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*Published by the Journal of Alzheimer's Disease*

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Journal of Alzheimer's Disease 62 (2018) 1319–1335
DOI 10.3233/JAD-170732
IOS Press



Review

# A Long Journey into Aging, Brain Aging, and Alzheimer's Disease Following the Oxidative Stress Tracks

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Accepted 28 November 2017

**Abstract**. The Editors of the *Journal of Alzheimer's Disease* invited Professor Patrizia Mecocci to contribute a review article focused on the importance and implications of her research on aging, brain aging, and senile dementias over the last years. This invitation was based on an assessment that she was one of the journal's top authors and a strong supporter of the concept that oxidative stress is a major contributor to several alterations observed in age-related conditions (sarcopenia, osteoporosis) and, more significantly, in brain aging suggesting a pivotal role in the pathogenesis and progression of one of the most dramatic age-related diseases, Alzheimer's disease (AD). Her first pioneering research was on the discovery of high level of 8-hydroxy-2'-deoxyguanosine (OH8dG), a marker of oxidation in nucleic acids, in mitochondrial DNA isolated from cerebral cortex. This molecule increases progressively with aging and more in AD brain, supporting the hypothesis that oxidative stress, a condition of unbalance between the production of reactive oxygen species and antioxidants, gives a strong contribution to the high incidence of AD in old age subjects. OH8dG also increases in peripheral lymphocyte from AD subjects, suggesting that AD is not only a cerebral but also a systemic disease. The role of antioxidants, particularly vitamin E and zinc, were also studied in longevity and in cognitive decline and dementia. This review shows the main findings from Mecocci's laboratory related to oxidative stress in aging, brain aging, and AD and discusses the importance and implications of some of the major achievements in this field of research.

Keywords: Aging, Alzheimer's disease, antioxidant, brain aging, dementia, mitochondria, oxidative stress, vitamin E

## INTRODUCTION

At the beginning of the 20th century, Alois Alzheimer first reported a case of a young patient with dementia whose brain contained characteristic histopathologic features defined as tangles and plaques [1]. The medical community has assimilated these observations and a diagnostic eponym, Alzheimer's disease (AD), has been assigned to such a condition [2]. For many years, memory loss and other cognitive deficits in the elderly have been considered as the result of the aging process and therefore called "senile dementia," whose prevalence and incidence increases exponentially with population aging. Expanding the definition of AD with the inclusion of those with senile dementia swelled the ranks of those diagnosed to such an extent that caused some to claim an "Alzheimerization" of dementia [3]. Nowadays, AD is the most diagnosed type of dementia representing a national health priority. For a long time, amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs) have been considered unquestionably the main cause of AD pathogenesis, but many other theories have been proposed, including

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ISSN 1387-2877/18/$35.00 

oxidative stress and neuroinflammation, to explain a still unknown disease.

## THE AMYLOID CASCADE AND THE TAUOPATHY HYPOTHESES

For many years, the amyloid cascade hypothesis has dominated AD thinking, modeling, and therapeutic approach. Amyloid proteins are beta-sheet proteins that can easily aggregate. Aβ is a proteolytic degradation product of a larger molecule called amyloid-β protein precursor (AβPP). Proteolysis by α-secretase can occur 83 amino acids from the AβPP intracellular carboxyl terminal. Alternatively, proteolysis by the enzyme β-secretase (BACE) cut 99 amino acids upstream of the AβPP carboxyl end. An enzyme complex, γ-secretase, further processes the remaining carboxyl end of α-secretase or β-secretase digested AβPP. This complex consists of the proteins presenilin 1, presenilin 2, nicastrin, APH-1, and PEN2 [4]. γ-secretase proteolysis does not uniformly occur at a single amino acid, although proteolysis either 57, 59, or 61 amino acids up from the AβPP carboxyl terminus is most common. The 40–42 (although it can be 38–43) amino acid segment, directly created by β- and γ-secretase proteolysis, is the Aβ peptide. The amyloid cascade hypothesis postulates an overproduction of Aβ, which leads to neuronal dysfunction and apoptosis causing AD clinical manifestations [5]. According to this hypothesis, amyloid accumulation represents the "upstream" event in AD pathogenesis. This point of view has been overcome by the possibility that soluble Aβ oligomers, more than mature amyloid plaques, are the key toxic moieties [6–8]. In fact, it has been demonstrated that amyloid oligomers may access intracellular organelles, including mitochondria, and compromise their function [9]. Amyloid deposition causes local inflammatory and immunologic alterations for a direct neurotoxicity with microglial recruitment and astrocyte activation. It is also associated with the release of cytokines, nitric oxide, and other radical species that can promote neuroinflammation and neurodegeneration. In addition to the amyloid cascade, intracellular NFTs are found in AD brain. They consist of hyperphosphorylated tau protein. Tau phosphorylation is regulated by a series of serine-threonine kinases [10], and tau protein interacts with microtubules of the cytoskeleton leading to the formation of NFTs. The interaction with cytoskeleton causes the destabilization of microtubules and consequently of intracellular trafficking of vesicles and organelles. Interestingly, NFTs correlate more closely with the severity of dementia than plaque counts [11–13]. The association of tangles with a variety of brain damage supports the "tauopathy" concept of neurodegeneration [14], although tauopathy as a primary cause of neurodegenerative diseases is currently demonstrable only in a subgroup of familial frontotemporal dementia. However, the recent failures of drugs targeting amyloid pathways have raised questions not only about this approach but also on the validity of the amyloid cascade hypothesis itself.

## OXIDATIVE STRESS AND DEMENTIA

Oxidative stress is a condition where reactive oxygen species (ROS) production exceeds the cellular antioxidant defense system. In the cell, the main protective system is represented by the antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPX), glutaredoxins, thioredoxins, and catalase, in addition to non-enzymatic antioxidant factors, such as vitamin E, vitamin C, vitamin A, uric acid, and carotenoids.

