EXPERT REVIEWS

Strategy to reduce free radical species in Alzheimer's disease: an update of selected antioxidants

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Alzheimer's disease (AD), characterized by progressive loss of memory, language, reasoning and other cognitive functions, including dementia, is characterized pathologically by the presence of senile plaques, neurofibrillary tangles and synapse loss. Increased oxidative/nitrosative stress, decreased antioxidants, mitochondrial damage and other factors play major roles in the development and progression of AD. Strategies to reduce pro-oxidant species to ameliorate AD pathology have been proposed with mixed results. In this review, we focus on the most recent *in vitro* and *in vivo* antioxidant approaches for removing oxidant species with relevance to AD, including *N*-acetyl-L-cysteine, vitamin D, vitamin E, ferulic acid, tricyclodecan-9-yl-xanthogenate, selenium and melatonin as therapeutic stratagems in AD management. In addition, we reviewed the most effective mitochondria targeted antioxidants such as coenzyme Q10 and lipoic acid. We suggest the use of multitargeted approaches by formulas containing one or more antioxidant compounds may be more promising than single-agent approaches.

KEYWORDS: Alzheimer's disease • antioxidants • dementia • free radicals • neurodegenerative

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders that cause dementia and affect middle- to old-aged individuals, with a prevalence that increases markedly after age 65. Sporadic AD accounts for approximately 95% of all AD cases and results from a complex array of etiological factors, such as head trauma, gender, education level, vascular disease, the presence of the gene, *apoE4* and general lifestyles, in addition to age. AD is often preceded by three stages of progression characterized by gradual increase of AD hallmarks starting from preclinical AD, to amnestic mild cognitive impairment (MCI) and early AD (EAD) [1,2]. Despite the progresses made in AD research in the past decades, the exact cause and pathogenesis of AD are not completely understood, and currently, there is no effective treatment for the disease.

Clinically, AD is characterized by progressive loss of memory, language, reasoning and other cognitive functions accompanied by concomitant behavioral, emotional and social deterioration, all leading to dementia. Pathologically, AD is characterized by the presence of senile plaques (SPs), neurofibrillary tangles, decreased synaptic density and brain atrophy, particularly in the hippocampus, amygdala and frontal cortex, consistent with cognitive and memory deficits observed [3]. The main component of SPs is amyloid β-peptide (Aβ), comprising 39–43 amino acids and generated by proteolytic cleavage of amyloid precursor protein (APP), a type I transmembrane protein, by β-secretase and γ-secretase. In the past, SPs have been perceived as the primary pathogenic element of AD; however, recent evidences provided insights into the notion that plaques may be an extracellular storage site for cells to deposit excess Aβ, suggesting that the real damaging agent, may be a much smaller aggregate form of Aβ(1–42) oligomers [4,5]. Research has shown that while plaques do not correlate with cognitive dysfunction in AD, soluble oligomers do [6]. Neurofibrillary tangles are formed by τ, a microtubule-associated protein, that if hyperphosphorylated is prone to aggregation becoming insoluble and losing its affinity for microtubules [3].

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Involvement of oxidative stress in AD development & progression

Abundant evidence supports the notion that oxidative/nitrosative stress (OS) has a major role in the pathogenesis of AD leading to the damage of vital cellular components such as proteins, lipids and nucleic acids [7–12]. Elevated levels of peroxidizable fatty acids, high requirement for oxygen, relative deficiency of antioxidant systems and richness in iron content make the brain extremely sensitive to oxidative stress [13,14]. Normal metabolism generates oxygen free radicals and other reactive oxygen species (ROS) that, even if potentially toxic, are part of several physiologic processes including signal transduction pathways. The body possesses an arsenal of protection against ROS toxicity, to neutralize these compounds and thus restore homeostasis. The antioxidant factors that form true protective systems of the body against free radicals are represented by antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (Gpx), glutaredoxins, thioredoxins and catalase, in addition to non-enzymatic antioxidant factors. A reduction in the antioxidant enzymatic system, as indexed by decreasing specific activity of the main antioxidant enzymes, has been demonstrated in AD [15]. Thus, increased levels of ROS could result not only from the increased production, but also from the depletion of the antioxidant system's capacity [12,13,16]. When ROS exceeds the capacity of the cell to terminate ROS, oxidative stress take place and oxidative damage to cell components ensues [11]. ROS can damage cell or organelle membranes directly (e.g., through lipid peroxidation), and can react with metals, nitrogen or carbon to form intermediates that react with proteins (e.g., through nitration, carbonylation and nitrosylation). Several types of free radicals exist, and the most common include superoxide anion (O_2^-), hydroxyl radical (HO), nitric oxide and peroxyl (ROO) radicals [17]. Though not free radicals, hydrogen peroxide (H_2O_2) and reactive α,β-unsaturated alkenes (such as 4-hydroxy-2-nonenal [HNE] and acrolein) are also ROS. The difference between free radicals, based on their structural and biochemical features, is crucial because it confers the compound its specific oxidative power, that is, its toxicity. As noted, Aβ peptides, together with altered mitochondrial function, the presence of trace metal ions such as iron and copper, have been identified as potential sources of oxidative stress [18–20].

The Aβ-induced OS hypothesis of AD states that Aβ oligomer accumulation and its consequent damage are among the principal causes of the increased OS observed in AD brain. The Aβ-induced oxidative stress hypothesis of AD places the majority of the causative effect of increased cellular oxidative stress associated with Aβ on Aβ(1–42) oligomers, as it is believed that only the oligomers are viable to insert into the lipid bilayer, wherein they may form α-helices to begin the proposed catalytic ROS production that may lead to the lipid peroxidation and protein oxidation found in AD [21]. Studies on AD transgenic animal models overexpressing Aβ confirmed the association between Aβ and OS suggesting the involvement of methionine 35 of Aβ in the mechanism of oxidative damage [21,22]. In turn, oxidizing conditions during AD cause protein cross-linking and aggregation of Aβ peptides and also contribute to aggregation of τ and other cytoskeletal proteins. Indeed, numerous studies have suggested that OS as result of the defects in antioxidant defense system promotes the production of Aβ in transgenic mice overexpressing APP mutant [23,24].

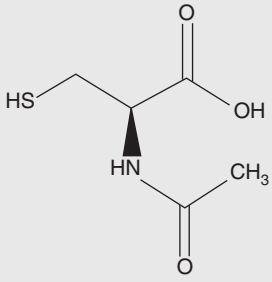
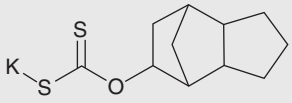
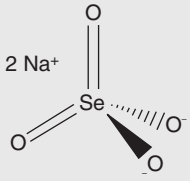
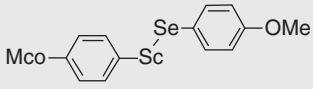
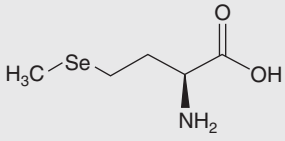
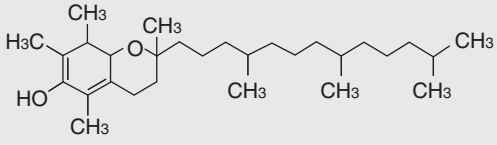
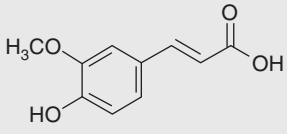
Noteworthy, broad studies have demonstrated that mitochondria dysfunction is an important factor involved in the pathogenesis of AD through the production of ROS [25–27]. Mitochondria are unique organelles that are essential for a variety of cellular functions including ATP synthesis, calcium homeostasis and cell survival and death. The mitochondrial respiratory chain is a major site of ROS production in the cell, and mitochondria are particularly vulnerable to oxidative stress. Free radical production is increased within the aging process, when both abnormalities in function and alterations of mitochondrial membrane integrity occur. Mitochondrial membrane defects are produced in turn by excess free radicals, the membrane structure being highly susceptible to lipid peroxidation.

A number of mitochondrial and metabolic abnormalities have been identified in neurons of AD compared with age-matched controls that most likely lead to increased ROS production and reduction in energy stores, thus contributing to the neurodegenerative process [28–30]. In addition, the dysregulation of transition metals such as copper (Cu), zinc (Zn) and iron (Fe) homeostasis can result in neurotoxic free radical production. Indeed, increased Fe(II) or Cu(I) can directly interact with hydrogen peroxide to produce hydroxyl radical, which leads to oxidative stress. Several studies reported abnormal levels of Cu, Zn and Fe in AD hippocampus and amygdala, areas showing severe histopathology alterations [25,31].

In addition, significant evidence has shown that among the different mechanisms known to participate in AD onset and progression, neuroinflammation emerges as a major regulatory and common factor. Neuroinflammation depends mainly on the activation of microglia and astrocytes as well as on increased levels of cytokines like IL-1β, TNF-α and TGF-β [32], all of which increase with aging [33–35]. Microglial cell cytotoxic activation and increase of inflammatory cytokines further induce the secretion of more cytokines and ROS [36]. The redox status modulates the participation of cytokines in signaling processes, which are critical mediators of OS, neuroinflammation and neurodegeneration [37,38]. OS, in turn, results in increased production of cytokines. Additionally, cytotoxic activation induces oxidative changes, creating a vicious cycle between oxidative stress and neuroinflammation [39].

