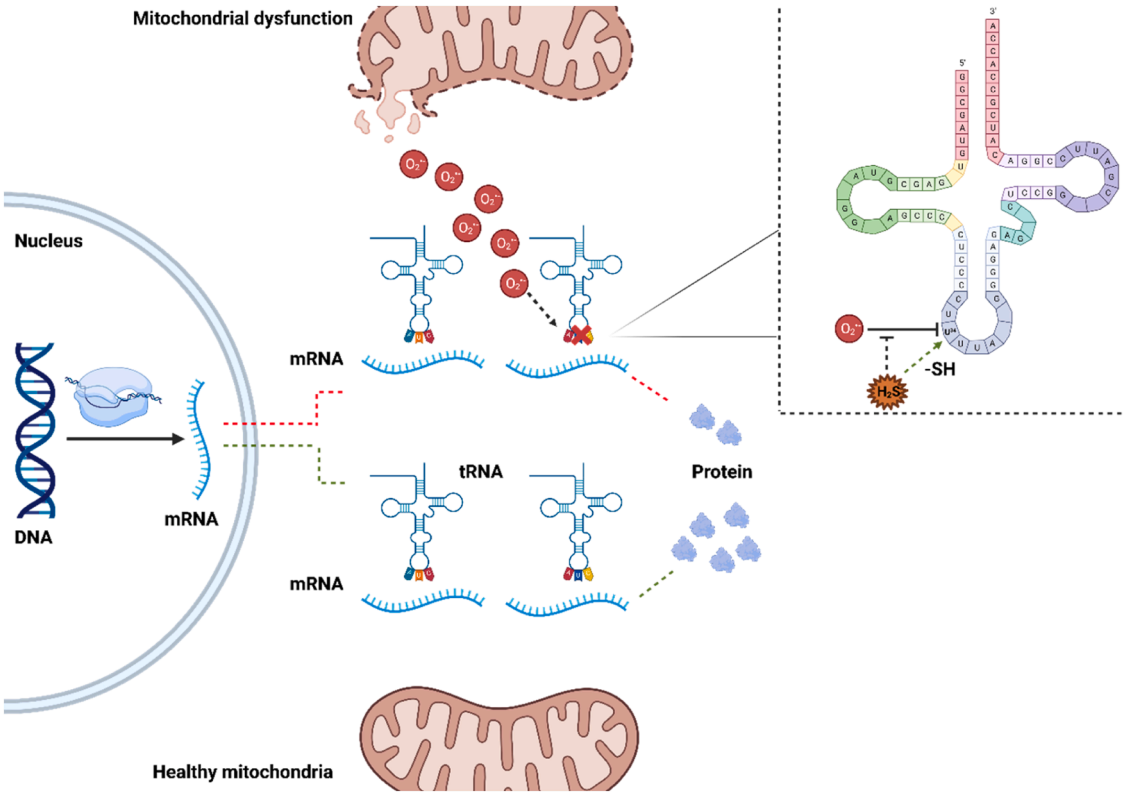

Fig. 4. Mitochondrial disease perturbs the epitranscriptome. Healthy mitochondrial environments in non-disease states allow normal transcriptional processes, where nucleotides within tRNA's are capable of binding to the mRNA reading frame for successful protein translation (bottom portion). Conversely, in settings of elevated oxidant production (i.e., PMD, top portion), the U_{34} region of tRNA undergoes oxidation that can impair translation fidelity and hinder protein expression. tRNA thiolation is a crucial modification for maintaining metabolic homeostasis, representing a therapeutic window for pharmacological H_2S interventions to potentially restore tRNA thiolation. *This figure was made using BioRender.com.*

Ample data supports the concept of RNA (mRNA and tRNA) oxidation that is caused by excessive ROS exposure across species. For example, translational capacity was reduced in *E. coli* exposed to high (4 mM) H_2O_2 concentrations, impairing the activity of theronyl-tRNA synthase limiting bacterial growth [213]. Equally, up to 50% of mRNAs (measured by semi-quantitative RT-PCR) from brain tissue of Alzheimer's disease patients displayed elevated oxidative damage [214], an event that was later shown to cause a decrease in protein expression [215]. In fact, the majority of literature surrounding RNA oxidation shows a common theme towards neurodegenerative disorders – an important note for the context of specific forms of neurodegenerative PMD's (i.e., Leigh syndrome, Friedreich's ataxia). Crucially, impairments in tRNA thiolation have been implicated in a range of mitochondrial diseases [216–220] as well as familial dysautonomia [221] and myoclonic epilepsy with ragged-red fibres [222]. With both mitochondrial tRNA and cytosolic tRNA known to exhibit RNA oxidation across diverse PMD's [223], therapeutic interventions that could ameliorate RNA insult could improve protein translation and, thus, reduce pathological outcomes.