The brain is highly susceptible to an oxidative imbalance due to its high-energy demand, high oxygen consumption, an abundance of easily peroxidable polyunsaturated fatty acids, high level of potent ROS catalyst iron, and a relative paucity of antioxidant enzymes, this latter more evident in AD brain [15]. Oxidative stress causes damage by lipid peroxidation of membranes, such as plasma and mitochondrial membranes, or oxidation of structural and enzymatic proteins, with irreversible modification of protein tertiary structure and function, and of nucleic acids. Furthermore, Aβ oligomers can insert into membrane bilayers leading to ROS production, followed by intracellular protein and nucleic acids oxidation.

Among products of lipid peroxidation, 4-hydroxy-2-trans-nonenal (HNE) is highly reactive and primarily produced in the brain via lipid peroxidation of arachidonic acid, a highly abundant omega-6 polyunsaturated fatty acids (PUFA) component of neuronal membranes. HNE may modify the ATP synthase, the final step in the production of ATP from electron transport chain (ETC) inside mitochondria [16]. The ETC is a very efficient enzymatic system for energy production but, physiologically, a small amount of superoxide anion ($O_2^{\circ -}$) is constantly produced due to electron leak, but antioxidants act to

protect the cell from its deleterious action [17]. In fact, $O_2^{\circ -}$ can be converted into nonradical derivate such as hydrogen peroxide ($H_2O_2$) either by a spontaneous dismutation reaction or catalyzed by the manganese superoxide dismutase that resides in the mitochondrial matrix [18]. $H_2O_2$ can be converted into hydroxyl radicals ($^{\circ}OH$) through the Fenton reaction. Similar to iron, copper also participates in the Fenton reaction, which exacerbates ROS production [19]. $^{\circ}OH$ can also be produced by a direct reaction of $O_2^{\circ -}$ with $H_2O_2$, a reaction known as the Haber–Weiss reaction.

Hence, mitochondria are prone to oxidative damage and particularly susceptible to $^{\circ}OH$-mediated oxidation, which plays a significant role in DNA oxidation.

To counteract an exaggerated production of ROS, mitochondria possess a very efficient antioxidant system, including glutathione peroxidase, catalase, and peroxiredoxin III, that can catalyze the electron transfer from NADPH to molecular oxygen and generate $O_2^{\circ -}$ [20]. Mitochondrial DNA (mtDNA) is particularly susceptible to oxidative damage because is localized near the ETC and is not surrounded by histones. All four bases (adenine, guanine, cytosine, and thymine) are susceptible to oxidative damage. The aging brain is characterized by an increased oxidative damage to mtDNA with the OH8dG formation, which is the most common marker of oxidative DNA damage. The simultaneous increased oxidation of mtDNA and deficiency of DNA repair could enhance the lesion to mitochondrial genome, potentially causing neuronal damages.

On this basis, it is reasonable that oxidatively mediated damage to biomolecules is extensively reported in AD, suggesting that oxidative stress plays a critical role in the disease pathogenesis [21]. As the main source of ROS generation and a major target of oxidative damage, progressive impairment of mitochondria has been implicated in aging and AD [22]. The interaction between oxidative stress and mitochondrial dysfunction likely forms a vicious downward spiral that amplifies the alterations observed in AD.

## THE MITOCHONDRIAL CASCADE HYPOTHESIS

Based on a large amount of data from *in vitro* and *in vivo* studies, in 2004 the mitochondrial cascade hypothesis was first proposed to explain AD pathogenesis [23]. It is generally accepted that mitochondrial function progressively declines along with age when compensation is no longer possible. In summary, the mitochondrial cascade hypothesis proposes that every single person has a genetically determined mitochondrial starting line, that together with environmental factors determine the age at which clinical disease may ensue. Thus, the "mitochondrial cascade hypothesis" places the mitochondrial dysfunction as the leading factor in the late-onset, non-autosomal dominant AD pathology cascade, underlying the individual genetic background able to regulate since birth its mitochondrial function and sustainability. For this reason, the rate at which age-related mitochondrial dysfunction proceeds differs among individuals. When the mitochondrial function declines and falls below a critical threshold, AD-typical dysfunction at the cellular level may ensue, including Aβ production, tau phosphorylation, synaptic degeneration, and oxidative stress [24–26]. Some authors suggested that mtDNA could predict inheritance; accordingly, epidemiologic studies demonstrated that although both parents may contribute to an individual's AD risk [27], the maternal contribution is higher as compared with paternal [28], and maternal inheritance may specifically affect mid-life memory performance [29].

The fact that mtDNA polymorphisms influence Aβ production and plaque deposition in animal models expressing AβPP transgene further support the mitochondrial cascade hypothesis [30]. Overall, it may be plausible that AβPP and Aβ homeostasis are finely regulated processes where bioenergetic metabolism plays a pivotal role.

## ALZHEIMER'S DISEASE: ONLY A NEURODEGENERATIVE DISEASE OR A SYSTEMIC DISEASE?

The fact that some mitochondrial defects observed in AD patients are not brain-limited, as shown by lower enzymatic activity, such as cytochrome oxidase, in mitochondria from peripheral cells (platelets and fibroblasts), strongly support the concept that AD is a systemic disease [31, 32].

In fact, many studies demonstrated that subjects affected by AD have systemic manifestations that accompany the central nervous system (CNS) dysfunction. The association of AD with some systemic manifestations suggests that it is a multifactorial disease affecting both the brain and the periphery, in which different causative pathways play/interplay

significant roles. Many factors that could increase the risk of developing AD have been identified, such as diabetes mellitus, obesity, and physical inactivity. Growing evidence is showing that physical activity may have a direct positive impact on brain health, not only by the regulation of cardiovascular and metabolic profile [33]. In fact, on one hand, physical activity may modulate cardiovascular risk factors, such as diabetes, hypertension, and stroke, that could, in turn, promotes dementia [34–37]; on the other hand, physical activity *per se* can increase the level of the brain-derived neurotrophic factor (BDNF) and stimulate neurogenesis [38, 39]. Physical activity is associated with larger brain volume, and this relationship could be mediated not only by BDNF but also by other neurotrophic factors not identified yet. Recent data showed that serum levels of BDNF are positively correlated with hippocampal volume, and that exercise is able to increase hippocampal levels of BDNF in animal models [40–43].

Furthermore, AD subjects also exhibit reduced VO2 peak (independent of dementia severity or physical function decline) correlated with brain atrophy [44], and imaging studies show that decreased aerobic fitness is associated with hippocampal atrophy in early AD [45].