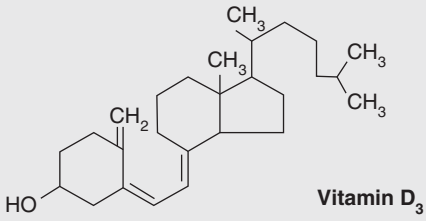
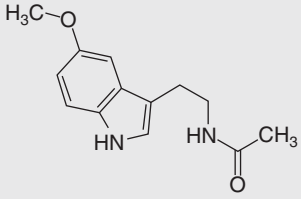
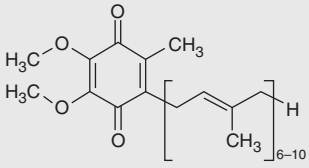
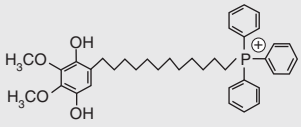
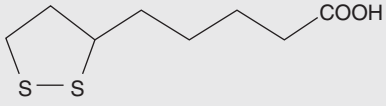
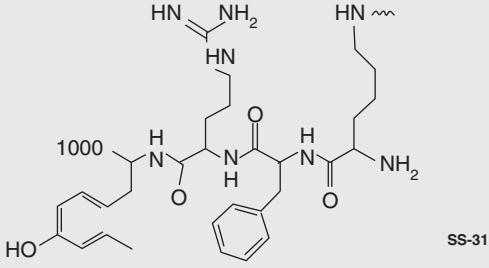
The effects of OS have been found as early as amnestic MCI and EAD in the progression toward AD. Studies from our and other laboratories have shown that OS markers for protein oxidation/nitration, such as protein carbonyls (PCs) and 3-nitrotyrosine (3-NT), are elevated in brains from subjects with MCI and EAD [12,40–42]. In agreement with the prominent role of Aβ peptide, regions of the brain rich in Aβ were reported to have increased levels of protein oxidation, while cerebellum known to be Aβ-poor does not [43]. Furthermore, high levels of free and protein-bound HNE (a product of lipid peroxidation)

Table 1. List, chemical structure and principal outcomes obtained employing the antioxidant molecules discussed in the review as therapeutic agents for Alzheimer's disease management.

Compound	Structure	Outcomes in AD treatment
N-Acetyl-L-cysteine		• Increased glutathione levels • Reduced protein oxidation • Modulation of Aβ processing and production via inhibition of γ-secretase • Reduced Aβ toxicity • Protection against apoptosis
D609		• Reduced ROS production • Reduced protein oxidation and lipid peroxidation • Reduced Aβ toxicity • Attenuated apoptosis
Sodium selenate		• Reduced Aβ production and plaque deposition via inhibition of α-secretase and γ-secretase • Reduced DNA and RNA oxidation • Amelioration of τ pathology via the activation of the PP2A and stabilization PP2A-τ complexes • Reversed learning and memory impairments • Mitigate the decrease of synaptic proteins in the hippocampus and cortex • Inhibited glial activation • Increased level of reduced glutathione • Increase activity of glycogen synthase kinase 3β
p,p´-Methoxyl-diphenyl diselenide		
L-Selenomethionine		
Vitamin E (α-tocopherol)		• Reduced lipid peroxidation and β-amyloid deposition in the brain • Reduced isoprostane levels in the brain • Decreased susceptibility of lipoproteins to oxidation • Lower plasma levels of total tocopherols, total tocotrienols and total vitamin E are associated with a higher risk of AD • Vitamin E supplementation reduced the *in vitro* oxidation of CSF lipoproteins
Ferulic acid		• Reduced amyloid deposition and IL-1β levels • Reversed behavioral deficits including defective hyperactivity, object recognition, spatial working and reference memory • Reduced brain parenchymal and cerebral vascular β-amyloid deposits as well as abundance of various Aβ species decreased Aβ production and reduced amyloidogenic APP proteolysis

Aβ: Amyloid β-peptide; AD: Alzheimer's disease; CSF: Cerebrospinal fluid; ETC: Electron transport chain; GSH: Glutathione; PP: Protein phosphatase; ROS: Reactive oxygen species; SOD: Superoxide dismutase; SS peptides: Szeto–Schiller peptides.

Table 1. List, chemical structure and principal outcomes obtained employing the antioxidant molecules discussed in the review as therapeutic agents for Alzheimer's disease management (cont.).

Compound	Structure	Outcomes in AD treatment
Vitamin D	 Vitamin D_3	• Reversed soluble Aβ and inflammatory issues • Reduced Ca^{2+}-mediated excitotoxicity • Regulates levels of glutathione • Improved phagocytosis of Aβ peptides and inhibited fibrillar Aβ-induced apoptosis • Chronic low vitamin D levels result in significant increases of proteins tyrosine nitration in brain
Melatonin		• Prevented Aβ-induced increases in intracellular Ca^{2+} and lipid peroxidation • Reduced protein nitration • Increased SOD, catalase and GSH levels • Prevented calyculin A-induced synaptophysin loss, memory retention deficits as well as hyperphosphorylation of τ and neurofilaments • Promoted PP2A activation
Coenzyme Q10		• Prevented a decrease in mitochondrial transmembrane potential • Reduced mitochondrial ROS generation • Attenuated Aβ overproduction and intracellular Aβ deposits in AD cells and mouse models • Reduced astrogliosis, synaptic loss and caspase activation in AD mouse models • Extended lifespan, delayed Aβ-induced paralysis, ameliorated depletion of the mitochondrial lipid cardiolipin and protected complexes IV and I of the ETC in *Caenorhabditis elegans* treated with Aβ
MitoQ		
Lipoic acid		• Ameliorated cognitive processes and declines in AD human brain and mouse models • Decreased mitochondrial oxidative stress and damage
SS peptides	 SS-31	• Protected AD mouse models from Aβ toxicity by: reducing Aβ-induced mitochondrial toxicity, increasing axonal transport of mitochondria in AD mouse models, enhancing synaptic viability in AD mouse models

Aβ: Amyloid β-peptide; AD: Alzheimer's disease; CSF: Cerebrospinal fluid; ETC: Electron transport chain; GSH: Glutathione; PP: Protein phosphatase; ROS: Reactive oxygen species; SOD: Superoxide dismutase; SS peptides: Szeto–Schiller peptides.

were found in AD brain as well as PCs and protein nitration in regions of the brain significantly associated with AD, including the hippocampus and parietal cortex [44–50].

The employment of redox proteomics to identify oxidatively modified brain proteins in AD and MCI revealed a number of oxidatively modified brain proteins that are associated with several key functions of the brain, such as ATP synthesis, energy metabolism and antioxidant response, suggesting that the alteration of these pathways by increased oxidative damage is involved in AD progression and pathogenesis [51,52]. Increased DNA and RNA oxidation have been reported in AD from early stages of the disease, with 8-hydroxy-2-deoxyguanosine and 8-hydroxyguanosine found elevated in AD hippocampus, frontal and occipital neocortex [7,53–55]. In addition, higher levels of protein-bound HNE, PCs, 3-NT, free HNE and malondialdehyde (MDA) have been described also in cerebrospinal fluid (CSF), blood and urine of AD patients when compared with healthy controls [56–59], supporting the involvement of oxidative stress and consequent oxidative damage at peripheral level.

Taken together, all the findings demonstrated that OS is a causative or at least collateral factor linked with several major

pathological processes in AD including Aβ-induced neurotoxicity, τ pathology, mitochondrial dysfunction and metal dyshomeostasis. Intriguingly, abnormal accumulation of Aβ and τ proteins leads to increased ROS production which in turn may further exacerbate Aβ and τ neurotoxicity, thus forming a vicious cycle that promotes the initiation and progression of AD. OS, either a primary or secondary event, is a common feature of several neurodegenerative disorders and markers of OS may overlap in some brain disorders [60–62]. The identification of common together with different OS-related deregulated pathways may help to decipher the complex 'redox' signature of degenerating neurons. Thus, removal of ROS or prevention of their formation may delay the onset or slow down the progression of AD by targeting a number of different molecular events implicated in AD.

The use of antioxidant strategies in AD

Based on the early occurrence of OS in AD patients, a number of trials have been performed in the last few years aimed at exploring the efficacy of antioxidant in AD and in MCI. Based on data derived from several observational and epidemiological studies, compounds with antioxidant activity have been proposed for prevention of cognitive decline and treatment of MCI or AD. Rationale for the use of glutathione-related agents, such as *N*-acetyl-L-cysteine (NAC), tricyclodecan-9-yl-xanthogenate (D609), selenium (Se) compounds and other common antioxidants such as vitamin E, ferulic acid (FA) as well the main results of relevant clinical trial is presented hereafter. Since mitochondria are both source and target of ROS, and since mitochondrial dysfunction is highly related to AD onset and progression, we report in this review some of the most effective mitochondria-targeted antioxidant strategies, represented by coenzyme Q10 (CoQ10), mitoQ, lipoic acid (LA) and Szeto–Schiller (SS)-peptides supplementation. In addition, in agreement with the close relationship between vitamin D and AD-mediated oxidative damage studies on vitamin D administration are discussed. The antioxidant molecules reviewed in this manuscript were selected according to the authors' direct experience as well as the literature relevant to these molecules. Table 1 provides the structures of and comments about the various antioxidant compounds relevant to studies of AD discussed in this review.

