CHAPTER SIX

Mitochondria and Reactive Oxygen Species in Aging and Age-Related Diseases

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Contents

Abstract

Aging has been linked to several degenerative processes that, through the accumulation of molecular and cellular damage, can progressively lead to cell dysfunction and organ failure. Human aging is linked with a higher risk for individuals to develop cancer, neurodegenerative, cardiovascular, and metabolic disorders. The understanding of the molecular basis of aging and associated diseases has been one major challenge of scientific research over the last decades. Mitochondria, the center of oxidative metabolism and principal site of reactive oxygen species (ROS) production, are crucial both in health and in pathogenesis of many diseases. Redox signaling is important for the modulation

of cell functions and several studies indicate a dual role for ROS in cell physiology. In fact, high concentrations of ROS are pathogenic and can cause severe damage to cell and organelle membranes, DNA, and proteins. On the other hand, moderate amounts of ROS are essential for the maintenance of several biological processes, including gene expression. In this review, we provide an update regarding the key roles of ROS—mitochondria cross talk in different fundamental physiological or pathological situations accompanying aging and highlighting that mitochondrial ROS may be a decisive target in clinical practice.

1. REACTIVE OXYGEN SPECIES AND AGING

Proposed in 1954, by Denham Harman, the free radical theory of aging (FRTA) was the first attempt to link aging and oxidative stress (Harman, 1956). Later on, in 1972, this theory was revised, and the same author developed the mitochondrial free radical theory of aging (MFRTA) (Harman, 1972; Schriner et al., 2005), which states that mitochondrial dysfunction and consequent increased reactive oxygen species (ROS) production result in a vicious cycle contributing to cellular damage and consequent cell death. Although his theory was initially received by his peers with indifference and sometimes rebuttal, we know nowadays that ROS are important during the aging process. In fact, these highly reactive oxygen-derived molecules produced during aerobic metabolism can interact with cellular components, causing cumulative oxidative damage along time that may thus plausibly reduce life span (Harman, 1956). Oxidative damage to DNA genomes, proteins, and lipids has been associated with elevated ROS production, mitochondrial function impairment, and ultimately cell senescence or death (Bokov et al., 2004; Sohal, Weindruch, 1996). Of particular importance, the close proximity between ROS production sites and mitochondrial DNA (mtDNA) can favor the accumulation of oxidative stress—associated DNA damages. Elevated ROS production has been correlated with mitochondrial oxidative damage, along with a reduction of mitochondrial copy number (Cocheme et al., 2011; Herbener, 1976; Lambert et al., 2007; Yen et al., 1989). These alterations are associated with an increased mutation rate of mtDNA in the brain, liver, and muscle fibers of aged individuals (Cahill et al., 2005; Corral-Debrinski et al., 1992; Fayet et al., 2002; Rahaet al., 2000; Yen et al., 1991). Interestingly, the establishment of the mutator mouse model allowed the demonstration of a direct correlation between an increased number of mtDNA mutations and a decreased mitochondrial respiratory chain activity (Trifunovic et al., 2004). For

instance, these alterations were accompanied by the development of typical symptoms of aging in humans, namely hair loss, weight and fat reduction, decreased bone density, and cardiomyopathy (Trifunovic et al., 2004).

Aging has been also associated with a decline of antioxidant defense efficiency, which together with increased ROS production significantly contributes to a manifestation of an oxidative stress state. This in turn can initially disturb enzyme activity through reversible oxidation of thiol groups, but which ultimately can lead to a more profound alteration in biomolecule structure and integrity (Freitas et al., 2016). Consistent with this, overexpression of antioxidant enzymes decreases ROS production and protects DNA from harmful ROS effects, which is associated with a prolonged life span in *Drosophila melanogaster* (Orr, Sohal, 1994; Schriner et al., 2005). Moreover, it has been found that long-lived mice strains possess higher level of antioxidant enzymes and have reduced oxidative damage of proteins and lipids (Pamplona et al., 2002; Rebrin, Sohal, 2004). Interestingly, the reduced oxidative damage in long-lived species could be explained by an adaptive mechanism of cysteine depletion in mitochondria (Moosmann, Behl, 2008).

Despite the numerous studies supporting Harman's ROS theory of aging, other discoveries are questioning a direct correlation between oxidative stress damages and the life span. Using *Caenorhabditis elegans* as a model, mitochondrial mutations had no effect on overall ROS despite an increase of mitochondrial superoxide level (Yanget al., 2010). Surprisingly, the above-mentioned study reported a positive correlation between mitochondrial oxidative stress and the extension of life span (antioxidants supplementation shortened life span of mutants). Similarly, a number of recent works using mice models have also questioned the validity of ROS as the cause of an aged phenotype. Lapointe and Hekimi showed that a reduced level of mitochondrial enzyme MCLK1 causes mitochondrial dysfunction manifested as a reduction of electrons transport through mitochondrial respiratory chain and decrease of tricarboxylic acid (TCA) cycle activity. All these events are accompanied by increased mitochondrial oxidative stress but decreased oxidative damage to cytosolic proteins and reduced level of isoprostanes in plasma (systemic biomarker of aging and oxidative stress) (Lapointe, Hekimi, 2008). Additionally, the silencing of antioxidant enzymes, such as mitochondrial SOD2 (manganese superoxide dismutase; also called MnSOD) and GPx-1 (glutathione peroxidase-1), did not affect longevity in spite of increased oxidative stress (Perez et al., 2009a; Zhang et al., 2009). It thus seems that there is not sufficient evidence to undermine

credibility of the FRTA; however, the contradictory studies have been rather supporting for a new recent theory named mitohormesis. According to this theory, moderate levels of mitochondrial ROS could activate compensatory mechanisms that protect cellular organelles from the deleterious effects of ROS and ultimately, delaying the appearance of an aging phenotype (Ristow, Zarse, 2010). For instance, moderately increased levels of ROS have been linked to an extension of longevity in *D. melanogaster* and in young mice (Copeland et al., 2009) (Basisty et al., 2016; Csiszar et al., 2008). The discovery that the reduction of elevated mitochondrial ROS levels protects against age-related decline in old mice (Basisty et al., 2016) implies that a decrease of ROS levels could be a determinant factor to delay progression of diseases parallel to the extension of life span in mammals in more advanced ages (Schriner et al., 2005). For example, administration of an antioxidant N-acetylcysteine (NAC) has been shown to prevent the loss of activity (observed during aging) of complexes I and IV (Miquel et al., 1995). Moreover, supplementation with antioxidant compounds selegiline and vitamin E (Vit-E) alone or in combined therapy showed to delay Alzheimer disease (AD) progression in human subjects (Sano et al., 1997). In conflict with the mentioned studies, some evidence reported that antioxidant therapies may not be universally beneficial in the prevention of age-related diseases. While Vit-E did not show to protect or delay Parkinson's disease (PD) progression (Parkinson Study, 1993), this antioxidant compound was even deleterious in AD patients (Lloret et al., 2009). The finding that not all patients respond similarly to the antioxidant therapy is consistent with the requisite of moderate level of ROS to induce stress resistance adaptation. As opposed to the controversial effects of dietary antioxidant compounds, caloric restriction (CR) is a promising therapeutic strategy able to retard or prevent aging in several species ranging from worms to humans (Hekimi, Guarente, 2003; Sohal, Weindruch, 1996). The mechanism underlying these effects is not completely understood. Although, evidence supported the role of ROS as inducers of mitochondrial oxidative stress adaptations, including a marked increase in mitochondrial function through peroxisome proliferator—activated receptor gamma coactivator 1-alpha (PGC-1α) and mitochondrial NAD—dependent deacetylase sirtuin-1 (SIRT1) activation (Nisoli et al., 2005). Likewise, endurance training may cause increased levels of ROS, which induce cellular signaling pathways associated to the function and turnover of mitochondria, hence contributing to the extension of life span (Lanza et al., 2008; Ristow, Zarse, 2010).

1.1 Intracellular Sources of Reactive Oxygen Species

Uncontrolled ROS production may lead to the oxidation of fundamental cellular components, such as proteins, phospholipids, and nucleic acids. Ultimately, ROS (hydroxyl radical, •OH; superoxide anion, $O_2^{\bullet-}$; hydrogen peroxide, H_2O_2; alkoxyl and peroxyl radicals, as well as singlet oxygen) can not only modify enzyme activity but also result in profound alterations in biomolecular structure. Cellular components can be also modified by products of free radical reaction intermediates such as peroxynitrite (formed by the reaction of nitric oxide with superoxide anion) or lipid hydroperoxides (prominent nonradical intermediates of lipid peroxidation produced by the reaction of a hydroxyl radical with unsaturated fatty acids). Although ROS are generally seen as harmful agents that need to be removed by detoxification mechanisms, the truth is that some of these species, most notably H_2O_2, play a physiological role in cell homeostasis, functioning as signaling molecules. This is still a controversial concept because the specificity of ROS action is unclear due to the high reactivity of some of the species toward many macromolecules, the covalent nature of modifications they bring, and the limited (in some cases) spatial effects of some of the species. Nevertheless, it is accepted that some ROS regulate their own demise through upregulation of ROS detoxification enzymes (D'Autreaux, Toledano, 2007). Under physiological conditions, ROS can act as mediators and regulators of cell metabolism, by interfering with the transmission of signals to and throughout the cell. Specific ROS such as H_2O_2 and $O_2^{\bullet-}$ are important second messengers in growth, differentiation, and cell death, activating proteins involved in cell division (mitogenic-activated protein) and participating in the immune response of the organism. By affecting the synthesis, release, or inactivation of the endothelium-derived relaxing factor (EDRF), ROS may cause the relaxation or contraction of the vascular wall. In addition, ROS can increase the permeability of the capillary walls and stimulate transport of glucose into cells and of serotonin into platelets (Droge, 2002). Furthermore, H_2O_2 regulates the expression of many genes, including AP-1, CREB, HSF1, NRF2, HIF-1, TP53, NF-κB, NOTCH, SP1, or SCREB-1 (Marinho et al., 2014; Sies, 2017). Finally, it has been demonstrated that peroxides may regulate the synthesis of prostanoids (Korbecki et al., 2013).

Mitochondria are considered one of the important sources of ROS and these, when produced extensively during pathological conditions, can evoke intracellular oxidative stress, leading to the aforementioned damage. ROS

overproduction in cells may cause disruption of tissue and organ function, leading to different pathologies or even premature death of the organism. Not surprisingly, mitochondria are both producers and targets of ROS. So far, several distinct sites of ROS production in mammalian mitochondria have been identified. The two sites that have been most extensively studied are complexes I and III of the mitochondrial respiratory chain, with the focus on the mechanistic role of the ubiquinone cycle in promoting univalent oxygen reduction (Brand, 2010; St-Pierre et al., 2002). Traditionally, complex II was not considered a source of ROS per se, instead it was described to contribute to their formation via its substrate, succinate. In many tissues, succinate plays a role in reverse electron transfer, the process in which electrons are transferred from succinate to ubiquinone via complex II and then back to complex I (Liu et al., 2002; Yankovskaya et al., 2003). Despite this, it has been suggested that complex II alterations with tissue aging would be responsible for $O_2^{\bullet-}$ production (Ishii et al., 2011). The hypothesis concerning the involvement of complex II in ROS production is discussed later in the context of diabetes (Nishikawa et al., 2000) and skin aging (Anderson et al., 2014). Moreover, it was suggested that mutation in complex II might also result in $O_2^{\bullet-}$ overproduction (Ishii et al., 2005). Additionally, Paddenberg et al. investigated the role of mitochondrial complex II in ROS production, showing that complex II plays an essential role during hypoxia. At reduced oxygen tension, catalytic activity of complex II switches from succinate dehydrogenase to fumarate reductase, with this alteration being associated with increased ROS production (Paddenberg et al., 2003a, 2003b; Yankovskaya et al., 2003). Reports indicate that the magnitude of the transmembrane electric potential regulates ROS generation by the respiratory chain (Korshunov et al., 1998), which has been shown to depend on the AMP-activated protein kinase (AMPK) activity (Weisova et al., 2012), whereas others presented evidence against this relationship between mitochondrial polarization and ROS production (Shabalina, Nedergaard, 2011).

It is important to note that mitochondria are not the only ROS-producing organelles in the cell. Microsomal enzymes, including the cytochrome P450 system (Bhattacharyya et al., 2014), peroxisomal enzymes, xanthine oxidase, polyamine oxidase, sarcosine oxidase, and different types of acyl-CoA oxidases (Bonekamp et al., 2009), as well as some plasma membrane enzymes (NADPH oxidase and lipoxygenase) (Bedard, Krause, 2007; Shintoku et al., 2017), have been identified as nonmitochondrial ROS generators. Despite the fact that Brown and Borutaite presented a number

of examples supporting the hypothesis that mitochondria are not the primary source of ROS (Brown; Borutaite, 2011), oxidative phosphorylation accounts for 90%–95% of cellular oxygen consumption. Although it is difficult to make an exact assessment because of frequent artifacts with the use of fluorescence-based redox-sensitive dyes, it is now considered that the initial idea that 1%–4% of oxygen consumption is converted into $O_2^{\bullet-}$ is wrong, as most of the original works were performed with mitochondrial inhibitors (Chance et al., 1979). More recent work brought down the value to 0.15%, with $O_2^{\bullet-}$ being generated at distinct topologies at the respiratory chain, notably at complex I and III (Quinlan et al., 2013; St-Pierre et al., 2002). Although it may seem a very small amount, 0.15% of total oxygen consumed represents a significant amount of $O_2^{\bullet-}$ produced and therefore should not be downplayed, when considering mitochondria as an ROS producer under physiological and pathological situations (Fridovich, 2004). Other documented sources of ROS in mitochondria include monoamine oxidase and dihydroorotate dehydrogenase (Cadenas, Davies, 2000; Lenaz, 2001). The former enzyme was previously demonstrated to be involved in oxidative damage in myocytes from patients with collagen V myopathies (Sorato et al., 2014). In addition, the flavoproteins acyl-CoA dehydrogenase and glycerol phosphate dehydrogenase can produce ROS in tissues during the oxidation of lipid-derived substrates (Lambertucci et al., 2008; St-Pierre et al., 2002). Both pyruvate and α-ketoglutarate dehydrogenase contain flavoenzyme dihydrolipoyl dehydrogenase subunits and are additional ROS sources (Starkov et al., 2004; Tahara et al., 2007). Mitochondria, as both generators and targets of ROS, accumulate some of the damage that can initiate a vicious circle of further ROS formation. The age-dependent handicapping of mitochondrial energetics is related to the accumulation of defective mtDNA and defective respiratory chain complexes that are prone to electron leakage (Linnane et al., 1989; Wei, 1992).

1.2 Mitochondria as a Source and Target for Reactive Oxygen Species in Aging: An Interventional Review

As mentioned above, approximately five decades ago, coincident with the postulation of the "FRTA" increased formation of ROS was proposed to be the major factor responsible for the aging process and decreased life span (Harman, 1956). The continuous generation of ROS by mitochondria throughout cell life produces an age-related chronic oxidative stress, especially on mtDNA, resulting in oxidative modification of bases or deletions

(Santos et al., 2013). As a consequence, mitochondria have been identified as key players in the aging process (Miquel et al., 1980). However, new findings in the last years suggested that ROS generation cannot be the initial trigger of the aging process, providing an alternative point of view to the Harman's hypothesis. One of the stronger evidence in this lack of mechanistical linkage is the lack of effect on life span of under- or overexpressing a large number and wide variety of genes coding for antioxidant enzymes (Perez et al., 2009a). In addition, a recent study showed that oxidative damage of cardiomyocytes did not positively correlate with age in human beings, although the samples were obtained from a restricted age span (<2 years old) (Huang et al., 2017). To have a thorough knowledge on this topic, we refer the readers to very relevant review articles (Gems, Partridge, 2008; Hekimi et al., 2011; Ristow, Schmeisser, 2011). In this section, we want to discuss the "canonical" association between ROS production and aging, with particular relevance on CR, which represents the most convincing intervention to delay aging and attenuate age-related disease in multiple species.

Mitochondrial function during aging has been described to decrease, especially at advanced ages. Different studies showed a decline at multiple levels, ranging from decreased activity of the respiratory chain and ATP synthase, Krebs cycle fluxes, oxidative alterations of cardiolipin, disrupted regulation, and defective mtDNA regulation and activity, among other described effects (de Almeida et al., 1989; Emelyanova et al., 2017; Petrosillo et al., 2009; Rottenberg, Hoek, 2017). A recent model describes a biphasic model in which an initial increase in mitochondrial function in middle age is followed by a fast decline at older ages (Baker, Peleg, 2017). As already mentioned, a significant number of studies in different model organisms suggest that inhibition of oxidative stress contributes to an increase in life span. Administration of Vit-E was previously shown to extend the life span of many animals, including the nematode *C. elegans* (Harrington, Harley, 1988); male mice receiving Vit-E from 28 weeks of age showed a 40% increased median life span, with a beneficial effect on aging-related decline in neurological performance and mitochondrial function. The activities of mitochondrial nitric oxide synthase and SOD2 decrease with age, whereat these effects are ameliorated by Vit-E treatment (Navarro et al., 2005). A class of Vit-E analogues, called tocotrienols, shows excellent antioxidant activity in vitro and have been suggested to suppress ROS production more efficiently than tocopherols (Schaffer et al., 2005); tocotrienols extend life span by reducing ROS damage (Collins et al., 2006). Despite this, antioxidants have had limited success in preventing the progression

of diseases involving mitochondrial oxidative damage probably because they distribute around the body, with only a small fraction being accumulated by mitochondria (Serviddio et al., 2011). To overcome this problem, lipophilic cations have been conjugated with several antioxidants to allow their specific accumulation inside mitochondria. MitoVitE is one of the first mitochondria-targeted antioxidants, rapidly taken up by mitochondria (Smith et al., 2003). In cerebellar granule cells, MitoVitE mitigated EtOH-induced accumulation of intracellular oxidants and counteracts suppression of glutathione peroxidase/glutathione reductase functions and overall cellular glutathione levels (Siler-Marsiglio et al., 2005). MitoQ was reported to significantly increase the life span of SOD-deficient flies and to improve their tolerance to paraquat stress, but it could neither increase the life span nor rescue the paraquat sensitivity of wild-type *D. melanogaster* (Magwere et al., 2006). Moreover, MitoQ was shown to be effective as an antioxidant when complex I-derived superoxide generation is already elevated due to disrupted electron flow, whereas it has a prooxidant role in intact cells with normal complex I activity. Consequently, MitoQ may be useful in the treatment of diseases originating from impairment of respiratory chain complex I due to oxidatively damaged mtDNA, when its targeted delivery to pathogenic tissues is ensured (Plecita-Hlavata et al., 2009). SkQ molecules, in which a plastoquinone molecule is bound to a positively charged carrier, have also been attempted in the context of delaying aging/senescence effects in cells and tissues, with positive effects being obtained in the eye, heart, and kidney (Skulachev et al., 2009).

For several years, coenzyme Q 10 (CoQ10), whose levels are affected during aging and neurodegenerative diseases, has been considered a key factor in the progression of aging-associated complications (Lopez-Lluch et al., 2010). In rats fed a diet enriched in polyunsaturated fatty acids (PUFAs), supplementation with CoQ10 produces significant increases of mean and maximum life span, attenuating oxidative alterations related to this specific kind of diet (Quiles et al., 2004). Furthermore, enrichment of cells with CoQ10 resulted in an ordering and condensing effect on cell membranes, leading to a decrease in ROS generation and to a protective benefit on DNA integrity (Tomasetti et al., 2001). On the other hand, different studies in *C. elegans* demonstrated that lowering CoQ10 content, by inactivating genes involved in ubiquinone biosynthesis (Asencio et al., 2003) or by dietary deprivation (Larsen, Clarke, 2002), induces a significant life span increase. This discrepancy may be explained by considering that life span extension occurs with moderately low levels of global CoQ10 content

(up to 50%), whereas severe CoQ10 depletion leads to developmental and reproductive inefficiency, with no extension in longevity. This interpretation is supported by the observation that the hallmark of CoQ10 deficiency syndrome is, obviously, a decreased CoQ10 concentration (about 30% or lower of the total coenzyme content, compared to healthy individuals) in human muscle and/or fibroblasts (Montero et al., 2007). Moreover, CoQ10 concentration progressively declines after the age of 40, and, in rodents, this drop occurs even under dietary CoQ10 supplementation, suggesting a higher CoQ10 consumption. Thus, although lifelong CoQ10 supplementation did not prolong or shorten the life span of either wild-type rats or mice (Lonnrot et al., 1998), it may help to prevent life span shortening due to cumulative oxidative insults.

Use of antioxidants or targeting antioxidants to mitochondria by conjugation to lipophilic cations is not the only strategy to reduce oxidative damage and prolong life span (Fig. 1). Dietary factors, including restriction of caloric intake, restriction of protein or methionine intake, or the ingestion of specific nutrients, have been shown to alter mitochondrial redox metabolism, cellular oxidative stress, and animal life span (Page et al., 2010). An inverse correlation between longevity and mitochondrial

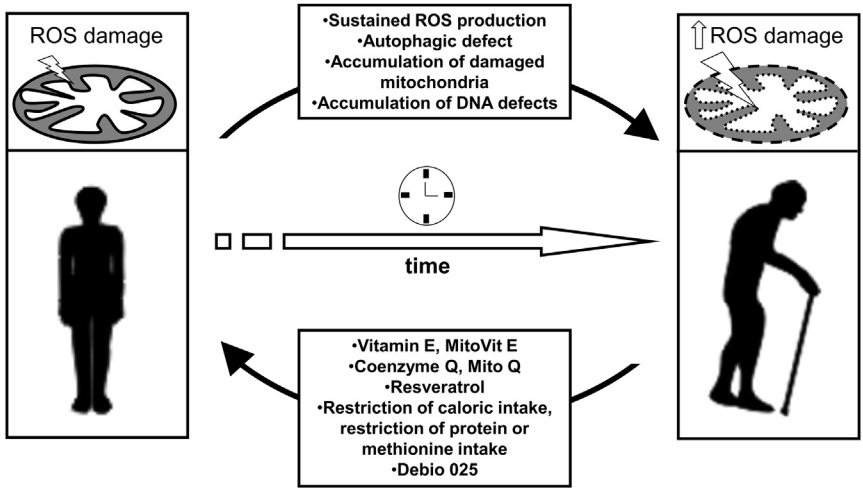

Figure 1 ***Potential role of mitochondrial ROS increase in aging.*** During aging, mitochondrial ROS production steadily increases, leading to mitochondrial damage and decreased life span. Here we report the major events contributing to aging (upper panel), or most important chemicals and experimental interventions, which may promote longevity (bottom panel).

ROS generation is demonstrated by three different studies in which H_2O_2 production by mitochondria isolated from the liver, skeletal muscle, and brain was reduced in calorie-restricted rats (Bevilacqua et al., 2004; Hagopian et al., 2005; Sanz et al., 2005). In particular, CR increased the efficiency of brain mitochondria in electron transfer in complex I, avoiding electron leak in that complex. Superoxide anion generated by complex I is specifically directed to the mitochondrial matrix, with consequent mtDNA damage and resulting bioenergetic deficits (Stefanatos, Sanz, 2011). The relationship between longevity and complex I is described in a report by Ayala et al., in which liver mitochondria of calorie-restricted rats demonstrated reduced levels of complex I (Ayala et al., 2007). Moreover, analysis of rat liver samples revealed a significant change in abundance in specific subunits of respiratory chain complexes I and IV with lifelong CR, allegedly to minimize the electron leak and subsequent ROS formation (Dani et al., 2010). Conversely, dietary restriction did not affect the activity of the oxidative-phosphorylation system or the mitochondrial H_2O_2 production in a similar rat strain (Valle et al., 2007). Thus, more work is required to confirm whether modulation of complex I levels represents one mechanism by which mitochondrial ROS production is reduced in parallel with extended longevity.

CR also reduces oxidative stress through a mechanism involving the mitochondrial deacetylase sirtuin-3, SIRT3. Expression of SIRT3 is increased during CR, and SIRT3 reduces cellular ROS levels by regulating SOD2 through deacetylation of two critical lysine residues, promoting its antioxidant activity (Qiu et al., 2010). However, there is some uncertainty regarding which acetylated lysine residue regulates SOD2 activity, and different groups proposed different site-specific regulation of SOD2 by SIRT3 (for a complete review on sirtuins and redox stressors, see (Webster et al., 2012)). Although the precise site(s) of regulation remains unclear, ROS levels are tightly controlled by SIRT3. Beyond SOD2, another important redox target of SIRT3 activity is represented by $NADP^+$-dependent isocitrate dehydrogenase 2 (IDH2), found in mitochondria that catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate. In response to CR, SIRT3 activates IDH2, thereby increasing NADPH levels in mitochondria. This in turn leads to an increased ratio of reduced-to-oxidized glutathione and decreased levels of ROS (Someya et al., 2010). The sites of SIRT3 deacetylation (K211 and K212) were found by Schlicker et al., who showed that in the presence of NAD^+ purified SIRT3, but not SIRT5, deacetylated IDH2 and increased its activity (Schlicker et al., 2008).

Importantly, IDH2 deacetylation/activation mediated by SIRT3 has been linked to age-related hearing loss, whereat a calorie restricted diet reduces the age-related loss of neurons and hair cells, whereas this effect is abrogated in SIRT3-deficient mice (Someya et al., 2010). Association between CR, SIRT3, and IDH2 sustains the concept that oxidative stress is a major component of aging and that nutrient status can regulate the cellular response to degenerative pathologies.

A report by Morselli et al., demonstrates how another component of the Sirtuin family, SIRT1, is required for the life span-prolonging effects of CR and resveratrol, through a mechanism that involves autophagy (Morselli et al., 2010). Dietary delivery of resveratrol increases mitochondrial abundance and aerobic capacity in cultured endothelial cells and mice (Lagouge et al., 2006). Interestingly, resveratrol is able to interact with different components of the respiratory chain: by competition with coenzyme Q, resveratrol is able to decrease complex III activity (Zini et al., 1999), and a binding site on complex V/ATP synthase has been observed (Gledhill, Walker, 2005). In two different cellular settings, cardiomyocytes and dopaminergic neurons, resveratrol protected against oxidative stress and was able to maintain mitochondrial membrane potential (MMP), with both effects directly related to resveratrol-dependent increase in SOD2 activity (Danz et al., 2009; Okawara et al., 2007). In fact, resveratrol supplementation in the context of a high-fat diet proved to be effective at elevating antioxidant capacity in the brain, resulting in an increase in both SOD2 protein levels and activity (Robb et al., 2008). Interestingly, recent data showed regulation of SIRT3 activity by SIRT1-mediated deacetylation, with aging demonstrated to be related with SIRT3 acetylation (Kwon et al., 2017). Considering that SIRT3 regulates several mitochondrial metabolic pathways (Hirschey et al., 2010; Pereira et al., 2012), this discovery sheds light on a cytosolic-mitochondrial sirtuin-based cross talk with important roles in mitochondrial alterations during aging.