Experimental studies suggest that also insulin pathway draws a clear trajectory from the peripheral to the central nervous system such as insulin treatment seems to enhance performance in subjects with AD. Insulin can cross the blood-brain barrier where it modulates several processes such as neurotransmission, cell survival, and amyloid trafficking [46, 47]. Cognitive decline and dementia are among the most common complications of diabetes mellitus (DM), while in turn, a rise in adiposity increases the incidence of DM. Insulin resistance (IR) links obesity, pre-diabetes, and diabetes, which leads to an increased risk of developing cognitive decline. Elevated adiposity precedes and accompanies DM and leads to IR, which in turn promotes hyperinsulinemia. Hyperinsulinemia, insulin resistance, and other related conditions such as hypertension, dyslipidemia, and sub-clinical inflammation are related to a higher risk of cognitive impairment and AD in diabetic patients.

In this context, inflammation plays certainly a role. Plasma cytokines are known to communicate with the brain, and circulating levels of peripheral cytokines have been shown to reflect cytokine levels in the brain. Some studies have also shown that inter-individual variations in peripheral cytokines levels are associated with individual differences in brain structures [48].

Recent studies show that metabolic state may affect nuclear DNA and neuronal plasticity with the regulation of chromatin structure, in particular by inducing histone modifications. A connection has been found between cellular metabolism, and neuronal plasticity with the neuronal function of enzyme acetyl-CoA synthetase 2 (ACSS2) as a chromatin-bound transcriptional coactivator that stimulates histone acetylation and gene expression. Acetyl-CoA metabolism is cell- and tissue-specific, and it is frequently dysregulated in malignant transformation. Optimal acetyltransferase activity requires an increased local acetyl-CoA to CoA ratio, which determines the catalytic activity and substrate specificity of histone acetyltransferases (HAT) enzymes. This finding suggests that histone acetylation can be controlled by changing levels of nuclear acetyl-CoA. Thus, chromatin-bound ACSS2 could provide acetyl-CoA to fuel HAT activity locally, instantaneously recycling CoA and could also recapture acetate from deacetylation reactions. Recently, it has been showed that specific chromatin binding by ACSS2 plays a crucial role in upregulation of immediate early genes with key functions in neuronal plasticity and long-term memory consolidation [49–51].

## ALZHEIMER'S DISEASE AND AGING: AN INSEPARABLE DUO?

Advancing age represents the strongest risk factor AD, but whether AD is a part of aging or whether aging is part of AD is still under debate. Along with aging, the structure and function of the brain change. Neurons are lost in the substantia nigra, mesial temporal region, and hippocampus; at the same time, the amount of neuritic plaques and NFTs increase. Cellular senescence is one of the main contributing factors to age-associated brain tissue dysfunction and represents the core feature of the so-called age-related changes (ARCs), leading to an overall reduction in brain volume and weight and enlargement of cerebral ventricles. ARCs arise from intrinsic/programmed and extrinsic/stochastic causes affecting neuronal viability and vulnerability. Among intrinsic factors, telomere shortening in neural stem cell—engaged in supporting brain tissues—plays certainly a role. Neural stem cells, in the dentate gyrus of the hippocampus and the subventricular zone beneath the lateral ventricles, are likely "replacement parts" for

damaged or dying neurons. The progressive loss of chromosome ends with each replication events leads to cellular apoptosis [52]. Their depletion over a lifetime may contribute to decline in brain structure and function [53]. In this context, oxidative stress increases while mitochondrial energy production decreases along with cellular aging. In this view, considering the fundamental role of mitochondria in cellular bioenergetics, the decline in mitochondrial function represents probably the pivotal factor of cell aging.

## STUDIES ON THE ROLE OF OXIDATIVE STRESS IN AGING AND AGE-RELATED DISEASES FROM OUR RESEARCH LABORATORY

The role of oxidative stress in aging, with a particular focus on brain aging, and in dementia have been the main topic of our research group since the 1990 s. We developed different techniques to measure markers of oxidative stress in tissues of aged subjects, including centenarians, and in subjects suffering from cognitive impairment or dementia and then applied our methodologies in epidemiological studies.

## OXIDATIVE STRESS IN BRAIN AGING AND DEMENTIA

### *Oxidative stress in the brain*

In 1993, in collaboration with Prof. M. Flint Beal at the Harvard Medical School, oxidized nucleoside 8-hydroxy-2'-deoxyguanosine (OH8dG) was measured in nuclear (nDNA) and in mtDNA isolated from three regions of cerebral cortex and from cerebellum of humans aged 42 to 97 years. The amount of OH8dG increased progressively with age in both nDNA and mtDNA; however, the rate of increase was much greater in mtDNA. In fact, there was a significant 10-fold increase for OH8dG in mtDNA as compared with nDNA in the entire group of samples, and a 15-fold significant increase in those older than 70 years. These results showed for the first time that there is a progressive age-related accumulation of oxidative damage to DNA in the human brain and that mtDNA is preferentially affected [54]. We hypothesized that such a damage contributed to the age-dependent increase in the incidence of neurodegenerative diseases. In fact, when we measured OH8dG in the brain of subjects with AD, we found a significant threefold increase for OH8dG in mtDNA in the parietal cortex of AD compared with controls. In the entire group of samples, there was a small significant increase in oxidative damage to nDNA and a highly significant threefold increase in oxidative damage to mtDNA in AD compared with age-matched controls. These results confirmed that mitochondrial DNA is particularly sensitive to oxidative damage, and showed that there is increased oxidative damage to DNA in AD, which contributes to the neurodegenerative process [55].

To better identify the oxidative stress damage in the brain, the OH8dG and levels of mitochondrial DNA 4977 bp depletion ($mtDNA^{4977}$) were determined from two brain areas of healthy subjects and AD patients. Postmortem brain tissue specimens were obtained from frontal and parietal cortices of six healthy subjects and seven patients affected by AD. The mean values either of $mtDNA^{4977}$ and OH8dG levels did not show significant differences between frontal and parietal areas in healthy or in the AD group. On the other hand, when controls and AD subjects were compared, the OH8dG level in the AD patients was six- to eightfold higher than in healthy subjects, whereas $mtDNA^{4977}$ percentage in the AD patients was about threefold lower than in the controls. A positive correlation between the age-related increases of $mtDNA^{4977}$ and of OH8dG levels was found in the brain of healthy individuals. On the contrary, in both brain areas of AD patients, $mtDNA^{4977}$ levels were very low in the presence of high OH8dG amounts. We explained these results assuming that the increase of OH8dG above a threshold level, as in AD, implies consequences for mtDNA replication and neuronal cell survival [56].