N-acetyl-L-cysteine

NAC is a derivative of cysteine containing an acetyl group that is attached to the nitrogen atom. NAC is de-amidated at the blood–brain barrier (BBB) and the resulting cysteine can reach brain parenchyma via amino acid transporters. Cysteine serves as the rate-limiting precursor for *de novo* synthesis of the powerful endogenous antioxidant glutathione (GSH). Studies on rodents showed protection from pro-oxidant species by NAC treatment through the increase of GSH levels [63,64]. Interestingly, NAC was also shown to be effective against Aβ toxicity by regulating several signaling pathways, such as Ras/ERK pathway or MLK3-MKK7-JNK3, protecting against apoptosis and playing a role in APP processing and Aβ formation [65–69]. Clinical trials on NAC supplementation 50 mg/kg/day demonstrated that it is safe, well-tolerated and potentially beneficial in AD management. Recent research and treatment studies on NAC characteristics in AD-related neurodegeneration sustained its favorable antioxidant properties [70].

In 2010, Huang *et al.* treated human double mutant *APP/PS1* knock-in mice 4- and 7-months old orally (2 mg/kg/day) with NAC for 5 months each. The results demonstrated overall that early administration of NAC protected APP/PS1 mice brains to a superior degree compared with initiation of NAC administration at a later age [71]. NAC was able to reduce protein oxidation as indexed by decreased PCs, decreased protein-bound HNE and decreased 3-NT in brain isolated from APP/PS1 mice at 9 months of age, and, to a lesser degree, from 12-month-old mice. In addition, NAC supplementation increased the activity and expression of GPx *in vivo*, resulting in more clearance of H_2O_2, while no alterations in glutathione reductase levels were observed in either age group, regardless of treatment. In a subsequent study, the authors analyzed mice groups by proteomics to identify specific target altered by neurodegenerative process, rescued by NAC treatment [72]. These researchers showed that both APP/PS1 at 9 and 12 months compared with wild-type littermates present differential expression of several proteins involved in energy-related pathways, excitotoxicity, cell cycle signaling, synaptic abnormalities, cellular defense and cellular structure. NAC supplementation of APP/PS1 mice was able to restore the basal expression of proteins found altered in comparison with wild-type mice, suggesting a beneficial approach for increasing cellular stress responses and for influencing the levels of energy- and mitochondria-related proteins in APP/PS1 mice.

In 2012, Hsiao *et al.* [73] treated 3-month-old APP/PS1 mice with NAC by intraperitoneal (ip.) injection (200 mg/kg/day) for 7 days. The authors observed that NAC was able to prevent social isolation-induced accelerated impairment of contextual fear memory and loss of hippocampal LTP through reduction of Aβ levels via inhibition of γ-secretase. In addition, NAC administration restored surface GluR1 to normal level, through the stabilization of p35, rescuing memory deficits.

In the same year, Head *et al.* [74] administered aged dogs (98–115 months), which naturally develop learning and memory impairments, human-type Aβ deposits and oxidative damage, with a medical food cocktail containing in addition to NAC and curcuminoids, epigallocatechingallate, R-alpha LA and piperine. Dogs treated with this medical food cocktail exhibited an improved spatial attention, but not in other measures of cognition. As well, no changes in both the extent of Aβ plaque accumulation and the analysis of soluble and insoluble Aβ(1–40) and Aβ(1–42) were observed.

In somewhat of an outlier study, Xu *et al.* [75] evaluated the effects of a 6-week combined treatment of aerobic exercise and NAC administration on 10-month-old APP/PS1 mice. NAC intake was calculated to be 5 mg/day combined with 2 h running/day. The authors observed a combined treatment of aerobic exercise plus antioxidant compound did not attenuate the

severe decline in spatial learning and memory, and did not mitigate Aβ deposition and production, oxidative stress, glial inflammation and synaptic loss, showing, overall, to not be effective in counteracting the pathophysiology in the moderate or mid stages of AD.

In 2014, Gamba *et al.* [76] demonstrated that neuronal cells pretreated with NAC (100 μM) 1 h before treatment with 24- or 27-hydroxycholesterol had reduced oxysterol-induced β-amyloidogenesis inhibition of BACE1 increase and Aβ deposition, supporting the concept that NAC represents an efficient inhibitor of oxysterols-induced Aβ toxic peptide accumulation in the brain.

With the rationale of using NAC to provide a precursor for GSH synthesis, NAC (600 mg/day) was included in two nutraceutical preparations tested on a multisite Phase II clinical trial [77,78] on AD and MCI individuals to evaluate improvements in cognitive performance. Both trials started in 2012 and are now completed but data are not available yet.

Tricyclodecan-9-YL-xanthogenate

D609 is a tricyclodecanol derivative of xanthic acid well known for its antiviral, antitumor and anti-inflammatory properties. D609 mechanisms of action are attributed to inhibiting phosphatidylcholine (PC)-specific phospholipase C and sphingomyelin synthase, thus affecting lipid second messengers 1,2-diacylglycerol and/or ceramide, cell cycle and cell proliferation [79]. Xanthogenate compounds are also potent antioxidants and Zn^{2+} chelators. First studies from the Butterfield laboratory have shown that pretreatment of primary hippocampal cells with D609 significantly attenuated Aβ(1–42)-induced cytotoxicity, intracellular ROS accumulation, protein oxidation, lipid peroxidation and apoptosis, while methylated D609, with the thiol functionality no longer able to form the disulfide upon oxidation, did not protect neuronal cells against Aβ(1–42)-induced oxidative stress [80]. In a subsequent study, the authors identified the specific targets of protein oxidation in neurons upon Aβ(1–42) treatments and also studied the protective effect of D609 on the identified oxidatively modified proteins. In the cells treated with Aβ(1–42) alone, four proteins significantly oxidized were identified glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, malate dehydrogenase and 14-3-3 zeta, and interestingly, pretreatment with D609 prior to Aβ(1–42) protected all the above-mentioned oxidized proteins against Aβ(1–42)-induced protein oxidation [81].

An *in vivo* study on synaptosomes isolated from gerbils, previously injected ip. with D609, were treated with the oxidants Fe^{2+}/H_2O_2 or 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH), demonstrated a significant reduction in ROS, levels of PC, protein-bound HNE and 3-NT compared with those in synaptosomes isolated from gerbils that were injected with saline, but treated with Fe^{2+}/H_2O_2 or AAPH [82].

In the same year, the effects of D609 (50 μM) were tested on Aβ(1–42)-treated gerbil synaptosomes, demonstrating its efficacy through the protection against Aβ(1–42)-induced loss of phospholipid asymmetry and against Aβ(1–42)-induced apoptosis [83]. These results supported the notion that D609 is able to protect neuronal cells against oxidative damage acting as a glutathione mimetic in the scavenging of hydroxyl radical. In 2006, synaptosomes isolated from gerbils injected ip. with D609 or with saline solution and treated *ex vivo* with Aβ (1–42) showed a significant decrease of oxidative stress parameters: ROS levels, protein oxidation (PC and 3-NT levels), lipid peroxidation (protein-bound HNE levels) and the levels of inducible nitric oxide synthase consistent with the hypothesis that D609 is a potent antioxidant able to scavenge free radicals generated by Aβ-peptide [84]. Brain mitochondria isolated from gerbils after ip. injection with D609 were subsequently treated *ex vivo* with the oxidants Fe^{2+}/H_2O_2, AAPH and AD-relevant Aβ(1–42). Mitochondria from the gerbils injected with D609 and subjected to these oxidative stress inducers showed significant reduction in levels of PCs, protein-bound HNE, 3-NT and cytochrome *c* release compared with saline-injected gerbils [85]. In addition, D609 treatment was able to maintain the GSH/glutathione disulfide ratio in oxidant-treated mitochondria, increasing the activity of glutathione S-transferase, Gpx and glutathione reductase. No human trial has been currently executed or proposed for the use of D609 in the clinical management of AD.

Selenium

Recent reports highlighted the involvement of Se in the development of neurodegenerative diseases including AD, playing different roles in the stages of progression [86]. Se status decreases with age and may contribute to decline in neuropsychological functions among aging people. A significantly increased risk of cognitive decline was recorded over 4 years in participants with low plasma Se at baseline [87]. Moreover, plasma Se levels are lower in AD patients when compared with healthy patients [88]. Se is known to provide protection from free radical-induced cell damage and is incorporated into proteins as the amino acid selenocysteine. Selenoprotein P (SelP), a Se transport protein, although produced predominantly in the liver and transported to the brain via plasma, was found at high concentrations in the brain suggesting an important physiological role in the CNS [89]. SelP has an important neuroprotective role, enhancing neuronal survival and preventing apoptotic cell death in response to Aβ-induced oxidative challenge [90]. Indeed, the reduction of SelP expression in neuronal cells by RNAi resulted in decreased viability, increased apoptotic cell death and in increased Aβ toxicity [90]. Although data correlating the supplementation of Se with cognitive improvement and AD pathophysiology are controversial, several authors demonstrated promising aspects of Se supplementation in AD patients and animal models. In 2009, Wistar rats were pretreated with sodium selenite, a salt of Se (0.1 mg/kg) or vehicle for 7 days and rats then were injected bilaterally with intracerebroventricular administration of streptozocin (ICV-STZ) (3 mg/kg) [91]. ICV-STZ-infused rats showed significant loss in learning and memory ability, a significant increase in thiobarbituric acid reactive species, PC and a significant decrease in

