Oxidative challenge in Alzheimer's disease: state of knowledge and future needs

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ABSTRACT

A large body of experimental and *postmortem* findings indicate that Alzheimer's disease (AD) is associated with increased oxidative stress (OxS) levels in the brain. Despite the current limitations of OxS assessment in living subjects, recent data suggest that oxidative challenge might increase early both in the central nervous system and peripheral fluids. The aim of this review was to provide an overview of the existing literature linking systemic OxS to brain OxS in AD. We firmly believe that continued research aimed at overcoming the methodological and design issues affecting the body of studies in this field is mandatory for successful development of an effective antioxidant-based treatment of AD.

BACKGROUND

Alzheimer's disease (AD) is the most frequent form of neurodegenerative disease associated with dementia in the older population.[1 2] Recent estimates on the prevalence of AD in the USA indicate that about five million individuals suffer from this disease.[1]

A corollary of evidence suggests that oxidative stress (OxS), occurring in the presence of an imbalanced cellular redox state,[3] might be an early feature of AD.[4–7] However, the definitive in vivo confirmation of an interaction between OxS-induced biological damage and AD development has not yet been obtained, mainly as a result of unsolved methodological issues.[8–10] Likewise, the few clinical trials conducted to assess the possible effect of antioxidant supplementation in slowing or preventing AD have yielded contradictory results.[11 12]

The purpose of this review is to describe and discuss the main findings of the current literature supporting the hypothesis that OxS might be a precocious central nervous system (CNS) as well as a systemic manifestation in patients with AD. Moreover, the importance of identifying reliable biomarkers for in vivo OxS assessment and the implications of marker development on the design of future human intervention and epidemiology AD studies will be discussed.

AD: DIAGNOSIS AND NEUROPATHOLOGICAL FEATURES

Two major forms of AD have been described: the early-onset familial form (occurring before 65 years), and the late-onset form, which accounts for more than 95% of all AD cases.[1] AD can be diagnosed with >90% confidence, based on clinical criteria, including medical history, physical examination, laboratory tests, neuroimaging and neuropsychological evaluation. At present, the conclusive diagnosis of this disease requires both clinical assessment of the disease and histopathological brain examination.[13] *Postmortem* brain tissue is examined for the presence of two neuropathological hallmarks of AD, extracellular amyloid-β (Aβ) peptide (Aβ1-40 and Aβ1-42) accumulated in extracellular senile plaques and intracellular neurofibrillary tangles (NFT) primarily comprising abnormal and hyperphosphorylated τ protein.[13]

The deposition of amyloid plaques and NFTs is estimated to start approximately 10–15 years before the earliest signs of cognitive impairment, a stage referred to as presymptomatic AD[14 15] (in this potential stage of AD, the patient presents as a fully functional individual in cognitive examinations[16]). The recent advances in imaging techniques, *in primis* structural MR, have also revealed a reduction in volumes in specific brain regions prior to the first signs of cognitive impairment.[17] Notably, the progress of this disease is chiefly associated with atrophy, reflecting neuronal shrinkage and death and synaptic and axonal loss.[18] These volumetric changes (along with neuropathological lesions) initially affect the hippocampus and entorhinal cortex, which have been widely observed in the brains of individuals with early-stage AD and mild cognitive impairment (MCI).[19] The latter is regarded as an intermediate state between normal aging and early AD[20] and is primarily characterized by short-term/long-term memory impairment, which at variance of dementia is not associated with disability in activities of daily living.[20 21] Patients with MCI represent a high-risk population; indeed, almost half of these individuals evolve to AD at a rate of approximately 10–15% per year, which is much greater than in control subjects.[20] As the brain area affected by atrophy expands, neurodegeneration progresses from the limbic area to cortical regions, particularly the parietal, prefrontal, and orbitofrontal regions,[17] where additional cognitive symptoms emerge and the full dementia syndrome becomes apparent.[22]

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Main hypotheses on AD pathogenesis

Despite years of intense research, a complete understanding of the pathogenic mechanisms underlying AD has not been achieved. Experimental and epidemiological data have resulted in the generation of several hypotheses regarding AD pathophysiology.

