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The mitochondrial free radical theory of ageing – Where do we stand?

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1. ABSTRACT

Understanding the molecular mechanisms underlying the ageing process may provide the best strategy for addressing the challenges posed by ageing populations worldwide. One theory proposing such molecular mechanisms was formulated 50 years ago. Harman *et al.* suggested that ageing might be mediated by macromolecular damage through reactions involving reactive oxygen species (ROS). Today, a version of the free radical theory of ageing, focusing on mitochondria as source as well as target of ROS, is one of the most popular theories of ageing. Here we critically review the status of key principles and concepts on which this theory is based. We find that the evidence to date shows that many of the original assumptions are questionable, while on some critical issues further refinements in techniques are required. Even so, it is becoming evident that mitochondria and mtDNA integrity may indeed be crucial determinants of organismal ageing. Implications for the prospect of successful interventions as well as evidence for and against efficacy of current therapeutic approaches are discussed.

2. INTRODUCTION

In 1954 Gerschman *et al.* suggested that oxygen free radicals are the toxic agents responsible for both oxygen poisoning and gamma irradiation damage (1). Two years later, Denham Harman first proposed that endogenous free radicals might cause macromolecular damage *in vivo* and suggested free radical-mediated damage as the causative agent for mutation accumulation, cancer and ageing (2).

In over fifty years since its inception, the free radical theory of ageing (FRTA) has undergone modifications and extensions, importantly with the suggestion that the most relevant source of reactive oxygen species (ROS) *in vivo* may be the superoxide radical produced as by-product of normal oxidative phosphorylation (OXPHOS) and that mitochondria themselves may be a major target for the accumulation of ROS-mediated damage (3-5). This realization has led to the formulation of the mitochondrial FRTA, variations of which will be discussed in more detail below. The FRTA in its various versions has been reviewed extensively elsewhere (6-10) and the purpose of the current article is to review critically some of the evidence that is said to support it, as well as to examine prospects for diminishing mitochondrial free radical damage.

2.1. The role of mitochondrial DNA

Mitochondrial DNA (mtDNA) has received particular attention as a putative target of ROS-mediated damage, because of its location in the mitochondrial matrix, since at least some mitochondrially-produced ROS are released into the matrix (3,4,11-13).

In its original form, the mitochondrial FRTA is based on four principles (6-13). First, that mtDNA, due to its location in the mitochondrial matrix, is subject to elevated levels of free radical attack. Second, that mtDNA, unlike nuclear DNA (nDNA) is "naked", that is, that it is not covered by protective structural proteins such as histones. The third assumption is that mitochondria lack the capacity to repair oxidative DNA damage. Together, these three assumptions suggest that the steady state burden of oxidative damage to mtDNA should be significantly higher than to nDNA.

ROS attack on mtDNA has the potential to facilitate formation of oxidative DNA base damage products such as 8-hydroxy-2'-deoxyguanosine (8OHdG) as well as strand breaks (14-16). Some of these DNA lesions are mutagenic; for instance the 8-hydroxyguanine DNA lesion can sometimes pair with adenine instead of cytosine, leading predominantly to GC to TA transversions (17-19). Oxidative damage to cytosine on the other hand mostly results in the formation of GC to AT transitions (20). Overall, GC to AT transitions and GC to TA transversions are the most commonly observed mutations resulting from oxidative DNA damage (reviewed in: (21)). Several components of the mitochondrial electron transfer chain (ETC) are encoded on mtDNA.

The fourth assumption underlying the mitochondria free radical theory, therefore, is that elevated levels of mtDNA mutations will further elevate ROS levels, and thereby facilitate mutation accumulation by negatively affecting the integrity of the ETC. This mechanism, in which a small amount of initial damage could initiate an exponential increase in further damage, constitutes a positive feedback mechanism and has been termed "The Vicious Cycle" (4, 22).

2.1.1. Mitochondrial DNA, dysfunction and the etiology of age-dependent degenerative processes – insights and questions

The mitochondrial FRTA, as described above, essentially proposes an exponentially accelerating degeneration of the mitochondria. In this process, ROS are both the initiator and the key mediator of the positive feedback mechanisms driving the process. The central role of free radicals in this model immediately suggests avenues of intervention based on anti-oxidants that scavenge mitochondrial ROS or on modulation of mitochondrial ROS production. However, before discussing the merits of some of these avenues, it is necessary to explore the experimental evidence to date (or lack thereof) for the assumptions underlying the mitochondrial FRTA.

2.1.2. Is the mitochondrial matrix a hostile environment for DNA?

Studies in isolated mitochondria have shown that complex III is the primary site for ROS release from the mitochondrion. However, there is evidence that these ROS are released into the inter-membrane space and thus away from the mitochondrial matrix (23). Other components of the mitochondrial ETC, however, such as the flavin mononucleotide group (FMN) of complex I, do release superoxide radicals into the mitochondrial matrix during normal respiration (24-26). Most data favouring mitochondria as the main source of ROS production have been obtained by studying isolated mitochondria in various states of respiration. The validity of these data has been challenged, particularly due to the fact that the ROS formation was often determined under non-physiological conditions (27). However, two recent studies have shown that the production of superoxide and hydrogen peroxide also occurs in isolated mitochondria from the skeletal muscle and brain, respectively, maintained under physiological conditions (28,29). In both studies, the ROS release originated mainly from complex I. Similarly, Kozlov and co-workers (30) detected increased ROS production in heart mitochondria isolated from aged animals relative to young controls. The authors concluded, however, that complex III was the likely source of ROS formation, which, based on the aforementioned data, might not pose a direct risk to the biomolecules in the mitochondrial matrix, such as mtDNA.

While the superoxide radical itself is not sufficiently reactive to directly damage DNA (14), it can facilitate DNA damage by interacting with free iron and hydrogen peroxide (H_2O_2) to form the more reactive hydroxyl radical, as well as by promoting the release of iron from iron-sulphur clusters e.g. in aconitase (15,31,32).

Potentially then, mtDNA located in the mitochondrial matrix may be subject to oxidative damage by endogenous ROS generated from complex I. Complex I, in turn, might be most prone to being affected by mtDNA mutations as seven out of the thirteen polypeptides encoded by mtDNA belong to this complex (33). Despite the extensive knowledge on mitochondria-associated ROS production *in vitro*, as well as evidence for their importance *in vivo* (discussed in section 2.1.6), it is still unclear what actual amounts of superoxide and hydroxyl radicals are produced *in vivo* in mitochondria. Advances in analytical methods such as electron paramagnetic resonance spectroscopy might help to answer this question (34).

Although there is evidence for an increase in iron, e.g. in the brain with ageing and in age-related disease (35), our knowledge of mitochondrial iron homeostasis and its potential accumulation is also still rudimentary. An interesting new insight regarding the importance of dysfunctional iron homeostasis in cellular stress originates from the identification of ferrihydrite as the main iron storage form in the mitochondria of cells from Friedrich's ataxia (FA) patients. Despite the fact that FA-affected humans do not suffer from a general iron overload and given the relative low reactivity of ferrihydrite, the authors of one study also reported the existence of potentially toxic ferrous iron in the mitochondria, which might cause a moderate though chronic oxidative stress (36).

2.1.3. Are mitochondria deficient in DNA repair and is mtDNA naked?

The idea that mitochondria may lack DNA repair capacity originally derived form the observation that ultraviolet light-mediated mtDNA damage is not repaired efficiently in mitochondria, suggesting that they lack nucleotide excision repair (NER) (37,38). However, it has since been shown that, unlike NER, base excision repair (BER) activity is present in mitochondria (37,39,40). Specifically, OGG1, the enzyme responsible for BER of 8OHdG, has been detected in rat liver mitochondria (41). Furthermore, the mitochondrial DNA repair machinery repairs 8OHdG as well as mtDNA strand breaks at least as efficiently as the equivalent system in the nucleus (39,42). Therefore, it is clear that, in contrast to the original and often repeated assumption, mitochondria are capable of rapid and efficient repair of oxidative DNA damage, especially 8OHdG.

There is also evidence that mtDNA is not as "naked" as was originally believed. For one it has long been known that mtDNA is not freely mobile inside the mitochondrial matrix, but bound to protein and anchored to the inner membrane (43). Furthermore, there is some evidence that mtDNA may be bound to multiple units of the mitochondrial transcription factor A (TFAM) and it has been suggested that TFAM may play the role of a histone-like protein in the context of mitochondria (reviewed in: (44)). Consistent with this idea, footprinting experiments also suggest that mtDNA is not entirely bare but bound to protective protein (45).

2.1.4. Is oxidative damage to mtDNA extensive *in vivo*?

The mitochondrial FRTA received a significant boost in 1988 when results by Richter *et al.* suggested that 8OHdG levels in mtDNA were 16 times higher than those in nDNA (13). However, it has since been appreciated that accurate quantification of oxidative lesions in DNA is extremely challenging. Problems include artifactual oxidation of DNA during extraction and sample preparation, as well as systematic differences between techniques employed (46-48). The extent of the problem, especially with respect to earlier methods, was illustrated by the European Standards Committee on Oxidative DNA damage study (ESCODD) (49,50). As part of this study, standardized tissue samples were distributed to 28 participating laboratories specialized in quantification of oxidative DNA damage. Despite efforts to standardize methods and minimize artifact, the reported values of 8OHdG in identical tissue samples spanned more than two orders of magnitude (49,50). In 1999 Beckman and Ames surveyed literature values for baseline levels of 8OHdG in both nDNA and mtDNA and found that values spanned over four orders of magnitude (48), suggesting that artifacts are indeed significantly confounding some published values.

