Evaluating The Potential of Hydrogen Sulfide to Improve Health with Age and Spaceflight

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Abstract

Ageing can be a debilitating process, reducing an organism's ability to function and interact with its environment. With the world's population increasing there is a pressing need to understand and augment the ageing process, allowing the elderly to remain functional for as long as possible. Ageing and its related diseases are closely associated with mitochondrial dysfunction. This thesis explored using novel hydrogen sulfide (H_2S) compounds to maintain the mitochondria throughout the lifespan of *C. elegans*.

Firstly, mitochondrially targeted H_2S was compared to H_2S without targeting. The compounds were administered to juvenile worms with movement rate and survival observed, to match similar H_2S experiments published. In addition to this the mitochondrial network fragmentation state and mitochondrial content was analysed. While both compounds improved lifetime movement rate, survival, and mitochondrial network state, mitochondrial targeting consistently expressed the largest elevation and was the only condition to also preserve mitochondrial content.

Secondly, both compounds were administered with delayed starting time points, starting from young adulthood, 2^{nd}, or 4^{th} day of adulthood. This was to simulate using the compounds as an ageing intervention rather than a lifetime supplement. Again, mitochondrial targeting showed the more pronounced benefits for lifetime movement rate, however neither compound affected survival. This shows an increase in the healthspan of the animals keeping them functional for longer.

Finally, having shown that H_2S improved the ageing symptoms terrestrially, mitochondrially targeted H_2S was administered to *C. elegans* during an accelerated ageing model using microgravity in spaceflight. The gene expression data provided evidence that untreated microgravity upregulated genes associated with energy generation, metabolism, and mitochondria. These are contributing factor to high oxidative damage and mitochondrially targeted H_2S was able to downregulate many of these genes, helping to

alleviate the damage. Microgravity also downregulated genes associated with calcium signalling, cell adhesion, ion transport and neural receptors. These factors contribute to motor control and other neurological declines. H_2S was able to upregulate many of these genes.

Overall, this thesis highlights that mitochondrially targeted H_2S maintained mitochondrial function throughout ageing and kept whole organisms functional for longer. This mitochondrial based H_2S is a viable pathway to be explored and exploited for ageing therapeutics for both natural and accelerated ageing models.

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Accomplishments

Publications

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Sudevan, S. Takiura, M. Kubota, Y. Higashitani, N. Cooke, M. Ellwood, R.A. Etheridge, T. Szewczyk, N.J. and Higashitani, A. (2019) Mitochondrial dysfunction causes Ca2+ overload and ECM degradation–mediated muscle damage in C. elegans. *Faseb*, 33, 9540-9550

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Poster Presentations

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Cooke, M. Deane, C, S. Pollard, A. Gaffney, C, J. Philips, B. Etheridge, T. and Szewczyk, N, J. (2018). C. elegans as a model for biology in space. Poster. The Physiological Society. Experimental Models in Physiology, Exeter, UK

Cooke, M. Gaffney, C, J. Torregrossa, R. Wood, M, E. Szewczyk, N, J. Whiteman, M. and Etheridge, T. (2019). Mitochondria-targeting hydrogen sulfide donors prolong healthspan in C. elegans. Poster. CLESCon 2019. Exeter, UK

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Cooke, M. Sinclair, G. Shamsul, A. Bolliand, Martinez de Bujo, M. A. Chabi, A. Zalasiewicz, M. Etheridge, T. and Cullen, D. (2019). BAMMsat on BEXUS proposal. ESTEC Selection Workshop, ESTEC, Netherlands.

Cooke, M. Slade, L. Gaffney, C. Torregrossa, R. Paling, B. Ellwood, R. Vanapalli, S. A. Szewczyk, N. J. Whiteman, M. and Etheridge, T. (2019). Mitochondria-targeting hydrogen sulfide extends life- and health-span in C. elegans: implications for primary mitochondrial disease. Mitochondrial Medicine Conference 2019. Cambridge, UK.

Cooke, M. Sinclair, G. Shamsul, A. Bolliand, Martinez de Bujo, M. A. Chabi, A. Zalasiewicz, M. Lucero, J. Tonina, T. Etheridge, T. and Cullen, D. (2020). BAMMsat on BEXUS. European virtual worm meeting 2020, Online.

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Travel Grant – ESA – BEXUS – 2019

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1. Chapter One: General Introduction

This thesis proposes to study the effect of supplementing H_2S on health with age and spaceflight. Therefore, in this introductory chapter will generally review the biology of H_2S, ageing, and spaceflight. More specific introductions to the individual studies will be provided in each study chapter.

1.1. Hydrogen Sulfide

1.1.1. History

The toxic effects of H_2S have been known since its discovery in 1777 and was termed a toxic pollutant, associated with causing disease states for most of its known existence, with early experimentation investigating toxicity in animal models (*1*). These experiments have led to a clear view of how varying concentrations of H_2S affect the body and produce clinical symptoms (*2*). At low levels, 0.01-3 parts per million (ppm) the human nose can detect an odour and at 1-5 ppm this odour is referred to as moderately offensive and has clinical effects on the body showing symptoms, including nausea, tearing of the eyes, headaches, and reduction in sleep (*2*).

The physiological significance of H_2S's chemical properties are important, as it appears to be conserved across many organisms, suggesting evolutionary conservation. This may stem from H_2S being a major atmospheric component 4 billion years ago, in an environment with no to little free atmospheric oxygen to use for energy production (*3, 4*). Due to this it is likely that H_2S had a large impact on early life and was used as an energy source (*5*). This form of energy production is known as lithotrophy and involves oxidation of reduced inorganic compounds, including H_2S, and the reduction of electron acceptors such as sulfur, or sulfate, this is a mechanism conserved in many present-day organisms, including mammals and *C. elegans (5-7)*.

This impact on early life has been retained and provides mechanisms for H_2S detoxification. One of these mechanisms to detoxify in a high H_2S concentration is for the organisms to use a class of enzymes called cysteine synthase (CYS), cysteine synthase-like 1 (*cysl-1)* produces a protein able to bind the H_2S with O-acetyl serine to form cysteine and acetate (*8*). A second mechanism for H_2S detoxifying is through *sqrd-1*, a homolog of succinate-coenzyme Q reductase (SQR) or sulfide-quinone reductase (*8*). A third gene used in detoxification is *ethe-1* used to produce sulfur dioxygenase and required for the oxidation of sulfur (S^0) to sulfate (SO^{2-}) (*8*). Sulfur dioxygenase proposed to de-persulfidate low molecular weight thiols such as glutathione or dihydrolipoate within the mitochondrial matrix, which would then allow free H_2S to persulfidate these molecules and lower the concentration (*9*).

Recent evidence has shown that low H_2S concentrations are beneficial to animal life and produced endogenously (*10, 11*). Under physiological conditions, H_2S forms a pool of H_2S, hydrosulfide (HS) and sulfide ions (S^{2-}) (Equation 1-1). The combination of all three parts of the equation is referred to as free sulfide pool. This ability to donate protons (hydrogen ions, H^+) means H_2S is an acid and when dissolved in water, under normal physiological pH (pH 7.4), only a third remains intact but is permeable through plasma membranes due to its affinity to lipophilic solvents (*10, 11*). These properties, along with its ability to be utilised as a regulated receptor-independent signalling molecule, without the need for secondary messengers, allows H_2S to be termed as the third gasotransmitter, joining nitric oxide (NO) and carbon monoxide (CO) (*10*).

$$H_2S \Leftrightarrow H^+ + HS \Leftrightarrow 2H^+ + S^{2-}$$

Equation 1-1. Dissociation of hydrogen sulfide into hydrosulfide (also called bisulfide) and sulfide.

1.1.1. In vivo Hydrogen Sulfide Production

As H_2S had such a substantial impact on early life, it is unsurprising that it still influences modern life in complex organisms, with humans and *C. elegans*

producing H_2S endogenously (Figure 1-1). In mammals, the majority of this is through key enzymes, cystathionine beta synthase (CBS) and cystathionine gamma lyase (Cystathionase - CSE) (*11, 12*).

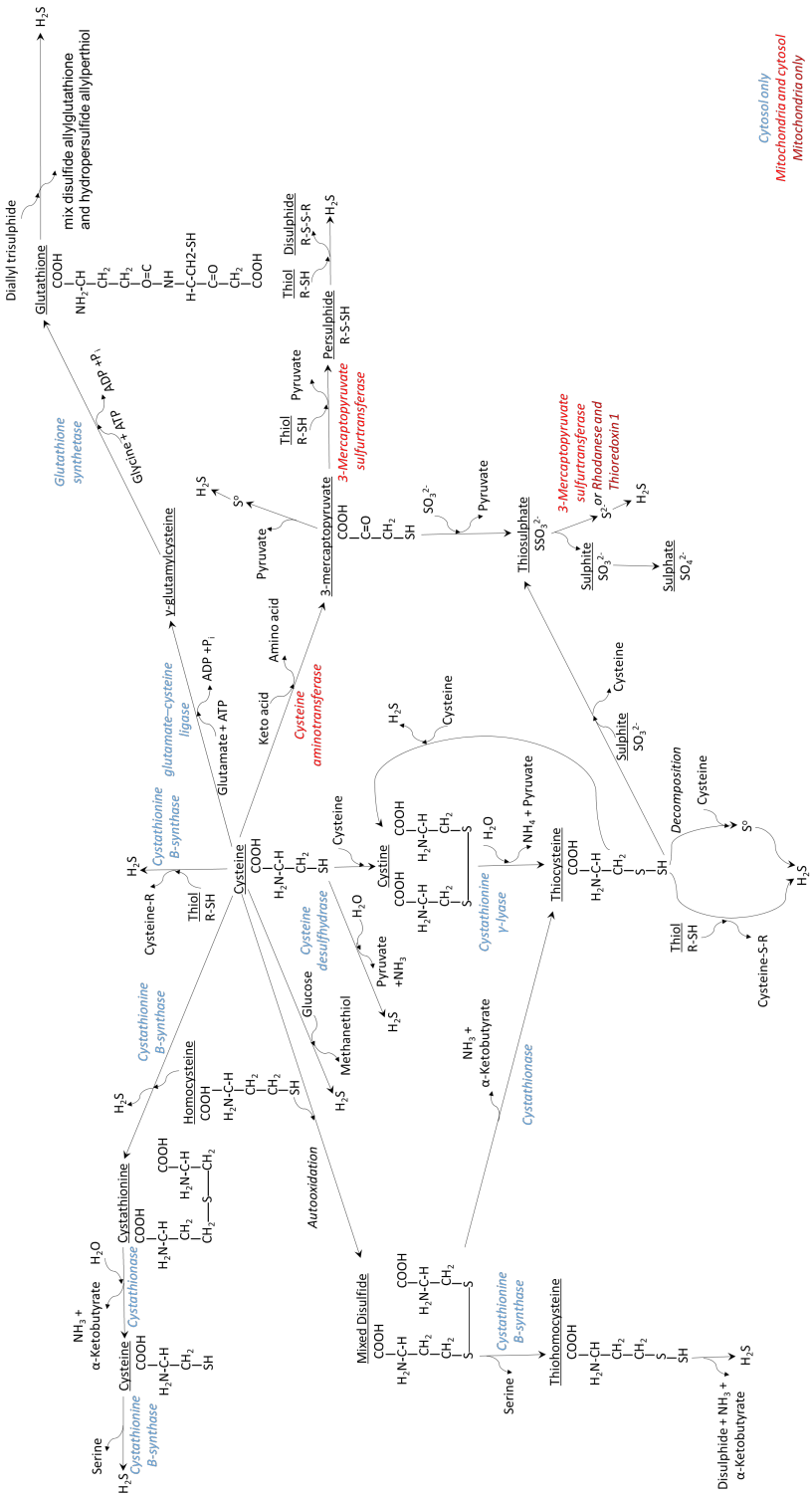


Figure 1-1. Endogenous production methods of H2S

1.1.1.1. Enzymic Production

There are some similarities between CBS and CSE, both require pyridoxal 5'-phosphate (PLP) as a cofactor and are located in the cytosol, where CSE is exclusively found but CBS can translocate to the mitochondria using a chaperone, mitochondrial heat shock protein 70 (mtHsp70) (*12-14*). Though only CBS has active sites for the two other gasotransmitters and the ability to regulate its own function through a redox sensor (*14*).

The other enzymes involved in H_2S production, are found more evenly distributed in both cytosol and mitochondria are cysteine aminotransferase (CAT) and 3-mercaptosulfurtransferase (3-MST) (*11*). 3-MST has a stronger association with the mitochondria than the cortisol, while CAT appears to be more evenly distributed (*15*). Much like CSE and CBS, CAT also uses PLP, however 3-MST uses zinc as a cofactor and works more as a sulfur carrier to produce sulfane sulfur, which is the same end product of CATs reaction (*14*).

Rhodanese (also known as thiosulfate sulfurtransferase) is one of the few enzymes that produce H_2S that are purely mitochondria based (*14*). All these enzymes involved in the production of H_2S reduce sulfur or extract it from sulfur containing molecules such as cysteine, thiocysteine, thiosulfate and persulfides, with cysteine being the main starting substrate (*10, 16*).

1.1.1.2. Non-enzymic Production

H_2S production also occurs non-enzymically in the mitochondria (Figure 1-1). (*11*). Mitochondrial 3-MST transfers sulfur from 3MP to sulfite (SO_3^{2-}) forming thiosulfate ($S_2O_3^{2-}$) but requires the assistance of reducing agents; thioredoxin (Trx), dihydrolipoic acid (DHLA) or dithiothreitol (DTT) (*11, 17*). Reducing agents Trx, DHLA and DTT also have a role in sulfite (SO_3^{2-}) based H_2S release (*18-21*).

1.1.2. Hydrogen Sulfide Mechanisms

As H_2S has such a complex endogenous production pathway retained from millennia of evolution, it stands to reason that it must also retain a function. H_2S is a signaling molecule with autocrine and paracrine effects (*22, 23*). The biological effects include roles in vascular tone, coagulation, neuromodulation, cytoprotection, metabolism, inflammation and apoptosis (*22*). These effects are thought to occur through persulfidation, reactive species scavenging, redox with metallic compounds and mitochondrial bioenergetics (*24-28*).

1.1.2.1. Persulfidation

H_2S decays to undetectable levels in 30 minutes but its effects last much longer, the method of action is a molecular 'switch' that has its effect exerted long after the gas has disappeared (*24, 25*). Persulfidation is the most likely mechanism, defined as H_2S post translationally modifying cysteine residues of proteins, particularly enzymes, receptors and ion channels, altering their properties and functions (*29*).

There are multiple factors that impact a proteins ability to be persulfidated, most importantly is cysteine accessibility (*30*). Cysteine is the least common amino acid and often found in active sites of proteins due to the sulfur containing thiol (-SH) groups high reactivity and affinity for metals, (*30*). At physiological pH cysteine has a low pKa, forming a strong acid, and exists as a thiolate anion ($R\text{-}S^-$), a strong nucleophile with the ability to neutralise reactive oxygen species (ROS) and form products like sulfenic acid (R-SOH) or sulfinic acid ($R\text{-}SO_2H$) (*31*).

Persulfidation changes protein properties and may protect cysteine from being irreparably oxidised under oxidative stress, which would alter its structure and cause decreased breakdown and/or function (*31*). Cysteine persulfidation turns thiol (-SH) to hydropersulfides (-SSH), increasing its nucleophilic ability and chemical reactivity resulting in stronger acidity and hydrogen sulfide donation (*31*). When hydropersulfides act as ROS scavenger, the result is

perthiyl radicals (R-SS•) which are more stable and so less reactive compared to thiyl radical (RS•) providing better protection to cellular components (*32*).

An additional factor affecting persulfidation is that H_2S is directly competing with nitric oxide (NO or $^{\bullet}NO$) for the persulfidation sites to S-nitrosylation, which has opposing effects to persulfidation, for instance preventing NF-κB activation (*33*). Nitrosylation is a faster reaction than persulfidation but is weaker, when NO and H_2S donors are applied together the initial effects of nitrosylation are overcome, suggesting that persulfidation outcompetes and inhibits nitrosylation (*34, 35*).

1.1.2.2. Reactive Species Scavenging

H_2S's has more links with NO and its radical intermediates than competing for active sites (*26, 36*). Early work has uncovered the H_2S can react to reactive nitrogen species (RNS), to form thionitrous acid (HSNO) within cells (*26*). This new discovery has demonstrated HSNO improves diffusion through membranes and alters the electron state of the NO when released (Figure 1-2) (*26*). H_2S and HSNO may be showing affects not just on its direct interaction with proteins but also by altering and regulating the effects and bioavailability of NO (*26, 36, 37*).

NO regulation is crucial to limit formation of free radicals capable of inducing oxidative stress, namely NO reacting with superoxide ($^{\bullet}O_2^-$) to form another RNS peroxynitrite ($ONOO^-$). An additional benefit of H_2S is direct scavenging of $ONOO^-$ limiting its oxidative damage by donating hydrogen ions and producing peroxynitrous acid (ONOOH) (*38*). Both ONOOH and its stronger oxidising metabolite nitric dioxide ($^{\bullet}NO_2$), further react with H_2S to form nitrite (NO_2^-), which can be used as a stable reservoir of NO or be used for nitrosation or nitration (*38, 39*).

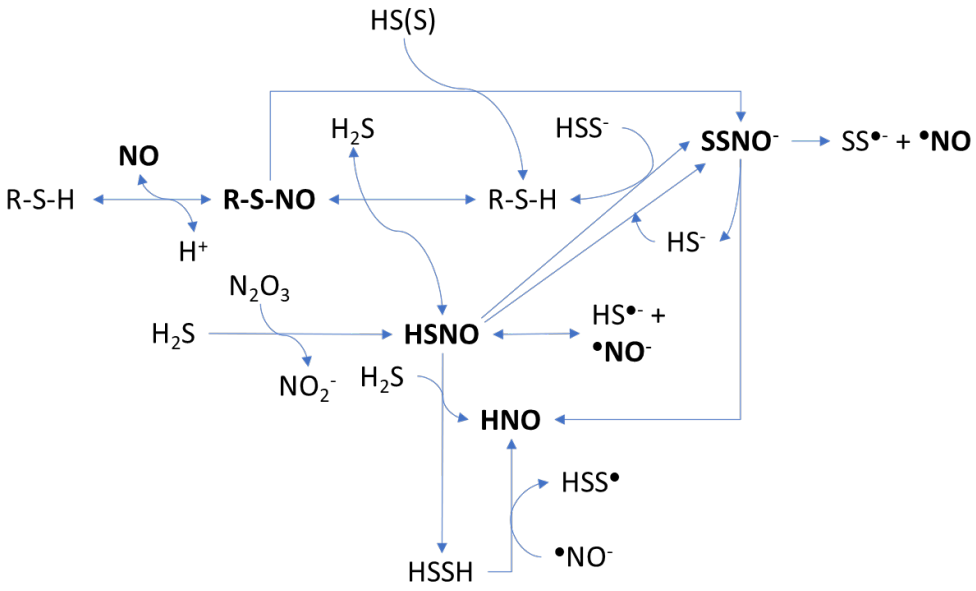


Figure 1-2 Production of HSNO and $SSNO^-$.

Other reactive species that can be scavenged are reactive oxygen species (ROS) (*38*). These include hydroxyl radical ($^{\bullet}OH$), superoxide ($^{\bullet}O_2^-$), and hydrogen peroxide (H_2O_2) (*38*). Hydroxyl reacting with H_2S forms a sulfur radical ($^{\bullet}S^-$) and water, this radical is able to either react with protons to form HSSH or in the presence of oxygen becoming dithionite ($S_2O_4^{2-}$) and after hydrolytic disproportionation produces thiosulfate ($S_2O_3^{2-}$) and bisulfite (HSO_3^-) (*38*). Superoxide similarly reacts with H_2S forming sulfur radical ($^{\bullet}S^-$) and hydrogen peroxide, this hydrogen peroxide further reacts with H_2S producing hydrogen disulfide (HSSH) and hydroxide (OH^-) (*38*). This ability to scavenge damaging oxidisers protects the cells from the oxidative damage, however it has been suggested that endogenous levels of H_2S are unlikely to be high enough to be the preferential target of the oxidants unless they both accumulate at high local concentrations, namely the mitochondrial (*38*).

1.1.2.3. Redox with Metallic Compounds

H_2S's negatively charged sulfur contributes not just with ROS scavenging but also to its nucleophilic abilities, making it strongly attracted to positively charged atoms such as metals (*27*). The proposed major metals are iron,

copper, and manganese, while the main metalloproteins are iron-heme, non-heme iron proteins, iron-sulfur, and zinc fingers (*27*). Metals and metalloproteins interactions with H_2S are all biologically viable but only heme proteins have been studied extensively (*27*).

When H_2S binds to ferric heme proteins (Fe^{3+}) an anion thiol ($-SH^-$) group is formed, creating ferric sulfide, and the H_2S can be released leaving both compounds in their original state (*40*). Alternatively, while bound the heme can be reversible reduced to ferrous heme (Fe^{2+}) depending on its polarity and generates an Fe^{2+}-$S^{\bullet}H$ radical, which can form disulfide compounds or elemental sulfur but if H_2S bonds with ferrous heme no radical forms (*40, 41*).

When H_2S binds to ferric iron it forms a stable bond to store H_2S (*27*). Given that this happens in hemoglobin, myoglobin and neuroglobin, proteins used as oxygen stores, suggests a possible functional link (*40, 41*). This function to detoxify by storing H_2S, transporting and releasing H_2S at tissue with high respiration demand, protecting against low O_2 concentrations, (*40, 41*). An additional benefit of H_2S binding to these globins is it reduces their affinity to oxygen, so localised increases of H_2S can induce O_2 release (*27*).

Other than hemoglobin, iron in the blood is also found in ferritin or free iron (*42*). Apoferritin is a protein produced by the body to store free iron by both binding and surrounding to produce a protein cage, this end product is a non-heme iron protein called ferritin (*43*). Interestingly, H_2S interacts with iron bound ferritin causing release by disruption of the apoferritin protein cage and freeing iron (*43*). Both ferric (Fe^{3+}) and ferritin bound iron catalyses the release of H_2S from cysteine, the H_2S then binds with the iron to form iron sulfide, used for the formation of iron-sulfur clusters, reducing iron toxicity and iron related ROS production (*42, 44, 45*).

Zinc fingers were originally thought to simply be persulfidated however, for the zinc to be released, freeing the cysteine residues, H_2S must directly bond

with the zinc which is bound to three cysteines and one histidine (*46*). By removing zinc from the proteins reduces the ability to bind to the RNA affecting its transcriptional and translational properties (*46*).

1.1.2.4. Iron-Sulfur Proteins and Mitochondrial Bioenergetics

H_2S's strong reactivity to metals and its application to metal containing proteins has a large impact on the mitochondria, as it stores metallic ions and imports unfolded metalloproteins for maturation (*47*). Further contributing to this, cysteine is the main endogenous source of H_2S and is found at three times the concentration in the mitochondria than in the cytosol (*13*). One of the major enzymes that catalyses its release, cystathionine γ-lyase (CSE), translocation to the mitochondria suggests than H_2S plays a significant role in mitochondrial function (*13*). CSE translocation is Ca^{2+} dependent, using translocase Tom20 as a motif, which binds at the N terminus and the first and third helix (*13*).

Mitochondria also has a role in the functions of H_2S through the electron transport chain (ETC). Through oxidising sulfide quinone reductase (SQR), an iron-sulfur protein part of the complex 2 family, allowing the fast removal of H_2S and form a defence against the substance's toxicity (Figure 1-3) (*6, 28, 48*). However, SQR varies in tissue, brain and liver produce little SQR while colon and heart have high expression (*6, 28, 48*). The expression of SQR in *C. elegans* is limited to the hypodermis, body wall muscle and pharynx (*8*). The expression in the pharynx is structurally and functionally similar to cardiac muscle, allowing comparison, but body wall muscle is a hybrid of smooth and skeletal muscle making it harder compare with other organisms (*49, 50*).

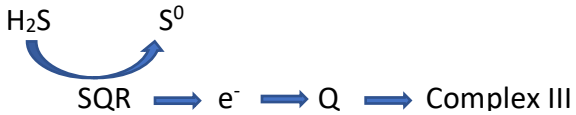


Figure 1-3. H_2S is oxidised as it feeds electrons into the mitochondrial transport chain.

The SQR related defence is limited, as after a certain threshold depending on SQR and sulfur extracting metabolites availability, inhibition of mitochondrial respiration occurs at complex IV (*6, 13*). As oxygen is outcompeted by H_2S at the iron and iron-copper cores of the complex (*51*). During this inhibition, electrons fed into the ETC are redirected towards complex II (*52*). This allows the reduction of fumarate into succinate for anaerobic oxidation of sulfide (*52*).

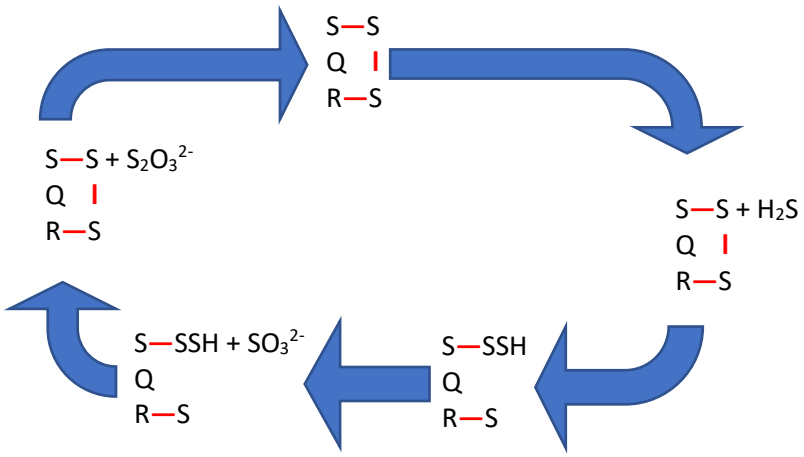


Figure 1-4. SQR persulfidation.

SQR is persulfidated by H_2S and then donates the sulfur to various acceptors to return to its original form.

Oxidation of sulfide is a source of electrons for the ETC, through SQR (*28, 53*). SQR's affinity for H_2S is stronger than complexes I and II's affinity for carbon derived substrates for electron donation and when SQR is used in this fashion complex I activity decreases (*6*). This is because electrons coming from NAD/complex I are competitors against those from SQR and the over reduced state in complex I inhibits the movement of electrons through the transport chain (*6*). When H_2S interacts with SQR it is turned into sulfane sulfur (thiosulfoxide, RR'S=S, S^0) by transferring its electrons to coenzyme Q, and S^0 is then able to interact with SQR using cyanide, sulfite (SO_3^{2-}) or sulfide (S^{2-}) as an acceptor (Figure 1-4) (*53*). By using another iron-sulfur protein, coenzyme Q, as an electron acceptor, the electrons are fed into the electron transport chain and able to produce ATP (*53*). The S^0 produced by this catabolic method, can involve the binding of oxygen and is the major source of thiosulfate ($S_2O_3^{2-}$) (*53-55*).

Moreover, H_2S persulfidates the α-subunit of ATP synthase at two cysteine residues, C244 and C294 (*56*). The data suggests that lack of persulfidation on both residues by cysteine mutation can reduce complex V activity to 50%, showing complex V needs H_2S to function at full capacity (*56*).

1.1.3. Hydrogen Sulfide Donors

With H_2S's role in cellular function established, research into activating its signaling pathways is emerging. This can be performed through supplementation in a variety of ways, which affects the rate of delivery, be it instant release or slow sustained (*57*). There are three main classes of H_2S donors: plant derived natural products, hydrolysis-based H_2S prodrugs and controlled-release H_2S prodrugs (*58*).

1.1.3.1. Plant Derived Natural Products

Large amounts of plant derived H_2S products are found in allium vegetables, such as onions and garlics, stored as organic sulfides S-allyl-cysteine (SAC), diallyl trisulfide (DATS), and diallyl disulfide (DADS) (*58, 59*). SAC unlike the diallyl sulfides are thought to not produce H_2S directly but rather mediate and increase endogenous production through increased CSE gene expression (*59*). When administered to mammals, organic diallyl polysulfides are converted into H_2S at endothelial and red blood cell (RBC) membranes by thiols on outside of the cell membrane but need the presence of glucose to maintain energy and reduced GSH substrate pools that are needed for the process (*60*). A small amount of diallyl polysulfides can move through the cell membrane, where nucleophilic substitution of the sulfide causes thiol/disulfide exchange with the thiols of glutathione releasing S-allyl-glutathione and allyl perthiol which both further reacts with reduced glutathione to produce H_2S and glutathione disulfide (GSSG) (*60*).

Though similar, DATS and DADS do possess key differences in their qualities, DATS release of H_2S is very fast taking minutes, creating short term doses, while DADS reaction is much slower taking hours and release much smaller, almost undetectable, amounts of H_2S (*61*). The high energy requirement and additional need for a GSH pool is not ideal for H_2S production as this does mean the pool is reduced while the compounds interact and hydrogen peroxide can be released through intermediate reactions for DATS based reactions (*61*). In addition to this, having the reaction occur in the blood with any thiol or nucleophile limits its use to effects on the vasculature and circulating immune cells, as the molecule is short lived and unlikely to penetrate deeply through the endothelium (*24, 60, 62*). With DATS releasing its entire supply of H_2S so quickly, keeping a therapeutic concentration throughout the body is difficult (*61, 63*). To be considered an effective therapeutic a compounds half-life should ideally be 12-48 hours to allow a reliable dosing regimen with a peak-to-trough ratio at a steady state (*63*). To ensure these efficacy and safety demands are met, synthetic compounds are often developed combining biological and chemical knowledge to adapt compound clearance and/or therapeutic component release (*63*).

1.1.3.2. Controlled Release H_2S Prodrugs

Thiol-Activated Donors

Synthetic compounds have been developed based on and improving the naturally occurring plant based DATS, by having H_2S released from the compound when interacting with thiols (-SH) (*64*). The chemistry behind these compounds is developed on nitrogen and sulfur bonds (N-S), which are highly reactive and so easily broken, allowing the possibility of H_2S donors attached to nitrogen-based compounds (*64*). This N-S bond can have variable stability based on the nitrogen group offering protection to the bond and N-(benzoyl)-thiobenzamide derivatives are the most commonly ones produced (*64*). The major benefit of this type of donor is H_2S is only released through interacting with thiols such as cysteine and not with other common nucleophile groups

such as hydroxyl (-OH) or amine (-NH_2) (*64*). This selective reactivity causes a slower release rate peaking under physiological conditions at around 18 minutes and sustained for about 2 hours (*64*). However, cysteine and thiols are found throughout the body, both within and outside cells so while this may improve tissue penetration and some may be released intercellularly in organs and intracellular, it does not improve specificity, target pathways or exhibit a suitable half-life.

Thiol-activated donors have the drawback of acylating other biological nucleophilic group containing molecules (*65*). This can be detrimental to cellular health on proteins acylation alters charge states disrupting protein-protein interaction, and on fatty acids targets proteins for the cellular membrane and disrupt cellular spatial control of signalling (*66*)

Thioamino Acids

Thioamino acids are fairly unique as they are very stable at a range of pH conditions but in the presence of bicarbonate and mild alkaline conditions will release H_2S (*65*). In this reaction, the carbon on bicarbonate replaces a hydrogen of the amide group on the thioamino acid, releasing water, and the hydroxyl group then reacts with the thiol group forming an ether to release H_2S (*65*). Caveat of this drug is this method uses the bicarbonate concentration in the blood to release the gas and so is not produced in the cells (*65*).

1.1.3.3. Hydrolysis Release Prodrugs

Inorganic sulfite salts such as sodium hydrosulfide (NaHS) and sodium sulfide (Na_2S) are both donors that are rapidly hydrolysed upon contact with water to produce H_2S and sodium hydroxide (NaOH) (*58*). The release peaks in under 20 minutes and becomes undetectable by 90 minutes, the rapid release of these drugs is more prone to cause negative effects due to toxicity (*57, 67*). Due to this more stable, hydrolysis releasing H_2S compounds have been developed to improve upon both the salts and the plant derived products (*58*).

Lawesson's Reagent Derivatives

Lawesson's reagent release is much slower than that of the salts, with 18% releases within an hour of being dissolved in buffer, the sustained ubiquitous release closer mimicking endogenous production and reducing toxicity (*68*). Derivatives of Lawesson's reagent have been developed, one commonly used in research is GYY4137 to form a much slower releasing donor, which will release H_2S for up to 7 days, reducing the toxicity when using matched concentrations of fast releasing donors (Figure 1-5) (*2, 57, 67, 69*). Though there are advantages to GYY4137 over NaHS, it is not without its disadvantages as the backbone of the drug will remain after H_2S is released with unknown effects (*65*). This compounds ubiquitous release means H_2S is produced in places where it may have limited or adverse effects before reaching therapeutic concentrations in the relevant areas within tissues or cells and so a more targeted release may be more beneficial.