Overexpression of SOD2 confers enhanced oxidative capacity and greater resistance against inducers of mitochondrial permeability transition (Silva et al., 2005). Moreover, flies with severe reductions in SOD2 expression exhibited accelerated senescence of olfactory behavior as well as precocious neurodegeneration and neuronal DNA strand breakage (Paul et al., 2007). Antioxidant supplementation, such as with Vit-E and Vitamin-C (Vit-C), reduces oxidative stress, improves SOD2 activity, with consequent positive muscle work in chronically loaded muscles of aged rats (Ryan et al., 2010). Furthermore, due to increased SOD2 expression, melatonin-treated

animals showed an increase in active mitochondria population and the ability to restore the mitochondrial potential of age-damaged neurons (Garcia-Macia et al., 2011). Interestingly, mice receiving intravenous SOD2-plasmid liposome prior to total-body irradiation show increased survival from the acute hematopoietic syndrome, and males demonstrate improved long-term survival (Epperly et al., 2011). Given these observations, it appears surprising that different publications reported the failure of SOD2 overexpression in prolonging life span (Perez et al., 2009b; Zhang et al., 2009), a discrepancy that future research on aging must address. Still, the controversy supports the notion that mitochondrial-produced ROS may have a duality of effects, which are at present difficult to fully understand.

SOD2 expression is also increased in p66Shc knockout mice, which exhibit prolonged life span (Haga et al., 2008). Other genetic mouse models of longevity have been reported, such as Ames and Snell dwarf mice and $Igf1r^{+/-}$ female mice (for a review, see Liang et al., 2003), and the increased life span of these models has been correlated to increased resistance to oxidative stress. To test the causative role of mtDNA mutations in aging, the mtDNA mutator mouse, which accumulates high levels of point mutations due to a proofreading deficiency of the mtDNA polymerase γ (POLG) has been developed. In this model, mtDNA mutations resulted in a variety of aging phenotypes, i.e., weight loss, alopecia, osteoporosis, anemia, reduced fertility, heart disease, progressive hearing loss and decreased spontaneous activity, but without inducing ROS production or increasing oxidative stress (Edgar, Trifunovic, 2009). This is not the case in p66Shc KO mice, in which oxidative stress plays a crucial role. p66Shc is localized to mitochondria in about 20% of fibroblasts of higher organisms, and oxidative stress promotes a translocation of part of the cytosolic pool of p66Shc to mitochondria (Orsini et al., 2004). Within mitochondria, inner mitochondrial membrane p66shc acts as a redox enzyme, with a consequent increment of ROS production and aging. The molecular route that leads to p66Shc activation and mitochondrial import was identified by our group in 2006 (Pinton et al., 2007). p66Shc must be phosphorylated at serine 36 to be active (Migliaccio et al., 1999), and this phosphorylation is mediated by PKCβ, a kinase of the PKC family, activated after an oxidative challenge. Once phosphorylated, p66Shc can be recognized by Pin1, a peptidyl-prolyl isomerase that induces cis-trans isomerization of phosphorylated Ser-Pro bonds, causing mitochondrial translocation of p66Shc (Pinton et al., 2007). At this point, p66Shc can exert its oxidoreductase

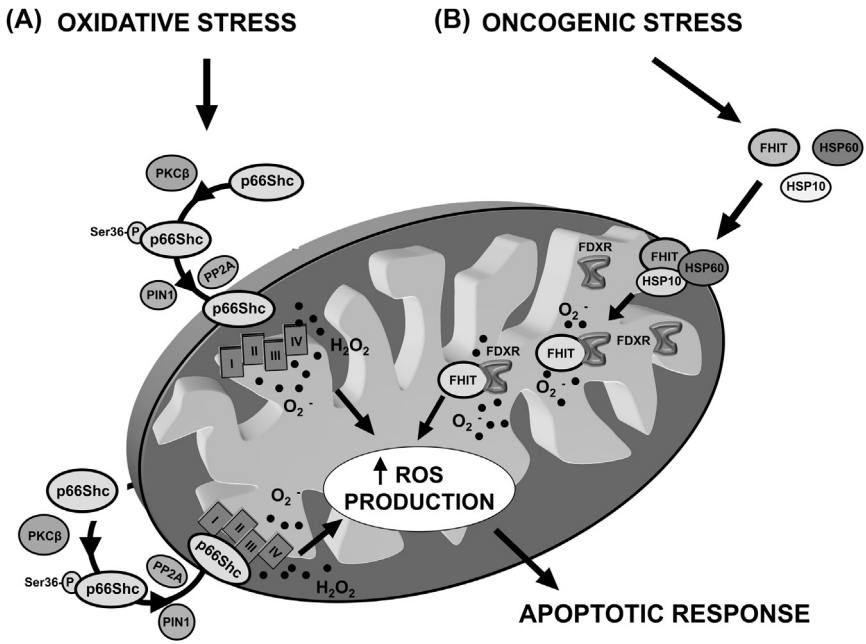

Figure 2 ***Schematic representations of two pivotal apoptotic molecular routes, involved during oxidative stress and oncogenic stress, respectively.*** (A) Oxidative stress-induced activation of PKCβ leads to phosphorylation of p66shc at Serine 36 residue, allowing translocation of protein to mitochondria by PIN1-dependent mechanism. The mitochondrial pool of p66shc oxidizes cytochrome c and catalyzes the reduction of O_2 to H_2O_2, inducing ROS production and successively apoptotic induction. (B) Oncogenic stress promotes the binding between FHIT and mitochondrial-import complex Hsp60/Hsp10. In mitochondria FHIT interacts with FDXR promoting ROS production and cytochrome c release, respectively, leading to apoptotic response.

activity, generating H_2O_2 and inducing the opening of the permeability transition pore (PTP) (Fig. 2). In turn, this event perturbs mitochondrial structure and function (as revealed by the reduced Ca^{2+} responsiveness and the alteration of mitochondrial three-dimensional structure (Pinton, Rizzuto, 2008)). A novel cyclophilin-binding agent, Debio 025, was demonstrated to inhibit cyclophilin D (a component of PTP) without having immunosuppressive effects (Ptak et al., 2008). It has been recently reported that Debio 025 was able to normalize mitochondrial function, muscle apoptosis, and ultrastructural defects in Col6a1$^{-/-}$ myopathic mice (Tiepolo et al., 2009), and it may represent a novel therapeutic opportunity to extend life span, minimizing oxidative stress—induced damages typical of aging.

1.2.1 ROS, Mitochondrial DNA, and Aging

Human mtDNA is a circular, double-stranded molecule consisting of 16,569 base pairs (for a review see (Lauri et al., 2014)). 13 mitochondrial proteins (subunits of the electron transport chain, ETC), 22 tRNA molecules, and 2 rRNA species are encoded in this DNA molecule. Depending on the cell type, there are a few to several hundreds of mitochondria per cell, and from a few to several (on average) mtDNA copies in each mitochondrion. This gives the number of about 10^3–10^4 mtDNA molecules per cell, on average (Lauri et al., 2014). The mtDNA is replicated by DNA polymerase γ that is encoded by the chromosomal *POLG* gene. All mtDNA molecules in one mitochondrion and in the cell can have exactly the same nucleotide sequence, which is called homoplasmy. However, occurrence of different variants of mtDNA (i.e., both wild-type and those containing point mutations or deletions) is referred to as heteroplasmy.

In evolution, mitochondria appeared as a result of a symbiosis between α-proteobacterium and a eubacterium, which was a milestone in the formation of eukaryotic organisms (Otten, Smeets, 2015 and references therein). Creation of mitochondria, which still, after billions of years of evolution, contain their own DNA, allowed producing a large amount of ATP in the cell. However, the price for this advantage is the production of large amounts of ROS, which can be deleterious for mitochondria and the whole cells. Recent analysis (Otten, Smeets, 2015) provided interesting conclusions about differences in evolutionary strategies of mtDNA between plants, fungi, and animals. Plants have much larger mtDNAs than animals, with fungal mitochondrial genomes being in between. In plant cells, mtDNA occurs in a low copy number while recombination is quite efficient. Moreover, anti-ROS mechanisms are efficient. Contrary to plants, animals have small mitochondrial genomes, recombination is very rare if any, and mtDNA is prone to mutagenesis. However, relatively high copy number of mtDNA per cell allows to compensate for effects of deleterious mutations in a few mtDNA copies, while providing opportunity to adapt animals, the active creatures, efficiently to various and changing environmental conditions. Nevertheless, the cost of such a strategy is accumulation of mutations in mtDNA during the life. As a consequence, mitochondrial diseases can appear in further generations. Since such diseases are generally rare, this is an acceptable event for a population, which benefits from increased adaptive possibilities. However, several mutations in mtDNA contribute also to aging of cells and organisms.

Again, this is not dangerous for a population, as aging occurs after the time of a reproductive peak, but has a serious consequence for elderly individuals.

The MFRTA hypothesis has been proposed over 60 years ago and has been reviewed many times (see, for example (Lee, Wei, 1997; Wei, 1998; Wei et al., 2001)). The main assumption of this theory is that mtDNA is not protected by histones, thus, it is more prone to lesions caused by ROS. Because mitochondria are one major source of ROS, actions of these agents lead to accumulation of mutations in mtDNA. This, in turn, may cause dysfunctions of ETC, which result in even more efficient production of ROS. Such a positive feedback, called also the vicious cycle, has been considered the major cause of mitochondrial destruction, then cell functions' inefficiency, and finally apoptosis of cells and aging of the organism (summarized by (Guest, Russell, 1992; Lee, Wei, 1997; Wei, 1998)). The importance of the role of ROS production in aging may be also supported by the observed age-related decreases in activities of several antioxidant enzymes, which could reflect overall accumulation of oxidized proteins with age (Stadtman, 1992, 2006). Nevertheless, even at the mature state of this theory, there were some problems that were difficult to explain. For example, considering that phenotypic manifestation of the mtDNA mutation occurs only when a threshold level is exceeded, usually 60%–80% heteroplasmy, depending on the type of mutation (Rossignol et al., 2003), it was difficult to explain how aging can be provoked by mutations in a low fraction of damaged or mutated mtDNA molecules of about 1%–5%, which was determined experimentally (Lee, Wei, 1997).

What kind of mutations occurs predominantly in mtDNA? There are both base pair substitutions and deletions (reviewed by (DeBalsi et al., 2017; Lauri et al., 2014)). Molecular mechanisms of appearance of such mutations in mtDNA have been excellently summarized and deeply discussed recently (Szczepanowska, Trifunovic, 2017), thus, they will be mentioned only shortly here. Nevertheless, one should also note that mtDNA depletion (a decrease in the copy number of mtDNA, not necessarily associated with mutations in this molecule; see, for example (Weglewska et al., 2005)) may also occur is cells of aging organisms. It was considered that formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), caused by ROS production by Complex I, is the major mutator causing base substitutions in mtDNA. ROS-mediated conversion of guanosine to 8-oxodG appears to be a particularly efficient process, and since 8-oxodG pairs with adenine instead of cytosine, the next replication round results in creation of the

G to T transversion. Moreover, ROS cause breaks in DNA strands, which is a prerequisite to formation of deletions. In fact, early studies on mtDNA in a mouse model indicated that a particular deletion of 4236 base pairs in mtDNA and 8-oxodG were abundant in mitochondria from old mice while absent in young animals (Muscari et al., 1996). Accumulation of mtDNA deletion of 4977 bp was reported to increase with age (between 32 and 82 years) in neurons from the human brain (Soong et al., 1992). It was supposed that mtDNA should be prone to ROS-mediated mutagenesis more than nuclear DNA because it is not protected by histones, while it is located close to the ROS production site, i.e., complex I in mitochondrion. However, more recent studies, in which the duplex sequencing method was used, allowing to detect one mutation per 10^7 DNA molecules, provided evidence against these early assumptions (Kennedy et al., 2013). When mtDNA was isolated from human brains of old (over 80 years old) and young individuals, the frequency of substitutions in DNA was about fivefold higher in elderly people. However, the frequency of the G to T transition, which is often used as a marker of ROS-mediated mutations, did not increase with age. On the other hand, in both young and old individuals, transition mutations predominated (Kennedy et al., 2013). Moreover, similar analyses performed in mice indicated that the frequency of mutations in mtDNA appears to be an order of magnitude lower than that reported in earlier studies with the use of significantly less sensitive methods (Vermulst et al., 2007). Such results suggest that errors made by DNA polymerase γ, the only DNA polymerase present in mitochondria (though encoded by nuclear DNA) and responsible for replication of mtDNA, may be responsible for most of the mutations in mtDNA appearing during the whole life.

Other results that are against the early MFRTA theory came from advanced microscopic studies. Contrary to previous assumptions, it appeared that mtDNA is not "naked," but instead, it is covered by the TFAM (transcription factor A) protein which together with DNA forms a nucleoid-like structure (Kukat et al., 2011). Moreover, it was reported that mtDNA may be separated from sites of ROS production in mitochondria due to microcompartmentalization of the matrix (Appelhans et al., 2012). In fact, by using sensitive methods for detection of mutations, it was found that frequencies of stable genetic changes in mtDNA are similar to those detected in nuclear DNA (Anson et al., 2000; Lim et al., 2005), rather than higher as presumed on the basis of earlier studies in which small amount of mtDNA was a limiting factor for detailed analyses.

The milestone in studies on mutations in mtDNA was construction of mice, which produce DNA polymerase γ with deficiency in the proof-reading function. Such mice constructed independently by two teams (Kujoth et al., 2005; Trifunovic et al., 2004) accumulate highly elevated number of mutations relative to wild-type animals, which allow detailed studies on mitochondrial mutagenesis in vivo. In these animals, no correlation could be found between number of accumulated mutations in mtDNA and oxidative stress markers (Kujoth et al., 2005). Rather, an increasing number of mutations in mtDNA correlate with aging without influence of the oxidative stress (Trifunovic et al., 2004). Subsequent studies indicated that accumulation of mutations in mtDNA by mice with mutator DNA polymerase γ occurred linearly with age, while production of ROS was at the level comparable to that in wild-type animals (Trifunovic et al., 2005). Therefore, one might suppose that errors made by DNA polymerase γ, rather than ROS-mediated DNA lesions, are the major cause of mtDNA mutations. It was proposed that during aging, their accumulation arise due to clonal expansions of particular mutated mtDNA molecules, rather than due to ongoing ROS-mediated mutation events (Wiesner et al., 2006).

This theory, alternative to MFRTA, received some additional experimental support. Studies on cultured fibroblasts indicated that the T414G mutation in mtDNA has little effect on ROS production and cell aging, which is against the MFRTA theory (Birket et al., 2009). On the other hand, another study on cultured fibroblasts demonstrated that a higher level of a deletion in mtDNA was accompanied with elevated concentrations of ROS (Quan et al., 2015). In fact, there is still an extensive debate on which theory is valid. Still some experimental results might suggest that the old MFRTA is valid, i.e., that ROS cause significant numbers of mtDNA lesions, which are accumulated in time due to defects in ETC and result in a constantly increasing ROS production, whereas other studies indicated that errors made by DNA polymerase γ during replication of mtDNA are the main source of mutations in the mitochondrial genome. The debate is ongoing, and definitely it is not finished. The reader is referred to recent review articles on this topic for detailed discussions on the mechanisms which lead to accumulation of mutations in mtDNA during aging (Bautista-Niño et al., 2016; Chih-Hao et al., 2013; DeBalsi et al., 2017; Edgar, Trifunovic, 2009; Kim et al., 2015; Kirkwood, Kowald, 2012; Lagouge, Larsson, 2013; Lauri et al., 2014; Lee, Wei, 2012; Pamplona, 2011; Szczepanowska, Trifunovic, 2017; Zapico, Ubelaker, 2013). In addition to the two theories mentioned in the preceding paragraph, a third one

has been proposed recently. Studies based on the deep sequence analysis indicated that in the absence of overproduction of ROS and with no accumulation of somatic mutations in mtDNA, the respiration defects associated with aging of human fibroblasts are still evident (Hashizume et al., 2015). Moreover, the previously reduced respiratory function could be restored if aging fibroblasts were reprogrammed by formation of iPSCs (induced pluripotent stem cells). In fact, reprogramming of cells could also recover some other phenotypes related to aging (Lapasset et al., 2011). Therefore, a hypothesis has been published that mitochondrial defects associated with aging may be due to epigenetic changes in nuclear genes, rather than due to accumulation of mutations in mtDNA (Hayashi et al., 2016).

In conclusion, three different theories have been proposed to explain the cause and the role of mutations in mtDNA in aging. It is documented that number of mutations in mtDNA increases significantly with age. However, it is still unclear whether the major cause of senescence might be (1) ROS-mediated lesions in mtDNA and the positive feedback leading to enhanced production of ROS and more and more elevated mutagenesis, (2) errors made by DNA polymerase γ and subsequent clonal expansion of mutated mtDNA molecules, or (3) epigenetic changes in nuclear genes affecting mitochondrial metabolism, with little contribution of mtDNA mutations. It is still possible that each of these mechanisms may contribute to the total picture of the senescence in relation to mtDNA and ROS.

1.3 Intracellular Defense Mechanisms Against ROS: Regulation on Aging and Disease

As protection against the deleterious effects of excessive ROS production, cells developed several enzymatic and nonenzymatic antioxidant defenses. The enzymatic system consists in a number of antioxidant enzymes such as superoxide dismutases, glutathione peroxidase, and catalase, localized in distinct cellular compartments. Superoxide anion is a by-product of oxidative phosphorylation, generated by the leak of electrons from the respiratory chain complexes. $O_2^{\bullet -}$, which is generated in the respiratory chain, especially in a pathological state (Ide et al., 1999; Raha et al., 2000), can be converted to H_2O_2 by SOD2 in the mitochondrial matrix or SOD1 (zinc—copper superoxide dismutase; also called Zn-CuSOD) in the intermembrane space of mitochondria (IMS) and cytosol (Sturtz et al., 2001). Subsequently, H_2O_2 can be converted to water by GPx (a selenocysteine-containing enzyme specific to organic peroxides) and peroxiredoxins (Prx 3 and 5; also controlling the level of peroxynitrite) directly in mitochondria (Cao et al., 2007).

Finally, H_2O_2 can diffuse through the mitochondrial membranes to the cytosol, where peroxisomal catalase or cytosolic GPx convert it to water. Besides the antioxidant enzymes, cells and specifically mitochondria also possess other nonenzymatic antioxidant system that comprises small molecules such as ascorbate (Vit-C), glutathione, tocopherol (Vit-E), retinoic acid, uric acid, pyridine nucleotides and thioredoxin, which also provide efficient cellular protection against excessive oxidative stress. These molecules can act directly as free radical scavengers or by modulating the activity of enzymatic systems (Lu et al., 2010). Examples of free radical scavengers include ascorbate and tocopherol (Lu et al., 2010). Ascorbate, present in aqueous phase, becomes a very stable and nonreactive radical and can be subsequently regenerated by pyridine nucleotide-dependent reductases. Tocopherol, present in the cellular lipid phase, is able to neutralize lipid peroxyl radicals, becoming a less reactive phenyl radical (Lu et al., 2010). Tocopherol radicals can then be regenerated by ascorbate. Uric acid is a strong scavenger of peroxynitrite in extracellular fluid but requires the presence of ascorbic acid and thiols for a complete scavenging (Nimse, Pal, 2015). Reduced glutathione, pyridine nucleotides, and thioredoxin work together with antioxidant enzymatic systems, donating reducing equivalents to neutralize ROS (Lu et al., 2010; Nimse, Pal, 2015). Melatonin, the sleep hormone produced by the pineal gland, was also demonstrated to have intrinsic antioxidant protective effects on mitochondria, especially by preventing cardiolipin oxidation and limiting the loss of activity of the mitochondrial respiratory chain (Paradies et al., 2010, 2017; Petrosillo et al., 2008).

The relationship between the MMP and mitochondrial ROS production has been described by Korshunov et al (Korshunov et al., 1997), as a potential mechanism to regulate oxidative stress. Increased ROS production occurring in the presence of mitochondrial hyperpolarisation relates to the higher NADH/NAD ratio indicating a more reduced NADH pool (Adam-Vizi, Chinopoulos, 2006; Aon et al., 2010; Kushnareva et al., 2002). A protection mechanism against excessive ROS production is proposed to be a mild uncoupling of MMP. This process is catalyzed both by a group of uncoupling proteins (UCP2-5) and free fatty acids (FFAs). In the case of hyperpolarization, mitochondrial carriers (e.g., adenine nucleotide translocase, dicarboxylate, and glutamate/aspartate carriers), UCPs, and FFA have been shown to be able to partly "discharge" the high proton gradient to a physiological level (Wieckowski, Wojtczak, 1997). It has been demonstrated that (mild) uncoupling of the MMP (by 10%) enables the

reduction of ROS generation by approximately 90% (Korshunov et al., 1997). Moreover, superoxide activates UCPs (UCP1, UCP2, and UCP3), causing increased proton leakage, mitochondrial depolarization, and decreased ROS production (Echtay et al., 2002; Mailloux, Harper, 2011). In the rat heart and skeletal muscle, UCPs have been proposed to remove the superoxide anion radical from the mitochondrial matrix (Wojtczak et al., 2011). There is an on-going debate regarding the physical and biological functions of UCP, a number of associations of UCPs against ROS production have up-to-date been proposed, elucidating this group of proteins as potentially significant in a number of pathophysiological situations (Bugger et al., 2011; Diao et al., 2008; Prakash et al., 2015). The role of mitochondrial uncoupling in preserving muscle fibers from the aging process was demonstrated by Amara et al. In this work, the authors demonstrated that mild uncoupling serves to protect mitochondrial function and contribute to the longevity of the most active muscle fibers, i.e., those with higher oxidative capacity (Amara et al., 2007). Interestingly, opposite effects were found in fibroblasts and yeast cells, where mild-uncoupling led to fibroblast senescence and decrease life span in yeast (Stockl et al., 2007).

It has been repeatedly demonstrated that a defective antioxidant defense system may lead to serious ROS-related human pathologies, often associated with the aging process, such as AD, PD, and diabetes. Oxidative stress caused by disruption of the antioxidant defense and excessive ROS production is closely linked to the pathogenesis of neurodegeneration (Uttara et al., 2009). For example, the levels of SOD1 in AD patients were significantly decreased. Interestingly, the levels of mitochondrial SOD2 and extracellular SOD3 were not changed (Murakami et al., 2011). These data can be supported by the observations that the antioxidant defense of cells from familial AD patients was weaker than in cells from healthy individuals (Cecchi et al., 2002). In another context, Hwang et al. presented evidence that catalase plays an important role in kidney protection during hyperglycemia. Deficiency of catalase, in catalase knockout (KO) mice accelerated diabetic nephropathy observed in streptozotocin-induced diabetes (Hwang et al., 2012). Liu et al. (2017) also demonstrated an increased ROS levels in oocytes from diabetic mice resulting from altered acetylation status of SOD2 in lysine 68. Also, supporting the antioxidant role of UCP and their importance for the development of diabetes, Robson-Doucette et al. (2011) demonstrated that UCP2 regulates ROS production and affects insulin and glucagon secretion by pancreatic cells. Broche et al. (2018) demonstrated

that UCP2 regulates pancreas development during embryogenesis through ROS-AKT mediated signaling pathway, evidencing the importance of the antioxidant role of the UCP in the development of diabetes.

Increased ROS formation is also a common intracellular stress that effectively leads to induction of autophagy, which acts as a protective mechanism, at least up to a certain degree (Martinet et al., 2009). The principal link between ROS and autophagy is the cysteine protease Atg4 (autophagy-related gene 4), which is a direct target of H_2O_2. Oxidation of cysteine promotes lipidation of LC3 (MAP1 light chain 3)/Atg8, essential for autophagosome maturation (Scherz-Shouval et al., 2007). On the other hand, it has been demonstrated that autophagy can be a crucial mechanism for preventing the accumulation of ROS by removing damaged mitochondria (Kroemer et al., 2010). This mitochondrial quality control is named mitophagy and serves to eliminate the fraction of damaged mitochondria normally suffering from mitochondrial membrane permeabilization. The recognition of this subset of mitochondria is provided by the involvement of the mitochondrial kinase PINK1 (PTEN-induced putative kinase protein 1). When mitochondria lose membrane potential, PINK1 rapidly accumulates on the mitochondrial surface, leads to the recruitment of the cytosolic protein Parkin, which mediates the ubiquitination of mitochondrial protein with consequent engulfment of damaged mitochondrial by membranes that then fuse with lysosomes (Narendra et al., 2010), a process called mitophagy. In fact, mitophagy has been considered a process by which mitochondrial quality is maintained during the course of aging, avoiding excessive oxidative damage to these organelles (Shi et al., 2017).

Although it exceeds boundaries of this review, it is worth to mention that a number of regulatory programs exist capable of modulating the intrinsic antioxidant defenses. Calorie restriction is known to be one of the strongest life span extending interventions and to have a positive impact in different pathologies (Colman et al., 2009). However, the mechanisms underlying CR remain largely elusive and have become the point of interest of many research groups. Among the proposed mechanisms, the activation of members of the Sirt2 family proteins has gained much interest (Rogina, Helfand, 2004). There are a number of experimental works that associate the activation of Sirt2 orthologues with upregulation of cellular antioxidant defense systems (Qiu et al., 2010; Rahman et al., 2009). Interestingly, the positive effects of CR were mimicked in mice through the administration of low dosages of the protonophore 2,4-dinitrophenol, suggesting that mild uncoupling (see above) can indeed be a protective strategy by

upregulating the antioxidant network (Caldeira da Silva et al., 2008). In addition, it has been proposed that CR is able to activate the Nrf2/antioxidant response element (ARE) pathway, inducing ROS detoxification systems, exert antiinflammatory effects, and, thereby, suppress initiation/progression of vascular disease (Ungvari et al., 2008). Next to CR, another important antioxidative intervention is physical activity. It is believed that the two mechanisms together are capable of restoring age-dependent reductions of critical endogenous protective mechanisms such as ischemic preconditioning (Abete et al., 2011). This adaptive mechanism in response to brief episodes of myocardial ischemia enables the reduction of cellular damage due to a prolonged ischemic insult (Murry et al., 1986). Increased ROS production observed on physical exertion may induce compensational increase of the antioxidant defense efficiency (Ji, 1993). Growing evidence indicates that exercise training can result in an elevation in the activity of antioxidant enzymes. Similarly, long-term physical activity is related to the increase of catalase, SOD, and glutathione peroxidase activities in muscles of trained animals (Ascensao et al., 2013; Goncalves et al., 2013; Ji, 1993; Laughlin et al., 1990; Powers et al., 1999).