1. Amyloid cascade hypothesis: the accumulation of Aβ in the brain is the primary pathogenic event, which then triggers the entire array of subsequent brain lesions.[23]
2. τ Hypothesis: τ hyperphosphorylation is the first event in AD.[24 25]
3. Mitochondrial cascade hypothesis: the impairment of brain mitochondria is the pathogenic trigger of AD neurodegeneration.[26]
4. Vascular hypothesis: AD develops first as a vascular disorder characterized by an abnormal reduction of cerebral blood flow.[27] Reduction in oxygen and nutrient supply leads to a neuronal energy crisis that, in turn, promotes the cellular and molecular neuropathology that defines AD.[27–30]

At present, the amyloid cascade is the most commonly held hypothesis, although concerns about the centrality of Aβ aggregation in senile plaques in AD pathophysiology have been repeatedly raised.[25–27] The difficulty of developing a unifying hypothesis about the pathogenesis of AD primarily reflects the multifactorial nature of this disease. Nevertheless, OxS is a factor that, regardless of the pathogenic trigger proposed, plays a role in AD pathophysiology.[24 31–33]

OXS: AN IMBALANCE LEADING TO BIOLOGICAL DAMAGE

Helmut Sies formulated the most inspired definition of OxS nearly three decades ago, describing this disturbance as "an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage."[3] This definition implies a pivotal concept: oxidants become harmful for cellular components only when these compounds overcome the antioxidant cellular defense system. Oxidants are primarily represented by reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive aldehyde species (RAS).[31] These species, although commonly described as 'dangerous', typically act in defense against pathogens[31 34] and as regulators of cellular metabolism.[35] However, most oxidants are free radicals forced (because of intrinsic instability) to react with and transform all biochemical constituents of living organisms.[31] Thus, even a slight modification of the cellular redox state, through dysfunctional defensive enzymes or depleted dietary micronutrients, has the potential to result in biomolecular damage.[3 31] Cumulative oxidative damage is a progressive and inevitable process associated with aging, and this progression is accelerated in several aging-related diseases, particularly cardiovascular diseases (CVD)[36] and neurodegenerative disorders.[37 38] Therefore, maintaining cellular 'redox homeostasis' might prevent the development of pathological status.[31]

Oxidants and antioxidants: the main sources, routes of formation, and the reaction of the major members of the two families

Free radicals are defined as an atom or a molecule containing at least one unpaired electron.[31] Superoxide anion ($O_2^{\cdot-}$) is regarded as the 'primary' ROS that further interacts with other molecular species, either directly or through the mediation of metal or enzyme catalyzed reaction, to form other 'secondary' ROS.[39] The intracellular increase in the superoxide radical primarily reflects mitochondrial dysfunction (with an increase in electron leak from complexes I and III of the electron transport chain) and the abnormal solicitation of both NADPH oxidase (NOX, present in both phagocytic and non-phagocytic cells) and xanthine oxidase (enzyme involved in purine catabolism).[31 34 40 41] Extremely high concentrations of superoxide and nitric oxide ($NO^{\cdot}$) are obtained during respiratory burst triggered through inflammatory responses.[42 43] Under these conditions, these two species react to form a highly reactive peroxynitrite anion ($ONOO^-$), which in turn induces lipid and protein oxidation and DNA fragmentation.[42] This deleterious process can be prevented through the highly efficient scavenging activity of both cytosolic and mitochondrial isoforms of superoxide dismutase (SOD) that dismutate $O_2^{\cdot-}$ to a 'less' reactive ROS, hydrogen peroxide (H_2O_2).[39 44] This is a critical step of the cellular redox pathway, since H_2O_2 can either be neutralized through other enzymes, such as catalase (CAT) or glutathione peroxidase (Gpx), or converted in the 'chief instigator' of OxS damage, that is, hydroxyl radical ($^{\cdot}OH$), through the metal-catalyzed Fenton reaction.[45 46] Hydroxyl radical is by far the most reactive ROS (half-life of 10^{-9} seconds), and this molecule indiscriminately reacts with all biomacromolecules.[46] Indeed, hydroxyl radicals trigger the lipid peroxidation cascade (leading to alterations in the biological properties of membranes, such as degree of fluidity, permeability, inactivation of receptors, etc), oxidative damage to proteins (alterations in folding and either the alteration or loss of enzymatic functions, etc), nucleic acids (damage to DNA and RNA), and carbohydrates.[31 47]