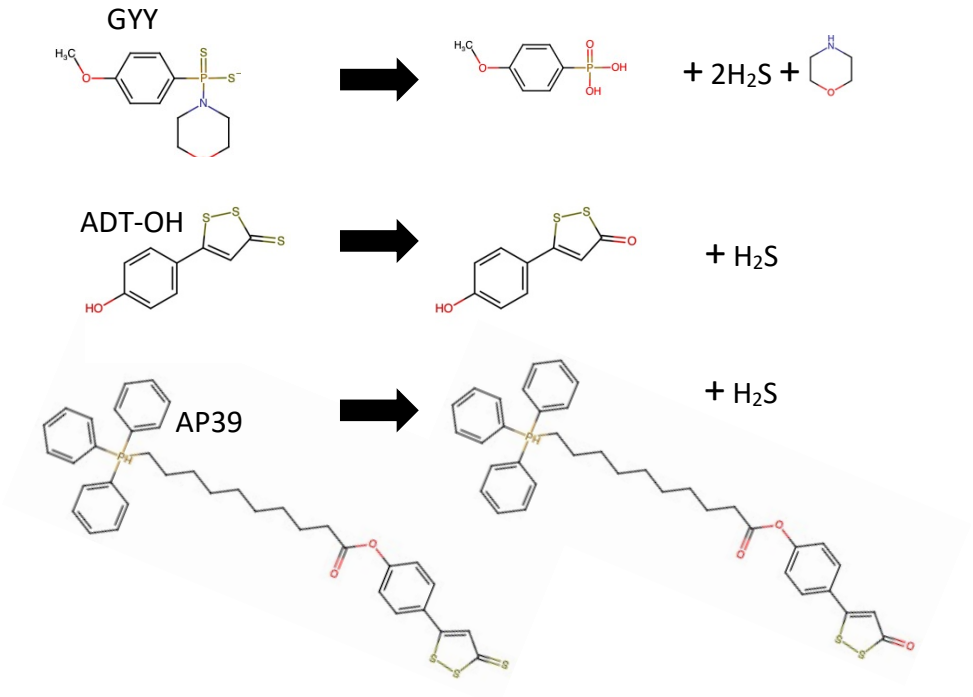


Figure 1-5. Release of hydrogen sulfide from donors.

Dithiolethiones

Dithiolethiones are a group of molecules found in the diet that contain sulfur which are known to persulfidate molecules and stimulate enzyme production

through Nrf2 activation (*70, 71*). The unique ability of this type of donor is that they show organ specificity towards the stomach, intestines, and bladder (*70, 71*). Due to this dithiolethione ADT-OH has also been used as moieties by attaching NSAIDS to them (*72*). These drugs then show a larger response at inhibiting angiogenesis at lower concentrations than parent drugs used individually or using in addition to H_2S gas and shows fewer side effects like gastrointestinal distress (*72*).

To target pathways and organelles within the cells ADT-OH has been bound to a mitochondrially targeting motif to create AP39, this donor has increased mitochondrial and reduced cytosolic release of H_2S, releasing for over 10 days in cell free standard cell culture medium at 37 ^oC through hydrolysis, and has potential for interventions in primary mitochondrial diseases, secondary mitochondrial conditions and reduce cytosolic side effects of other donors (Figure 1-5) (*73, 74*). As the mitochondria have many links with H_2S, through ATP generation in the electron transport chain, reduced ROS damage through production and possibly direct scavenging, and its involvement in the metalloproteome, metal storage, and metabolism, makes targeting slow releasing H_2S to the mitochondria a more efficient and safe, with the ability to directly target mitochondrial pathways (*63, 73, 75*). With AP39's mitochondrial targeted H_2S helping with mitochondrial dysfunction, this infers that dysfunction linked with multiple tissues in multiple diseases including, ageing, mitochondrial diseases, metabolic diseases, diabetes and obesity, neurodegenerative diseases, cardiovascular diseases, infection, and cancer, can all be therapeutically treated using this compound (*73, 75, 76*).

1.2. Ageing – Cellular and Physiological

1.2.1. Ageing and Muscle Decline

Ageing is a complex and poorly understood process that causes multiple physiological changes and phenotypes, while understanding of the

physiological and cellular causes of ageing are improving, effective treatments and interventions are still lacking (*77*). Causes are largely linked to oxidative stress and inflammation, with the mitochondria proposed as a therapeutic target, suggesting a potential use for mitochondrially targeting H_2S compound AP39 (*78*). Frailty in the ageing population is thought be at about 4% for the over 65s and raise to 26% in 20 years, this in conjuncture with projected models of the worlds over 65 years of age population estimating to more than double by 2050 from 702.9 million in 2019 to 1,548.9 million in 2050, due to increased survival rates of the elderly and suggests the portion of the population that enters the low functional frail state is increasing (*79, 80*). With humanity living longer and generating an increasingly ageing population, with the global percentage of over 65's increasing by 1.8% in the last decade (2010-2020), and consequently there is a strong need for healthy ageing (*81*). This estimated NHS cost of the muscle weakness based frail population was £2.5 billion in 2019, creating a pressing need to delay this process to help offset this economic burden (*82*).

Frailty is the consequence of age-related decline in physiological systems and homeostasis, creating an accumulative deficit in resilience, resulting in vulnerability of large health declines in response to minor stressors (*79*). Biologically elderly people often have reduced muscle mass, losing 1-2% per year after the age of 50, and at a cellular level have increased extracellular fluid and reduced cell mass (*77, 83*). There is an overlap between mitochondria and muscle decline in both sarcopenia and frailty, resulting in the physical aspects of the frailty phenotypes, presenting an opportunity for H_2S (*78, 84*). The crossover in diagnosis includes loss of muscle mass, low gait speed, low physical activity and quick exhaustion, which are linked with increased risk of disability and death, and all traits that can be measured both in humans and animal models, (*79, 84*).

1.2.2. Health span vs Lifespan

Increasing the lifespan of ageing individuals often results in an increase in the frail spectrum of life and therefore increases the incidence of chronic and degenerative diseases (*78*). There are many risk factors that affect the onset of frailty and not all result in life threatening illness making the mechanisms difficult to ascertain, but symptoms demonstrate an inability to properly respond and adapt to stressors (*78*). Investigating the transition of health to ageing and frail has revealed links with increasing inflammation and oxidative stress (*78*). Supporting and conserving the ability to control oxidative and inflammatory stressors with therapeutics may lead the way to healthier ageing and increase the quality of life for the ageing population. The mitochondrial theory of ageing places the mitochondria as the source and target of ageing inducing ROS (*85*). Limiting mitochondrial ROS damage and maintaining function is believed to improve health span and frailty (*85*).

With sarcopenia characterised was an impairment of muscle components and functionality, this allows a *C. elegans* model of ageing muscle decline to be used to estimate sarcopenia and its associated frailty though movement rate decline (*86*). Previous studies have used DEX induced sarcopenia in cell culture showing a reduction in H_2S production proteins CSE and CSB, and H_2S supplementation in the form of 3-mercaptopyruvate and shown protection against sarcopenic phenotype, with increased viability and protection against oxidative stress (*86, 87*). A *C. elegans* model has also shown improvements in lifespan and lifetime movement rate when dosed from L1, but H_2S has not been used as a therapeutic *in vivo* during natural ageing (*86, 87*).

1.2.3. Mitochondrial Function

Mitochondrial health, function and its reaction to stress is strongly linked with healthspan and can be used as a predictive tool in ageing research models (*88*). This can be done through basal oxygen consumption rates and ATP-linked

respiration measurements and thought to be related with epigenetic reprograming in later life which promotes healthy ageing (*88*).

Mitochondria have multiple functions some of which are tissue specific, in muscle mitochondria help regulate atrophy through catabolic signaling pathways, or growth from the stimulus of exercise to activate mitochondrial biogenesis and mitophagy (*89*). For proper function, 1200 mitochondrial proteins need to be produced and regulated but only 13 of these are coded for in the mitochondrial DNA, the remainder are from the nuclear DNA and transported to the mitochondria (*89*). To ensure proteins enter the correct mitochondrial compartment, importation machinery are required, and to help maintain proteostasis, misfolded and oxidized proteins are actively degraded (*89*).

1.2.3.1. ATP and Reactive Oxygen Species Production

Energy is produced in the cells via the creation of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and produce more than 90% of the energy used by the cell (Figure 1-6) (*90, 91*). One of the links with mitochondria and ageing is the production of mitochondrially damaging oxidative stress during this process (*92*). ROS is directly linked with mitochondrial efficiency and substrate usage, as electrons move through the ETC they produce instability in protein complexes and can be released to O_2 to produce superoxide anions ($^{\bullet}O_2^-$), which can be transformed into hydrogen peroxide (H_2O_2) or hydroxyl radicals ($^{\bullet}OH$) (*93*). ROS is produced at electron transfer sites on complexes and transporters, at exposed electron dense iron-sulfur centres (*93, 94*).

Complex I is the main source of ROS production in the mitochondria, with great debate over flavin mononucleotide, iron-sulfur clusters, or NAD radicals being the major contributor (*93*). Though complex II has minimal effect on the ETC due to low succinate levels in tissues, but at low pH the succinate dehydrogenase can dissociate from the complex, exposing an electron dense

iron-sulfide cluster to produce ROS (*93*). Alternatively, if the CoQ pool becomes overloaded, electrons can move from complex II to complex I in reverse electron transport to produce ROS at complex I CoQ and NADH binding sites (*93, 94*).

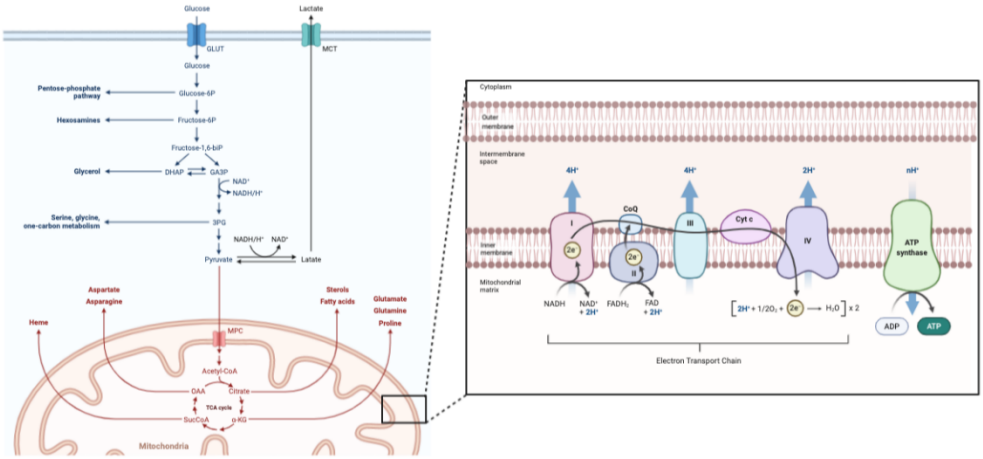


Figure 1-6. Internal compartments of the mitochondria and the components of the electron transport chain. Created with BioRender.com.

As cells and organisms age, there is a decline in mitochondrial function, which includes the production of ATP. This decline can in part be attributed to mutations of the mitochondria, often either genetic or from accumulation of oxidative damage, which can be organ sensitive and specific (*95, 96*). The heart has the highest mitochondrial respiration rates and lipid peroxidation (LPO), whereas metabolism and xanthine oxidase (XO) was highest in the liver but showed little LPO (*97*). This infers that the liver using a non-mitochondrial enzyme XO and producing ROS in the cytosol at the highest rate, to counter this it has developed higher levels of cytosolic SOD containing copper rather than manganese (*97*). The most striking revelation from this is when combining this information with the fact that most of livers reduced function in ageing is linked to declining cardiovascular function reducing liver blood flow, and that there are no ageing specific liver diseases, means that mitochondrial ROS production is the more significant than cytosolic in ageing and frailty (*98, 99*).

Given that there are hundreds to thousands of mitochondria per cell, mitochondrial damage and mutations must exceed a threshold within the network to cause dysfunction within the cell (*96*).

1.2.3.2. Apoptosis

To keep the mitochondrial network functional, the ETC production of ATP and ROS needs to be tightly regulated, as ROS damage to the mitochondria causes more ROS production (*95, 96*). Damaged and low functional producers of ATP are destroyed by mitophagy and as damage accumulates in the mitochondrial network or nuclei DNA, results in apoptosis. Apoptosis is required for normal cell turn over and tissue homeostasis (*100*).

Models of ageing have shown reduced apoptosis can be beneficial to tissue function and increase lifespan (*100*). During ageing cell turnover rate changes depending on tissue, increased apoptosis is associated with immune, skeletal and cardiac muscle and neural tissue, while senescent and cancerous cells are resistant, allowing them to accumulate (*100*). While defective cells need to be removed and is an important mechanism for pathogen and cancer defence, chronic inflammation can induce apoptosis and necrosis in healthy cells (*101*).

In ageing chronic inflammation occurs, which includes an increase of both plasma TNF-α and Fas suggesting that with ageing apoptosis may be induced in healthy cells (*102-104*). Inhibiting this abnormal apoptotic signalling may provide a therapeutic method to improve health in ageing. Using an ischemia model on cardiac cells, H_2S has shown the ability to decrease the activation of proapoptotic pathways (*105*). This was through two methods, firstly inhibiting caspase 9 activity, stopping its activation of effector caspases, and secondly improves the expression of apoptosis inhibitor Bcl-2 (*105*). H_2S also produces a decrease in proinflammatory effects through decreasing p38 mitogen-activated protein kinase phosphorylation and translocation, blocking the positive feedback loop of inflammation caused increased apoptosis (*105*).

1.2.3.3. Mitochondrial maintenance

Optimal mitochondrial function relies on a complex network of mitochondria (*89*). Though severely damaged mitochondrial network can cause cell death, this can be delayed or offset by maintaining the network health and structure using quality control mechanisms of fusion, fission and mitophagy (*89, 95*).

In high energy demanding and mildly stressful conditions, mitochondria use fusion to merge, distributing damage components and compensate their defects (*89, 95*). This reallocates resources such as metabolites, proteins, mtDNA, and mtRNA (*89, 95*). This enables the newly fused mitochondrion to have improved functionality and ATP generation compared to the combined output pre-fused (*89, 95*).

Fission happens in the alternative scenarios, occurring in nutrient excess and low stress or very high stress events like apoptosis (*95*). Fission events are required for isolating mitochondrial damaged or ineffective ATP production machinery that consume ATP to maintain their membrane potential (*89*). This is through a disparity in the daughter mitochondrion, one daughter is hyperpolarised compared to the originator, and the sister is hypopolarised (*95*). This is due to the hypopolarised mitochondrion possessing the damaged components, which if passing a damage threshold of mtDNA mutation, high ROS, low ATP production and membrane potential, are marked for destruction by mitophagy (*95, 96*). Many fission and mitophagy events resulting in an unhealthy and fragmented network (*89*). Interestingly H_2S switches the mitochondrial dynamics in favor of fusion and improved bioenergetics (*106*).

Mitophagy is a complex process that starts with the activation PINK1 and Parkin (*96*). Parkin is composed of many parts with the key components including, ubiquitin, Ring1, and an active site containing cysteine that can be persulfidated (*106-108*). Persulfidation of Parkin leads to enzyme activation

and clearance of damaged proteins and in combination with PINK1 mitophagy, shows H_2S can help maintain the mitochondrial network (*106*). This increased clearance improves cellular health as damaged components are removed and when applied to unhealthy mitochondria should reduce the ROS and inflammation produced in cells and lead to safer, more efficient bioenergetics (*109*).

Loss of mitochondria often leads to muscular atrophy and is thought to be a mechanism of age related sarcopenia (*89*). The damage can be accelerated through ROS overcoming antioxidant defenses and further increasing ROS production, exacerbating the damage and muscle loss (*89, 95*). Along with decreased mitochondrial number and muscle atrophy, sarcopenia also shows decreased expression of mitochondrial complexes I, III and IV, decreasing mitochondrial efficiency and ATP production (*110*). Complexes I, III and IV all have mitochondrially coded components, while the nuclear encoded components of complexes II and V show no decrease in expression, suggesting that the decrease in function is intensified by a decrease in mitochondrial DNA regulation (*110*). The loss of these complexes causes a reduction in membrane potential and with the decrease in mitochondrial content indicates the mitochondria have increased damaged and are being removed from the system (*110*). Reduced mitochondrial content contributes to diminished functionality in exercise, with lower maximal oxygen consumption, decreased ATP content and ROS production (*110*). Given the role of H_2S in mitochondrial function and maintenance, it is an ideal target for therapeutic intervention.

1.2.4. Basis of Chronic Diseases in Ageing

The combined low level of chronic oxidative stress and inflammation that occurs in ageing and has been termed “inflammaging”, leading to cell senescence and death, this damage slowly accumulates to reduce the function of tissues, leading to many age-related conditions and an increase in morbidity and mortality (*111*). Reducing inflammaging may lead to healthier ageing and

delay age related diseases (*111*). H_2S altering the reactive oxygen species (ROS) pool, which involves direct and indirect mechanisms and which in turn can affect the inflammatory response, while also directly influencing and reducing inflammatory mediators (*57, 112-114*).

One of the alarming outcomes of this type of oxidative stress is senescence, a state of irreversible growth arrest and apoptotic resistance, which is be induced by various types of DNA damage and/or mitochondrial dysfunction (*111, 115*). While at low levels senescence is needed for wound healing and tumor suppression but the danger of senescent cell accumulation is their resistance to cell death, leading to an accumulation of irregularly functioning cells in tissues and their unregulated pro-inflammatory secretions causing chronic inflammation, tumor formation, and stem cell exhaustion (*115*). Senescence's inflammatory activity is correlated with the symptoms of biologically ageing individuals, weight loss, muscle loss and weakness, and fragility (*115*). Given H_2S's ability to decrease oxidative stress and inflammation, and high oxidative stress and inflammation decrease H_2S production, it appears a prime candidate therapeutic for ageing and senescence (*57, 112, 113, 115-118*).

1.2.4.1. Oxidation

The free radical theory states that as an organism ages, damage is caused by an imbalance in the redox protection, as the pool of ROS in the system increases (*119*). This imbalance can either be due to an increase in ROS products or a decrease in antioxidants (*119*). In response to the damage, inflammatory markers are produced and as H_2S has multiple anti-inflammatory effects, alleviate the damage (*119*). As the damage accumulates there are several effects on the body including damage to DNA, nucleic or mitochondrial, and reduced lipid membrane fluidity, causing chronic low-grade inflammation to and senescence (*111, 120, 121*).

Though H_2S can decrease DNA damage through antioxidant effects, it has also been shown at high concentrations to increase the damage, causing cell cycle arrest in the G_1 phase and apoptosis (*122*). It should be noted that this method used 10 – 75 μM NaHS and followed a dose dependent trend so should be taken into consideration when comparing to other H_2S donors (*122*). When mitochondrial targeted donor AP39 is used at nanomolar concentrations; total protein, mitochondrial protein and mitochondrial DNA was protected from oxidative stress damage (*73*).

H_2S can directly interact with ROS due to its high reactivity and being strong reducing agent can directly scavenge inflammatory inducing oxidants like •NO, peroxynitrite ($ONOO^-$), hypochlorous acid (HOCL), superoxide and hydrogen peroxide (*57, 112, 113, 116*). H_2S also has indirect antioxidant effects, which are cytoprotective by improving the reduced glutathione (GSH) to oxidised glutathione (GSSH) ratio and increased expression of thioredoxin-1 (Trx-1) which is linked to protecting against apoptosis (*112-114*). This results in scavenging free radicals and reducing lipid peroxidation, this lowers the damage that would be caused to DNA, proteins, lipids and membranes (*112*). Another method is through persulfidation of p66Shc, which is an upstream activator of mitochondrial redox signalling, at cysteine-59 blocking the site of its phosphorylation by protein kinase C-ßII ($PKC_{ßII}$) and protein phosphatase 2 (PP2A) (*123*). By persulfidating p66Shc, it does not translocate to the mitochondria and does not perform its function of increases oxidative phosphorylation, which would result in increased ROS production (*123*). H_2S is also able to persulfidated superoxide dismutase (SOD) at cysteine-111, SOD is able to turn two superoxide into hydrogen peroxide and oxygen, persulfidation increases the stability of the molecule and reduces aggregation (*124*).

Much like nuclear DNA, Mitochondrial DNA (mtDNA) is also susceptible to oxidative damage over time and has multiple repair mechanisms to fix the damage caused (*125*). If the damage is too great the mitochondria start a chain reaction that leads to apoptosis, however this can be prevented by reducing

the production of oxidative stress which is the original cause and an inactivator of the repair enzyme formamidopyrimidine glycosylase (*125*). H_2S increases Akt phosphorylation leading to increased glutathione production which can scavenge ROS and protect both mtDNA and their repair proteins (*126*).

H_2S assists in the regulation of both the production of ROS in the mitochondria and neutralizing ROS that is produced (*112, 123*). This results in the protection of the cells form oxidative damage and senescence, one of the major causes of ageing (*111, 127*).

1.2.4.2. Inflammation

Chronic inflammation is common in ageing related diseases, such as hypertension, diabetes, atherosclerosis, and cancer (*128*). There are also lesser known ageing specific causing of chronic low grade inflammation, such as cell debris and immunoglobulin accumulation, which triggers the innate immune response (*128*). A common cause of ageing related inflammation is through senescent cells (*111*). These cells produce a senescence associated secretory phenotype (SASP), which include proinflammatory interleukins (IL-6, IL-8, and IL1α), tumour necrosis factor (TNF), chemokines (monocyte chemoattractant protein 2, and macrophage inflammatory protein 1 α), in addition to proteases and reactive species (*115, 129*).

Inflammation can be induced by administering high or toxic doses (200μM to 1000μM) of H_2S donor sodium hydrosulfide (NaHS), which instantly releases H_2S into solution (*57*). This dose on macrophages results in an increased production of proinflammatory markers IL-1β, IL-6, NO, PGE2 and TNF-α, and also increase NF-κB activation, which counteracted its initial anti-inflammatory effects (*57*). This isn't surprising as 200 to 1000 μM equates to between 6.82 ppm and 34.1 ppm which in humans would start to show physiologically relevant negative effects, irritation and inflammation and survival curves of NaHS show significant cell death after 60 μM (*2, 69*).

However, when a slow releasing donor GYY4137 is used at the same concentrations it does not produce this effect, suggesting that a slower sustained release rate has a more beneficial effect, mimicking enzymic release in cells (*57*).

With the initially available data in mind, it is easy to see why H_2S has been viewed as toxic and not investigated for beneficial or potential therapeutic effects however, recent studies are showing it is a more complicated subject. H_2S has been seen to increase or decrease in response to many diseases and is mostly thought to be involved in the inflammatory response (*57*). H_2S has a varied role in inflammation, at low concentrations inhibits the formation of PGE_2, NO, TNF-a, IL-1β, IL-4 and IL-6, which are inflammatory mediators (*57, 117, 118*). NF-kB activation can be inhibited by H_2S at low doses and also increases IL-10 release which result in an anti-inflammatory response (*57*).

If H_2S is administered after senescent cell formation, then it will still exhibit beneficial effect and delay fragility. H_2S can inhibit the formation of proinflammatory SASP factors IL-6, IL-8, and IL1α (*57, 117, 118, 129*). This will protect surrounding cells in the microenvironment from inflammatory damage which can cause paracrine pro-cancer and senescence (*129*).

1.2.4.3. Muscle Decline

As humans age, there is a decline in cardiovascular health and skeletal muscle mass (*130, 131*). Skeletal muscle mass is lost as we age which accelerates rapidly after the age of 60 (*131*). There is a link between mass and function, as 3% loss of mass can equate to 10% loss of strength (*132*). Though arguably strength is more important than mass, as strength has an inverse relationship with mortality regardless of age (*133*).

A large part of retaining skeletal muscle mass is proteostasis which is disrupted by ageing resulting in muscle wasting called sarcopenia (*134, 135*). H_2S is able

to protect against muscle wasting, caused by stimuli such as hyperhomocysteinemia which increases with age due to this proteostasis disruption, but does not induce muscle growth by itself and there appears to be no data directly correlating H_2S and muscle mass or strength as organisms age (*136, 137*). Hyperhomocysteinemia, at the time of writing this, lacks any treatment and produces an imbalance in the ROS system, increasing oxidative damage leading to ER stress and inducing cell death, which H_2S can alleviate (*138*).

Ageing's proteostasis disruption also causes an accumulation of protein aggregates, resembling those induced by stresses such as hypoxia, H_2S reduces this through production of F-box proteins, which breakdown harmful aggregates in a model of induced hypoxia (*139, 140*). Through the same proposed mechanisms, decreasing the ROS pool, and increasing activity and expression of antioxidants, H_2S is able to protect against ischemic injury in skeletal muscle (*141*).

Protection of skeletal muscle from diverse types of damage is key to health and healthy ageing. H_2S causes a shift in skeletal muscle adaptation to exercise and is produced by 3-MST, CBS and CSE (*28, 142, 143*). At maximal exercise with 5 ppm H_2S, there is a shift to anaerobic exercise at a lower work load, leading to lactate build-up and interestingly, this did not change power output or heart rate (*28*). This metabolic shift when induced maximal exercise is thought to be derived from a reduction in citrate synthase, limiting the Krebs cycle, suggesting that H_2S is altering energy pathways (*28*). When the concentration is doubled to 10 ppm during sub-maximal exercise, cellular oxygen uptake is decreased without changes in lactate levels or metabolic enzymes activities, though there is a slight trend towards increased lactate and lactate dehydrogenase and a decrease in cytochrome C oxidase (complex IV) (*28*). It may be that during exercise there is a decrease in H_2S production as lactate dehydrogenase is able to catalyse 3-MST's substrate 3-mercaptopyruvate to 3-metcaptolactate, reducing the pool available for H_2S

production (*142, 144*). Exercises high energy demand causes increased mitochondrial respiration and muscle damage, causing ROS accumulation to which H_2S acts as a scavenger and uncouples mitochondria to lower ROS production rate (*28, 145*). The muscle then needs to increase H_2S production, via CSE expression, to counter and recover the depletion (*28, 145*).

Skeletal muscle is a highly active tissue, requiring an abundance of ATP (*146*). H_2S affects oxygen consumption rate (OCR) in a bell-shaped curve manner, inhibiting complex IV and inducing mitochondrial uncoupling without significantly affecting ATP production (*28, 51, 147*). When Complex I is inhibited, H_2S allows electrons to enter the transport chain, and increase maximal electron flow and OCR, solely through electron acceptance of SQR allowing ATP synthesis (*6, 28*). This leads to H_2S preserving skeletal muscle mitochondrial integrity under low oxygen and nutrients stress by inhibiting complex IV inducing hypothermia, leading to tissue preservation, and altering oxygen and nutrient distribution due to changes in the vasculature (*28, 148*).

1.3. Spaceflight and Ageing Induce Similar Biological Changes

1.3.1. Biological Adaptations to Spaceflight

Spaceflight missions are extremely dangerous as it requires surviving an extreme and dangerous environment, containing microgravity and high amounts of solar and cosmic radiation, with the radiation becoming more dangerous in deep space due to moving past the protection of Earth's magnetic field. This deep space radiation is currently inhospitable with NASA stating that the radiation doses traveling to Mars would exceed safe radiation limits (*149, 150*).

The negative adaptations or maladaptation's associated with microgravity closely match the observations and symptoms seen with ageing on earth (*151,*

152). Due to this, spaceflight is considered a model for accelerated ageing, showing similar declines for neurological, cardiovascular function and develop sarcopenia and osteoporosis, with countermeasures having little effect (*151, 152*). Changes of biomechanics in space is largely affected by the changes in health and function of muscle and the neuronal system controlling them (*151, 152*).

1.3.2. Common Biological Features of Spaceflight and Ageing

There are multiple risks involved in spaceflight and many of the health declines and maladaptation's are often associated with observations and symptoms of ageing on earth (*151, 152*). The reduced gravity also reduces the skeletal muscle mass and strength, linked with a decrease in mechanosensitive enzymes, which when coupled with neuromuscular adaptations severely reduce functionality (*152*). This muscular decline is linked partially to protein homeostasis alterations possibly due to reduced nutrition intake and decreased mechanical load, resulting in less energy availability and contributors to ageing at a rate ten times faster than earth (*153, 154*).

At the mitochondrial level, spaceflight increases oxidative stress and alters the expression of three apoptotic related genes, while proapoptotic gene *VDAC2* was up-regulated, both proapoptotic *CASP9* and antiapoptotic *MnSOD* was down-regulated (*155, 156*). This increase in oxidative stress stems from a combined increase of ROS production and a decrease in measured antioxidant power, combined activity of redox-active antioxidants, though this study did not delve into individual antioxidants a separate investigation has shown down-regulation of *CuZnSOD* and *CAT* genes along with total glutathione levels (*155, 157*). An additional finding was an increase in NO and protein nitration, suggesting that H_2S production and influence was reduced due to, persulfidation sites being outcompeted and saturated, and H_2S not inhibiting

iNOS overexpression, though no direct H_2S related measure were performed (*157, 158*).

When looking into mitochondrial gene enrichment, one overlapping collection of genes sets were found across four cell types, fibroblasts, endothelial cells, primary T cells, and hair follicles (*156*). This collection contained four overlapping pathways essential for energy generation, mitochondrial ATP synthesis, mitochondrial electron transport, oxidative phosphorylation (OXPHOS), and hydrogen ion transmembrane transportation (*156*). This study also used network analysis to reveal the link between the mitochondrial stress and proteostasis failure (*156*). This link is through altered gene expression for mitochondrial proteins, interestingly while the mtDNA expression is increased the nDNA expression is decreased, the data suggests that the oxidative damage is causing reduced nDNA expression (*156*). This imbalance of mitochondrial proteins peaks at 9-fold higher expression after a year of spaceflight and appears to influence muscle and liver cells differently (*156*). Muscle cells exhibit decreases in carbohydrate and fatty acid related metabolic pathways and liver the opposite, this metabolic shift may be a result of muscle wasting and the livers response to it, which has shown similar responses on in ageing sarcopenia (*156*). In both spaceflight and ageing sarcopenia this metabolic shift caused increased ROS production causing mitochondrial damage leading to inflammation (IFN-γ, IL-1 and TNF-α), together with lipid accumulation and proteostasis failure (*156*). This contributes to the development of age-related diseases, cardiovascular disease, osteoporosis, and immune dysregulation (*156*).

1.3.1. H_2S a Potential Spaceflight Therapeutic

Given H_2S's role in ageing and disease, it has potential to be used as a therapeutic no just in terrestrial ageing but also in spaceflights accelerated ageing. H_2S has been proposed as a therapeutic against the radiation seen in spaceflight and for long term spaceflight, such as for mars missions, used to

induce hibernation in astronauts but neither has been tested (*159, 160*). In simulated spaceflight, H_2S donor GYY4137 was able to preserve bone structure in rats (*161*). Further adding to the need to test H_2S in spaceflight, a research group looking into spaceflight and sulphur metabolism found that extracts for the liver of space flown mice had a decrease in sulfur-based antioxidants that was partially mitigated after exposure to 1 g centrifugation (*162*). With this evidence that H_2S is reduced in spaceflight and ageing, with multiple benefits if supplemented, there is a pressing need to start understanding the practical implications of H_2S dosing.

1.4. Conclusion

H_2S is a simple gaseous molecule with complex biological effects. At high concentrations there are many negative biological impacts of H_2S but at low concentrations can have positive effects and help manage diseases. This has been shown in many conditions, especially effective in those linked to age as H_2S production reduces with age. Multiple types of H_2S donors, with altering release mechanisms and motifs, allow for targeted outcomes. However, many H_2S donors are not ready to be administered to humans and so testing on model organisms is required. *C. elegans* are an ideal model for this kind of research, due to the highly conserved signalling and metabolic pathways. Many discoveries about H_2S have been shown using *C. elegans,* such as lifespan extension with possible mechanisms and this would be difficult to perform in other organisms.

Having reviewed the literature, H_2S and the pathways related to its production have been shown to decrease in ageing and many other diseases it is yet be tested as an ageing intervention. The ageing studies available have used Qabazand *et al.* 2014 as a template where H_2S dosing started from L1 to provide an extended lifespan. While this is an important study giving a strong indication of the importance H_2S and provides a base to build upon, it is not specific to ageing as the data cannot distinguish if this outcome is due to

altered developmental changes at larval stages or due to improvements throughout ageing.

This thesis is aimed to identify health and mitochondrial changes during the ageing process of *C. elegans*, how H_2S donating compounds effect these health measures and determine if mitochondria is key to H_2S health effects. This health outcome was explored through augmented lifetime movement rates to assess muscle function and survival, while general mitochondrial function and content was explored through mitochondrial network and citrate synthase analysis. These experiments will increase our understanding of mitochondrial function during ageing and have important implications for therapeutics and interventions as to how this can be augmented using H_2S to promote healthier ageing.

In addition, this thesis also evaluated the effect of H_2S on accelerated ageing model of microgravity on spaceflight. Currently interventions to counteract the accelerated ageing and other maladaptations induced have little effect. This thesis has explored key *C. elegans* genes in the energy generation and cellular signaling in response to spaceflight linked to health declines and how H_2S has potential to counteract and improve function in flight.

2. Chapter Two: General Methods

2.1. C. elegans

2.1.1. Strains

Caenorhabditis elegans strains and OP50 are available from Caenorhabditis Genetics Center (CGC). Strains used for H_2S survival and movement experiments were N2 (wild type) and CB5600 (*ccIs4251 I; him-8 (e1489) IV*) with GFP fusion proteins localised to mitochondria and nuclei was used for the mitochondrial imaging experiments (*163*) (*163*).

2.1.2. OP50 Escherichia coli (E. coli) Culturing

OP50 culture was grown in LB broth from a single colony streaked from a LB agar plate (*164*). A single OP50 colony was cultured using an inoculation loop sterilised in a flame, dipped in OP50 in LB broth or from a single colony from a previous LB plate and spread as a small patch on LB agar near the edge of the agar plate. Loop was re-sterilized and used to spread three lines along the edge of the plate starting at the patch of OP50. Loop was sterilized and used to make three more streaks starting by passing through the end of the previous set of streaks, this process was repeated one or two times, the after sterilizing the loop a single streak passes through near the end of the last set of streaks. The plate was sealed in parafilm and allowed to grow over night at 37^{o}C and store at 4^{o}C for 1 month. A single colony was then transferred off the agar plate using a sterilised loop into 25ml of LB broth and allowed to grow over night at 37^{o}C spinning at ~180rpm in an orbital shaker. The OP50 broth can be stored for 2 weeks at 4^{o}C and added to the centre of the plate using a sterile pipette.