To further support our observations on the role of oxidative stress in AD brain, we evaluated membrane fluidity, as a marker of peroxidation of membrane unsaturated fatty acids, in mitochondria extracted from brain tissue of normal aged and AD and evaluated also the effect of peroxidizing conditions on this biological parameter. Mitochondrial membrane fluidity was measured by means of fluorescence polarization of the lipophilic probe DPH. Mitochondrial membrane fluidity was significantly lower in AD compared to controls both when all samples were pooled as well as in different cerebral region with the exception of cerebellum. Under peroxidizing conditions ($FeCl_2$ and $H_2O_2$), membrane fluidity was further reduced in controls, particularly in mitochondria extracted from parietal and temporal areas. This

did not happen when we tested mitochondria from AD brains, suggesting that, in this case, it was not possible to get further lipid peroxidation [57]. These observations suggest that a different susceptibility to oxidative damage exists in mitochondria of different brain areas, what was in agreement with our previous results concerning the evaluation of oxidative stress in mitochondrial DNA. In fact, when we related mitochondrial membrane fluidity with mtDNA damage, as measured by OH8dG content, in AD brain we found a parallel increase of viscosity in mitochondrial membrane and of the amount of OH8dG in mtDNA, suggestive of a relationship between these biological markers of oxidative stress and so of a significant role of oxidative stress in AD pathogenesis [58].

The hypothetical dangerous effect of oxidative stress on mtDNA was also observed in diseases other than AD. In postmortem brain specimens of Huntington's disease, a twofold increase of OH8dG in mtDNA was found in the parietal and slightly less in the frontal cortex compared to controls, suggesting that oxidative damage to mtDNA plays an important role also in other neurodegenerative diseases [59].

### *Oxidative stress in peripheral lymphocytes and in plasma molecules*

A significant amount of evidence supports the hypothesis that AD is not only a cerebral but also a systemic disease and that alterations observed in the brain could also be detected in the periphery. This issue has been recently brilliantly reviewed [60], summarizing several studies performed in the last years. From our side, we decided to evaluate if oxidative stress biomarkers could be detected in peripheral cells of subjects with AD, and we choose lymphocytes as a model for this research.

In a first study, we measured OH8dG in nuclear DNA extracted from peripheral lymphocytes of old age subjects with AD and in healthy controls. We found a higher concentration of this biomarker of oxidative stress in AD and, what was of particular interest, the amount of OH8dG increased along with age in controls, supporting again the role of aging as the main risk factor of those alterations leading to an increased risk of AD [61]. These data were later confirmed in another study where, in AD, OH8dG in nDNA of peripheral lymphocytes was inversely correlated with plasma concentration of several lipophilic antioxidants, such as lycopene, lutein, alpha- and beta-carotene. These findings suggest that lymphocyte OH8dG DNA content in patients with AD reflects a condition of increased oxidative stress possibly related to a poor antioxidant status [62].

Since mitochondria have shown such a fundamental role as a site of production of ROS as well as a target of their dangerous action, we set up a method to extract mitochondria from peripheral lymphocytes to examine more deeply and in detail any peculiar oxidative damage. In collaboration with Prof. Butterfield, at the University of Kentucky, measurements of biomarkers of oxidative stress on lipids and oxidative and nitrosative stress on proteins were performed in mitochondria extracted from lymphocytes of subjects suffering from AD and healthy age-matched controls. For the first time, increased levels of three biomarkers—namely, protein carbonyls, protein-bound HNE, and protein-resident 3-nitrotyrosine—were detected in AD, reinforcing the active role of oxidative damage in AD not only in the brain but also in peripheral cells [63]. This was true also in mild cognitive impairment (MCI), where all the three above-cited biomarkers were higher in lymphocyte mitochondria compared to controls. All biomarkers showed a significant negative correlation with the Mini-Mental State Examination (MMSE) score. Interestingly, they also negatively correlated with the antioxidants tocopherols and tocotrienols (vitamin E family). A further proteomic approach showed alterations in some proteins (thioredoxin-dependent peroxide reductase, myosin light polypeptide 6, and ATP synthase subunit beta) that might be important in the progression and pathogenesis of AD [64].

More recently, we studied the activity and expression of aconitase 2 ($ACO_2$), a mitochondrial enzyme of the Krebs cycle, that is particularly sensitive to free radical damage. Mitochondria were extracted from lymphocytes of old age subjects with AD or MCI and in age-matched cognitively healthy controls. We found a reduced $ACO_2$ activity in AD and MCI compared to controls. In the overall study population, $ACO_2$ activity was significantly correlated with the plasma levels of the antioxidants vitamin E, vitamin A, vitamin C, and uric acid as well as with the MMSE score. We hypothesized that the reduced activity was due to oxidative damage of the enzyme, linked to the paucity of antioxidants, but we also observed a reduced expression of $ACO_2$ in MCI and AD, that can contribute to a dysfunction in the Krebs cycle. Therefore, it is conceivable that the reduction of $ACO_2$ activity in mild AD, already present in the predementia phase (i.e., MCI), might promote

energy unbalance and foster the increase in oxidative and nitrosative stress, which then promotes Aβ accumulation, synaptic dysfunction, and neuronal death [65].

Lack of antioxidants seems to have an important role in determining oxidative alterations in dementias. In another study conducted not only in AD but also in vascular dementia (VaD), we found a significant reduction in water-soluble (vitamin C and uric acid) and lipophilic (vitamin E, vitamin A, carotenoids including lutein, zeaxanthin, beta-cryptoxanthin, lycopene, alpha- and beta-carotene) antioxidant micronutrients in plasma of AD and VaD compared to cognitively healthy subjects. This poor antioxidant status was associated with higher levels of biomarkers of lipid peroxidation [malondialdehyde (MDA)] and of protein oxidation (immunoglobulin G levels of protein carbonyls and dityrosine) in patients compared to controls. Thus, independently of its nature (vascular or degenerative) dementia was associated with the depletion of a broad spectrum of antioxidant micronutrients and with increased protein oxidative modification [66]. All these results suggested a fundamental role of nutrition and of food or supplement antioxidants in the prevention of several age-related diseases that we started to investigate more in details in successive studies and reported in comprehensive reviews [67–69].