The accumulation of ROS, particularly superoxide, results in biological damage when the responses from enzymes and non-enzymatic molecules with antioxidant properties is inappropriate.[3 31] Defense mechanisms against reactive species essentially involve: (1) preventive mechanisms; (2) repair mechanisms; and (3) scavenging actions.[31 37] The most effective strategies for preventing oxidative damage are dismutation (through SOD) and the reduction of hydrogen peroxide or lipid hydroperoxides (ROOH) through enzymes that use endogenous nucleophiles, such as glutathione and thioredoxin.[48] The repair of damaged biomolecules is afforded through various proteinases, lipases, and DNA enzymes. Finally, according to Forman *et al*,[48] only SOD, α-tocopherol, and most likely glutathione reach a suitable concentration in vivo and/or react with a sufficiently high-rate constant as effective radical scavengers. Indeed, most free radicals react, at nearly the same rate constant, with several low-molecular weight antioxidants and the biomolecules to be protected. To overcome these kinetic constraints, these antioxidants should be at intracellular concentrations that are difficult to achieve in vivo.[48] However, the beneficial effects of some dietary micronutrients are undeniable. Indeed, there are several types of polyphenols that potently augment endogenous antioxidant mechanisms via alterations in gene expression.[35 48]

However, when the concentrations of endogenous antioxidants are insufficient to directly quench radicals, reflecting the kinetic limitations described above, low-molecular antioxidants, such as ascorbic acid, β-carotene, uric acid, and protein thiols, represent a diverse array of important antioxidants that act in synergy. These molecules are also essential for recycling α-tocopherol from tocopheryl radical in membranes and lipoproteins, chelating iron and other pro-oxidant metals.[31 48]

Biomarkers of OxS: a longstanding issue

The measurement of OxS in living individuals is challenging, mainly because the direct assessment of reactive species requires time-consuming and expensive approaches that are not feasible for clinical applications.[49] The alternative to these methods is the indirect assessment of ROS through fingerprinting, that is, the quantification of the byproducts of oxidative biomolecular damage.[8 31 50] Among these potential indicators, a gold-standard biomarker for the quantification of OxS in the periphery (serum/plasma or urine) has not yet been validated. Indeed, none of the currently available indices of OxS can fulfill the widely accepted definition of biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention."[9]

The development and application of various potential markers for measuring OxS in biological fluids are a currently evolving research area. A multitude of indicators of OxS have been employed in population-based studies thus far, although there has been little direct comparison between different methods using identical samples.[8] In addition, the available results appear inconsistent and variable, most likely reflecting the lack of accuracy and comparability of different analytical methods; hence, it is extremely difficult to provide absolute reference values for the specific markers in humans.[8 51] A crucial advance in this framework is the application of multilaboratory validation studies to determine the reliability of markers used for non-invasive measurement of OxS in humans, which has previously been undertaken in animals.[51 52]

An additional concern regarding OxS assessment must be highlighted. Indeed, the detection of high levels of OxS in the blood/urine of patients with a given disease (eg, AD) may not be the definitive evidence of the implication of reactive species in the development of such a pathology.[8] Increased OxS might reflect potential concomitant pathologies (eg, CVD, diabetes, and liver diseases) and subclinical pathological conditions that induce low-grade inflammation (eg, atherosclerosis and arthritis).[8 53] Moreover, some large epidemiological studies have identified a number of demographic and physical lifestyles and dietary factors that markedly affect the systemic redox balance, even in the absence of a full-blown disease.[54–57] The most common examples of these factors are older age, gender, race, as well as overall and central obesity (the latter being more associated with OxS[58 59]), smoking, alcohol abuse, and nutrition (particularly the intake of dietary antioxidants[42]). The failure to identify these factors (through anamnesis, detection of disease markers, assessment of anthropometrics, etc) or an underestimation of the impact of these factors in data analysis could considerably affect the reliability of the conclusions drawn from these data, as exhaustively outlined in two previous reviews.[60 61]

Fortunately, although conclusive analytical and pathophysiological validations have not as yet been achieved, there are several markers that possess the characteristics of chemical stability, easy accessibility, and the reproducibility required for a 'reliable' index of OxS status.[8 51 62] In this review, we will describe the use of these markers in studies of AD.

Classification of the most assessed peripheral markers of oxidative stress

In general, markers of OxS can be classified as molecules modified through interactions with reactive species in a defined microenvironment and molecules of the antioxidant system that change in response to increased pro-oxidative burden.[8 51] Traditionally, most markers of oxidative damage reflect ROS/RAS/RNS attack on polyunsaturated fatty acids DNA and protein.[8]

Markers of lipid peroxidation

The most widely employed index of lipid peroxidation in blood and urine is that of thiobarbituric acid-reactive substances (TBARS), extensively used because of the simplicity of their assessment.[8] However, this simple assay lacks specificity and precision.[51 63] The TBARS method has been frequently employed to measure malondialdehyde (MDA), a secondary product of lipid peroxidation. However, this RAS can only be reliably measured through mass spectrometry (MS) and ancillary techniques of chromatographic separation.[63 64] In addition to MDA, one of the main peroxidation products studied as an oxidative biomarker with distinct biological effects, α-β unsaturated aldehyde 4-hydroxynonenal (4-HNE), derived from the oxidation of ω-6 fatty acid, has also been used as a biomarker. This RAS efficiently binds covalently to proteins, peptides, nucleic acids, etc.[65]