2.2. Media and Drug Preparation

M9 was made a minimum of 1 day prior to experiment. Ingredients are 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl and H_2O to 1 litre, sterilized by autoclaving (*164*). When cooled 1 ml 1 M $MgSO_4$ was added and store at 4^{o}C for 1 month.

Before making NGM agar the following was made; 1M $CaCl_2$, 1M $MgSO_4$, 1M KPO_4 and 5ml/ml cholesterol in ethanol (*164*). All were autoclaved but cholesterol and the same stock was used for the entirety of each individual experiment. NGM plates were made 5 days before use, by mixing 3g NaCl, 2.5g peptone, 17g agar and 975ml ddH_2O, autoclaved for 50min and cooled to 55^oC. Aseptically 1ml of 5mg/ml cholesterol, 1M $CaCl_2$, 1M $MgSO_4$ and 25ml of KPO_4 buffer was added and well mixed. 2ml of the NGM solution was pour into each 35mm petri dishes using a 25ml pipette. After being left at room temperature for 2-3 days to dry and checked for contamination, 200μL OP50 in LB broth was added to middle of plates and left to dry for 2 days, finally 100μl drug was added on top of OP50 and left for one day to dry before use.

Drugs concentrations were added to have final concentration on top of dry OP50-seeded 35mm NGM plates, final volumes including 2ml NGM agar and 200μl OP50 volume and 100μl drug/control. For final concentration of 100nM AP39 at 0.05% DMSO was made from a stored stock of 200μM in 100% DMSO, this was diluted in autoclaved double distilled water to 2.3μM at 1.15% DMSO which is added to the agar/OP50 plate for 100nM at 0.05% (*165*). The control for AP39 was DMSO diluted in the same way as AP39 to match the final DMSO concentration on these agar plates. Alternatively, for 100nM AP39 at 0.01%, a 1mM stored stock was made and this was used to produce 2.3μM AP39 at 0.23% DMSO, when added to plate makes a final concentration of 100nM AP39 at 0.01% DMSO. GYY4137 was made fresh to 2.3mM in autoclaved double distilled water and added to agar plates for 100μM, GYY4137's control was autoclaved double distilled water (*165*). AP39 and GYY4137 are made inhouse at the University of Exeter Medical School (*165*). Both AP39 and GYY4137 hydrolyse on contact with water so AP39 was stored in 100% DMSO and GYY4137 stored as a powder, once water was added to each compound the solution was added to plates within 30 minutes and excess was disposed of. *C. elegans* were added to plates after 24 hrs and transferred every 48 hrs, and so

used and discarded within the 7+ days of sustained release for both compounds (*67, 74*).

2.3. Synchronization of L1 Animals using Gravity Flotation

Animals were roughly age-synchronised by life stage from mixed population of animals that were washed with 3ml of M9 and aspirate several times from a nearly starved large stock 9cm NGM agar plate containing large numbers of animals (*166*). This solution was then transfer into two 1.5ml or 2ml Eppendorf's or a single 10ml falcon tube. After being left for 2.5min to gravity sync, the larger animals sink to the bottom of the container and the smaller and lighter larval stage 1 (L1) animals remain at the top. From the top portion, 10-20μl was taken and pipetted onto a glass slide to count the number of L1 animals under a microscope, this was repeated 2-3 times to take the average and find the volume needed for between 30-20 animals per plate. After the correct volume was added to the plates they were then checked and any older animals or dauers present were removed either with a pipette or picked with a needle. Plates were parafilm sealed and incubated at 20^{o}C for 58 hours allowing the animals to reach young adult. Animals were transferred every 48 hours to separate from progeny and to maintain constant food and drug source.

2.4. Assessment of Physiology and Subcellular Compartments in *C. elegans* Muscle

2.4.1. Survival assay

Survival analysis was started from pre-egg laying young adulthood at day 0 (*163*). Worms were assessed for movement, pharyngeal pumping and response to touch, and a negative response to all assessments was defined as dead. Animals were censored if accidently killed by the experimenter or if the

animal desiccated itself on the side of the Petri dish. Survival statistical analysis was performed using log-rank (Mantel-Cox) test with Bonferroni's correction for multiple comparisons of compound in GraphPad Prism 9.0. P value of < .05 was considered significant.

2.4.2. Movement Assay - WMicroTracker

One of the definitions for ageing is the time related biological deterioration of functions needed for survival (*167*). This can be measured through muscle decline and as an established index of animal health across the life course, movement rate was analysed every four days using the WMicroTracker movement assay (*163, 168*). *C. elegans* were washed using room temperature M9 into Eppendorf's or falcon tubes and washed 3-4 times to remove E. coli and any debris. The washes used 1ml M9 and spun down or gravity synchronised before removing as much supernatant as possible. Then 1ml fresh M9 was added, and the wash was repeated. After the final spin 500μl of the supernatant was removed and three to five 20μl mixed drops from the solution were added to a microscope slide and a count of the number of animals was performed to take the average and use this to determine the volume needed for 10 animals per well. This volume was then pipetted out, using tips coated in 0.1% Triton x-100 and the tip cut off to allow adults to flow unencumbered, into a 96 well flat bottom plate. Each run used 6 wells per condition and animal number was counted per well and adjusted accordingly. The final well volume was adjusted using M9 for a final volume of 100μl per well and blank wells using 100μl M9 without animals to remove background values. Raw values were adjusted for background values and worm number to produce movement rate per worm per well. Movement statistical analysis was analysed as area under the curve, which was then analysed using an ordinary one-way ANOVA using Bonferroni's correction for multiple comparisons of compound in GraphPad Prism 9.0 (*169*). P value of < .05 was considered significant.

2.4.3. Imaging of Mitochondrial Network in Body Wall Muscle

On assay day, a minimum of 20 animals were picked from the plates into 20μl of M9 on a microscope slide (*163*). After placing a coverslip, the animals were imaged covering enough animals to represent the majority of what was on the plate imaging as many animals as possible. The imaging was performed as quickly as possible to limit photo bleaching. The microscope used was an Olympus CKX41 using a GFP filter (460-600nm, blue light excitation) at 40X magnification and software Olympus cellSens Dimension 1.13. Assessment of mitochondrial network structure was determined to be networked, have minor fragmentation with disorganised and small infrequent gaps in the network, moderate fragmentation with large and frequent gaps in the network or severe fragmentation where mitochondrial networks were no longer recognizable. (*163, 170*). Mitochondrial network analysis was performed by manually scoring images into categories of networked, disorganised, moderately fragmented and severe (*163*). For each time point the percentage of animals in each category was calculated and analysed using two-way ANOVA with factors of compound and time with Bonferroni's correction for multiple comparisons in GraphPad Prism 9.0 (*163*). P value of $< .05$ compared to wildtype was considered significant.

2.5. Citrate Synthase Assay

Citrate synthase assay is used to as a marker for mitochondrial health, through the correlation with mitochondrial content and biosynthesis (*171*). This was performed on homogenised whole worm samples, containing 50 animals (*172*). Homogenisation was performed on ice using a tissue homogeniser, for 3 mins at 250 rpm. Sample was spun at 22000 g at 4^{o}C and supernatant was transferred. Citrate synthase master mix was incubated at 25^{o}C for 5 mins and blank reading obtained at 412 nm for 2 minutes. Sample was added to preincubated master mix and read for 3 minutes. Citrate synthase analysis was

performed using two-way ANOVA with factors of compound and time with Bonferroni's correction for multiple comparisons in GraphPad Prism 9.0 (*172*). P value of < .05 was considered significant.

2.6. Software and Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9.0 and Microsoft Excel. Images were captured using Olympus cellSens Dimension 1.13 software. Movement data was produced using wMicroTracker system software. P values of 0.05 were used for criteria of statistical significance in all analysis with factors of compound, time and post hoc analysis used Bonferroni correction. Survival analysis was performed using log-rank (Mantel-Cox) test with Bonferroni's correction for multiple comparisons. Movement analysis was analysed as area under the curve, which was then analysed using an ordinary one-way ANOVA using Bonferroni's correction for multiple comparisons. Mitochondrial network analysis was performed by manually scoring images into categories of networked, disorganised, moderately fragmented and severe. For each time point the percentage of animals in each category was calculated and analysed using two-way ANOVA with factors of compound and time with Bonferroni's correction for multiple comparisons. Citrate synthase analysis was performed using two-way ANOVA with factors of compound and time with Bonferroni's correction for multiple comparisons.

3. Chapter Three: Supplementing Larval *C. elegans* with Hydrogen Sulfide Improves Health in Late Life

3.1. Introduction

3.1.1. Ageing, Mitochondria and Hydrogen Sulfide

One of the definitions for ageing is the time related biological deterioration of functions needed for survival (*167*). This can lead to several phenotypes, but a common occurrence is a decline in muscle function which is strongly linked with health and mortality (*173*). In humans, skeletal muscle atrophy caused by ageing is commonly termed sarcopenia and cost the NHS up to £6221 per patient annually resulting in a total annual cost in excess of £2.5 billion (*82*). While a poorly understood stochastic process, symptoms of ageing are often linked with mitochondrial dysfunction (*174*). Mitochondria dysfunction in ageing has been studied since 1963 and is often linked with a decline in mitochondrial enzyme activity (*175*). One way to increase the activity of enzymes is through posttranslational modifications, such as persulfidation (*176*). Persulfidation, also called sulfhydration, is specific to hydrogen sulfide (H_2S) and is a posttranslational modifications of cysteine residues on proteins (*177*). This requires H_2S to bond to the thiol group (-SH) of proteins to form a persulfide group (-SSH), the remodelling of the structure alters the chemical properties, often changing the activity of enzymes (*177, 178*). There are many examples of this, including persulfidating ATP synthase, to keep the enzyme in a physiologically active state (*56*). An additional H_2S benefit is electron donation to the electron transport chain (ETC) through SQR for ATP generation and neutralising the harmful ROS produced by ATP production of the ETC (*8, 38*). This preserves the mitochondria and maintains the health and function of the mitochondrial network, protecting against apoptosis and cellular senescence (*100, 111, 120, 121*). While H_2S is historically perceived as a toxic

gas, there is evidence immerging that there are benefits to the gas, including its endogenous production to maintain cellular and mitochondrial health and is now considered a gasotransmitter (*179*). The majority of endogenous H_2S is produced by two key enzymes: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), with amino acid cysteine as the major substrate.

H_2S is naturally produced in the body with plasma sulfide ranging from 30 – 300 μmol/L and its production is known to decrease with age, with altered production rates seen in many other chronic diseases (*180, 181*). Research has begun to supplement and restore this loss, to understand how health is affected. H_2S can be administered as a gas but require constant administration so donor compounds are being developed (*180, 181*). Commercially available compounds such as sodium hydrosulfide (NaHS) and sodium sulfide (Na_2S) are fast, almost instant releasing donor on contact with water (*182*). However, this does not mimic *in vivo* release and is difficult to maintain working concentrations and avoiding acute toxicity(*183*). For H_2S to be an effective and translate into clinical practice, its half-life, thermal and aqueous stability need to be improved with controlled release sufficient to maintain and sustain its therapeutical level (*183*).

Due to this, compounds have been developed to alter the release rate, to produce slow releasing and target specific compounds. One example of this is GYY4137, a slow releasing compound working through hydrolysis on contact with water. *Caenorhabditis elegans* have shown that the use of GYY4137 has a moderate but beneficial effect on lifespan, this may be through protection against oxidative, endoplasmic reticulum and thermal stress (*87*). This compound is able to ubiquitously and slowly release H_2S at reliable levels for over seven days on contact with water, requiring no biological interaction (*67*). Other donors have been produced with more specific release patterns, such as [10-oxo-10-[4-(3-thioxo-3H-1,2-dithiol-5-yl)phenoxy]decyl]triphenyl-phosphonium (AP39) used in this study, with the same characteristics as GYY4137, slow sustained release over serval days on water contact and

requiring no biological interaction, but with the addition of mitochondrial targeting motif (*73*). A mitochondrial targeting motif has been selected as H_2S has strong links with mitochondrial evolution, integrity, and function, and has shown both health benefits and detriments in healthy and disease models.

The effect on mitochondria can be harmful in high concentration by irreparably inhibiting complex IV (*184*). However, at low doses H_2S can feed electrons into the electron transport chain without the need for glycolysis or the Krebs cycle to produce NADH and FADH typically needed to supply electrons (*56*). This is by persulfidating complex II and then releasing the sulfur in the form of sulfite. H_2S also keeps ATP synthase in its physiologically active state by persulfidating its alpha subunit. Both these features suggest that H_2S may be used to support the function of mitochondria.

3.1.2. *C. elegans* Model and H_2S

C. elegans are a useful biological model for human diseases, especially with ageing research (*185*). This is due to their short lifespan, well documented ageing processes, and large similarities with humans with regards to key metabolic and signalling pathways. There are simple and straightforward ways to measure general health as *C. elegans* age, using survival and movement assays. Both these assays are highly influenced by mitochondrial and muscle function, and are highly correlated with ageing, morbidity, and mortality.

This study has investigated and compared slow and sustained releasing H_2S donors, GYY1437 with ubiquitous release and new mitochondrial targeting AP39. Using the mitochondrial targeting release of H_2S to increase the understanding of the mitochondrial specific H_2S influences *C. elegans* survival and health, and this targeted release type reduces toxicity and other negative impacts. A previous GYY4137 *C. elegans* study was performed from L1 larval state and to accurately compare GYY4137 with AP39 this study has followed the same model (*87*).

3.2. Results

3.2.1. H_2S Compounds GYY 100 μM and AP39 100 nM Extended Lifespan

The first investigation was into the survival effect in wild-type *C. elegans* across the lifespan, to determine the effects of untargeted GYY4137 and mitochondrial targeted AP39 H_2S supplementation on ageing, when dosed from L1 larval stage. Compound concentrations were selected from previous publications, GYY4137 100 μM (*67*) and AP39 100 nM (*186*). Wildtype shows a maximal lifespan of 20 days post adulthood, and a mean lifespan of 11 days (Figure 3-1A and C). GYY4137 100 μM was used as an internal untargeted H_2S positive control, known to have a reproducible extension in lifespan (*187*).

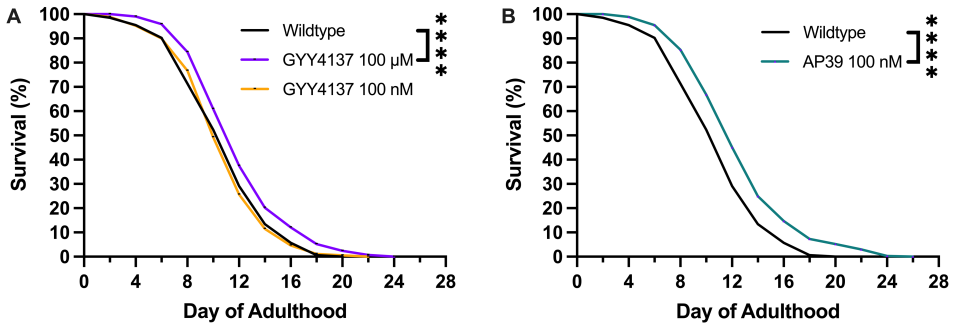


Figure 3-1. H2S donors extended the lifespan of wild-type C. elegans.

a *Survival curves of N2 wild types treated with vehicle control H_2O (n = 621) and H_2S donor GYY4137 at 100 μM (P < 0.0001; n = 627) and 100 nM (ns; n = 339).* ***b*** *Survival curves of N2 wild types treated with vehicle control H_2O (n = 621) and H_2S donor AP39 100nM (P < 0.0001; n = 638). Day 0 corresponds to young adult and are maintained at 20°C on compounds from L1 larvae to death. Survival curves were plotted from a minimum of three runs by the Kaplan-Meier method and survival values compared using log-rank (Mantel-Cox) test with Bonferroni* post hoc *test for multiple comparisons using GraphPad Prism v9. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

In this study, GYY4137 100 μM increased the survivability of *C. elegans* (P < 0.0001) by extending maximal lifespan by four days and mean lifespan by one day (Figure 3-1A). Mitochondrially targeted compound AP39 100 nM also showed a survival improvement comparted to wildtype (P < 0.0001) and showed greater improvements than the positive control GYY4137 (100 μM).

The maximal lifespan increased from wildtype by six days and mean lifespan by one and a half days (Figure 3-1B). With AP39 exhibiting strong effects at a thousand-fold lower concentration than GYY4137, we explored the effect GYY4137 has at the match concentration of 100 nM. With this matched dose GYY4137 100 nM lost its effect on lifespan extension and survivability improvement (Figure 3-1B). The motif of AP39 improved its H_2S release in the mitochondria, which improved the efficiency on lifespan which implies that both H_2S and lifespan are directly related to mitochondrial function.

3.2.2. H_2S Compounds GYY3147 100 μM and AP39 100 nM Increased Movement Rate and Improve Healthspan

Though healthspan is an important output, it does not show the quality of function of the animals exhibits throughout the lifespan. To determine if H_2S compound's extension in lifespan also translates to an increase movement function, healthspan was examined through health related and functional muscular outputs of habitual movement rates of the animals (*173, 188*). The typical movement rate for wildtype animals increases from young adult and peaks within the first few days of adulthood and declines to sub-basal levels as the animals age, this rate of decline depicts overall health of the animals. AP39 100 nM improved movement outcome compared to wildtype, showing an overall increase in movement across the lifespan ($P<0.0001$) showing an increase in the area under the curve across the lifespan (Figure 3-2). GYY4137 100 μM also exhibited increased movement rate vs wildtype ($P<0.01$), though this was to a lesser extent than AP39 100 μM but were not significant to each other (Figure 3-2). This is an indicator that H_2S donation was beneficial to *C. elegans* but AP39 100 nM with its mitochondrial release, improved the health of the *C. elegans* more effectively, enhancing their lifespan and delaying the effects of ageing.

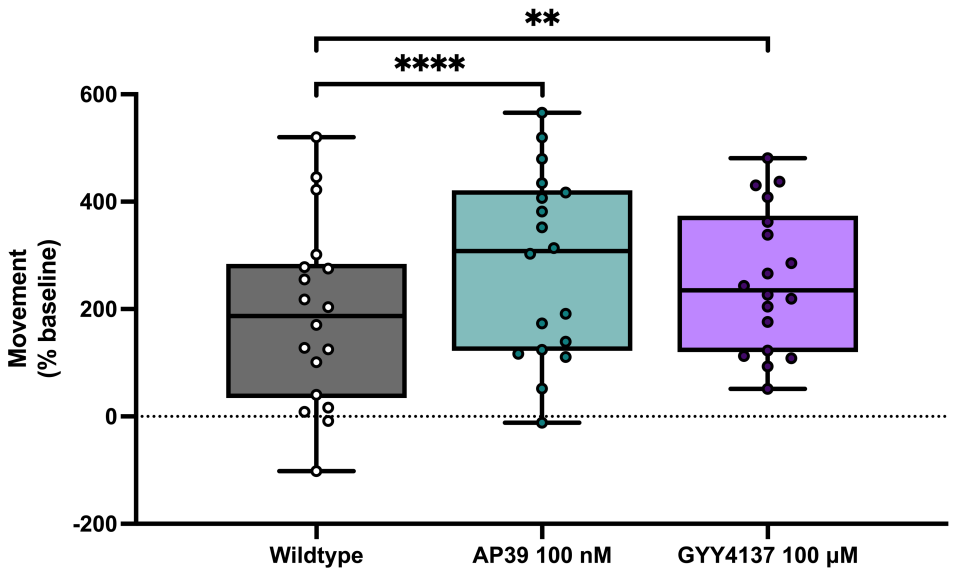


Figure 3-2. AP39 100 nM maintained habitual movement rate across age.

*AP39 100 nM increases and maintains movement rate across the lifespan of N2 wildtype animals (P < 0.001). GYY4137 100 µM showed an increase from wildtype (P < 0.001) but shows no significance to AP39. Day 0 corresponds to young adult and are maintained at 20 °C and dosed from L1 larvae to death, data expressed as area under the curve of 18 wells per group (worm number = 88–90 per group) every four days from day 0 adulthood till day 16 of adulthood, across three runs ± min/max showing all points representing area under curve, one-way ANOVA, with Bonferroni post hoc multiple comparisons using GraphPad Prism v9. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.2.3. H_2S Compounds GYY 100 μM and AP39 100 nM Maintained Mitochondrial Structure and Integrity

Muscle is a highly active tissue requiring significant quantities of ATP produced by the mitochondria and changes in muscle function is linked with mitochondrial health. To assess if the improved muscular function from AP39 100 nM is due to its mitochondrial effects and targeting, characterised of alterations in mitochondrial structure was conducted (*89*). *C. elegans* mitochondrial network structure and health were scored as one of four categories ranked from least to most severe, well networked, mildly fragmented, moderately fragmented, and severely fragmented. Body wall muscle cells of individual animals were ranked based on network status and then combined to categorise that individual animal. Each worm was scored in the most severe category that contained more than 30% of cells. Only AP39

100 nM (P < 0.001) maintained a healthy network of mitochondria for longer across the lifespan and with large improvements of 46% more worms in this category at days 2 and 17% at day 4 (P < 0.0001 and P < 0.05 respectively) (Figure 3-3A and C). Though GYY4137 failed to show an overall effect at maintaining mitochondria in a well networked structure, it does show an improvement of 28% more works presenting as well networked at day 2 (P < 0.0001) which declines to control match controls from day 4 onwards.

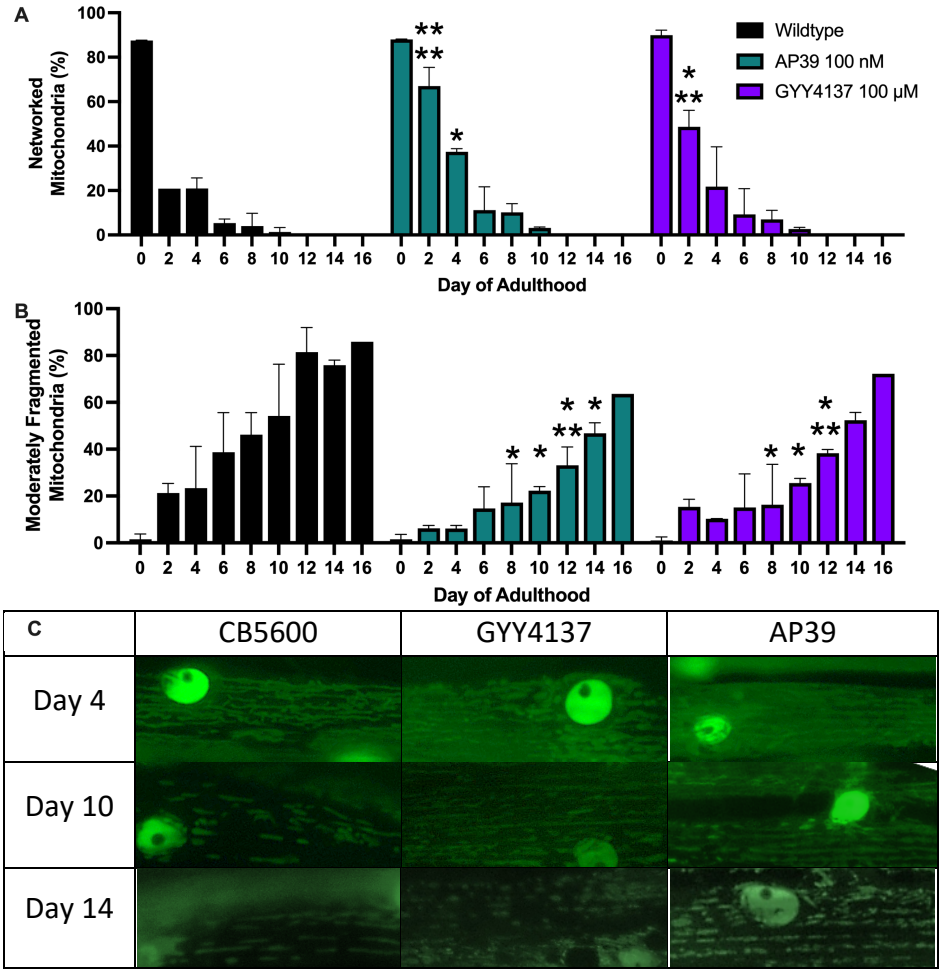


Figure 3-3. H_2S compounds maintain mitochondrial structure.

***a** 100 nM AP39 maintains the percentage of well networked mitochondria (P < 0.001). **b** Both 100 µM GYY4137 and 100 nM AP39 decrease the percentage of moderately fragmented mitochondria (Both P < 0.0001). Day 0 corresponds to young adult and are maintained at 20°C dosed from L1 larvae to death. Data expressed as mean from two independent runs ± SD, Two-way ANOVA,* factors time and compound, *with Bonferroni* post hoc *multiple comparisons test using GraphPad Prism v9. **c** Representative images of C. elegans at Day 4, Day 10 and Day 14 of adulthood. Significance expressed as timepoint wildtype vs compound *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

When evaluating the percentage of animals presenting with moderately fragmented mitochondria, both GYY4137 100 μM ($P < 0.0001$) and AP39 100 nM ($P < 0.0001$) delayed the fragmentation of mitochondria compared to control animals (Figure 3-3B and C). This is particularly true where at day 8, 46% of the wildtype population expressed moderate fragmentation and this increased to 81% by day 12, and within this period the compounds consistently retained a less fragmented network and kept moderate fragmentation lower by a minimum of 29% ($P < 0.05$). Though GYY4137 100 μM and AP39 100 nM both retained less moderately fragmented mitochondria within the population from day 8, AP39 100nM extended this protection for longer than GYY4173 100 μM, which loses this effect at day 12 but AP39 100 nM kept the networks structural preservation till day 14 of adulthood. This suggests that targeting H_2S through AP39 100 nM helps improve the structural integrity of the mitochondrial network more so than untargeted H_2S delivery of GYY4137 100 μM, further adding to the interpretation that the effect of H_2S on health and lifespan in *C. elegans* may, at least in part, be mitochondria related. The decline in mitochondria network structure depicts an absence in fission/fusion homeostasis (*189*). The gaps in the network, manifesting as fragmentation, indicate that there is more fission than fusion. As fission plays a large part in removing damaged components of mitochondria from the network, which are then destroyed through mitophagy. High fission rates are a necessary requirement for apoptosis, as the fragmented network results in a metabolic dysfunction and the more fragmented the network the unhealthier the cell and thus more likely to be culled. With more muscle cells removed the animals become weaker and develop sarcopenia. The compounds maintaining the network indicate a better ability to maintain homeostasis and a reduced likelihood of mitophagy and apoptosis, however if the mitochondria are low functional this may be a hinderance.

3.2.4. AP39 100 nM Maintained Citrate Synthase Activity

Visualising mitochondrial structure gives a good indication of fission and fusion ratio, as a these are required to remove damaged mitochondria and maintain the health of those remaining in the network. However, this does not depict the functional capacity and health of the mitochondria, and how this will affect the cell's ability to function. Citrate synthase activity is routinely used as a marker to depict the mitochondrial health through their substantial correlation with content and biosynthesis (r = 0.84, R_C = 0.80, Larsen et al. 2012) and moderate correlation with cristae area (r = 0.73, R_C = 0.67 Larsen et al. 2012) (*171*).

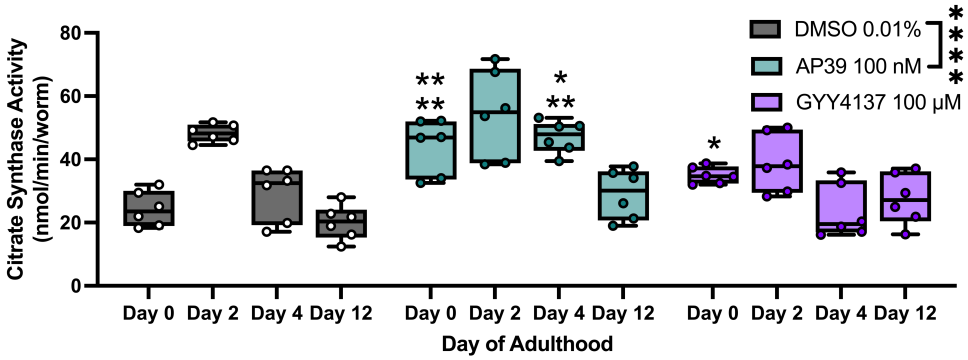


Figure 3-4. AP39 100nM maintain citrate synthase activity.

*AP39 maintains a higher lifetime citrate synthase activity (P < 0.0001). GYY4137 100 µM doesn't have a lifetime effect but does increase baseline levels. Citrate synthase is a marker for mitochondrial content. Day 0 corresponds to young adult and are maintained at 20°C and dosed from L1 larvae to death. Data expressed as mean from two repeats and 3 replicates ± SEM, Two-way ANOVA, factors time and compound, with post hoc Bonferroni multiple comparisons analysis using GraphPad Prism v9. Significance expressed as timepoint wildtype vs compound *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

As with networked structure, GYY4137 100 μM showed no improvement across the lifespan (Figure 3-4) and exhibits a pattern similar to the well networked mitochondrial structure, where initially there was an improvement. At day 0 GYY4137 100 μM shows a slight increase of 11 nmol/min/worm compared to control (P < 0.05) and matches wildtype control by day 2. This translates into structural decline, which also matches wildtype control at day 4 onwards (Figure 3-3A), which is unsurprising given that movement rate also

is unaffected at day 4 (Figure 3-2). Mitochondrial targeting of the H_2S compound with AP39 100 nM shows an improvement ($P < 0.0001$), particularly at day 0 ($P < 0.001$) and on day 4 ($P < 0.001$). AP39 100 nM also shows a similar trend on day 4 with that of the well networked mitochondria and movement data (Figure 3-3A and Figure 3-2A), as AP39 100 nM has a citrate synthase activity rate increased by 18 nmol/min/worm. AP39 100 nM shown increased mitochondrial content, either by needing to remove fewer mitochondria as they are failing less or by an increased ability to replace the ones that are removed, this feeds into the better maintained network of mitochondria and translates up into healthier cells and organism, allowing the body wall muscle cells to function for longer. The greater function of mitochondria and cells for longer in the lifespan demonstrates that AP39 100 nM delays the onset of sarcopenia and health decline.

3.3. Discussion

By studying the effects of H_2S on lifespan and health of *C. elegans*, it has been shown that through optimal dosing with specific compounds, H_2S supplementation has positive impacts on health and ageing. As both compounds, GYY4139 and AP39, have different methods of release and targeting motifs, there is potential that AP39's mitochondrial targeting is one of the reasons that it has a stronger effect on lifespan extension (*190*). This specificity may cause a reduced amount of H_2S in the cytosol, which potentially reduces the accumulation of toxic concentrations, reduced persulfidation of targets that are potentially irrelevant or detrimental to health, or outcompeting other gasotransmitters needed for signalling in the cytosol (*191*). When matching the concentration of GYY4137 to that of AP39's 100 nM, there is no effect on lifespan extension. However, at the higher concentration of 100 μM, GYY4137's slow H_2S release is shown to have similar effects, possibly by exerting similar mitochondrial H_2S concentrations and leading to similar beneficial effects to AP39 100 nM (*192*). As the effects of AP39 100 nM on lifespan are still greater than GYY4137 100 μM, there are potentially

negative cytosolic effect reducing the benefits on lifespan or still minor difference in concentration.

GYY4137 has previously been studied in *C. elegans* ageing, also showing increased lifespan and healthspan, validating the results in this experiment (*87*). The study found that blocking H_2S production by knockout of *mpst-1* resulting in a shorter lifespan, which H_2S supplementation was able to restore (*87*). Using wildtype, it was found that the gene expression of 18 ageing related and 31 stress related genes, increasing many downstream of DAF-16 (*dod* genes) and glutathione related genes, suggesting a genetic basis to improved longevity and antioxidant activity (*87*).

Ageing slowly results in the loss of tissue and organ function, which is speculated to be caused by oxidative stress from both reactive oxygen and nitrogen species (*193*). This loss of function can lead to age related diseases and frailty, age-related decline in physiological systems and homeostasis accumulating deficit in resilience, antioxidant therapies are thought to help delay the frailty and improve quality of life in ageing (*193*). The mitochondrial production of ATP causes ROS production as a natural by-product of normal function (*93*). Production of ROS is intrinsically linked to membrane potential, ROS production can be reduced by creating a small amount of proton leak resulting in a 18% decrease in membrane potential inhibits 90% of ROS production, whereas a small increase in membrane potential generates a large ROS production (*93, 194*). As a small drop in membrane potential has such protective effects with little effect on ATP production it has been proposed as a method of therapeutic intervention (*93*).

Using 50 and 100 μM H_2S donor ADT-OH targeting the ETC uncouples the mitochondria via SQR (*195*). This was performed with higher than optimal dose, as ADT-OH is toxic at 12.5 μM, and ATP production was not affected up to 35 μM with a sharp 20 -15% drop at 40 μM (*195, 196*). This drop in ATP production is through reverse electron transport induced with high membrane

potential and increases superoxide generation suggesting the uncoupling is ROS dependant when using high H_2S (*195*). AP39 is based on ADT-OH and using a lower targeted 100 nM dose neither ATP or ROS production is affected until stress was applied, this has been tested using glucose oxidase to induce oxidative stress (*197*). During stress events AP39 causes ATP levels to be maintained and lowers oxidative damage sustained (*197*). When applying this to ageing, healthy cells and mitochondria should not be affected until stress events occur, the AP39 protection should lower oxidative damage and help the cells recover and maintain their function. This increased resilience to stress should delay age related frailty and senescence, causing an improvement in healthspan.