Dietary antioxidant supplementation is another strategy to boost antioxidant defenses in different cell types, with possible positive impacts during aging. Phenolic and thiol compounds, flavonoids, and carotenoids are examples of antioxidant compounds that can be obtained from fruit, vegetables, spices, grain, and herbs (Nimse, Pal, 2015). A well-known dietary antioxidant supplement is resveratrol. Resveratrol is a stilbenoid phenolic compound, with its antioxidant capacity giving it several possible therapeutic applications (Cho et al., 2017b; Ko et al., 2017) (Li et al., 2017; Sawda et al., 2017; Truong et al., 2017), including age-related diseases (Lange, Li, 2017; Li et al., 2017; Navarro-Cruz et al., 2017) and cancer (Deus et al., 2017). In fact, resveratrol appears to improve mitochondrial function and biogenesis in skeletal muscle of aged animals (Muhammad, Allam, 2017). Also, due to its beneficial effects on mitochondria, resveratrol was proposed as a promising nutraceutical supplement in the treatment of mitochondrial disorders (De Paepe, Van Coster, 2017). Also, due to its antioxidant properties, flavonoids have demonstrated neuroprotective actions against neurodegeneration (Frandsen, Narayanasamy, 2018). Equally, high dietary intake of carotenoids appears to reduce the risk of stroke and stroke mortality (Bahonar et al., 2017). The effects of some approaches have been summarized in a recent review by Suski et al. (2011). However, although having good antioxidant properties, some

compounds are not able to reach mitochondria, where the majority of ROS are formed. Thereby, to observe any effect, a larger amount of those compounds is required, which may lead to undesirable side effects. Thus, new strategies are required to improve the delivery of those compounds.

Another interesting antioxidant strategy can be assigned to heme oxygenase-1 (HO-1) (Otterbein, Choi, 2000). The expression of this enzyme is increased as a response to oxidative and heat stress and its role is to degrade free heme, originating from the denaturation and proteolysis of hemoproteins, to release biliverdin, carbon monoxide, and iron (Baranano et al., 2002; Liu et al., 2006; Morse, Choi, 2005). The iron released by HO-1 increases the synthesis of ferritin, what minimizes the probability of Fenton reaction initiation. Furthermore, biliverdin can be converted by the enzyme biliverdin reductase into bilirubin, a potent antioxidant, capable of protecting cells from 10,000-fold higher concentrations of H_2O_2 (Morse, Choi, 2005). Activation of HO-1 was described already as a possible mechanism by which different natural compounds delay skin aging (Park et al., 2016).

In summary, although cells possess several mechanisms of protection against oxidative stress, impairment in mitochondrial function and antioxidant defenses systems during the normal aging phenotype promote an imbalance between ROS formation and cleansing, increasing cellular oxidative stress. It remains to be determined which are the best strategies to decrease cell and mitochondrial oxidative stress that accompanies the aging process without disturbing the physiological role of ROS. Such strategies may pass through the modulation of intrinsic antioxidant mechanisms.

1.4 Mitochondrial Morphology, Calcium Homeostasis, and Dynamics in Aging

1.4.1 Mitochondrial Dynamics

Unlike ROS, mitochondrial dynamics has only recently been studied as a possible player in aging, and specific links between these three processes have been scarcely documented. Thus, here we will give only a brief overview of the studies giving insight into the relationship between mitochondrial dynamics and aging and attempt to highlight the possible trends, underlying mechanisms and links with ROS production. Mitochondrial morphology in living cells is heterogeneous and can range from small spheres to interconnected tubules (Rizzuto et al., 1998) (Fig. 3). Growing evidence indicates that mitochondrial morphology is critical for the physiology of the cell, and changes in mitochondrial shape have been related to many different processes such as development, neurodegeneration,

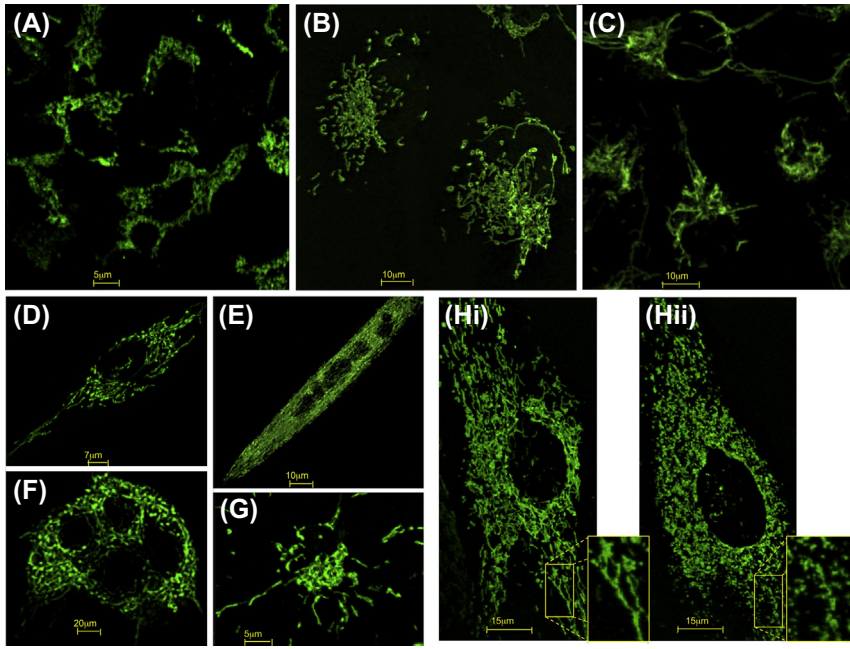

Figure 3 ***Mitochondrial network displays huge heterogeneity and shape rearrangements.*** Examples of mitochondrial network are shown in both immortalized [(A) HEK, (B) Cos7, (C) IB3] and primary cultured cell lines (D) human fibroblast, (E) rat myotube, (F) rat adipocyte, (G) rat oligodendrocyte progenitor). Rearrangement of mitochondrial network is also a typical adaptation to stress stimulus such as oxidative stress as shown for mouse embryonic fibroblast before (Hi) and after (Hii) exposure to H_2O_2.

calcium (Ca^{2+}) signaling, ROS production, cell division, and apoptotic cell death (Cereghetti, Scorrano, 2006). Mitochondrial dynamics in a broad sense involves the processes of fission, fusion, mitochondrial movement or transport, and interactions with other organelles (Benard, Rossignol, 2008). Mitochondrial fission and fusion regulate mitochondrial morphology, branching, and network formation and also determines individual mitochondrion size. Mitochondrial transport determines mitochondrial localization and overall distribution within the cell, this is especially important in highly polarized and large cells, such as neurons or oocytes (Barnhart, 2016; Frederick, Shaw, 2007; Mishra, Chan, 2014; Pernas, Scorrano, 2016). In the last two decades, we have learned intricate molecular details about these processes and their regulation, which now appears to be concertedly controlled by cellular activity. Mitochondria can associate with different cytoskeletal filaments to facilitate intracellular movement.

In mammalian cells microtubule filaments and dynein/kinesin motors are often used for movement purposes, but actin and actin nucleation factors recently emerged as essential players in determining mitochondrial positioning (Barnhart, 2016; Frederick, Shaw, 2007; Kanfer, Kornmann, 2016; Melkov, Abdu, 2018; Pathak et al., 2010). Moreover, mitochondrial interactions with the cytoskeleton are also important to link localization and movement with mitochondrial shape, by interactions with an elaborated machinery on the outer and inner mitochondrial membrane (OMM and IMM) accomplishing the fusion and fission of mitochondria (Chakrabarti et al., 2017; Hatch et al., 2016; Korobova et al., 2014; Korobova et al., 2013; Manor et al., 2015; Prudent, McBride). The cyclic rearrangement of the interconnected dynamic mitochondrial network, by individual mitochondria constantly undergoing fission and then fuse with each other has been studied in much molecular detail, but we still have no consensus on the functional consequences, the purpose and the exact regulation of the entire process (Cho et al., 2017a; Kanfer, Kornmann, 2016; Lee et al., 2016a; Misgeld, Schwarz 2017; Pernas, Scorano, 2016; Yamada et al., 2016). Fusion is an event where the outer and inner membrane of a mitochondrion fuses with the outer and inner membrane of another mitochondrion, respectively, allowing the matrix content of the two mitochondria to mix freely and form a single mitochondrion, whereas fission is the reverse of this event, and the two processes are regulated independently. Intriguingly, fission occurs preferably at sites where mitochondria interact with other organelles, in particular the endoplasmic reticulum (ER) (Friedman et al., 2011). These interactions involve a particular machinery present in subdomains of the ER (mitochondria-associated membranes—MAMs), further interacting with the actin cytoskeleton and the fission machinery of the OMM (Cho et al., 2017a; Hatch et al., 2016; Korobova et al., 2013; Kraus, Ryan, 2017; Li et al., 2015b; Manor et al., 2015; Moore et al., 2016). OMM fission is then promoted by dynamin-related protein 1 (Drp1), a dynamin-related GTPase, and Drp1 is recruited to the mitochondria by fission protein 1 (Fis1), mitochondrial fission factor (Mff), mitochondrial dynamics protein of 49 kDa (Mid49), and 51 kDa (Mid51) (Losón et al., 2013). While the molecular details of IMM fission is less known, both OMM and IMM fusion, facilitated by three dynamin-related GTPases, by mitofusin 1 and 2 (Mfn1 and Mfn2) located on the outer membrane, and by optic atrophy 1 (Opa1) located on the inner membrane (Chen et al., 2003; Cipolat et al., 2004), is reasonably well characterized. With interactions with all components of mitochondrial dynamics, the ER—mitochondrial interaction sites

are thus central hubs for a concerted regulation of both intracellular networks, probably executed by cellular and mitochondrial Ca^{2+} signals (Chakrabarti et al., 2017), and other processes regulating cell activity and shape (Shao et al., 2015; Wales et al., 2016). Here, an important link between local ROS production in this subdomain and mitochondrial dynamics has also been recently demonstrated (Debattisti et al., 2017; Norton et al., 2014). Interestingly, MAMs appointed to the modulation of calcium (Bononi et al., 2017; Kuchay et al., 2017; Marchi et al., 2018) and ROS (Verfaillie et al., 2012) signaling in health and disease appear to be altered in aged mice hearts in which Ca^{2+} transients, NAD(P)H regeneration, glutathione levels, and ER—mitochondria contact sites are significantly reduced compared with the young ones with a concomitant increase in ROS generation and mitochondrial protein oxidation (Fernandez-Sanz et al., 2014). Interestingly, recently it was demonstrated that knockdown of MCU and inositol 1,4,5-trisphosphate receptor type 2 (ITPR2), both involved in the accumulation of calcium in mitochondria, resulted in senescence escape, indicating the role of mitochondrial calcium accumulation in senescence induction (Wiel et al., 2014). Similarly, lower number of contacts between mitochondria and the ER in senescent human fibroblasts (Fig. 4) can also be responsible for the compromised mitochondrial calcium uptake in senescent cells. However, additional studies are needed to validate the alterations in the number of contacts between mitochondria and ER during aging or senescence to identify which factors have the highest influence of the regulation of Ca^{2+} fluxes through mitochondria—ER contacts sites in aging cells. The structure and function of MAM in the aspect of aging and senescence has been recently reviewed by Janikiewicz et al. (2015).

1.4.2 Mitochondrial Dynamics and Life Span in Model Organisms

Mitochondrial dynamics has been linked to existing pathways that regulate life span in *C. elegans*. It was shown that mitochondrial trafficking in distal neuronal processes declines progressively with age, and long-lived daf-2 mutants with reduced insulin signaling (IIS) show resistance to this decline (Morsci et al., 2016). Neuron-specific activation of CREB-regulated transcriptional coactivator 1 (CRTC-1), which promotes mitochondrial network fragmentation, is able to suppresses both AMPK and calcineurin-mediated life span extension in *C. elegans* (Burkewitz et al., 2015). Similarly, inactivation of *C. elegans* Drp1 significantly enhanced the ability of IIS to extend life span (Yang et al., 2011), suggesting a correlation between increased fusion/fission ratio and life span extension. Certain mutant bacteria

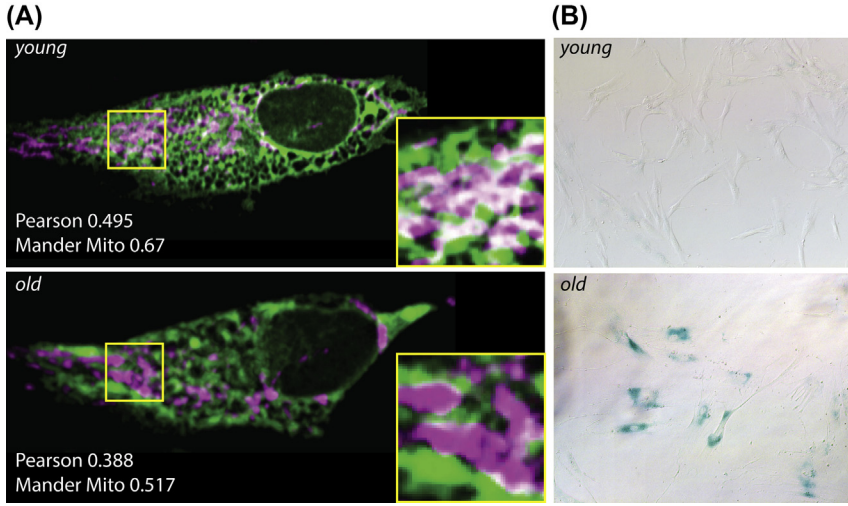

Figure 4 ***The number of contact sites between mitochondria and the ER in young and senescent human fibroblasts.*** (A) Image of the endoplasmic reticulum (ER) (green) and mitochondria (red) in young and senescent human fibroblasts. Maximum intensity projections of confocal micrographs from young and old fibroblasts expressing mitochondria-targeted Cherry (magenta) and ER-targeted GFP (green) contacts sites are represented by colocalization areas (white). Colocalization extents were quantified using Pearson's and Mander's coefficients. (B) Activity of senescence-associated β-galactosidase in young and senescent human fibroblasts. Cells referred as "young" fibroblasts were at 4th passage, and the "old" ones (senescent) at 16th passage.

of the *C. elegans* microbiome are able to extend life span via increased secretion of the polysaccharide colanic acid, and this extension is dependent on Drp1 and mitochondrial fission (Han et al.). Thus, existing pathways of life span extension such as the AMPK and IIS pathways are closely linked to mitochondrial dynamics but the causative role of any of these processes has not yet been demonstrated and is being debated. Indeed, it has been suggested that mitochondrial mass and fragmentation do not affect life span and are merely changes associated with aging (Regmi et al., 2014). However, it is still possible that mitochondrial dynamics plays important roles in the regulation of aging when working in coherence with or as part of other aging pathways such as IIS or metabolic regulation of life span via the AMPK pathway.

Fusion and fission balance has also been studied in fungal and *Drosophila* models. In *Podospora anserina* and *Saccharomyces cerevisiae*, deletion of dynamin-related protein 1 (Dnm1p), mediating fission, retards aging and extends life span (Scheckhuber et al., 2006). A double deletion mutant of

Saccharomyces cerevisiae where Dnm1 (the yeast orthologue of Drp1) and Mgm1 (the yeast orthologue of Opa1), are both deleted, contain wild-type like filamentous mitochondria, but a decrease in mitophagy and replicative life span (Bernhardt et al., 2015). While these models would support a generalization that fusion is associated with increased life span, the *Drosophila* model shows opposite trend. Upregulating Drp1 expression in midlife extends *Drosophila* life span; this is likely linked to autophagy as autophagy is required for the life span extending effect to occur (Rana et al., 2017). Similarly, overexpression of parkin in *Drosophila* extended life span and reduced the level of *Drosophila* orthologue of mitofusin, mitochondrial assembly regulatory factor (Marf), which typically increases with age in flies (Rana et al., 2013).

Overall, from these studies it appears that there is no clear association between either fusion or fission and life span, and promoting either process will have an effect that depends on the signaling and metabolic context. Importantly, a few studies pointed to the importance of the interplay between autophagy and mitochondrial-specific mitophagy in determining cellular homeostasis, affecting life span of whole organisms. This suggests that it is rather the homeostatic function of mitochondrial quality control events, mediated by mitochondrial fusion and fission, bearing the importance for determining or influencing the aging process. Indeed, in mammalian models it is now well documented that any disturbance of the homeostatic circuit maintaining the functional mitochondrial network has a profound effect on the healthy life span of the animals. Mice knockouts for essential components of mitochondrial dynamics (Pernas, Scorrano, 2016) develop pleiotropic symptoms in many organ systems reflecting loss of cellular function or the ability to cope with cellular stress, leading to indicators of early aging or leading to at least a reduced healthy life span. For example, Fis1 KO mice develop multiple early aging signs including lordokyphosis, lack of vigor, inability to accumulate fat, reduced ability to tolerate stress, perturbed Ca^{2+} dynamics, and decreased life span (Uzhachenko et al., 2017). Transmembrane protein 135 (TMEM135) is a protein likely involved in mitochondrial fission, and mice with mutated TMEM135 display abnormal mitochondrial dynamics and accelerated aging in the retina as well as pathologies observed in age-dependent retinal diseases (Lee et al., 2016b). Furthermore, it has been long known that mTOR plays an important role in aging in a range of different organisms (Johnson et al., 2013). mTORC1 has been linked to regulation of mitochondrial dynamics by stimulating translation of mitochondrial fission process 1 (Mtfp1), promoting

mitochondrial fission. Potent active-site mTOR inhibitors promote mitochondrial fusion over fission events (Morita et al., 2017).

We have to emphasize that the cellular consequences of perturbed mitochondrial dynamics are often associated with loss of redox homeostasis (Abeti et al., 2011) and increased ROS production. In this context, ROS is often synonymous with cellular damage (Röth et al., 2014; Willems et al., 2015), but mitochondrial or ER stress–related ROS production might play a role in negative feedback regulation of organelles homeostasis, e.g., by altering gene expression via mitohormetic and mitochondrial unfolded protein responses (Shpilka, Haynes, 2017; Yun, Finkel, 2014).

1.5 Mitochondrial Dynamics and Age-Related Diseases in Humans

Mammalian models, such as mice with genetically altered mitochondrial dynamics, develop symptoms that resemble human age-related disease, affecting critical organs (nervous system, liver, and endocrine and cardiovascular systems) or increasing incidence of cancer (Altieri, 2017; Mishra, Chan, 2014; Pernas, Scorrano, 2016; Senft, Ronai, 2016; Youle, van der Bliek, 2012). Most deaths in old age are still due to diseases and it is not possible to extend life span without reducing the effects of age-related diseases. Here, we summarize a series of studies that investigated the relationship between mitochondrial dynamics and age-related diseases.

Cardiovascular disease is a range of diseases whose incidence of occurrence increases with age and is one of the leading causes of death in the developed world (Rapsomaniki et al., 2014). In humans and mice, heart failure is linked to decreased mitochondrial fusion, fragmentation of the mitochondrial network, and lower levels of Opa1 expression (Chen et al., 2009). In *Drosophila*, Marf and Opa1 are essential for proper cardiomyocyte function and fusion defects are associated with cardiomyopathy (Dorn, Scorrano, 2010). Opa1 mutation heterozygotes have late-onset cardiomyopathy in mice (Chen et al., 2012a). Furthermore, unbalanced Opa1 processing and a decrease in mitochondrial fusion are linked to fragmentation results in heart failure in mice (Wai et al., 2015). It has been shown that endothelial cells (HUVECs) maintain a tubular mitochondrial network, but senescent cells have more elongated, interconnected mitochondria, and the change in mitochondrial morphology is caused by downregulation of Fis1 and Drp1 (Mai et al., 2010). However, another group found that in HUVECs, mitochondria of old cells showed a significant and equal decrease of both fusion and fission activity (Jendrach et al., 2005). These results are relatively

consistent and suggest that mitochondrial fusion, especially Opa1, is vital for proper heart function and preventing heart failure. This seems to be conserved throughout flies, mice, and humans. Expanding on this, it would be interesting to see if overexpressing of Opa1 or increasing mitochondrial fusion can improve declining heart function. In addition, differentiation of arterial smooth muscle cells and of cardiomyocytes is dependent of reduction of fusion, thus prone to disorders related to altered mitochondrial fusion/fission ratios (Chalmers et al., 2016; Kasahara et al., 2013). Altered ROS production was also found associated with disturbances of mitochondrial dynamics in this disease (Chen et al., 2012a; Mai et al., 2010).

Neurodegenerative diseases such as AD and PD are probably the most prevalent age-related neurodegenerative diseases whose risks increase dramatically with age (Fjell et al., 2014; Rodriguez et al., 2015). AD and PD have been linked to mitochondrial dynamics and mitophagy, as parts of the mitochondrial quality control machinery, and supposed to play important roles in these diseases (Chenet al., 2009). The vast literature on the overall importance of mitochondrial quality control in neurodegenerative disease has been recently reviewed, thus here we will focus on the less covered specific functions of mitochondrial fusion and fission. A large number of studies found that increased mitochondrial fission and fragmentation are linked to AD. Tau mice have higher levels of fission proteins Drp1, Fis1, and lower levels of fusion proteins Mfn1, Mfn2, Opa1 compared with WT mice (Kandimalla et al., 2018). In AD, amyloid-β (Aβ) is linked to Drp1-induced excessive mitochondria network fragmentation in AD progression (Reddy et al., 2017). Drp1 interacts with Aβ and phosphorylated tau, leading to mitochondrial fragmentation, abnormal mitochondrial dynamics, and synaptic damage (Manczak et al., 2011; Manczak, Reddy, 2012). Aβ precursor protein transgenic (APP) mice have significantly decreased anterograde mitochondrial movement, increased mitochondrial fission, and decreased fusion in neurons (Calkins et al., 2011). Furthermore, reducing levels of Drp1 decreases the amount of soluble Aβ production in AD progression and protects against Aβ-induced mitochondrial and synaptic toxicities in AD progression and pathogenesis (Manczak et al., 2016). However, sporadic AD is associated with a significantly lower level of Drp1 in fibroblasts (Wang et al., 2008a). Decreased Drp1 levels and mitochondrial localization, as well as reduced stomatin-like protein 2 (STOML2) and Mfn2 fusion protein levels, are observed in the fibroblasts of sporadic AD patients (Martín-Maestro et al., 2017). In cells overexpressing APP, a fragmented

mitochondria structure and abnormal distribution is observed. Moreover, levels of Drp1 and Opa1 were significantly decreased, whereas levels of Fis1 were significantly increased, and increasing Drp1 and Opa1 were both able to rescue some mitochondrial defects (Wang et al., 2008b). Thus, current evidence fails to reach consensus, but since alterations in ER–mitochondrial contacts, which appear as central organizer of fusion and fission events (see above), are implicated in AD (Area-Gomez, Schon, 2017; Filadi et al., 2017), one can speculate that deregulation of these membrane fusion and fission events is linked to disturbances of the ER–mitochondrial contact sites with variable outcome. Then again, mitochondrial ROS has been described as culprit for neuronal damage (Angelova, Abramov, 2018), but it also appears the local ROS in the contact sites is also a cellular signal at this interface and regulatory hub (Debattisti et al., 2017). Similarly, altered fusion/fission ratios accompany PD. In respiratory chain–deficient dopaminergic neurons fragmentation of the mitochondrial network and impaired anterograde axonal transport of mitochondria have been observed (Sterky et al., 2011). Alpha-synuclein (αS) has an inhibitory function on membrane fusion. On increased expression in cultured cells and in *C. elegans*, αS shift mitochondrial shape toward reduced fusion, leading to a fragmented mitochondrial structure (Kamp et al., 2010). Elegant studies demonstrated the ROS dependence of neuronal dysfunction in vivo in dopaminergic (DA) neurons (Guzman et al., 2010).

Another system affected by degenerative age-related symptoms is muscle. Muscle wasting is a hallmark of aging and the primary reason of declining physical abilities with age (Kalyani et al., 2014). Mitochondrial network in skeletal muscle has a complex and seemingly rigid organization, but it has been shown that mitochondrial fission is important for muscle atrophy to occur (Romanello et al., 2010). However, in aged muscle in mice, intermyofibrillar mitochondria in skeletal muscle were longer and more branched, suggesting increased fusion and/or decreased fission, and mitochondrial fusion index (Mfn2-to-Drp1 ratio) was significantly increased in aged muscles (Leduc-Gaudet et al., 2015). In human participants, the levels of the fusion protein Opa1 were lower in muscle from elderly subjects; however, no changes were detected in Mfn2, Drp1, or Fis1 among the groups (Joseph et al., 2012). Thus, studies on muscle atrophy do not have consistent results (Joseph et al., 2012; Leduc-Gaudet et al., 2015), and proper understanding of the organizational principles of the skeletal muscle mitochondrial network will be required to reveal the connection between muscle growth and function in aging.

Metabolic disorders are inherently linked to mitochondrial dysfunction, which can follow alterations in mitochondrial dynamics. This can be extended to insulin resistance and type 2 diabetes mellitus, which are strongly linked to aging and incidence of occurrence increases with age (Facchini et al., 2001; Meigs et al., 2003). While both pathologies have been linked to mitochondrial dysfunction (Mootha et al., 2003), the participation of mitochondrial dynamics is less known in the pathomechanism. Increased mitochondrial network fragmentation and Fis1 expression is observed in venous endothelial cells of type 2 diabetes mellitus patients compared to control (Shenouda et al., 2011). A shift toward mitochondrial fission with reduction of fusion protein, mainly Mfn2, has been associated with reduced insulin sensitivity and inflammation in obesity and insulin resistance development (Putti et al., 2015), which can be associated with altered ROS production.

Finally, although not primarily caused by degenerative age-related processes such as the abovediscussed diseases, in cancer, age is a major risk factor. The role of mitochondria in tumorigenesis is extensively researched (Gasparre et al., 2017; Valcarcel-Jimenez et al. 2017), and a few studies established links between mitochondrial dynamics and cancer cell biology. Some suggest a link between mitochondrial dynamics and development and metastasis (Altieri, 2017; Caino et al., 2016; Senft, Ronai, 2016), but not cancer causation. As such, the link between cancer and mitochondrial dynamics is less relevant toward aging for the purpose of this review but nonetheless could yield interesting insight into cellular transformation.