reduced GSH, GPx and GR and ATP in the hippocampus and cerebral cortex and choline acetyltransferase in hippocampus. Se supplementation significantly ameliorated all alterations induced by ICV-STZ in rats [91]. Se has been associated with the reduction of Aβ production and of Aβ induction. Lovell *et al*. in 2009 tested the potential antioxidant characteristics of organic Se against Aβ-induced OS in APP/PS1 mice treated with Se-enriched diet (Sel-Plex) showing lower levels of Aβ plaque deposition and significantly decreased levels of DNA and RNA oxidation [92]. In addition, Sel-Plex-treated mice showed a significant increase in GPx activity compared with mice on a normal diet. Treatment of human neuroblastoma cells (SH-SY5Y expressing Swedish APP mutant) with Se significantly reduced Aβ(1–40), Aβ(1–42) and secreted APPβ production by reducing Aβ producing β-secretase and γ-secretase activities [93]. In addition, the lipid peroxidation product HNE-induced transcription of β-secretase (BACE1) was blocked by Se. Further studies have determined that sodium selenate can ameliorate τ pathology [94,95]. Acute treatment of either neuroblastoma cells or normal aged mice rapidly reduced τ protein phosphorylation [95]. Sodium selenate-treated transgenic TAU441 mice had significantly lower levels of phospho- and total τ in the hippocampus and amygdala compared with controls and exhibited significantly improved spatial learning and memory on the Morris Water Maze task. In parallel, chronic oral treatment of Se to two independent τ transgenic mouse strains with NFT pathology, P301L mutant pR5 and K369I mutant K3 mice, reduced τ hyperphosphorylation and completely abrogated NFT formation, improved contextual memory and motor performance and prevented neurodegeneration [94]. Both, studies showed that Se-mediated reduced NFT formation involved the activation of the serine/threonine-specific protein phosphatase (PP) 2A and stabilization of PP2A-τ complexes.

Cornelli [96] tested in 2010 a cohort of 52 patients affected with moderate probable AD taking donepezil, a formula (Formula F) to counteract OS containing among the most common antioxidants also Se. The results showed a slight improvement in Mini Mental State Examination, 2nd edition in patients administered donepezil plus formula F.

In 2010, Pinton *et al*. [97] investigated the possible neuroprotective effect of p,p′-methoxyl-diphenyl diselenide $(MeOPhSe)_2$ in a mouse model of sporadic dementia of Alzheimer's type produced by ICV-STZ. Experimental results showed that ICV-STZ caused learning and memory deficits in mice and increased acetylcholinesterase (AChE) activity, while $(MeOPhSe)_2$ was able to reverse the learning and memory impairments induced by STZ and to protect against the increased AChE activity. The authors then analyzed the antioxidant activity of $(MeOPhSe)_2$ in sporadic dementia of Alzheimer's type mice, demonstrating protection against increased reactive species and the reduction of glutathione levels, as well as increases in SOD and glutathione S-transferase activities caused by STZ in whole brain [98]. The same organoselenium compound was then tested on male Wistar rats that received ICV/STZ [99]. Similar to data obtained in previous studies [97,98], these researchers showed that $(MeOPhSe)_2$ dietary supplementation reverted memory impairment, reverted oxidative stress and normalized AChE activity in the STZ group, strongly supporting the rationale for a potential therapeutic effect of the $(MeOPhSe)_2$-supplemented diet in AD management [99].

In 2013, Kiliaan *et al*. tested the favorable effects of a multinutrient diet on the APP/PS1 mouse model of AD [100–102]. The multinutrient intervention known as Fortasyn™ Connect, comprised of docosahexaenoic acid, eicosapentaenoic acid, uridine-mono-phosphate, choline, phospholipids, folic acid, vitamins B6, B12, C, E and Se, was able to decrease hippocampal Aβ levels and amyloid plaque burden and improve searching behavior and swim efficiency in APP/PS1 mice [100,102]. The medical food Souvenaid® (Nutricia N.V., Zoetermeer, The Netherlands) containing Fortasyn Connect, has been investigated in several randomized controlled trials (i.e., S-Connect) [103–109], showing controversial results in mild-to-moderate AD patients [110].

In 2014, Song *et al*. [111] investigated the potential of selenomethionine (Se-Met), an organic form of Se, in the treatment of cognitive dysfunction and neuropathology of triple transgenic AD (3xTg-AD) mice. Four-month-old 3xTg-AD mice treated with Se-Met for 3 months demonstrated increased Se level related to significant improvements in cognitive deficit. Se-Met treatment in 3xTg-AD was able to reduce the level of total and phospho-τ, mitigate the decrease of synaptic proteins in the hippocampus and cortex, inhibit glial activation, increase the level of reduced glutathione and increase the activity of glycogen synthase kinase 3β and PP2A, both involved in τ phosphorylation [111]. This study confirms the potential role of Se supplementation in improving AD-related cognitive deficit through the reduction in τ expression and hyperphosphorylation, amelioration of inflammation and restoration of synaptic proteins and antioxidants.

The PREADViSE trial recruited a subsample (n = 7547) of participants ages 62 and over from the NIH National Cancer Institute-sponsored Selenium and Vitamin E Cancer Prevention Trial from 130 participating clinical sites in the USA, Canada and Puerto Rico [112,113]. The specific aims of PREADViSE were to determine the effect of Se and vitamin E used in combination or alone on the incidence of AD primarily and on the incidence of other neurodegenerative diseases secondarily. A third aim was to investigate the features of normal cognitive aging in a validation subsample. The study is currently ongoing and the annual screening portion of the PREADViSE study will be ending in early 2014 [114].

Vitamin E

Natural vitamin E includes two groups of closely related fat-soluble compounds, the tocotrienols (TCTs) and tocopherols (TCPs), each with the four analogs, α, β, γ and δ. All vitamin E analogs possess a 6-membered, aromatic chromanol ring structure and a side chain. The TCPs have a phytol chain, whereas the TCTs have an unsaturated side chain with double

bonds at the 3′, 7′ and 11′ positions of the hydrocarbon tail. α-Tocopherol is the most abundant congener found in nature, has the most potent biological activity and corrects human vitamin E deficiency symptoms [115]. When produced synthetically, it is composed of eight stereoisomers with the RRR-α-tocopherol as the most biologically active form [116]. The most abundant sources of vitamin E are vegetable oils, which typically contain all four tocopherol congeners (α, β, γ and δ) in varying proportions [117]. Other important sources are nuts and seeds such as sunflower seeds [117]. Based on its lipophilicity, vitamin E is considered to be one of the major chain-breaking antioxidants preventing the propagation of oxidative stress, especially in biological membranes [118].

The effect of a diet deficient or supplemented with vitamin E has been extensively studied. Vitamin E has been shown to cross the BBB and to accumulate at therapeutic levels in the CNS, where it is able to lower lipid peroxidation and β-amyloid deposition [119]. Vitamin E also rescues neuronal damage and β-amyloid deposition in the brain indexed by reduced isoprostane levels [120]. Moreover, quite recently, it was reported that plasma concentrations of different vitamin E forms are related to the diagnosis of AD and MCI in elderly subjects, thus strengthening the hypothesis of a possible use of vitamin E as a biomarker in AD [121]. Indeed, the relation of all plasma vitamin E forms and markers of vitamin E damage (α-tocopherylquinone, 5-nitro-γ-tocopherol) to MCI and AD was examined. Compared with cognitively normal subjects, AD and MCI patients had lower levels of total TCPs, total TCTs and total vitamin E [121]. In multivariable polytomous logistic regression analysis, both MCI and AD cases had 85% lower odds to be in the highest tertile of total TCPs and total vitamin E, and they were, respectively, 92 and 94% less likely to be in the highest tertile of total TCTs than the lowest tertile [121]. Further, both disorders were associated with increased vitamin E damage. Low plasma TCPs and TCTs levels are associated with increased odds of MCI and AD [121].

Interestingly, the joint evaluation of MRI and plasma vitamin E measures enhanced the accuracy of differentiating individuals with AD and MCI from control subjects [122]. This combination of measures also identified 85% of individuals with MCI who converted to clinical AD at follow-up after 1 year. Plasma levels of TCPs and TCTs together with automated MRI measures can help to differentiate AD and MCI patients from control subjects, and to prospectively predict MCI conversion into AD [122].

Furthermore, mitochondria isolated from MCI lymphocytes showed increased oxidative stress that correlated with altered levels of a number of vitamin E components, suggesting that increased oxidative stress markers in the peripheral system may potentially reflect brain damage and could potentially serve as a biomarker for progression, diagnosis or treatment of AD [123].

However, the therapeutic effects mediated by the administration of vitamin E are still unclear due to conflicting results. Many studies have shown a decrease in vitamin E levels in aging and dementia with a correlation to memory loss [124,125]. Supplementation of vitamin E does increase levels of this vitamin in AD and decreases susceptibility of lipoproteins to oxidation [126]. It is not clear as to whether vitamin E improves cognition. Vitamin E alone or in combination with other vitamins and minerals showed no association with dementia [127–129], while others noted a correlation with improved cognitive performance [130–134]. Because no data are available about the antioxidant status of subjects before and after treatment, it is still unclear if vitamin E efficacy is or is not related to this aspect, with a population more or less sensitive according to redox status. Vitamin E may be detrimental to those whose oxidative stress parameters are high and do not improve with supplementation. It is possible that upon oxidation, vitamin E is reactive unless recycled to the reduced form. Approaches to solve this problem aimed to restore vitamin E antioxidant properties took advantages from combination treatments, in which vitamin E was associated with other nutritional supplements. However, the choice of the substances and of the doses to use remains a challenge.