Given that AP39 showed stronger benefits than GYY4137 and H_2S having proven mitochondrial roles, it must be this mitochondrial link that provides this improvement (*127*). Ageing and the mitochondria are linked through dysfunction in mitophagy, apoptosis and senescence (*89, 95, 127*). The improved mitochondrial structure and content with the use of H_2S does point to the conclusion that the mitochondrial network is either under less stress or in a better position to handle the stress (*95*). This may be due to a reduction in ROS damage, either through reduced ROS production or increased direct and indirect scavenging, minimising the amount of mitochondria lost to mitophagy and allows the cells to use fusion to repair the network (*95*). Preserving the network will reduce both apoptosis and senescence induction in cells, both of which contribute to ageing and inflammation (*127*). This ability to improve or maintain mitochondrial health has implications in primary and secondary mitochondrial diseases, including ageing and sarcopenia.

The data from this study concludes that H_2S is able to improve the health of *C. elegans* during ageing. The therapeutic viability of H_2S works more effectively when targeting the mitochondria using AP39, rather than the ubiquitous release of GYY4137. This has been shown through improved mitochondrial health through ageing. Given ageing's strong links to muscular atrophy and

mitochondrial defects, improving on these defects with targeted H_2S compounds may improve the quality of life in the elderly.

4. Chapter Four: Supplementing Adult *C. elegans* with Hydrogen Sulfide Improves Health but Does Not Extend Lifespan

4.1. Introduction

In the last chapter data was presented from experiments using two similar hydrogen sulfide (H_2S) donating compounds. Both these compounds release H_2S on contact with water consistently over several days, with the difference being that GYY4137 has ubiquitous release and AP39 targets the mitochondria. These methods for this experiments matched that of previous H_2S ageing publications (*87*), dosing *C. elegans* from L1, which while shows the importance of H_2S supplementation does not effectively evaluate it as an ageing intervention as the alterations could be from developmental alterations or ageing improvements.

This study examined health outcomes of survival and movement rate when dosing from specific timepoints in adulthood, young adult, 2nd, and 4th day of adulthood. The results distinguished any developmental effects from ageing augmentations, to determine that H_2S can be supplemented to improve muscle function in adult muscle decline. Proving H_2S improves post development muscle decline paves the way for intervention in many conditions that induce muscle atrophy, such as ageing and microgravity.

4.1.1. Importance of Development Vs Ageing

H_2S may function differently based on the organisms age and function, meaning there may be developmental effects producing the extension in healthspan and administering post development may produce no effect. Preeclampsia is a hypertensive syndrome which unfortunately is a major contributor to fetal morbidity and mortality (*198*). H_2S producing enzyme CSE has been proven to be found in the placenta and reduced in preeclampsia

causing reduced H_2S production, suggesting H_2S may have strong developmental effects (*198*). Also contributing to fetal survival, H_2S regulates maternal-fetal interface immune hemostasis by suppressing the immune system, reducing unexplained recurrent spontaneous abortion (*199*). Preterm neonates have higher H_2S turnover than full term, which is expressed as an increase over the first three days post birth and may contribute to mortality but is stable through childhood and adolescence at 53 ±15 μM/L, ages 7-19 years old (*200, 201*). Interestingly this increases at adulthood, 30 ±7 years, to 75 ±13 μM/L but decreases by ages 53 ±9 to 0.37 ±0.021 (*202, 203*). All these studies used different methods to determine H_2S levels, so it is difficult to truly compare however, if the general trend is to be believed H_2S increases from childhood into adulthood and then lowers into old age.

A decrease in H_2S production during development, as shown using CSE knockout mice, shows H_2S is necessary for proper growth and survival (*204*). These mice had limited growth reduced total body, liver, white adipose fat, kidney, and heart weight (*204*). This is linked to H_2S's mitochondrial effects as mitochondrial diseases also exhibit reduced growth rates, in runting and stunting syndrome the mitochondria metabolic pathways have abnormal gene expression resulting in reduced ATP production and dwarf chicken (*205*). Other mitochondrial diseases studied (melas, pyruvate dehydrogenase deficiency, Kearns Sayre Syndrome, Leigh's Disease, Complex 1, 2, and 3 deficiency, Optical Atrophy 1 gene deletion, and Pearson syndrome) also affect child height and weight, with children being short and thin (*206*). In *C. elegans* with GYY4137 as a H_2S donor show increased body length and area, suggesting that these worms have an increase ATP pools for growth (*87*). Given that increased weight are linked to increased cognitive ageing, supplying H_2S from an early age may lessen its beneficial effect (*207*). Interestingly and perhaps more relevant, height has a role in cognitive ability and taller people experience less cognitive decline with age, suggesting that H_2S from an early age could improve ageing (*207, 208*). Due to this supplying H_2S donors from a young age

may be masking or creating new effects that will not occur if supplied in adulthood or old age.

Mitochondrial dysfunction can occur at any age, having varying effects at childhood and old age due to the role mitochondria have on growing and development in childhood (*206, 209*). There are many disease states that reduce mitochondrial function, some acquired during the lifetime and others are inherited. Sarcopenia for example is an acquired mitochondrial disease that develops during old age and associated with numerous negative health complications which can increase mortality and reduce quality of life (*209*). In humans the disease develops in patients 40 years and older and continues to progress throughout the remaining lifespan, with 60% of muscle lost in patient in their 80's (*209*). This age-related disease is poorly understood and lacks a universal definition and standardized testing but to some extent, can be functionally managed with increased exercise in a population as old as 74 years (*209*). There is consensus that factors such as malnutrition, inflammation, oxidative stress, disrupted proteostasis and dysregulation of apoptosis are important in the progression (*210*). This produces an altered phenotype of developing as a depletion of fast-twitching type 2 muscle fibers and satellite cells, a muscle cell precursors, with increased fibration and fatty deposits (*209*). Muscle mass can be quantified through the lifespan and linked to life expectancy, previously muscle loss had been used to indicate clinical sarcopenia (*121, 209*). However, muscle mass poorly indicated functional capacity and therefore there has been a shift into using functional measurements to diagnose (*121, 209*). In humans, this is by using strength and motility often through grip strength and walking speed, in addition to muscle mass but in research models like *C. elegans*, this can be done using movement rate to determine healthspan (*209, 211*). Though sarcopenia is diagnosed by its muscular effects, they are highly influenced by motor neurons health (*212*). A decline in motor neuron health, like with muscle, is highly related to mitochondrial health, and sarcopenia is regulated by mitochondria causing cell death in both cell types, reducing muscle function by denervation or reduced

muscle cell number (*212*). Mitochondria induce this process when stressed and often are degraded through mitophagy but in some instances such as with ageing causes transition permeability pores to open (*212*). This releases the contents of the mitochondria and initiates the apoptosis cascade and triggering cell death (*212*). With enough cell death and reduced number of satellite cells to replace the lost tissue, results in sarcopenia (*212*). Intervention is needed to decrease the damage done through ageing and reduce cellular death to keep organs in a high functional stage for as long as possible.

4.1.2. Current Ageing Interventions

Intervention is often started after symptoms of ageing appear and focus on treating the symptoms and not the cause, unfortunately true "anti-ageing" is a fallacy and there is no way to reverse or stop the ageing process and the damage of age is often not fully recoverable (*213*). Therefore, interventions for reducing the symptoms that cause frailty and improving the quality of life as ageing progresses is so important.

4.1.2.1. Lifestyle Changes

Our ability to move around and interact with the world plays a huge part in both physical and mental health (*214*). Loss of muscle mass can play a large part of this and one of the most common intervention for sarcopenia is adjusting the nutritional intake and activity levels of the patient (*215*). This is because it is easier, cheaper and often safer for clinicians to prescribe lifestyle changes than pharmaceuticals (*215*).

There are studies showing that there is a correlation between low protein intake and low activity, leading to sarcopenia and increased intake can improve these conditions, in addition to alleviating inflammatory and catabolic conditions (*215*). Research into protein intake with the elderly shows that animal-based protein which contains 1.7% cysteine and the so called 'fast'

whey protein containing 2.8% cysteine is more effective than 'slow' casein protein having 0.3% cysteine (*216-218*). These fast and slow proteins can be further improved by further supplementing with essential amino acid leucine, omega-3 fatty acid and vitamin D (*216, 219*). In the elderly timing of intake is also paramount, with consumption most effective immediately after exercising, ideally at midday (*216*). Plasma cysteine levels reduce with age and supplementation increases muscular glutathione, peak power, and work levels (*220*). Given that cysteine is a H_2S precursor, H_2S supplementation should also interact with these pathways and increase these outcomes in the elderly.

Exercise improves quality of life by improving muscle mass and function, with recommended 30 – 60 minutes of moderate or 20 – 30 vigorous intensity exercise session daily (*210, 216*). In disease states exercise increases CSE expression and contributes to the return of healthy H_2S (*145, 221*). However, this can be impractical in bedridden patients and ineffective in astronauts and so pharmaceutical supplementation has also been developed (*216, 222*). Pharmaceutical intervention is mainly focused on myostatin inhibition, angiotensin-converting enzyme (ACE) inhibitors, testosterone and ghrelin (*219*).

4.1.2.2. Mitochondrial Interventions

There are several mitochondrial base therapeutics that are able to help with the symptoms of ageing and helping to reduce the related mitochondrial dysfunction (*223*). Some of compounds are antioxidants and treat neurodegenerative disease, kidney disease, and heart failure (*223*). Reducing ROS production decreasing damage to the cell and limiting apoptosis and inflammation, while also protecting the proteins from oxidation and maintaining proteostasis. The ability of ROS and mitochondria to affect inflammation and other age-related symptoms present an interesting target for intervention.

4.1.3. Hydrogen Sulfide: Future Ageing Intervention

Exercise is the most effective form of intervention for the symptoms of ageing and muscle atrophy, however this is not a cure or possible in all situations, for example bed bound or post-operative patients. In spaceflight even with strict exercise programs, muscle still atrophies aggressively with current protocols having no effect on the muscle loss (*224*). Due to this there is a need for pharmaceuticals that can safely and reliably help improve quality of life prevent the progression of sarcopenia and muscle atrophy, both in muscle mass and function. Given the mitochondrial role in energy and ROS production, apoptosis, and muscle atrophy, it is a prime target for ageing intervention. Endogenous hydrogen sulfide (H_2S) production decreases with age and may have a role in the decrease in muscle mass and function with age (*181*). Hydrogen sulfide increases ATP production by persulfidating SQR of complex II in the electron transport chain, allowing proton transfer across the inter mitochondrial membrane without the use of adenine dinucleotides form the Krebs cycle or glycolysis (*74*). The increase in ATP availability allows more body movement and exercise, and maintains mitochondrial membrane potential, keeping them healthy (*74*). Hydrogen sulfide has been shown to maintain muscle mass and function in a diabetic mouse model and is likely to do the same with ageing based sarcopenia (*225*). Due to this a mitochondrial targeted H_2S compound AP39 presents as an ideal candidate for treating ageing diseases like sarcopenia.

4.2. Results

4.2.1. Effect of Dosing at Young Adulthood

For this study, *C. elegans* had compound dosing start at vary life stages to decipher the effectiveness of untargeted H_2S compound and mitochondrially targeted AP39 when administered at different life stages. This was to help show how effective the compounds are as an ageing or muscle atrophy

therapeutic and when in the lifespan to administer and still effective. This was performed by applying compounds at day 0 young adult, day 2 or day 4 of adulthood through till the end of life.

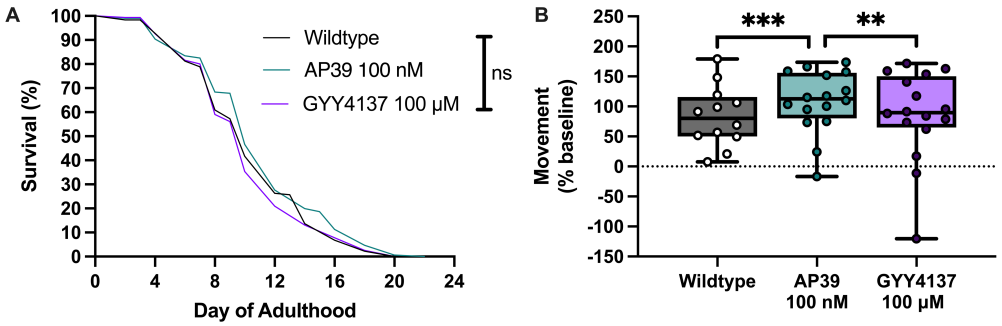


Figure 4-1. H_2S donors improve healthspan of Day 0 young adult wild-type C. elegans.

***a** Survival curves of wildtype, GYY4137 (ns) and AP39 (ns) with dosing commencing at day 0. Day 0 corresponds to young adult and are maintained at 20°C on compounds from day 0 young adult till death. Survival curves were plotted from three independent runs (n = 298–301 per group) by the Kaplan-Meier method and survival values compared using log-rank (Mantel-Cox) test with Bonferroni post hoc test for multiple comparisons using GraphPad Prism v9. **b** AP39 100 nM increases and maintains movement rate across the lifespan of N2 wildtype animals and is improved compared to GYY4137 (P < 0.01 and 0.001 respectively) movement when dosing started at day 0, while GYY4137 has no effect (ns). Day 0 corresponds to young adult and are maintained at 20 oC and dosed from L1 larvae to death, data expressed as area under the curve of 11-16 wells per group (worm number = 101-115 per group) every four days from day 0 adulthood till day 12 of adulthood, across three runs ± min/max showing all points* representing area under curve*, one-way ANOVA, with Bonferroni post hoc multiple comparisons using GraphPad Prism v9. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

Dosing from day 0 young adult with untargeted compound GYY4137 was ineffective at increasing the lifespan or healthspan of the animals (Figure 4-1A and B). However, while AP39 showed no effect on lifespan, movement rate was increased (P < 0.001), resulting in an improved healthspan. AP39's increase in healthspan without an extension in lifespan and is perhaps due to the alterations from the switch from growth and to maintenance and reproduction. In mice, endogenous H_2S peaks during reproductive age and if this is true in *C. elegans*, the animals at day 0 are reaching, or at, peak endogenous H_2S production (*226*). When at peak production and with the addition of GYY4137 H_2S supplementation, cellular and cytoplasmic levels may reach the toxic threshold. This contrasts with when dosed from L1 larval stage

as the animals do not show this effect, as they have developed methods of dealing with the additional H_2S at a juvenile age by utilising and detoxifying H_2S but without these already in place, the adult worms are prone to low-grade chronic toxicity until tolerance is achieved. Mitochondrial targeted AP39 at a much lower dose would be less likely to reach toxic threshold, especially as the mitochondria are the main source of toxification.

4.2.2. Effect of Dosing at Second Day of Adulthood

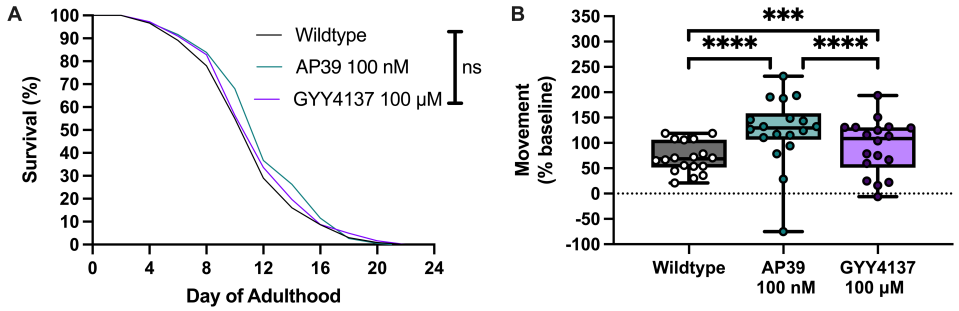


Figure 4-2. AP39 extend the lifespan and movement rate of wild-type C. elegans on the second day of adulthood.

a *Survival curves of wildtype, GYY4137 (ns) and AP39 (ns) with dosing commencing at day 2. Day 2 corresponds to 48 hrs post young adult and are maintained at 20°C on compounds from day 2 of adulthood till death. Survival curves were plotted from three independent runs (n = 296-299 per group) by the Kaplan-Meier method and survival values compared using log-rank (Mantel-Cox) test with Bonferroni post hoc test for multiple comparisons using GraphPad Prism v9.* ***b*** *AP39 100 nM increases and maintains movement rate across the lifespan of N2 wildtype animals (P < 0.001) movement when dosing started at day 2 and again is a stronger effect compared to GYY4137 (P < 0.001), while GYY4137 has a reduced effect compared to wildtype (P < 0.01). Day 2 corresponds to 48hrs post young adult and are maintained at 20°C and dosed from day 2 of adulthood till death. Data expressed as area under the curve of 12-18 wells per group (worm number = 115–119 per group) every four days from day 0 adulthood till day 12 of adulthood, across three runs ± min/max showing all points* representing area under curve*, one-way ANOVA, with Bonferroni post hoc multiple comparisons using GraphPad Prism v9. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

Dosing from the second day of adulthood also results in no improvement to lifespan but do show improvements in both AP39 and GYY4137's healthspan (P < 0.001 and P < 0.01 respectively) (Figure 4-2A and B). This suggests that H_2S

only has an effect at increasing lifespan before young adulthood, though may still be of use in congenital mitochondrial diseases. The continued improvement healthspan is slightly improved from day 0, possibly due endogenous H_2S and the decline in egg laying.

4.2.3. Effect of Dosing at Fourth Day of Adulthood

Unsurprisingly dosing from day 4 resulted in no effect on healthspan (Figure 4-3A and B). The mean difference in movement is reduced in both conditions thought still significant (AP39 $P < 0.001$, GYY4137 $P < 0.05$). Producing the lowest improvement on healthspan, dosing from day 4 lowered effects may be linked to the end of egg laying or a decline in mitochondrial number and health reducing the effectiveness of the compounds.

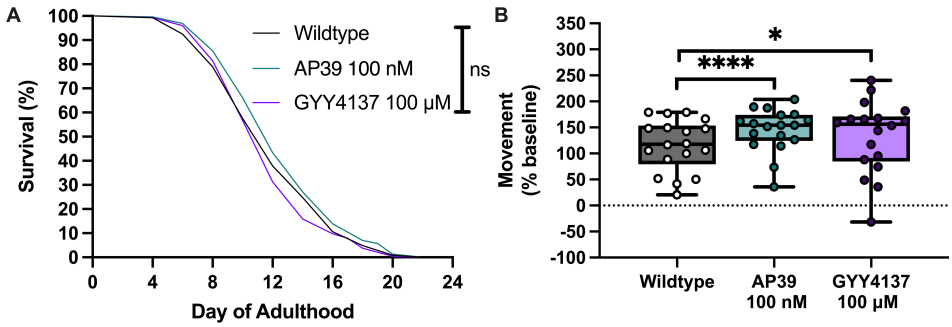


Figure 4-3. AP39 extend the lifespan and movement rate of wild-type C. elegans.

a *Survival curves of wildtype, GYY4137 (ns) and AP39 (ns) with dosing commencing at day 4, however AP39 does show improvement compared to GYY4137 ($P < 0.01$). Day 4 corresponds to 96 hrs post young adult and are maintained at 20°C on compounds from day 4 of adulthood till death. Survival curves were plotted from three independent runs (n = 295-300 per group) by the Kaplan-Meier method and survival values compared using log-rank (Mantel-Cox) test with Bonferroni post hoc test for multiple comparisons using GraphPad Prism v9.* ***b*** *AP39 100 nM ($P < 0.001$) and GYY4137 ($P < 0.5$) have improved effects on movement across the lifespan of N2 wildtype animals when dosing started at day 4. Day 4 corresponds to 96 hrs post young adult and are maintained at 20°C and dosed from day 4 of adulthood till death. data expressed as area under the curve of 18 wells per group (worm number = 114–116 per group), every four days from day 0 adulthood till day 16 of adulthood, across three runs ± min/max showing all points representing area under curve, one-way ANOVA, with Bonferroni post hoc multiple comparisons using GraphPad Prism v9. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

4.3. Discussion

Extended lifespan has potential to cause an extention of a low functional and frail state, while not affecting the young and healthly period of life, this prolongs the elderly stage of life with low functional. To discern the effectiveness of the H_2S compounds protective effects, this study investigated when in the lifespan the start of dosing can occur and still show positive effects. The *C. elegans* were administered a compound regime starting at day 0 young adult, day 2 or day 4 of adulthood until death. We have discovered that through optimal dosing with specific compounds, H_2S supplementation can have positive impacts on function throughout life and ageing when administered before a compound specific time frame.

As both compounds, GYY4139 and AP39, release H_2S in a slow and sustained manner but have different methods of targeting motifs, it may be that AP39 with its mitochondrial targeting motif, is one of the reasons that it has a stronger effect on healthspan extension despite having an active dose a thousand fold lower than ubiquitous releasing GYY4137, (*190*). This mitochondrial specificity may cause a reduced amount of H_2S in the cytosol, which potentially stops the accumulation of toxic concentrations, reduced interactions with targets that are potentially irrelevant or detrimental to health, or through outcompeting other gasotransmitters needed for signalling in the cytosol (*191*). AP39 may be a promising and viable candidate for mammalian ageing and preservation of physical as in a diabetes induced sarcopenia rat model, NaHS was able to help maintain muscle mass and strength (*225*).

The late dosing of AP39 delivers more promising effects than GYY4137 in healthspan for *C. elegans* but neither compound affected the lifespan of the animals. The null result for survival means an increase in healthspan and reduced muscle decline and ageing symptoms. Increased physical activity with age is often seen as a positive indicator for survivability, if this is not the case

for worms dosed from days 0, 2, or 4, then lifespan extension could be an effect resulting from interactions specific to growing larvae or a slight detriment from dosing at the later life stages. Days 0 through 4 are most likely to be peak or near peak of endogenous H_2S production (*226*). With the animals already producing high amounts of the gasotransmitter, it is unlikely that more is required and means the supplementation from the compound causes low grade toxicity and reduces the health of the animals. This negative impact on health can then be negated by the continued supplementation when the endogenous production decreases below the optimal concentration but have altered the lifespan of the animals.

Mitochondria have an influential role in symptoms of ageing and muscle decline, though the mechanisms appear elusive and difficult to predict due to being a multi factor process from the complex interaction of genetics, lifestyle, environmental factors, and chance (*227*). Mitochondrial function and its role in age-related muscle decline are seen through alterations in several processes including bioenergetics, dynamics, biogenesis, mitophagy, and protein homeostasis leading to loss of function (*228*). These mitochondrial alterations are seen in both the muscles and neurons and if left unabated the severity may develop into mitochondria generated apoptosis causing muscle wasting and denervation (*212*). Mitochondrial ageing leads to stress and dysfunction, often induced through damage to the mitochondrial DNA from reactive oxygen species (ROS), this makes mitochondria an ideal candidate for interventions and inhibit the dysfunctions before they become too severe (*212*). In *C. elegans,* muscle function decline starts with the mitochondrial membrane potential declining at day 2 and is subsequently followed by mitochondrial fragmentation as defective mitochondrial components are segregated by fission and removed from the network by mitophagy (*163, 228*). This increase in mitophagy occurs over the first 2 to 4 days of adulthood and is matched by alterations in proteostasis, as ATP levels drop there is an increased total animal protein degeneration (*163, 228*). This decline in proteostasis leads to sarcomere decline, occurring later between days 4 and 6, suggesting

mitochondrial decline leads to a muscular decline after a damage threshold (*163*).

The effects of the NaHS, a fast releasing and untargeted H_2S compound, has been shown to increase mitochondrial function and health through higher ATP production in hepatocytes (*56*). This increase in ATP availability and H_2S having antioxidant effects can keep mitochondria and cells in a healthier state and helps maintain mitochondrial membrane potential (*56*). Targeted H_2S reduces the effects of oxidative stress on renal cells pre-treated with 100 nM AP39 and when comparing this to the effect of GYY4137 100 μM shows similar or improved effects at countering induced oxidative stress (*190, 197*). This antioxidative protection translated to *C. elegans,* explains the increased mitochondrial health shown through network structural integrity and the delayed symptoms of age and sarcopenia in animals by protecting body wall muscle, neuronal, and cells in other organs. Though *C. elegans* show a very narrow window of healthy mitochondrial structure and function in ageing, this may not be the case when translated into mammals, due to their ability to improve and regenerate mitochondria function with exercise (*229*). If AP39 was to be administered after the symptoms of sarcopenia or muscle decline develop and an exercise program adhered to, or routinely given after middle age when mitochondria start to decline, then AP39 may have a huge effect on mammalian and even human ageing and mitochondrial function.

Translating and comparing human ageing with *C. elegans* using biomarker like citrate synthase to analyse mitochondrial function, measuring the decline and effects of the compounds many suggest key stages to target through the lifespan. *C. elegans* show around a 60% drop from peak citrate synthase activity between days 2 and 4, in humans the peak is 20 years and drops ~25% by the 80th year (*230*). It is therefore difficult to track at what point the mitochondrial level needs to drop before activity is lost and so may not directly translate into humans, or human regenerative capacity means H_2S's effect may be retained unless in a severe disease state (*230*). This said, day 4 of *C. elegans*

lifespan many equate to an elderly human mitochondrial and physical function. Taking this further, *C. elegans* have an egg laying period of around 2.5-4 days, after this period the worms could be considered the equivalent to human postmenopausal occurring between the age of 40-61 years and is associated with the onset of sarcopenia (*231-235*). If AP39 shows strong protective effects when used around this time frame, then it could be used as a supplement for muscle degeneration much like calcium and vitamin D is used for bone density loss (*236*). Post menopause causes mitochondrial dysfunction with increased oxidative stress, inflammation and a reduction in metabolism and mitochondrial related proteins, β-enolase (ENO3), glycerol-3-phosphate dehydrogenase (GPD1), NADH dehydrogenase (ubiquinone), Fe-S protein 2 (NDUFS2), and cytochrome b-c1 complex subunit Rieske (UQCRFS1) (*235, 237*). This reduction in post menopause mitochondrial health can be reduced with the use of AP39 if administered with early symptoms.

4.4. Conclusion

The data presented suggests that the use of mitochondrially targeted H_2S compound AP39 improves symptoms of ageing, including muscle and mitochondrial dysfunction and can improve biological function in ageing *C. elegans*. The improvements maintain a higher muscular function via improving mitochondrial function which has been shown through maintenance of mitochondrial structure and content. The ability of these compounds to improve symptoms of ageing in *C. elegans* and the retained effects seen in mammalian models suggest they can provide a possible therapeutic pathway in humans. Effective dosing and timings maybe required for these H_2S compounds and, in *C. elegans,* can be effectively administered before the symptoms and profuse mitochondrial damage has occurred.

5. Chapter Five: Supplementing Space Flown *C. elegans* with Hydrogen Sulfide Prevents Spaceflight Induced Changes in Neuronal Gene Expression

5.1. Introduction

The previous experimental chapters presented data from experiments using two similar hydrogen sulfide (H_2S) donating compounds. Both these compounds release H_2S on contact with water consistently over several days, with the difference being that GYY4137 has ubiquitous release and AP39 targets the mitochondria. These included dosing *C. elegans* from L1, young adult, 2nd and 4th day of adulthood, which showed the importance of H_2S supplementation as an ageing intervention and eliminated developmental effects. With the data showing H_2S treatment improved health with ageing and given the similar biological changes included with spaceflight and ageing, the next experiments examined if H_2S treatment improved health with spaceflight.

This study examined gene expression data from an accelerated ageing model using microgravity. The results revealed the major genes that likely have roles in muscular atrophy may be related to cellular signaling and energy homeostasis, many of which H_2S donor AP39 reversed. Proving H_2S improves gene expression related to muscle decline adds further evidence for its use as an intervention in many conditions that induce muscle atrophy, such as ageing and microgravity.

5.1.1. Spaceflight Overview

Space flight has fascinated people for generations, but biological missions have been limited to low earth orbit (LEO), but deep space still remains relatively untested for earth biology. Microgravity's symptoms closely mimic that of

accelerated ageing, this causes astronauts to slowly lose function after long missions and creating huge risks if other planetary bodies are to be explored and colonised (*151, 152*). Reducing spaceflight ageing symptoms is imperative for astronaut health.

Spaceflight is a harsh, inhospitable, and expensive environment, with biological missions on the ISS costing $120k/kg payload plus $130k/hr for astronaut time (*238, 239*). There is also very little access available, with volume, weight, time, and health/safety limitation (*239*). To make human health research more difficult, there are very few astronauts in space at any one time, currently on Expedition 66 there is a seven person crew, creating very small groups available (*240*). Due to this model organisms like *C. elegans* are use, allowing more invasive and tightly controlled experiments with much larger population sizes, with ICE FIRST having 41,000 animals at the end of flight (*241*). This also allows experiment that would not be safe or ethical in humans such as multigenerational studies and experimental pharmaceutics (*242*).

5.1.2. Biological Adaptations to Spaceflight

The negative adaptations or maladaptation's associated with microgravity closely match the observations and symptoms seen with ageing on earth (*151, 152*). Due to this, spaceflight is considered a model for accelerated ageing, showing similar declines for neurological, cardiovascular function and develop sarcopenia and osteoporosis, with countermeasures having little effect at countering these effects (*151, 152*). Changes of biomechanics in space is largely affected by the changes in health and function of not just the muscle but also the neuronal system controlling them.

5.1.2.1. Flight and Ageing Skeletal Muscle Loss

Skeletal muscle mass and strength are reduced with microgravity, with up to 20% decrease in mass and 30% decrease in strength over a month, while in ageing 3% if strength is lost per year (*243, 244*). While the decreased strength throughout the entire body may be partially due to disuse, this is not the entire cause, for example cardiac deconditioning through bedrest disuse is associated with altered *Trp53* expression however this is not the case with spaceflight (*245, 246*). It is unfortunate that disuse and bedrest have similar functional outcomes to spaceflight as it has led to the misconception that they have the same molecular signatures, more research is needed into the molecular alterations of spaceflight to find cause and interventions. Adding to this knowledge, a study comparing the proteomics analysis of over 7,500 proteins has found only 3 of 177 proteins down regulated in bedrest are in the 187 proteins downregulated in spaceflight, showing the two methods are not entirely comparable (*247*).

In flight, muscle dysfunction is linked with a decrease in mechanosensitive enzymes which are proteins that change their conformation due forces such as gravity, like myosin heavy chain (MHC), this decrease in MHC is also observed in ageing related muscle loss (*152, 248, 249*). This is also indicative of extreme deconditioning at a rate seen after 6 days of flight, matched by bedrest or limb suspension after 6 weeks, and due to reduced maintenance partially due to a disruption in protein homeostasis (*153, 248, 250*). This proteostasis dysregulation alters the muscle phenotype, down-regulating slow twitch type I and increasing fast twitch type IIx, resembling deconditioning on Earth (*248, 251*).

This deconditioning also expresses through damage to the muscle through loss of anchoring proteins for actin and myosin filaments, without these anchors the filaments decrease and cells become disorganised (*248*). Decreasing the loss of these anchoring proteins may provide a therapeutic target to limit the muscle degeneration. Integrins are key to holding cells together, and affect the

health and mobility of the cells (*252*). During spaceflight specific subunits in a variety of ways, integrin subunits *ina-1* and *pat-3* show increased expression while *pat-2,* an *C. elegans* ortholog for integrin subunit α5 and integrin subunit α8, has decreased expression (*242*). Aged muscle also expressed a decrease in integrin, this has been shown on satellite cells during migration showing a decrease in cell recovery and maintenance (*253*). A decrease in these integrins within skeletal muscle could cause muscular structural disruption and reduced neural impulse transmission, through decreased adhesion and recruitment. Lack of cell adhesion and mechano-sensing of muscles and ligaments would mean the individual muscle cells need to increase contractile force to still produce an effect.

5.1.2.2. Metabolism

Mitochondria in spaceflight are low functional, showing low ATP production and greater oxidative stress (*254-256*). In microgravity, skeletal muscle expresses less total RNA, and as with ageing exhibits no alteration in mitochondrial complex cytochrome C oxidase expression but the activity is severely reduced (*257, 258*). Mitochondrial stress and dysfunction, shown through induced ROS production, leading to mitochondrial unfolded protein response (UPR^{mt}) activation, to inhibiting the oxidative phosphorylation pathway, reducing ROS production but also ATP production (*259*). Long term UPR^{mt} activation, like in spaceflight and ageing, is a risk considering its role in deleterious mtDNA accumulation and is likely to eventually contribute to mitochondrial dysfunction, and UPR^{mt} inducing transcription of CHOP causes apoptosis activation further adding to the deterioration (*260-262*). Protection of mitochondria seems a prime target for spaceflight therapeutics and given that H_2S has a proven role in maintaining the mitochondrial network and function in ageing makes it a potential and untested candidate.

5.1.3. H_2S and Spaceflight

Having shown how H_2S can improve healthy ageing by targeting the mitochondria, H_2S may also prove beneficial at maintaining mitochondrial health and energy production in spaceflight, which mimics ageing and diabetes on earth (*225, 263*). Maintaining energy production should in turn help maintain the functional biomechanical and neurological deficits seen in flight, protecting against spaceflight related maladaptations (*263-265*).

5.2. Methods

5.2.1. Strains and Culture

Worms were flown in spaceflight mission Molecular Muscle Experiment (MME). This mission was designed to improve the understanding of gene expression change in spaceflight and how potential interventions may rectify some of this change. *C. elegans* strain used in spaceflight was PD55:*ccIs55* (*unc-54::lacZ*) V, with and without H_2S donor AP39. Worms were gravity synchronised or bleached (20% bleach in 2N NaOH) to obtain eggs (*163*). When grown to L4/young adult stage, approximately 300 animals were transferred to 6-well tissue culture dishes in 1.2 mL of S-Basal with freeze-dried OP50 (LabTie) and 400 µM FUdR. After an overnight incubation at 20°C, 5.3 mL S-Basal containing freeze-dried OP50 and 400 µM FUdR was added to each well and loaded the entire volume (6.5 mL) into polyethylene flight culture bags on December 2nd 2018 (*266*) (Figure 5-1). Culture bags were then placed into protective experiment cassettes (Kayser Italia). Samples were in stowage (8-13°C) until they reached the ISS. Launch of SpaceX CRS-16 occurred on December 5th 2018, and docking to the ISS occurred on December 8th 2018. On December 9th 2018, samples were transferred to 20°C for five days on the ISS. After the five days, samples were transferred to −80°C and kept frozen until return to Earth.