2. MITOCHONDRIAL DYSFUNCTION AND INCREASED ROS-RELATED/ACCOMPANIED PATHOLOGIES IN THE CONTEXT OF AGING

In this section, we provide some examples of pathological situations illustrating important role of ROS, oxidative stress, and mitochondrial dysfunction in the pathogenesis of described below abnormalities in the context of aging.

2.1 Liver, Mitochondria, and Aging

The process of aging develops in parallel with a gradual deterioration of cell functions, including the hepatocytes, in the body. The liver is a vital organ with a full battery of crucial functions that include the regulation of cholesterol, bile acid, triglyceride, protein, glucose, and energy metabolism, as well as detoxification, and production of bile. This latter function is essential for

digestion and absorption of intestinal cholesterol, triglycerides, and fat-soluble vitamins and is involved in hepatic secretion of three lipid components, i.e., bile acids, cholesterol, and phospholipids, which helps the body to excrete excessive cholesterol into the feces (Di Ciaula et al., 2017; Wang et al., 2017b). Although the liver has a major capacity to naturally regenerate, aging induces progressive "physiological" changes of the liver in the structural and functional aspects (Table 1). Whether the liver function becomes seriously compromised in elderly subjects remains largely contradictory because few studies have examined the aging process of the liver from a structural and functional point of view (Schmucker, 1998). The aging liver, however, indeed shows an impaired ability to counteract the hepatic insults, which is a situation often experienced by the elderly.

It has been repeatedly demonstrated that mitochondria play a critical role in driving the age-dependent oxidative lesions with ROS increase (Ames et al., 1995; Pamplona et al., 1998; Sastre et al., 1996). Liver mitochondria contain *manganese*-dependent superoxide dismutase (SOD) with antioxidant function. With aging, however, endogenous, mitochondria-derived free radicals might overwhelm the endogenous defensive response. Bejma et al. (2000) investigated the effects of aging and an acute bout of exercise on intracellular oxidant generation, lipid peroxidation, protein oxidation, and glutathione (GSH) status in the heart and liver of young adult (8 month) and old (24 month) male rats. In the whole liver homogenates and in the mitochondria (and also the heart), the rate of dichlorofluorescin oxidation, an indication of intracellular oxidant production, was higher in the homogenates of aged rats. Lipid peroxidation was also increased in the aged liver and exercised aged heart. Both electron transport chain and NADPH oxidase were two major sources of the age-related increase in oxidant production. In turn, in the study of Kujoth et al. (2005) the aging process was evaluated in mice expressing a proofreading-deficient version of the mtDNA polymerase γ. Mice accumulated mtDNA mutations while displaying features of accelerated aging. Of note, the accumulation of damage, i.e., mutations and deletions, of mtDNA was not associated with increased markers of oxidative stress in the liver mitochondria (i.e., H_2O_2 production and protein carbonyls, F2-isoprostanes, 8-hydroxy-2′-deoxyguanosine) or a defective cellular proliferation but was associated with the induction of apoptotic markers (i.e., cleaved caspase-3), particularly in tissues characterized by rapid cellular turnover such as the liver. Notably, CR is the only nutritional intervention that retards aging and the accumulation of mtDNA mutations

Table 1 Effect of Aging on Changes of Liver Mass and Function.

Finding(s)	Change(s)	Reference
Liver mass	Decrease by 20%–40%	Schmucker (2005), Wynne et al. (1989)
Perfusion and blood flow	Decrease by up to 50% after age 30	McLean and Le Couteur (2004)
Accumulation of hepatic dense body compartment (lipofuscin)	Increase	Gregg et al. (2012), Schmucker (1998), Cogger et al. (2014), Schmucker (2005)
Hepatocyte size (macrohepatocytes)	Increase	Schmucker (1998), Watanabe et al. (1978)
Polyploidy	Increase	Schmucker (1998), Watanabe et al. (1978)
Pseudocapillarization	Increase	Cogger et al. (2014), McLean et al. (2003)
Functional liver function tests	Decrease	Hall et al. (2005), Rahmioglu et al. (2009)
Albumin synthesis	Decreased (animal studies) Unchanged (human studies)	Anantharaju et al. (2002) Fu and Nair (1998)
Serum albumin	Minor decrease	Tietz, et al. (1992)
Hepatic uptake of HDL1-cholesterol	Decrease	Bravo et al. (1994)
LDL receptor	Decrease	Miller (1984), Schmucker (2005)
serum LDL–cholesterol levels	Increase	Anantharaju et al. (2002), Miller (1984)
Liver steatosis, inflammation, fibrosis, anisokaryosis, cellular senescence	Increase	Gregg et al. (2012)
Accumulation of oxidation products	Increase	Gregg et al. (2012)
Cytochrome P450	Decrease by 30%	Sotaniemi et al. (1997)
Drug metabolic clearance	Slower by 20–40%	Turnheim (2003)
Synthesis of vitamin K–dependent clotting factors	Decrease	Froom et al. (2003)
Gallbladder bile	Increased cholesterol saturation index	Valdivieso et al. (1978), Wang (2002)
Mitogen-activated protein kinase activity	Decrease	Schmucker (2005)
Number of binucleated hepatocytes	Increase	Gan et al. (2011), Premoli et al. (2009)

(Aspnes et al., 1997) with reduction of mitochondria-mediated apoptotic pathways (Cohen et al., 2004; Shelke, Leeuwenburgh, 2003).

ROS production increases with aging in state 3 when mitochondria isolated from old rats are supplemented with succinate. This finding could be explained by the defective suppression of H_2O_2 production, i.e., an example of mitochondrial ROS production, during the energy transition from state 4 to state 3. Also, levels of 8-oxodG in the biological macromolecule mtDNA increase with age in old animals. Notably, this increase is abolished by CR, with a positive effect on the aging rate (Lopez-Torres et al., 2002). In senescent rats from the ages of 28–60 and to 92 weeks, the mitochondrial mass of liver remains unchanged throughout ages, but the aging process is paralleled by increased (36%–45%) content of the oxidation products, i.e., thiobarbituric acid–reactive substances and protein carbonyls. These changes are associated with a progressive decrease in critical enzymes for mitochondrial function, i.e., -47% mitochondrial nitric oxide synthase, -46% SOD2, -30% complex I, and -24% complex IV, in old rats compared with young adult rats. However, liver mitochondria from young to old rats do not differ for fragility and water permeability (Navarro, Boveris, 2004). Age-associated decrease of mtRNA is another event involving also the liver (Anantharaju et al., 2002), whereas damaged mitochondrial proteins increase membrane stiffness (Pamplona et al., 1998). In addition, PUFAs could be easily damaged by ROS (Anantharaju et al., 2002). Thus, the accumulation of oxidized and carboxymethylated proteins in the mitochondrial matrix during senescence concomitant with defective degradation of abnormal matrix proteins affects the ability of aging mitochondria to respond to additional stress (Bakala et al., 2003).

A summary of established damages in the mitochondria of aging liver is depicted in Table 2. These changes include oxidative lesions in the mtDNA (Ames et al., 1993), oxidation of mitochondrial lipids, increased levels of long-chain PUFA, decreased membrane phospholipid peroxidability and decreased Δ9-desaturase activity coefficient leading to decreased levels of 16:1 and 18:1 fatty acids with less membrane stability. Additional mechanisms include increased apoptotic pathways and increased inner mitochondrial phospholipase A_2 activity (Laganiere, Byung, 1993; Pappu et al., 1978). The content of cytochrome oxidase is associated with loss of enzymatic activity (Wilson, Franks, 1975), whereas malondialdehyde accumulation increases (Von Zglinicki et al., 1991).

In all organs of the body, the aging process develops with progressive unbalanced response of the immune system where proinflammatory

Table 2 Damaging Effects of Aging on Mitochondria of the Hepatocytes

Finding(s)	Change(s)	Reference
mtDNA damage	Oxidative lesions	Ames et al. (1993), Lopez-Torres et al. (2002)
mtDNA mutations	Increased apoptosis Unchanged oxidative stress	Kujoth et al. (2005)
Mitochondrial lipid oxidation	Decreased membrane phospholipid peroxidability. Decreased Δ9-desaturase activity coefficient. Decreased levels of 16:1 and 18:1 fatty acids. Decreased membrane stability Increased amount of polyunsaturated fatty acids (in cardiolipin) Increased inner mitochondrial phospholipase A_2 activity	Laganiere and Byung (1993) Pappu et al. (1978)
Content of cytochrome oxidase	Loss of enzymatic activity	Wilson and Franks (1975)
Malondehaldeide accumulation	Increase	Von Zglinicki et al. (1991)
Mitochondrial number	Decrease	Cogger et al. (2014), Gan et al. (2011), Premoli et al. (2009)
Intracellular oxidant production (dichlorofuorescin)	Increased (whole tissue, mitochondria) Mediated by electron transport chain and NADPH oxidase	Bejma et al. (2000)

cytokines, chemokines, and ROS are not adequately counteracted by antioxidants molecules. The liver is enriched with enzymes with antioxidative functions such as mitochondrial SOD2, SOD1, cytosolic glutathione peroxidase, and peroxisomal catalase (Anantharaju et al., 2002). As already mentioned, aging has been shown to be associated with increased oxidative stress, likely due to decreased capacity to eliminate toxic substrates, i.e., metabolically generated superoxide radical. In particular, aging is paralleled by increased production of superoxide anion, hydrogen peroxide, and

hydroxyl radical, with all these molecules driving the oxidative protein damage in the liver. The mitochondria play a key role in this scenario leading the oxidative lesions with age (Ames et al., 1995; Pamplona et al., 1998; Sastre et al., 1996). Few studies have attempted to link the oxidative stress to cell injury (Zhang et al., 2003) examined the effects of hypertermic challenge on levels of ROS, oxidative injury, changes in redox status, and DNA-binding activation of critical stress response transcription factors, i.e., activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) in old and young rats. Compared with young rats, old rats show greater oxidative damage with sustained ROS levels through 24 h, higher malondialdehyde (MDA) and 4-hydroxy-2-noneal (4-HNE) levels as marker of lipid peroxidation products, as well as hepatocyte damage, i.e., monocyte infiltration, sinusoidal congestion, hepatocellular vacuolization, and diffuse necrosis. Moreover, the ratio of GSH to glutathione disulfide (GSSG) as a marker of hepatic redox status is significantly lower in old rats than in young rats. The effect on peroxidation of PUFAs in old animals might influence the cellular membrane permeability and membrane leakage. In addition, aging impairs the intracellular redox buffering mechanisms, e.g., antioxidants and glutathione, which, by definition, prevent ROS accumulation. Additional damage includes age-related decrease in DNA base excision repair in mouse hepatocytes (Intano et al., 2003), and increased level of oxidatively damaged DNA in the livers of old mice and rats in comparison to young animals (Hamilton et al., 2001). Increased oxidative stress in old animals appears to be paralleled by the enhanced induction of the antioxidant enzyme heme oxygenase via the transcription factor NF-kB (Lavrovsky et al., 2000). The study by Ikeyama and colleagues (Ikeyama et al., 2003) showed that aging, in rats aged 24–26 months, is associated with elevated basal H_2O_2 and epidermal growth factor (EGF)-induced gadd153 gene expression, as proapoptotic marker, in the livers compared with that in young animals. Moreover, there might be a variable change in the antioxidant family members with aging. Thomas et al. (2002) used gene array analysis to find that both aged rat and human liver samples display increased expression of redox and detoxification enzymes, i.e., the glutathione S-transferase GST, UDP-glucuronosyltransferase, and cytochrome P-450 enzyme families.

The excision repair cross-complementation group 1 $(Ercc1)^{-/\Delta}$ murine model hosts a rare human progeroid syndrome caused by inherited defects in DNA repair and premature aging (Gregg et al., 2012). In the 5-month-old $Ercc1^{-/\Delta}$ mice that are similarly to the old

wild-type group, at the ages of 24–35 months, the livers show architectural and inflammatory damages, elevated liver enzymes, and decreased albumin. Of note, there is a significant increase in oxidative damage in *Ercc1*$^{-/\Delta}$ and old wild-type liver, with a detection of lipid peroxidation and senescence products, i.e., lipofuscin, lipid hydroperoxides, and acrolein (Gregg et al., 2012).

Thus, increased oxidative stress and reduced tolerance to oxidative stress with age can initiate the further signaling pathways that drive cellular dysfunction and reduced stress tolerance in older organisms (Schmucker, 2005; Thomas et al., 2002).

2.1.1 Lipids, Mitochondria, and Aging

Lipid accumulation, i.e., triglycerides and cholesterol, greatly promotes aging in the liver (Ghosh et al., 2012; Petersen et al., 2003; Slawik, Vidal-Puig, 2006), whereas phospholipids remain quantitatively unchanged (Schneeman, Richter, 1993). A typical scenario in this respect is age-related increase in the metabolic syndrome with age (Ford et al., 2002), leading to the progressive redistribution of adipose tissue from subcutaneous sites to visceral ones (Tchkonia et al., 2010), and the increased prevalence of nonalcoholic fatty liver disease (NAFLD) as part of the age-dependent process of fat redistribution in nonadipose tissues. The process of ectopic fat deposition and lipotoxicity, therefore, initiates/perpetuates the damage in the liver, heart, skeletal muscle, and pancreas, thus leading to increased cardiovascular risk, the metabolic syndrome and, in turn, to further enhance liver steatosis (Floreani, 2007; Slawik, Vidal-Puig, 2006; Tchkonia et al., 2010; Tran et al., 2008). Of note, this process also referred to as "inflammaging" can also develop in lean but metabolically obese subjects (Tchkonia et al., 2010; Vecchie et al., 2017). NAFLD refers to the presence of excess fat in the liver, i.e., hepatic steatosis, when more than 5% accumulation of triglycerides occurs in the hepatocytes (Krawczyk et al., 2010). NAFLD encompasses the spectrum of liver abnormalities which range from simple steatosis, i.e., nonalcoholic fatty liver (NAFL), to steatohepatitis (NASH), and to (likely cryptogenic) cirrhosis, and has a potential progress to hepatocellular carcinoma (HCC) (Brunt et al., 2015). NAFLD is one of the most common liver disorders worldwide (Krawczyk et al., 2010), with a 20%–50% prevalence according to ultrasonographic and computed tomography (CT) imaging across population studies (Williams et al., 2011; Zelber-Sagi et al., 2006). Pathogenic factors of NAFLD include a genetic background (Krawczyk et al., 2013), enhanced uptake of FFAs (long-chain fatty acids,

LCFA), increased de novo lipogenesis, decreased fatty acid β-oxidation, and/or decreased synthesis or secretion of very low-density lipoproteins (VLDL) (Cohen et al., 2011).

Overall mechanisms accounting for the age-related increase of hepatic steatosis include accumulation of ROS and DNA damage (Aravinthan et al., 2013), hypercholesterolemia (Bonomini et al., 2013), decreased autophagy (Amir, Czaja, 2011), activation of NF-κB signaling that is the key regulator of inflammatory responses (Franceschi et al., 2000), metabolic dysfunction (Rodriguez et al., 2007), telomere shortening (Tomás-Loba et al., 2013), sedentary lifestyle (Breitling et al., 2009), and cigarette smoking (Booth et al., 2011). Aging also induces liver damage, increases proinflammatory M1 macrophage polarization, and enhances inflammatory response typical of NASH (Fontana et al., 2012). Notably, NAFLD appears to decline in very elderly subjects (Koehler et al., 2012) and this finding becomes more apparent when liver fibrosis and NASH are more advanced (van der Poorten et al., 2013), as observed in elderly patients (Koehler et al., 2012; Noureddin et al., 2013). Different protective mechanisms might be responsible for the age-dependent decrease of liver steatosis and include increased serum adiponectin levels (van der Poorten et al., 2013), the onset of portosystemic shunting (Nosadini et al., 1984), metabolic changes of mitochondria (Caldwell, Crespo, 2004), and inflammatory and catabolic state of cirrhosis (McCullough, Raguso, 1999), as well as collagen deposition in the liver (Noureddin et al., 2013). In the serum, levels of cholesterol, HDL cholesterol, and triglycerides also increase with age. This trend is reverted in individuals older than 90 years old (Tietz et al., 1992). Notably, LDL—cholesterol metabolism rate is decreased by over 30% with age, as a consequence of a decrease in expression of LDL receptors (Miller, 1984). Aging significantly enhances the progression of NAFLD to NASH, and to fibrosis, leading to the conditions that predispose to increase mortality in elderly subjects with NAFLD (Regev, Schiff, 2001). Age-dependent decline of fatty acid β-oxidation and reduced expression of hepatic nuclear receptor peroxisome proliferator—activated receptor may be potential mechanisms (Sanguino et al., 2004). An additional mechanism is the age-dependent increase of the β-adrenergic signaling that is able to drive liver steatosis (Ghosh et al., 2012; Katz et al., 1993). In addition, p300-dependent regulation of chromatin structure during aging is responsible for the activation of five key genes that govern triglyceride synthesis in the liver (Jin et al., 2013). Markers of hepatocyte senescence are also associated with NAFLD (Aravinthan et al., 2013; Park et al., 2010). The link between increased intrahepatic diacylglycerol (DAG) and PKCε activation

is essential, as LCFA are oxidized at both mitochondrial and extramitochondrial sites in the hepatocytes. Excessive incorporation of LCFA will increase ROS and mediate hepatocellular and mitochondrial injury (Diogo et al., 2011; Grattagliano et al., 2011, 2013; Zhu et al., 2017).

Mitochondria contribute to the progression of NAFLD (Grattagliano et al., 2004b), a condition characterized by increased predisposition toward prooxidant insults (Pessayre, Fromenty, 2005). One pathway that limits excessive fat accumulation in the liver is the increased mitochondrial oxidation of LCFA, a step associated with an impaired respiration (Fromenty, Pessayre, 1995). Fatty degeneration exposes hepatocytes to a higher risk of oxidative damage, although a number of adaptive metabolic mechanisms have been described (Grattagliano et al., 2003; Yang et al., 2000). Mechanisms include expression of intracellular sensors and signaling molecules for lipid metabolism and oxidative stress pathways (Merriman et al., 2006; Sanyal et al., 2001). Impairment of these systems may have important pathogenic roles in NAFLD progression, including disturbed ATP synthesis (Cortez-Pinto et al., 1999). mtDNA levels, protein expression, and activity of respiratory complexes are also decreased in liver mitochondria (Haque, Sanyal, 2002; Perez-Carreras et al., 2003), pointing to a role for oxidative stress mechanism.

Hepatic steatosis indeed causes cellular damage and ROS production. Our group has shown that rat hepatoma FaO cells loaded with oleate/palmitate to mimic liver steatosis resulted in higher production of ROS and lipid peroxidation, stimulation of catalase activity, and activation of NF-κB. Lipid droplet accumulation also increased levels of peroxisome proliferator—activated receptors (PPARs) and sterol regulatory element—binding protein-1c (SREBP-1c) (Vecchione et al., 2016). In the intact hepatocyte HepG2 cells incubated with saturated fatty acids (a model resembling NASH), mitochondrial function was depressed together with inhibition of mtDNA gene expression and accelerated degradation of respiratory chain subunits (Garcia-Ruiz et al., 2015). In line with these results, we recently showed that sequential exposure of hepatocytes to high concentrations of fatty acids (FAs) and TNF-α mimics in vitro the progression of NAFLD from simple steatosis to steatohepatitis. Several damages were observed at a mitochondrial level and elsewhere in the hepatocyte (reduced hepatocyte viability, increased apoptosis and oxidative stress, reduction in lipid droplet size, and upregulation of I-κB kinase—interacting protein and adipose triglyceride lipase expressions). Notably, silybin, the extract of the milk thistle seeds counteracted the FA-induced mitochondrial damage, increased the

mitochondrial size and improved the mitochondrial cristae organization; stimulated mitochondrial FA oxidation; reduced basal and maximal respiration and ATP production in steatohepatitis hepatocytes, stimulated ATP production in steatotic cells, and rescued the FA-induced apoptotic signals and oxidative stress in steatohepatitis hepatocytes (Vecchione et al., 2017). In steatotic livers, ROS formation is increased at the mitochondrial respiratory chain level and determines oxidation of unsaturated lipids (Grattagliano et al., 2003, 2008; Yang et al., 2000). The activity of complex I of the respiratory chain is also reduced (−35%) in mitochondria from fatty livers and is associated with changes in state 3 respiration (Petrosillo et al., 2007); hydrogen peroxide generation and oxidized cardiolipin are significantly increased (Grattagliano et al., 2008; Petrosillo et al., 2007). ROS affect the mitochondrial complex I activity by oxidizing cardiolipin, which is required for the function of this enzyme complex (Paradies et al., 2002).

Oxidation, glutathionylation, and nitrosylation of mitochondrial proteins occur as a response to oxidative stress and result in posttranslational modification of proteins by carbonyl and disulfide formation or by thiol nitrogen exchange. Factors contribute to a block of the electron flow in the respiratory chain resulting in subsequent generation of ROS. This vicious circle involves ROS-mediated antioxidant depletion and the deficient capacity of mitochondria to inactivate ROS (Grattagliano et al., 2003). Ultimately, protein and lipid oxidation, and cytokine production are increased. Hepatocytes react to fat deposition with an early increase of GSH and thioredoxin to prevent lipid and protein oxidation (Grattagliano et al., 2008). Also, increases of protein mixed disulfides (PSSG), nitrates, and nitrosothiols are consistent with both prooxidant protein modifications and increased nitric oxide (NO) synthesis. A critical role for mitochondrial GSH in the development of NASH was in fact proposed (Garcia-Ruizet al., 2006). GSH depletion sensitizes hepatocytes to inflammatory cytokines and TNF-α. Mitochondrial GSH content declines more rapidly than cytosolic GSH, suggesting mitochondria as specific early target for oxidative changes (Grattagliano et al., 2008).

Other pathogenic factors, including nitric oxide, play a role for the progression of liver steatosis and appearance of fibrosis. Thioredoxin, a redox active protein regulates of PSH/PSSG ratio. Thioredoxin is actively involved in the regulation of NO activity via cleavage of nitrosothiols (Nikitovic, Holmgren, 1996; Stoyanovsky et al., 2005), which are formed by conjugation of NO with free thiols and oppose dangerous reactions such as peroxynitrite formation. Nitrosothiols also act as intracellular

messengers that control mitochondrial functions (Arnelle, Stamler, 1995; Grattagliano et al., 2004a). Major alterations of thioredoxin levels have been observed with ongoing liver steatosis and have been associated with PSSG and nitrosothiols formation (Garcia-Ruiz et al., 2006). Increased peroxynitrite formation is associated with a variety of interactions, including protein nitration and generation of nitrotyrosine (Sanyal et al., 2001).

Additional mechanisms of mitochondrial damage include increased production of angiotensin II associated with oxidative stress. Animals with elevated endogenous angiotensin II levels display mitochondrial alterations with reduced β-oxidation and consequent decreased mitochondrial palmitate oxidation, decreased enzymatic activities, and expression of mitochondrial proteins, including cytochrome *c*, cytochrome *c* oxidase subunit 1, and TFAM. Administration of angiotensin II receptor blockers or superoxide dismutase/catalase mimetic treatment improves these abnormalities (Wei et al., 2009). Fatty livers show about 35% decrease of catalytic β-F1 subunit of the F0F1-ATP synthase. The process of aging might also influence other pathways in the steatotic liver. Under starvation, mitochondrial oxidative injury is exacerbated to a greater extent in fatty livers. In the steatotic liver, fasting induces a further decrease of the ATP levels, which is accompanied by a 70% fall of the catalytic β-F1 subunit. These changes may account for the observed reduction in the synthesis of ATP (Fernández-Checa et al., 1998). Liver steatosis is also favored by prolonged intake of drugs such as amiodarone or valproate (Berson et al., 1998), and aging is a strong risk factor for medication-induced damage. Amiodarone or valproate accumulates in mitochondria and induces inhibition of fatty acid oxidation and electron transfer chain (Berson et al., 1998). In support of the key role of mitochondria in NAFLD, recent data indicated the critical role of the mitochondrial pyruvate carrier MPC, a heterologous complex made of MPC1 and MPC2 proteins in the inner mitochondrial membrane (Colca et al., 2017). The complex is required for the entry of pyruvate that is synthetized in the cytosol, in the mitochondrial matrix, where it will be further metabolized. MPC might become therefore the target for treatment of several metabolic and inflammatory diseases, namely diabetes (Chen et al., 2012b; Colca et al., 2013; McCommis et al., 2015), and even NASH (McCommis et al., 2016).

Although the abovedescribed observations suggest that fatty livers have compromised mitochondrial function, there are several evidences that the initial stages of the NAFLD condition are associated with an increase in mitochondrial mass, with or without increased mitochondrial fatty acid

oxidation, and which serves as an adaptation response to excessive accumulation in the liver. This was elegantly demonstrated in human liver biopsies by Koliaki et al. (2015). This paper showed that when compared with isolated mitochondria from lean individuals, their counterparts from obese humans with or without NAFLD had 4.3- to 5.0-fold higher maximal respiration rates, despite similar mitochondrial content. In opposition to this, and despite the fact that NASH patients featured higher hepatic mitochondrial mass, a 31%–40% lower maximal respiration associated with greater hepatic insulin resistance, mitochondrial uncoupling, and leaking activity was described.

Initial adaptation to a fatty-rich environment contributes to enhance fatty acid oxidation, which means that hypothetically, accelerating the rate of fatty acid burn by mitochondria could be an effective therapeutic strategy. In fact, a liver-targeted mitochondrial protonophore was described to promote a mild depolarization of the inner membrane, contributing to increase electron transfer and fatty acid oxidation. In rodent models for NAFLD and diabetes type 2 (T2D), this approach appears to reverse hypertriglyceridemia, hepatic steatosis, insulin resistance, and hyperglycemia (Perry et al., 2013). Another approach involved adenovirus-mediated liver expression of a malonyl-CoA-insensitive CPT1A (CPT1mt) in a high fat/high sugar animal model, with the ultimate objective of accelerating mitochondrial fatty acid β-oxidation. This approach was able to reverse insulin resistance and glucose intolerance, although not affecting steatosis (Monsenego et al., 2012). The important role of mitochondria in the context of NAFLD and its progression to NASH is well demonstrated by two recent findings. One of them demonstrates that mtDNA, a proinflammatory molecule per se (Boyapati et al., 2017; Zhang et al., 2016), when released from fatty liver hepatocytes, causes liver inflammation by TRL-9 activation (Garcia-Martinez et al., 2016), which can be an important component of the transition between NAFLD and NASH. In another interesting development, it was recently demonstrated that when mitochondria isolated from hepatoma cells were injected into rodents with fatty liver, the phenotype was improved. Because exogenous mitochondria were tagged with green-fluorescence protein (GFP), it was possible to demonstrate accumulation in the mouse liver, lung, brain, muscle, and kidney. How mitochondria entered the different cells and were able to maintain the integrity and restore metabolic activity was not explained.