At present, gas chromatography (GC)-MS methods to analyze 4-HNE adducts show the highest sensitivity compared with other quantification techniques, such as western blotting and high-pressure liquid chromatography (HPLC).[8 65] Recently, an ELISA test was proposed as a potential alternative to these time-consuming and labor-consuming methods, but the appropriateness of this assay has not been validated in blood samples.[66]

Similar considerations apply for the assessment of F_2-isoprostanes (F_2-iso), a family of prostaglandin F2α compounds generated in vivo through the non-enzymatic ROS-catalyzed peroxidation of esterified arachidonic acid.[8 67] F_2-iso and other isomers (derived from different PUFAs) are widely considered the most reliable serum and urinary markers of lipid peroxidation.[68] The specificity and sensitivity of F_2-iso as an OxS marker, however, strictly depend on the method used to assess this prostaglandin-like compound.[67 68] In this regard, the most reliable analytical method (but also the least applicable in clinical routine) is GC/MS, while the easiest and most accessible method (although less accurate and occasionally misleading) is an ELISA approach.[8 51]

Hydroperoxides (ROOH) are prominent non-radical intermediates of the oxidative modification of all unsaturated phospholipids and glycolipids, cholesterol, and (to a

minor extent) amino acids and carbohydrates.[8] ROOH are primarily formed during the propagation phase in the lipid peroxidation cascade through the reaction of the highly reactive peroxyl radical ($ROO^{\cdot}$) with a lipid molecule (figure 1).[31] Reflecting diverse sources of origin, hydroperoxides reach high concentrations in the blood, and monitoring them does not require highly sensitive analytical procedures. The most commonly used assays for these determinations are based on the spectrophotometric assessment of the products formed through reactions between a chromogenic substrate and hydroperoxides. However, previous studies and reviews[8 51] have concluded that, although presenting some positive features (low cost, simplicity, etc), none of the currently available methods afford an accurate and specific in vivo assessment of these lipid peroxidation byproducts.

Markers of protein and nucleic acid oxidation

Proteins are highly sensitive targets for ROS/RAS/RNS because of the overall abundance of these molecules in biological systems.[8 62] The primary markers derived from the oxidation of these macromolecules are carbonyls, nitrotyrosine and the least specific advanced oxidized protein products (AOPP).[62 69 70] Among these, carbonyls are now considered the most reliable markers, but only when they are measured through MS or HPLC.[3 5 53] Simpler, but less reliable, spectrophotometric or ELISA-based assays are also now available for large-scale determination.[71] Conversely, 3-nitrotyrosine (formed from a reaction with peroxynitrite or nitrogen dioxide) is widely considered as a promising early marker of protein oxidation in AD-related neurodegenerative processes and is extremely difficult to measure in serum or urine because of the high instability of this compound in these fluids.[8 70]

Studies have shown that cellular DNA and RNA damage results from radical species generated under different conditions, and a number of assays have been developed to oxidatively measure modified bases in several biological specimens.[72] The most used markers are derived from the

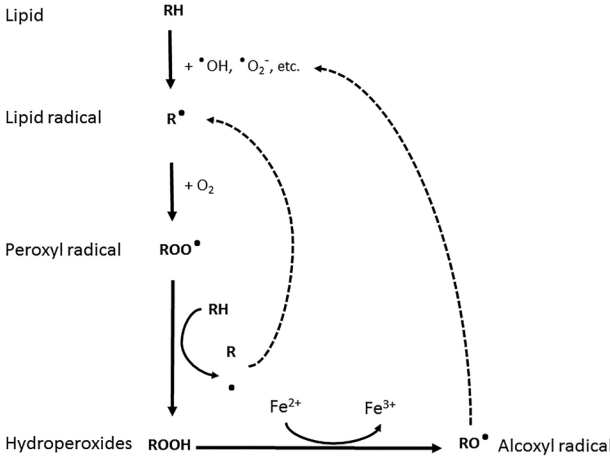

Figure 1 Initiation and propagation phases of lipid peroxidation cascade reaction. Hydroperoxides are relatively stable intermediates of lipid peroxidation, but in the presence of iron or copper in reduced form can form a highly reactive radical (alcoxyl radical), which is able to damage lipids and other biomolecules.