These challenges are particularly relevant with respect to mtDNA because yields of mtDNA are typically low and because mtDNA may be more susceptible to *ex vivo* artifacts than nDNA (48,51,52). Given these complications, it is not surprising that the actual extent of oxidative modification to mtDNA *in vivo*, both in terms of absolute value and relative to nDNA, is controversial.

Thus some authors find elevated levels of oxidative damage in mtDNA relative to nDNA (13, 53-55), while others did not observe such differences (46,48,56). Given the known artifacts and the fact that the differences in data between methods and laboratories are many times larger than the proposed differences between nDNA and mtDNA, it has to be concluded that evidence to date is insufficient to prove that levels of oxidative damage in mtDNA are more extensive than in nDNA. The situation is similar with respect to the question as to whether oxidative mtDNA damage increases with age, with some authors reporting age-dependent increases (55,57-60) while others do not see any such increase (61,62).

On the other hand, indirect evidence for the involvement of ROS-mediated mtDNA damage in age-dependent mtDNA mutagenesis has recently been reported. Using a highly sensitive mutation capture assay (63), Vermulst *et al.* were able to show that the accumulation of mtDNA point mutations in both brain and heart of wild type (WT) mice follows exponential kinetics with age (64). This agrees with earlier data for mtDNA deletions, which also showed exponential increases in mtDNA mutation burden with age (65). An exponential kinetic can be interpreted to imply a positive feedback mechanism, where the rate of mutation at any given time point is in some way a function of the mutation burden accumulated up to this point in time. Furthermore, the mutations detected in WT mice by Vermulst *et al.* were found to be over 80% GC to AT transitions, with most of the remaining mutations being

GC to TA transversions (64). This mutational spectrum agrees with that seen as a consequence of oxidative DNA damage and is therefore consistent with a causative role of ROS-mediated mtDNA damage (21,66). However, such consistency does not *prove* a free radical mediated mechanism. In contrast to these observations in mice, it has recently been noted that the mutational spectrum observed in some mutational hotspots of human mtDNA is more consistent with polymerase errors (67).

In summary, upon closer inspection it appears that most of the original factors motivating the suggestion that mtDNA may be highly susceptible to oxidative damage require at least qualification.

2.1.5. Is there evidence for a vicious cycle?

The plausibility of the Vicious Cycle hypothesis is supported by experiments in cultured human neurons where disruption of the ETC by RNA interference (RNAi) can result in significantly increased oxidative DNA damage and ROS production (68). Similarly, temporary *in vivo* chemical inhibition of complex I by rotenone leads to irreversible damage to rat brain mitochondria associated with increased ROS generation (69). Even though absolute levels of mtDNA point mutations detected by Vermulst *et al.* (64) are very low (< 0.2 per mtDNA molecule), the exponential kinetics observed are consistent with the idea of the Vicious Cycle.

To directly investigate the functional consequences, including effects on ROS production and oxidative stress, of mtDNA point mutation accumulation *in vivo*, a number of knock-in mouse models carrying error prone mtDNA polymerase have been generated (70-72). The first such transgenic mouse model, expressing a proofreading-deficient mtDNA polymerase specifically in the heart, has been shown to accumulate both mtDNA point mutations and deletions in this tissue faster than WT mice (72). The transgenic mice developed cardiomyopathy as a consequence of increased cell death in cardiomyocytes but showed no evidence of increased protein carbonyl levels, a measure of oxidative protein damage (73,74), decreased aconitase activity or decreased levels of glutathione in their heart (75,76). Using a purely qualitative assay based on southern blot following formamidopyrimidine DNA glycosylase (fpg) digest of mtDNA, Mott *et al.* did not detect an age-dependent increase in oxidative damage burden to mtDNA in hearts of transgenic mice (75).

Two different homozygous mtDNA mutator mouse models, expressing proofreading-deficient mtDNA polymerase globally, showed dramatically increased mtDNA mutation burdens and exhibited a range of pathologies commonly associated with ageing as well as suffering from significantly shortened lifespans (70,71). Kujoth *et al.* (70) compared mutator and WT mice in terms of tissue (skeletal muscle, liver) levels of F_2-isoprostanes, a widely used biomarker of oxidative lipid damage (77,78), as well as of 8OHdG in DNA and RNA from liver and found no evidence for globally increased oxidative damage. In the same study, mitochondrial protein carbonyl levels in heart and liver as well as H_2O_2 production from isolated liver mitochondria were not significantly different between mutator and WT mice (70).

Apart from a slight elevation in protein carbonyls and some evidence for the activation of glutathione peroxidase expression Trifunovic *et al.* also did not detect evidence for increased oxidative stress in the tissues of the second mutator mouse model (79). However, it is interesting to note that they found comparable levels of free radical production in Mouse Embryonic Fibroblast (MEF) cells derived from mutator and WT embryos, despite the former cell line being almost completely deficient in respiration (79). It has been pointed out that such data imply that the amount of ROS produced by mutator mouse-derived cells per molecule of oxygen consumed would actually be much higher compared to control cells (44). However, given the known complexities and artifacts relating to the evaluation of ROS production in cell culture ((80) and discussed below), it is also possible that this result mainly reflects the methodological difficulties intrinsic to the determination of ROS levels using *in vitro* assays.

In summary, none of the mouse models for increased mtDNA mutations provides evidence for increased oxidative stress. Instead, these mice show signs of increased cell death (70,79). It appears that there is little evidence that increased mtDNA point mutation burden does indeed lead to globally increased oxidative stress, even though highly elevated mutation burden leads to severe pathology associated with activation of cell death pathways.

On the other hand, the mutator mice show that high levels of mtDNA mutations are associated with ageing or at least with phenotypes resembling several aspects of ageing. Is this relevant for normal ageing? The obvious question is, how abundant *in vivo* mtDNA mutations are during normal ageing. Age-dependent mtDNA mutation accumulation has been extensively investigated in tissue of animals as well as humans. Levels of specific mtDNA point mutations affecting the non-coding control region of mtDNA increase dramatically with age and can expand to over 50% of mtDNA molecules (81,82). Such mutations are highly tissue specific (e.g. specific point mutations commonly occur in muscle but are undetectable in fibroblasts and *vice versa*), do not diminish mitochondrial OXPHOS capacity and some of them may in fact be beneficial functional adaptations (83).

In contrast, evidence from WT animals in the mutator mouse study (discussed above), suggests that levels of mtDNA point mutations affecting the coding region of mtDNA, and potentially leading to disruption of normal ETC function, remain extremely low even in very old organisms (64). For comparison, heterozygous mtDNA mutator mice in one study were able to sustain a 500-fold increase in mtDNA point mutation burden, without exhibiting any features of pathology or premature senescence (Figure 1) (64). This latter result has been interpreted as evidence that mtDNA point mutations

themselves do not accumulate sufficiently during normal mouse ageing to become limiting to lifespan (64).

However, a number of open questions remain that need to be considered before this conclusion can be made with full confidence. For one, mutations in the mutator mice originate early in life due to polymerase errors and there is little evidence for further age-dependent accumulation. In contrast, in WT mice, mtDNA mutations are rare in early life but accumulate exponentially during ageing (64). This implies significant differences in both the mechanism and dynamics of mtDNA mutation accumulation between WT and mutator mice. Mechanisms by which this could matter are discussed below.

Secondly, it has been noted that some of the phenotypes observed in the mutator mice seem to indicate involvement of tissues with high mitotic activity (spleen, testes, epidermis) rather than of post-mitotic tissues as would be expected in true accelerated ageing (84). At this stage it is therefore unclear if the mutator mice are suitable models for the functional effects of normal age-dependent loss in mtDNA integrity (84-86).

Using PCR techniques, mtDNA deletions have also been detected in different tissues from diverse organisms (87-92). Inherited mtDNA deletions, with high abundance, are thought to underlie certain rare diseases, including myopathies and encephalomyopathies (93). Furthermore, the level of mtDNA deletions in heart increases significantly with age (92) and also in brain (87,94,95). It has been suggested that a correlation between deletion levels and levels of 8OHdG may exist in heart mtDNA, suggesting that oxidative damage to mtDNA may be an initiating event in *de novo* formation of mtDNA deletions as well (96). A possible mechanism for this may be oxidative damage to DNA polymerase, as ROS-mediated DNA damage itself does not normally cause deletions (97). However, as in the case of mtDNA point mutations, abundance of deletions in bulk tissue is very low, usually remaining significantly below 1% of total mtDNA even in very old animals (88,95,98). Consistently, with the relatively low tissue levels of both point mutations and deletions, most studies of global OXPHOS capacity and enzyme activity in cells and tissue from both primates and rodents detect some functional decline with age but this decline is tissue specific and in most cases relatively mild with high inter-individual variability (99-105).

In summary, many of the original assumptions leading to the suggestion that mtDNA may be subject to extensive oxidative damage require qualification and there is, to date, no convincing evidence that oxidative damage to mtDNA is indeed extensive *in vivo*. It is unclear how functionally significant is *in vivo* mtDNA damage at the order of 1 8OHdG lesion per 10^5 bases. The available evidence suggests that oxidative mtDNA damage could be a causative agent for mtDNA point mutations and may also be involved in *de novo* formation of mtDNA deletions. Consistent with the conclusion that steady state levels of oxidative damage to mtDNA may in fact be low, both deletions and point mutations in the mtDNA coding region remain low in most bulk tissues, even in very old animals. While unphysiologically high mtDNA mutations levels are clearly pathogenic, their low abundance in normal animals raises questions regarding their functional relevance in the context of normal ageing.