For ground control samples, we performed the same synchronization protocol as for spaceflight samples and we loaded C. elegans into the polyethylene flight culture bags on December 5, 2018. Ground control bags were placed into experiment cassettes and exposed them to the same time frame/temperatures as spaceflight samples but maintained on Earth. Temperature conditions experienced by spaceflight samples were recorded by iButton digital thermometers (iButtonLink) placed inside cassettes and allowed us to expose ground control samples to similar conditions. Detailed temperature recordings from spaceflight and control samples are available in the Harvard Dataverse repository.

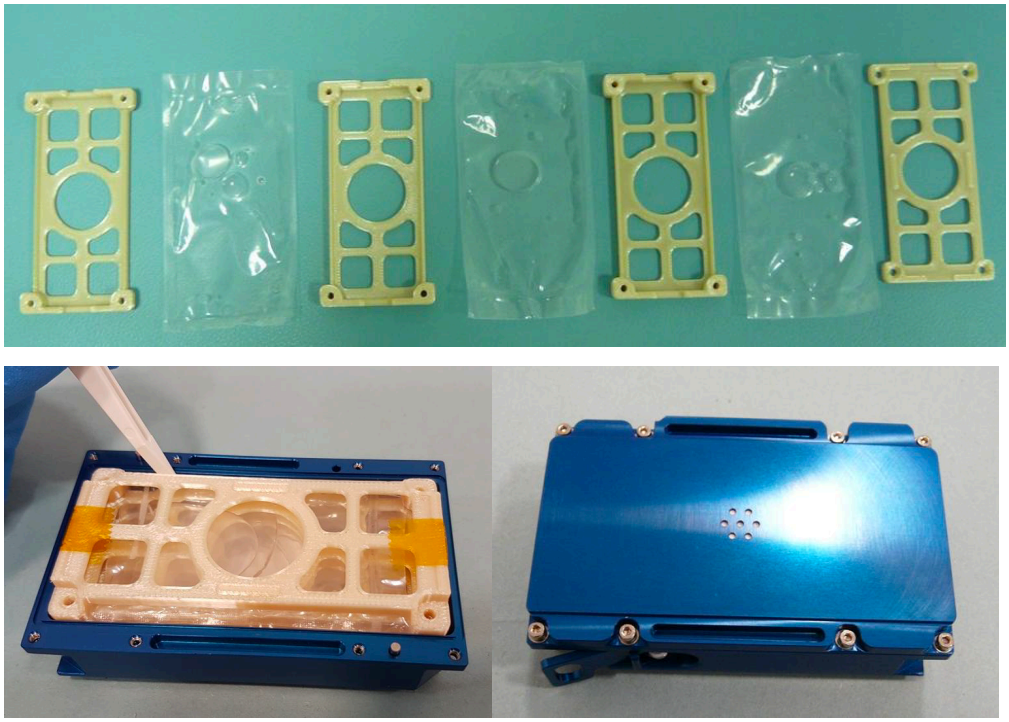


Figure 5-1. Hardware used in MME spaceflight.

5.2.2. Sample Processing

Microarray analysis was carried out as described in ICE FIRST experiment, up-regulation was defined as log2 fold change (LFC) increase of LFC > 0.26 (FC > 1.2) and down-regulation was defined as LFC < -0.32 (FC < 0.8) (*267*). Post-flight worm densities provided RNA for one microarray hybridization per sample. Samples were thawed and washed with M9 buffer before five repeated freeze

thaw cycles. RNA was extracted using Isogen RNA purification kit, concentration and purity was determined by Agilent 2100 Bioanalyzer. Gene expression was analysed using the Affymetrix GeneChip C. elegans Genome Array.

5.2.3. Data Analysis

Analysing the fold change of ground control PD55 *C. elegans* compared to of PD55 *C. elegans* flown with and without H_2S donor AP39, we assessed if H_2S was capable of nullifying or reversing the maladaptation's presented due to spaceflight. Microarray data analysis was carried out as described in ICE FIRST experiment, up-regulation was defined as log2 fold change (LFC) increase of LFC > 0.26 (FC > 1.2) and down-regulation was defined as LFC < -0.32 (FC < 0.8) (*267, 268*).

5.3. Results

5.3.1. AP39 Alters Gene Expression Changes Induced by Spaceflight

When comparing to ground control, untreated spaceflight showed 10,145 genes having altered expression, likewise the number of genes altered with administration of AP39 inflight is 10,443 (Figure 5-2A). Comparing these groups showed 7,320 genes overlapped between control flight and AP39 flight (Figure 5-2A). Of these control flight altered genes, when AP39 was administered 3,617 (36%) of these genes were unaffected. The remaining 6,528 (64%) genes showed AP39 enhanced up-expression in 1,698 (17%) genes inflight and enhanced 678 (7%) genes down-expressed inflight. Interestingly, AP39 increased the expression of 2,837 (28%) genes down-expressed in flight, of which 1,677 (17% of 10,145) genes showed a reversal of change, a significant increase with AP39 compared to ground control. Additionally, AP39 decreased 1,315 (13%) genes up-expressed in flight, of which 576 (6% of

10,145) genes showed significant decreased expression with AP39 compared to ground control.

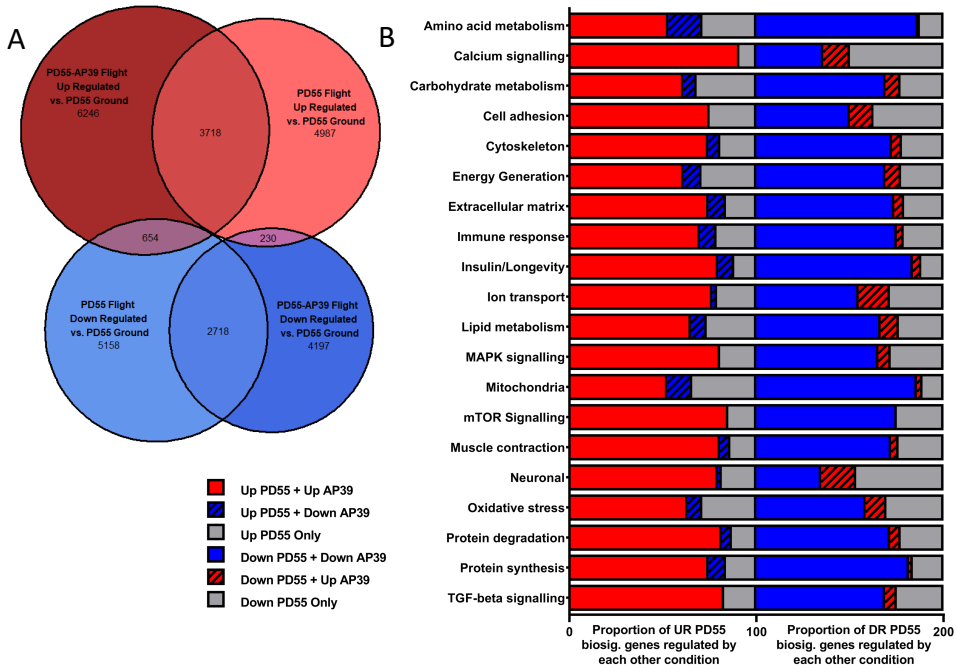


Figure 5-2 Gene expression change vs. ground control during flight with and without AP39 100 nM.

a *Venn diagram comparing PD55 flight vs ground and PD55-AP39 flight vs ground and showing overlapping Gene expression change. Data shown as number of genes showing significance using log2 fold-change expressed as -0.32 > LFC > 0.26 vs ground control.*

b *GO, Reactome and KEGG database terms to create biosignatures of interest. Each signature looks at proportion of genes regulated in-flight with PD55 condition are also regulated in flight with AP39 100 nM.*

To determine possible effects of this on the animals, genes were grouped by function, and expression change was visualised (Figure 5-2B). When averaging the combined functional changes, the majority of the gene function expression change enhances the flight change, increasing up-regulation (74%) or decreasing down-regulation (66%) and many are unchanged (20% and 26% respectively), however there is some reversal of change with 6% of up-regulated being reduced and 8% of down-regulated increasing.

5.3.1. Up-regulated Changes in Flight are Down-regulated with AP39 in Spaceflight

Gene functional groups up-regulated inflight which flown AP39 down-regulated and had the largest changes were amino acid metabolism (18%), mitochondrial (13%), and energy generation (9%).

Table 5-1. Amino acid metabolism related gene expression change in flight with AP39 100 nM.
a *Amino acid metabolism related gene expression is up-regulated in flight but down-regulation when flown with AP39. Significance shown as down-regulation -0.32 > LFC > 0.26 up-regulation vs ground control* ***b*** *Amino acid metabolism related gene expression is up-regulated with flight, but lower expression is seen with AP39. Significance shown as a difference in PD55 flight vs ground and PD55-AP39 flight vs ground less than -0.32.*

A. Gene Symbol	LFC: PD55 Flight versus PD55 Ground	LFC: PD55-AP39 Flight versus PD55 Ground	Delta LFC: PD55 Flight and PD55-AP39 Flight
gst-19	**0.95**	**-1.29**	-2.24
gst-27	**0.35**	**-0.72**	-1.08
gst-6	**0.57**	**-0.41**	-0.99
gst-8	**0.52**	**-0.46**	-0.99
acox-1.3	**0.34**	**-0.98**	-1.31
acox-1.6	**0.67**	**-0.44**	-1.11

B. Gene Symbol	LFC: PD55 Flight versus PD55 Ground	LFC: PD55-AP39 Flight versus PD55 Ground	Delta LFC: PD55 Flight and PD55-AP39 Flight
gst-13	0.69	-0.19	**-0.88**
gst-17	1.00	-0.31	**-1.30**
gst-22	0.94	0.33	**-0.61**
gst-29	1.64	0.88	**-0.76**
gst-42	0.42	-0.24	**-0.65**
alh-12	0.85	-0.01	**-0.86**
alh-3	0.88	0.49	**-0.39**
alh-8	0.48	0.12	**-0.36**

Four of the altered amino acid metabolism-based genes are glutathione s-transferases (GST) which showed a reversal in gene expression from flights up-regulated to AP39 down-regulated with five additional GST genes which were up-regulated in flight and reduced with AP39 inflight (Table 5-1). GST increases in ageing, to compensate for decreased antioxidant potential and increased ROS generation, GST is used in the mercapturic acid pathway to produce pyruvate from glutathione, detoxify cells using glutathione, and correlates to tissue damage (*269-271*). This could suggest an increase in tissue damage from ROS and/or toxins due to a shift in energy metabolism away from glycolysis (*272*).

In addition to this acyl-coenzyme A oxidase (ACOX) and aldehyde dehydrogenase (ALH) both have genes that react to AP39, suggesting an alteration in metabolism from sugars to fatty acids and amino acids (*273, 274*). Coupled with this, the mitochondrial linked functional group shows alterations in genes related to insulin resistance through glycerol metabolism (C55B6.1),

lipid catabolism (pcca-1), and aspirate catabolism (got-2.2) (Table 5-2) (*275-278*). This combined data shows a significant switch of inflight energy generation and increased ROS production, which AP39 alleviates.

Table 5-2. Mitochondria related gene expression change in flight with AP39 100 nM.

a *Mitochondria related gene expression is up-regulated in flight but down-regulation when flown with AP39. Significance shown as down-regulation -0.32 > LFC > 0.26 up-regulation vs ground control.* ***b*** *Genes related to mitochondria are up-regulated with flight, but lower expression is seen with AP39. Significance shown as a difference in PD55 flight vs ground and PD55-AP39 flight vs ground as less than -0.32.*

A. Gene Symbol	**LFC: PD55 Flight versus PD55 Ground**	**LFC: PD55-AP39 Flight versus PD55 Ground**	**Delta LFC: PD55-AP39 Flight versus PD55 Flight**	**B. Gene Symbol**	**LFC: PD55 Flight versus PD55 Ground**	**LFC: PD55-AP39 Flight versus PD55 Ground**	**Delta LFC: PD55-AP39 Flight versus PD55 Flight**
C55B6.1	0.52	-0.76	-1.28	*sqst-2*	0.76	0.34	-0.41
pcca-1	0.60	-0.58	-1.18	*sqst-3*	2.78	1.27	-1.51
got-2.2	0.71	-0.43	-1.14	*sqst-4*	3.45	1.42	-2.03

The energy generation biosignature group has three genes in the dehydrogenase short chain (DHS) family with reversed expression and an additional three with reduced expression (Table 5-3). In addition to this, 11 genes in the cytochrome P450 (CYP) family further adds to the data showing an alteration in metabolism, as both family of proteins have important roles in phase 1 metabolism, defined as reduction, oxidation, or hydrolysis reactions (*279*). The DHS family are NAD(P)H dependant oxidoreductases showing roles in lipid, amino acid, and carbohydrate metabolism, particularly aldehydes and ketones (*275, 279-282*). This suggesting that NAD(P)H is being used outside of the electron transport chain in a stress response to detoxify and protect against oxidative stress at the cost of an energy deficit (*275, 279-282*). While cytochrome P450 is used to oxidise fatty acids and lipophilic toxins (*275, 279*).

This ROS causing metabolic shift affects mitochondria, shown through the large increases in three genes in the sequestosome related family (SQST) during flight and has some of the largest decreases with AP39 (Table 5-2). SQST proteins are predicted to have a role in mitophagy, likely due to damaged mitochondria caused by increased ROS pools inflight and AP39's decreased expression suggest a level of protect (*155, 157, 275*).

Taken together, the data shows flight negatively alters metabolism and oxidative phosphorylation. Possibly by a switch in the source of energy generation, breaking down proteins to free amino acids for the metabolic demands of the cells. This in turn damages the cell and mitochondria leading to increased mitophagy and puts greater strain on the remaining mitochondria, leading to increased cell death. Fortunately, the data shows that AP39 can help relieve some of the stress on the cells and presumably improve the health of the animals.

Table 5-3. Energy generation related gene expression change in flight with AP39 100 nM.

a *Energy generation related gene expression is up-regulated in flight but down-regulation when flown with AP39. Significance as down-regulation -0.32 > LFC > 0.26 up-regulation vs ground control.* ***b*** *Energy generation related gene expression is up-regulated with flight, but lower expression is seen with AP39. Significance shown as a difference in PD55 flight vs ground and PD55-AP39 flight vs ground as less than -0.32.*

A. Gene Symbol	LFC: PD55 Flight versus PD55 Ground	LFC: PD55-AP39 Flight versus PD55 Ground	Delta LFC: PD55-AP39 Flight versus PD55 Flight
acox-1.3	0.34	-0.98	-1.31
acox-1.6	0.67	-0.44	-1.11
dhs-14	0.33	-0.62	-0.96
dhs-28	0.61	-0.47	-1.07
dhs-3	0.41	-0.67	-1.08

B. Gene Symbol	LFC: PD55 Flight versus PD55 Ground	LFC: PD55-AP39 Flight versus PD55 Ground	Delta LFC: PD55-AP39 Flight versus PD55 Flight
cyp-13A1	1.71	1.30	-0.41
cyp-13A12	0.28	-0.06	-0.35
cyp-25A1	0.72	0.17	-0.54
cyp-25A2	0.86	0.11	-0.74
cyp-32B1	1.52	1.17	-0.35
cyp-33C7	0.98	0.64	-0.34
cyp-35A2	0.68	-0.02	-0.70
cyp-35A3	0.40	0.05	-0.35
cyp-35B3	1.24	0.00	-1.24
cyp-37B1	1.34	0.65	-0.69
cyp-43A1	0.71	0.31	-0.40
dhs-2	1.16	0.13	-1.03
dhs-20	0.74	-0.11	-0.85
dhs-21	0.48	0.00	-0.48

5.3.1. Down-regulated Changes in Flight are Up-regulated with AP39 in Spaceflight

In contrast, the largest gene functional group changes that are down-regulated inflight and AP39 up-regulated are neural (19%), ion transport (17%), calcium signalling (14%), and cell adhesion (13%).

Of these neural genes, 148 are Serpentine Receptor classed, also known as G-Protein Coupled Receptors (GPCR) or seven-transmembrane receptors (7-TM), showing reversal of flight induced down-regulation, and a further 423 genes

are down-regulated inflight and show delta increase of more than 0.26 between PD55 flight and PD55-AP39 flight (Table 5-4). These receptors are found on the cell membrane and cause physiological responses to a variety of stimuli called ligand, from environmental senses, such as light, odours and taste, to internal signals through peptides, proteins, neurotransmitters, lipids, and ions (*283*).

Table 5-4. Expression changes in G-Protein Coupled Receptor (GPCR) during spaceflight with and without 100 nM AP39.

GPCR related gene expression is down-regulated with flight, but higher expression is seen with AP39. Significance shown as a difference in PD55 flight vs ground less than -0.32 LFC and PD55-AP39 flight vs ground as more than 0.26 LFC.

Gene Symbol	LFC: PD55 Flight versus PD55 Ground	LFC: PD55-DMSO Flight versus PD55 Ground	Gene Symbol	LFC: PD55 Flight versus PD55 Ground	LFC: PD55-DMSO Flight versus PD55 Ground	Gene Symbol	LFC: PD55 Flight versus PD55 Ground	LFC: PD55-DMSO Flight versus PD55 Ground
sra-18	-0.75	0.26	*srh-220*	-0.88	1.61	*srw-26*	-0.79	0.43
sra-20	-0.41	1.04	*srh-222*	-0.79	0.53	*srw-36*	-1.81	1.71
sra-8	-1.46	0.61	*srh-235*	-0.88	2.39	*srw-44*	-0.56	0.81
srab-13	-0.73	0.84	*srh-240*	-0.49	0.96	*srw-7*	-0.44	0.79
srab-18	-1.57	1.36	*srh-245*	-0.64	0.39	*srw-73*	-0.57	0.62
srab-3	-0.96	1.23	*srh-261*	-0.87	0.73	*srw-79*	-0.73	1.08
srb-7	-0.51	0.30	*srh-264*	-0.80	0.62	*srw-95*	-0.41	0.34
srbc-13	-0.56	1.69	*srh-293*	-0.88	0.54	*srx-112*	-0.71	0.80
srbc-16	-0.67	1.54	*srh-31*	-1.46	0.59	*srx-13*	-0.88	1.40
srbc-17	-0.54	1.18	*srh-40*	-0.59	0.70	*srx-4*	-0.70	1.72
srbc-2	-0.50	0.79	*srh-8*	-1.55	0.40	*srx-48*	-0.80	0.43
srbc-21	-0.68	0.74	*srh-95*	-0.77	0.46	*srx-54*	-1.21	0.87
srbc-22	-1.13	1.88	*sri-17*	-0.82	0.61	*srx-56*	-0.47	0.65
srbc-25	-1.53	0.69	*sri-31*	-0.71	1.32	*srx-6*	-0.70	0.31
srbc-36	-0.72	0.31	*sri-33*	-0.81	0.85	*srx-62*	-1.74	0.48
srbc-45	-0.91	0.92	*sri-60*	-0.55	0.84	*srx-66*	-0.87	0.67
srbc-56	-1.15	0.34	*sri-61*	-1.12	0.34	*srx-68*	-0.97	0.50
srbc-8	-0.59	0.37	*sri-7*	-0.65	0.59	*srx-84*	-0.71	2.74
srd-16	-0.36	0.34	*srj-18*	-0.44	0.27	*srxa-6*	-0.82	1.63
srd-29	-0.43	0.30	*srj-20*	-1.91	0.40	*srz-101*	-0.95	1.51
srd-38	-1.29	0.97	*srj-25*	-0.80	0.39	*srz-42*	-0.35	0.58
srd-42	-0.88	0.82	*srj-38*	-0.88	1.05	*srz-63*	-0.60	0.50
srd-48	-0.59	0.50	*srj-57*	-0.50	0.67	*srz-67*	-0.82	1.61
srd-68	-0.78	0.42	*srj-6*	-0.61	1.25	*srz-72*	-0.56	0.60
sre-13	-0.95	0.29	*srj-8*	-0.76	1.33	*srz-83*	-0.77	1.04
sre-15	-0.69	0.76	*srj-9*	-1.03	0.50	*str-112*	-0.74	1.02
sre-26	-0.39	1.39	*srm-6*	-0.34	0.57	*str-113*	-0.64	1.30
sre-3	-1.07	0.50	*srsx-10*	-0.89	0.86	*str-114*	-1.09	0.54
sre-56	-2.09	0.35	*srsx-11*	-0.63	1.18	*str-116*	-0.71	0.50
srg-20	-0.45	0.31	*srsx-14*	-0.49	1.87	*str-145*	-0.82	0.63
srg-26	-0.81	0.31	*srsx-33*	-0.74	0.65	*str-165*	-0.88	0.88
srg-29	-0.37	0.54	*srt-5*	-0.84	0.70	*str-166*	-0.79	1.29
srg-39	-0.76	0.33	*srt-50*	-0.35	1.38	*str-188*	-0.72	0.34
srg-50	-0.38	0.73	*srt-56*	-0.88	0.30	*str-19*	-0.73	0.49
srg-51	-0.34	1.58	*srt-64*	-0.84	1.76	*str-205*	-0.61	0.34
srg-53	-1.04	1.46	*srt-67*	-1.15	0.48	*str-214*	-0.79	1.17
srg-57	-0.95	0.54	*srt-73*	-1.50	0.89	*str-221*	-0.47	1.32
srg-61	-0.78	1.84	*srt-9*	-1.11	0.69	*str-226*	-1.47	0.30
srh-109	-0.90	1.00	*sru-1*	-0.77	0.50	*str-227*	-0.74	0.52
srh-111	-1.15	0.98	*sru-27*	-0.59	1.12	*str-230*	-0.71	0.88
srh-127	-0.45	0.44	*sru-44*	-0.88	1.64	*str-244*	-1.04	0.67
srh-141	-0.77	0.50	*srv-11*	-0.68	0.31	*str-246*	-0.46	1.84
srh-165	-0.88	1.16	*srv-17*	-0.53	1.33	*str-249*	-0.36	2.50
srh-18	-0.50	1.03	*srv-28*	-0.63	1.22	*str-25*	-0.70	0.72
srh-180	-0.66	0.68	*srv-31*	-0.74	0.50	*str-250*	-0.60	0.38
srh-195	-0.58	0.64	*srw-103*	-1.40	0.84	*str-261*	-0.88	0.98
srh-200	-1.30	0.50	*srw-120*	-0.92	0.96	*str-56*	-0.48	0.79
srh-206	-0.51	0.28	*srw-136*	-0.64	0.70	*str-82*	-1.28	0.35
srh-218	-0.80	0.31	*srw-23*	-0.98	1.09	*str-9*	-0.38	0.46
						str-92	-0.74	3.08

Table 5-5. Ion transport and calcium related gene expression change in flight with AP39 100 nM.

a *Ion transport and calcium signalling related gene expression is down-regulated in flight but up-regulation when flown with AP39. Significance as down-regulation -0.32 > LFC > 0.26 up-regulation vs ground control.* ***b*** *Ion transport and calcium signalling related gene expression is down-regulated with flight, but higher expression is seen with AP39. Significance shown as a difference in PD55 flight vs ground and PD55-AP39 flight vs ground as more than 0.26.*

A. Gene Symbol	LFC: PD55 Flight versus PD55 Ground	LFC: PD55-AP39 Flight versus PD55 Ground	Delta LFC: PD55-AP39 Flight versus PD55 Flight	B. Gene Symbol	LFC: PD55 Flight versus PD55 Ground	LFC: PD55-AP39 Flight versus PD55 Ground	Delta LFC: PD55-AP39 Flight versus PD55 Flight
lgc-17	-0.55	1.21	1.77	*lgc-12*	-0.48	-0.19	0.28
lgc-18	-0.60	0.50	1.11	*lgc-16*	-0.71	0.10	0.81
lgc-5	-0.57	1.16	1.73	*lgc-27*	-1.14	-0.83	0.32
lgc-7	-0.36	0.78	1.15	*lgc-46*	-0.65	-0.03	0.62
egas-2	-0.68	0.84	1.52	*lgc-48*	-0.68	0.05	0.73
ncx-9	-0.34	0.73	1.07	*lgc-52*	-0.71	0.04	0.75
twk-36	-0.47	0.60	1.07	*lgc-6*	-1.90	-0.26	1.63
unc-103	-0.50	0.32	0.81	*ant-1.2*	-0.88	0.05	0.93

In *C. elegans* there are over a thousand GPCR receptors, making up five percent of their genes, which have been placed into families and classes based on genetic similarities (*283*). Many of these receptors have recently been discovered in 2018 and so there is little information about their ligand and expression (*284*). This said, there is still much that can be inferred from the expression data, as the overall receptor expression is reduced due to microgravity, its highly probable that the worms were experiencing alterations in their ability to sense and adapt to changes to their internal and external environment (*263*). Astronauts also experience sensory sensitivity abnormalities through senses such as smell and taste (*263*). This altered perception could be on the macro level, with alterations in sensing the external environment through light, pressure, and chemotaxis, or microenvironment based, reduced cell to cell or internal cellular level with communication perception changed. To further add to this, egas-2, linked to sodium transport, twk-36, a potassium leak enabler, unc-103, voltage-gated potassium channel enabler, and eleven ion transport alteration genes related to ligand-gated ion channels (LGC) are also similarly altered (Table 5-5). This is shown through four ion transport genes reversed and seven with improved expression with AP39,

suggested lower ion flow and membrane potential inflight that AP39 alleviates (Table 5-5) (*285*).

Investigating ion flow and calcium signalling, adenine nucleotide translocator (ANT) ant-1.2 shows a large decrease inflight while also showing the large AP39 recovery (Table 5-5). This protein is mitochondrial related and is predicted to have ATP:ADP antiporter activity (*275*). Reduction in ANT would reduce the mitochondria's ability to keep up with ATP demands as ATP would be trapped in the mitochondria and ADP in the cytosol (*286*). Given neurons metabolic rate using 20% of the bodies oxygen, any metabolic and mitochondrial changes are likely to have a huge effect on their health and function (*287*). In addition to this function, ANT also plays a role in formation of mitochondrial permeability transmembrane pore (MPTP), when cyclophilin-D binds to ANT a calcium binding site is exposed and is used to create a conformational change to become a nonspecific pore (*286*). The pore opening is key for movement of matrix ions and metabolites through the membrane and is linked to either high ROS pools or calcium accumulation in the matrix to prevents toxic calcium overload (*286*). However, high ROS pools in spaceflight cause a detrimental loss of mitochondrial calcium contributing to increased apoptosis (*286*). Calcium loss is key for neural function as calcium is used in Na^+/Ca^{2+} exchangers like NCX-9 for Na^+ entry at the cost of Ca^{2+} being removed and contributes to current generation (*288, 289*). With mitochondrial Ca^{2+} leaking into the cytosol and subsequently be released from the cell, the mitochondria/cell to have less reserve during neuronal action potentials (*288, 289*). Improper calcium handling is thought to be a contributor to cellular ageing and senescence, particularly in neurons (*290, 291*). Mitochondrial contribution to calcium homeostasis is controlled by the electron gradient passively drawing in the positively charged calcium to create a calcium store and released due to stimulus (*291*). The increase in mitochondrial calcium exporters reduces Krebs cycle activity, due to its calcium dependence (*291, 292*). This coupled with the electron gradient decreasing in ageing and spaceflight, all evidence points to a

decline in neuronal and mitochondrial function, and energy production which AP39 improves (*291, 292*).

Table 5-6. C-type lectins gene expression change in flight with AP39 100 nM.

a *C-type lectins gene expression is down-regulated in flight but up-regulation when flown with AP39. Significance as down-regulation -0.32 > LFC > 0.26 up-regulation vs ground control.* ***b*** *C-type lectins gene expression is down-regulated with flight, but higher expression is seen with AP39. Significance shown as a difference in PD55 flight vs ground and PD55-AP39 flight vs ground as more than 0.26.*

A. Gene Symbol	LFC: PD55 Flight versus PD55 Ground	LFC: PD55-AP39 Flight versus PD55 Ground	Delta LFC: PD55-AP39 Flight versus PD55 Flight	B. Gene Symbol	LFC: PD55 Flight versus PD55 Ground	LFC: PD55-AP39 Flight versus PD55 Ground	Delta LFC: PD55-AP39 Flight versus PD55 Flight
clec-105	-0.88	1.26	2.14	*clec-168*	-1.91	-0.98	0.93
clec-134	-0.81	0.52	1.33	*clec-18*	-0.78	0.04	0.82
clec-162	-2.16	0.75	2.91	*clec-183*	-0.88	0.05	0.93
clec-163	-0.76	0.27	1.03	*clec-189*	-0.79	0.14	0.94
clec-171	-0.76	0.97	1.73	*clec-198*	-0.43	-0.06	0.37
clec-186	-0.83	0.35	1.18	*clec-207*	-3.08	-2.20	0.88
clec-191	-0.94	0.63	1.57	*clec-21*	-1.22	-0.86	0.37
clec-193	-0.58	0.97	1.55	*clec-211*	-0.85	0.04	0.89
clec-202	-0.75	1.58	2.32	*clec-212*	-0.57	0.05	0.62
clec-206	-0.69	0.48	1.18	*clec-213*	-0.81	0.09	0.89
clec-234	-0.75	0.63	1.39	*clec-214*	-0.70	0.07	0.77
clec-256	-1.24	2.12	3.36	*clec-22*	-1.39	-0.58	0.81
clec-262	-0.74	4.60	5.34	*clec-238*	-0.58	0.06	0.64
				clec-239	-0.52	0.08	0.60
B. Gene Symbol	**LFC: PD55 Flight versus PD55 Ground**	**LFC: PD55-AP39 Flight versus PD55 Ground**	**Delta LFC: PD55-AP39 Flight versus PD55 Flight**	*clec-240*	-1.09	-0.33	0.76
				clec-241	-0.77	0.01	0.77
				clec-242	-0.88	-0.46	0.43
				clec-243	-0.44	0.10	0.54
clec-100	-0.77	0.12	0.89	*clec-244*	-0.74	0.15	0.88
clec-104	-0.70	-0.09	0.61	*clec-257*	-0.63	-0.16	0.46
clec-108	-3.10	-2.24	0.86	*clec-261*	-1.68	-1.05	0.63
clec-110	-0.76	0.05	0.81	*clec-263*	-0.88	0.05	0.93
clec-111	-0.82	-0.07	0.75	*clec-266*	-0.42	-0.15	0.27
clec-114	-0.88	0.09	0.97	*clec-3*	-0.49	-0.02	0.48
clec-115	-0.77	0.15	0.92	*clec-38*	-0.63	0.04	0.67
clec-116	-0.82	0.02	0.84	*clec-45*	-2.29	-1.99	0.30
clec-121	-0.79	0.05	0.85	*clec-46*	-1.29	-0.16	1.13
clec-129	-0.88	0.05	0.93	*clec-58*	-0.75	0.16	0.91
clec-130	-3.00	-1.37	1.62	*clec-6*	-0.66	0.03	0.69
clec-132	-1.75	-0.82	0.93	*clec-68*	-0.39	0.05	0.44
clec-133	-2.17	-1.73	0.44	*clec-86*	-1.09	-0.57	0.52
clec-138	-2.42	-1.57	0.85	*clec-92*	-0.93	-0.63	0.30
clec-140	-1.85	-0.20	1.65	*clec-93*	-0.70	0.20	0.89
clec-141	-2.24	-1.11	1.13	*clec-94*	-1.08	-0.69	0.39
clec-156	-0.75	-0.11	0.65	*clec-96*	-1.88	-0.96	0.92
clec-157	-1.55	-0.75	0.80	*clec-97*	-1.17	-0.70	0.47

Abnormal calcium handling also affects cell to cell adhesion, there are 67 c-type lectins (CLEC) downregulated by spaceflight and affected by AP39. Of these 13 have reversed down expression and 54 with down expression improved with AP39. CLEC cell to cell binding is calcium dependant and found to be strongly expressed in neural tissue (*293-295*). The loss of this binding is detrimental not only to synaptic cleft and neuromuscular junction organisation and function, but also neural interactions with micro ganglia, astrocytes, and oligodendrocytes (*293-295*). Another neuronal based cell adhesion protein

family affected is through four versions of Zwei Ig domain proteins (ZIG) and have roles in synaptic organisation, suggesting cell adhesion in neurons are severely affected (*275*). Decreased adhesion creates a larger distance between cells, altering the efficiency of signals, this increased area lowers concentration and slows diffusion, creating slower and weaker signals (*296*). This change is associated with neural inflammation and ROS production (*296*). This ROS can cause axon outgrowth, normally modulated by CLEC-38, leading to spaceflight causing neural abnormalities that AP39 alleviates (*297, 298*).

Evaluating the data shows a strong link between ROS production from the altered mitochondrial metabolism and the negative gene expression change in spaceflight. AP39 in many cases rectifies these maladaptation's due to its mitochondrial targeting and H_2S's ability to protect mitochondria from oxidative damage and improve electron transport chain efficiency.

5.4. Discussion

The gene expression data from a single population of *C. elegans* on ground or flown with or without AP39 has shown that in response to spaceflight many genes related to neuronal health and function are down-regulated, while metabolic processes are up-regulated, some of which can be reversed with the use of AP39.