ROS-induced modification of guanosine of DNA (hydroxy-2′-deoxyguanosine, acronym: 8-OHdG) and RNA (hydroxy-2′-guanosine, acronym: 8-OHG), which can be accurately measured through ELISA (particularly in urine, where these molecules are more stable compared with other fluids), although MS technology is considered the gold standard.[73]

INVOLVEMENT OF OXS IN AD PATHOGENESIS

Several lines of evidence from in vitro and animal studies suggest that OxS might be an early event in AD pathogenesis.[5–7 74 75] According to these experimental observations, oxidative burst could precede and/or contribute to the formation of Aβ deposits and NFT and plays a role in AD-related neurodegeneration. Evidence of a primary role for OxS in disease development has also been provided through *postmortem* examination, revealing fingerprints of oxidative challenge in MCI and AD.[76–79] In contrast, clinicoepidemiological studies on peripheral markers of OxS have generated inconsistent findings.[10 80–84]

Evidence of the early involvement of OxS in AD and potential mechanisms linking OxS with disease pathogenesis

Extensive oxidative damage has been observed in vulnerable neurons that do not yet show any pathological hallmarks of AD.[85] As stressed in the next paragraph, oxidatively modified biomolecules have been observed in MCI and AD *postmortem* brain tissue.[86 87] In vivo experiments on transgenic mouse models support the concept that oxidative burden temporally precedes the deposition of Aβ and other typical neuropathological traits of AD.[5]

In an exhaustive review, Cai *et al*[88] suggested that OxS might play a role in the generation and deposition of Aβ in brain parenchyma by diverse mechanisms. Briefly, the formation of Aβ occurs through two sequential cleavages of amyloid precursor protein (APP), elicited through β-secretase and γ-secretase. Accumulating experimental evidence from transgenic animal models[4 75] suggests that OxS significantly increases the catalytic activity of these two enzymes, which in turn augments Aβ production. The deposition of Aβ in the brain could reflect a compromised blood–brain barrier (BBB),[89] and OxS could contribute to damaging BBB either directly[32] or through the activation of metalloproteinases.[88 90] As a consequence of an alteration in BBB permeability, Aβ clearance from the brain might be delayed, and Aβ influx from cerebrospinal fluid (CSF) might be increased.[88]

The factors that contribute to elevated ROS levels most likely include aging, genetic factors, environmental exposure to contaminants (eg, pesticides), metal dyshomeostasis, etc.[39 91–93] This deleterious increase in reactive species most likely involves impaired neuronal mitochondria, which have been consistently observed as an early and prominent feature of AD.[24 26] Mitochondrial dysfunction could reflect reduced energetic substrates or oxygen delivery to the highly active neurons following chronic brain hypoperfusion (from stroke or heart failure)[27 32] or the defective expression of glucose receptors in the BBB.[29] The ensuing reduction of ATP synthesis is the precursor of a biochemical cascade (altered protein and lipid synthesis, signal transduction breakdown, etc), eventually leading to

OxS[30] and cellular hypometabolism, with the latter preceding any clinical manifestations of AD.[27] Moreover, according to Morris *et al*[94] and Kaminsky *et al*,[95] this form of dementia might be associated with several systemic manifestations (ie, obesity, diabetes, chronic inflammation, insulin resistance, etc), which are well-recognized factors with the potential to negatively influence peripheral redox balance.[8 31 36 54 55 57]

Excess ROS generation might also influence the clinical progression of AD. Indeed, it has been suggested that these noxious species mediate the neurotoxicity of the Aβ soluble oligomers (ie, the precursors of insoluble fibrils of amyloid plaques)[74] and hyperphosphorylated τ.[96] Specifically, experimental data have shown that Aβ oligomers, mainly those formed from the Aβ1-42 isoform, directly generate H_2O_2 through copper-dependent SOD-like activity.[6 31 74] Copper present in high levels in amyloid plaques reacts with hydrogen peroxide to form highly reactive hydroxyl free radicals.[7] The excessive generation of ROS reflects the interaction between Aβ oligomers and neuronal and microglial and NOX,[34 97] with the latter presumably activated through increased intracellular free calcium.[98] Furthermore, these aberrant oligomers could also induce the release of pro-inflammatory and pro-oxidant cytokines from astrocytes and microglia[97] and alter ROS production in mitochondria through modulating the activity of enzymes, such as Aβ-binding alcohol dehydrogenase, or impairing the activity of respiratory chain complexes (eg, cytochrome oxidase, figure 2).[99 100] Moreover, increasing evidence suggests that OxS might also be involved in the hyperphosphorylation and polymerization of the τ protein.[101] Indeed, the neurotoxic action of neurofibrillary deposits in the brain is depressed or induced through the downregulation or upregulation, respectively, of mitochondrial SOD.[96]