Supplementation of vitamin E and an antioxidant that is water soluble, capable of recycling vitamin E and increasing redox thiol status, such as inducing NAC or γ-glutamylcysteine ethyl ester upregulation, may have more consistent positive effects on dementia, providing protection in the lipid membrane and inside the cell. According to this, treatment with vitamin C plus a medium dose of vitamin E decreased oxidative damage and improved performance on tests of spatial memory; these benefits were not seen with vitamin C plus a high dose of vitamin E [135]. In 2012, it was reported that the combination of 800 IU/day of vitamin E (α-tocopherol) plus 500 mg/day of vitamin C plus 900 mg/day of α-LA (E/C/ALA), did not influence pathways related to amyloid and τ pathology, whereas E/C/ALA did result in a significant decrease in CSF levels of F2-isoprostanes, consistent with antioxidant effects in the brain [136]. Furthermore, increased decline on the Mini Mental State Examination and a trend in this direction on the Alzheimer's Disease Co-operative Study-Activities of Daily Living in the E/C/ALA group raised a concern that this combination could adversely affect cognition in AD [136]. The lack of correlation of changes in these measures with changes in CSF biomarkers suggests that the cognitive changes may not be due to worsening of AD-related pathology [136]. Although a mechanism is uncertain, this cognitive finding raises a caution and will need to be carefully monitored if long-term studies are planned.

In another recent study, it was demonstrated that the combined supplementation of AD patients with vitamins E (400 IU/day) and C (1 g/day) is capable of increasing vitamin concentrations in CSF, which results in a clear reduction of *in vivo* oxidation of CSF lipoproteins after 1 month, and a possible antioxidant effect after 12 months [137]. Antioxidant supplementation was unable to significantly slow cognitive decline in AD over a period of 1 year in this small open-label study [137]. Although this result seems discouraging, additional larger double-blind treatment and prevention studies over a

longer period of time are needed to reliably assess whether antioxidants might have a long-term effect on the course of AD, especially when given in early or preclinical stages of the disease. In the absence of positive results from such trials, the treatment of AD with vitamin E and vitamin C cannot be recommended without further investigation.

In randomized controlled trials, high doses of α-tocopherol have usually been used, while a balanced intake of different vitamin E congeners can be more effective in terms of neuroprotection. Indeed, epidemiological evidence suggests that the protective effect of vitamin E against AD can be due to the contribution of its different forms, while intake of high doses of α-tocopherol can decrease the bioavailability of the other congeners potentially increasing mortality risk [138–140].

Despite the above, high doses of vitamin E have not been shown to slow cognitive decline even following co-administration of selegiline [141]. Similarly, in one of the latest trials in human with vitamin E (the TEAM-AD VA cooperative randomized trial), the effects of the association of vitamin E plus memantine in mild-to-moderate AD were evaluated. The results showed that among patients with mild–to-moderate AD, α-tocopherol (2000 IU/day) compared with placebo resulted in slower functional decline [142]. There were no significant differences in the groups receiving memantine alone or memantine plus α-tocopherol [142]. These findings suggest a possible benefit of α-tocopherol in mild-to-moderate AD by slowing functional decline and decreasing caregiver burden.

As previously reported in section on 'Selenium' above, the PREADViSE trial is a still ongoing clinical intervention study evaluating the efficacy of vitamin E and Se for AD prevention. It is an ancillary study to Selenium and Vitamin E Cancer Prevention Trial (a large prostate cancer prevention trial) whose blinded results of the first year were presented in 2012 as an exposure study. Despite that no data are available with regard to the redox status of the participants, once unblinded, the RCT and exposure study data will have the potential to yield new information on long-term exposure to antioxidant supplements under controlled conditions [112].

FA & its derivatives

FA ((*E*)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid)) is a common polyphenolic compound most abundant in vegetables, especially artichokes, eggplants (~90% of total polyphenols) and in maize bran (~3.1% of total polyphenol content) [143–145]. During the last decades, scientists focused their attention on FA, especially for its direct antioxidant properties as a free radical scavenger as well as for its ability to induce cell stress response [146,147], even if, perhaps due to the poor bioavailability when administered *per os* [146,147], the neuroprotective effects produced by FA *in vitro* were not completely replicated in *in vivo* models [66,146,147].

FA has previously been reported to have free radical scavenging activity toward hydroxyl radical, peroxynitrite, superoxide radical and oxidized low-density lipoprotein [148–150]. The hydroxyl group in the FA can readily form a resonance-stabilized phenoxy radical and is key to its antioxidant property [150–152]. FA can also protect biological membranes from lipid peroxidation and neutralized peroxyl and alkoxyl radicals [153].

Recent results show that chronic (for 6 months from the age of 6–12 months) oral administration of FA at a dose of 5.3 mg/kg/day significantly enhanced the performance in novel-object recognition task, and reduced amyloid deposition and IL-1β levels in the frontal cortex of APP/PS1 mouse model of AD [154]. Interestingly, of the two doses of FA used in this study (5.3 and 16 mg/kg/day), the beneficial effects were observed only at the lower dose suggesting that FA neuroprotective effects are dependent on the dosage range [154]. This observation is not surprising and is consistent with the well-known notion about the pro-oxidant effects induced by almost all the antioxidant molecules at high concentrations [155–163].

In a similar work, APP/PS1 mice that underwent oral FA (30 mg/kg/d) treatment for 6 months showed reversed behavioral deficits including defective: hyperactivity, object recognition, spatial working and reference memory [164]. Furthermore, brain parenchymal and cerebral vascular β-amyloid deposits as well as abundance of various Aβ species including oligomers were decreased in FA-treated APP/PS1 mice [164]. These effects occurred with decreased cleavage of the β-carboxyl-terminal APP fragment, reduced β-site APP cleaving enzyme 1 protein stability and activity, attenuated neuroinflammation and stabilized oxidative stress [164]. In the same study, mutant human APP-overexpressing murine neuron-like cells treated with FA (1.5–12.5 μM) reportedly have significantly decreased Aβ production and reduced amyloidogenic APP proteolysis [164]. Collectively, these results highlight that FA is a β-secretase modulator reinforcing its therapeutic potential against AD pathology.

However, only few achievements were obtained aimed to increase FA bioavailability in the brain and thus, despite its potential use in AD, the research on this molecule needs major efforts. Esterification of the acid group increases lipophilicity allowing for increased brain bioavailability and the ability to protect cell membranes from oxidative stress [165]. The ethyl ester derivative of FA (FAEE) (ethyl 4-hydroxy-3-methoxycinnamate) has been shown to have both anti-inflammatory and antioxidant properties as stated for FA [148,149,166]. The presence of the ester group in FAEE makes this compound more lipophilic, thereby increasing its ability to cross cell membranes, which are rich in lipids. Especially in the case of brain the added lipophilicity of FAEE is helpful in allowing a better transport of FAEE across the BBB. This property makes FAEE a better potential antioxidant with respect to brain compared with FA [167].

Interestingly, caffeic acid (CA) and its esters were generally more active than FA and its derivatives in terms of their ability to reduce oxidative stress [168]. Indeed, CA alkyl esters (methyl, ethyl, propyl and butyl esters, 0.2–25 μM) had higher antioxidant activities, lower redox potential and higher lipophilicity compared with their parent compound and had the ability of protecting PC12 cells against oxidative damage in a dose-

dependent manner [168]. On the other hand, FA esters are weaker antioxidants than CA derivatives (IC_{50} threefold higher than CA derivatives) and although their lipophilicity was higher than FA, they did not show neuroprotective effect [168]. These findings showed that optimization of the antioxidant activity and lipophilicity of these phenolic compounds can lead to the production of more efficient antioxidants.