5.4.1. Reproducibility of Gene Expression Data

The MME spaceflight mission is not the first to fly *C. elegans* and analyse the gene expression, as ICE FIRST and CERISE have also performed this but under different conditions (*267, 299-301*). In these experiments the worms were flown in chemically defined medium (*C. elegans* maintenance media, CeMM) for ICE-FIRSE, in liquid NGM containing OP50 and the current MME expriment used OP50 suspended in S-Basal (*266, 267, 299-301*). Each food source affects the worms differently with CeMM having worms grow smaller and lowering

the oxygen consumption rate vs OP50 S-Basal, and liquid NGM also growing longer compared to NGM agar (*267, 299-301*). With food sources affecting body morphology there is also alterations in gene expression, for example liquid NGM causes genes *myo-3* and *hlh-1* to both show an increased expression compared to NGM agar (*299*). Due to this variability it is difficult to pinpoint the cause of the differences in gene expression between the spaceflight missions (Table 5-7). To add to the differences in methodologies, ICE FIRST used N2 wildype with a 11 day spaceflight (2 days at 12 ^{o}C, 9 days at 20 ^{o}C), while CERISE was 4 or 8 days microgravity to produce a second generation also using N2 wildtype, and MME used PD55 strains and flew for 5 days (*266, 267, 299-302*).

Table 5-7 Examining differences in gene expression between current and past spaceflights. (267, 299-301)

Gene Symbol	MME		ICE FIRST		CERISE		Gene Symbol	MME		ICE FIRST		CERISE	
acox-1.1	↑	*0.2*	↑	1.13			*abu-7*	↑	0.16	↓	*-1.24*	↓	-0.63
acox-3	↑	*0.28*	↑	1.28			*abu-6*	↑	0.01	↓	*-2.18*	↓	-0.78
acs-7	↑	*0.6*	↑	1.12			*C41G11.1*	↑	0.3	↓	*-1.21*		
C23H5.8	↓	*-0.13*	↓	-1.06			*cha-1*	↑	0.31	↓	*-4.32*		
C35A5.3	↓	*-1.3*	↓	-1.2			*F14D2.7*	↑	1	↓	-1.46		
C36C5.12	↑	*1.8*	↑	1.37			*F15A4.6*	↓	-0.26	↑	1.3		
cyp-32B1	↑	*1.52*	↑	1.25			*F19B2.5*	↓	-0.44	↑	1.51		
dim-1	↓	*-0.07*	↓	-0.53	↓	−0.11	*F57A8.4*	↑	0.75	↓	-3.32		
F17H10.1	↑	*0.49*	↑	1.11			*gar-3*	↑	0.14	↓	*-0.23*		
fat-7	↓	*-1.07*			↓	-0.38	*ins-35*	↑	1.81	↓	*-2*		
flp-17	↑	*0.61*	↑	1.67			*lipl-5*	↑	1.02	↓	*-1.21*		
glc-4	↓	*-0.29*	↓	-0.62			*lgc-54*	↑	*0.23*	↓	-4.32		
glr-1	↓	*-0.14*	↓	-4.32			*msrp-7*	↓	-0.12	↑	*1.06*		
hsp-16.41	↑	*0.2*	↑	1.36			*myo-3*	↑	0.44	↓	*-0.34*	↓	-0.89
hsp-70	↑	*0.98*	↑	1.23			*shk-1*	↑	0.46	↓	*-3.32*		
K07C11.7	↓	*-0.24*	↓	-1.67			*sir-2.1*	↓	-0.1	↑	*0.03*	↑	0.53
pqn-78	↓	*-0.07*	↓	-3.08	↓	-0.8	*T14A8.2*	↑	0.76	↓	*-1.23*		
ule-4	↓	*-0.43*	↓	-1.16			*ugt-41*	↓	-0.33	↑	*1.21*		
unc-17	↓	*-0.47*	↓	-0.32			ugt-42	↓	-1.11	↑	*1.07*		
							unc-54	↑	0.57	↓	*-0.45*	↓	-0.49
							unc-87	↑	0.75	↓	*-0.34*	↓	-0.25

The alterations in worm *myo-3* may be related to worm size. Dauer worms fed and then experiencing 4 day spaceflight produced smaller worms however, 40 days of microgravity produced much larger worms (*301, 303*). Low *myo-3*, *unc-53* and *unc-87* expression after 11 days post adulthood in CeMM (ICE FIRST) would be old age for the animals and with microgravity further accellerating the ageing, thus the expression is unexpected of an elderly animal. The CERISE 4 days post dauer experiment also showed the expected down-regulation despite the worms growing to adulthood in flight. However, in MME the

worms were grown to adulthood and placed in cold storage preflight, lowering their metabolism and slowing their growth. It is estimated that after 5 days of flight the animals may only be biologically 6 days post adulthood but despite the used of FuDR prodgeny were produced and growing and so may have produced anomalous results (*302*). Unfortunatly without confirmation on the size of the MME worms or number of progeny little can be concluded from this.

Overexpression of *abu* genes are linked with the immune response to live bacteria and pathogenic infection causing ER stress, this could suggest that the MME animals are infected by bacteria, as does increases of *C41G11.1* and *lipl-5* (*275, 304, 305*). CeMM contains no bacteria and CERISE presents little data about the used OP50, however LabTie freeze-dried OP50 has reduced viability but is not killed, providing a possible OP50 infection route or bacterial contamination (*266, 267, 299-302, 306*). CeMM's alteration in growth is linked to dietary restriction or partial starvation and so many of the differences may be food source related (*307*). This can be seen through *ins-35* being increased similar to the diabetes phenotype experience by humans, and like *F14D2.7*, *T14A8.2*, *shk-1,* being up-regulated and *msrp-7* down-regulation in MME opposing CeMM growth, due to being regulated by the daf-2/daf-16, which is linked to stress response and dietary restriction (*300, 308, 309*). *F19B2.5* is linked to growth and its expression peaks around day 6, suggesting that this increase may be to do with timing of end of flight and the combined effects of microgravity and food source on the growth of the animals and the delay in peak/trough (*310*). Similarly *F57A8.4* and *sir-2.1* are linked with dietary restriction but not directly related to *daf-16* expression, with *F57A8.4* decreasing and *sir-2.1* increasing with starvation (*300, 311*). Dietary restriction also affects glr-1 through *daf-7* increasing expression and inhibiting glr-1 (*312, 313*). Gar-3 has cross talk with eat-2 and has important roles controlling pharangeal pumping and food intake (*314*). Eating liquid CeMM is easier for the pharynx and so pumping is less work, so expression of *gar-3* would be reduced.

5.4.2. Neuronal and Muscular GCPR in Spaceflight and Ageing

Many of the genes altered in spaceflight affect neuronal function through receptor down-regulation, ion transport and calcium signalling, and cell adhesion. In both ageing and spaceflight there is an alteration in calcium handling and may be a contributing factor to the altered GPCRs expression (*315-317*). While there are many factors that affect the progression of this condition, outcomes involve decreased sarcoplasmic reticulum calcium content and calcium ion channels resulting in reduced calcium mediated signalling as higher stimulation is needed to induce synaptic depression (*316, 318*). This inability to signal efficiently with calcium, coupled with abnormal synaptic cleft adhesion in spaceflight, affects both neural and muscular function, promoting both fine motor control and neurological dysfunction, presenting as balance, memory, and concentration difficulties (*316, 318, 319*). With ageing reduced cellular calcium is also mirrored in the lowered serum calcium levels and contributes to the reduction in bone mass, through an imbalance in osteoclast and osteoblast coupling in bone remodelling, leading to bone resorption, however in spaceflight this bone resorption and strict diets keep serum calcium levels within range and the body excretes large amounts to limit hypercalcinaemia (*317, 320*). If the mechanism is conserved the cause is either alterations within the cell at a genetic level in response to microgravity/ageing or a calcium independent factor at the microenvironmental level such as vitamin D and/or magnesium deficiencies (*321-323*).

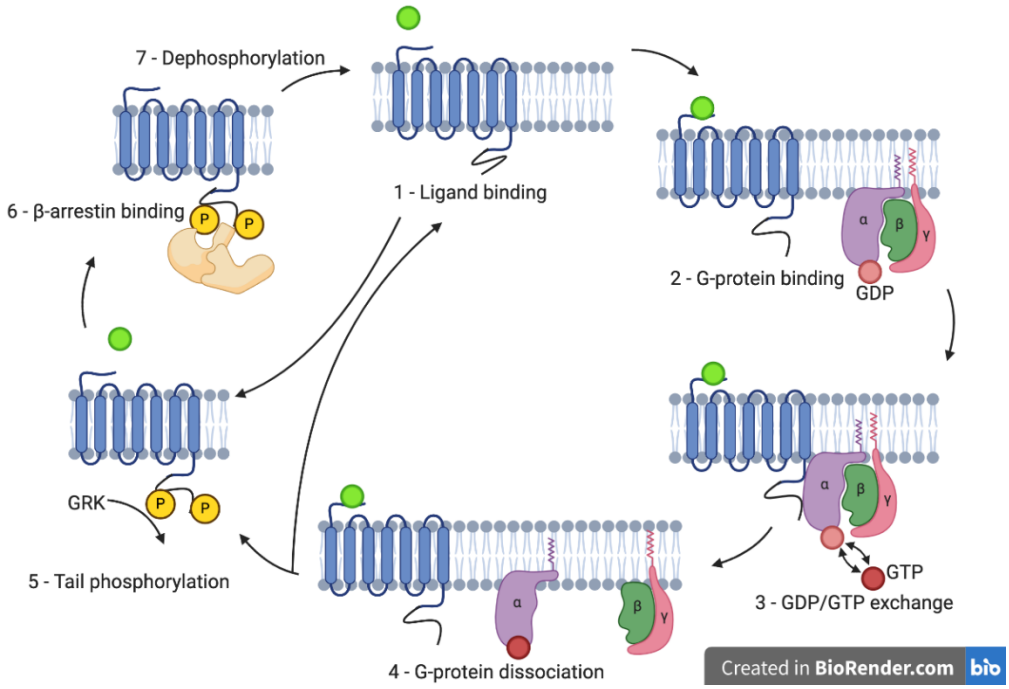


Figure 5-3. Classical G-Coupled Protein Receptor (GCPR) activation and signalling.

1 – Ligand binds to GCPR causes conformational changes to the receptor, exposing binding sites and leading to either process 2 or 5. 2 – G-protein containing subunits α, β, and γ binds to receptor causing the conformational change of the G-protein. 3 – Guanosine diphosphate (GDP) is exchanged with guanosine triphosphate (GTP). 4 – G-protein α subunit dissociated from βγ subunits and leaves the receptor freeing for the restarting at path 1 or through path 5. 5 – GPCR kinases (GRK) phosphorylate the receptor tail releasing the ligand from the external binding site. 6 – The phosphate sites attract and bind to β-arrestin and desensitising the receptor by blocking G-protein binding and recruit calthrin for internalisation/endocytosis. 7 – Endocytic vesicles are trafficked for either degradation by lysosomes or recycled by endosomes, involving dephosphorylation by protein phosphatase 2A (PP2A) returning receptor to deactivated state and returning to plasma membrane.

Spaceflight causes elevated levels of calcium available in the serum, GPCRs signalling could initially be under activated due to excessive amounts of calcium activating calmodulin, which is also up-regulated in spaceflight (*283, 320, 324, 325*). This is further contributed to by a slight increase in spaceflight expression GRK genes *grk-1* and *grk-2* both having slight 0.11 and 0.27-fold increases respectively from ground control. By calmodulin increasing GRK phosphorylating the ligand tail of the GPCR inactivating it and marking for β-arresting to internalise in vesicles to either be recycled or if inactivation is prolonged marking it for degradation (Figure 5-3) (*326, 327*). GPCR activation uncouples the G protein subunits and activate secondary and tertiary effects

including activating adenylate cyclase pathway to open Ca^{2+} channels and increase intra cellular Ca^{2+} (*328*). As spaceflight has altered calcium handling, it could be that the down-regulation is a mechanism in response to this reducing intracellular Ca^{2+} and protect the cells from Ca^{2+} overload (*329*).

With mitochondrial targeted H_2S donors improving the GPCR expression, this could be in part due to a downstream adaptation to improved mitochondrial and sarcoplasmic reticulum calcium handling (*75, 330*). AP39's inhibition of mitochondrial permeability transition pore and ryanodine receptors to keep calcium ions within the organelles, decreasing oxidative stress associated with pore opening while also reducing risk of calcium overload, helping to improve ion handling of the cell, increasing general health of cells and the efficiency of the neurons, motor neurons and muscle cells (*75, 330*). Unfortunately, the data is end of flight and so we can't measure key time points and show when these adaptations occur, and the available data has no way to test the true efficiency of the neural impulses and check motor function. However, this does pave a route for future experiments to where motor and neuronal function can be measure in space and compared to control and terrestrially aged animals.

Another tissue type that experiences maladaptation in spaceflight and also expresses 14 of the AP39 enhanced neuronal GCPR is coelomocyte, macrophage like cells that are part of the worms innate immune system, and this may correspond well with the 26 up-regulated GCPR and known to be affected by various types of bacterial and fungal infections (*331*). In humans the immune system is hindered in spaceflight as monocytes, prerequisite of macrophages, struggle to remain mobile and signal effectively, contributing to infections, hypersensitivity and rashes, and reactivation of latent viruses that become prevalent in astronauts (*332, 333*). Increasing the expression of these GCPRs in immune activating neurons ASI and ASH, and coelomocytes which all play a role in C. elegans immunity suggests that either flight decreases the immune systems function and AP39 increases the health and ability of the

animal's immune system, or the worms were infected with a bacterial infection before and during the flight. If the former and is as in terrestrial experiments, H_2S helps mediate the improvements in inflammation and immune cell secretions to reduce tissue damage through oxidative stress and apoptosis (*334*).

5.4.1. Cytoskeletal and Adhesion in Spaceflight and Ageing

Other cytoskeletal and G-protein related signalling appear to be an interaction between Gα's switch region I and β3 or β1 integrin, to regulate cell shape and proliferation (*335*). Integrin is a critical component of focal adhesion complex, this complex contains many parts, which allows cross talk in the signalling pathway resulting in smooth muscle contraction (*336*). GPCR's Ca^{2+} signalling is enhanced by integrins inhibiting cAMP/KPA and activating G proteins and ERK signalling (*336*). The remodelling functions of GPCR in disease states like asthma cause increased extracellular matrix protein deposits, altered integrin expression and play a role in muscle contraction (*336*). GPCR's also act upstream of integrins, by Gα activating Ras homolog family member A (RhoA), and increasing the expression of extracellular matrix protein Cysteine-rich angiogenic inducer61 (Cyr61), which when up-regulate and binds to integrins to induce mitogen-activated protein kinase (MAPK) pathway (*337*). Cyr61 is an extracellular matrix associated signalling protein with autocrine and paracrine effects induces processes linked to adhesion through focal adhesion formation, cytoskeletal structure thought lamellipodia and filopodia formation (*337*).

Fluid shear stress (FSS) is measured by endothelial cells through mechano-sensor PECAM-1, and GPCRs (*338*). GPCR comes in many types and adhesion type GPCR latrophilin (ADGRL3 or LPHN) can be activated within seconds of sheer stress change (*338*). Latrophilin's effect are through the α -subunits of the G protein that contain amino acid lysine at position 307, which is a key

component of the binding interface between the G-protein and GPCR (*338*). In spaceflight the change in fluid distribution reduces sheer stress but has little effect on LPHN genes lat-1 and a minor effect on lat-2, which with the use of AP39 change from 0.025 and 0.248-fold flight control to 0.0485 and 1.467-fold, respectively. VE-cadherin forms a complex with PECAM-1 and interacts with an α-subunit G-protein in response to oxidative stress and inflammation (*339*). This causes Src activation and VE-cadherin phosphorylation and internalisation, which destabilises adherens junctions (*339*). GPCRs are a diverse group of receptors and can have opposing effects depending on the type, importunately the data available from the spaceflight and the current lack of testing of GPCRs in *C. elegans* makes it difficult to determine the types and functions of what has occurred in flight.

As many of *C. elegans* GPCRs are severely down-regulated in spaceflight and either salvaged or up-regulated with the administration of AP39. This suggests that cellular signalling is preserved in spaceflight with the compound which maintains the cytoskeletal structure, cellular signalling and preserve neuromuscular function. However, without functional outputs in spaceflight it is difficult to ascertain if these effects and expression changes affect the phenotype.

5.4.2. Metabolic changes with AP39 in spaceflight

There are also alterations in growth and energy metabolism in spaceflight, with H_2S supplementation reversing some of these. With energy metabolism being a large process taking place in multiple compartments of the cell, the ability to control and improve the process in spaceflight would make stronger healthier *C. elegans*. Some examples are alterations of receptors on the cell membrane F40G9.8 reduced by -0.81 log fold change (LFC) inflight and increased to 2.73 LFC with AP39 vs ground, this receptor is thought to be a transmembrane receptor with tyrosine kinase activity (*275*). Its activation positively regulates

the MAPK and ERK1/2, which are known for influencing survival decisions of the mitochondria, increasing the membrane potential and colocalising with Bcl2 for pro-survival but also increased Caspar activation and decreasing oxidative phosphorylation for pro-apoptosis (*340*). ERK1/2 also promotes survival through proliferation and differentiation related gene expression using transcription factor activation, particularly Elk1 (*340*). ERK1/2 has interactions with the cytoskeleton, by directly binding to actin and microtubules, and indirectly through calponin (*340*). These interactions help regulate ERK1/2 activity by restricting entry into the nucleus for transcription factor activation (*340*). This has interesting implications as it requires an intact cytoskeleton so remodelling or degradation of the cytoskeleton would promote ERK1/2 transcription factor activation (*340*). ERK1/2 also enhances myosin light chain kinase activity, to enhancing motility and improving muscle contraction (*340*).

Also on the cell membrane is insulin/EGF-receptor L domain protein-59 (irld-59) lowered to -0.82 LFC inflight and increased to 1.54 LFC with AP39 vs ground (*275, 341*). Unlike DAF-2, an orthologue of insulin like growth factor 1 receptor (IGFR) and insulin receptor (INSR), irld-proteins doesn't contain tyrosine kinase domains, and are proposed to be part of an atypical insulin receptor complexes (*341*). This decrease in glucose uptake is matched by an increase in lipid metabolism through eleven cytochrome P450 (CYP) family genes increasing on average by 0.95 LFC decreasing by 0.56 LFC with AP39 administration, and seven dehydrogenase short chain (DHS) family genes changing from 0.62 to -0.29 LFC. Spaceflight is associated with symptoms of age-related type II diabetes at a subclinical state, showing insulin resistance and a metabolic shift to lipid metabolism (*342, 343*). This is dangerous as β-oxidation becomes overloaded transforming nutritional intake into fat stores causes large fat storage in lipid droplets to protect the mitochondria but at the expense of non-alcoholic fatty liver disease (*343*).

Mitochondrial components altered and rectified in spaceflight using H_2S involve the expression of *C14B9.10* and *C09E9.1*. *C14B9.10* produces a protein containing domains for ubiquinol-cytochrome C reductase subunit 9 a component for mitochondrial complex 3, the increase from -0.88 to 1.20 LFC suggests flight causes an energy deficit that AP39 improves through the electron transport chain and ATP production (*275*). Adding to this C09E9.1's ability to regulate membrane potential and changing from -0.62 to 0.88 shows multiple ways AP39 improves electron transport chain efficiency and mitochondrial health (*275*).

Protecting mitochondrial from reactive oxygen species (ROS) produced by the electron transport chain (ETC) is important as it damages lipids and proteins, and also negatively regulates ATP production. *Y46E12A.3* changes from -1.31 to 1.26 when AP39 is used in flight, it contains a thioredoxin-like fold, which acts as a nucleophile to reduce oxidised antioxidant proteins such as peroxiredoxin back into their native state to continue their ability to remove ROS (*344, 345*).

As flight reduces the antioxidant capacity of the cell's mitochondria, causing damaged organelles and are removed through mitophagy which is up-regulated in flight through SQST genes, expressing an average change of 2.33 to 1.01 LFC using AP39. SQST genes are key to mitophagy independent of the classical PINK1/parkin pathway (*346*). The SQST genes are used to ubiquitinate the mitochondria and mark for mitophagy, this is controlled differently to the PINK1 model, which is due to loss of dynamin 1-like (DNM1L) resulting in loss of division and creating large mitochondrial, leading to the view that PINK1 may be regulating mitochondrial dynamics and function rather than mitophagy (*346, 347*). However, the damage mechanism for SQST is still unproven but does show selectivity for depolarised mitochondria and accounts for 80% of ubiquitination (*346, 347*). There are varying reports using SQST knockout, with some observing no difference in mitophagy due to overlapping functions with parkin, but early stages show less PINK1

accumulation at mitochondria, but has little effect as there are four other autophagy receptors to compensate the loss (*346, 347*). Overexpression of SQST has very varying results, having involvement in invasion and metastasis of cancer, accelerating amyotrophic lateral sclerosis, protection from prion disease, and protection from drug-induced ROS and inflammation (*348-351*). It is worth nothing that its expression is also increased in ageing and caused my mitochondrial dysregulation and so while it may be protective, its increase in spaceflight should be viewed as a marker of mitochondrial stress (*351, 352*).

5.5. Future Directions

In the previous chapters it has been shown that H_2S produces healthier ageing by interacting with the mitochondria. This was shown through survival, movement, and mitochondrial network and content assays. Spaceflight being a form of accelerated ageing, producing weak musculature and altering metabolism, also shows AP39 helps some of the adaptive gene expression responses to microgravity (*153*).

In this chapter, many genes that are altered in spaceflight can be improved with H_2S/AP39. Interestingly H_2S altered the up-regulation of those related to mitochondria, metabolism and energy generation thought to be related to the highly ROS producing pathways of lipid oxidation. Adding to this, genes related to the function of highly energy demanding tissues, neurons and muscular, are also altered.

However, gene expression outputs give very little data about the health and quality of life of the animals. To obtain this data similar outputs are needed to those in the chapters before. Unfortunately, present hardware available does not allow the accurate collection of survival, movement, or fluorescent marker data in flight and so to determine if the gene expression change does relate to improved health inflight new hardware need to be developed.

6. Discussion

6.1. Summary

H_2S is an endogenously produced gasotransmitter, with reduced production associated with many diseases and supplementation alleviating symptoms (*10, 11, 74, 75*). While research into ageing and H_2S, using GYY4137 have shown positives, the study was flawed as the supplementation started at larval stage, rather than an appropriate stage of adulthood, causing effects on developmental to combine with effects on ageing (*87*). In addition to this the study implicated antioxidant activity of H_2S as a likely mechanism through paraquat use to oxidatively damage mitochondria but were unable to determine if H_2S caused mitochondrial protect.

To expand our knowledge and determine the efficiency of H_2S as an ageing therapeutic to promote healthier ageing, this research was designed to firstly compare GYY4137 with novel mitochondrial targeted H_2S donor AP39, both of which release H_2S consistently for several days on contact with water and required no biological processing. The first key findings from study one in this thesis demonstrated H_2S supplementation from L1 improved muscle and mitochondrial function and protection throughout the lifespan and produced healthier ageing. The improvements expressed through lifetime movement rate and survival matched the available literature on GYY4137 and targeted donor AP39 expressed further improvement (*87*). To add to the investigation, this study analysed ageing mitochondrial function through mitochondrial network visualisation and citrate synthase activity to show key improvements in mitochondrial function through ageing, with mitochondrial targeting having a clear improvement, cementing mitochondrial H_2S as a crucial participant in healthy ageing.

The second study took this further by using GYY4137 and AP39 in adulthood to negate developmental effect, this study had the unexpected result of not

improving survival but produced increased healthspan, with AP39 providing the greater effect. This added to the evidence that has consistently shown that mitochondrial targeting of the H_2S delivery improved the effects regardless of administration delay and required a much smaller dose.

With AP39 improving the symptoms of terrestrial ageing, this led to the natural progression to study three, testing on an accelerated ageing model through microgravity experienced in spaceflight. Both accelerated and natural ageing, show symptoms of decreased function and mitochondrial function, which was the inspiration that led to the use of AP39 as a potential therapeutic for spaceflight maladaptations. The third study in this thesis has shown AP39 reverses many of the gene expression changes seen in microgravity and likely improving neuro and muscular communication and function, while improving mitochondrial function and energy production pathways to reduce oxidative stress.

6.2. Limitations and Future work

6.2.1. Study Limitations

Ageing research is naturally very slow as it requires monitoring organisms from birth through the entire natural lifespan. *C. elegans* have a relatively short lifespan but each experiment in triplicate can take many months to complete. Due to this there was not time within the allotted duration to complete the citrate synthase and mitochondrial network analysis in the delayed dosing experiment. This makes it difficult to comprehensively conclude what the effects on the mitochondria are.

There are also hardware limitations of the WMicroTracker. This equipment used 10 worms per well and counted movement as breaks through the two light beams shining vertically in each well. The automation of thrash assays reduces any unconscious bias but also means movement counts are only

shown if the worms happen to move through the sections of the wells containing the light beams, but the worms are more prone to circling the edge, potentially reducing the sensitivity. This does not invalidate the results as during the 10+ years the device has been in the market is has been extensively validated and has over 100 publications, but other movement behaviour readouts are lost.

The gene expression data showed many changes during accelerated ageing but it is difficult to be certain of the reliability of the data as with a single replicate p value cannot be obtained. The data also suggests that the FUdR, used to stop progeny production, was not completely effective but to what degree cannot be determined. Adding to this, *C. elegans* microgravity experiments are very rare, with previous spaceflight missions having all used different methods and timings to improve and perfect the model, making direct comparisons is complicated. Standardisation with increased repeats and replicates of *C. elegans* spaceflight missions needed for the subject to produce clear results.

6.2.2. Future Work

While this thesis has provided evidence that H_2S improves biological function throughout ageing, it does not provide the mechanisms and signalling pathway behind the effects, which could be examined with proteomics work. By measuring which proteins H_2S interacts with and is persulfidating using low-pH quantitative thiol reactive profiling, persulfidation sites could be explored (*353*). This ratio of specific protein persulfidation measured over the lifespan and combined with genetically altered *C. elegans* to produce individual proteins without sites available for persulfidation, by changing the cysteine site to selenocysteine, would provide more targeted sites for intervention. Selenocysteines can be used to in place of cysteine and still retain functionality but cannot be persulfidated (*354*). This combined with the alterations in lifespan, movement and mitochondrial function could be measured to determine which proteins are the main contributors to the improvements.

An additional limitation of this study is although it has measured mitochondrial related values, citrate synthase for mitochondrial content and analyses network structure, key functions of the mitochondria were not measured such as ATP generation and the related ROS production. Combining ATP generation with ROS detection and oxidative damage markers would show how mitochondrial efficiency is altered and if this also translates to lower oxidation damage, a potential cause of symptomatic ageing.

Future work on this topic could include other model organisms, as while the compounds work well for ageing in *C. elegans*, it would be beneficial to also know if this translates into other more complex organisms. While AP39 is not yet approved for human testing other mammalian models could be used, namely rats and mice. Ageing research is not often performed on these animals due to the time constraints needed, both living around 2.5 years. However, if the study harnessed accelerated ageing techniques and is limited to suitable cutoff points for muscle and mitochondrial function, the compounds could be using for in vivo mammalian ageing research.

In addition to this, improving the data output for the microgravity accelerated ageing model is needed. The opportunity to perform spaceflight is rare and expensive, so more data per flight is needed to make the science cost effective. Current *C. elegans* missions have been centered on gene expression changes but with improved hardware, containing cameras and fluorescent technology, would permit more data to be captured. The additional movement, survival, growth, and inflight gene expression using GFP changes throughout the flight would allow the spaceflight community to perform much more complicated experiments and enhance the rate that spaceflight biology can improve. Current spaceflight missions are performed on the ISS but can be automated and flown as free flying CubeSats using commercial off the shelf components, controlled by simple programs run on raspberry pi. Increasing flight

opportunities is key to creating an accessible flight environment and the progression of space biology.

6.3. Implications of Findings

There is a growing need for ageing therapeutics, as the population is ageing and is a major risk factor of chronic disease (*355*). Ageing has largely been attributed to oxidative damage, leading to cellular senescence and chronic inflammation (*355*). H_2S has a growing body of evidence demonstrating its ability to reduce oxidative damage and inflammation but not to alleviate ageing symptoms and promote healthier ageing during a natural ageing process (*32*). With the mitochondria being implicated as the source of the increased ROS production in ageing, this thesis used a mitochondrially targeted donor AP39 and compare this to the commercially available GYY4137 with its ubiquitous release (*73, 87, 355*). As the only difference between the donors is organelle targeting any differences seen in experimental measures is due to changes in mitochondrial and cytosolic concentrations. With AP39 showing the greater healthspan throughout ageing, suggests that the mitochondrial H_2S is improving the antioxidant capacity of the cells through increased reducing power at the source of ROS and further limiting the oxidative damage (*87*).

While AP39 has been used for various diseases in many organisms, from cell lines through to large mammals like rats and pigs, but at the time of writing there are no clinical trials (*74, 197, 356*). There have been trials of H_2S donating prodrugs, with seven compounds completing at least phase 1, one withdrawing, and Clopidogrel and Zofenopril have FDA approval (*357*). As these clinical trials prove the general concept of H_2S donating is safe for human and this thesis has shown the benefits of AP39, there should be no reason not to pursue using AP39 in clinical trials to show improvement in human ageing and ageing symptoms.

The clinical implications of AP39 may result in decreased frailty in the elderly population, this would be through promoting healthier ageing and helping with comorbidities. With H_2S being studied for the use for treatment of inflammatory diseases and the Covid-19 pandemic, AP39 may be necessary to help treat not only the initial infection but also "long covid", both of which are more serious in the elderly (*358, 359*). Work has shown that plasma H_2S is predictive and protective against Covid-19's cytokine storm, and with H_2S increasing antioxidant activity also protects against the oxidative stress infection produces and limits viral replication through GSH (*358*). GSH interacts with ACE2 and transmembrane serine protease 2 to reduce virus entry into the host cell (*358*). With long covid, mitochondrial oxidative stress has been implicated as the cause of its chronic fatigue syndrome and antioxidants are being observed at potential therapies (*360*). AP39's mitochondrial targeting makes it a prime therapeutic for long covid and potentially cement it as a therapeutic for chronic diseases, including those associate with ageing.

While longer lifespans has implications with exacerbating overpopulation, climate change, social-economic issues, and large-scale pandemic disasters, medicine must be both moral and pragmatic. With the world in its current state, increasing lifespan would be disastrous for both humanity and the planet. The focus of ageing research must be on increasing healthspan so the human population can be as productive as possible for as long as possible, else the above issues would create a tipping point for the life on this planet as we know it. The social implications of increasing the ratio of healthy and functional to unhealthy and nonfunction of an organism's lifespan has clear benefits. This said, there are moral implications for the misuse as the reality is that all pharmaceuticals can be for benefit and harm. H_2S is no exception to this, with its clear and well-defined toxic effects if abused, and even when being used for benefitting health, it may still cause harmful with unknown side-effects.

However, H_2S compounds are likely to be safe if controlled and used for low dose therapeutics given that supplementation is already used though garlic

extract and FDA has approved specific compounds (*357*). If H_2S is to be used as a prognostic maker and supplement when blood concentrations are low, then H_2S measurements need to be made available to diagnose and monitored to limit risk of overdose. Currently these tests are not readily available and are unlikely to be made available without a pressing need to invest in the technology and infrastructure, requiring other branches of science and biotechnology to collaborate and aid in its advancement. In the short term the drugs and technology are likely to remain in highly specialised industries like spaceflight or military, where physical health is both crucial and at risk, and easy to package compounds are take preference over bulky and perishable natural supplements. As these industries develop the need and technology for H_2S detection, targeted H_2S compounds will become more popular and accepted as an intervention.

H_2S has shown to be a versatile and useful tool in promoting healthy ageing but unfortunately it is unlikely that there is a single mechanism or solution to this increase in healthspan. H_2S offers a potential step to improving the health of the ageing population worldwide but to reach this goal, a better understanding of the mechanisms and molecular alterations that occur in the ageing process is necessary, creating combination treatments that are affordable and applicable for everyone.

7. References

1. Y. Zhao, T. D. Biggs, M. Xian, Hydrogen Sulfide (H2S) Releasing Agents: Chemistry and Biological Applications. *Chemical Communications*, (2014).
2. T. L. Guidotti, Hydrogen Sulfide: Advances in Understanding Human Toxicity. *International Journal of Toxicology* **29**, 569-581 (2010).
3. R. E. Dickerson, Chemical Evolution and the Origin of Life. *Scientific Ameican* **239**, 70-87 (1978).
4. W. Heinen, A. M. Lauwers, Organic sulfur compounds resulting from the interaction of iron sulfide, hydrogen sulfide and carbon dioxide in an anaerobic aqueous environment. *Origins of life and evolution of the biosphere* **26**, 131-150 (1996).
5. N. R. Pace, A Molecular View of Microbial Diversity and the Biosphere. *Science* **276**, 734-740 (1997).
6. E. Lagoutte *et al.*, Oxidation of hydrogen sulfide remains a priority in mammalian cells and causes reverse electron transfer in colonocytes. *Biochim Biophys Acta* **1797**, 1500-1511 (2010).
7. E. Tomitsuka *et al.*, Direct evidence for two distinct forms of the flavoprotein subunit of human mitochondrial complex II (succinate-ubiquinone reductase). *J Biochem* **134**, 191-195 (2003).
8. M. W. Budde, M. B. Roth, The response of Caenorhabditis elegans to hydrogen sulfide and hydrogen cyanide. *Genetics* **189**, 521-532 (2011).
9. V. Tiranti *et al.*, Loss of ETHE1, a mitochondrial dioxygenase, causes fatal sulfide toxicity in ethylmalonic encephalopathy. *Nat Med* **15**, 200-205 (2009).
10. R. Wang, Two's company, three's a crowd: can H2S be the third endogenous gaseous transmitter? *FASEB J* **16**, 1792-1798 (2002).
11. P. Kamoun, Endogenous Production of Hydrogen Sulfide in Mammals. *Amino Acids* **26**, 243-254 (2004).
12. H. Teng *et al.*, Oxygen-sensitive mitochondrial accumulation of cystathionine beta-synthase mediated by Lon protease. *Proc Natl Acad Sci U S A* **110**, 12679-12684 (2013).
13. M. Fu *et al.*, Hydrogen sulfide (H2S) metabolism in mitochondria and its regulatory role in energy production. *Proc Natl Acad Sci U S A* **109**, 2943-2948 (2012).
14. J. L. Wallace, R. Wang, Hydrogen sulfide-based therapeutics: exploiting a unique but ubiquitous gasotransmitter. *Nat Rev Drug Discov* **14**, 329-345 (2015).
15. R. Miyamoto, K. Otsuguro, S. Yamaguchi, S. Ito, Contribution of cysteine aminotransferase and mercaptopyruvate sulfurtransferase to hydrogen sulfide production in peripheral neurons. *J Neurochem* **130**, 29-40 (2014).
16. M. H. Stipanuk, P. W. Beck, Characterization of the Enzymic Capaticy for Cysteine Desulphhydration in Liver and Kidney of the Rat. *Biochemical Journal* **206**, 267-277 (1982).
17. Y. Mikami *et al.*, Thioredoxin and dihydrolipoic acid are required for 3-mercaptopyruvate sulfurtransferase to produce hydrogen sulfide. *Biochemical Journal* **439**, 479-485 (2011).
18. A. J. L. Cooper, Biochemistry of Sulfur Containing Amino Acids. *Annual Review of Biochemistry* **52**, 187-222 (1983).
19. K. R. Olson *et al.*, Thiosulfate: A Readily Accessible Source of Hydrogen Sulfide in Oxygen Sensing. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **305**, 592-603 (2013).