2.1.6. Do mitochondria matter?

Does this mean that mitochondrial ROS production and mtDNA integrity are unimportant in the context of organismal ageing? Not necessarily, since there is considerable evidence suggesting that mitochondrial ROS are important determinants of organismal ageing rates. A large body of literature shows that mitochondrial superoxide production rates correlate with rates of ageing in different species (106-115). Recent data suggest that this conclusion remains valid, even when confounding factors such as body size and phylogeny are considered (116,117). Consistent with these observations are data showing that oxidative damage burden (measured as 8OHdG) in mtDNA but not nDNA may be inversely correlated with lifespan (118,119); but yet again the questions of accurate methodology arise. Direct evidence for the relevance of mitochondrial ROS production for mammalian ageing is provided by knock-in mouse models expressing human catalase targeted either to the peroxisome, nucleus or mitochondria. Only the expression of catalase targeted to mitochondria (MCAT-mice) resulted in decreases in levels of oxidative damage and caused a highly significant increase in both median and maximum lifespan (up to 21%) (120). Consistent with a causative role of ROS for *de novo* mtDNA point mutations *in vivo*, MCAT-mice accumulated less than 50% of the mtDNA point mutations in their hearts than age matched WT mice (Figure 1) (64). In contrast to the supportive evidence from MCAT mice, heterozygous $MnSOD^{+/-}$ knockout mice do not show accelerated ageing phenotypes, despite reportedly suffering from elevated 8OHdG levels in both nDNA and mtDNA (121). They do, however, experience higher rates of cancer and it is as yet unclear if these mice actually suffer from elevated levels of mtDNA mutations. Further investigations into mitochondrial ROS production, ROS detoxification and redox signaling, mtDNA repair pathways and their effect on age-dependent mtDNA integrity will be required to resolve these contradictions.

More correlative evidence comes from the observation that animals under caloric restrictions (CR) produce significantly less mitochondrial superoxide, accumulate less mtDNA deletions and experience less degenerative disease as well as extended mean and maximum lifespans compared to animals fed *ad-libitum* (122,123). However, superoxide does not itself damage DNA (14) and the extension of lifespan as well as the prevention of age-dependent disease associated with CR may involve many hormonal, physiological and biochemical alterations (e.g. perhaps changes in iron metabolism (15)), in addition to the reduction of superoxide production and oxidative damage (124). The exact sequence of events and role, in terms of cause and consequence, of ROS in these processes is still unclear.

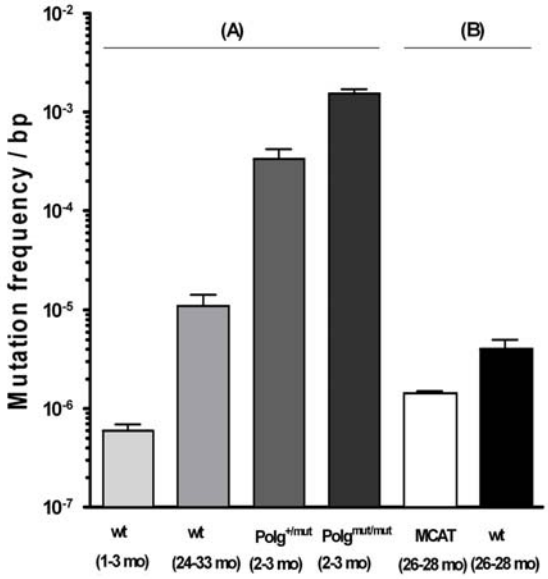

Figure 1. (A) Mutational burden to mtDNA in brain tissue of wild type (wt) mice compared to heterozygous ($Polg^{+/mut}$) and homozygous ($Polg^{mut/mut}$) mutator mice. While there is clear age-dependent increase in mtDNA point mutations in wt-mice, heterozygous mutator mice experience dramatically elevated levels of mtDNA point mutations, relative even to 10-fold older wt-mice, without apparently suffering from accelerated ageing phenotypes or mitochondrial pathology. (B) Reduced mutational burden to mtDNA in heart tissue of MCAT mice over-expressing catalase in their mitochondria compared to age-matched wt-mice. All data redrawn from (64).

2.1.7. Mosaicity is a key aspect of pathology associated with age-dependent mtDNA mutations accumulation

An important insight into the possible functional role of low-level mtDNA mutations in ageing comes from single cell studies carried out in a range of post-mitotic tissues, particularly in skeletal muscle. In humans, sarcopenia, characterized by atrophy and loss of skeletal muscle fibres with age, causes loss of up to 40% of muscle mass by the age of about 80 years (125,126). Immunohistochemical analysis of ageing muscle reveals a clear age-dependent increase in the number of fibres exhibiting abnormalities in their mitochondrial ETC. This includes fibres negative for complex IV (cytochrome c oxidase / COX) staining (COX^{-} fibres) (127-129). *In situ* hybridization shows that these ETC abnormalities are associated with mtDNA deletions (130-132). Even though the total abundance of deletions remains low in bulk tissue (88), fibre bundle analysis reveals high focal abundance of deletions in those fibre segments exhibiting ETC abnormalities and atrophy (133-135).

Using laser capture micro-dissection and quantitative PCR on axial serial sections of muscle fibres, it is possible to follow disease progression along the length of individual fibres and to elucidate the sequence of events that ultimately lead to cell death and muscle fibre loss. This analysis reveals that mtDNA deletions clonally expand within individual muscle fibres, first leading to progressively worsening ETC abnormalities, followed by muscle fibre splitting, atrophy and ultimately loss of whole fibres (135,136). Based on the observed propensity of such fibres, it has been estimated that about 15% of fibres in aged rat muscle contain a region of severely compromised ETC function (135).

Clonally expanded mtDNA point mutations affecting tRNA genes have similarly been shown to be strongly correlated with COX deficiency in human muscle fibres (137). In the example of skeletal muscle, high mosaicity and clonal expansion of mutated mtDNA provide a mechanism by which relatively low bulk tissue levels of mutant mtDNA can be causative for the age-dependent functional and structural decline commonly observed in selected areas of this tissue. The fact that the worst affected muscle fibres are eventually lost may also explain why the overall abundance of mtDNA deletions in bulk muscle does not rise above a certain level. Mutant mtDNA, negatively affecting the ETC, could simply be lost from the tissue as affected cells die.

Focally distributed mtDNA mutations are a feature also seen in other post mitotic tissues. In two independent single-cell studies of human cardiomyocytes, 15% and 25%, respectively of cells from the oldest patients contained mtDNA deletions (138,139). In the same studies, mtDNA deletions were practically absent in cardiomyocytes from young individuals.

In the brain, neurons in the *substantia nigra* (SN) are thought to be subject to elevated levels of oxidative damage and accumulate orders of magnitude more deletions than do neurons in other brain regions (87,98,140). It has been suggested that the higher oxidative burden in the SN may be a consequence of higher demands on energy metabolism in SN dopamine neurons (141). In old individuals, up to 30% of neurons in the SN region show defects in their mitochondrial respiratory activity and these cells are associated with high levels of clonally expanded deletions (87,142,143). In addition to the high oxygen demand, destruction of the neuromelanin-iron system has the potential to further amplify oxidative stress levels. ROS might cause both the release of soluble melanin (termed melanin-free acid) and iron from melanin scaffold. Complexes of iron and melanin-free acid, in addition to the presence of dopamine render dopaminergic nigral neurons particularly susceptible to the occurrence of oxidative stress (144). Whether this pathomechanism also includes enhanced iron mobilization in mitochondria *in vivo* is an interesting, but currently unproven hypothesis. L-dopa is considered to be the gold standard treatment for Parkinson's disease (PD) (145). At the same time, L-dopa has been suspected to cause neurotoxicity. This assumption is supported by data from Alam *et al.* showing in PD patients an increase in oxidative protein damage in the SN and in brain regions other than those typically affected in PD (146). This increase in oxidative damage might partly originate from the formation of cytotoxic L-dopa-cysteinyl adducts, which are elevated in the SN of PD patients (147). The ELLDOPA study, which for the first time explored the

dose-response effect of L-dopa in PD patients, however, favours a neuroprotective mode of action for L-dopa (148).

Overall, the aggregate burden of sporadic mtDNA point mutations in brain increases significantly with age and correlates negatively with mitochondrial enzyme (cytochrome c oxidase) activity (149,150). In the colon, clonally expanded mtDNA point mutations have also been identified as the cause of cytochrome *c* oxidase deficient crypts (151,152).

In each of the above cases, mutant mtDNA, potentially but not necessarily originating from ROS-mediated mtDNA damage, undergoes clonal expansion until the whole cell becomes deficient in normal ETC function whilst levels of mtDNA deletions overall in the tissue remains low.

Various models for this clonal expansion have been proposed, but the exact mechanisms driving the expansion process are as of yet poorly understood (153-157). Interestingly, CR has been shown to reduce ROS production from mitochondrial complex I and to decrease formation of *de novo* mtDNA deletions. On the other hand, there is evidence that CR does not retard clonal expansion of existing mtDNA deletions (122,123). It may be that ROS-independent clonal expansion is a mechanism (possibly the main mechanism) by which low levels of mtDNA mutations focally accumulate to levels sufficient to cause cellular and ultimately tissue dysfunction.

2.2. How can mtDNA mutations be a causative agent of age-dependent tissue dysfunction?

It has been increasingly appreciated that ROS are not purely detrimental by-products of metabolism but also play an important role in signaling, particularly between mitochondria and nucleus and in the control of cell death and proliferation pathways (reviewed in: (7,15,158)). How exactly ROS can escape from mitochondrial and cytosolic antioxidant defenses and modulate nuclear gene expression is, as yet, unexplained.