2.1.2 Bile Mitochondria and Aging

Bile is an aqueous solution containing organic solutes, inorganic electrolytes, and trace amounts of proteins. The three classes of biliary lipids include

unesterified cholesterol, phospholipids, and bile acids (i.e., primary bile acids: cholic and chenodeoxycholic acid; and secondary bile acids derived from 7α-dehydroxylation of the primary bile acids in the liver and by intestinal bacteria in the ileum and colon: deoxycholic, lithocholic, ursodeoxycholic, sulfolithocholic, and 7α-oxo-lithocholic acids) (Di Ciaula et al., 2017). Earlier studies have shown that hepatobiliary function as assessed by bile flow and bile acid secretion decline with age in male inbred rats (Schmucker et al., 1985). Moreover, a number of liver enzymes are involved and affected by aging (Schmucker, 2001). Choi et al. (1987) have found a significant decrease in the enzymatic activity of cholesterol 7α-hydroxylase in rats 5–32 weeks of age. In the study by Wang (2002), gallstone-susceptible C57L mice and resistant AKR mice of both genders split into young adult, older adult, and aged groups (8, 36, and 50 weeks of age, respectively) were each fed a lithogenic diet for 8 weeks. Increasing age augments biliary secretion and intestinal absorption of cholesterol, whereas reducing hepatic synthesis and biliary secretion of bile acids. Einarsson et al. (1985) analyzed biliary lipid composition in 60 healthy lean and gallstone-free subjects of various ages and both genders. They found a positive correlation between age and cholesterol saturation index and a negative correlation with bile acid synthesis and the pool size of the cholic acid. A role for a decline in the enzymatic activity of cholesterol 7α-hydroxylase and 7α-hydroxylated cholesterol (as a marker of bile acid synthesis) has been advocated in humans by the study of Bertolotti et al. (1993). By contrast, Valdivieso et al. (1978) found increased proportion of biliary cholesterol and lithogenic index in gallbladder bile of elderly female Chilean patients without significant changes in bile acid metabolism.

Additional age-dependent changes in lipid metabolism include a decrease of hepatic clearance of HDL-cholesterol in older rats (Bravo et al., 1994). Lipophilic and secondary bile acids, moreover, are able to change the membrane composition of hepatocyte mitochondria (where the inner membranes harbor the cytochrome P450 oxidoreductase system). Thus, changes in the membrane fatty acid composition lead to a decrease of the activity of the mitochondrial enzyme system; this step makes enzymes, defective in handling free radicals that originate during the normal process of energy production and detoxification. As a consequence, free radicals may attack membrane PUFAs to initiate and propagate lipid peroxidation, leading to formation of aldehydic lipid peroxidation products (Pandey, Shukla, 2000).

2.1.3 Gallstones, Mitochondria, and Aging

Increasing age is associated with increased risk of gallbladder stones of either types, i.e., cholesterol and pigment stones (Palasciano et al., 1989; Portincasa et al., 2006; Portincasa, Wang, 2015, 2016). It is estimated that the prevalence of gallstone disease is about 50% in females aged 70–75 and in males aged 80–85 (Diehl, 1991; Sama et al., 1990). Pigment stones account for about 20%–25% of all gallstones and form as a consequence of abnormalities in bilirubin metabolism arising in the gut–liver axis. The most common risk factors are hemolytic anemias, liver cirrhosis, Crohn's disease, or extended ileal resection, biliary infection, cystic fibrosis, vitamin B12/folic acid–deficient diets, and genetic factors due to UGT1A1 mutation. Age per se represents a risk factor for such biliary diseases. Cholesterol gallstones represent about 75%–80% of all gallstones in western societies and are due to at least five pathogenetic defects: (1) genetic factors and *LITH* genes; (2) hepatic hypersecretion of biliary cholesterol, leading to nonphysiological and sustained supersaturation of gallbladder bile with cholesterol; (3) accelerated phase transitions of cholesterol in bile; (4) hypomotile gallbladder harvesting the immune-mediated inflammation, as well as hypersecretion and accumulation of mucin gel in the lumen; and (5) increased absorption of cholesterol at the small intestinal enterocyte level (Wang et al., 2017a; Wang et al., 2017b; Wang and Portincasa, 2017, pp. 1–676).

A more detailed analysis of the elegant study by Wang (2002) found that on the lithogenic diet, cholelithiasis prevalence, gallbladder size (i.e., a marker of gallbladder hypomotility and stasis), biliary lipid secretion rate, and HMG-CoA reductase activity (i.e., a marker of cholesterol biosynthesis in the liver) are significantly greater in C57L mice of both genders compared to those in AKR mice. The activity of cholesterol 7α-hydroxylase (i.e., a marker of hepatic bile acid synthesis) is significantly lower in C57L mice than in AKR mice. Of note, increasing age augments biliary secretion and intestinal absorption of cholesterol, while reducing hepatic synthesis and biliary secretion of bile acids, and decreasing gallbladder contractility. Putting these data together, it is clear that all the abovementioned age-dependent factors greatly increase susceptibility to cholesterol cholelithiasis in C57L mice. Mitochondria might play a role in some steps of lithogenesis. Biliary proteins and their redox status have been demonstrated by our group in gallstone patients undergoing cholecystectomy and serial analyses of bile composition and crystallization (Grattagliano et al., 2009). The role of gallbladder contractility during fasting and postprandially is essential in preventing stasis of cholesterol supersaturated bile and precipitation of either

cholesterol crystals or bilirubinate pigments (Di Ciaula et al., 2017; Portincasa et al., 1994, 2004, 2006). Mitochondrial Ca^{2++} handling is implicated in spontaneous rhythmic activity in smooth muscle and interstitial cells of Cajal. Indeed, disruption of the MMP (by carbonyl cyanide 3-chlorophenylhydrazone, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, rotenone, and antimycin A) reduced or eliminated action potentials, Ca^{2++} flashes, and Ca^{2++} waves in typical of the gallbladder smooth muscle. Data suggest that mitochondrial Ca^{2++} handling accounts for spontaneous electrical activity, a step involved in gallbladder tone and motility (Balemba et al., 2008). Gallbladder mitochondria are also involved in other pathways such as drug-mediated effects on apoptosis during tumorigenesis in cells and mice (Bao et al., 2014; Li et al., 2015a; Liu et al., 2013; Shu et al., 2014; Wang et al., 2014; Weng et al., 2014) and treatment with oxysterols causing cytochrome *c* release in the dog (Seo et al., 2004). Both aspects are potentially linked to the aging process of the gallbladder with or without stone-formation ability. Accumulation of gut microbiota—derived secondary bile acids in the aging, hypomotile gallbladder, moreover, by virtue of their lipophilic action, might change the membrane composition of and contribute to gallbladder carcinogenesis (Pandey, Shukla, 2000).

Epidemiological and clinical investigations have clearly demonstrated that during the aging process, a person progressively loses the ability to maintain normal physiological functions due to structural alteration of cells or dysfunction of vital organs such as the liver. Aging is a major risk factor for most chronic hepatobiliary disorders because the volume and blood flow of the liver progressively reduce with age. As the elderly population is increasing due to an extended life span, the number of elderly patients with complicated hepatobiliary diseases has grown in the past decades. New and effective strategies for enhancing liver functions should be extensively investigated to improve life quality of human beings.

2.2 Copper Toxicity in Age-Related Diseases and Wilson Disease

The redox active transition metals, copper and iron, are fundamental for vital enzymes (Festa, Thiele, 2011; Winter et al., 2014). However, on overload they might become cell toxic if uncontrolled redox activity occurs. Here, we restrict ourselves to the detailed discussion of copper. Aqueous free copper (and iron) may catalyze the formation of hydroxyl radicals ($^{\bullet}OH$) via Fenton— and Haber—Weiss-based chemistry causing the subsequent damage to proteins, DNA, and lipids that result in cell death

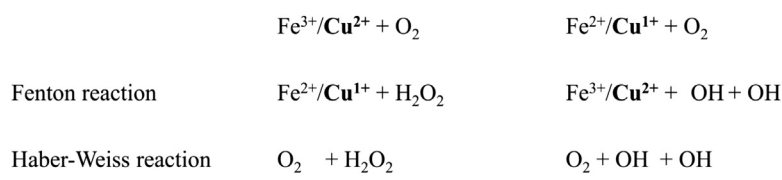

Figure 5 ***Fenton and Haber–Weiss reaction.*** In the presence of catalytic amounts of trace metals (such as iron and copper), highly reactive hydroxyl radicals (˙OH) are generated from hydrogen peroxide via the Fenton and Haber–Weiss reaction.

(Fig. 5) (Leonard et al., 2004). However, under physiological conditions, this redox activity is prevented by the tight incorporation of copper into the active sites of various vital enzymes that results in the absence of intracellular free copper pools (Kaim, Rall, 1996; Lippard, 1999; Rae et al., 1999; Rubino, Franz, 2012). At present, 54 copper-binding or transporting proteins have been identified in the human proteome. Together they ensure the safe transport of the transition metal to the target enzymes (Blockhuys et al., 2017). Protein copper binding occurs mainly via cysteine residues (e.g., metallothionein) but can also be performed by histidine (e.g., ceruloplasmin) or methionine (e.g., copper transporter Ctr1) residues (Koch et al., 1997). In cells, the most important copper-dependent enzyme resides in mitochondria, the cytochrome *c* oxidase (complex IV) that is responsible for proper cell respiration and oxidative phosphorylation (Wainio et al., 1959). In serum, the transition metal is mainly present tightly bound to the ferroxidase ceruloplasmin (around 70%), with a small part (around 30%) available as so-called "free" or "loosely bound" copper associated to amino acids or albumin (Linder, 2016).

Under pathological situations, the tight regulation of copper uptake, distribution and excretion can be disturbed leading to increased free copper levels that are highly detrimental to cells. Age-related diseases such as AD, PD, diabetes, cardiovascular diseases, and cancer have been suggested to be associated with increased serum free copper levels (Brewer, 2010). For example, in AD, high free copper serum levels (e.g., due to increased copper uptake via drinking water) were linked to an increased risk for cognitive decline (Sparks, Schreurs, 2003; Squitti, Polimanti, 2013). It was shown that copper interacts with the characteristic beta-amyloid plaques resulting in increased ROS production and neuronal death (Huang et al., 1999). Besides this "direct" impact of copper on AD progression, copper was also found to oxidize low-density lipoprotein receptor–related protein responsible for the efflux of beta-amyloid from the brain (Singh et al., 2013).

However, there are also conflicting studies reporting a reduced beta-amyloid production by addition of dietary copper in mice (Bayer et al., 2003) and a correlation between cognitive decline and low copper plasma concentrations in patients with mild to moderate AD (Pajonk et al., 2005). Additionally, high serum copper levels may be associated with an increased risk for several types of cancer, e.g., lung cancer, neoplastic kidney tissue, leukemia, and hepatocellular carcinoma (Hrgovcic et al., 1973; Karcioglu et al., 1978; Mateo et al., 1979; Poo et al., 2003). Whether high copper levels are a cause or "bystander" effect of these malignancies is still under investigation. Thus, at present, future studies have to further substantiate the link between free copper and age-related diseases.

In contrast to the mentioned diseases, copper overload (especially in the liver) is a well-established cell-death causative feature in Wilson disease (WD) (Bearn, 1953; Cumings, 1948). In WD, the hepatocyte demise is linked to copper-induced mitochondrial dysfunction. WD is an autosomal inherited disorder caused by mutation(s) in the gene encoding for the copper transporting ATPase ATP7B (Gitlin, 2003; Tanzi et al., 1993). In hepatocytes, this protein facilitates the transport of excess copper into the bile (Bull et al., 1993). In WD patients, the dysfunction of ATP7B results in the accumulation of massive amounts of copper, primarily in the liver and brain. If untreated, WD is fatal (Liver., 2012).

Mitochondria were identified as first responders in WD patients and in WD animal models. In WD, mitochondria present with dramatically increased copper levels (humans (Sokol et al., 1994), animal models (Lichtmannegger et al., 2016; Zischka et al., 2011)), structural abnormalities ranging from distorted cristae structure to electron-dense inclusions and detachment of the inner and outer membrane (humans (Sternlieb, 1968, 1992; Sternlieb, Feldmann, 1976), animal models (Huster et al., 2006; Lichtmannegger et al., 2016; Roberts et al., 2008; Sternlieb et al., 1995; Yurkova et al., 2011; Zischka et al., 2011)), decreased ETC complex activities (humans (Gu et al., 2000), animal models (Roberts et al., 2008; Sauer et al., 2011)), and a reduced ATP production capacity (animal models (Lichtmannegger et al., 2016)).

These structural and functional alterations are accompanied by the direct attack of copper on mitochondrial protein/peptide thiol residues (Nakamura, Yamazaki, 1972; Zischka et al., 2011). Moreover, treating intact liver mitochondria with copper (in the presence of reducing agents such as DTT or GSH) resulted in WD mitochondrial phenotypes (Zischka et al., 2011). This latter finding questions the hypothesis that ROS (resulting from

copper-based Fenton reaction) are primarily causative for mitochondrial structure changes, although such ROS might be relevant in subsequent mitochondrial destruction (Lichtmannegger et al., 2016; Zischka et al., 2011). It rather appears that thiol residues not only in scavenging proteins such as metallothionein but also in vulnerable enzymes such as the ATP synthase (complex V) are prime copper targets (Duncan et al., 2006; Yagi, Hatefi, 1987). Detrimental outcomes by such mitochondrial functional depletion together with persistent copper overload may consequently pave the way for further mitochondrial and subsequently cellular destruction.

Studies in WD patients described a reduced antioxidative defense system (reduced GSH, GST, SOD1/2) (Bruha et al., 2012; Nagasaka et al., 2006; Summer, Eisenburg, 1985), decreased levels of Vit-E in the serum (that correlated with free copper serum level) (von Herbay et al., 1994), and mtDNA deletions (Mansouri et al., 1997). Additionally, aconitase activity, a classical mitochondrial marker for ROS damage to proteins was reduced (Gu et al., 2000). These findings were further confirmed by studies in WD animal models. Here, an increased lipid peroxidation (Kumar et al., 2016; Ohhira et al., 1995; Rui, Suzuki, 1997; Samuele et al., 2005; Yamada et al., 1992; Yamamoto et al., 1999), DNA damage (Chung et al., 1999; Nair et al., 1996; Yamamoto et al., 1993; Yu et al., 2016), decreased antioxidative defense system (GPx, GSH) (Kumar et al., 2016; Samuele et al., 2005; Yamamoto et al., 1999), and reduced free protein thiols were described (Samuele et al., 2005; Zischka et al., 2011). Thus, oxidative damage is clearly associated with WD. However, it needs to be emphasized that these features mostly appear in WD patients or animal models at severe liver damage, i.e., at late disease stages.

Importantly, diverse therapy strategies addressing increased oxidative stress in WD were evaluated in animal models, in particular the LEC rat. Here, a surplus of Vit-E resulted in later onset of hepatitis and reduced lipid peroxidation in male rats compared to control animals (Yamazaki et al., 1993). In contrast, Hawkins et al. described no effect of Vit-E and β-carotene but a delayed onset of jaundice and decreased mortality in animals with enforced administration of proline (80%) and Vit-C (65%) (Hawkins et al., 1995). Another strategy used by Yamashita et al. (1996) was the use of the spin-trapping antioxidant phenyl butyl nitrone that resulted in reduced lipid peroxides and delayed hepatitis and mortality. This finding was further underlined by studies of Asanuma et al. (2007), who described delayed hepatitis, reduced lipid peroxidation, and decreased

oxidative DNA damage in treated animals, while the liver copper level was unaltered. Additionally, unsaturated fatty acids, e.g., linolenic acid and linoleic acid, reduced the incidence and onset of hepatitis (Shibata et al., 1999) and increased animal survival by stimulation of bile acid synthesis (Du et al., 2004). However, curcumin (Frank et al., 2003), quercetin, and phytic acid (Kitamura et al., 2005) failed to increase survival or reduce liver damage. In contrast, DL-alpha lipoic acid, a dithiol-containing cofactor of the pyruvate dehydrogenase complex of mitochondria, reduced liver damage and increased the antioxidative defense capacity by upregulation of glutathione peroxidase and reductase (Yamamoto et al., 2001). Additionally, NAC, a prodrug to cysteine and therefore a precursor for glutathione, reduced liver copper and overall liver damage mainly due to metal chelation rather than ROS scavenging (Kitamura et al., 2005). Additionally, fermented brown rice (Shibata et al., 2006) and coffee (Katayama et al., 2014) were tested to reduce liver damage in LEC rats. Both treatment options were able to prolong survival. Whereas fermented brown rice reduced the incidence of hepatitis, coffee delayed the onset of hepatitis in treated animals compared with untreated rats.

In conclusion, the redox-active transition metal copper may be highly detrimental to cells on toxic overload. While such a role is currently under debate in age-related diseases such as AD, there is ample evidence of its destructive power on hepatic mitochondria in WD. With respect to the toxic mode of action of copper on mitochondria, susceptible thiol residues appear as early copper targets. In agreement, treatment regimens that affect the intracellular thiol status (DL-alpha lipoic acid, NAC) were able to increase survival by delaying hepatitis onset or decreasing hepatitis incidence. In contrast, radical scavengers that would counteract a (Fenton chemistry–based) ROS burden (e.g., Vit-E, phenyl butyl nitrone) only showed slight effects on WD progression. Nevertheless, such oxidative stress damage may be present in WD patients and animal models with late-stage disease.

2.3 Mitochondria, Mitochondrial-Associated Membranes, ROS, and Diabetes in Aging

Type 2 diabetes is multifactorial disorder characterized by chronic hyperglycemia due to impaired insulin secretion from pancreatic β-cells and insulin resistance in target tissues. Nowadays, it is estimated that up to 70%–90% of patients with T2D are overweight or obese with aging being an important contributing factor (Al-Goblan et al., 2014). Particularly, excessive lipid intake or age-related prolonged ectopic fat deposition in tissues is believed

as the primary reason for insulin resistance development, the islet dysfunction, and disease progression (Janikiewicz et al., 2015; Szymanski et al., 2017). There is now ample evidence that diabetes and obesity-related metabolic dysfunction can accelerate the progression of other age-related diseases and pathologies in both mouse models of disease and normally aging mice (Butterfield et al., 2014; Farr et al., 2008; Morrison et al., 2010). For example, several reports have shown high fat diets, or insulin resistance, can accelerate learning and memory loss as well as neurodegeneration in different mouse models of AD (Kadish et al., 2016; Knight et al., 2014; Maesako et al., 2015; Morrison et al., 2010; Petrov et al., 2015). It is thus possible that obesity-related insulin resistance may be accelerating the aging progression (Salmon, 2016), and mitochondria and oxidative stress may play important role in this process.

Mitochondria-associated membranes are important hubs for insulin signaling due to several proteins that were detected at the MAM location, including protein kinase AKT, mTORC2, phosphatase, and tensin homologue deleted on chromosome 10 (PTEN) (Tubbs, Rieusset, 2017); however, such interconnection awaits further investigations. As T2D is associated with alterations in lipid metabolism and ER—mitochondria contact sites foster lipid species exchange between these organelles, it is a favorable hypothesis that MAM integrity and action participate to lipotoxicity in diabetes. In fact, MAM integrity was required for insulin signaling and it was altered in palmitate-induced insulin-resistant HuH7 hepatic cells, as well as in liver of leptin-deficient ob/ob mice and high-fat and high-sucrose fed mice. Furthermore, disruption of MAM integrity by genetic or pharmacological inhibition of MAM-residing protein cyclophilin D induced insulin resistance in animals and led to aberrant insulin signaling in human primary hepatocytes. Enhancement of MAM formation restored hepatic insulin signaling and action of HuH7 cells and in diabetic mice (Tubbs et al., 2014). In addition, an abnormal increase in MAM formation was reported in livers of ob/ob, and high-fat fed mice, alongside mitochondrial calcium overload, compromised mitochondrial capacity, and augmented oxidative stress (Arruda et al., 2014).

Oxidative stress is considered one of the major risk factors in the onset and progression of T2D. Since the 1960s (Giugliano et al., 1996) hyperglycemia has been hypothesized to contribute to oxidative stress either by direct production of ROS (Nishikawa et al., 2000) or by altering the redox balance. It has been demonstrated that hyperglycemia induces an increased polyol pathway flux and increased intracellular formation of advanced

glycation end products and contributes to an oxidative stress environment by activating PKC and causing overproduction of superoxide via mitochondrial ETC (Brownlee, 2001). As emphasized in this review, mitochondria play an important role in the maintenance of cellular redox status, thereby acting as an ROS and redox sink and limiting NADPH oxidase activity. The main sources of mitochondrial ROS under physiological conditions are complexes I and III, which produce $O_2^{\bullet -}$ mainly on the matrix side, where it is rapidly transformed into H_2O_2 by SOD2. Brownlee and colleagues proposed that the mitochondrial transport chain plays a key role in hyperglycemia-induced overproduction of superoxide and in the development of secondary complications such as endothelial dysfunction (Brownlee, 2005). Using ρ^0 cells lacking functional ETC, they demonstrated that the effect of hyperglycemia on ROS production was completely lost. Another study by Nishikawa discovered that T2D alters the primary site of superoxide generation such that complex II becomes the primary source of electrons contributing to superoxide formation under diabetic conditions (Nishikawa et al., 2000). Yu et al. (2006) found that dynamic changes in mitochondrial morphology are an important factor contributing to ROS overproduction under high glucose (HG) conditions. Mitochondria become rapidly fragmented in HG concentrations with a concomitant increase of ROS. These findings suggest that mitochondrial dynamics may influence ROS overproduction in diabetes, obesity, and other related disorders.

Conventional antioxidants neutralize ROS on a one-to-one basis, whereas hyperglycemia-induced overproduction of superoxide is a continuous process. Riley (Salvemini et al., 1999) proposed a novel type of antioxidant, a catalytic antioxidant, such as SOD/catalase mimetic, which works continuously, similar to the enzymes for which these compounds are named. However, SOD is not yet in widespread use in human clinical medicine due to some obstacles: none of the three human SODs possess the necessary pharmacological properties to make it a clinically useful therapeutic agent (McCord, Edeas, 2005).

Another source of ROS in diabetes is NADPH oxidase. This enzyme has been implicated as a major source of ROS generation in the vasculature in response to high glucose and advanced glycation end products (Thallas-Bonke et al., 2008). This study suggests that blockade of NADPH oxidases is a valid intervention to consider for combating established diabetic nephropathy. A study by Li and Shah (2003) supports the theory that NADPH is a mediator of diabetic complications and its action can be suppressed by a variety of PKC inhibitors, implicating this family of kinases in the regulation of

hyperglycemia-induced NADPH oxidase activity. Production of ROS from mitochondria has received great attention, and it is now becoming clear that it may be regulated under physiological conditions and plays and important role in redox signaling. A recent work by Darley-Usmar and colleagues (Chacko et al., 2010) hypothesize that MitoQ, the most-studied mitochondria-targeted antioxidant, decreased mitochondrial ROS production and showed beneficial effects in diabetic nephropathy. In this study, $Ins2^{+\backslash -AkitaJ}$ mice were selected as a model of diabetes, showing a dysfunction in pancreatic β-cells, to test the potential benefits of MitoQ therapy. This mouse model develops insulin resistance over time and has many of the characteristics of chronic hyperglycemia (Barber et al., 2005; Bugger et al., 2008). Of the potential compounds available, the ubiquinone analog MitoQ has received particular attention, as it is orally bioavailable, has low toxicity, and reaches concentrations of 200–700 pmol/g of weight in the tissue of a number of organs, including the kidney. The treatment seems to protect mitochondrial redox signaling from hyperglycemia-induced alterations and would thereby ameliorate diabetic nephropathy. Another study by Wang and colleagues proposes the use of vanadium compounds in therapeutic treatment of diabetes and in cancer prevention (Zhao et al., 2010).

Mitochondria also play an important role in β-cell function as insulin release in response to glucose levels and in the sensing of oxygen tension in the carotid body and pulmonary vasculature, two events involved in diabetes pathologies (Duchen, 2004). One of the hypotheses for induction of β-cell dysfunction focuses on changes in the expression and function of UCP2. The inner mitochondrial membrane UCPs are thought to be major facilitators of uncoupled respiration and may modulate the pathophysiology of diabetes (Sack, 2006). It has been proposed that UCP activity and expression contribute to an increase in superoxide formation under diabetic conditions (Krauss et al., 2003). UCP2 is thought to negatively regulate glucose-stimulated insulin secretion by reducing the amount of ATP produced (Fig. 6). Another member of this family, UCP3, seems to have a positive role in T2D. This isoform, very enriched in skeletal muscle, is downregulated in pathologic condition and conversely increased in response to exercise training. UCP3 confers resistance against oxidative stress and the genetic deficiency of UCP3 in primary skeletal myocytes results in excess ROS levels under normoxic and hypoxic conditions (Luet al., 2008). To test whether the induction of UCP3 could modulate insulin sensitivity, a skeletal muscle transgenic mouse line harboring the human UCP3 gene was created (Clapham et al., 2000). These mice are hyperphagic and lean

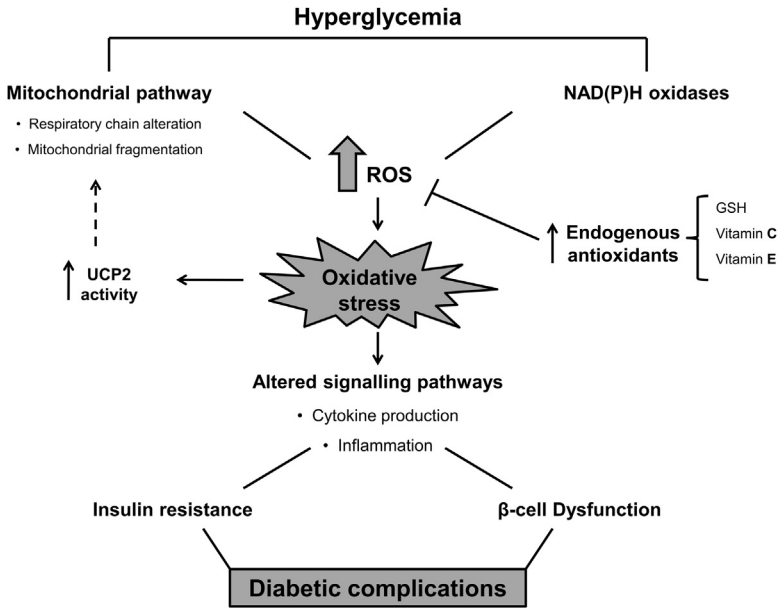

Figure 6 ***Putative pathways linking oxidative stress, mitochondrial dysfunction, and hyperglycemia.*** *GSH*, glutathione; *ROS*, reactive oxygen species; *UCP2*, uncoupling protein-2.

with diminished adipose tissue mass, a phenotype consistent with uncoupled mitochondrial oxidative phosphorylation. Despite this, knockout mice for UCP3 showed some divergent effects, i.e., glucose tolerance improvement, suggesting how the beneficial role of UCP3 may be skeletal muscle specific (for an intriguing discussion on molecular manipulation of mitochondrial metabolism and diabetes, see Pagel-Langenickel et al., 2010).