20. G. N. L. Jameson, Iron, cysteine and Parkinson's disease. *Monatshefte für Chemie* **142**, 325-329 (2011).
21. Y. Saidu, Physicochemical features of rhodanese: A review. *African Journal of Biotechnology* **3**, 370-374 (2004).
22. N. V. Zaichko, A. V. Melnik, M. M. Yoltukhivskyy, A. S. Olhovskiy, I. V. Palamarchuk, Hydrogen sulfide: metabolism, biological and medical role. *Ukr Biochem J* **86**, 5-25 (2014).
23. K. R. Olson, A theoretical examination of hydrogen sulfide metabolism and its potential in autocrine/paracrine oxygen sensing. *Respir Physiol Neurobiol* **186**, 173-179 (2013).
24. B. H. Tan, P. T. Wong, J. S. Bian, Hydrogen sulfide: a novel signaling molecule in the central nervous system. *Neurochem Int* **56**, 3-10 (2010).
25. L. F. Hu, M. Lu, Z. Y. Wu, P. T. Wong, J. S. Bian, Hydrogen sulfide inhibits rotenone-induced apoptosis via preservation of mitochondrial function. *Mol Pharmacol* **75**, 27-34 (2009).
26. M. R. Filipovic *et al.*, Chemical characterization of the smallest S-nitrosothiol, HSNO; cellular cross-talk of H2S and S-nitrosothiols. *J Am Chem Soc* **134**, 12016-12027 (2012).
27. K. Ono *et al.*, Redox chemistry and chemical biology of H2S, hydropersulfides, and derived species: implications of their possible biological activity and utility. *Free Radic Biol Med* **77**, 82-94 (2014).
28. S. Veeranki, S. C. Tyagi, Role of hydrogen sulfide in skeletal muscle biology and metabolism. *Nitric Oxide* **46**, 66-71 (2015).
29. G. Meng, S. Zhao, L. Xie, Y. Han, Y. Ji, Protein S-sulfhydration by hydrogen sulfide in cardiovascular system. (2017).
30. S. M. Marino, V. N. Gladyshev, Analysis and functional prediction of reactive cysteine residues. *J Biol Chem* **287**, 4419-4425 (2012).
31. B. D. Paul, S. H. Snyder, The conversion of H2S to sulfane sulfur: authors' response. *Nature Reviews Molecular Cell Biology* **13**, 803 (2012).
32. M. Iciek *et al.*, S-sulfhydration as a cellular redox regulation. *Biosci Rep* **36**, (2015).
33. N. Sen *et al.*, Hydrogen sulfide-linked sulfhydration of NF-κB mediates its antiapoptotic actions. *Mol Cell* **45**, 13-24 (2012).
34. R. Wang, Gasotransmitters: growing pains and joys. *Trends Biochem Sci* **39**, 227-232 (2014).
35. Z. Altaany, Y. Ju, G. Yang, R. Wang, The coordination of S-sulfhydration, S-nitrosylation, and phosphorylation of endothelial nitric oxide synthase by hydrogen sulfide. *Sci Signal* **7**, ra87 (2014).
36. M. M. Cortese-Krott *et al.*, Key bioactive reaction products of the NO/H2S interaction are S/N-hybrid species, polysulfides, and nitroxyl. *Proc Natl Acad Sci U S A* **112**, E4651-4660 (2015).
37. H. Liu, M. N. Radford, C. T. Yang, W. Chen, M. Xian, Inorganic hydrogen polysulfides: chemistry, chemical biology and detection. *Br J Pharmacol* **176**, 616-627 (2019).
38. S. Carballal *et al.*, Reactivity of hydrogen sulfide with peroxynitrite and other oxidants of biological interest. *Free Radic Biol Med* **50**, 196-205 (2011).
39. A. W. DeMartino, D. B. Kim-Shapiro, R. P. Patel, M. T. Gladwin, Nitrite and nitrate chemical biology and signalling. *Br J Pharmacol* **176**, 228-245 (2019).
40. T. V. Mishanina, M. Libiad, R. Banerjee, Biogenesis of reactive sulfur species for signaling by hydrogen sulfide oxidation pathways. *Nat Chem Biol* **11**, 457-464 (2015).

41. R. Pietri *et al.*, Factors controlling the reactivity of hydrogen sulfide with hemeproteins. *Biochemistry* **48**, 4881-4894 (2009).
42. J. Yang *et al.*, Non-enzymatic hydrogen sulfide production from cysteine in blood is catalyzed by iron and vitamin B. *Commun Biol* **2**, 194 (2019).
43. W. Chen *et al.*, H2S Activated Drug Release from Protein Cages. *ACS Appl Mater Interfaces* **9**, 33571-33575 (2017).
44. J. Yang, N. Bithi, C. Link, Y. Saunthararajah, C. Hine, Iron Catalyzed Hydrogen Sulfide and Its Prevalence during Oxidative Events in Sickle Cell Disease. *Blood* **134**, 3534-3534 (2019).
45. T. L. Stemmler, E. Lesuisse, D. Pain, A. Dancis, Frataxin and mitochondrial FeS cluster biogenesis. *J Biol Chem* **285**, 26737-26743 (2010).
46. M. Lange *et al.*, Direct Zinc Finger Protein Persulfidation by H2S Is Facilitated by Zn2+. *Angewandte Chemie International Edition* **58**, 7997-8001 (2019).
47. A. Atkinson, D. R. Winge, Metal acquisition and availability in the mitochondria. *Chem Rev* **109**, 4708-4721 (2009).
48. D. Linden *et al.*, Sulphide quinone reductase contributes to hydrogen sulphide metabolism in murine peripheral tissues but not in the CNS. *Br J Pharmacol* **165**, 2178-2190 (2012).
49. G. R. Francis, R. H. Waterston, Muscle organization in Caenorhabditis elegans: localization of proteins implicated in thin filament attachment and I-band organization. *J Cell Biol* **101**, 1532-1549 (1985).
50. M. Pilon, C. Morck, Development of Caenorhabditis elegans pharynx, with emphasis on its nervous system. *Acta Pharmacol Sin* **26**, 396-404 (2005).
51. J. P. Collman, S. Ghosh, A. Dey, R. A. Decréau, Using a functional enzyme model to understand the chemistry behind hydrogen sulfide induced hibernation. *Proc Natl Acad Sci U S A* **106**, 22090-22095 (2009).
52. M. Goubern, M. Andriamihaja, T. Nubel, F. Blachier, F. Bouillaud, Sulfide, the first inorganic substrate for human cells. *FASEB J* **21**, 1699-1706 (2007).
53. M. R. Jackson, S. L. Melideo, M. S. Jorns, Human sulfide:quinone oxidoreductase catalyzes the first step in hydrogen sulfide metabolism and produces a sulfane sulfur metabolite. *Biochemistry* **51**, 6804-6815 (2012).
54. E. Łowicka, J. Bełtowski, Hydrogen sulfide (H2S) - the third gas of interest for pharmacologists. *Pharmacol Rep* **59**, 4-24 (2007).
55. D. G. Searcy, HS-:O2 oxidoreductase activity of Cu,Zn superoxide dismutase. *Arch Biochem Biophys* **334**, 50-58 (1996).
56. K. Módis *et al.*, S-Sulfhydration of ATP synthase by hydrogen sulfide stimulates mitochondrial bioenergetics. *Pharmacol Res* **113**, 116-124 (2016).
57. M. Whiteman *et al.*, The Effect of Hydrogen Sulfide Donors on Lipopolysaccharide-Induced Formation of Inflammatory Mediators in Macrophages. *Antioxidants and Redox Signaling* **12**, 1147-1154 (2010).
58. Y. Zheng, X. Ji, K. Ji, B. Wang, Hydrogen sulfide prodrugs-a review. *Acta Pharm Sin B* **5**, 367-377 (2015).
59. R. Tocmo, D. Liang, Y. Lin, D. Huang, Chemical and biochemical mechanisms underlying the cardioprotective roles of dietary organopolysulfides. *Front Nutr* **2**, 1 (2015).
60. G. A. Benavides *et al.*, Hydrogen sulfide mediates the vasoactivity of garlic. *Proc Natl Acad Sci U S A* **104**, 17977-17982 (2007).
61. D. Liang, H. Wu, M. W. Wong, D. Huang, Diallyl Trisulfide Is a Fast H2S Donor, but Diallyl Disulfide Is a Slow One: The Reaction Pathways and Intermediates of Glutathione with Polysulfides. *Org Lett* **17**, 4196-4199 (2015).

62. E. Germain, J. Auger, C. Ginies, M. H. Siess, C. Teyssier, In vivo metabolism of diallyl disulphide in the rat: identification of two new metabolites. *Xenobiotica* **32**, 1127-1138 (2002).
63. D. A. Smith, K. Beaumont, T. S. Maurer, L. Di, Relevance of Half-Life in Drug Design. *J Med Chem* **61**, 4273-4282 (2018).
64. Y. Zhao, H. Wang, M. Xian, Cysteine-activated hydrogen sulfide (H2S) donors. *J Am Chem Soc* **133**, 15-17 (2011).
65. Z. Zhou *et al.*, Thioglycine and L-thiovaline: biologically active H_2S-donors. *Bioorg Med Chem* **20**, 2675-2678 (2012).
66. E. Thinon, H. C. Hang, Chemical reporters for exploring protein acylation. *Biochem Soc Trans* **43**, 253-261 (2015).
67. Z. W. Lee *et al.*, The slow-releasing hydrogen sulfide donor, GYY4137, exhibits novel anti-cancer effects in vitro and in vivo. *PLoS One* **6**, e21077 (2011).
68. L. Li *et al.*, Anti-inflammatory and gastrointestinal effects of a novel diclofenac derivative. *Free Radic Biol Med* **42**, 706-719 (2007).
69. Y. Huang *et al.*, Hydrogen sulfide, a gaseous transmitter, stimulates proliferation of interstitial cells of Cajal via phosphorylation of AKT protein kinase. *Tohoku J Exp Med* **221**, 125-132 (2010).
70. Y. Zhang, G. B. Gordon, A strategy for cancer prevention: stimulation of the Nrf2-ARE signaling pathway. *Mol Cancer Ther* **3**, 885-893 (2004).
71. R. Munday *et al.*, Synthesis, biological evaluation, and structure-activity relationships of dithiolethiones as inducers of cytoprotective phase 2 enzymes. *J Med Chem* **53**, 4761-4767 (2010).
72. J. S. Isenberg *et al.*, Modulation of angiogenesis by dithiolethione-modified NSAIDs and valproic acid. *Br J Pharmacol* **151**, 63-72 (2007).
73. B. Szczesny *et al.*, AP39, a novel mitochondria-targeted hydrogen sulfide donor, stimulates cellular bioenergetics, exerts cytoprotective effects and protects against the loss of mitochondrial DNA integrity in oxidatively stressed endothelial cells in vitro. *Nitric Oxide* **41**, 120-130 (2014).
74. D. Gerő *et al.*, The novel mitochondria-targeted hydrogen sulfide (H2S) donors AP123 and AP39 protect against hyperglycemic injury in microvascular endothelial cells in vitro. *Pharmacol Res* **113**, 186-198 (2016).
75. Q. G. Karwi *et al.*, AP39, a mitochondria-targeting hydrogen sulfide (H2S) donor, protects against myocardial reperfusion injury independently of salvage kinase signalling. *Br J Pharmacol* **174**, 287-301 (2017).
76. R. K. Olsen, N. Cornelius, N. Gregersen, Redox signalling and mitochondrial stress responses; lessons from inborn errors of metabolism. *J Inherit Metab Dis* **38**, 703-719 (2015).
77. R. N. Baumgartner, Body composition in healthy aging. *Ann N Y Acad Sci* **904**, 437-448 (2000).
78. T. Fulop *et al.*, Aging, frailty and age-related diseases. *Biogerontology* **11**, 547-563 (2010).
79. A. Clegg, J. Young, S. Iliffe, M. O. Rikkert, K. Rockwood, Frailty in elderly people. *Lancet* **381**, 752-762 (2013).
80. D. o. E. a. S. A. United Nations, Population Division. *World Population Ageing 2019: Highlights* **ST/ESA/SER.A/430**, (2019).
81. D. o. E. a. S. A. United Nations, Population Division (2019). (United Nations, https://population.un.org/wpp/DataQuery/, 2019), vol. 2022.
82. R. Pinedo-Villanueva *et al.*, Health Care Costs Associated With Muscle Weakness: A UK Population-Based Estimate. *Calcif Tissue Int* **104**, 137-144 (2019).

83. E. Curtis, A. Litwic, C. Cooper, E. Dennison, Determinants of Muscle and Bone Aging. *J Cell Physiol* **230**, 2618-2625 (2015).
84. R. Dodds, A. A. Sayer, Sarcopenia and frailty: new challenges for clinical practice. *Clin Med (Lond)* **16**, 455-458 (2016).
85. D. F. Dai, Y. A. Chiao, D. J. Marcinek, H. H. Szeto, P. S. Rabinovitch, Mitochondrial oxidative stress in aging and healthspan. *Longev Healthspan* **3**, 6 (2014).
86. L. Micheli *et al.*, Beneficial Effect of H2S-Releasing Molecules in an In Vitro Model of Sarcopenia: Relevance of Glucoraphanin. *International Journal of Molecular Sciences* **23**, 5955 (2022).
87. B. Qabazard *et al.*, Hydrogen sulfide is an endogenous regulator of aging in Caenorhabditis elegans. *Antioxid Redox Signal* **20**, 2621-2630 (2014).
88. S. Maglioni, D. F. Mello, A. Schiavi, J. N. Meyer, N. Ventura, Mitochondrial bioenergetic changes during development as an indicator of C. elegans health-span. *Aging (Albany NY)* **11**, 6535-6554 (2019).
89. V. Romanello, M. Sandri, Mitochondrial Quality Control and Muscle Mass Maintenance. *Front Physiol* **6**, 422 (2015).
90. I. R. Sweet *et al.*, Regulation of ATP/ADP in pancreatic islets. *Diabetes* **53**, 401-409 (2004).
91. R. B. Mounsey, P. Teismann, Mitochondrial dysfunction in Parkinson's disease: pathogenesis and neuroprotection. *Parkinsons Dis* **2011**, 617472 (2010).
92. M. P. Murphy, How mitochondria produce reactive oxygen species. *Biochem J* **417**, 1-13 (2009).
93. D. B. Zorov, M. Juhaszova, S. J. Sollott, Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiol Rev* **94**, 909-950 (2014).
94. F. Scialò, D. J. Fernández-Ayala, A. Sanz, Role of Mitochondrial Reverse Electron Transport in ROS Signaling: Potential Roles in Health and Disease. *Front Physiol* **8**, 428 (2017).
95. R. J. Youle, A. M. van der Bliek, Mitochondrial fission, fusion, and stress. *Science* **337**, 1062-1065 (2012).
96. N. Sun, R. J. Youle, T. Finkel, The Mitochondrial Basis of Aging. *Mol Cell* **61**, 654-666 (2016).
97. S. P. Patel, S. S. Katyare, A comparative study of reactive oxygen species (ROS) related parameters in rat tissues. *Indian J Clin Biochem* **21**, 48-53 (2006).
98. I. H. Kim, T. Kisseleva, D. A. Brenner, Aging and liver disease. *Curr Opin Gastroenterol* **31**, 184-191 (2015).
99. A. Floreani, Liver diseases in the elderly: an update. *Dig Dis* **25**, 138-143 (2007).
100. J. Tower, Programmed cell death in aging. *Ageing Res Rev* **23**, 90-100 (2015).
101. Y. Yang, G. Jiang, P. Zhang, J. Fan, Programmed cell death and its role in inflammation. *Mil Med Res* **2**, 12 (2015).
102. S. Jiang *et al.*, Associations of plasma-soluble fas ligand with aging and age-related macular degeneration. *Invest Ophthalmol Vis Sci* **49**, 1345-1349 (2008).
103. S. P. Cullen, S. J. Martin, Caspase activation pathways: some recent progress. *Cell Death Differ* **16**, 935-938 (2009).
104. H. Bruunsgaard, P. Skinhøj, A. N. Pedersen, M. Schroll, B. K. Pedersen, Ageing, tumour necrosis factor-alpha (TNF-alpha) and atherosclerosis. *Clin Exp Immunol* **121**, 255-260 (2000).

105. A. Sivarajah *et al.*, Anti-apoptotic and anti-inflammatory effects of hydrogen sulfide in a rat model of regional myocardial I/R. *Shock* **31**, 267-274 (2009).
106. D. Petrovic, E. Kouroussis, T. Vignane, M. R. Filipovic, The Role of Protein Persulfidation in Brain Aging and Neurodegeneration. *Front Aging Neurosci* **13**, 674135 (2021).
107. C. Gladkova, S. L. Maslen, J. M. Skehel, D. Komander, Mechanism of parkin activation by PINK1. *Nature* **559**, 410-414 (2018).
108. A. Voigt, L. A. Berlemann, K. F. Winklhofer, The mitochondrial kinase PINK1: functions beyond mitophagy. *J Neurochem* **139 Suppl 1**, 232-239 (2016).
109. J. M. Yuk, P. Silwal, E. K. Jo, Inflammasome and Mitophagy Connection in Health and Disease. *Int J Mol Sci* **21**, (2020).
110. A. Hiona *et al.*, Mitochondrial DNA mutations induce mitochondrial dysfunction, apoptosis and sarcopenia in skeletal muscle of mitochondrial DNA mutator mice. *PLoS One* **5**, e11468 (2010).
111. L. Zuo *et al.*, Inflammaging and Oxidative Stress in Human Diseases: From Molecular Mechanisms to Novel Treatments. *Int J Mol Sci* **20**, (2019).
112. H. L. Wei, C. Y. Zhang, H. F. Jin, C. S. Tang, J. B. Du, Hydrogen sulfide regulates lung tissue-oxidized glutathione and total antioxidant capacity in hypoxic pulmonary hypertensive rats. *Acta Pharmacol Sin* **29**, 670-679 (2008).
113. Y. Kimura, Y. Goto, H. Kimura, Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. *Antioxid Redox Signal* **12**, 1-13 (2010).
114. S. Jha, J. W. Calvert, M. R. Duranski, A. Ramachandran, D. J. Lefer, Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: role of antioxidant and antiapoptotic signaling. *Am J Physiol Heart Circ Physiol* **295**, H801-806 (2008).
115. R. Di Micco, V. Krizhanovsky, D. Baker, F. d'Adda di Fagagna, Cellular senescence in ageing: from mechanisms to therapeutic opportunities. *Nat Rev Mol Cell Biol* **22**, 75-95 (2021).
116. M. Whiteman *et al.*, Novel Neuromodulator Hydrogen Sulfide: an Endogenous Peroxynitrite 'Scavenger'? *Journal of Neurochemistry* **90**, 765-768 (2004).
117. Li *et al.*, Hydrogen Sulfide is a Novel Mediator of Lipopolysaccharide-induced Inflammation in the Mouse. *The FASEB Journal* **19**, 1051-1207 (2005).
118. H. Cao *et al.*, Hydrogen Sulfide Protects Against Bleomycin-induced Pulmonary Fibrosis in Rats by Inhibiting NF-kB expression and Regulating Th1/Th2 Balance. *Toxicology Letters* **224**, 387-394 (2014).
119. A. P. Wickens, Ageing and the free radical theory. *Respir Physiol* **128**, 379-391 (2001).
120. H. Y. Chung *et al.*, Molecular inflammation: underpinnings of aging and age-related diseases. *Ageing Res Rev* **8**, 18-30 (2009).
121. S. Dalle, L. Rossmeislova, K. Koppo, The Role of Inflammation in Age-Related Sarcopenia. *Front Physiol* **8**, 1045 (2017).
122. R. Baskar, L. Li, P. K. Moore, Hydrogen sulfide-induces DNA damage and changes in apoptotic gene expression in human lung fibroblast cells. *FASEB J* **21**, 247-255 (2007).
123. Z. Z. Xie *et al.*, Sulfhydration of p66Shc at cysteine59 mediates the antioxidant effect of hydrogen sulfide. *Antioxid Redox Signal* **21**, 2531-2542 (2014).
124. M. D. de Beus, J. Chung, W. Colon, Modification of cysteine 111 in Cu/Zn superoxide dismutase results in altered spectroscopic and biophysical properties. *Protein Sci* **13**, 1347-1355 (2004).

125. B. S. Mandavilli, J. H. Santos, B. Van Houten, Mitochondrial DNA repair and aging. *Mutat Res* **509**, 127-151 (2002).
126. S. G. Spassov *et al.*, Hydrogen Sulfide Prevents Formation of Reactive Oxygen Species through PI3K/Akt Signaling and Limits Ventilator-Induced Lung Injury. *Oxid Med Cell Longev* **2017**, 3715037 (2017).
127. E. Latorre, R. Torregrossa, M. E. Wood, M. Whiteman, L. W. Harries, Mitochondria-targeted hydrogen sulfide attenuates endothelial senescence by selective induction of splicing factors *HNRNPD* and *SRSF2*. *Aging (Albany NY)* **10**, 1666-1681 (2018).
128. F. Sanada *et al.*, Source of Chronic Inflammation in Aging. *Front Cardiovasc Med* **5**, 12 (2018).
129. J. P. Coppé, P. Y. Desprez, A. Krtolica, J. Campisi, The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* **5**, 99-118 (2010).
130. J. B. Strait, E. G. Lakatta, Aging-associated cardiovascular changes and their relationship to heart failure. *Heart Fail Clin* **8**, 143-164 (2012).
131. I. Janssen, S. B. Heymsfield, Z. M. Wang, R. Ross, Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. *J Appl Physiol (1985)* **89**, 81-88 (2000).
132. B. H. Goodpaster *et al.*, The loss of skeletal muscle strength, mass, and quality in older adults: the health, aging and body composition study. *J Gerontol A Biol Sci Med Sci* **61**, 1059-1064 (2006).
133. A. García-Hermoso *et al.*, Muscular Strength as a Predictor of All-Cause Mortality in an Apparently Healthy Population: A Systematic Review and Meta-Analysis of Data From Approximately 2 Million Men and Women. *Arch Phys Med Rehabil*, (2018).
134. M. Sandri, Protein breakdown in muscle wasting: role of autophagy-lysosome and ubiquitin-proteasome. *Int J Biochem Cell Biol* **45**, 2121-2129 (2013).
135. C. Nelke, R. Dziewas, J. Minnerup, S. G. Meuth, T. Ruck, Skeletal muscle as potential central link between sarcopenia and immune senescence. *EBioMedicine* **49**, 381-388 (2019).
136. A. Majumder *et al.*, Hydrogen sulfide alleviates hyperhomocysteinemia-mediated skeletal muscle atrophy via mitigation of oxidative and endoplasmic reticulum stress injury. *Am J Physiol Cell Physiol*, (2018).
137. Y. Yao *et al.*, Effect of advanced age on plasma homocysteine levels and its association with ischemic stroke in non-valvular atrial fibrillation. *J Geriatr Cardiol* **14**, 743-749 (2017).
138. A. Majumder, M. Singh, A. K. George, S. C. Tyagi, Restoration of skeletal muscle homeostasis by hydrogen sulfide during hyperhomocysteinemia-mediated oxidative/ER stress condition. *Can J Physiol Pharmacol* **97**, 441-456 (2019).
139. E. M. Fawcett, J. M. Hoyt, J. K. Johnson, D. L. Miller, Hypoxia disrupts proteostasis in Caenorhabditis elegans. *Aging Cell* **14**, 92-101 (2015).
140. S. Ayyadevara *et al.*, Proteins that accumulate with age in human skeletal-muscle aggregates contribute to declines in muscle mass and function in. *Aging (Albany NY)* **8**, 3486-3497 (2016).
141. J. T. DU *et al.*, Hydrogen sulfide is endogenously generated in rat skeletal muscle and exerts a protective effect against oxidative stress. *Chin Med J (Engl)* **126**, 930-936 (2013).
142. T. Ubuka *et al.*, Metabolism ofL-cysteine via transamination pathway (3-mercaptopyruvate pathway). *Amino Acids* **3**, 243-252 (1992).

143. N. C. Chen *et al.*, Regulation of homocysteine metabolism and methylation in human and mouse tissues. *FASEB J* **24**, 2804-2817 (2010).
144. J. Ohta, T. Ubuka, Reduction of 3-mercaptopyruvate in rat liver is catalyzed by lactate dehydrogenase. *Acta Med Okayama* **43**, 89-95 (1989).
145. D. Wu *et al.*, Treadmill exercise increases cystathionine γ-lyase expression and decreases inflammation in skeletal muscles of high-fat diet-induced obese rats. *International Journal of Clinical and Experimental Medicine* **9**, 23119-23126 (2016).
146. J. Bangsbo, P. Krustrup, J. González-Alonso, B. Saltin, ATP production and efficiency of human skeletal muscle during intense exercise: effect of previous exercise. *Am J Physiol Endocrinol Metab* **280**, E956-964 (2001).
147. C. Szabo *et al.*, Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms. *Br J Pharmacol* **171**, 2099-2122 (2014).
148. S. Srinivasan, N. G. Avadhani, Cytochrome c Oxidase Dysfunction in Oxidative Stress. *Free Radic Biol Med* **53**, 1252-1263 (2012).
149. F. A. Cucinotta, "Radiation Risk Acceptability and Limits," (NASA, 2010).
150. L. C. Simonsen, C. Zeitlin, "Briefing to NAC HEO/SMD Joint Committee Meeting “Mars Radiation Environment – what have we learned?”," (2017).
151. A. R. Hargens, L. Vico, Long-duration bed rest as an analog to microgravity. *J Appl Physiol (1985)* **120**, 891-903 (2016).
152. R. Rabin *et al.*, Effects of spaceflight on the musculoskeletal system: NIH and NASA future directions. *FASEB J* **7**, 396-398 (1993).
153. Y. Honda, S. Honda, M. Narici, N. J. Szewczyk, Spaceflight and ageing: reflecting on Caenorhabditis elegans in space. *Gerontology* **60**, 138-142 (2014).
154. K. Otsuka *et al.*, Anti-aging effects of long-term space missions, estimated by heart rate variability. *Sci Rep* **9**, 8995 (2019).
155. H. P. Indo *et al.*, Changes in mitochondrial homeostasis and redox status in astronauts following long stays in space. *Sci Rep* **6**, 39015 (2016).
156. W. A. da Silveira *et al.*, Comprehensive Multi-omics Analysis Reveals Mitochondrial Stress as a Central Biological Hub for Spaceflight Impact. *Cell* **183**, 1185-1201.e1120 (2020).
157. L. Qu *et al.*, Protective effects of flavonoids against oxidative stress induced by simulated microgravity in SH-SY5Y cells. *Neurochem Res* **35**, 1445-1454 (2010).
158. W. Hua *et al.*, Cardioprotection of H2S by downregulating iNOS and upregulating HO-1 expression in mice with CVB3-induced myocarditis. *Life Sci* **93**, 949-954 (2013).
159. M. P. Schoenfeld, R. R. Ansari, A. Nakao, D. Wink, A hypothesis on biological protection from space radiation through the use of new therapeutic gases as medical counter measures. *Med Gas Res* **2**, 8 (2012).
160. C. A. Nordeen, S. L. Martin, Engineering Human Stasis for Long-Duration Spaceflight. *Physiology (Bethesda)* **34**, 101-111 (2019).
161. M. Yang *et al.*, Treatment with hydrogen sulfide donor attenuates bone loss induced by modeled microgravity. *Can J Physiol Pharmacol* **97**, 655-660 (2019).
162. R. Kurosawa *et al.*, Impact of spaceflight and artificial gravity on sulfur metabolism in mouse liver: sulfur metabolomic and transcriptomic analysis. *Sci Rep* **11**, 21786 (2021).

163. C. J. Gaffney *et al.*, Greater loss of mitochondrial function with ageing is associated with earlier onset of sarcopenia in C. elegans. *Aging (Albany NY)* **10**, 3382-3396 (2018).
164. T. Stiernagle, Maintenance of C. elegans. *WormBook*, 1-11 (2006).
165. R. A. Ellwood *et al.*, Mitochondrial hydrogen sulfide supplementation improves health in the C. elegans Duchenne muscular dystrophy model. *Proc Natl Acad Sci U S A* **118**, (2021).
166. L. A. Zdinak *et al.*, Transgene-coded chimeric proteins as reporters of intracellular proteolysis: starvation-induced catabolism of a lacZ fusion protein in muscle cells of Caenorhabditis elegans. *J Cell Biochem* **67**, 143-153 (1997).
167. T. Flatt, A new definition of aging? *Front Genet* **3**, 148 (2012).
168. Y. Fardghassemi, A. Tauffenberger, S. Gosselin, J. A. Parker, Rescue of ATXN3 neuronal toxicity in Caenorhabditis elegans by chemical modification of endoplasmic reticulum stress. *Dis Model Mech* **10**, 1465-1480 (2017).
169. R. C. Gagnon, J. J. Peterson, Estimation of confidence intervals for area under the curve from destructively obtained pharmacokinetic data. *J Pharmacokinet Biopharm* **26**, 87-102 (1998).
170. S. Sarasija, K. R. Norman, Analysis of Mitochondrial Structure in the Body Wall Muscle of *Caenorhabditis elegans*. *Bio Protoc* **8**, (2018).
171. S. Larsen *et al.*, Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiol* **590**, 3349-3360 (2012).
172. F. Macedo *et al.*, Lifespan-extending interventions enhance lipid-supported mitochondrial respiration in Caenorhabditis elegans. *FASEB J* **34**, 9972-9981 (2020).
173. E. J. Metter, L. A. Talbot, M. Schrager, R. Conwit, Skeletal muscle strength as a predictor of all-cause mortality in healthy men. *J Gerontol A Biol Sci Med Sci* **57**, B359-365 (2002).
174. E. Marzetti *et al.*, Mitochondrial dysfunction and sarcopenia of aging: from signaling pathways to clinical trials. *Int J Biochem Cell Biol* **45**, 2288-2301 (2013).
175. M. ROCKSTEIN, K. F. BRANDT, Enzyme changes in flight muscle correlated with aging and flight ability in the male housefly. *Science* **139**, 1049-1051 (1963).
176. G. R. Smith, D. P. Shanley, Modelling the response of FOXO transcription factors to multiple post-translational modifications made by ageing-related signalling pathways. *PLoS One* **5**, e11092 (2010).
177. R. Wang, Two's Company,Three's a Crowd: Can H2S be the Third Endogenous Gaseous Transmitter? *The FASEB Journal* **16**, 1792-1798 (2002).
178. S. Kubo *et al.*, Hydrogen sulfide inhibits activity of three isoforms of recombinant nitric oxide synthase. *Toxicology* **241**, 92-97 (2007).
179. Y. Kaneko, Y. Kimura, H. Kimura, I. Niki, L-cysteine inhibits insulin release from the pancreatic beta-cell: possible involvement of metabolic production of hydrogen sulfide, a novel gasotransmitter. *Diabetes* **55**, 1391-1397 (2006).
180. K. R. Olson, Is hydrogen sulfide a circulating "gasotransmitter" in vertebrate blood? *Biochim Biophys Acta* **1787**, 856-863 (2009).
181. Y.-H. Chen *et al.*, Endogenous Hydrogen Sulfide in Patients With COPD. *Chest* **128**, 3205-3211 (2005).
182. C. R. Powell, K. M. Dillon, J. B. Matson, A review of hydrogen sulfide (H. *Biochem Pharmacol* **149**, 110-123 (2018).
183. H. Ali, C. Opere, S. Singh, In vitro-controlled release delivery system for hydrogen sulfide donor. *AAPS PharmSciTech* **15**, 910-919 (2014).