Cells suffering from clonally expanded mtDNA mutations affecting the ETC are likely to experience decreased electron transfer efficiency. Such changes might increase intra-mitochondrial oxygen concentration, since less oxygen is consumed, as well as promoting a more reduced ETC. This should increase ROS formation, which, coupled with lower ATP levels, can promote cell death pathways (159,160).

As in the case of sarcopenia, mtDNA mutations may therefore promote age-dependent tissue dysfunction in post-mitotic tissue by triggering the loss of irreplaceable cells (161,162). Increased cellular and organismal susceptibility to stressful stimuli and challenges is one of the defining characteristics of the ageing process in all species that experience ageing.

This model is supported by *in vitro* experiments with cells carrying an inherited mtDNA disease (163), as well as by data from transgenic mouse models, such as the mutator mice, which often (but not always) show increased cell death as a prominent phenotype of genetic interventions negatively affecting mtDNA integrity (reviewed in: (162)).

ROS and redox signaling have been shown to affect diverse processes such as energy homeostasis, stress resistance, inflammation, mitogenesis and the threshold of cell death (7,15,158). Progressive loss of mtDNA integrity, associated with changes in energy homeostasis and redox status, can potentially affect a wide range of cellular processes. They may therefore lead to disruption of tissue homeostasis through additional mechanisms such as pro-inflammatory signaling.

Alternative mechanisms have also been proposed, which would allow low levels of focally distributed mutant mtDNA to be causative for significant tissue dysfunction (133,164-167). Some of these hypotheses propose mechanisms by which cells harboring mtDNA mutations could be actively toxic to the surrounding tissue (165,166). One such theory, termed “reductive hotspot hypothesis”, is based on the assumption that cells could compensate for ETC-deficient mitochondria by reducing molecular oxygen through the plasma membrane redox system, thereby potentially producing extracellular superoxide and increasing oxidative burden to the surrounding tissue (165,167). An alternative hypothesis, put forward by Mott *et al.* in 2005 (164), suggests that mitochondrial mutations lead to transcription of misfolded mitochondria-encoded proteins, which then interact with mitochondrial mediators of cell death. According to this hypothesis, due to the delicate balance between pro- and anti-death mediators, even a small number of aberrant transcripts could potentially be sufficient to cause cell death (164).

3. THE CASE FOR TARGETING DETERMINANTS OF BASAL AGEING-RATE INSTEAD OF INDIVIDUAL AGE-DEPENDENT DISEASES

The ageing populations in the industrialized nations today live under conditions where extrinsic mortality is low. The leading causes of morbidity and mortality in these countries are therefore chronic degenerative diseases. Consequently, large amounts of resources are expended in efforts to find treatments or effective preventative measures for each of these diseases (168). However, since age itself is the single most important risk factor for most of these degenerative diseases, treatment or prevention of any one condition in an ageing population is likely to result in it being rapidly replaced by another, perhaps equally devastating condition. As populations age, interventions aimed at treatment or prevention of specific diseases therefore will have increasingly diminishing effects on the overall mortality as well as the associated cost to health care systems.

For comparison, a 20% increase in lifespan, such as has been observed in the mitochondrial catalase knock-in MCAT-mice (discussed in section 2.1.6), would be more than five times greater than the effect expected if all forms of cancer were eliminated from the human population

(169,170). It is interesting to note that in the MCAT mice both cardiac pathologies and cataract formation were also delayed, suggesting a reduction in age-dependent diseases as well as in basic ageing rate (120). The dominance of the rate of ageing over specific disease processes was further elegantly demonstrated in 2004 by comparing the time of onset and rate of progression of neurodegeneration resulting from a range of functionally equivalent mutations in orthologous genes across five mammalian species with maximum lifespans between 3.5 years (mouse) and 122 years (human) (161). The analysis of these data reveals that the rate of neurodegeneration in each case scales with the maximum lifespan despite being based on identical molecular mechanisms. In response to functionally identical mutations, faster ageing species progress through identical chains of neurodegeneration at proportionally higher speed than slower ageing species (161). Cell loss through activation of cell death pathways is the crucial event in each of the diseases considered.

As discussed above, mitochondria and mitochondrial ROS production are intricately linked to the threshold for, and execution of, cell death pathways and mitochondrial modulation of these pathways may therefore be an important factor explaining the observed scaling effect (161). The possibility that mitochondrial ROS production and loss of mtDNA integrity may be important determinants of age-dependent cell death and inflammation as well as basal organismal ageing rate, make mitochondria prime targets for the development of therapies aimed at preventing or alleviating age-dependent degenerative processes. Once tools capable of specifically modulating mtDNA integrity *in vivo* are available, testing the effect of reduced mtDNA mutation burden on lifespan will provide more answers to the question of whether or not mtDNA damage is relevant for ageing.

4. STRATEGIES

There is evidence that oxidative damage to proteins, lipids and DNA accumulates moderately with age in a wide variety of tissues (171-176) although the overall rise is quite small. Also, some of the damage detected shows patterns that are inconsistent with random, stochastic damage. For instance, oxidative modification to protein often preferentially affects a small number of conserved targets (reviewed in: (15)). One of the predictions made based on the original FRTA is that antioxidants should retard organismal ageing. As discussed above, it appears that ROS might indeed be causative for at least some of the initial mtDNA mutation events. However, there is currently very little evidence for the massive and random increase in oxidative damage originally proposed by the Vicious Cycle theory. Furthermore, many age-dependent diseases are themselves associated with increased oxidative damage (15,35). It can therefore be challenging to differentiate cause and consequence in terms of ROS-mediated macromolecular damage and ageing (7). This has important implications for any intervention strategy based on modulating mitochondrial ROS.

The MCAT-and CR mice (64,122,123) provide some evidence that ameliorating ROS-mediated damage, specifically to mitochondria, may protect mtDNA and extend lifespan. However, according to the evidence summarized above, ROS-mediated *de novo* mtDNA mutagenesis is probably a relatively rare event. The clonal expansion mechanism implies that strategies aimed at modulating or scavenging mitochondrial ROS can only be expected to affect *de novo* mutagenesis but are unlikely to be effective once mtDNA mutations have occurred. Such strategies would therefore require life-long interventions and would become less and less effective later in life. Modulating mtDNA repair pathways is a little explored avenue to affecting mtDNA integrity and may deserve more attention. Most traditional strategies discussed below have aimed at modulating mitochondrial ROS or OXPHOS capacity.

4.1. Mitochondrial nutrients

It is crucial to maintain mitochondria in a non-deteriorating equilibrium during ageing. The integrity of mitochondria depends on a plethora of biomolecules, some of which, in addition to their endogenous biosynthesis, can be directly supplied in food or dietary supplements. Based on their importance for mitochondrial bioenergetics, the elevation of L-carnitine (LC)/acetyl-L-carnitine (ALC), coenzyme Q10 (CoQ10) and lipoic acid (LA) levels via dietary or pharmaceutical interventions has been suggested as a promising healthy-/anti-ageing strategy.

Prior to discussing the efficacy of these so-called mitochondrial nutrients (mt-nutrients) in affecting ageing and age-dependent diseases, we concisely summarize 1) some background information on LA, ALC, CoQ10 and LA, 2) their possible modes of action and 3) evidence for their potential health-benefits that can be derived from human intervention studies.

4.1.1. L-carnitine / acetyl-L-carnitine

4.1.1.1. Background

The quaternary ammonium compound L (-)-carnitine (LC) is ubiquitous in nature. Humans synthesize LC from its precursors, the essential amino acids lysine and methionine, in the kidney, brain, and particularly the liver at an approximate daily rate of 1.2 μmol/kg body weight. Most of LC, however, is found in the human skeletal muscles. LC biosynthesis is closely associated with the outer mitochondrial membrane (177, 178).

LC acts as an essential factor for the β-oxidation of long-chain fatty acids in the mitochondria (Figure 2). Consequently, in states of LC deficiency, the main energy source of the body, i.e. fat, cannot be utilized for the generation of ATP (177). LC is present in almost any food. In contrast to plant foods, however, foods of animal origin contain much more LC, which exists either in free or acetylated form, i.e. bound to fatty acids. ALC accounts for about 5-30% of total LC in food (177,179). Although little is known about the LC requirements (if any) during the life cycle, several studies indicate an age-dependent decline in