Cells and tissues contain antioxidant defense mechanisms, which maintain their redox balance and aid in preventing the accumulation of ROS. T2D is associated with reduced levels of antioxidants such as GSH, Vit-C, and Vit-E (Jain, 1998). Thus, antioxidant therapy has been of great interest as a means to combat oxidative stress in diabetic patients. However, these treatments lack broad therapeutic effect across the patient population (Ceriello, Testa, 2009). Nevertheless, the current data rise an important, and rather unexplored, question of whether antioxidant treatment could be used in obese patients with metabolic dysfunction to prevent the progression of additional comorbidities or slow the acceleration of the aging process caused by insulin resistance and chronic inflammation.

2.4 Mitochondria, ROS, Cardiovascular Pathology, and Aging

Cardiovascular diseases (CVDs) are multifactorial, but several lines of evidence suggest that mitochondrial dysfunction contributes to their pathophysiology. The underlying mechanisms appear to involve not only damage to the organelle and loss of bioenergetic function but also disruption of mitochondrion-dependent redox-signaling pathways. Mitochondria are considered as a major source of ROS, and loss of control of their formation leads to mitochondrial oxidative damage and dysfunction; mitochondria are also important targets for ROS. ROS can lead to the activation of pathways that control cell differentiation and apoptosis, both of which are mechanisms of particular relevance to CVDs. Indeed, mitochondrial oxidative damage has been implicated in a range of degenerative conditions that include CVDs, namely atherosclerosis, hypertension, heart failure, and ischemia/reperfusion (I/R) injury (Fig. 7) (Delles et al., 2008; Di Lisa, Bernardi, 2006; Madamanchi, Runge, 2007; Misra et al., 2009).

Some of the most compelling evidence that mitochondrial ROS is a causative agent in the development of CVDs in vivo comes from experiments using transgenic mice to alter expression of mitochondrial antioxidant proteins. Initial experiments using genetic knockouts showed that mice lacking SOD2 produce huge amounts of mitochondrial ROS and develop cardiomyopathy within the first weeks of birth (Li et al., 1995; Schriner et al., 2005). Nowadays, it is widely accepted that deficiencies in mitochondrial antioxidants and/or regulatory proteins that modulate mitochondrial oxidant production promote the onset of CVDs. In a recent work Nox4, a member of the NADPH oxidases (Nox) family expressed primarily in the mitochondria in cardiac myocytes, was reported to be a major source of superoxide production in the cardiovascular system. Nox4 mediates cardiac hypertrophy and heart failure in response to pressure overload. Upregulation of Nox4 increased mitochondrial superoxide thereby directly mediating oxidative stress, mitochondrial dysfunction, and myocardial cell death during pressure overload-induced cardiac hypertrophy. Because expression of Nox4 is upregulated by cardiac stress, including pressure overload, heart failure, and aging, it could become an ideal target for pharmacological interventions to in the heart (Kuroda et al., 2010). Another study shows sex differences in the phosphorylation of mitochondrial proteins, including aldehyde dehydrogenase 2 (an enzyme that detoxifies ROS-generated aldehyde adducts) and α-ketoglutarate dehydrogenase

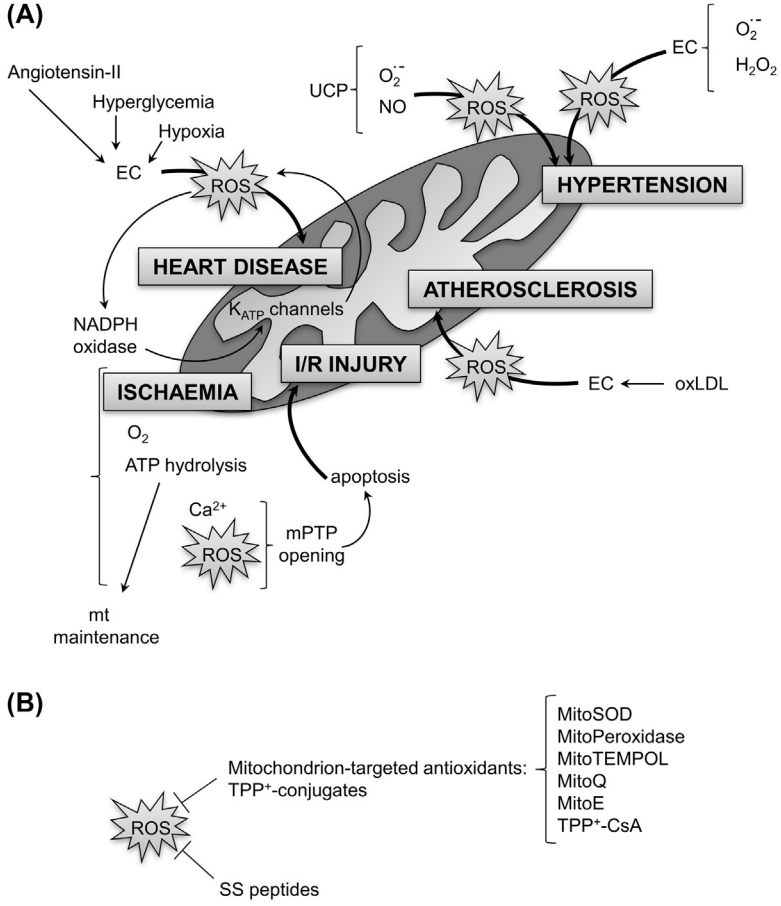

Figure 7 ***ROS as causative agents in cardiovascular diseases (CVDs) and therapeutic approaches targeting mitochondrial oxidative damage.*** (A) Principal mechanisms of ROS production and subsequent mitochondrial dysfunction leading to CVDs. Mitochondria participate in the pathogenesis of hypertension: increased blood pressure has been associated with an excessive endothelial cells (EC) production of superoxide ($O_2^{\bullet-}$) and H_2O_2; UCP1 expression also increases $O_2^{\bullet-}$ production and decreases the availability of NO. ROS production by endothelial mitochondria contribute to heart disease: angiotensin-II, hyperglycemia, or hypoxia increases mitochondrial ROS production in EC, which then stimulates the NADPH oxidase; moreover ROS, produced by the NADPH oxidase, activate mitochondrial KATP channels, suggesting a possible feedback amplification system. Exposure of endothelial cells to oxidized lipids (oxLDL) induces ROS formation, which has a pivotal role in atherogenesis. During ischemia O_2 is lacking and mitochondria hydrolyze ATP to maintain the mitochondrial membrane potential ($\Delta\Psi$); the cardiac cell strives to maintain ATP production, this eventually results in mitochondrial Ca^{2+} overload, mitochondrial depolarization, and increases the generation of ROS. Restoration of blood flow will help to restore ATP levels, but the damaged mitochondria generate enormous amounts of ROS during reperfusion and promote

(one of the major sources of ROS generation), resulting in a reduced production of ROS and cardioprotection in females (Lagranha et al., 2010). In addition, mtDNA damage is increased in cardiovascular tissues of CVD patients, and it has been established that mtDNA mutations lead to increased ROS production (Aliev et al., 2002).

ROS may be both a cause and an effect of hypertension because increased blood pressure has been associated with an excessive endothelial production of superoxide and H_2O_2 in both animal models and humans with increased blood pressure (Puddu et al., 2008). The participation of mitochondria in the pathogenesis of hypertension is also suggested by the involvement of UCPs in experimental and human hypertensive states (Table 2). In mice with doxycycline-inducible expression of UCP1 in arterial walls, UCP1 expression increases superoxide production and decrease the biological availability NO, causing increased blood pressure (Bernal-Mizrachi et al., 2005). In addition, a common polymorphism of the UCP2 gene has been associated with hypertension (Ji et al., 2004); however, as the hypertension-associated allele was reported to increase transcription of the UCP2 gene, and UCP2 is known to diminish ROS production and emission from mitochondria (Teshima et al., 2003), oxidative stress seems unlikely to be a link between the polymorphism and hypertension. ROS also underlie much of the endothelial cell (EC) damage related to heart disease (Davidson, 2010). Damage to the endothelium contributes to the development of atherosclerosis and hence to possible myocardial infarction and subsequent heart failure. EC has relatively little dependence on oxidative phosphorylation for ATP production; however, endothelial mitochondria are centrally involved in maintaining the fine regulatory balance between mitochondrial Ca^{2+} concentration, ROS production, and NO. Moreover, a general principle appears to be emerging in which mitochondrial ROS

mitochondrial permeability transition pore (mPTP) opening and activation of apoptosis, triggering ischemia/reperfusion (I/R) injury. (B) Approaches to deliver drugs to the mitochondria and prevent ROS-induced mitochondrial oxidative damage. Compounds conjugated to the lipophilic triphenylphosphonium cation (TPP^+) can be delivered selectively into the mitochondrial matrix in a potential-driven manner; a series of mitochondria-targeted antioxidants have been designed to decrease superoxide (MitoSOD), hydrogen peroxide (MitoPeroxidase), ferrous iron (MitoTEMPOL), lipid peroxidation (MitoQ, MitoE), and preventing mPTP opening (TPP^+-CsA). Recently, Szeto and Schiller (SS) peptides targeting the inner mitochondrial membrane have been developed; they concentrate in a potential independent manner and possess intrinsic mitoprotective activities.

signals to other cellular sources, triggering ROS production from these. For example, angiotensin-II, hyperglycemia, or hypoxia, each increases mitochondrial ROS production in EC, which then stimulates NADPH oxidase via activation of mitogen-activated protein kinase (MAPK). Furthermore, a "reverse" pathway, in which ROS produced by NADPH oxidase leads to increased mitochondrial ROS production (via activation of mitochondrial K_{ATP} channels, matrix swelling, and alkalization) may also exist, suggesting a feedback amplification system (Daiber, 2010). These data demonstrate that ROS production by endothelial mitochondria contribute to heart disease, therefore targeted scavenging of ROS may have various protective and beneficial effects on the heart. Interestingly, ROS play a pivotal role in atherogenesis, which is one of the major factors in the development of heart failure. Oliveira et al. (2005) have shown that mitochondria from atherosclerosis-prone hypercholesterolemic LDL receptor knockout mice have oxidative phosphorylation efficiency similar to that of control mice, while their net production of ROS and susceptibility to developing membrane permeability transition are both higher. Indeed, it has been demonstrated that exposure of endothelial cells to oxidized lipids such as oxLDL induces ROS/reactive nitrogen species (RNS) formation (Zmijewski et al., 2005). Recently, overexpression of mitochondrial thioredoxin (Trx2) in the endothelium has been proven to protect from atherosclerosis (Zhang et al., 2007).

ROS production is also a feature of ischemia and is implicated in the pathogenesis of I/R injury (Misra et al., 2009). In acute myocardial infarction, two distinct types of damage occur to the heart: ischemic injury and reperfusion injury. The first results from the initial loss of blood flow and the second from the restoration of oxygenated blood flow. To maintain the MMP when O_2 is lacking, mitochondria hydrolyze ATP; this activity destroys any available ATP, favors Ca^{2+} accumulation, and increases the generation of ROS. During ischemia, ROS are generated in the myocardium, at complexes I and III of the ETC, and primarily formed by the degradation of adenosine. Increased ROS production (initiated during ischemia and exacerbated upon reperfusion) coupled with increased cellular [Ca^{2+}] are thought to be the main causes of reperfusion injury. The combined effects of ROS and elevated [Ca^{2+}] lead to the opening of the PTP, which ultimately induces apoptosis (Solaini, Harris, 2005). Ischemic preconditioning (IPC) is a very effective way of protecting the heart from reperfusion injury. This process involves one or more short nonlethal cycles of I/R that protect the heart against a subsequent prolonged period of ischemia. It seems clear that IPC protects the heart by reducing oxidative stress during

I/R, and that this decreases PTP opening. However, the signaling pathways involved in mediating these effects have yet to be elucidated and several possibilities exist. For example, UCP2 overexpression in rat neonatal cardiomyocytes confers tolerance to oxidative stress by diminishing mitochondrial Ca^{2+} overload and reducing ROS generation, suggesting that UCP2 may mitigate ischemia-reperfusion injury and be a mechanism of cardioprotection (Teshima et al., 2003). Other data demonstrate that nitrite (NO_2^-), a stable endocrine pool of nitric oxide that is selectively reduced to NO in ischemic conditions, mediates cardioprotection after I/R. The mechanism involves the inhibition of mitochondrial complex I by S-nitrosation, leading to the inhibition of electron transport and subsequent decrease in mitochondrial ROS generation, which limits apoptosis and cytotoxicity at reperfusion (Shiva et al., 2007). Another possible mechanism involved mitochondrial ROS formation catalyzed by p66Shc; hearts from p66Shc knockout mice display decreased ROS production and decreased myocardial injury caused by postischemic reperfusion. In particular the absence of this protein involved in mitochondrial ROS formation prevented the oxidative attack of structural components of cardiomyocytes, such as lipids and proteins (Carpi et al., 2009).

Today, oxidative stress remains an attractive target for cardiovascular prevention and therapy. A general therapy for decreasing mitochondrial oxidative damage should be effective as a future therapy for CVDs. As the mitochondrial respiratory chain in the IMM is considered as important intracellular source of ROS, a specific action of antioxidants on the mitochondrial respiratory chain may constitute an important mechanism of cardiovascular protection. For example, uncouplers such as dinitrophenol are protective, as they decrease MMP and thus decrease the activity of the mitochondrial Ca^{2+} uniporter, whereas F1FO inhibitors such as oligomycin can decrease wasteful ATP hydrolysis. Unfortunately, these two agents show complementary deleterious effects: uncouplers increase ATP hydrolysis and F1FO inhibitors increase MMP and, presumably, increase mitochondrial Ca^{2+} uptake, thus they are used only as research tools. Other studies seeking to counteract the deleterious effects of ROS have shown that antioxidants such as Vit-E, CoQ10, and NAC decrease mitochondrial oxidative damage in different models. Despite, a number of preclinical and clinical lines of evidence, studies testing the effects of classical antioxidants such as Vit-C, Vit-E, or folic acid in combination with Vit-E have been disappointing. Vit-E or CoQ is quite lipophilic and tends to be retained in cell membranes and subsequently fail to achieve significant intracellular

concentrations. NAC fails to provide significant antioxidant effect, presumably due to its low lipid solubility and tissue distribution. As these compounds do not significantly accumulate within mitochondria, their effectiveness remains limited. A number of low-molecular weight catalytic antioxidants, generally called SOD mimetics, have been shown to provide some protection against I/R injury (Gianello et al., 1996). Unfortunately, again, while these SOD mimetics are cell permeable, they do not selectively target mitochondria.

Recent drug development efforts have focused their attention on reducing mitochondrial oxidative stress using mitochondrion-targeted antioxidants, which show potential as future therapies for CVDs (Fig. 7). MitoVit-E, one of the first TPP^+ (triphenylphosphonium cation) conjugates, has been shown to successfully decrease ROS production and apoptosis in bovine aortic EC exposed to oxidative stress (Dhanasekaran et al., 2004) but was ineffective against hypoxic–ischemic striatal injury in neonatal rats (Covey et al., 2006). One disadvantage of Vit-E is that it is not a catalytic antioxidant and its scavenging activity is not regenerated. In contrast, MitoQ consists of a TPP^+ covalently attached via an aliphatic linker to a ubiquinone derivative; after detoxifying an oxidant species, it is regenerated by the respiratory chain. MitoQ concentrates several 100-fold within the mitochondria, is orally bioavailable, distributes to various organs, including the heart, without adverse effects in rats or humans, and decreases mitochondrial oxidative damage in rodent models of cardiac I/R injury (Adlam et al., 2005). Administration of MitoQ to stroke-prone hypertensive rats improves endothelial function and attenuates cardiac hypertrophy (Graham et al., 2009). Also a cyclosporine A (CsA) derivate, specifically targeted to mitochondria by conjugation to a TPP^+, has been designed (Malouitre et al., 2010); CsA is well known to protect the heart from reperfusion injury by preventing PTP opening; however, side effects associated with PTP inhibitors limit their therapeutic potential. An alternative compound, called SkQ1, when perfused through isolated heart preparations or fed to rats, was able to reduce ischemia-induced arrhythmia and infarct size, despite the use of a concentration that was an astounding six orders of magnitude lower than that of MitoQ (Bakeeva et al., 2008). On the other hand, the accumulation of these lipophilic cations in the mitochondrial matrix can disrupt MMP and inhibit mitochondrial respiration and ATP production. As a result, the therapeutic index of these molecules is rather low, with toxic concentrations being only ~10-fold greater than effective concentrations. The utility of TPP^+-conjugated antioxidants may also be

limited by their requirement of MMP for mitochondrial uptake, especially considering that diseased mitochondria are unlikely to have normal MMP. The SS peptides, a novel class of mitochondrial-targeted antioxidant (see above, Section vii), appear to hold great promise in the setting of anticipated ischemic intervals and may be used for minimizing I/R injury during angioplasty, coronary bypass surgery, cardiac surgery, and organ transplantation. SS-02, SS-31, and SS-20 have been reported to reduce myocardial ischemia—reperfusion injury in ex vivo and in vivo studies, and in vivo studies showed that SS-02 and SS-31 both reduced cardiac infarct size. A recent study showed that mitochondrial targeted peptide SS-31 ameliorates angiotensin-induced cardiomyopathy through the reduction of mitochondrial ROS and provided a strong rationale for investigating the clinical application of SS-31 for treatment or prevention of hypertensive cardiovascular diseases (Dai et al., 2011).

Furthermore, physical activity is another condition that increases antioxidant capacity in the heart by augmenting ROS scavenging enzymes such as catalase, superoxide dismutase, and glutathione peroxidase (Linke et al., 2005; Powers et al., 1994; Roh et al., 2016; Somani et al., 1995). Heat-shock proteins (HSPs) play important role in cellular defense against oxidative stress. It was shown that physical training induced HSP70 and HSP27 expression in trained old rats compared with sedentary old and young rats partially counterbalanced the heart age-related effects in the antioxidant system without altering peroxidation levels (Rinaldi et al., 2006). The antioxidant action of exercise seems to be dependent on exercise protocol. While endurance exercise exacerbates oxidative stress and cardiac remodeling, acute or moderate aerobic exercise training preserves cardiac health and prevents remodeling by maintaining myocardial defense system through stabilizing NRF2-antioxidant signaling (Narasimhan, Rajasekaran, 2016). Although aging hearts exhibit reduced NRF2-dependent antioxidant mechanisms, exercise training in aged mice increases NRF2 activity and induces of its electrophile-responsive/antioxidant-responsive elements (EpRE/ARE) target pathway to near-normal levels seen in young counterparts (Gounder et al., 2012; Narasimhan, Rajasekaran, 2016). NRF2 also *trans*-activates genes of the antioxidant response (Kang et al., 2005) and is coactivated by PGC-1α during oxidative stress (Aquilano et al., 2013). Long- and short-term endurance exercise increases PGC-1α expression in cardiac muscle (Bayod et al., 2012; Safdar et al., 2011) and induces mitochondrial biogenesis (Chinsomboon et al., 2009; Safdar et al., 2011). Besides these, cardioprotective effects of PGC-1 are mediated through its

ROS-lowering effects because PGC-1α induces glutathione peroxidase 1 and superoxide dismutase 2 and protects neural cells in culture from oxidative-stressor-mediated death (St-Pierre et al., 2006). Moreover, Ferrara et al., 2008 demonstrated that exercise training, which significantly increases activity of SIRT1, a factor that plays important role in antioxidant pathways, could counteract age-related systems impairment, by activation of antioxidant systems and DNA repair and disability (Cacciatore et al., 2004), suggesting therefore a possible role of exercise training in conditioning life span. Like SIRT1, SIRT3 protects against oxidative stress, in large part through FOXO3a-dependent mechanisms that induce superoxide dismutase and catalase (Sundaresan et al., 2009). Notably, these two sirtuins are upregulated during exercise in the heart and are positive modulators of PGC-1α activity (Palacios et al., 2009; Planavila et al., 2011). The enriched oxidative status caused by exercise training leads to number of beneficial effects, e.g., decreased arterial stiffness, improved endothelial function and metabolic and clotting setting, and reduced body weight (Corbi et al., 2012). Other findings derived from clinical studies show that regular physical activity decreases cardiovascular comorbidity and mortality in adult and in elderly by restoring the protective effect of ischemic preconditioning and partially contrasting loss of antioxidant defense in the aged heart (Corbi et al., 2012).

2.5 Mitochondria, ROS, Inflammation, and Aging

Recent studies highlight the important role of mitochondria, especially mitochondrial ROS (mtROS) production, as an upstream messenger in the induction of proinflammatory signaling. Inflammation initiates as a defensive immune response to pathogenic stimuli. This inflammatory response is a protective mechanism induced by the organism to remove the injurious stimuli and to promote the repair of damaged tissues. Dysregulation of the inflammatory response is observed in a variety of human diseases, including diabetes, neurodegeneration, and cancer. Activation of inflammatory processes starts through the formation of the inflammasome: a high-molecular weight multiprotein complex consisting of scaffold proteins and the adaptor protein ASC that recruits and binds caspase-1 (the initiator inflammatory caspase-1. There are four subfamilies of inflammasomes depending on the sensor molecule: NLRP3, NLRP1, NLRC4 (NLR family, CARD domain containing 4), and AIM2 (absent in melanoma 2) (Schroder, Tschopp, 2010). On activation, inflammasomes trigger the proteolytic maturation of proinflammatory cytokines, such as the potent

IL-1β, to engage the immune defenses. Due to its association with numerous inflammatory diseases, the NLRP3 inflammasome is currently the most fully characterized (Martinon et al., 2009).

A variety of danger signals, exogenous as well as endogenous, can activate the NLRP3 inflammasome, although the mechanisms of activation are poorly understood (Baroja-Mazo et al., 2014; Tschopp, 2011). Recent evidence suggests that mitochondria may integrate these distinct signals and deliver information to NLRP3 inflammasomes. Zhou et al. (2011) found that mtROS are key signals that directly trigger NLRP3 inflammasome activation. These authors artificially induced mtROS production, either by blocking key enzymes of the respiratory chain (complex I or complex III) or by inhibiting mitophagy/autophagy, and resulted in an increased amount of IL-1β secretion. In accordance with these findings, Nakahira et al. (2011) observed the fundamental role of autophagy in regulating mtROS production, demonstrating that decreased autophagy impairs mitochondrial homeostasis, increases mtROS levels and leads to NLRP3 inflammasome activation and IL-1β production. Moreover, treatment with the antioxidant 4-amino-2,4-pyrrolidinedicarboxylic acid (APDC) blocked NLRP3 inflammasome activation and IL-1β secretion (Zhou et al., 2011). Consistent with these observations, defective mitophagy increased inflammasome activation. In fact, macrophages treated with 3-methyladenine (3MA) or silenced of the autophagy regulator beclin 1 and autophagy protein 5 (ATG5) showed a hyperactivation of NLRP3 and IL-1β release on stimulation with monosodium urate (MSU) crystals and nigericin due to the accumulation of damaged mitochondria and increased ROS generation, while resveratrol treatment attenuated this effect (Wu et al., 2016).

The link between NLRP3 inflammasomes and mitochondria is further confirmed by the subcellular localization of NLRP3 and ASC. Under resting conditions, NLRP3 is localized in the ER but relocates to MAMs and mitochondria after inflammasome stimulation. Similarly, ASC proteins translocate from the cytosolic fraction to mitochondria and MAM after stimulation (Zhou et al., 2011). Based on these findings, Zhou et al. proposed a model in which NLRP3 acts as a sensor of mitochondrial status. This model was confirmed by evidence that NLRP3 and ASC are required for the release of mtDNA into the cytosol, a key step for caspase-1 activation and IL-1β secretion (Nakahira et al., 2011). Additional evidence of the intimate relationship between mitochondria and inflammasome is sustained by the role of mitochondrial Ca^{2+} uniporter (MCU). Modulation of MCU

expression has been shown to regulate the activation of NLRP3 inflammasome induced by complement complex (Triantafilou et al., 2013) or by *Pseudomonas aeruginosa* in lung epithelial cells, preventing mitochondrial ROS production (Rimessi et al., 2015).

In contrast, other data reveal a distinct role for ROS in inducing inflammatory cytokines through a transcriptional-dependent mechanism, rather than through direct activation (van de Veerdonk et al., 2010). Following this theory, Bulua et al. demonstrated that mtROS influence the transcription of proinflammatory cytokines through the regulation of the MAPK pathway (Bulua et al., 2011; Kamata et al., 2005). It was found that patients with an autoinflammatory syndrome (TRAPS, TNF receptor-associated periodic syndrome) possessed increased basal levels of mtROS in cells that are responsible for production of IL-6 and TNF-α, independent of the NLRP3 inflammasome activation. Indeed, increased levels of IL-6 and TNF-α were observed after inflammation induction in the absence of NLRP3, caspase-1, or IL-1R. All of these findings underscore a common pathway identifying mitochondria as a crucial source of ROS, driving inflammation through mechanisms either inflammasome-dependent or inflammasome-independent. MtROS in particular, but also other key factors in inflammation induction such as NLRP3 and IL-1β, might be therapeutically targeted for treating inflammatory diseases. It has already been demonstrated that blocking excessive ROS with antioxidants reduces inflammation. Treatments with mitochondria-targeted antioxidants such as Mito-TEMPO (Trnka et al., 2009) or MitoQ (Murphy, Smith, 2007; Villalba et al., 2010), scavengers specific for mtROS, have been shown to reduce the secretion of IL-1β or IL-6 in vitro (Bulua et al., 2011; Nakahira et al., 2011). Such findings are promising and warrant further study in controlled clinical trials to confirm the in vivo efficacy of antioxidants.