In this scenario, stearic acid (SLN)- and stearyl ferulate (SLN-SF)-based solid lipid nanoparticles in which FA was entrapped, were synthesized with the aim to increase the amount of intact FA that can be delivered to cells [169]. The idea of using FA, also as structural constituent of these lipid nanoparticles, was due to the significant role of FA alkyl derivatives in the protection of the encapsulated compounds as observed in different types of FA-based drug carriers [170–172]. In particular, SLN-SF-FA (0.1–200 μM) displayed greater efficacy (EC_{50}) and potency (maximal activity) against AAPH- and NADPH/ADP-Fe^{3+}-induced lipid peroxidation than SLN-FA or FA or FAEE [169]. The improved antioxidant capacity observed for SLN-SF-FA with respect to FA and FAEE highlights the potential of this new system for FA delivery in terms of antioxidant effects based at least on two conceivable explanations: the final effect could be due to the sum of the single effects produced by SF and FA alone; SF could protect FA from oxidation, and then FA is responsible for the final effect [169]. In our opinion, this promising approach could represent a novelty in the wide view of the methods aimed to improve FA bioavailability, and the need to produce FA entrapped within SLN could be related to the flexible use of this formulation in clinical settings. Indeed, SLN might be either administered by parenteral routes or orally, depending on their dimension, thus putting the basis for the use in acute or chronic conditions. In particular, this SLN-SF-FA could be administered by an intranasal route thus making these formulations potentially useful to prevent free radical damage in the brain. Last but not least, FA and tacrine hybrids were synthesized. Tacrine was the first cholinesterase inhibitor approved by the US FDA for the treatment of AD. The neurotransmitter acetylcholine (ACh) is decreased in AD. By inhibiting the main degrading enzyme of ACh, levels of ACh could be stabilized for neurotransmission. However, tacrine alone induces hepatotoxicity and does not address oxidative stress found in AD. Conversely, several FA/tacrine hybrids were found to have both antioxidant properties against peroxyl radicals and AChE inhibitor effects [173]. One of these compounds, the tacrine-6-ferulic acid (T6FA), was recently reported to prevent Aβ-induced AD-associated pathological changes both *in vitro* and *in vivo*. Indeed, T6FA (2–50 μM) significantly inhibited auto- and AChE-induced aggregation of Aβ(1–40) *in vitro* and blocked Aβ(1–40)–induced cell death in PC12 cells [174]. Furthermore, T6FA (2 and 20 mg/kg/day for 21 days intra gavage) significantly improved the cognitive ability along with increasing choline acetyltransferase and SOD activity, decreasing AChE activity and MDA level in C57 BL/6J mice following the intracerebroventricular injection of Aβ(1–40) [174].

Finally, with regard to the potential mechanisms that mediate T6FA-associated neuroprotective effects, it was reported that T6FA (3–30 μM) potently prevent OS-induced cell death in HT22 cells through the activation of the Nrf2/ARE pathway and the upregulation of HO-1 protein levels [175].

Vitamin D

Vitamin D is present in two major forms. Vitamin D2 (ergocalciferol) is present in plants, yeast and fungi [176,177], while vitamin D3 (cholecalciferol) can be obtained from animal sources such as oily fish and egg yolk [176–178]. Vitamin D3 is also synthesized endogenously in the skin upon ultraviolet light exposure. Sunlight exposure to the skin results in a photochemical conversion of 7-dehydrocholesterol to pre-vitamin D, which then rapidly converts to cholecalciferol. This process is self-limiting to prevent toxicity [176,178]. Vitamin D3 is transported in the blood via vitamin D-binding protein. In the liver, vitamin D3 is converted to calcidiol, 25-hydroxyvitamin D (25-OH vitamin D), followed by further conversion to calcitriol, 1α,25-dihydroxyvitamin D (1α,25-$(OH)_2$ vitamin D), primarily in the kidneys, where it helps to regulate calcium homeostasis [179,180]. The 1α,25-$(OH)_2$ vitamin D can now act on its receptor, the vitamin D receptor, in many target tissues, including the intestine, kidney, bone and brain [181].

Vitamin D deficiency has long been associated with osteoporosis, brittle bones and muscle weakness, but recently low levels of vitamin D have been linked to increased overall mortality [182,183]. The elderly represent those at greatest risk of age-related cognitive decline and neurodegenerative disorders [184]. Recent retrospective studies on elderly human subjects provide correlative evidence that those with vitamin D deficiency have a much higher incidence of cognitive impairment than those with normal vitamin D levels [185,186]. Thus, it seems that vitamin D deficiency may accelerate cognitive decline in aging [187]. A recent meta-analysis also shows that patients with AD typically have lower serum concentrations of vitamin D [188]. Interestingly, it was reported that 1α,25$(OH)_2$ vitamin D helped: to reverse soluble Aβ and inflammatory issues [189] as well as Ca^{2+}-mediated excitotoxicity [180]; to reduce biomarkers of brain aging associated with Ca^{2+} dyshomeostasis [190]; and to regulate levels of glutathione, a primary antioxidant in the brain, by modulating γ-glutamyltranspeptidase activity [191].

In addition, because immune defects in Aβ phagocytosis and degradation underlie Aβ deposition and inflammation associated with increased OS levels in AD brain, better understanding of the relation between Aβ phagocytosis and inflammation could lead to promising preventive strategies. For this reason, in 2013 the effects mediated by the administration of 1α,25-$(OH)_2$ vitamin D3 (well known for its immune modulator properties), in peripheral blood mononuclear cells of AD patients and controls were evaluated. 1α,25$(OH)_2$ vitamin D3 improved phagocytosis of Aβ peptides by AD macrophages and inhibited fibrillar Aβ-induced apoptosis. The action of 1α,25$(OH)_2$ vitamin D3 depended on the nuclear vitamin D and the protein disulfide isomerase A3 receptors [189].

Furthermore, 1α,25(OH)$_2$ vitamin D3 treatment in mixed neuron-glial cell cultures increased the mRNA levels of 27 genes, among which 17 genes were related to neurodegenerative and psychiatric diseases or brain morphogenesis [192]. Notably, 10 of these genes encode proteins potentially limiting the progression of AD such as vitamin D receptor, lipoprotein lipase, complement component 3 (C3) and lecithin cholesterol acyltransferase [192]. These data provide support for a role of 1α,25(OH)$_2$ vitamin D3 in brain disease prevention. The possible consequences of circannual or chronic vitamin D insufficiencies on a tissue with a low regenerative potential such as the brain should be considered.

In another interesting study, the direct interaction between vitamin D binding protein (DBP) and Aβ was confirmed in the brain of AD patients and transgenic AD model mice by immunoprecipitation assay and immunohistochemical double-staining methods. Moreover, atomic force microscopic examination revealed that DBP reduced Aβ aggregation *in vitro*. DBP also prevented Aβ-mediated death in a cultured mouse hippocampal HT22 cell line. Finally, DBP decreased Aβ-induced synaptic loss in the hippocampus and rescued memory deficits in mice after injection of Aβ into the lateral ventricle. These results provide converging evidence that DBP attenuates the harmful effects of Aβ conceivably together with a reduction of OS levels, by a direct interaction and suggest that DBP is a promising therapeutic agent for the treatment of AD [193].

In 2012, the importance of vitamin D in driving dementia was highlighted by the finding from Annweiler *et al.* showing that low 25-OH vitamin D concentrations were associated with MCI status in older non-demented community-dwellers with subjective memory complaint [194].

In 2013, a study from the Butterfield group reported for the first time that a chronic low-vitamin D diet and consequential low levels of vitamin D in the bloodstream result in significant increases in tyrosine nitration in brain proteins, alterations in glucose metabolism and mitochondrial changes in brain of elderly rats, an animal model of brain in older human subjects [195]. In addition, preliminary results of the same study showed impaired learning and memory functions in low vitamin D animals, thus providing biochemical evidence to support the conclusions that suggest that chronic lower dietary vitamin D levels can cause brain damage. In contrast, adequate or high dietary vitamin D led to normal learning and memory performance in aged rats, consistent with the notion that sufficient or higher serum vitamin D levels may have direct and/or indirect antioxidant properties and be beneficial to modulate damaging effects of brain aging [195].

Despite these encouraging lines of evidence, solid results supporting the link between vitamin D and oxidative stress in the brain and their involvement in AD neuropathology are still to be determined.

In 2011, in a RCT the effects of high-dose vitamin D followed by nasal insulin on memory and disability in mild-to-moderate AD were examined. All participants took low-dose vitamin D (1000 IU/day) throughout. After run-in (8 weeks), subjects were randomized to additional high-dose D/placebo for 8 weeks, followed immediately by randomization to nasal insulin (60 IU four times a day)/placebo for 48 h. Neither cognition nor disability changed significantly after high-dose of vitamin D thus suggesting that high-dose vitamin D provides no benefit for cognition or disability over low-dose vitamin D in mild-to-moderate AD [196]. However, it is necessary to recall that pathology occurs about 20 years prior to clinical symptoms in AD, so translational studies even in mild-to-moderate AD may not prove beneficial since neuronal loss is simply too extensive.

With the aim to provide a synergistic mechanism, which could be helpful to slow/rescue AD pathology, the AD-IDEA trial was designed [197]. That is a unicenter, double-blind, randomized, placebo-controlled, intent-to-treat, superiority trial, in which all participants receive memantine 20 mg once daily – titrated in 5 mg increments over 4 weeks – and each one was randomized to one of the two treatment options: either vitamin D3 (one 100,000 IU drinking vial every 4 weeks) or placebo (administered at the same pace) [197]. The AD-IDEA trial seeks to provide evidence on its efficacy in limiting cognitive and functional decline in AD, and, being an ongoing trial, the results will be available in the near future.

Melatonin

N-Acetyl-5-methoxytryptamine (melatonin) is a lipophilic hormone that is mainly produced and secreted at night by the pineal gland. Melatonin was first reported to be an efficient endogenous antioxidant in 1993 by Reiter *et al.* [198–200]. It is found in all organisms, including bacteria, plants, insects and vertebrates [201,202]. Because melatonin is also ingested in foodstuffs such as vegetables, fruits and herbal medicines, from a nutritional point of view, melatonin may also be classified as a vitamin [199,203,204].

Melatonin possesses several unique advantages. First, its solubility in both lipids and water allows melatonin to be easily distributed into the cell. Second, its ability to cross the BBB allows melatonin to enter the CNS [205]. Evidence demonstrated that melatonin levels decrease during the aging process and that patients with AD have more profound reductions of this hormone. Zhou *et al.* reported that CSF melatonin levels were significantly decreased in AD patients [206].