## OXIDATIVE STRESS IN MUSCLE AND IN BONE DISEASES

Besides cerebral tissue, muscle is also mainly formed by post-mitotic cells, which are liable to accumulate oxidative damage over time, and both account for a large share of the body's total oxygen consumption at rest. So, we supposed that what we had observed in the brain could be also detectable in muscle and based on this assumption, we examined three well-established markers of oxidative damage to DNA (OH8dG), lipids (MDA), and proteins (carbonyl groups), respectively, in muscle biopsies obtained from healthy subjects aged 25 to 93 years. An increase of OH8dG and MDA was detectable after the age of sixty and also carbonyl groups increased, although not significantly [70].

In a further series of experiments in muscle specimens from vastus lateralis of young and old healthy subjects of both sexes, the increase of oxidative damage in DNA and lipids was confirmed in the elderly, more evident in men than in women, together with a reduction in the activities of the antioxidant enzymes catalase and glutathione transferase [71].

Taken together, these data strongly supported the impact of oxidative stress also in the aging of muscle and sarcopenia, that still represents a strong risk factor of weakness and falls in the elderly and, as a consequence, of severe disability.

We found alteration due to oxidative damage also in muscle specimens from subjects suffering from chronic fatigue syndrome (CFS), a still poorly understood condition characterized by long-lived, disabling fatigue associated with deficits in short-term memory, impairments in concentration, sleep disturbances, and skeletal muscle pain. In particular, we detected a marked oxidative damage in DNA and lipids but also an increased activity of the antioxidant enzymes catalase, glutathione peroxidase, and transferase, with higher plasma levels of total glutathione in patients compared to age-matched controls. This suggests an increased production of free radicals in CFS that the organism tries to counteract increasing the endogenous antioxidant activities and justifying an organic origin of CFS, that is too often consider a psychological disease [72].

In old-age subjects, a pro-oxidant status was also observed in a common disease such as osteoporosis. In fact, in a population of postmenopausal women, living independently, in good health and nutritional status, and affected by mild or no disability, those who suffered from osteoporosis had consistently lower plasma levels of vitamin C, vitamin E, vitamin A, and uric acid compared to controls. Despite this, none of the subjects belonging to the two groups had levels below the normal vitamin C and vitamin E ranges. The activities of antioxidant enzymes in plasma (SOD and GPX) and erythrocytes were significantly lower in osteoporotic than in controls. The study showed the negative effect of low levels of antioxidants in bone mass and fracture risk, as demonstrated in epidemiological studies. Of note, vitamin C is a cofactor in the maturation of collagen. We explained these observations supposing an inadequate antioxidant intake that can affect osteoclastogenesis [73].

## OXIDATIVE STRESS IN CEREBROVASCULAR DISEASES

An increased production of free radicals has been constantly observed in the brain in both ischemic and hemorrhagic stroke. Therefore, it is conceivable that in these acute conditions a deficiency in antioxidants

may reduce resistance against the damage, leading to a status of oxidative stress [74].

We investigate this hypothesis evaluating the antioxidant profile in plasma of subjects suffering from a stroke, in order also to establish the impact of the antioxidant status on the prognosis and to evaluate a potential effect of antioxidants in stroke therapy.

In subjects with cortical stroke, we found higher levels of lipid hydroperoxides (measured as cholesteryl ester hydroperoxides, CEOOH), with a peak at day-five after the acute event, compared to subjects with lacunar infarcts. This was accompanied by reduced levels of the antioxidant vitamin C, or ascorbic acid that did not differ significantly between groups. CEOOH levels correlated with severity of the stroke, as evaluated by the NIH stroke scale and the Glasgow coma scale, and with stroke volume and site—higher in total anterior than in posterior cerebral syndrome [75]. In a subsequent study, several plasma enzymatic (SOD, GPX, and erythrocyte SOD (eSOD) activities) and non-enzymatic (vitamins A, E, C and uric acid) antioxidants were serially measured in subjects suffering from acute ischemic stroke of recent onset on admission and at specific time points in the first week. Antioxidant levels in patients on admission were compared with those of age- and sex-matched controls. The mean antioxidant levels and activities in patients on admission were lower than those of controls with a gradual increase over time. Patients with the worst early outcome (death or functional decline) had higher vitamin A and uric acid plasma levels and lower vitamin C levels and eSOD activity than those who remained functionally stable, suggesting that the majority of antioxidants are reduced immediately after an acute ischemic stroke, possibly as a consequence of increased oxidative stress, and that a specific antioxidant profile is associated with a poor early outcome [76]. Also, other lipophilic antioxidants such as carotenoids were found significantly lower and MDA higher after stroke in the same population, with a lower amount of lutein in those with a poor early outcome [77].

All these observations suggested that vascular damage can be modulated by antioxidant status and this not only in ischemic but also in hemorrhagic stroke, where vitamin C plasma levels were significantly correlated with the severity of the neurological impairment, as assessed by the Glasgow Coma Scale and the NIH Stroke Scale, as well as with the major diameter of the lesion [78]. Vitamin C was also found lower in plasma of oldest-old subjects with hyperhomocysteinemia suffering from stroke [79] suggesting that this vitamin could have a significant effect in protecting against oxidative stress due to ischemic or hemorrhagic stroke. For this reason, we conducted a multicentric study named AVASAS (Aspirin versus Ascorbic acid plus Aspirin in Stroke) study to evaluate the effect of vitamin C added to standard therapy with aspirin regarding clinical outcomes and plasma biomarkers of oxidative stress. In the acute phase and along with time until the end of the three months of therapy, we found lower levels of 8,12-isoprostanes $F_{2\alpha}$-VI, a specific marker of lipid peroxidation, and higher levels of vitamin C in those treated with the association compared to aspirin alone, while the functional outcomes were similar between groups [80]. The relationship between high levels of oxidative damage biomarkers and low antioxidants in plasma was revealed also in studies on conditions at risk for both cerebrovascular and neurodegenerative diseases such as diabetes [81], atherosclerosis [82, 83], congestive heart failure [84], a definite risk for AD in the elderly [85], suggesting that vascular risk factors can act as so through oxidative stress mechanisms particularly in the oldest-old [86–88], who appears a sub-group of population more sensitive than younger to oxidative stress [89]. The relationship between reduction of antioxidant defenses and aging seemed more and more intriguing. Therefore, the question was: if aging is the major risk factor for AD and antioxidants decrease with aging, could we modulate the risk for AD with antioxidants, like diet or supplementation? To answer this question, we decided to study a particular aged population: the centenarians.