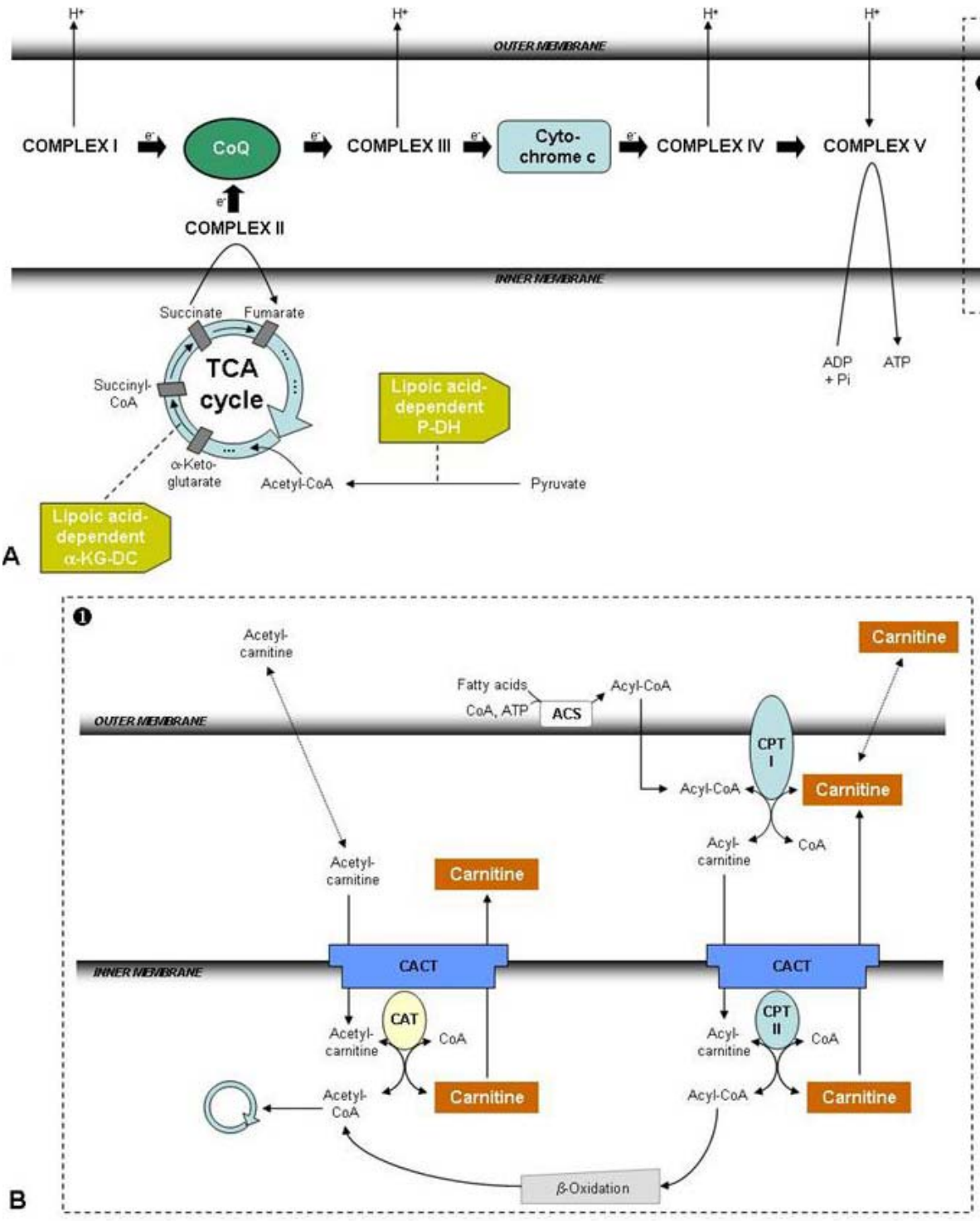

Figure 2. Location and key function of mitochondrial nutrients (for details see text). (A) Coenzyme Q10 (CoQ) transfers electrons from complexes I and II to complex III. Lipoic acid (LA) in the form of lipoamide acts as a cofactor of the mitochondrial enzymes alpha-ketoglutarate decarboxylase (α-KG-DC) and pyruvate dehydrogenase (P-DH). LA is also required for catalyzing the turnover of branched chain alpha-keto acids (not shown). (B) Carnitine facilitates the transfer of long chain fatty acids across the outer and inner mitochondrial membrane (ACS = acylcoenzym A synthetase; CACAT = carnitine carnitine acylcarnitine translocase; CAT = carnitine aceyltransferase; CPT = carnitine palmitoyl transferase). Diagram adapted from (23,177,241).

the LC levels in various tissues, not only in animals but also in humans (179.180).

4.1.1.2. Possible mode of action

4.1.1.2.1. Effects on mitochondrial bioenergetics

As previously mentioned, a hallmark of the ageing process is the decline in mitochondrial enzyme activity. Sub-chronic supplementation of aged (>22 months old) rats with LC (300 mg/kg) prevented the age-dependent decline in activities of both individual TCA cycle enzymes as well as of complexes I and IV of the ETC in brain, thus maintaining their levels comparable to those found in brain tissue derived from young (3-4 months old) animals. Noteworthy, LC treatment did not stimulate any significant changes in young animals (181). The effects of LC supplementation on mitochondrial enzyme activities are not limited to the brain but also extend to other tissues, such as the skeletal muscle and heart (182).

A brain-region specific increase in cytochrome oxidase (complex IV) activity in both synaptic and non-synaptic mitochondria has also been reported upon administration of ALC to rats (183,184). In the same set of animals the activities of TCA cycle enzymes, particularly that of citrate synthetase, decreased in all assessed brain regions, indicating different modulatory effects of ALC in comparison to LC with respect to brain energy metabolism. Changes in the acetylation status of lysine residues have been suggested as one mode of action of ALC on mitochondrial enzyme activities (185).

Moreover, ALC i.v. administration at a dose of 500-750 mg/kg evoked significant regional increases in glucose metabolic rate in rats, whereas co-supplementation of acetate and LC did not show any effect on glucose utilization (186). As indicated above, ALC functions as a shuttle of acyl groups, including acetyl groups for acetylcholine synthesis, between the mitochondria and the cytosol. Hence, it is interesting to note that ALC enhanced glucose metabolism most markedly in the subcortical cholinergic regions. Similar effects of ALC on the energy supply have been reported by Al-Majid and co-workers (187), who noted a 50% increase in the hippocampal ATP levels of rats treated (i.p.) with 300 mg/kg ALC. The same treatment regime facilitated significant protection from ischemia-induced oxidative stress and cell damage in the hippocampus. Oral supplementation of rats with 1.5% (w/v) of ALC for 1 month led to a significant restoration of the age-dependent decline in oxygen consumption and metabolic parameters, such as gluconeogenesis, in the perfused liver model. However, the up-regulation in oxygen consumption has been suggested to be due to an increase in acyl-CoA-synthetase activity rather than an enhanced maximal oxygen capacity (188). Interestingly, under similar experimental conditions, the same group reported not only elevated skeletal muscle respiration in the aged, ALC-treated rats but also found significantly more mitochondrial protein mass (189). As ALC is rapidly hydrolyzed to LC, it is difficult to determine whether the observed effects following i.p. or i.v. ALC administration are due to ALC itself. Likewise, data on the activity of orally-administered ALC must be interpreted with caution as ALC, though absorbed intact in the jejunum, already starts to become deacetylated to LC in intestinal cells, where LC in turn is only partly re-acetylated to ALC. In summary, due to the rapid metabolism of ALC, it is difficult to draw a clear line between the effects linked to LC vs. ALC (190, 191).

4.1.1.2.2. Effects on antioxidant defense

Although LC and its derivatives (namely ALC and propionyl-carnitine, PLC) do not possess strong direct antioxidant activity (as is predictable from their chemical structures (15)), their potential to indirectly modulate antioxidant defense has been repeatedly reported in the literature. In parallel to restored activity of TCA enzymes and mitochondrial complexes, improved brain antioxidant status, as indicated by increased levels of glutathione (GSH), ascorbic acid and alpha-tocopherol, as well as reduced accumulation of lipofuscin, protein carbonyls and lipid peroxidation products (thiobarbituric acid reactive substances, TBARS) has been shown in several brain regions in rats sub-chronically treated with 300 mg/kg LC (192). Although widely used, measuring TBARS must not be considered to quantitative reflect lipid oxidative damage, simply because TBARS formation is confounded by a vast array of compounds (15). The significance of changes in ascorbate levels in animals that can synthesize their own ascorbate is also hard to decide.

The administration of 400 mg/kg of ALC to SAMP8 (senescent accelerated-prone mouse) mice three times a week up to 4 months of age significantly lowered brain hydroperoxide levels. Of note, in ALC-treated animals, the age-dependent deficit in learning and memory performance was significantly ameliorated (193). Bearing in mind the different phenotypes of SAMP strains (194), which might be due to mechanisms other than the aging process itself, the SAMP model might be a valuable tool for studying chronic, degenerative diseases.

In two studies the effect of PLC in comparison to LC on the antioxidant status and oxidative stress-related biomarkers in heart and aorta of spontaneously hypertensive vs. normotensive rats has been investigated (195,196). Spontaneously hypertensive animals generally benefited from both PLC and LC treatment. In contrast, in the aortas of healthy animals PLC supplementation evoked only negligible effects, whereas LC in particular exerted an unfavorable metabolic impact evident by the aggravation of NADPH-stimulated superoxide production and a decrease in eNOS expression. Based on the latter observation, one might speculate that the health beneficial impact of LC treatment is limited to conditions of malfunction (such as in ageing and disease), but is possibly not helpful for preventing the onset of age-related functional decline in healthy individuals.

On the other hand, LC has the potential to increase the amount of other antioxidants such as alpha-tocopherol in rat liver (197). In contrast, short-term ALC

supplementation was found to not only lower vitamin C levels in hepatocytes (but not heart tissue) of both young and aged rats but also to increase the formation of the lipid peroxidation marker MDA, measured by gas chromatography/MS (an acceptable biomarker of an end product of lipid peroxidation), in the liver of old rats (198). Again, effects on hepatic ascorbate biosynthesis must be considered in interpreting changes in ascorbate levels.

Despite the enormous interest in the application of LC, ALC and PLC for the prevention and/or amelioration of oxidative stress- and thus age-related malfunction, little is known about the actual effect of LC, ALC and PLC on the antioxidant status and oxidative stress levels in humans. In agreement with the aforementioned animal studies, elevated plasma levels of retinol and alpha-tocopherol were reported for women orally taking 680 mg/day LC for 3 weeks. Concomitantly, the plasma load of "lipid peroxides" was significantly reduced (199), although the significance of the latter observation is unclear as the error-prone TBARS method was used. When measuring 8OHdG levels by HPLC with electrochemical detection (HPLC-ECD) in rat brain, Haripriya *et al.* detected significantly reduced mtDNA damage in aged animals supplemented with LC (200). HPLC-ECD is currently considered the best method for the quantification of 8OHdG, although major uncertainties still exist regarding the exact 8OHdG background levels (47).