Chronic inflammation is associated with physiological and pathological aging (Franceschi et al., 2007). Jurk et al. demonstrated that *nfkb1* −/− mice show premature aging. In particular, their data suggest that chronic low-grade inflammation can accelerate aging via ROS-mediated exacerbation of telomere dysfunction and cell senescence (Jurk et al., 2014). Recently, the NLRP3 inflammasome has been shown to be implicated in several aging-related diseases such as gout, T2D, obesity, and cancer. AMP-activated protein kinase (AMPK) plays a pivotal role in the control of metabolic events involved in the pathophysiology of aging. Nevertheless, it has emerged as an important integrator of inflammation signaling (Cordero et al., 2018). In fact, many of the various AMPK-dependent

pathways regulate NLRP3 inflammasome activation during aging. Aging has a negative effect on the quality of immune responses, which increases the frequency and severity of infectious diseases. Recent results demonstrate the role of inflammation and oxidative stress in age-related changes of immune cell survival factors in the bone marrow with an accumulation of IFN-γ, TNF, and ROS, which induce IL-15 and IL-6 expression. The treatment with the ROS scavengers NAC and Vit-C reduces cytokine levels, suggesting that antioxidants may be beneficial in counteracting immunosenescence by improving immunological memory in old age (Pangrazzi et al., 2017). Furthermore, osteoarthritis, a whole-joint degenerative multifactorial disorder, is influenced by oxidative stress and aging. Cartilage of osteoarthritis patients has significantly more ROS-induced DNA damage than normal cartilage and this damage is mediated by IL-1β. The overproduction of ROS in osteoarthritis regulates intracellular signaling processes, such as chondrocyte senescence and apoptosis, extracellular matrix synthesis, and degradation along with synovial inflammation and dysfunction of the subchondral bone (Lepetsos, Papavassiliou, 2016).

All these findings identify the ROS-mediated inflammation as a possible target in the treatment of a wide range of aging-related diseases, suggesting the use of antioxidants, both natural and synthetic.

2.6 Mitochondria, ROS, Cell Death, and Aging

Apoptosis, the process that allows multicellular organisms to eliminate unnecessary, dangerous, or damaged cells without evoking inflammation or tissue damage, takes place in a wide number of physiological and pathological events (Green, Kroemer, 2004). Its efficacy for removing hazardous cells is circumvented in viral diseases and neoplasia by specific molecular routes. On the other hand, the progressive occurrence of apoptosis in non-proliferating cells has been proposed as the basis for a number of degenerative disorders, as well as in progressive loss of organ function during aging (Green et al., 2011). Thus, understanding the control mechanisms of apoptosis is a major goal for the development of new therapeutic approaches. Mitochondria, traditionally considered the powerhouses that supply energy to the cells, are also considered vessels filled with an array of weaponry that can be unleashed to promote the apoptotic signaling cascade, resulting in the demise of the cell. Apoptotic stimuli cause the release of proapoptotic mitochondrial mediators into the cytoplasm (cytochrome *c*, AIF, Smac/DIABLO). Their assembly with cytosolic proteins forms a complex (apoptosome) that recruits and activates caspases leading

the cell into apoptotic death (Adams, Cory, 2002). The molecular mechanism of this release is unclear but most likely requires the activity of a large-conductance channel, known as the PTP, which remains again an elusive molecular entity (Lepetsos, Papavassiliou, 2016). Main regulators of the PTP include voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (OMM), adenine nucleotide translocase (ANT) in the inner mitochondrial membrane (IMM), cyclophilin D in the mitochondrial matrix, and more recently the mitochondrial phosphate carrier (PiC) (Leung et al., 2008). Whereas, hexokinase II (HKII), mitochondrial creatine kinase (CK), benzodiazepine receptor (PBR), and Bcl-2-family members (Bcl-2, Bcl-x_L, and Bax) are currently included as putative regulatory components. However, despite the use of multiple methodologies, the identity and the number of PTP's components are still elusive, and it remains to be clarified whether ANT and VDAC are the main components of pore or accessory. As showed using isolated mitochondria from mice knockout for ANT or VDAC and CypD, where a permeability transition response to the classical inducer calcium (Ca^{2+}) was monitored, suggested a limited role for these proteins (Baines et al., 2007; Basso et al., 2005; Kokoszka et al., 2004; Nakagawa et al., 2005). Could be, that the structure of pore depends on the tissue as well as the pathophysiological state (Halestrap, 2009). In fact, four homologous genes, whose expression is not only tissue specific but also vary according to the pathophysiological state of the cell, encode ANT. Similarly VDAC, where the different isoforms appear to rely on their ability to engage protein—protein interactions with different partners complicating VDAC's contribution to cell death, strictly dependent from isoform and stimulus (Cheng et al., 2003; Rapizzi et al., 2002). These and other PTP proteins are targets for ROS, and oxidative modifications of those proteins containing thiol groups will significantly impact PTP activation and thus mitochondrial ions fluxes, as mentioned later (Zoratti, Szabo, 1995) (Costantini et al., 1996; Kowaltowski et al., 2001).

In response to proapoptotic stimuli, including ROS and Ca^{2+} overload, the PTP assumes a high-conductance state that allows the deregulated entry of small solutes into the mitochondrial matrix along their electrochemical gradient (Morciano et al., 2015). The opening of the PTP induces mitochondrial swelling, and these large-scale alterations of organelle morphology may allow the release of the caspase cofactors into the cytosol (Yang, Cortopassi, 1998). Evidences by which PTP opening may accelerate aging can represent the link between PTP-driven apoptosis, ROS, and senescence. It is reported that PTP may be the culprit of the progressive

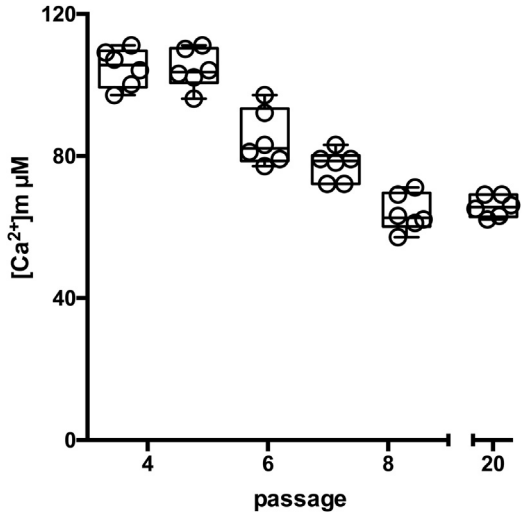

Figure 8 ***Mitochondrial calcium uptake as a function of cell culture passage number.*** Representative quantitation of mitochondrial Ca^{2+} uptake elicited by ATP 100 μM n mouse embryonic fibroblasts (MEFs) from low and high passage rates. Mitochondrial calcium concentration ($[Ca^{2+}]_m$).

age-dependent NAD^+ depletion inside mitochondrial matrix (Schriewer et al., 2013) affecting DNA repair mechanisms and the protective sirtuin pathway (Merksamer et al., 2013) prompting the cell to senescence and death. This relationship is not univocal; indeed also aging could favor PTP opening (Rottenberg, Hoek, 2017) giving rise to an intricate loop. Interestingly, an extracellular agonist–stimulated Ca^{2+} uptake by mitochondria in mouse embryonic fibroblasts (MEFs) is gradually decreased with culture time (Fig. 8). Moreover, many reports described dysregulations in Ca^{2+} homeostasis and MMP in aged cells in terms of calcium buffering thresholds, which significantly decrease (Gant et al., 2015; Pandya et al., 2015) and a lowered MMP (Sugrue, Tatton, 2001). These changes intensify PTP opening events in senescent cells (Paradies et al., 2013) and the liaison with age-dependent mitochondrial ROS accumulation is very strong as the oxidation of channels and transporters such as MCU (Dong et al., 2017) in which the oxidation of the reactive thiol represented by Cys-97 showed constitutive channel activity and a consequent mitochondrial calcium overload), the mitochondrial calcium uptake 1 (MICU1) and phospholipids contribute to the progressive damage and dysregulation of proteins and to the exchange of radical species between mitochondria and cytosol, where during aging, it could become the main exchange route.

Recently, it has been proposed a multistep nature of the PTP complex opening involving first, a disassembly of ATP synthase dimers and second, a correct rearrangement of the C-ring in the IMM (Bonora et al., 2017; Morciano et al., 2017). This is coherent with previous findings in which a reduced dimerization status of ATP synthase was detected in aging cells, thus prompting them to cell death (Daum et al., 2013). Indeed, it is reported that aging is intimately related to a decline of mitochondrial functions (Sun et al., 2016) and young, middle-aged and senescent cells own important mitochondrial differences in terms of IMM integrity and organization, as well as the oxidative state of proteins, mtDNA, and phospholipids; for instances, it has to be remarked that ATP synthase in young cells is mainly detected in dimeric and oligomeric structures (Daum et al., 2013) to fully support the energetic requirement of the cell and the correct curvature of the IMM. Recent findings award the synthasome assembly to PTP modulators, such as CypD and generally, to conditions preventing mitochondrial permeability transition (MPT) (Beutner et al., 2017); on the other hand, in senescent mitochondria, a highly dynamic transition from dimers to monomers occur (Daum et al., 2013).

Investigation of the molecular routes of apoptosis has revealed the important role of mitochondria in decoding oxidative insults (Fig. 2). In fact, oxidative stress and altered mitochondrial function have consistently been proposed to be major determinants of life span, as shown by several studies with transgenic overexpression—antioxidants systems (Parkes et al., 1998; Sun et al., 2008), or as shown by experiments with fundamental genes for mitochondrial redox regulation thioredoxins and glutaredoxins (Chen et al., 2002; Choksi et al., 2011; Diotte et al., 2009; Enoksson et al., 2005; Kim et al., 2010; Nagy et al., 2008; Stanley et al., 2011). Indeed, mouse embryonic fibroblast cells (MEFs), from which p66shc has been completely depleted, are resistant to oxidative stress induced apoptotic death (Migliaccio et al., 1999). This death is p53-dependent, and knockout of either p53 or p66shc causes resistance, suggesting that p66shc is downstream of p53 in the pathway (Trinei et al., 2002). In agreement with this finding, mice with p66shc completely knocked out are resistant to paraquat and live about 30% longer than controls. The relationship between mitochondria and p66shc emerged by the effective localization of the protein to the organelle: part of the cytosolic pool translocates to mitochondria (Orsini et al., 2004). In the intermembrane space (IMS), p66shc binds cytochrome *c* and acts as a redox enzyme, generating H_2O_2, in turn inducing the opening of the PTP and cellular apoptosis. ROS production by p66shc appears to

be a specialized function whereby electrons are subtracted from the ETC to catalyze the partial reduction of molecular oxygen (Giorgio et al., 2005). The redox activity of p66shc explains the decrease in ROS levels observed in p66shc knockout cells (Migliaccio et al., 2006) and is also responsible for an altered mitochondrial metabolism under basal conditions, characterized by lower oxygen consumption (Nemoto et al., 2006). Clear and specific references about p66shc and its aging activity will be addressed in detail in a next section, emphasizing pivotal molecular proteins involved in p66shc's signal transduction as new targets of pharmacological therapy.

The tumor suppressor Fhit protein characterizes another pivotal mitochondrial proapoptotic route (Rimessi et al., 2009) associated with oxidative-stress induction. It is absent or reduced in many types of human tumors including lung, esophagus, stomach, kidney, and cervical carcinomas (Croce et al., 1999). Fhit is encoded by the FHIT gene, located within a fragile region of chromosome 3 of the human genome, and is frequently altered in cancers and inactivated in cancer-derived cell lines (Inoue et al., 1997). Direct evidence from Fhit-deficient cancer cells shows that chemotherapy-induced cell death is accompanied by a mild response to production of ROS, suggesting that Fhit-deficiency could negatively influence treatment outcome. In addition, Fhit loss has been considered as a mechanism to delay cell aging due to its ability to overcome oncogene-induced senescence (Waters et al., 2014); indeed, it is known that oncogene activation generates, most of the time, irreversible DNA damage allowing senescence or apoptosis pathways in preneoplastic cells. In this key passage, observations made in $Fhit^{+/+}$ and $Fhit^{-/-}$ MEF cells, Fhit function is highly dependent on its DNA "caretaker" role prompting p53 deregulation and avoiding senescence once lost (Miuma et al., 2013). Although Fhit is identified as a cytosolic protein, it may sort to mitochondria where it interacts with ferredoxin reductase (fdxr), a flavoprotein transactivated by p53 (Trapasso et al., 2008). Through interaction with chaperones Hsp60 and Hsp10, mitochondrial Fhit modulates electron-transfer from NADPH via the activity of fdxr. The Fhit/fdxr complex generates ROS and increases Ca^{2+} uptake into mitochondria, potentiating the effects of apoptotic agents (Rimessi et al., 2009). The sorting of Fhit to mitochondria is now recognized as essential to its tumor suppressor actions in apoptosis induction. Together, these results identify a mitochondrial signaling step at the center of the mechanisms of the anticancer action of Fhit and draw attention to the importance of pharmacologically regulating its intracellular sorting and organelle activity, as

confirmed by the overexpression of mitochondrial-targeted Fhit-chimera in tumor cells (Rimessi et al., 2009).

In recent years, an interesting role in regulating cell destiny has been identified for autophagy (the catabolic pathway for degradation of intracellular proteins and organelles via the lysosome) (Klionsky et al., 2016; Scherz-Shouval et al., 2007). Autophagy is mainly activated by nutrient starvation and it plays a dual role: not only it is primarily a survival mechanism but it also leads to cell death, thus possibly acting as an alternative to apoptosis (Levine, Kroemer, 2008). In contrast to the well-documented prosurvival function of autophagy, some examples of ROS-induced autophagy linked to cell death have been described in tumor cells. Treatments with rotenone and TTFA (inhibitors of complex I and II, respectively) in cancer cell lines induced autophagy-dependent cell death, displaying increased mitochondrial ROS production. Use of siRNA against autophagy genes or the autophagy inhibitor 3-MA produces reversal of the effects of autophagic cell death (Chen et al., 2007b). Today, the precise role of autophagy in cancer is strongly debated, but it remains a potentially important area of development for new therapeutic interventions. Scientific evidence supports both tumor promoting and suppressive functions for autophagy. The exact role of it during cancer progression depends on tumor type, context, and stage. Although genetic evidences confirm a role for autophagy as a tumor suppressor mechanism, it can also promote the maintenance of models of ovarian cancer and gastrointestinal stromal tumor (Guo et al., 2011; Gupta et al., 2010; Lu et al., 2008). The requirement for autophagy becomes more apparent in later stages as tumor cells cope with microenvironmental stresses encountered during progression and metastasis (Roy, Debnath, 2010). The tumor suppressive functions are most apparent during tumor initiation, where H-ras^{12v} induces different autophagic responses depending on the duration of oncogene overexpression. After 48 h of Ras overexpression, autophagy inhibits cell proliferation, whereas a longer time of oncogene overexpression, cell proliferation was enhanced by autophagy (Wu et al., 2011). Growing evidence suggests that in different pathological contexts, cross talk between apoptosis and autophagy takes place; this is intimately interconnected during stress responses, and anti- or prosurvival effects have been shown in cancer or neurodegenerative diseases, respectively (Codogno, Meijer, 2005; Kroemer et al., 2007). For this reason, researchers have a marked interest in developing pharmacological regulators of autophagy as alternative therapeutic approaches to counter the dysfunctional apoptotic response during pathological conditions. On the other

hand, in healthy conditions, growing (but not yet direct) evidences link autophagy to aging revealing this pathway as cure-all for longevity and thus, a landmark for therapeutic manipulations. These observations were made in many systems such as yeast, *S. Cerevisiae*, *Drosophila* and ultimately in mammals with concordant findings that describe a reduced age-dependent activation of autophagy in mammals (Kaushik et al., 2012; Vittorini et al., 1999), in in vitro senescent cultures and isolated organs from old rodents (Cuervo, Dice, 2000). In addition, the disruption of a type of autophagy, classified as chaperone-mediated autophagy, promoted cell sensitivity to stress, a condition dramatically associated to decreased longevity (Massey et al., 2006). Another proof by which autophagy could be deeply involved in an extended life span derive from protein overexpression studies in which increased levels of ATG5, ATG7, ATG8 and SIRT1 autophagy proteins prevented ER stress and oxidative insult-induced cell death, improved glucose tolerance and clearance, and preserved motor function (Lee et al., 2008; Pyo et al., 2013; Yang et al., 2010). Although all these observations support the hypothesis that some forms of basal autophagy could ameliorate aging-associated dysregulated pathways, further efforts are required to get direct and final evidences.

Redox regulation by moderate levels of ROS is also observed in autophagy. It is generally accepted that mitochondria play a fundamental role in ROS-mediated autophagy regulation (Azad et al., 2009; Kim et al., 2007a; Kirkland et al., 2002; Kissova et al., 2006; Xu et al., 2006). A specialized form of the autophagy process, called mitophagy, degrades defective mitochondria. Mitophagy helps to maintain a healthy population of mitochondria in the cell (Rimessi et al., 2013) and thus reduces oxidative damage (Scherz-Shouvalet al., 2007). Increases in cellular ROS lead to loss of MMP, which is considered a trigger for mitophagy (Kim et al., 2007b). Under serum deprivation, a typical decrease in MMP is observed in hepatocytes prior to engulfment by autophagosomes, whereas cyclosporin A (PTP inhibitor) prevents this depolarization and the autophagosomal proliferation (Elmore et al., 2001; Rodriguez-Enriquez et al., 2009). Indeed, a recent study showed that mitochondria provide the membrane source for autophagosome biogenesis during the autophagy process (Hailey et al., 2010). Tracking photolabeled mitochondria showed that fusion and fission events permitted the segregation of abnormal mitochondria, which were then degraded by mitophagy (Twig et al., 2008), and that the profission protein Fis1 triggered mitophagy only when associated with mitochondrial dysfunction (Gomes, Scorrano, 2008).

2.7 Mitochondrial Dysfunction and Oxidative Stress in Age-Related Neurodegenerative Diseases

Links between mitochondria and neurodegeneration have been reported for several years, with the first hints stemming from studies of classical mitochondrial disorders. Interestingly, the incidence of neurodegenerative diseases increases with aging, which means that some of the mitochondrial phenotypes found in aged individuals are similar to some mitochondrial hallmarks found in neurodegeneration. In this type of disease, mtDNA mutations lead to impairment of mitochondrial respiration, ATP synthesis, reduction in MMP, and increased ROS production (DiMauro, Hirano, 2009). These defects usually cause a bioenergetic deficit in tissues with the highest energy demand, particularly the central nervous system and muscular tissue, causing neurological disorders, ataxia, muscle weakness, stroke-like episodes, epilepsy, and other disease types. Similarly, deregulation of mitochondrial physiology and increased oxidative stress, often at an early stage, have both been correlated with the most common neurodegenerative diseases including AD, PD, Huntington's disease (HD), or Amyotrophic Lateral Sclerosis (ALS), among others (Lin, Beal, 2006). In all of these pathologies, mitochondria appear to be central hubs of the pathophysiological process, due to their impaired ability for ATP synthesis and for an increased production of ROS (Szeto, 2006). In fact, alterations of mitochondrial function and increased oxidative stress were found in patients affected by these pathologies.

Loss of neurons in the hippocampus and neocortex combines with two brain alterations in AD: the accumulation of senile plaques (composed of amyloid-β, Aβ) and neurofibrillary tangles (made of the hyperphosphorylated protein tau). The principal risk factor for AD is age, but there are also cases of autosomal dominant familial forms caused by mutations in amyloid precursor protein (APP), presenilin-1 (PS1), or −2 (PS2). Mitochondrial abnormalities, such as alterations of TCA cycle enzymes, and reduced activity of respiratory complexes I, III, and IV were found in postmortem brains of patients with AD (Bosetti et al., 2002; Gibson et al., 1998; Lin, Beal, 2006; Reddy, 2006; Reddy, Beal, 2008). Direct evidence of oxidative stress was obtained in the brains of early-stage AD animal models by using in vivo electron paramagnetic resonance (EPR) imaging with methoxycarbamyl-proxyl (MCP) as a redox-sensitive probe (Fang et al., 2016), with these defects being associated not only with cognitive defects but also with mitochondrial dysfunction. It has been demonstrated that Aβ25-35

and Aβ1-40 induce apoptotic cell death in cerebral endothelial cells, and Aβ-neurotoxicity is associated with mitochondrial dysfunction (increased generation of ROS, excitotoxicity, apoptosis, and inflammation) (Mattson, 2000). Moreover, AD-associated metabolic alterations have been described to alter mitochondrial dynamics in the neurons, causing interference with local ATP and calcium gradients (Correia et al., 2016), as well as alterations in cardiolipin species, which may explain alterations in mitochondrial respiratory chain activities and increased ROS generation (Monteiro-Cardoso et al., 2015). SIRT3, a mitochondrial sirtuin has recently been shown to be decreased in the context of AD, leading to p53-mediated negative effects on mtDNA transcription and resulting in inhibition of respiration and increased oxidative stress (Lee et al., 2017). As referred above, SIRT3 is involved in the regulation of mitochondrial respiration and oxidative stress through regulation of protein acetylation and SOD2 expression/activity (Pereira et al., 2012).

Rhein and colleagues showed that convergence of Aβ and tau on mitochondria with associated defects in mitochondrial complexes I and IV cause disturbances in the respiratory and energy system of ^{triple}AD mice. The same work describes that age-related oxidative stress leads to dysfunctional energy metabolism and consequently to neuronal death (David et al., 2005; Rhein et al., 2009). Other in vitro and in vivo studies by Keil et al. (2004) demonstrated that Aβ induces mitochondrial adaptation and failure in a vulnerable and dose-dependent pattern, with NO being involved in these processes.

APP has an unidentified mitochondrial-signal sequence that targets it to mitochondria. Aβ interacts with Aβ-binding alcohol dehydrogenase (ABAD), and this interaction leads to release of cytochrome *c* and an increase of ROS generation (Lustbader et al., 2004). Yet, it has not been revealed if APP could be processed to Aβ directly within mitochondria or if processing precedes Aβ translocation. The finding that AD patients display impaired mitochondrial APP translocation and accumulation between OMM and IMM as well as evidences showing the presence of γ secretase in the mitochondrial matrix suggest that mitochondria could be a processing site for APP and that this event is fundamental for the onset of pathology and increased oxidative stress (Devi et al., 2006; Pavlov et al., 2011). Recently, it was proposed that overexpression of Aβ modifies the activity mitochondrial fusion and fission proteins. In particular, an increase of Fis1, a decrease of DLP1 (fission proteins), and a decrease of the fusion protein OPA1 were measured. All these alterations lead to mitochondrial fragmentation,

reduction of MMP and increase in ROS generation, most likely resulting from deficient quality control mechanisms (de Moura et al., 2010; Wang et al., 2008b).

It was proposed that the "mitochondrial cascade" might provoke AD development due to accumulation of mutations in mitochondrial genes, which causes insufficient respiratory chain accompanied with increased ROS production, and results in more and more severe mtDNA damage that leads to severe oxidative stress, stimulating AB toxicity (Swerdlow et al., 2014). Although it was not possible to identify any mitochondrial mutations causing AD, an increased number of point mutations in mtDNA isolated from brains of AD patients, relative to controls, has been reported (Lin et al., 2002). However, recent studies indicated such mutations appear to be effects of errors during the mtDNA replication process rather than be caused by ROS (Hoekstra et al., 2016). In contrast to AD, PD is characterized by tremor, rigidity, postural instability, and bradykinesia. PD is caused by degeneration of the dopaminergic neurons of the substantia nigra, combined with accumulation of α-synuclein- and ubiquitin-containing inclusions, called Lewy bodies, in the surviving neurons (de Moura et al., 2010). Disordered protein handling/degradation, mitochondrial dysfunction, and increased oxidative stress have been shown to be correlated with sporadic and familial PD as well as Parkinsonism due to exposure to toxins or pesticides (Dagda et al., 2009).

Several neurotoxins that affect dopaminergic neurons act at the mitochondrial level and induce oxidative stress in that organelle. One toxin is the heroin contaminant 1-methyl-4-phenyl-1,2,3,6-tetrahydroxypyridine (MPTP), which produces a Parkinsonian phenotype (Przedborski, Jackson-Lewis, 1998). MPTP is converted to $MPDP^+$ and then to the active metabolite MPP^+ by astrocytes and acts as an inhibitor of complex I of the mitochondrial ETC (Langston et al., 1983), promoting cytochrome *c* release from the IMM (Banerjee et al., 2009). Interestingly, both MPTP and its metabolites, $MPDP^+$ and MPP^+, reveal also mutagenic properties (Ulanowska, Wegrzyn, 2006) (Ulanowska et al., 2007). Thus, one can speculate that they might potentially cause also mutations in mtDNA, accelerating the disease development. Other compounds that inhibit complex I and contribute to PD development include the pesticides rotenone and paraquat, commonly used in farming. The latter acts by causing degeneration of dopaminergic neurons and increased oxidative stress (Cicchetti et al., 2005). Chronic exposure to rotenone leads to dopaminergic degeneration and formation and aggregation of α-synuclein and ubiquitin, as well as

oxidative damage and caspase-dependent cell death (Rego, Oliveira, 2003). Moreover, rotenone itself is widely used as a tool for the generation of PD models in *Drosophila*, mice, and rats. The appearance of cytoplasmic inclusions containing alpha synuclein and ubiquitin is reported in the different biological models, as well as dopaminergic neuron degeneration and impairment of locomotor activity in animal models, resembling the human pathology (Blandini, Armentero, 2012; Coulom, Birman, 2004; Inden et al., 2011). It was reported how rotenone administration induces ATP depletion, increased oxidative stress and death in a neuroblastoma model. Interestingly, ATP depletion in that model, induced by exposure to 2-deoxyglucose did not induce cell death, suggesting that ROS production is the critical event in rotenone-induced toxicity. Moreover, these effects were abolished by overexpression of the rotenone insensitive respiratory complex I subunit ND1 derived from yeast (Sherer et al., 2007). Nonetheless, it has been shown that NDUFS4 KO mice were completely insensitive to rotenone-induced neurodegeneration (Choi et al., 2008). The results obtained appear to suggest that there are more than one binding site for rotenone in mitochondrial Complex I (Fendel et al., 2008), with the most relevant appearing to be ND1 (Murai et al., 2007). Moreover, a redox-active dopamine analogue, 6-hydroxy-dopamine (6-OHDA), induces the death of dopaminergic neurons and leads to an increase of free radicals, abolishing α-synuclein's role.