The concept of a primary role for OxS as a mediator of NFT and Aβ neurotoxicity is further supported by the potential high vulnerability of neurons to reactive species insults. Indeed, neurons have a long life, relatively low levels of endogenous antioxidants (particularly glutathione), high levels of polyunsaturated fatty acids (PUFA, the primary target of reactive species), high levels of pro-oxidant metals, and the conspicuous use of oxygen, which enhance the 'physiological' extent of ROS derived by mitochondria.[91 102] In this scenario, even a moderate increase in the normal rate of ROS formation could lead to detrimental consequences, as demonstrated through the extensive accumulation of oxidative damage.

Markers of OxS in human studies: brain (*postmortem* tissue and CSF) versus peripheral fluids (blood and urine)

Higher concentrations of carbonyls and 3-nitrotyrosine have been repeatedly detected in neocortical/hippocampal *postmortem* tissue samples from patients with AD compared with controls.[77 103] Butterfield *et al*[78] and other groups have shown that protein oxidation could be a precocious event in AD pathogenesis, as the quantity of carbonyls is significantly increased in the hippocampi or in the

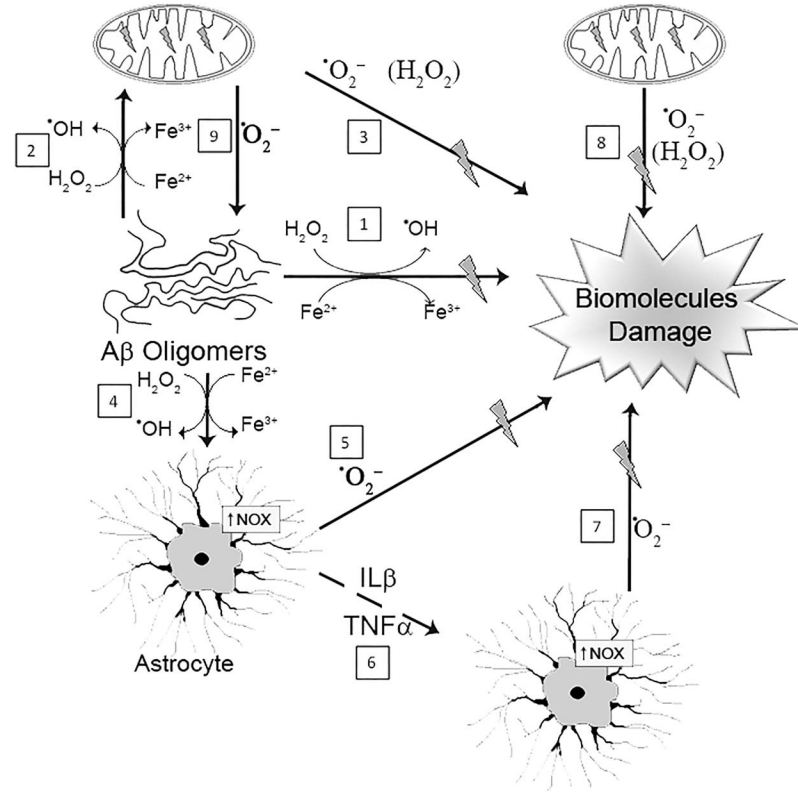

Figure 2 Sources of reactive oxygen species (ROS) in the Alzheimer's disease (AD) brain. Soluble amyloid-β (Aβ) oligomers and mitochondrial dysfunction are the main sources of ROS and related biomolecular damage in the brain tissue of patients affected by AD. Once formed, the Aβ oligomers are able to generate oxygen peroxide (H_2O_2), which acts with the reduced transition metals (Fe^{2+} or Cu^{+}) and produces hydroxyl radicals ($^{\cdot}OH$). In turn, this noxious radical is able to: directly damage biomolecules (1); cause damage to neuronal mitochondria (2) and, consequently, a large leak of superoxide anion ($O_2^{\cdot-}$) and oxygen peroxide from the respiratory chain (3); directly activate microglial NOX (NADPH oxidase) (4) (and promote production of superoxide anion (5)) and/or induce the release of pro-inflammatory cytokines from astrocytes and microglia (6), which in turn can promote the release of ROS from other glial cells (7). Mitochondrial dysfunction, and consequent emanation of ROS from the organelle to cellular constituents (8), may also occur prior to Aβ oligomer accumulation (and be one of the causes of their formation (9)) (IL, interleukin; TNF, tumour necrosis factor).