4.1.1.3. Human intervention studies in ageing and age-related diseases

LC and its derivatives are useful in the treatment of primary carnitine deficiencies. The general outcome of these studies has been comprehensively summarized elsewhere (201,202).

As carnitine levels tend to drop with age and may *possibly* be insufficient to meet increasing demand as a function of the ageing process, the potential and efficiency of LC, ALC and PLC to affect the pathology of age-dependent diseases have received considerable attention (203). PLC, which is highly specific for the skeletal and cardiac muscle, has been used for the treatment of cardiovascular disease (CVD) (204). Initial small-scale studies have indicated a significant effect of PLC intake on exercise capacity in CVD patients (204). A multicenter study further supports the improvement of exercise duration following PLC treatment in chronic heart failure patients under stable drug therapy. Noteworthy, patients with relatively preserved myocardial function, and hence only moderate deconditioning, benefited most from the PLC intervention (205). When assessing in centenarians the impact of oral LC treatment (2g daily; 6 months) on physical and mental fatigue as well as on cognitive function in a placebo-controlled, randomized, double-blind study, Malagnarnera *et al.* found a significant improvement in all three parameters **(**206)**.** However, based on the assumption that ALC enters the human body and the observation that ALC is transported faster than LC across the blood-brain-barrier in a saturable, sodium-dependent process, ALC has been the carnitine derivative of choice for studying possible ALC-induced cognitive benefits in aged humans (185,207). A trend for less deterioration with ALC treatment was found, especially in patients who were less cognitively impaired at baseline (185). However, due to the small number of patients involved in these studies and the rather moderate effects of ALC on cognitive status, further studies are needed in order to determine the efficacy of ALC in dementia.

4.1.2. Coenzyme Q10

4.1.2.1. Background

Coenzyme Q (also known as ubiquinone) is composed of a 2,3-dimethoxy-5-methylbenzoquinone nucleus and a side chain consisting of isoprene units (208). Coenzyme Q10 (CoQ10) is the most abundant ubiquinone derivative found in human tissues and body fluids where it exists in different forms based on the redox states of its quinone head: (208-210):

1. fully oxidized form: ubiquinone (Q)
2. partly reduced, free radical form: ubisemiquinone (.QH)
3. fully reduced form: ubiquinol (QH2)

The potential health beneficial effects of CoQ10 are mainly linked to its key role in mitochondrial bioenergetics (Figure 2) and possibly to its direct and indirect antioxidant activity (208,211), although other functions of CoQ10, e.g. in the modulation of gene expression, have also been proposed (212). The maintenance of mitochondrial function and removal of ROS have both been suggested to account for CoQ10's putative preventive and therapeutic effects in cardiovascular, neurodegenerative and mitochondrial diseases (202,209,213). As plasma CoQ10 levels are normally not significantly affected by common foods, supplementing with purified CoQ10 is needed to increase the CoQ10 concentration in the blood (209).

The $CoQ10H_2$/CoQ10 ratio, although susceptible to pre-analytical variation (such as time period since blood sampling), has been proposed as a possible biomarker for *in vivo* oxidative stress (214,215). When analyzing plasma samples of healthy men, Wada *et al.* found a small though significant increase in oxidized CoQ10 in older subjects (aged 20-39: 3.1±0.9%; 40-59: 3.6±1.2%; >60: 4.7±1.6%) (216) while $CoQ10H_2$ levels were age-independent. Whereas the authors of the latter study concluded an association between the CoQ10 redox status and chronological age, it remains to be elucidated whether these small changes in CoQ10 plasma levels are biologically relevant in ageing and the onset of chronic, degenerative diseases.

4.1.2.2. Possible mode of action

4.1.2.2.1. Effects on mitochondrial bioenergetics

The transport of electrons across the inner mitochondrial membrane forms the basis of mitochondrial respiration and ATP generation. CoQ10 plays a fundamental role in this cellular process by carrying electrons from complexes I and II to complex III (217).

The heart is particularly rich in CoQ10 due to its high ATP demand. Myocardial dysfunction is commonly found in ageing subjects and is often aggravated during interventions that induce aerobic or ischemic stress (218). Daily i.p. treatment of old rats (35 months) for 6 weeks (4 mg/kg of CoQ10) significantly improved heart oxygen consumption and recovery of cardiac work in comparison to untreated animals (218). Similarly, the tissue samples of patients (>70 years) receiving 300 mg CoQ10 per day (7 days) prior to cardiac surgery showed more efficient mitochondrial respiration and greater recovery from hypoxia in comparison to those of control subjects. These effects appear to be directly linked to the increase in the CoQ10 content of the tissue and, consequently, of the mitochondria (218).

As mitochondrial dysfunction is a hallmark of neurodegeneration, the efficiency of CoQ10 treatment to restore and/or maintain brain mitochondrial bioenergetics has been repeatedly tested. However, the correct interpretation of data, like those given below, obtained in rodents supplemented with CoQ10 is complicated by the fact that in mice and rats CoQ9 is significantly more abundant than CoQ10 (209). Still, results from animal studies indicate that CoQ10 supplementation is able to attenuate ATP depletion in the brain following the administration of neurotoxins, such as malonate, 3-nitropropionic acid, MPTP or aminoxy-acetic acid (reviewed in: (210)). Nonetheless, only little is known as to whether CoQ10 treatment exerts direct effects on the brain mitochondria. In fact, it is commonly held that CoQ10 intake does not elevate endogenous CoQ10 levels, except in the plasma and liver. In contrast to this notion, elevated levels of CoQ10 have been found in the brain tissue of rats after the oral administration of CoQ10 (200 mg/kg; 2 months) and water-miscible CoQ10 (150 mg/kg; 4 and 13 weeks), respectively (219,220). In a more recent study, Sohal *et al.* (2006) reported increased CoQ10 levels in liver, heart, kidney and skeletal muscle homogenates as well as in isolated mitochondria of mice under long-term supplementation with various concentrations of CoQ10 (221). Despite the lipophilic nature of CoQ10, no signs of CoQ10 enrichment in the brain were observed in this study. Moreover, the activities of mitochondrial ETC oxidoreductases and state 3 respiration, determined at 19 or 25 months of age, were unaffected by CoQ10 administration in all tested mouse tissues and no effect on lifespan was observed (221). This is in agreement with earlier reports obtained in mice (222,223) and *Drosophila melanogaster* (*D. melanogaster*) that also failed to demonstrate any effect on mean and maximum lifespan (224). In fact, feeding CoQ10 at a concentration of 1.25 mg/ml medium to *D. melanogaster* caused not only an increased ROS production but also a reduction in lifespan (225). Considering the important pro-oxidant effects of CoQ10 by producing superoxide and H_2O_2, mainly via the CoQ10-semiquinone intermediate, ROS production in CoQ10 (over-)saturated organisms may be likely to occur (226). Likewise, studies performed with CoQ10 in *Caenorhabditis elegans (C. elegans*), generally considered to be a good model for studying oxidative stress-modulated ageing processes in post-mitotic tissues, have been conflicting (217,227).

4.1.2.2.2. Effects on antioxidant defense

Ubiquinol ($CoQH_2$), present in biological membranes and lipoproteins, is capable of inhibiting lipid peroxidation by scavenging peroxyl radicals. Furthermore, $CoQH_2$ has been implicated 1) in the interception of reactive nitrogen species, such as nitric oxide and peroxynitrite (228) and 2) in the regeneration of alpha-tocopherol by reducing alpha-tocopheroxyl radicals, an effect that has been suggested to be biologically more important than the direct scavenging of free radicals (15). Whereas strong antioxidant effects of CoQ10 have been reported in several cell culture experiments (229), a study conducted in rabbit corneal keratinocytes indicates that CoQ10 prevents cell death independently of its free radical scavenging property (230). Another potentially interesting effect of CoQ10 derives from its suggested interaction with mitochondrial uncoupling proteins (UCPs). The activation of UCPs is generally considered to lower the burden of mitochondrial ROS production (210,231).

Studies on the impact of CoQ10 on antioxidant status and defense in animals and especially humans, however, have been less conclusive. CoQ10-supplemented rats fed a diet rich in polyunsaturated fatty acids showed higher content and/or activity of CoQ10, alpha-tocopherol, and catalase in the heart. CoQ10 feeding also ameliorated the age-dependent decline in mitochondrial function and lowered hydroperoxide levels *ex vivo*, assessed by the ferrous oxidation xylenol orange assay, in heart mitochondria. These results indicate that CoQ10 can sometimes exert direct as well as indirect antioxidant effects *in vivo* (232). However, as mentioned above, Sohal *et al.* (2006) have been unable to detect any beneficial impact of the long-term CoQ10 intake on mitochondrial bioenergetics (221). In fact, despite the tissue accumulation of CoQ10 (except in the CNS), the carbonyl content, glutathione redox state of tissues, and activities of superoxide dismutase, catalase, and glutathione peroxidase were unaltered in liver, kidney, skeletal muscle, heart, and brain (221). On the other hand, significant antioxidant protection has been reported for lipoproteins isolated from both CoQ10-suplemented animals and human subjects (214,233), indicating that CoQ10 has the potential to positively affect the homeostasis of at least one suggested key trigger of atherosclerosis (i.e. oxidized lipoproteins) *in vivo*. Whether the same holds true for body tissues, especially the brain, cannot be concluded from currently available data.