## ANTIOXIDANTS IN CENTENARIANS

Healthy centenarians can be considered a good example of "successful aging", and although genetic factors influence their longevity, environmental aspects are also of great importance and, among them, diet is probably one of the most influent. Since aging is associated with oxidative stress, and antioxidant defense systems were developed by the organism to guarantee its survival, we decided to evaluate the antioxidant status in centenarians to understand if this could influence such an extreme longevity. To this purpose, we measured a broad set of enzymatic (SOD, eSOD, and GPX activities) and non-enzymatic (vitamins A, C, E; carotenoids, and uric acid) antioxidants in plasma of a group of healthy centenarians comparing them with other three groups aged <60 years, 61–80 years, and 81–90 years. The most striking

findings were the highest levels of vitamin A and vitamin E in centenarians compared to all the other groups, that, instead, after exclusion of centenarians from regression analysis, showed a significant decrease of all non-enzymatic antioxidants and increase of plasma SOD and eSOD activities along with age [90]. So, the main characteristic of centenarians seemed to be the high levels of lipid soluble vitamins, A and E, but when we analyzed more in details the characteristics of our centenarian population, that lived in different geographic areas of Italy, surprisingly we found that those living in Sardinia island—famous for being one of the regions in the world with the highest number of centenarians—had high plasma levels of uric acid but not high level of vitamin A and vitamin E as the centenarians from the other regions. Thus, probably in this particular group of Sardinian centenarians genetic, rather environmental factors, seem to play a significant role in longevity compared to other Italian populations [91]. This is a plausible explanation, considering that Sardinia has been quite isolated from the rest of Italy for a long time and its demographic characteristics are peculiar as, for example, the ratio between male and female centenarians, that is different from the rest of Italy [92].

## THE WAR AGAINST OXIDATIVE STRESS: ANTIOXIDANTS IN POPULATION STUDIES

All the studies we performed in old age subjects with different type of chronic age-related diseases lead to the same conclusion: a status of poor antioxidant levels detected in plasma is associated with a higher risk to be affected, strongly supporting the idea that a good food intake of antioxidants results in a better health condition and protection from several diseases. If this is true, and probably obvious, in young population in order to prevent future diseases, and also in subjects definite as young-old (65–75 years of age), it is less evident if changing habits, including diet, in the old (75–84 years) and oldest-old (over 85 years) populations can have benefit in terms of physical and mental health.

In a study on healthy elderly subjects who underwent a diet very rich in fruit and vegetables, compared to age-matched subjects with a poor intake of both, we found high levels of plasma lipophilic antioxidants in the first group, together with lower levels of the oxidative by-products MDA and protein carbonyls. These differences were maintained after age correction, suggesting that, no matter how old a subject is, a long-term balanced diet with high content of fruits and vegetables prevents, at least in part, the formation of oxidatively damaged molecules [93]. A further interventional study in old-age subjects who consumed a high rich diet of fruits and vegetables for almost one year, not only plasma antioxidants were higher and oxidative stress biomarkers lower compared to low-intake age-matched group, but they also showed, what is noteworthy, better cognitive functions as evaluated by a large neuropsychological battery [94]. Therefore, a good antioxidant status seems to be protective against cognitive decline and, in fact, in subjects with MCI, we detected a similar depletion of plasma antioxidant as in AD when compared with age-matched cognitively healthy controls. It was particularly interesting the observation that, although the non-enzymatic antioxidants were low, there was not a parallel induction of activity of antioxidant enzymes, such as SOD or GPX, not only in AD but also in MCI [95]. Hence, with MCI a condition at risk for conversion to AD, a diet containing high levels of antioxidants may correct an antioxidant imbalance and prevent or delay a further deterioration toward dementia. Furthermore, measurements of peripheral markers of oxidative stress could be useful in detecting those subjects more responsive to a supplement of antioxidants, an aspect not properly explored in trials with, for example, vitamin E that failed to protect against AD [96, 97]. In fact, the effect of supplementation should be evaluated only in those lacking a specific micronutrient, an approach that should not be proposed indiscriminately. Since vitamin E was used in the treatment of MCI and of AD, we decided to investigate more in detail its role in population studies. In fact, revising the few randomized clinical trials with vitamin E, we found that only α-tocopherol was used as treatment, while under the umbrella of the term vitamin E several other natural forms are present, namely α-, β-, γ-, δ-tocopherol and α-, β-, γ-, δ-tocotrienol. α-Tocopherol is a prevalent form in the organism, but the other forms have unique antioxidant and anti-inflammatory properties that are also higher than α-tocopherol in prevention and therapy of chronic diseases [98]. Thus, their role in cognitive impairment was worthy to be examined in depth.

To this purpose, we measured plasma levels of the eight vitamin E forms as well as of α-tocopherylquinone, αTQ (byproduct of oxidation of α-tocopherol) and of 5-nitro-γ-tocopherol, 5NγT

(byproduct of nitrosative damage of γ-tocopherol), in a large number of free-living elderly (over 65) who participated in the Conselice study [99], a population-based Italian study that evaluated the incidence of dementia after a four-year follow-up. The prevalence of dementia at baseline was highest among participants with higher concentrations of δ-tocopherol and αTQ while in the longitudinal analysis a lower risk of dementia was detected in those in the middle tertile of plasma γ-tocopherol at baseline [100]. So, in this old-age population, both prevalent and incident cognitive impairment was associated with plasma concentrations of non α-tocopherol forms of vitamin E, suggesting that dietary interventions, more than supplementation with α-tocopherol, may affect the risk of cognitive impairment in the elderly.