A new player which has been described to have an important role in an early stage of PD is the mitochondrial LON protease. It was recently determined that Lon protease expression increased in the ventral mesencephalon of MPTP-treated animals, in the same time frame as the appearance of oxidized proteins and dopaminergic cell loss. The authors also observed a loss of Lon activity by ROS and carbonylation in α-ketoglutarate dehydrogenase (KGDH), aconitase, or subunits of respiratory chain complexes (Bulteau et al., 2017). Interestingly, not only Lon operates as a mtDNA and protein quality control protein (Pinti et al., 2016; Sepuri et al., 2017) but it also was recently shown to relocate to mitochondrial-associated membranes on different stimuli (Polo et al., 2017), suggesting that ROS may affect mitochondrial−ER interactions in PD through inactivation of Lon, besides avoid its quality control activity.

Recent studies demonstrated that mutations of different genes involved in mitochondrial function or with antioxidant activities cause familial PD. The leucine-rich repeat kinase 2 (LRRK2) is the most commonly mutated gene in the familial and sporadic type of PD (Kachergus et al., 2005),

whereas DJ-1, parkin, and PINK-1 are involved in autosomal recessive Parkinsonism (Dagda et al., 2009; Kitada et al., 1999).

Relatively high levels of mutant mtDNA, particularly with deletions, were reported in dopaminergic neurons of elderly people and in PD patients (Bender et al., 2006) (Kraytsberg et al., 2006; Lin et al., 2012). Studies on animals gave quite similar results, i.e., accumulation of deletions in mtDNA in brains, particularly in substantia nigra, of PD models (Dolle et al., 2016; Parkinson et al., 2014; Tzoulis et al., 2016). Moreover, depletion of mtDNA was also reported in this disease (Grunewald et al., 2016). Thus, it is possible that mtDNA dysfunction might contribute to development of PD.

In HD, the abnormal expansion of polyglutamine repeats in the Huntingtin (HTT) protein (above 40 repeats) causes aggregation of the unfolded protein that leads to neuronal degeneration in the cortex and striatum. Similarly to PD, alterations of mitochondrial respiratory complexes (especially II and III) were found in *postmortem* brain samples (Damiano et al., 2010). Furthermore, altered MMP in lymphoblasts from patients and augmented lactate production in the brain suggest a connection between mitochondrial dysfunction and HD. In addition, inhibitors of respiratory complex II, such as 3-nitropropionic acid and malonate, induce an HD pathological phenotype in animals (Beal, 1994). Mutated HTT is sufficient to decrease ATP synthesis and impair respiratory chain activity, whereas overexpression of respiratory complex II subunits is enough to recover mitochondrial function and sensitivity to apoptosis in neurons expressing an 82 glutamine HTT (Benchoua et al., 2006). The physiological role of HTT is still under debate, although it has been shown to colocate with the OMM and to regulate mitochondrial trafficking along axons (Chang et al., 2006). Interestingly, HTT facilitates PTP opening during Ca^{2+} stimulation in neurons and promote ROS production on 3-NP exposure in cybrids (Ferreira et al., 2010), suggesting that mutated HTT could have a role in the regulation of ROS metabolism. In fact, the PTP has been proposed as a major factor for mitochondrial damage in HD (Quintanilla et al., 2017).

Increased levels of DNA lesions and mutations, both base pair substitutions and deletions of larger DNA fragments, were reported in nuclear and mitochondrial genomes of HD patients and in animal models of this disease (summarized in Ayala-Pena, 2013). This suggests that mutations in DNA, including those in mtDNA, might be important factors in development of HD. The problem of mtDNA depletion is more complicated, as different groups reported either decreased (Liu et al., 2008; Petersen et al., 2014)

or increased (Chen et al., 2007a) levels of mtDNA in HD patients relative to controls. Recent studies, based on testing biological material from a relatively large population, indicated higher levels of mtDNA in leukocytes, but depletion of mtDNA in fibroblasts of HD patients relative to healthy controls (Jędrak et al., 2017). Therefore, it was suggested that both size of the study group, and particularly the kind of investigated tissue, as well as some methodological and technical details important for adequate measurement of mtDNA levels, might be responsible for differences in results published by various groups (Jedrak et al., 2017). Nevertheless, it appears that levels of mtDNA might be changed in HD patients, while either decreased or increased, depending on the tissue. Because mtDNA depletion has been suggested to occur in the brain of HD patients due to ROS-generated DNA damage, and due to evident accumulation of mutations in mtDNA and nDNA, a model of progression of HD, driven by accumulation of mtDNA lesions caused by toxicity of mutant HTT, and resultant enhanced production of ROS that cause mtDNA depletion, has been proposed (Ayala-Pena, 2013). According to this model, mtDNA dysfunction leads to deficiency in mitochondrial functions and subsequent neurodegeneration.

Similar connections between mitochondria and neurodegeneration are present in ALS. Alterations of mitochondrial structures and number, as well as defects, in respiratory chain complexes have been observed in post-mortem samples of spinal cord. Genetic causes of ALS are still poorly understood. Approximately 90% of ALS cases are sporadic, and of the remaining 10% (familial ALS) only 20% are attributed to genetic disorders. These cases are linked to mutations that alter SOD1 activity. SOD1 is considered one of the most important cellular scavengers of the cytoplasmic superoxide anion. Recently, SOD1 presence has been confirmed in mitochondria, particularly in the OMM and the IMS, where it may exert a scavenging activity. Apparently, its localization to the IMS is fundamental for its correct maturation (Reddehase et al., 2009) and this observation gains special relevance in the context of ALS. It is well known that mutant SOD1 found in patients generates toxic aggregates (Bruijn et al., 1998). Recently, mutant SOD1 was discovered to specifically aggregate in mitochondria and induce mitochondrial impairment and induction of apoptosis (Cozzolino et al., 2009). Although SOD1 mutations affect only a small fraction of ALS patients, it is plausible that similar mechanisms involving mitochondria and oxidative stress are responsible for the induction of pathogenesis in most of the patients.

2.8 Is Preventing Mitochondrial Oxidative Stress With Antioxidants Effective?—The Example of Neurodegenerative Diseases

As a natural consequence of the multiple connections between mitochondria, oxidative stress, and neurodegeneration, several strategies have been developed directed at reducing oxidative stress and recovering from the pathological phenotype. Several compounds with antioxidant properties have been shown to reduce oxidative stress and increase cell survival in in vitro systems (Fig. 9).

Lipoic acid (LA), a cofactor for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, and N-acetyl-cysteine decrease mitochondrial oxidative stress in fibroblasts derived from AD patients. LA used in a combination with acetyl-L-carnitine (ALCAR, a membrane permeable form of the mitochondrial acetyl carrier carnitine) protected primary cortical neurons against apoptosis induced by 4-hydroxy-2 nonenal (Abdul, Butterfield, 2007).

Coenzyme Q 10 (CoQ10), a fundamental cofactor in the respiratory chain with elevated antioxidant properties, was able to protect human neuroblastoma cells from paraquat- and rotenone-induced mitochondrial dysfunction and cell death. Similarly, the mitochondria-targeted form, MitoQ, prevented cell death in fibroblasts from patients affected by Friedreich's Ataxia (FA). This molecule possesses elevated antioxidant properties, preserves mitochondrial functions, and reduces ROS formation even in Rho zero cells (lacking mtDNA).

Other mitochondria-targeted antioxidants are potentially interesting. MitoE, an analogue of Vit-E, shows elevated scavenging activity in fibroblasts from FA patients. MitoQ and LPBNAH, a derivative of PBN, protected neuroblastoma cells from H_2O_2-induced oxidative stress and Aβ1-42 toxicity.

Antioxidant-based strategies also appear widely effective in animal models of neurodegenerative disease. Natural antioxidant-like CoQ, Vit-E, creatine, and green tea polyphenols showed protective effects in mouse models of both PD and AD (Beal, 2003). LA significantly increased survival in HD mice models N171-82Q and R6/2 (Andreassen et al., 2001) and also reduces oxidative stress in aged rats and reduces memory impairment in aged mice (Quinn et al., 2007). The combination of LA and ALCAR reduced aging-related mitochondrial damage in rats and promotes neuron survival during glutamate-induced toxicity (Nagesh Babu et al., 2011).

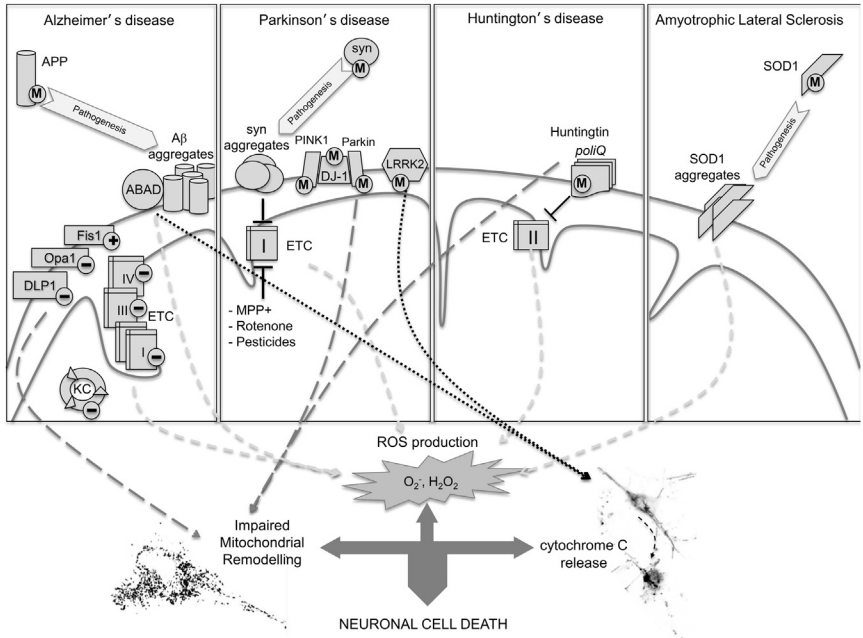

Figure 9 ***Mitochondrial-related alterations in principal neurodegenerative disorders.*** In Alzheimer's disease amyloid peptides (Ab) can aggregate to mitochondria and interact with Ab alcohol dehydrogenase (ABAD) to induce cytochrome C release and ROS production. Contemporary altered activity of Krebs cycle enzymes (KC), complexes of respiratory chain, and proteins involved in mitochondria fusion and fission leads to impaired mitochondrial remodeling and ROS production. Similar readouts on mitochondrial physiology are observed in Parkinson's disease. Aggregation of mutant synuclein (syn) impair activity of respiratory complex I similarly as many pro-parkinsonian compounds. In this scenario should be added the activity of mutant proteins such as PINK1, DJ-1, Parkin, LRRK2 that impair mitochondrial modeling and recycling as promoting induction of cytochrome C release. In Huntington disease mutant huntingtin (carrying poliQ expansion) can impair activity of II respiratory complex (with consequent ROS production) and mitochondria transport along filaments in axons. Also superoxide dismutase 1 (SOD1) mutations causing ALS can induce appearance of toxic mitochondrial aggregates that lead to improved toxic ROS production. Altered protein or OXPHOS complexes activities are shown with a plus or minus symbol in a circle, mutant proteins instead are marked by an M in a circle.

Ginkgo biloba extract seems to be of therapeutic benefit in the treatment of mild dementia of different etiology, especially as regards AD (Janssen et al., 2010). Leuner and colleagues suggest that mitochondrial protection and reduction of oxidative stress are important components of the neuroprotective activity of Ginkgo biloba extract (Leuner et al., 2007).

A novel class of cell-permeable small peptides that selectively partition into the inner mitochondrial membrane and possess intrinsic mitoprotective activities have been developed and proposed as a novel class of mitochondria-targeted antioxidants. These novel peptides were originally designed by Szeto and Schiller and have been designated SS peptides. This class of molecules is characterized by a structural motif that alternates aromatic residues and basic amino acids. Contrary to MitoQ and MitoE, these aromatic-cationic peptides are taken up by mitochondria but are not delivered into the matrix. Furthermore, uptake is not dependent on MMP (the extent of uptake was only reduced by ~10−15% in mitochondria that were depolarized with FCCP) and is not limited to mitochondria with normal MMP. This is a major advantage when dealing with diseased mitochondria with a reduced MMP (Zhao et al., 2004). Animal studies indicated that these novel mitochondria-targeted peptides have excellent pharmacokinetic properties and are relatively free of toxicity, suggesting that they may have enormous therapeutic potential. Several different compounds have been tested and most of them display antioxidant properties (SS-02, SS-19, SS-31) and have been demonstrated to inhibit mitochondrial ROS production, prevent mitochondrial swelling, and neuronal cell loss in animal models of ALS (Petri et al., 2006) and PD (Yang et al., 2009). Interestingly, the SS-20 peptide that does not show antioxidant properties appears to be potent in protecting neuronal loss in a mouse model for Parkinson's disease.

Despite the collective success of antioxidant strategies in recovering the pathological phenotype in cells and animal systems, convincing clinical results are still lacking. In 1997, Sano et al. published one of the first clinical studies testing compounds that protect against mitochondria-mediated oxidative stress (Sano et al., 1997). This study was a double blind, placebo-controlled, randomized, multicenter trial in patients with moderate severity AD. This study tested both selegiline (an inhibitor of monoamine oxidase) and α-tocopherol (Vit-E) for 2 years in a total of 341 patients. Unfortunately, no significant benefits were observed.

More recently, MitoQ, considered one of the most promising mitochondria-targeted antioxidants, was tested in a Phase 2 trial on PD patients. The double blind, placebo-controlled study was conducted for 12 months in 128 patients, but failed to provide evidence that MitoQ could ameliorate the pathological condition (Snow et al., 2010).

Clinical failure for the tested antioxidant therapies could be explained by many reasons. First, the inability to measure mitochondrial damage and contributors to oxidative damage may be due to low bioavailability of the

compound within the brain because of difficulties in crossing the blood–brain barrier. Second, the therapy is usually administered to patients in advanced stages of the pathology, in a condition under which the scavenging activity of the compound might not be sufficient to reverse the phenotype (as this may be due to a different, specific mechanism, at this stage already independent of oxidative stress).

Together with mitochondrial dysfunction and oxidative stress, another characteristic common in most neurodegenerative diseases is the presence of aggregated proteins. Examples include Aβ in AD, α-synuclein and ubiquitin in PD, mutant SOD1 in some forms of ALS, and mutant HTT in HD. As already cited, in most of the cases, protein aggregates accumulate also in mitochondria, promoting the increase of oxidative stress and impairment of mitochondrial functions.

Interestingly, in rats, administration of epoxomicin or PSI, two proteasomal inhibitors, leads to generation of an animal model of Parkinson's disease (McNaught et al., 2004). It is well known that proteasomal degradation requires ATP, while elevated levels of oxidative stress impair correct protein folding and, in the case of AD, promote BACE overexpression and accumulation of Aβ (Tamagno et al., 2005). A model could be envisioned in which the presence of protein aggregates can promote mitochondrial dysfunction and ROS generation. This would promote protein misfolding and impairment of proteasomal activity, initiating a cycle leading to neuronal death and progression of the pathology.

It should be considered that even if altered oxidative stress is a common feature of neurodegeneration, ROS might not be generalized as toxic components, but rather act as proper signaling molecules. The pathways of MAPK (mitogen-activated kinase), PI3K (phosphoinositide 3-kinase), and PKCs are ROS-sensitive and could promote cell proliferation in the presence of some oxidants (Giorgi et al., 2010; Hole et al., 2010). In the presence of ROS, the PI3K pathway can induce the nuclear respiratory factor 2, whereas PKCs can sustain brain remodeling and synaptogenesis after stroke (Sun et al., 2008). Moreover, ROS also mediate cell survival through the activation of HIF1a and NF-κB. HIF1a results in glycolytic switch and reduction of respiratory protection during stroke or hypoxia conditions (Siddiq et al., 2005) or in HD neurodegeneration animal models, whereas NF-κB maintains the expression of antiapoptotic factors such as GADD45B and XIAP (Pahl, 1999).

Thus, it should be considered that in an oxidative environment, such as found in brain cells from patients with neurodegenerative disease, systems

are likely adapting in an attempt to survive. Administration of antioxidants could help cells to recover their equilibrium but could also be helpful for stimulating pathways that manage ROS metabolism. From this point of view, the stimulation of Nrf2 is a promising target. This protein is normally present at low levels in the cytosol, bound to the Cul3-based E3 ligase adaptor KEAP1. Under oxidative stress, KEAP1 undergoes conformational changes, leaving Nrf2 free to move within the nucleus. In the nucleus, it binds to ARE and exerts its transcriptional activity to promote the expression of antioxidant genes such as NQO1, SOD1, and GST (Nguyen et al., 2009).

To date, sulforaphane is considered one of the most potent compounds for promoting Nrf2 activation. Sulforaphane is usually obtained from cruciferous vegetables, converted from glucosinolate or glucoraphanin. Sulforaphane is able to oxidize cysteine residues on KEAP1, leading to Nrf2 activation. In an animal model of stroke, administration of sulforaphane or carnosic acid (another Nrf2 activator) led to neuroprotection and improvement of neurological functions (Satoh et al., 2008). Furthermore, modulation of Nrf2 improved neurological impairment in animal models of AD, HD, or after administration of the pro-Parkinsonian drug MPTP (Calkins et al., 2005; Kanninen et al., 2009).

Investigating antioxidant strategies as therapeutic interventions in neurodegenerative disease are a promising area of inquiry, but convincing clinical results remain elusive. The development of such strategies should involve compounds that act on pathways controlling intracellular ROS metabolism, including adjuvants of antioxidant compounds, as well as compounds modulating proteasomal activity (Fig. 10).

3. CONCLUSIONS

In addition to the well-characterized energy-producing functions, mitochondria are an important intracellular source of ROS. Multiple mitochondrial functions and interconnections exist between aerobic energy metabolism, generation of ROS, activation of the apoptotic pathways, and other fundamental homeostatic and signaling pathways (e.g., Ca^{2+} homeostasis, lipid and nucleotide synthesis). Thus, mitochondrial impairment determines various degrees of energy failure and deregulation of ROS production. An in-depth investigation of all these effects will be the prerequisite to identify effective strategies to counteract the deleterious

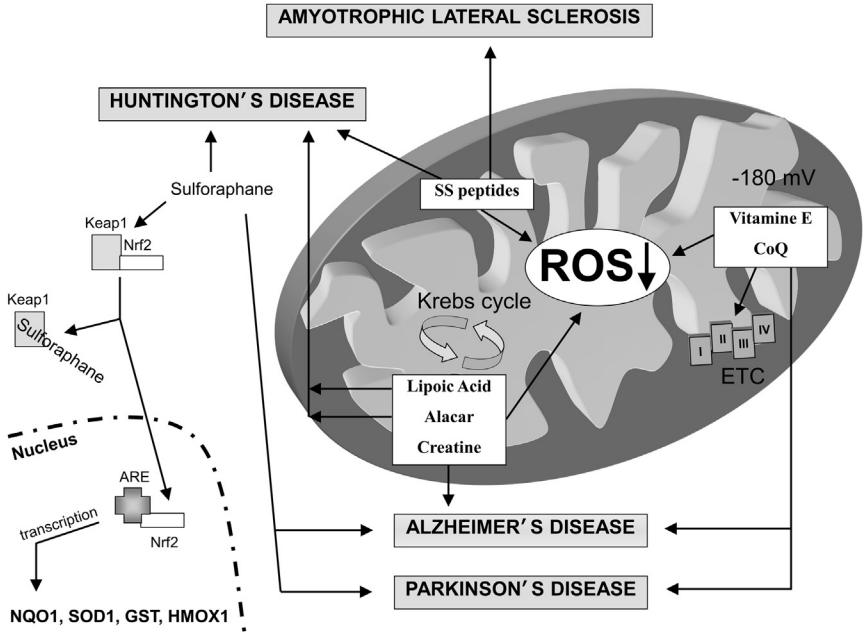

Figure 10 ***Schematic view of various compounds that reduce oxidative stress in diseases mentioned in the text and their correlation with neurodegenerative disorders.*** Mitochondrial selective compounds can act in directly buffering ROS production, such as Szeto–Shiller peptides (SS), vitamine E or coenzyme Q (CoQ), and derivatives. Buffering of ROS production could by obtained by inhibiting interaction between Keap1 and the nuclear respiratory factor 2 (NRF2). Restoration of mitochondrial functions has been also obtained by the use of drugs targeting Kreb's cycle, such as lipoic acid, acetyl-l-carnitine (ALCAR), or creatine.

and multiple consequences of mitochondrial malfunctioning. The comprehension of the mechanisms regulating mitochondrial physiology and homeostasis, and in particular the control of mitochondrial ROS production, may have a significant impact for the development of novel therapies for the treatment of a wide variety of human diseases.

However, it should be noted that many clinical trials using antioxidants (in conditions such as cardiovascular diseases, cancer, diabetes, and neurological degenerative diseases, as well as to slow the aging process) have provided contradictory results. Thus, although the experimental evidence for an antioxidant therapy is quite promising, further validation work is required. Despite the unresolved questions about the parallel role of ROS in oxidative damage or as signaling molecules, the causal link of mitochondria impairment in aging and associated diseases is unequivocal.

ABBREVIATIONS

3MA	3-Methyladenine
4-HNE1	4-Hydroxy-2-noneal
6-OHDA	6-Hydroxy-dopamine
8-oxodG	8-Oxo-7,8-dihydro-2′-deoxyguanosine
Aβ	Amyloid-beta
ABAD	Ab-binding alcohol dehydrogenase
AD	Alzheimer's disease
ANT	Adenine nucleotide translocase
AIF	Apoptosis-inducing factor
ALS	Amyotrophic Lateral Sclerosis
AHL	Age-related hearing loss
ALCAR	Acetyl-1-carnitine
AMPK	AMP-activated protein kinase
AP-1	Activator protein-1
APDC	4-Amino-2,4-pyrrolidinedicarboxylic acid
APP	Amyloid precursor protein
ARE	Nrf2/antioxidant response element
Ca^{2+}	Calcium ion
$[Ca^{2+}]c$	Cytosolic calcium concentration
$[Ca^{2+}]m$	Mitochondrial calcium concentration
CoQ10	Coenzyme Q 10
CPT1mt	Malonyl-CoA-insensitive CPT1A
CR	Caloric restriction
CRTC 1	CREB-regulated transcription coactivator 1
CsA	Cyclosporine A
CT	Computed tomography
CVDs	Cardiovascular diseases
DA	Dopamine
DAG	Intrahepatic diacylglycerol
Dnm1p	Dynamin-related protein 1
Drp1	Dynamin-related protein 1
DTT	Dithiothreitol
EC	Endothelial cell
EDRF	Endothelium-derived relaxing factor
EGF	Epidermal growth factor
EPR	Electron paramagnetic resonance imaging
ER	Endoplasmic reticulum
***(Ercc 1)*$^{-/\Delta}$**	The excision repair cross-complementation group 1
ETC	Electron transport chain
Fdxr	Ferredoxin reductase
FA	Friedreich's Ataxia
FAs	Fatty acids
FFA	Free fatty acids
Fis1	Fission protein 1
FRTA	Free Radical Theory of Aging
GFP	Green-fluorescence protein
GPx	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione S-transferase

HCC	Hepatocellular carcinoma
HD	Huntington's disease
HUVECs	Human umbilical vein endothelial cells
HG	High glucose
HK	Hexokinase
HO-1	Heme oxygenase-1
H_2O_2,	Hydrogen peroxide
HSPs	Heat-shock proteins
HTT	Huntingtin
IDH2	$NADP^+$-dependent isocitrate dehydrogenase 2
IIS	Insulin signaling
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IPC	Ischemic preconditioning
iPSC	Induced pluripotent stem cells
I/R	Ischemia/reperfusion
ITPR2	Inositol 1,4,5-trisphosphate receptor type 2
JNK	c-Jun NH2-terminal kinase
KO	Knockout
LA	Lipoic acid
LC3	MAP1 light chain 3
LCFA	Long-chain fatty acids
LRRK2	Leucine-rich repeat kinase 2
MAMs	Mitochondria-associated membranes
MAPK	Mitogen-activated protein kinase
(Marf)	Mitochondrial assembly regulatory factor
MDA	Malondialdehyde
MEFs	Mouse embryonic fibroblast cells
Mff	Mitochondrial fission factor
Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
MFRTA	Mitochondrial Free Radical Theory of Aging
MGM1	Dynamin-like GTPas
Mid49	Mitochondrial dynamics protein of 49 kDa
Mid51	Mitochondrial dynamics protein of 51 kDa
MMP	Mitochondrial membrane potential
MnSOD	Manganese-dependent superoxide dismutase
MPC	Mitochondrial pyruvate carrier
MPP^+	1-Metyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	Mitochondrial DNA
Mtfp1	Mitochondrial fission process 1
mTOR	Mammalian target of rapamycin kinase
NAC	N-acetylcysteine
(NAFL)	Nonalcoholic fatty liver
NAFLD	Nonalcoholic fatty liver disease
NASH	Steatohepatitis
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
Nox	NADPH oxidases
$O_2^{\bullet-}$	Superoxide
$^{\bullet}OH$	Hydroxyl radical

OMM	Outer mitochondrial membrane
$ONOO^-$	Peroxynitrite
Opa1	Optic atrophy 1
PGC-1α	Peroxisome proliferator—activated receptor gamma coactivator 1-alpha
PD	Parkinson's disease
PBR	Peripheral benzodiazepine receptor
Pin1	Peptidyl—prolyl isomerase
PINK1	PTEN-induced putative kinase protein 1
PKC	Protein kinase C
PiC	Mitochondrial phosphate carrier
POLG	DNA polymerase γ
PPARs	Peroxisome proliferator—activated receptors
Prx	Peroxiredoxins
PS	Presenilin
PSSG	Protein mixed disulfide
PTP	Permeability transition pore
PUFAs	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SIRT	Mitochondrial NAD^+—dependent deacetylase sirtuin
SOD1	Zn—Cu superoxide dismutase
SOD2	Manganese superoxide dismutase
SOD3	Superoxide dismutase 3
SREBP-1c	Sterol regulatory element—binding protein-1c
SS peptides	Szeto—Schiller peptides
STOML2	Stomatin-like protein 2
T2D	Type 2 diabetes
TCA	Tricarboxylic acid
TFAM	Transcriptor factor A
TMEM135	Transmembrane protein 135
TPP^+	Triphenylphosphonium cation
Trx2	Thioredoxin
UCP	Uncoupling proteins
Vit-C	Vitamin C
Vit-E	Vitamin E
VLDL	Very low-density lipoproteins
VDAC	Voltage-dependent anion channel
WD	Wilson disease

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