4.1.2.3. Human intervention studies in ageing and age-related diseases

Myocardial tissues and plasma of CVD patients have been reported to be lower in CoQ10 than in healthy adults. Since the 1960s, the effect of CoQ10 on cardiovascular health has been tested in cardiomyopathy and congestive heart failure patients in more than 20 studies. Although no CVD-beneficial effect of CoQ10 treatment has been found in several small-scale intervention studies, a recent meta-analysis concludes that

CoQ10 is able to enhance the systolic function in chronic heart failure, particularly in less diseased hearts, possibly due to a greater number of salvageable myocytes (234). For this function, however, it seems to be necessary to not only correct CoQ10 plasma levels to normal ranges but to achieve CoQ10 plasma concentrations above the normal range, presumably in order to facilitate maximal tissue uptake (235). Less convincing evidence, however, exists regarding the effect of CoQ10 in ischemic heart disease (236).

In the case of neurodegeneration, there is evidence from animal studies that suggest CoQ10 may act as a neuroprotectant (237,238). Although CoQ10 deficiency was shown in humans suffering from neurodegenerative diseases, oral CoQ10 treatment in Parkinson's disease and Huntington's disease patients caused only a very modest decrease in neurological symptoms. Patients with Friedreich's ataxia benefited from CoQ10 treatment (in combination with vitamin E) by improved cardiac and skeletal muscle bioenergetics, whereas at the same time no favorable impact on neurological parameters was observed (239). Currently, no data from clinical trials exist that would support the use of CoQ10 for the prevention or treatment of AD (240).

4.1.3. Alpha-lipoic acid

4.1.3.1. Background

Like LC and CoQ10, lipoic acid (LA) is a naturally occurring substance, which is most often covalently bound to the ε-amino group of lysine residues. LA is commonly found in foods of plant (e.g. spinach) and animal (e.g. heart) origin and it has been suggested that LA is synthesized in the mitochondria from octanoic acid and a sulphur source (241-243).

The oxidative decarboxylation of pyruvate, alpha-ketoglutarate and branched-chain alpha-keto acids takes place within the mitochondria and crucially depends on LA (in the form of lipoamide) as an enzymatic cofactor (Figure 2). While transferring acyl groups from the enzyme complex to another biomolecule, LA becomes reduced to dihydrolipoic acid (DHLA), which, in the presence of lipoamide dehydrogenase, can be reoxidized to LA with the formation of NADH (243).

Free LA has been suggested to be the most important therapeutic form of LA. Whereas normal food intake is only associated with marginal levels of free LA, oral intake of purified LA has been shown to lead to a significant but only short-term rise in free LA plasma concentration (243). Aside from participating in the regulation of mitochondrial energy metabolism by carrying electrons to NAD^+, the redox couple LA/DHLA in its free, non-protein-bound form exerts significant impact on the cellular oxidative stress balance *in vitro*. The question remains, to what extent free LA/DHLA act as direct antioxidants *in vivo* or whether its putative efficacy originates from maintaining and regenerating the levels of other ROS-scavengers, such as glutathione and vitamin C (242,244).

4.1.3.2. Possible mode of action

4.1.3.2.1. Effects on mitochondrial bioenergetics

The effects of LA administration (i.v.) on the mitochondrial enzyme activity of young and aged rats have been assessed in several studies. At a daily dose of 100 mg/kg for 14 days, aged animals treated with LA revealed significantly lower (sometimes no) decline in the activity of various TCA enzymes as well as of complexes I and IV in liver and kidney in comparison to control animals (245). The same LA dose applied for 30 days led to significantly higher activities of TCA enzymes and complexes I, II, III and IV of aged rat brain mitochondria (246). Similar to previously discussed mt-nutrients, LA exerted only marginal beneficial effects in young animals.

Aside from alpha-ketoglutarate dehydrogenase, which directly depends on LA as a cofactor, the action of various other mitochondrial enzymes improved in the LA-treated animals. This indicates that LA might indeed exert other cellular functions, e.g. by affecting the antioxidant status, leading to improved mitochondrial bioenergetics.

In contrast to rats, post-mortem brain samples obtained from aged subjects as well as AD and vascular dementia (VD) patients revealed no decrease in SDH activity, suggesting species specific differences in the age-dependent alteration of mitochondrial bioenergetics (247). However, the LA-dependent pyruvate-dehydrogenase (PDH) complex activity is decreased in the AD and VD brain. Surprisingly, PDH activity could only be stimulated with LA in VD but not in AD post-mortem brain samples. Furthermore, the observation that the SDH activity did not differ under either basal or stimulated conditions between the controls, AD, and VD samples, suggests that 1) that loss of mitochondria is unlikely to explain the reduced PDH activity and 2) selective vulnerability of PDH (and possibly other mitochondrial enzymes) may be due to the direct or indirect, disease-dependent structural and/or functional enzymatic impairment (248).

As mitochondrial biogenesis heavily depends on the regulatory action of PGC-1-alpha (peroxisome proliferators-activated receptor gamma co-activator-1 alpha), the observation that LA (like, e.g., resveratrol) is able to stimulate PGC-1-alpha signaling and hence increase mtDNA and protein content, offers an exciting new approach for better exploring the possible effects of LA in the prevention of mitochondrial dysfunction (249).

4.1.3.2.2. Effects on antioxidant defense

LA and DHLA have been described as the most versatile antioxidant pair, based on the fact that either both or at least one of the two compounds can react with all important free radicals and other ROS (241,250,251). Moreover, LA and DHLA might drive the recycling of other exogenous and endogenous antioxidants, such as vitamins C and E, CoQ10, and GSH (241,252).

Evidence that LA exerts antioxidant activity *in vivo* is mainly derived from the quantification of oxidative stress-related biomarkers. Plasma and organ samples obtained from animals supplemented with LA showed

enhanced GSH (253,254) and vitamin C and E levels (255-258), reduced production of ROS (255), lowered burden of lipid (257,259) and of nuclear DNA oxidation (255) as well as reduced mtDNA damage (246). Methods applied in the aforementioned publications include HPLC with electrochemical detection for measuring DNA damage (8-oxo-dG), the use of the fluorescent dye DCF for assessing ROS production as well as the application of the ferrous oxidation with xylenol orange assay for measuring lipid hydroperoxide levels. Although several pitfalls might affect the outcome of all these methods (15), the data are suggestive overall of antioxidant effects.
On the other hand, prooxidant activity can occur *in vitro*, mainly for DHLA (250). Little is known about the potentially deleterious effects of LA/DHLA in animals and humans *in vivo*. Whereas sub-chronic LA treatment of aged rats caused an *increase* in protein oxidation and nitration in brain (but not muscle) (259), the lifespan-extending effect of LA in *D. melanogaster (*260) and *C. elegans (*261) point to favorable metabolic alterations of LA in association with the ageing process.

4.1.3.3. Human intervention studies in ageing and age-related diseases

In the 1960s, LA was used for the first time as a therapeutic agent in patients with liver cirrhosis, intoxication symptoms and especially diabetic polyneuropathy (243). The use of LA in diabetics for the prevention of polyneuropathic complications and oxidative stress-associated damage is supported by limited clinical data (262-264). The antioxidant effects of LA even persisted in diabetic patients with albuminuria and poor glycemic control (265). In contrast, the efficacy of LA for the prevention and/or symptomatic treatment of chronic, age-dependent CVD and dementia still need to be established. A Cochrane review published in 2007 concludes that LA cannot be recommended for patients with cognitive impairment due to the unavailability of any scientifically sufficient double-blind, randomized, placebo-controlled trial (266).

Much is known about the versatile impact of LA/DHLA *in vitro* and on animal models of CVD (267). Still, as LA supplementation has only been evaluated in a limited number of small-scale human trials, at present no conclusions regarding efficacy of LA interventions for the amelioration of CVD-linked symptoms and pathological mechanisms can be drawn (267).

4.2. Combined vs. single supplementation with mitochondrial nutrients

As LC/ALC, CoQ10 and LA all participate in the control of mitochondrial bioenergetics and antioxidant defense, it has been suggested that the combination of two or more mt-nutrients might exert superior efficacy in comparison to single compound treatment. Unfortunately, only a few authors have attempted to discriminate between the effects of single vs. combined supplementation with mt-nutrients in terms of oxidative stress-related biomarkers and, e.g., cognitive performance, such as learning paradigms. Hagen *et al.* (198) demonstrated in rats that the concomitant administration of ALC and LA negated both the age-related decline in liver vitamin C levels and the increase in lipid peroxidation (assayed by using a gas chromatography-MS method for pentofluorophenyl-derivatized MDA), indicating that these two nutrients might act synergistically (198). When further exploring the impact of ALC, LA and ALC+LA on age-related changes in rats, the same group found in some but not all brain oxidative stress- and cognition-related parameters a superior activity for ALC+LA supplementation in contrast to either ALC or LA treatment alone (172, 268). However, as ALC treatment alone has the potential to evoke oxidative stress in the form of lipid peroxidation and to reduce the levels of the antioxidant vitamin C in both young and old rats (see above (198)), it is difficult to estimate whether ALC+LC treatment indeed exerts superior effects due to synergistic or additive action or whether some of LA's efficacy is consumed in order to reverse the negative metabolic outcome of single ALC supplementation.

Similarly, careful re-evaluation of 2 sets of data (Figure 3) recently presented by another group challenges the notion that the co-administration of LA and, in this case, LC generates superior effects in comparison to single mt-nutrient intake (182,269). LC treatment ameliorated the age-related decline in heart mitochondrial enzyme activity (Figure 3C) in both studies and the authors argue that supplementing old rats for 30 days with LC and LA causes a further increase in mitochondrial enzyme activity that is significantly different from the effects evoked by single LC intake. However, the effects exerted by LC+LA treatment in study II appear not to be at all different from those seen in study I, which only assessed the effects of LC treatment (21 days). Based on the information provided, animal age, route of drug application as well as dosage of LC and LA were the same in both studies, thus minimizing the effect that differences other than the marginal difference in study duration might be responsible for the data inconsistency. However, the oral supplementation of LC in combination with N-acetyl cysteine and S-adenosylmethionine improved cognitive performance and reduced aggression in adult, human ApoE4-expressing mice. In this case, WT mice also benefited from the treatment in terms of enhanced cognitive function (270). Taken together, there is currently only limited evidence that the co-treatment mt-nutrients might exert additive or even synergistic health beneficial effects *in vivo* and the variation in results between different laboratories is a cause for concern.