The same set of molecules were measured in plasma of cognitively healthy subjects, older than eighty years, participating in the Kungsholmen study, a community-based study on aging and dementia based in a district of Stockholm. Plasma samples were collected at baseline and incidence of dementia evaluated after a six-year follow-up. After correction for several confounders, subjects with total tocopherols, total tocotrienols, and total vitamin E plasma levels in the highest tertile had a reduced risk of developing AD compared to those in the lowest tertile. When considering each form, the risk of incident AD was lower in subjects with levels of β-tocopherol in the highest tertile compared to the lowest, with a trend of protective effects for α-tocopherol, α-tocotrienol, and β-tocotrienol. Overall, this study suggests that low plasma levels of vitamin E in late life may be involved in the development of AD but that the protective activity seems to be related to the combination of different forms, that can be guaranteed by dietary intake, more than by supplementation with only α-tocopherol [101].

We applied a similar study protocol also to a Finnish population from the Cardiovascular Risk Factors, Aging and Dementia (CAIDE) study, characterized by a longitudinal design with several follow-ups every five years, with the aim to detect the incidence of several chronic diseases in relationship with midlife risk factors. In a subsample of this population, we evaluated the eight forms of vitamin E as well as the biomarkers of their oxidative or nitrosative damage in collected plasma samples of subjects clinically re-evaluated after seven to ten years. The study revealed an association between higher serum levels of γ-tocopherol, β-tocotrienol, and total tocotrienols and reduced risk of dementia. Furthermore, an elevated index of vitamin E nitrosative damage, i.e., the 5NγT/γ-tocopherol ratio, indicating a consumption of γ-tocopherol in free radical reactions, was associated with an increased risk of cognitive impairment [102].

In a further study on subjects with MCI or AD enrolled, together with cognitively healthy age-matched subjects, in the AddNeuroMed project, a multicenter European longitudinal study on the detection of biomarkers for AD, measurement of the vitamin E molecules previously described, showed that high plasma levels of tocopherols and of tocotrienols are associated with reduced odds of MCI and AD. In both groups, reduced levels of α-tocopherol and γ-tocopherol, as compared to controls, were associated with increased indexes of their utilization due to reaction with free radicals, suggesting that vitamin E depletion could be due to its increased utilization in oxidative and nitrosative stress events [103]. In a sub-study on this population, plasma concentrations of all forms of vitamin E and of markers of their oxidative/nitrosative damage were combined with structural magnetic resonance imaging (MRI) measures to evaluate the accuracy in differentiating individuals with AD and MCI from cognitively intact control (CTL) subjects. The joint evaluation of MRI and plasma vitamin E measures enhanced the accuracy of differentiating individuals with AD and MCI from CTL subjects: 98.2% (sensitivity 98.8%, specificity 97.7%) for AD versus CTL, and 90.7% (sensitivity 91.8%, specificity 89.5%) for MCI versus CTL. This combination of measures also identified 85% of individuals with MCI who converted to clinical AD at follow-up after one year [104].

Also, in a functional neuroimaging study with $^{18}$F-FDG PET, which evaluates cerebral glucose utilization, performed in subjects with subjective cognitive impairment, MCI and mild AD, brain glucose metabolism progressively decreased bilaterally in posterior temporoparietal and cingulate cortices across the three groups and, interestingly, the extracellular SOD activity measured in plasma was positively correlated with glucose metabolism in a large area of the left temporal lobe. Extracellular SOD is mainly bound to the endothelial surface of the arterial wall, while a small amount is released into the plasma, being in equilibrium with the endothelial component. Several studies showed that extracellular SOD is involved in regulating cerebral flow by maintaining the balance between nitric oxide and superoxide in the sub-endothelial space. Therefore, reduced activ-

ity, and probably amount, of this enzyme might contribute to the reduction of blood flow, and then metabolism, we observed in the brain [105].

In summary, most of the cross-sectional and longitudinal studies focused on oxidative stress in aging, brain aging, and dementia is quite concordant on its pivotal role as a cause of many structural and functional alterations in all these conditions, but, on the other hand, most of the clinical trials based on the use of antioxidants failed in finding beneficial effects. Why?

## COULD WE PREVENT COGNITIVE DECLINE AND OXIDATIVE STRESS DAMAGE WITH ANTIOXIDANTS AND/OR DIETARY HABITS?

Much evidence has shown, along with time, the role of micronutrients in protecting against several diseases, in our case those associated with aging, but most of the interventional studies were not successful. Vitamin E is paradigmatic to this purpose. After a great enthusiasm when vitamin E use was associated with a slower decline in AD [106], all the following studies failed in finding beneficial effects of vitamin E supplementation in AD and MCI. But many biases are present in most of them [107]. The first is the treatment with only α-tocopherol, which at high dose reduces or blocks the absorption of all the other essential tocopherols and tocotrienols, probably causing a deleterious biochemical imbalance in the organism. Furthermore, there was not a baseline evaluation of vitamin E status, so it is conceivable that many subjects did not suffer from vitamin deficiency and they were over/useless-treated. This is not a trivial consideration since, for example, we know that, without a good level of vitamin C, the increase in α-tocopherol can modify the redox balance, with an increase in oxidized forms of α-tocopherol that cannot be re-reduced by ascorbic acid. However, this is just an example of possible alteration we could cause without a profound knowledge of biochemical pathways. From this perspective, in a study conducted in a broad healthy community-dwelling old subjects, we found a progressive decline with age of another micronutrient, zinc (Zn), with the lowest levels in nonagenarians, who showed inadequate plasma levels in more than 60% [108]. Few weeks of supplementation increased Zn plasma levels in subjects with low but not in those with normal levels at baseline, suggesting that supplementation is beneficial mainly, or only, in the state of deficiency [109]. Therefore, if we want to treat patients with vitamins or other nutraceuticals, as frequently suggested also for cognitive impairment and AD [110], we need to know better their physiological actions and interactions, chemical properties and side effects. Every proposed nutraceutical must be studied as a new drug, passing through all phases of a clinical trial, with a rigorous flowchart.