In eukaryotes, the wobble uridine (U_{34}) of three tRNAs specific for lysine ($tRNA^{Lys}_{UUU}$), glutamate ($tRNA^{Glu}_{UUC}$), and glutamine ($tRNA^{Gln}_{UUG}$) possess a methoxycarbonylmethyl (mcm^5) group at position 5 and a thiol modification (s^2) at position 2 [224]. The addition of a thiol group (termed thiolation) onto U_{34} has been shown to prevent ribosomal frameshifting [225] and ensure accurate protein translation at an optimal rate [226], suggesting this modification acts to improve translational accuracy. Although little work has been done on pharmacological H_2S interventions and RNA modifications, previous work showed that sulfur amino acid treatments restored tRNA thiolation [227], and that sulfur metabolism pathways control thiolation status – a pathway recently shown to be downregulated in the genetically inherited muscle disease DMD [90]. Altogether, RNA oxidation and restoration *via* thiolation represents a largely neglected aspect of pharmacological PMD research. Where substrates of H_2S synthesis have been shown to restore tRNA thiolation (an important event for amino acid homeostasis [227]), pharmacological H_2S compounds (particularly, mitochondrial donors for mitochondrial tRNA) could ameliorate RNA damage occurring from PMD pathology and should be investigated further.

12. Conclusions and directions for drug exploration

Despite its known stimulatory and cytoprotective roles within mitochondria, H_2S has been largely overlooked as a potential therapeutic treatment for PMD. H_2S can directly stimulate mitochondrial respiration, bypass PMD-defective ETC subunits to uphold mitochondrial function and post-translationally modify the activity and stability of cysteine containing proteins, all candidate therapeutic mechanisms for these heterogeneous diseases. We recommend that future research into H_2S treatments in PMD focus on utilising targeted compounds with more clinical likelihood (i.e., mitochondria-targeted H_2S compounds), in order to test more physiologically relevant doses of H_2S that subjugates any uncertainties that arise from supra-physiological H_2S exposures. These targeted donors can provide potent electron reduction and post-translational regulation of mitochondria at doses orders of magnitude lower (from 1000 – 10,000-fold) than conventional H_2S salt-based donors. Utilising these compounds reduces the need for exposures that could be cytotoxic if translated into the clinic. As such, development/use of more clinically appropriate molecules that encompass more acceptable drug-like properties such as Lipinski [228], Veber compliancy [229] and compound solubility need to be more widely considered if the therapeutic potential of H_2S in PMD is to progress. Additionally, exploration of these novel compounds in pre-clinical models should be

of priority, as the molecular mechanisms underlying salt-based compounds could be distinct from those effective at physiologically relevant doses.

Beyond PMD, mitochondrial dysfunction is an appreciated hallmark of ageing [230], and declines in transcriptional and proteome-wide mitochondrial signatures, as well as functional capacity, is a feature of ageing across species. Similarly, mitochondrial dysfunction is implicated in numerous conditions beyond inherited mutations in mitochondrial genes, such as sarcopenia and muscular dystrophy [20,90,197], diabetes [231] and obesity [232] - major burdensome contributors on healthcare systems. More recently, mitochondrial dysfunction has been implicated in the severity of COVID-19 symptomology [233], and may play roles in the development of long COVID [234]. Thus, exploring clinically relevant H_2S treatments in mitochondrial pathologies beyond PMD could provide new, unexplored treatment options for age-related and infection-induced conditions. The acceleration of drug development for use in rare/orphan diseases could provide valuable human clinical data to establish the safety and efficacy of these new drugs for use in wider mitochondria-associated pathologies. Given the high doses of non-targeted sources of sulfide (e.g., GYY4137, NaSH), putative natural sources of sulfide (DATS, DADS) and existing drugs (thiosulfate) required to elicit any therapeutic effect, future directions should focus on evaluating mitochondria-targeted sulfide delivery molecules with drug-like characteristics and potency at several orders of magnitude greater than these non-targeted approaches, limiting off-target effects and toxicity. Importantly, these experiments could begin to pave the way for taking mitochondrial H_2S molecules into the clinic.

CRediT authorship contribution statement

Luke Slade: Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Colleen Deane:** Writing – review & editing. **Nathaniel Szewczyk:** Writing – review & editing. **Timothy Etheridge:** Writing – review & editing, Funding acquisition, Conceptualization. **Matt Whiteman:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of Competing Interest

M.W. has intellectual property (patents awarded and pending) on slow-release sulfide-generating molecules and their therapeutic use. M. W. is CSO of MitoRx Therapeutics, Oxford, U.K, developing organelle-targeted molecules for clinical use. L.S, C.S.D, N.J.S and T.E declare no competing interests.

Data availability

This review article contains no datasets generated or analysed during the current study

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