In 1997, it was reported for the first time that melatonin (10 μM, for 24 h) prevented death of cultured neuroblastoma cells exposed to Aβ [207]. Melatonin also averted Aβ-induced increased intracellular Ca^{2+} and lipid peroxidation [207], thus suggesting melatonin as a potential therapeutic approach in AD. Since then, several steps forward have been made with regard to the effects mediated by melatonin against oxidative stress-induced damage in AD as reviewed in [208]. Interestingly, Matsubara *et al.* reported that early (beginning at 4 months of age), long-term (lasting from 4 to 11.5 months of age) administration of melatonin partially inhibited the expected time-dependent elevation of Aβ and reduced the abnormal nitration of proteins in Tg2576 transgenic mice [209]. Similarly, long-

term melatonin administration prevented the abnormal upregulation of apoptotic markers and alleviated memory impairments in APP695 transgenic mice [210].

Quite recently, melatonin (50 mg/kg, for 3 days, ip.) reportedly significantly reduces ROS production in astrocytes, lymphocytes and hepatocytes of Aβ (25 μg, ip.)-injected mice by increasing the levels of scavenging enzymes, SOD, catalase and GSH compared with the untreated group [211]. Immunohistochemistry study revealed that melatonin prevented the activation of glial fibrillary acidic protein in neocortex and transcription factor NF-κB in liver and neocortex of Aβ-injected mice [211]. Melatonin also prevented the loss of dopamine and its degradation products [211]. Thus, while melatonin may be a potential therapeutic agent in the prevention of oxidative stress associated with Aβ and AD, it reportedly also can prevent dopamine turnover induced by Aβ.

In a subsequent study, decreased serum melatonin was associated with: development of spatial memory deficits; τ hyperphosphorylation at multiple sites; activation of GSK-3β and protein kinase A, as well as suppression of PP1 [212]. Prominent oxidative damage and organelle lesions, demonstrated by increased expression of endoplasmic reticulum (ER) stress-related proteins including BiP/GRP78 and CHOP/GADD153, decreased the number of rough ER and free ribosomes, thinner synapses and increased SOD and monoamine oxidase were also observed [212]. Simultaneous supplementation of melatonin partially arrested the behavioral and molecular impairments [212], suggesting that melatonin deficiency may be an upstream effector responsible for the AD-like behavioral and molecular pathologies along with mechanisms associated with ER stress.

The positive effects of melatonin on OS-mediated development of AD pathology were also associated with its ability to modulate PP2A and PP1 activities. Indeed, as recently highlighted, inhibition of PP2A and PP1 by calyculin A, induced AD-like hyperphosphorylation of τ together with spatial memory retention impairment. Conversely, administration of melatonin ip. for 9 consecutive days before injection of calyculin A could prevent calyculin A-induced synaptophysin loss, memory retention deficits as well as hyperphosphorylation of τ and neurofilaments [213]. Furthermore, melatonin partially reversed the phosphorylation of the catalytic subunit of PP2A at Tyrosine 307 (Y307), a crucial site negatively regulating the activity of PP2A, and reduced the levels of MDA, a marker of OS, induced by calyculin A [213]. These results suggest that melatonin could serve as a potential therapeutic agent for preventing AD-like pathological changes and behavioral abnormality via modulating the activity of PP2A and OS.

Very recently, in 2012, the combined effects mediated by physical exercise and melatonin (10 mg/kg, for 6 months, p.o.) in 3xTg-AD male mice aged from 6 to 12 months, therefore modeling moderate to advanced phases of AD pathology, were assayed [214]. Analysis of behavior and brain tissue at termination showed differential patterns of neuroprotection for the two treatments. Both treatments decreased soluble Aβ oligomers, whereas only melatonin decreased hyperphosphorylated τ [214]. Melatonin was effective against the immunosenescence that 3xTg-AD mice present. Both treatments protected against cognitive impairment, brain OS and a decreased mitochondrial DNA [214]. Interestingly, only the combined treatment was effective against the decrease of mitochondrial complexes [214]. Therefore, melatonin plus physical exercise may exert complementary, additive or even synergistic effects against a range of disturbances present in AD.

Another feature of AD patients is the appearance of severe circadian rhythm disruptions, whose causes are still not fully known, although reduced systemic melatonin levels may contribute to these effects, since melatonin is an effective chronobiotic and antioxidant with neuroprotective properties. To clarify this aspect, the effects of long-term treatment with melatonin or the selective melatonin MT1/MT2 receptors agonist ramelteon, on circadian system function, hippocampal oxidative stress and spatial memory performance in the APPswe/PS1 mouse model of AD were evaluated with surprising outcomes. The results have indicated that many of the circadian and behavioral parameters measured, including OS markers, were not significantly affected in these AD mice, thus partially revising the idea about the effects of reduced melatonin levels in AD [215]. Notwithstanding these findings, brain tissue analysis revealed significant reduction in hippocampal protein oxidation both in melatonin- and ramelteon-treated mice [215]. These results suggest that not all aspects of the circadian system are affected in the APPswe/PS1 mice. Therefore, care should be taken when extending the results obtained in Tg mice to develop new therapies in humans. This study also revealed the complexity in the therapeutic actions of melatonin and ramelteon in this mouse model of AD.

As cited above for FA, tacrine-melatonin hybrids were also designed and synthesized as new multifunctional drug candidates for AD. These compounds may simultaneously palliate intellectual deficits and protect the brain against both Aβ peptide and OS. Indeed, these new molecules are potent and selective inhibitors of hAChE with IC_{50} values in the nanomolar and picomolar ranges (5×10^{-9} to 8×10^{-12} M), and are therefore 70- to 43,000-fold more potent than tacrine [216]. These agents also showed *in vitro* greater antioxidant properties than trolox [216]. Moreover, they inhibited Aβ self-aggregation and were at least as potent as propidium [216]. In human neuroblastoma cells, they showed protective properties against damage caused by Aβ and by mitochondrial free radicals [216]. Finally, these molecules have low toxicity and would be able to penetrate the CNS to reach their cerebral targets [216].

Despite the numerous positive effects found both *in vitro* and *in vivo* animal models, neither melatonin showed positive effects when administered to AD subjects. Even in this case we do not have any data available on OS levels, but the failure of clinical trials in terms of cognitive or AD-associated symptoms improvements may be related to the caveat stated above regarding AD pathology occurring much earlier than clinical symptoms. Indeed, in the last two clinical trials with human melatonin alone (5 mg/day, for 10 weeks, from Monday to Friday [217]) or (8.5 mg/day immediate release plus 1.5 mg/day

sustained release, for 10 days [218]) did not improve sleep, circadian rhythms or agitation [217,218]. The lack of efficacy may be related to the absence of a true treatment effect [217,218] or to the high physiologic dose of melatonin used in [218].

Mitochondria-targeted antioxidant molecules

Mitochondrial dysfunction has long been associated with neurodegenerative disease. Accumulating evidence indicates that mitochondrial abnormalities and oxidative damage are early events in AD [219]. In AD, mitochondrial impairment correlates with increased oxidative damage, altered mitochondrial DNA and cytochrome oxidase levels [220]. Moreover, emerging evidence indicates that an imbalance of mitochondrial dynamics is involved in the pathogenesis of AD since the expression of several proteins involved in mitochondrial fission and fusions were affected in postmortem AD brains, leading to abnormal redistribution of mitochondria [221,222]. APP and Aβ have been shown to cause decreased activity of mitochondrial respiratory chain complexes, decreased activity of several mitochondrial metabolic enzymes and also to induce ROS production [223,224]. Even mitochondria isolated from peripheral lymphocytes in AD and amnestic MCI patients demonstrate elevated oxidative stress, and expression proteomics identified differential levels of metabolic and other enzymes similar to the case in brain [60,61]. Such metabolic and oxidative compromise may thereby render neurons susceptible to excitotoxicity and apoptosis, and, besides, dysfunction of mitochondria has been reported to alter APP metabolism, to increase the intraneuronal accumulation of Aβ-peptide and to enhance the neuronal vulnerability [225]. Therefore, mitochondrial-targeted antioxidants might represent a valuable tool able to modify the pathogenesis of neurodegeneration decreasing mitochondrial oxidative damage and protecting from mitochondrial dysfunctions. To date, the most effectiveness molecules to counteract mitochondrial OS have been CoQ10 and mitoQ, LA and SS peptides.

CoQ10 & mitoQ

CoQ10 acts as a potent antioxidant, blocks apoptosis by inhibiting the PTP and is a co-factor of mitochondrial uncoupling proteins [226]. *In vitro* and cellular studies showed that CoQ10 pretreatment prevents a decrease in mitochondrial transmembrane potential and reduces mitochondrial ROS generation [219]. Moreover, several studies have been conducted using CoQ10 in *in vivo* models. Aged PS1 transgenic mice fed with CoQ10 for 60 days partially attenuated Aβ overproduction and intracellular Aβ deposits [227]. However, it must be mentioned that idebenone, a synthetic analog of CoQ10, failed to slow cognitive decline in AD patients during clinical trials [222].