superior and middle temporal gyri[103] of MCI subjects compared with age-matched and sex-matched controls. In contrast with the results from postmortem analyses, a recent meta-analysis did not report any significant evidence of higher protein oxidation levels in the serum of patients with AD compared with healthy individuals.[80] Indeed, with a few exceptions,[104 105] no observational studies have reported significant changes in the serum concentration of AOPP, carbonyls, or 3-nitrotyrosine in relation to the occurrence of AD.[80 81 106 107]

High levels of biomarkers of oxidative DNA damage in circulating lymphocytes from patients affected with AD[108–110] have been reported in a small number of studies. In contrast, Abe *et al*[111] reported that the higher (fivefold compared with controls) levels of 8-OHG observed in the CSF of patients with AD were not observed in the serum of these individuals. The results from *postmortem* studies are much more consistent than those conducted in living individuals. Specifically, Mecocci *et al*[112] observed an increase of 8-OHdG in mitochondrial and nuclear DNA in three regions of the cortex of patients with AD compared with controls. Moreover, Wang *et al*[113] showed higher levels of this marker in nuclear DNA from frontal and temporal lobe tissues and mitochondrial DNA from temporal lobe tissues in MCI compared with age-matched control subjects.

A number of studies have shown increased levels of F_2-iso in frontal and temporal cortical areas or in the CSF of patients with AD compared with healthy individuals or patients affected with other neuropsychiatric diseases[114–117] (table 1). Markesbery *et al*[79] observed precocious lipid peroxidation in AD pathogenesis and reported higher F_2-iso levels in the frontal, parietal, and occipital lobes in MCI and AD compared with controls, whereas no significant differences were present between MCI and late AD. *Postmortem* CSF levels of F_2-iso in patients with AD were positively associated with the severity of neurodegeneration, as assessed through histopathological examination.[118] However, alterations in the blood/urine concentration of F_2-iso in living patients with AD are inconsistent.[82–84] Other isoprostane-like compounds, such as D4-isoprostanes, E4-isoprostanes, and F4-isoprostanes (or neuroprostanes), might be promising indicators of brain damage, given that these isoforms are derived from the oxidation of the major PUFA in the CNS docosahexaenoic acid (DHA).[117] Interestingly, neuroprostanes levels were more elevated in the temporal and occipital lobes of patients with MCI and AD [79] and in the CSF[119] of patients with AD compared with controls. Unfortunately, data concerning this marker in the blood/urine from patients with AD remain scarce.

A large body of data have been gathered through measurements of peripheral MDA, 4-HNE, and hydroperoxides.[80 81 107 120–125] A meta-analysis of 29 studies revealed that MDA was significantly increased in the plasma/serum of patients with AD and MCI compared with controls,[80] thereby reflecting the results obtained in brain tissue.[103 126] A similar trend has also been described for 4-HNE, which was increased both in the serum[127] and brain[128–130] of patients with AD and MCI[129]. Moreover, an association between higher serum levels of hydroperoxides and AD was observed in a previous study involving one of the largest population samples (422 participants) examined in studies on this topic.[131] To the best of our knowledge, there are no published studies that evaluate hydroperoxide levels in autopsied brains or CSF samples.

Table 1 summarizes the results of some of the largest studies evaluating OxS markers in brain tissue and CSF or peripheral fluid samples from patients with MCI or AD.

Some, but not all, lipid peroxidation markers are elevated in the peripheral fluids of patients with AD: potential explanatory hypotheses

Considering the data discussed in the previous paragraph, the markers most consistently altered in the peripheral fluids of patients with AD are those derived from lipid peroxidation. These byproducts, at variance with oxidized proteins, are small lipophilic molecules that freely cross the

Table 1 OxS markers in peripheral fluids and brain of patients with MCI or AD

	Brain (or CSF)		Periphery	
OxS markers	**AD**	**MCI**	**AD**	**MCI**
F_2-iso	(79) ↑; (115) ↑ (116) ↑; (117) ↑ (118) ↑; (119) ↑ (133) ↑	(79) ↑	(82) ~ ; (83) ↑ (84) ~ ; ↑	(83) ~;(84) ~
F_4-iso	(79) ↑; (120) ↑	(79) ↑		
4-HNE	(129) ↑; (131) ↑;	(130) ↑	(128)↑	
Hydroperoxides	–	–	(81) ↑; (132) ↑	
MDA (or TBARS)	(104) ↑; (127) ↑	(104) ↑	(121) ↑; (122) ↑ (123)↑; (124) ↑ (126) ↑;(128) ~	(124) ↑
Carbonyls	(104) ↑	(78)↑ (104)↑	(105) ↑; (106) ↑ (107) ~; (108) ~	(106) ↑; (108) ~
3-nitrotyrosine		(77) ↑	(105) ↑	
8-OHG	(112) ↑		(112) ~	
8-OHdG	(113) ↑	(114) ↑	(109) ↑*; (110) ↑* (111) ↑*	