4.3. Antioxidants – some general comments

For antioxidant therapy to provide significant protection versus mtDNA oxidative damage, the antioxidants utilized would have to be present in close proximity to the mtDNA in sufficiently high concentrations to scavenge ROS species with high efficiency. Even though there is some evidence for protection of mtDNA by classic antioxidants (55,60,271) and mt-nutrients (see above), any antioxidant strategy is likely to benefit from a "targeted" approach whereby the antioxidant moiety is selectively accumulated in mitochondria.

This conclusion is supported by the fact that intervention studies, aimed at retarding ageing of mammals

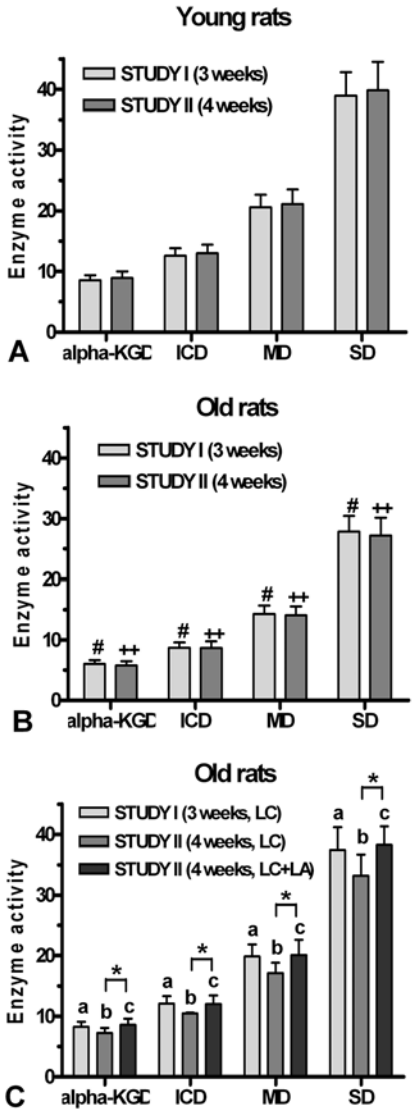

Figure 3. Effect of L-carnitine (LC) vs. LC + lipoic acid (LA) treatment on the enzymatic activity of alpha-ketoglutarate dehydrogenase (alpha-KGD), isocitrate dehydrogenase (ICD), malate dehydrogenase (MD) and succinate dehydrogenase (SD) in the hearts of young and aged rats. Data were taken from Kumaran *et al.* (182) and Savitha *et al.* (269). Study I: supplementation with LC (300 mg/kg) for 21 days; study II: supplementation with LC (300 mg/kg) for 30 days as well as co-supplementation with LC (300 mg/kg) and LA (100 mg/kg) for 30 days (# = significantly (sign.) different from young control animals (study I); ++ = sign. different from young control animals (study II); a = sign. different from old control animals (study I); b = sign. different from old control animals (study II); c = sign. different from old control animals (study II); * = sign. different from old LC-treated animals (study II)). Note the similar effects of LC (study I) vs. LC+LA (study II) supplementation as well as the presumably superior effect of single LC treatment (study I vs. study II) on enzymatic activities in aged animals.

using classical (non-targeted) antioxidant compounds, have consistently failed to show clear effects on maximum lifespan (10,15,223,272,273). However, an alternative explanation is that most of the compounds explored may not have much antioxidant effect *in vivo*, as is true of most supplementary antioxidants in humans (274,275). This is not to say that classical antioxidant treatment cannot be beneficial in the context of age-dependent diseases. Many age-dependent pathologies including neurodegenerative diseases such as Alzheimer's and Parkinson's disease are associated with increased inflammation and oxidative damage (15,35). If suitable *in vivo* antioxidants, that is, compounds that actually work as such *in vivo* and are able enter the brain, could be identified, these conditions may well benefit from antioxidant treatment

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A class of mitochondria-targeted antioxidants has been developed by conjugating a lipophilic cation to a range of antioxidant moieties (276). One such compound, mitoQ, a mitochondria-targeted derivative of ubiquinone has been shown to accumulate within mitochondria several hundred-fold (276,277). Other strategies for delivering compounds specifically to mitochondria have also been described (278). However, it should be noted that "antioxidants" can sometimes be potential pro-oxidants, the difference being one of environment (15). This is a potential problem, especially in the context of the mitochondrion, because of the close proximity of multiple electron donors and acceptors. In this context, it is interesting to note that in some systems mitoQ has been shown to undergo redox-cycling, acting as a pro-oxidant, actually increasing superoxide production and cell death (279,280).

Finally, the fact that ROS are important signaling molecules, amongst other things affecting cell growth and proliferation, complicates the picture further because indiscriminately reducing ROS to unphysiologically low levels raises (at least theoretically) the possibility of unintended side effects e.g. decreased cell proliferation (7,158).

Despite these challenges, the ability to target antioxidants, probes and ETC modulators specifically to mitochondria opens up new possibilities in manipulating mitochondrial function and ROS production *in vivo*, making this an exciting area of development.

5. SUMMARY AND PERSPECTIVE

Given the evidence that mitochondrial degeneration and loss of mtDNA integrity are involved in age-dependent degenerative processes, mitochondria make an attractive target for attempts to prevent or delay age-dependent pathologies and maybe even modulate basal ageing rates.

Most such approaches, both theoretical and experimental, to date have been based on the assumption that reducing or scavenging mitochondrial ROS will protect mitochondrial function and mtDNA integrity. As discussed,

attempts to achieve this in praxis have involved mt-nutrients as well as classical and some targeted antioxidants. Several *in vitro* studies have shown that mt-nutrients and targeted antioxidants are able to prevent and even rescue cells from the deleterious impact of mitochondria-impairing agents.

Data obtained in cell culture experiments, however, are prone to misinterpretation (80, 281) as culturing animal and human cells is a highly complex process readily paving the way for the generation of artifacts. For example, too low or too high cell plating efficiency has profound effects on the generation of a healthy population of the same cells and thus on the experimental outcome. Furthermore, the usage of different cell-culture media can lead to irrelevant positive or negative results based on the interaction of the culture-medium with the test substance, which, among others, might cause the unexpected production of oxidative stress (80, 282). The occurrence of artifacts is further aggravated by the use of supra-physiological concentrations of the compound of interest (283). Furthermore, the fact that cells are routinely cultured under severely hyperoxic conditions might cause a disproportionate production of ROS (80).

In vivo data show that animals treated with mt-nutrients often display enhanced mitochondrial bioenergetics and improvements in parameters of age-dependent functional decline. In contrast to this proven efficacy to maintain and partially rescue mitochondrial energy production, there is as yet little evidence that the available mt-nutrients or antioxidants can prevent mtDNA damage and lower mutation burden *in vivo* (200,246). Further work with mt-nutrients as well as with novel targeted antioxidants will show if any of these compounds is indeed an effective mitochondrial antioxidant, that is, if any of them significantly reduce oxidative damage to mitochondria and particularly mtDNA *in vivo*. A more fundamental question is whether lifelong reduction of oxidative mtDNA damage can indeed reproduce the phenotype observed in the MCAT-mice, that is, if it translates into significantly reduced *in vivo* mtDNA mutation burden and extended lifespan. Whether or not it does, such work will provide important further insights into the mechanisms underlying age-dependent mitochondrial and organismal deterioration. Aside from anecdotal evidence (284) there is currently no convincing data justifying the routine supplementation of humans with mt-nutrients for the prevention of age-related diseases, let alone to delay the ageing process itself.

An alternative approach for modulating mitochondrial ROS production is the manipulation of mitochondrial membrane potential, for instance through mild activation of uncoupling (285,286). A moderate reduction in the mitochondrial membrane potential leads to significantly reduced leakage of ROS that might translate into lower burden of mtDNA damage. Ultimately, however, any approach aimed at reducing ROS burden by scavenging of ROS or "metabolic tuning" will be complicated by the complexity of the evolved feedback systems, illustrated for instance by the dual role of ROS in signaling. Any such attempt to change a limited number of desired parameters without causing undesired side- or compensatory effect is intrinsically challenging in the context of a complex and highly interconnected network. Furthermore, prevention of ROS-mediated damage will always be imperfect and the ROS-independent clonal expansion mechanism may eventually amplify *any* mtDNA damage to physiologically relevant levels.

Targeting damage directly instead of attempting to modulate its accumulation rate has been suggested as one solution, as this approach does not evoke possibly detrimental effects due to unforeseen cellular responses likely to occur during metabolic tuning (287). Another class of strategies aimed at ameliorating lifelong accumulation of oxidative damage is based on enhancing endogenous antioxidant or detoxification systems or by activating turnover and repair pathways. Such activation of endogenous defense capacities has for instance been shown to occur in response to hormesis (reviewed in: (288)) as well as in connection with genetic interventions known to modulate ageing (289–290). Pharmacological strategies may exploit this endogenous capacity by either directly interfering with signaling along these pathways or by mimicking stressors without causing actual damage. Future approaches will likely be based on our emerging understanding of the mechanism underlying CR, clonal expansion of mtDNA mutations, mtDNA repair, mitogenesis and mitochondrial turnover.

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