MitoQ is a triphenylphosphonium-linked ubiquinone derivative that concentrates several 100-fold in mitochondria due to the large mitochondrial membrane potential and converts H_2O_2 to H_2O and O_2, reducing toxic insults from free radicals in the mitochondria [228,229]. MitoQ is oriented with the TPP+ moiety near the membrane surface accessing the membrane core to act as a chain breaking antioxidant and allowing reduction and recycling of mitoQ to ubiquinol by complex II [230]. In several cell models, mitoQ has demonstrated protective effects by reducing free radicals, decreasing oxidative damage and maintaining mitochondrial functions [222,231]. In mouse neuroblastoma (N2a) cells, mitoQ exerted protection from toxicity. The 3xTg mouse model of AD diet supplemented with mitoQ for 5 months showed protection toward cognitive decline as well as oxidative stress, Aβ accumulation, astrogliosis, synaptic loss and caspase activation in their brains [232]. Recent studies on *Caenorhabditis elegans* administered mitoQ and then treated with Aβ showed extended lifespan, delayed Aβ-induced paralysis, ameliorated depletion of the mitochondrial lipid cardiolipin and protection of complexes IV and I of the electron transport chain [233]. A Phase I clinical trial in AD patients also has been performed using mitoQ supplementation, which showed overall good pharmacokinetic behavior, while Phase II studies were performed only on PD patients obtaining no significant changes on any measure of PD progression [219].

Lipoic acid

LA is the cofactor of two mitochondrial enzymes: pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. LA is a powerful antioxidant working in the recycling of other antioxidants such as vitamin C and E and glutathione [234]. Oxidative damage of LA in human plasma has been observed in AD and MCI patients [235]. LA requires lipoamide dehydrogenase to reduce the S-S bond in this moiety, and in AD brain HNE is bound less to LA due to damaged lipoamide dehydrogenase [235]. Studies on LA supplementation (600 mg LA/day) showed, in AD patients, amelioration of cognitive decline [236], while chronic dietary LA reduces deficits in hippocampal memory of aged Tg2576 mice [237] and the SAMP8 mouse model of AD [238]. The use of LA in combination with other antioxidant has been object of several subsequent studies. LA given with NAC was shown to decrease mitochondrial-related oxidative stress in fibroblasts isolated from AD patients. Similarly, pretreatment of cortical neurons with LA and acetyl-L-carnitine (ALCAR) protected cortical neuronal cells from 4-hydroxy-2-nonenal-mediated oxidative stress and neurotoxicity [239]. Aged rats administered with LA and ALCAR showed a significant reduction of damaged mitochondria and an increase of intact mitochondria, as well as, ApoE4 mice that reported the improvement in cognitive performance [240]. Clinical trials on ALCAR alone or in association with donepezil or rivastigmine showed beneficial effects on both clinical and psychometric tests in MCI and mild AD patients [241,242]. Currently, a Phase II clinical trial on LA plus Ω-3 fatty acids administered for 18 months to AD patients is ongoing [243].

SS peptides

The SS peptides are small cell permeable antioxidant peptides that target mitochondria. These include four peptides (SS02, SS19, SS20 and SS31) that are able to scavenge H_2O_2 and $ONOO^-$, and inhibit lipid peroxidation [244,245]. Compared

with the other three SS peptides, SS-31 has remarkable effectiveness as an antioxidant molecule due to its extensive cellular uptake and selective partitioning into mitochondria, where it is localized to the site of ROS production and protects against mitochondrial oxidative damage and further ROS production [246]. Several studies by the Reddy laboratory on Tg2576 mice treated with Aβ showed that SS31 has the potential of reducing Aβ-induced mitochondrial toxicity, increasing axonal transport of mitochondria and enhancing synaptic viability and overall protection of neurons from Aβ toxicity [221,247–249].

Expert commentary

Although extensive studies led to increased knowledge of the neurobiology of AD, yet the definitive causes remain indefinite and several therapies have failed. None of the drugs currently used for the treatment of AD has proved successful at disease modification, possibly because they do not treat the underlying causes of the disease, or possibly many trials begin too late in the course of the disease or do not take into account basal redox status. Therefore, at present, prevention appears to still be a more likely option. Human epidemiological studies support the idea that there is an inverse relationship between antioxidant levels and intake and cognition function and development of AD. Indeed, oxidative stress is a well-recognized risk factor for age-associated cognitive decline and is widely considered to be a common underlying aspect in the complex pathogenesis of several neurodegenerative diseases, including AD. Recent evidence gained from human autopsy and animal studies even indicate that this phenomenon is an early event and might have a functional role in the pathogenesis of AD. Thus antioxidant therapy, as one of the promising therapeutic strategies for AD, has been studied for years. Antioxidants such as vitamin E, vitamin C and Se reportedly may scavenge intracellular and extracellular oxygen radicals. Nonetheless, most antioxidant drugs show general success in animal models but are less beneficial in human trials. If we look at these negative results, it is worth mentioning that they all lack some important information and do not unequivocally support a protective effect. These unsatisfactory findings open a number of questions that should be addressed to develop alternative and effective approaches. Do we have to re-visit the oxidative stress hypothesis of AD? Does OS play a functional role but is a secondary event in the pathophysiology of AD? Were 'the most powerful' antioxidants, at the right dose, for the right time selected for use? Based on the current experimental evidence, we can only partially answer these critical questions. Large heterogeneity in study design, differential control of confounding factors such as genetics, smoking, physical activity, basal redox state, energy intake, insufficient assessment of cognitive function, may contribute to these inconsistent findings. Considering that AD is both multifactorial and heterogeneous, future trials should include multiple intervention arms either by treatment with antioxidant cocktails and co-administration of antioxidants with other promising therapeutic options. In fact, focusing on a single compound may be inadequate, and a group or a panel of antioxidants may need to be considered. Moreover, repeat measurements of a panel of antioxidant biomarkers are needed to avoid random temporal fluctuations of single measurements. Such temporal variation may significantly affect the association between antioxidant biomarkers and cognitive outcomes. As a final comment, more extensive life-course studies are needed for better understanding of the association between antioxidant intake and decreased age-related cognitive decline, due to the possibility that protective effects may be observed over the lifespan rather than only in old individuals. The way toward effective therapy appears to be a long and complex process, but with different therapeutic strategies under investigation, a successful disease-modifying therapy of AD may become feasible in the future.

Five-year view

The use of antioxidant strategies for the treatment of OS-driven AD development has advanced in the last decade. The test of singular antioxidant compounds on animal model of the disease highlighted the potential efficacy of removing pro-oxidant species from pathological brain. However, translation to clinical trials did not yield the hypothesized results. Recent studies moved toward the use of formulas containing compounds able to modify several aspects of the disease. In this view, the synergistic use of drugs targeting different underlying causes of AD is expected to produce favorable results. A critical aspect that needs to be taken into consideration for the design of therapeutic approaches involving antioxidants is surely the redox status of the patient that could negatively affect the hypothesized outcomes. Indeed, due to the Janus face of antioxidant molecules to be both neuroprotective and neurotoxic depending on dosage as well as recycling and degradation processes, it became a real need to set reliable mutitargeted approaches. With regard to the dosage, new drug delivery systems aimed to overcome the limitation linked to bioavailability of the antioxidant compound, including, for example, solid lipid nanoparticles, liposomes or nanoemulsions, should be extensively evaluated. These systems might be helpful for at least two reasons: these approaches allow the reduction of the therapeutic dose used to obtain the neuroprotective effects and thus avoid the possible side effects related to an altered pharmacokinetics; these approaches allow the direct targeting into the brain. Finally, since OS being an early event in AD development, the use of antioxidants needs to be started before the appearance of clinical symptoms of AD even if this goal is difficult to achieve due to the lack of reliable diagnostic criteria in the early phases of the disease.

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Key issues

- Oxidative/nitrosative stress (OS) has a major role in the pathogenesis of Alzheimer's disease (AD) leading to the damage of vital cellular components such as proteins, lipids and nucleic acids.
- Compounds with antioxidant activity have been proposed for prevention of cognitive decline and treatment of AD and its early stages.
- *N*-Acetyl-L-cysteine showed protection against pro-oxidant species and amyloid β-peptide (Aβ) toxicity though the increase of glutathione levels in animal models and humans.
- Tricyclodecan-9-yl-xanthogenate was able to reduce reactive oxygen species, protein oxidation and glutathione/glutathione disulfide ratio in *ex vivo* studies.
- Selenium supplementation in AD animal models and patients demonstrated promising effects in recovering endogenous antioxidants and reducing Aβ production.
- Vitamin E is able to lower lipid peroxidation and β-amyloid deposition; however, its efficacy might be related to the redox status of the patient.
- Ferulic acid protects biological membranes from lipid peroxidation and neutralized peroxyl and alkoxyl radicals and is a modulator of β-secretase activity *in vitro* and *in vivo*.
- Chronic low dietary-derived vitamin D levels in the bloodstream are associated with increased protein tyrosine nitration, alterations in glucose metabolism and mitochondrial changes in the brain.
- *N*-Acetyl-L-cysteine, selenium and vitamin E are currently used in ongoing multitargeted clinical trials in combination with other drugs with the rationale to ameliorate OS-induced AD pathology.
- Melatonin reduced OS only in *in vitro* and *in vivo* animal models by reducing Aβ levels and τ phosphorylation, but failed to improve AD-associated symptoms when administered to humans.
- Mitochondria being both source and objective of reactive oxygen species represent a valuable therapeutic target; supplementation with mitochondria-focused antioxidants, such as coenzyme Q10 or lipoic acid alone or in formulas, is currently of high interest.

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