184. C. E. Cooper, G. C. Brown, The inhibition of mitochondrial cytochrome oxidase by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide: chemical mechanism and physiological significance. *J Bioenerg Biomembr* **40**, 533-539 (2008).
185. M. Markaki, N. Tavernarakis, Modeling human diseases in Caenorhabditis elegans. *Biotechnol J* **5**, 1261-1276 (2010).
186. B. C. Fox *et al.*, The mitochondria-targeted hydrogen sulfide donor AP39 improves health and mitochondrial function in a C. elegans primary mitochondrial disease model. *J Inherit Metab Dis* **44**, 367-375 (2021).
187. B. Qabazard *et al.*, C. elegans aging is modulated by hydrogen sulfide and the sulfhydrylase/cysteine synthase cysl-2. *PLoS One* **8**, e80135 (2013).
188. J. H. Hahm *et al.*, C. elegans maximum velocity correlates with healthspan and is maintained in worms with an insulin receptor mutation. *Nat Commun* **6**, 8919 (2015).
189. L. L. Xie *et al.*, Mitochondrial network structure homeostasis and cell death. *Cancer Sci* **109**, 3686-3694 (2018).
190. S. Le Trionnaire *et al.*, The synthesis and functional evaluation of a mitochondria-targeted hydrogen sulfide donor, (10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5-yl)phenoxy)decyl)triphenylphosphonium bromide (AP39). (2014).
191. A. Giuffre, J. B. Vicente, Hydrogen Sulfide Biochemistry and Interplay with Other Gaseous Mediators in Mammalian Physiology. *Oxid Med Cell Longev* **2018**, 6290931 (2018).
192. L. A. Montoya, M. D. Pluth, Organelle-Targeted H2S Probes Enable Visualization of the Subcellular Distribution of H2S Donors. *Anal Chem* **88**, 5769-5774 (2016).
193. I. Liguori *et al.*, Oxidative stress, aging, and diseases. *Clin Interv Aging* **13**, 757-772 (2018).
194. S. S. Korshunov, V. P. Skulachev, A. A. Starkov, High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett* **416**, 15-18 (1997).
195. J. Jia *et al.*, SQR mediates therapeutic effects of H2S by targeting mitochondrial electron transport to induce mitochondrial uncoupling. *Sci Adv* **6**, eaaz5752 (2020).
196. F. Cai *et al.*, ADT-OH, a hydrogen sulfide-releasing donor, induces apoptosis and inhibits the development of melanoma in vivo by upregulating FADD. *Cell Death Dis* **11**, 33 (2020).
197. A. Ahmad *et al.*, AP39, A Mitochondrially Targeted Hydrogen Sulfide Donor, Exerts Protective Effects in Renal Epithelial Cells Subjected to Oxidative Stress in Vitro and in Acute Renal Injury in Vivo. *Shock* **45**, 88-97 (2016).
198. K. Wang *et al.*, Dysregulation of hydrogen sulfide producing enzyme cystathionine γ-lyase contributes to maternal hypertension and placental abnormalities in preeclampsia. *Circulation* **127**, 2514-2522 (2013).
199. B. Wang *et al.*, Trophoblast H2S Maintains Early Pregnancy via Regulating Maternal-Fetal Interface Immune Hemostasis. *J Clin Endocrinol Metab* **105**, (2020).
200. R. M. Dyson *et al.*, A role for H2S in the microcirculation of newborns: the major metabolite of H2S (thiosulphate) is increased in preterm infants. *PLoS One* **9**, e105085 (2014).
201. Y. G. Ding *et al.*, [Range of plasma hydrogen sulfide in children]. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* **28**, 714-716 (2006).

202. R. Wu, W. Z. Yao, Y. H. Chen, B. Geng, C. S. Tang, [Plasma level of endogenous hydrogen sulfide in patients with acute asthma]. *Beijing Da Xue Xue Bao Yi Xue Ban* **40**, 505-508 (2008).
203. E. A. Peter *et al.*, Plasma free H2S levels are elevated in patients with cardiovascular disease. *J Am Heart Assoc* **2**, e000387 (2013).
204. S. Mani, G. Yang, R. Wang, A critical life-supporting role for cystathionine γ-lyase in the absence of dietary cysteine supply. *Free Radic Biol Med* **50**, 1280-1287 (2011).
205. H. Li *et al.*, Runting and Stunting Syndrome Is Associated With Mitochondrial Dysfunction in Sex-Linked Dwarf Chicken. *Front Genet* **10**, 1337 (2019).
206. S. Wolny, R. McFarland, P. Chinnery, T. Cheetham, Abnormal growth in mitochondrial disease. *Acta Paediatr* **98**, 553-554 (2009).
207. C. Guven, W. S. Lee, Height, aging and cognitive abilities across Europe. *Econ Hum Biol* **16**, 16-29 (2015).
208. V. H. Pereira *et al.*, Adult Body Height Is a Good Predictor of Different Dimensions of Cognitive Function in Aged Individuals: A Cross-Sectional Study. *Front Aging Neurosci* **8**, 217 (2016).
209. J. D. Walston, Sarcopenia in older adults. *Curr Opin Rheumatol* **24**, 623-627 (2012).
210. K. Mikkelsen, L. Stojanovska, M. Polenakovic, M. Bosevski, V. Aposto lopoulosa, Exercise and mental health. *Maturitas* **106**, 48 - 56 (2017).
211. A. Bansal, L. J. Zhu, K. Yen, H. A. Tissenbaum, Uncoupling lifespan and healthspan in Caenorhabditis elegans longevity mutants. *Proc Natl Acad Sci U S A* **112**, E277-286 (2015).
212. S. E. Alway, J. S. Mohamed, M. J. Myers, Mitochondria Initiate and Regulate Sarcopenia. *Exerc Sport Sci Rev* **45**, 58-69 (2017).
213. L. Hayflick, Aging: The Reality: "Anti-Aging" Is an Oxymoron. *The Journals of Gerontology: Series A* **59**, B573-B578 (2004).
214. K. V. Chang, T. H. Hsu, W. T. Wu, K. C. Huang, D. S. Han, Is sarcopenia associated with depression? A systematic review and meta-analysis of observational studies. *Age Ageing* **46**, 738-746 (2017).
215. F. Landi, C. Sieber, R. A. Fielding, Y. Rolland, J. Guralnik, Nutritional Intervention in Sarcopenia: Report from the International Conference on Frailty and Sarcopenia Research Task Force. *J Frailty Aging* **7**, 247-252 (2018).
216. J. Bauer *et al.*, Evidence-based recommendations for optimal dietary protein intake in older people: a position paper from the PROT-AGE Study Group. *J Am Med Dir Assoc* **14**, 542-559 (2013).
217. C. J. Rasmussen, in *Nutritional Supplements in Sports and Exercise*. (Humana Press, Totowa, NJ, 2008), pp. 369-407.
218. G. Wu *et al.*, Composition of free and peptide-bound amino acids in beef chuck, loin, and round cuts. *J Anim Sci* **94**, 2603-2613 (2016).
219. K. Sakuma, A. Yamaguchi, Recent advances in pharmacological, hormonal, and nutritional intervention for sarcopenia. *Pflugers Arch* **470**, 449-460 (2018).
220. W. Dröge, Oxidative stress and ageing: is ageing a cysteine deficiency syndrome? *Philos Trans R Soc Lond B Biol Sci* **360**, 2355-2372 (2005).
221. B. Seifi, A. Sajedizadeh, M. Kadkhodaee, M. Ranjbaran, Long-term exercise restores hydrogen sulfide in the kidney and contributes to exercise benefits in 5/6 nephrectomized rats. *Clin Exp Hypertens* **41**, 87-91 (2019).
222. A. Leblanc *et al.*, Bisphosphonates as a supplement to exercise to protect bone during long-duration spaceflight. *Osteoporos Int* **24**, 2105-2114 (2013).

223. M. P. Murphy, R. C. Hartley, Mitochondria as a therapeutic target for common pathologies. *Nat Rev Drug Discov* **17**, 865-886 (2018).
224. P. Comfort *et al.*, Effects of Spaceflight on Musculoskeletal Health: A Systematic Review and Meta-analysis, Considerations for Interplanetary Travel. *Sports Med* **51**, 2097-2114 (2021).
225. M. S. Bitar *et al.*, Hydrogen Sulfide Donor NaHS Improves Metabolism and Reduces Muscle Atrophy in Type 2 Diabetes: Implication for Understanding Sarcopenic Pathophysiology. *Oxid Med Cell Longev* **2018**, 6825452 (2018).
226. K. Eto, H. Kimura, The production of hydrogen sulfide is regulated by testosterone and S-adenosyl-L-methionine in mouse brain. *J Neurochem* **93**, 1633 (2005).
227. O. H. Franco *et al.*, Changing course in ageing research: The healthy ageing phenotype. *Maturitas* **63**, 13-19 (2009).
228. F. Bellanti, A. Lo Buglio, G. Vendemiale, Mitochondrial Impairment in Sarcopenia. *Biology (Basel)* **10**, (2021).
229. S. Ghosh *et al.*, Reduction in reactive oxygen species production by mitochondria from elderly subjects with normal and impaired glucose tolerance. *Diabetes* **60**, 2051-2060 (2011).
230. K. R. Short *et al.*, Decline in skeletal muscle mitochondrial function with aging in humans. *Proc Natl Acad Sci U S A* **102**, 5618-5623 (2005).
231. L. Byerly, R. C. Cassada, R. L. Russell, The life cycle of the nematode Caenorhabditis elegans: I. Wild-type growth and reproduction. *Developmental Biology* **51**, 23 - 33 (1976).
232. D. J. Feistel *et al.*, A Novel Virulence Phenotype Rapidly Assesses Candida Fungal Pathogenesis in Healthy and Immunocompromised Caenorhabditis elegans Hosts. *mSphere* **4**, e00697-00618 (2019).
233. M. Tatar, Reproductive aging in invertebrate genetic models. *Ann N Y Acad Sci* **1204**, 149-155 (2010).
234. M. C. Wang, H. D. Oakley, C. E. Carr, J. N. Sowa, G. Ruvkun, Gene pathways that delay Caenorhabditis elegans reproductive senescence. *PLoS Genet* **10**, e1004752 (2014).
235. V. Messier *et al.*, Menopause and sarcopenia: A potential role for sex hormones. *Maturitas* **68**, 331-336 (2011).
236. N. Di Daniele *et al.*, Effect of supplementation of calcium and vitamin D on bone mineral density and bone mineral content in peri- and post-menopause women; a double-blind, randomized, controlled trial. *Pharmacol Res* **50**, 637-641 (2004).
237. M. Gueugneau *et al.*, Proteomics of muscle chronological ageing in post-menopausal women. *BMC Genomics* **15**, 1165 (2014).
238. Z. S. Patel *et al.*, Red risks for a journey to the red planet: The highest priority human health risks for a mission to Mars. *npj Microgravity* **6**, 33 (2020).
239. NASA, M. Johnson, Ed. (2021), vol. 2022.
240. N. A. a. S. Administration. (2021), vol. 2022.
241. N. J. Szewczyk *et al.*, Description of International Caenorhabditis elegans Experiment first flight (ICE-FIRST). *Adv Space Res* **42**, 1072-1079 (2008).
242. A. Higashibata *et al.*, Microgravity elicits reproducible alterations in cytoskeletal and metabolic gene and protein expression in space-flown. *NPJ Microgravity* **2**, 15022 (2016).
243. O. J. Juhl *et al.*, Update on the effects of microgravity on the musculoskeletal system. *NPJ Microgravity* **7**, 28 (2021).

244. D. J. Wilkinson, M. Piasecki, P. J. Atherton, The age-related loss of skeletal muscle mass and function: Measurement and physiology of muscle fibre atrophy and muscle fibre loss in humans. *Ageing Res Rev* **47**, 123-132 (2018).
245. A. Kumar, C. G. T. Tahimic, E. A. C. Almeida, R. K. Globus, Spaceflight Modulates the Expression of Key Oxidative Stress and Cell Cycle Related Genes in Heart. *Int J Mol Sci* **22**, (2021).
246. J. M. Memme, A. N. Oliveira, D. A. Hood, The importance of TP53/p53 in regulating the mitophagy-lysosomal machinery in muscle following disuse. *Autophagy Reports* **1**, 75-78 (2022).
247. M. Murgia *et al.*, Signatures of muscle disuse in spaceflight and bed rest revealed by single muscle fiber proteomics. *PNAS Nexus* **1**, (2022).
248. A. M *et al.*, Putative Receptors for Gravity Sensing in Mammalian Cells: The Effects of Microgravity. *Applied Sciences* **10**, 2028 (2020).
249. P. Balagopal, O. E. Rooyackers, D. B. Adey, P. A. Ades, K. S. Nair, Effects of aging on in vivo synthesis of skeletal muscle myosin heavy-chain and sarcoplasmic protein in humans. *Am J Physiol* **273**, E790-800 (1997).
250. J. L. Andersen, T. Gruschy-Knudsen, C. Sandri, L. Larsson, S. Schiaffino, Bed rest increases the amount of mismatched fibers in human skeletal muscle. *J Appl Physiol (1985)* **86**, 455-460 (1999).
251. D. Pette, R. S. Staron, Mammalian skeletal muscle fiber type transitions. *Int Rev Cytol* **170**, 143-223 (1997).
252. M. D. Boppart, Z. S. Mahmassani, Integrin signaling: linking mechanical stimulation to skeletal muscle hypertrophy. *Am J Physiol Cell Physiol* **317**, C629-C641 (2019).
253. H. Collins-Hooper *et al.*, Age-related changes in speed and mechanism of adult skeletal muscle stem cell migration. *Stem Cells* **30**, 1182-1195 (2012).
254. X. W. Mao *et al.*, Spaceflight environment induces mitochondrial oxidative damage in ocular tissue. *Radiat Res* **180**, 340-350 (2013).
255. H. Schatten, M. L. Lewis, A. Chakrabarti, Spaceflight and clinorotation cause cytoskeleton and mitochondria changes and increases in apoptosis in cultured cells. *Acta Astronaut* **49**, 399-418 (2001).
256. L. Shi *et al.*, Spaceflight and simulated microgravity suppresses macrophage development via altered RAS/ERK/NFκB and metabolic pathways. *Cell Mol Immunol*, (2020).
257. M. K. Connor, D. A. Hood, Effect of microgravity on the expression of mitochondrial enzymes in rat cardiac and skeletal muscles. *Journal of Applied Physiology* **84**, 593-598 (1998).
258. G. Paradies, F. M. Ruggiero, G. Petrosillo, E. Quagliariello, Age-dependent decrease in the cytochrome c oxidase activity and changes in phospholipids in rat-heart mitochondria. *Arch Gerontol Geriatr* **16**, 263-272 (1993).
259. P. Liu, D. Li, W. Li, D. Wang, Mitochondrial Unfolded Protein Response to Microgravity Stress in Nematode Caenorhabditis elegans. *Sci Rep* **9**, 16474 (2019).
260. Y. Tian, C. Merkwirth, A. Dillin, Mitochondrial UPR: A Double-Edged Sword. *Trends Cell Biol* **26**, 563-565 (2016).
261. J. E. Aldridge, T. Horibe, N. J. Hoogenraad, Discovery of genes activated by the mitochondrial unfolded protein response (mtUPR) and cognate promoter elements. *PLoS One* **2**, e874 (2007).
262. S. Angeli *et al.*, The mitochondrial permeability transition pore activates the mitochondrial unfolded protein response and promotes aging. *Elife* **10**, (2021).

263. J. Vernikos, V. S. Schneider, Space, gravity and the physiology of aging: parallel or convergent disciplines? A mini-review. *Gerontology* **56**, 157-166 (2010).
264. M. R. Deschenes, M. H. Wilson, W. J. Kraemer, Neuromuscular adaptations to spaceflight are specific to postural muscles. *Muscle Nerve* **31**, 468-474 (2005).
265. T. P. Stein *et al.*, Energy expenditure and balance during spaceflight on the space shuttle. *Am J Physiol* **276**, R1739-1748 (1999).
266. A. K. Pollard *et al.*, Molecular Muscle Experiment: Hardware and Operational Lessons for Future Astrobiology Space Experiments. *Astrobiology* **20**, 935-943 (2020).
267. F. Selch *et al.*, Genomic response of the nematode Caenorhabditis elegans to spaceflight. *Adv Space Res* **41**, 807-815 (2008).
268. J. A. Pérez-Valencia *et al.*, Angiogenesis and evading immune destruction are the main related transcriptomic characteristics to the invasive process of oral tongue cancer. *Sci Rep* **8**, 2007 (2018).
269. G. J. Beckett, B. J. Chapman, E. H. Dyson, J. D. Hayes, Plasma glutathione S-transferase measurements after paracetamol overdose: evidence for early hepatocellular damage. *Gut* **26**, 26-31 (1985).
270. A. J. Cooper, J. T. Pinto, Cysteine S-conjugate beta-lyases. *Amino Acids* **30**, 1-15 (2006).
271. P. K. Maurya, S. I. Rizvi, Age-dependent changes in glutathione-s-transferase: correlation with total plasma antioxidant potential and red cell intracellular glutathione. *Indian J Clin Biochem* **25**, 398-400 (2010).
272. P. Mohanty *et al.*, Both lipid and protein intakes stimulate increased generation of reactive oxygen species by polymorphonuclear leukocytes and mononuclear cells. *Am J Clin Nutr* **75**, 767-772 (2002).
273. A. Nene, C. H. Chen, M. H. Disatnik, L. Cruz, D. Mochly-Rosen, Aldehyde dehydrogenase 2 activation and coevolution of its εPKC-mediated phosphorylation sites. *J Biomed Sci* **24**, 3 (2017).
274. T. E. Cullingford, D. A. Eagles, H. Sato, The ketogenic diet upregulates expression of the gene encoding the key ketogenic enzyme mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase in rat brain. *Epilepsy Res* **49**, 99-107 (2002).
275. WormBase website, http://www.wormbase.org, release WS279, date 2020.
276. P. Wiklund *et al.*, Insulin resistance is associated with altered amino acid metabolism and adipose tissue dysfunction in normoglycemic women. *Sci Rep* **6**, 24540 (2016).
277. S. Sookoian *et al.*, Serum aminotransferases in nonalcoholic fatty liver disease are a signature of liver metabolic perturbations at the amino acid and Krebs cycle level. *Am J Clin Nutr* **103**, 422-434 (2016).
278. L. Rahib, N. K. MacLennan, S. Horvath, J. C. Liao, K. M. Dipple, Glycerol kinase deficiency alters expression of genes involved in lipid metabolism, carbohydrate metabolism, and insulin signaling. *Eur J Hum Genet* **15**, 646-657 (2007).
279. D. Gems, J. J. McElwee, Broad spectrum detoxification: the major longevity assurance process regulated by insulin/IGF-1 signaling? *Mech Ageing Dev* **126**, 381-387 (2005).
280. K. L. Kavanagh, H. Jörnvall, B. Persson, U. Oppermann, Medium- and short-chain dehydrogenase/reductase gene and protein families : the SDR superfamily: functional and structural diversity within a family of metabolic and regulatory enzymes. *Cell Mol Life Sci* **65**, 3895-3906 (2008).

281. P. Pumirat *et al.*, The role of short-chain dehydrogenase/oxidoreductase, induced by salt stress, on host interaction of B. pseudomallei. *BMC Microbiol* **14**, 1 (2014).
282. M. A. Bitew *et al.*, SdrA, an NADP(H)-regenerating enzyme, is crucial for Coxiella burnetii to resist oxidative stress and replicate intracellularly. *Cell Microbiol* **22**, e13154 (2020).
283. J. A. Brzostowski, A. R. Kimmel, Signaling at zero G: G-protein-independent functions for 7-TM receptors. *Trends Biochem Sci* **26**, 291-297 (2001).
284. B. Vidal *et al.*, An atlas of Caenorhabditis elegans chemoreceptor expression. *PLoS Biol* **16**, e2004218 (2018).
285. N. Unwin, Neurotransmitter action: opening of ligand-gated ion channels. *Cell* **72 Suppl**, 31-41 (1993).
286. M. Franco, E. Chávez, O. Pérez-Méndez, Pleiotropic effects of thyroid hormones: learning from hypothyroidism. *J Thyroid Res* **2011**, 321030 (2011).
287. O. Kann, R. Kovács, Mitochondria and neuronal activity. *Am J Physiol Cell Physiol* **292**, C641-657 (2007).
288. J. Lytton, Na+/Ca2+ exchangers: three mammalian gene families control Ca2+ transport. *Biochem J* **406**, 365-382 (2007).
289. M. H. Kim, N. Korogod, R. Schneggenburger, W. K. Ho, S. H. Lee, Interplay between Na+/Ca2+ exchangers and mitochondria in Ca2+ clearance at the calyx of Held. *J Neurosci* **25**, 6057-6065 (2005).
290. E. C. Toescu, M. Vreugdenhil, Calcium and normal brain ageing. *Cell Calcium* **47**, 158-164 (2010).
291. V. Nikoletopoulou, N. Tavernarakis, Calcium homeostasis in aging neurons. *Front Genet* **3**, 200 (2012).
292. B. J. Berry, M. Kaeberlein, An energetics perspective on geroscience: mitochondrial protonmotive force and aging. *Geroscience* **43**, 1591-1604 (2021).
293. A. Aspberg, C. Binkert, E. Ruoslahti, The versican C-type lectin domain recognizes the adhesion protein tenascin-R. *Proc Natl Acad Sci U S A* **92**, 10590-10594 (1995).
294. J. J. Siew, Y. Chern, Microglial Lectins in Health and Neurological Diseases. *Front Mol Neurosci* **11**, 158 (2018).
295. M. Bhimreddy, E. Rushton, D. L. Kopke, K. Broadie, Secreted C-type lectin regulation of neuromuscular junction synaptic vesicle dynamics modulates coordinated movement. *J Cell Sci* **134**, (2021).
296. H. Liu *et al.*, Fundamental Characteristics of Neuron Adhesion Revealed by Forced Peeling and Time-Dependent Healing. *Biophys J* **118**, 1811-1819 (2020).
297. V. J. Milton *et al.*, Oxidative stress induces overgrowth of the Drosophila neuromuscular junction. *Proc Natl Acad Sci U S A* **108**, 17521-17526 (2011).
298. Y. Salzberg *et al.*, Synaptic Protein Degradation Controls Sexually Dimorphic Circuits through Regulation of DCC/UNC-40. *Curr Biol* **30**, 4128-4141.e4125 (2020).
299. S. Harada *et al.*, Fluid dynamics alter Caenorhabditis elegans body length via TGF-β/DBL-1 neuromuscular signaling. *NPJ Microgravity* **2**, 16006 (2016).
300. Y. Honda *et al.*, Genes down-regulated in spaceflight are involved in the control of longevity in Caenorhabditis elegans. *Sci Rep* **2**, 487 (2012).
301. A. Higashibata *et al.*, Microgravity elicits reproducible alterations in cytoskeletal and metabolic gene and protein expression in space-flown Caenorhabditis elegans. *NPJ Microgravity* **2**, 15022 (2016).

302. R. Laranjeiro *et al.*, Spaceflight affects neuronal morphology and alters transcellular degradation of neuronal debris in adult *Caenorhabditis elegans*. *iScience* **24**, 102105 (2021).
303. A. K. Gravely *et al.*, Levels of acid sphingomyelinase (ASM) in Caenorhabditis elegans in microgravity. (2020).
304. K. A. Haskins, J. F. Russell, N. Gaddis, H. K. Dressman, A. Aballay, Unfolded protein response genes regulated by CED-1 are required for Caenorhabditis elegans innate immunity. *Dev Cell* **15**, 87-97 (2008).
305. M. C. Berg, Ph.D., University of California, Berkeley, Ann Arbor (2017).
306. Molgen. (2020), vol. 2022.
307. N. J. Szewczyk, E. Kozak, C. A. Conley, Chemically defined medium and Caenorhabditis elegans. *BMC Biotechnol* **3**, 19 (2003).
308. R. G. Tepper *et al.*, PQM-1 complements DAF-16 as a key transcriptional regulator of DAF-2-mediated development and longevity. *Cell* **154**, 676-690 (2013).
309. T. P. Stein, M. D. Schulter, G. Boden, Development of insulin resistance by astronauts during spaceflight. *Aviat Space Environ Med* **65**, 1091-1096 (1994).
310. D. David-Rus, In the search of molecular signature of sarcopenia in C. elegans. *arXiv preprint arXiv:1212.1928*, (2012).
311. S. Bamps, J. Wirtz, F. R. Savory, D. Lake, I. A. Hope, The Caenorhabditis elegans sirtuin gene, sir-2.1, is widely expressed and induced upon caloric restriction. *Mech Ageing Dev* **130**, 762-770 (2009).
312. M. Fletcher, D. H. Kim, Age-Dependent Neuroendocrine Signaling from Sensory Neurons Modulates the Effect of Dietary Restriction on Longevity of Caenorhabditis elegans. *PLoS Genet* **13**, e1006544 (2017).
313. A. McGehee, The GLR-1 phenotypes of the daf-7(e1372) allele are not temperature sensitive. *MicroPubl Biol* **2019**, (2019).
314. Y. J. You, J. Kim, M. Cobb, L. Avery, Starvation activates MAP kinase through the muscarinic acetylcholine pathway in Caenorhabditis elegans pharynx. *Cell Metab* **3**, 237-245 (2006).
315. J. D. Sibonga *et al.*, Recovery of spaceflight-induced bone loss: bone mineral density after long-duration missions as fitted with an exponential function. *Bone* **41**, 973-978 (2007).
316. A. Wnorowski *et al.*, Effects of Spaceflight on Human Induced Pluripotent Stem Cell-Derived Cardiomyocyte Structure and Function. *Stem Cell Reports* **13**, 960-969 (2019).
317. D. a. C. Institute of Medicine Committee to Review Dietary Reference Intakes for Vitamin, in *Dietary Reference Intakes for Calcium and Vitamin D,* A. C. Ross, C. L. Taylor, A. L. Yaktine, H. B. Del Valle, Eds. (National Academies Press (US) Copyright © 2011, National Academy of Sciences., Washington (DC), 2011).
318. A. Herraiz-Martínez *et al.*, Ageing is associated with deterioration of calcium homeostasis in isolated human right atrial myocytes. *Cardiovasc Res* **106**, 76-86 (2015).
319. D. Datta *et al.*, Age-related calcium dysregulation linked with tau pathology and impaired cognition in non-human primates. *Alzheimers Dement* **17**, 920-932 (2021).
320. S. M. Smith *et al.*, Space flight calcium: implications for astronaut health, spacecraft operations, and Earth. *Nutrients* **4**, 2047-2068 (2012).
321. M. Kouhnavard *et al.*, Effects of Vitamin D and Calcium Supplementation on Micro-architectural and Densitometric Changes of Rat Femur in a Microgravity Simulator Model. *Iran Red Crescent Med J* **16**, e18026 (2014).

322. S. M. Smith, S. R. Zwart, Magnesium and Space Flight. *Nutrients* **7**, 10209-10222 (2015).
323. M. Barbagallo, N. Veronese, L. J. Dominguez, Magnesium in Aging, Health and Diseases. *Nutrients* **13**, (2021).
324. C. V. Carman, T. Som, C. M. Kim, J. L. Benovic, Binding and phosphorylation of tubulin by G protein-coupled receptor kinases. *J Biol Chem* **273**, 20308-20316 (1998).
325. W. Yongqing Liu and Eugenia, Transcriptional Analysis of Normal Human Fibroblast Responses to Microgravity Stress. *Genomics, Proteomics & Bioinformatics* **6**, 29-41 (2008).
326. J. Wang, C. Gareri, H. A. Rockman, G-Protein-Coupled Receptors in Heart Disease. *Circulation Research* **123**, 716-735 (2018).
327. I. Böhme, A. G. Beck-Sickinger, Illuminating the life of GPCRs. *Cell Communication and Signaling* **7**, 16 (2009).
328. D. V. Predescu *et al.*, G Protein-Coupled Receptors (GPCRs)-Mediated Calcium Signaling in Ovarian Cancer: Focus on GPCRs activated by Neurotransmitters and Inflammation-Associated Molecules. *Int J Mol Sci* **20**, (2019).
329. Y. Gao, Y. Arfat, H. Wang, N. Goswami, Muscle Atrophy Induced by Mechanical Unloading: Mechanisms and Potential Countermeasures. *Front Physiol* **9**, 235 (2018).
330. L. Tomasova *et al.*, Effects of AP39, a novel triphenylphosphonium derivatised anethole dithiolethione hydrogen sulfide donor, on rat haemodynamic parameters and chloride and calcium Cav3 and RyR2 channels. *Nitric Oxide* **46**, 131-144 (2015).
331. Q. Tahseen, Coelomocytes: Biology and Possible Immune Functions in Invertebrates with Special Remarks on Nematodes. *International Journal of Zoology* **2009**, 13 (2009).
332. S. Littleton, D. Ludwick. (2015).
333. B. E. Crucian *et al.*, Immune System Dysregulation During Spaceflight: Potential Countermeasures for Deep Space Exploration Missions. *Front Immunol* **9**, 1437 (2018).
334. M. A. Rahman *et al.*, The Role of Host-Generated H2S in Microbial Pathogenesis: New Perspectives on Tuberculosis. *Front Cell Infect Microbiol* **10**, 586923 (2020).
335. K. Baumann, Integrin's new partner. *Nature Reviews Molecular Cell Biology* **11**, 164-164 (2010).
336. C. M. Teoh, J. K. Tam, T. Tran, Integrin and GPCR Crosstalk in the Regulation of ASM Contraction Signaling in Asthma. *J Allergy (Cairo)* **2012**, 341282 (2012).
337. C. T. Walsh, D. Stupack, J. H. Brown, G protein-coupled receptors go extracellular: RhoA integrates the integrins. *Mol Interv* **8**, 165-173 (2008).
338. K. Tanaka *et al.*, Latrophilins are essential for endothelial junctional fluid shear stress mechanotransduction. *bioRxiv*, 2020.2002.2003.932822 (2020).
339. H. Gong *et al.*, Evidence of a common mechanism of disassembly of adherens junctions through Gα13 targeting of VE-cadherin. *J Exp Med* **211**, 579-591 (2014).
340. I. Wortzel, R. Seger, The ERK Cascade: Distinct Functions within Various Subcellular Organelles. *Genes Cancer* **2**, 195-209 (2011).
341. O. Hobert, The neuronal genome of Caenorhabditis elegans. *WormBook*, 1-106 (2013).

342. B. W. Tobin, P. N. Uchakin, S. K. Leeper-Woodford, Insulin secretion and sensitivity in space flight: diabetogenic effects. *Nutrition* **18**, 842-848 (2002).
343. K. R. Jonscher *et al.*, Spaceflight Activates Lipotoxic Pathways in Mouse Liver. *PLoS One* **11**, e0152877 (2016).
344. J. L. Pan, J. C. Bardwell, The origami of thioredoxin-like folds. *Protein Sci* **15**, 2217-2227 (2006).
345. M. Laskowski *et al.*, What do we not know about mitochondrial potassium channels? *Biochim Biophys Acta* **1857**, 1247-1257 (2016).
346. T. Yamada, T. M. Dawson, T. Yanagawa, M. Iijima, H. Sesaki, SQSTM1/p62 promotes mitochondrial ubiquitination independently of PINK1 and PRKN/parkin in mitophagy. *Autophagy* **15**, 2012-2018 (2019).
347. A. Poon *et al.*, The role of SQSTM1 (p62) in mitochondrial function and clearance in human cortical neurons. *Stem Cell Reports* **16**, 1276-1289 (2021).
348. Z. Y. Zhang, S. Guo, R. Zhao, Z. P. Ji, Z. N. Zhuang, Clinical significance of SQSTM1/P62 and nuclear factor-κB expression in pancreatic carcinoma. *World J Gastrointest Oncol* **12**, 719-731 (2020).
349. S. Mitsui *et al.*, Systemic overexpression of SQSTM1/p62 accelerates disease onset in a SOD1. *Mol Brain* **11**, 30 (2018).
350. Y. Xu *et al.*, Overexpression of p62/SQSTM1 promotes the degradations of abnormally accumulated PrP mutants in cytoplasm and relieves the associated cytotoxicities via autophagy-lysosome-dependent way. *Med Microbiol Immunol* **203**, 73-84 (2014).
351. F. Alegre *et al.*, Role of p62/SQSTM1 beyond autophagy: a lesson learned from drug-induced toxicity in vitro. *Br J Pharmacol* **175**, 440-455 (2018).
352. B. Wu *et al.*, Aldehyde dehydrogenase 2 activation in aged heart improves the autophagy by reducing the carbonyl modification on SIRT1. *Oncotarget* **7**, 2175-2188 (2016).
353. L. Fu *et al.*, Direct Proteomic Mapping of Cysteine Persulfidation. *Antioxid Redox Signal* **33**, 1061-1076 (2020).
354. A. A. Turanov *et al.*, Regulation of Selenocysteine Content of Human Selenoprotein P by Dietary Selenium and Insertion of Cysteine in Place of Selenocysteine. *PLoS One* **10**, e0140353 (2015).
355. W. MacNee, R. A. Rabinovich, G. Choudhury, Ageing and the border between health and disease. *Eur Respir J* **44**, 1332-1352 (2014).
356. S. Juriasingani *et al.*, Subnormothermic Perfusion with H2S Donor AP39 Improves DCD Porcine Renal Graft Outcomes in an Ex Vivo Model of Kidney Preservation and Reperfusion. *Biomolecules* **11**, (2021).
357. G. Gojon, G. A. Morales, SG1002 and Catenated Divalent Organic Sulfur Compounds as Promising Hydrogen Sulfide Prodrugs. *Antioxid Redox Signal* **33**, 1010-1045 (2020).
358. V. Citi *et al.*, Anti-inflammatory and antiviral roles of hydrogen sulfide: Rationale for considering H2S donors in COVID-19 therapy. *Br J Pharmacol* **177**, 4931-4941 (2020).
359. S. J. Cho, H. W. Stout-Delgado, Aging and Lung Disease. *Annu Rev Physiol* **82**, 433-459 (2020).
360. E. Wood, K. H. Hall, W. Tate, Role of mitochondria, oxidative stress and the response to antioxidants in myalgic encephalomyelitis/chronic fatigue syndrome: A possible approach to SARS-CoV-2 'long-haulers'? *Chronic Dis Transl Med* **7**, 14-26 (2021).