However, it is also more probable that a well-balanced diet is the most reasonable approach to guarantee the best strategy to maintain a good health status with all nutrients obtained at a more equilibrated concentration.

To this purpose, it is noteworthy that nutrition and lifestyle are the most important contributors to a long and healthy life also through telomere biology modulation. Telomeres are long sequences of nucleotides at the end of our chromosomes, forming with specific proteins complex, an "end caps" which preserve genome stability and lead a cell to divide correctly. Extremely short or dysfunctional telomeres can be repaired by the enzyme telomerase, that working as a reverse transcriptase, adds nucleotides at the end of each chromosome promoting its stability.

Lifestyle factors known to modulate aging and age-related diseases might also affect telomerase activity [111]. Obesity, insulin resistance, and cardiovascular disease processes, which are related to oxidative stress and inflammation, have all been linked to alteration in telomere/telomerase system, which leads to cellular senescence [112]. Cellular senescence, the state of irreversible cell-cycle arrest, in turns contributes to age-related loss of tissue function and the production and secretion of reactive oxygen species as well as bioactive peptides collectively known as the senescence-associated secretory phenotype. Cellular senescence in the brain contributes to AD pathogenesis and may represent a link between the aging process and disease progression [113].

## CONCLUSIONS

What is the leading factor in AD pathogenesis remains unknown and Aβ deposition may be an expression of upstream alterations. Among them, oxidative stress has a crucial role, as summarized in Tables 1 and 2. Oxidative damage may impair the cells in their structure and function, being cause and effect of a mitochondrial reduced activity. The damage is not confined to the brain but also evident in peripheral cells and tissues. Thus we have

Table 1
Levels of oxidative stress biomarkers in brain tissue and peripheral tissues and cells

| Oxidative stress | Brain tissue |
|---|---|
| 8-hydroxy-2'-deoxyguanosine (OH8dG) | Measured in nDNA and mtDNA from cerebral cortex and cerebellum in humans: higher values with aging [54], threefold increase in subjects with AD compared to controls [55]. |
| | High levels of OH8dG in patients with Huntington's disease [59]. |
| DNA 4977bp depletion (mtDNA $^{4977}$) | Determined in two brain areas: mtDNA $^{4977}$ levels were lower in subjects with AD than controls [56]. |
| | **Peripheral lymphocytes and lymphocyte mitochondria** |
| 8-hydroxy-2'-deoxyguanosine (OH8dG) | Measured in nDNA extracted from peripheral lymphocytes: higher levels in subjects with AD than controls [61]. |
| Protein carbonyls, protein-bound HNE and protein-resident 3-nitrotyrosine | Measured in mitochondria extracted from lymphocytes: higher levels in AD and MCI subjects than controls [63, 64]. |
| Aconitase 2 ($ACO_2$) | Measured in mitochondria extracted from lymphocytes: reduced $ACO_2$ activity in AD and MCI subjects compared to controls [65]. |
| | **Skeletal muscle** |
| 8-hydroxy-2'-deoxyguanosine (OH8dG) | Increased in healthy subjects with age >60 years [70]. |
| | Increased in subjects with chronic fatigue syndrome [72]. |
| Carbonyl groups | Increased in healthy subjects with age >60 years [70]. |
| Malondialdehyde (MDA) | Increased in healthy subjects with age >60 years [70]. |
| | Increased in subjects with chronic fatigue syndrome [72]. |

Table 2
Antioxidants and dementia risk in population studies

| Antioxidants | |
|---|---|
| | **Population studies** |
| SOD, eSOD and GPX | – In the Italian Centenarian study the levels of vitamin A, vitamin E, SOD and eSOD activities was higher in centenarians than younger subjects [95]. |
| Vitamin A, C, E, carotenoids and uric acid | |
| Vitamin E (all eight forms) | – In the Conselice study the prevalence of dementia highest with higher concentrations of δ-tocopherol and α-tocopherol [99, 100]. |
| | – In the Kungsholmen study lower AD risk in highest tertile of β-tocopherol compared to the lowest. Protective effects for α-tocopherol, α-tocotrienol and β-tocotrienol [101]. |
| | – In the Cardiovascular Risk Factors, Aging and Dementia study (CAIDE) higher levels of γ-tocopherol, β tocotrienol and total tocotrienols associated with reduced risk of dementia [102]. |

to reconsider AD as a systemic disease. Nevertheless, a pro-oxidant status can be counterbalanced by an adequate antioxidant status, better guaranteed by a healthy diet rather than uncontrolled supplementations. Many studies are still needed to evaluate how we can modulate diet, and more in general lifestyle, to offer a physical and mental healthy life, reduce the risk of chronic diseases and contain disability. The major strength of our research is the broad spectrum of biological models we used, from cells and tissues to population, from *in vivo* to postmortem studies. In the latter, although it is conceivable that agonic status might determine oxidative alterations in the brain, specific anatomopathological findings did not reveal such an influence [114, 115]. As a main weakness, we have to consider that it was not always possible to correlate in the same subjects peripheral, and brain oxidative biomarkers, and that not all molecules related to the redox status have been measured simultaneously. The latter is undoubtedly a valuable aspect, especially when we want to test the validity of supplementation in subjects at risk. However, this is also the main limit of the few clinical trials with vitamin E in MCI, where the antioxidant status before supplementation has not been considered [116–118], suggesting the need for a more appropriate approach in the next future.

In conclusion, in this review, we have tried to summarize a long-lasting research activity that aimed to figure out how oxidative stress can explain some of the several modifications observed in aging and in age-related diseases. However, the journey to understand so many, different, and, sometimes, contradictory results seems still long. However, not unsatisfactory. Therefore, we would like to conclude this part of the journey joking with the beginning

of a famous song (not mentioning the end of the lyric, too pessimistic for any traveling researcher).

*Well we know where we're going*
*But we don't know where we've been*
*And we know what we're knowing*
*But we can't say what we've seen*
*And we're not little children*
*And we know what we want*
*And the future is certain*
*Give us time to work*

Talking Heads

## ACKNOWLEDGMENTS

We would like to thank Prof. Maria Cristina Polidori and Dr. Francesca Mangialasche for their long-lasting friendship and collaboration to produce many of the presented results.

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-0732r1).

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