*Markers detected in circulatory lymphocytes.
AD, Alzheimer's disease; CSF, cerebrospinal fluid; 4-HNE, 4-hydroxynonenal; MDA, malondialdehyde; MCI, mild cognitive impairment; OHG, hydroxy-2′-guanosine; OHdG, hydroxy-2′-deoxyguanosine; OxS, oxidative stress; TBARS, thiobarbituric acid-reactive substances.

BBB, even when this barrier is not damaged, and subsequently diffuse into the blood. Indeed, the level of these compounds should be considerably higher in the brain and CSF of patients with AD to become detectable in the systemic circulation. In a pioneering study, Montine reported that the difference in the mean concentration of CSF F_2-iso between the AD and controls was 28 pg/mL, which could only reflect the oxidation of arachidonic acid (10% of the brain lipid content)[132]; this surplus is then diluted 20–80 times in the blood, becoming undetectable even by the most sensitive methods. These considerations might account for the mixed results obtained with this superior marker of OxS.[82–84]

The most reasonable explanation for the increase in lipid oxidation markers, particularly lipid hydroperoxides, may be the induction of peripheral lipid peroxidative cascades through ROS. While the most noxious free radicals (hydroxyl and superoxide) are too unstable to translocate from the brain without reacting within neuronal tissue, H_2O_2 and hydroperoxides are stable enough to travel from the brain to the blood.[31] In the presence of even small concentrations of free iron or copper ions in reduced form, both species generate two highly reactive species, hydroxyl and alcoxyl radicals (figure 1), which in turn initiate and/or accelerate the peroxidative modification of lipids, such as the plasmatic cholesterols and phospholipids of circulating cells.[31 48] Some recent observations are consistent with these considerations: (1) circulating copper levels are increased in patients with AD[133 134]; (2) ceruloplasmin enzymatic activity, promoting the incorporation of the free pro-oxidant Fe^{2+} into transferrin, is decreased in AD[80 134]; (3) AD has often been associated with an increase in oxidized-low density lipoprotein (LDL) level[106] and a loss of erythrocyte membrane stability, reflecting the oxidative modification of structural lipids.[135]

Abnormal levels of lipid peroxidation byproducts in the serum of patients with AD could also result from systemic processes and manifestations that often accompany (and mutually influence[67]) CNS dysfunctions, such as impaired glucose and lipid metabolism (type 2 diabetes and insulin resistance are risk factors for AD) and chronic inflammation. Increases in inflammatory cytokines[136] and altered energy metabolism are also associated with OxS.[94]

Alterations in the peripheral levels of antioxidants: co-factors for systemic OxS in AD

The propagation of oxidative damage occurs rapidly when the antioxidant system affords an inadequate buffer.[3 31] Previous studies[80 81 124 131 137] have shown low serum concentrations of dietary-derived and endogenous low-molecular non-enzymatic antioxidants, such as uric acid, vitamin E, and vitamin C, in patients with AD or MCI, compared with healthy controls. Although they rarely reach extracellular and intracellular concentrations sufficient to scavenge radicals,[48] these compounds act synergistically to prevent, or slow down, the perpetuation of oxidative damage to biological molecules.[3 31] However, it is difficult to ascertain whether the low levels of exogenous antioxidants might be secondary to excessive consumption (reactions with radicals) or low dietary intake (or malabsorption syndromes).

A more concrete contribution to abrogate the systemic propagation of ROS damage is obtained through enzymatic activities; these proteins, by definition, are much faster and more efficient ROS scavengers than low-molecular weight antioxidants.[48] Most importantly, the modulation of the activity of these enzymes depends on both genetic factors and interactions with micronutrients procured through the diet (eg, polyphenols).[48 138 139] Nonetheless, the most effective scavengers of ROS (CAT, Gpx and SOD) have a predominantly intracellular localization, and thus they cannot be used as serum indicators of systemic antioxidant status. On the contrary, there is increasing evidence that the measurement of some antioxidant enzymes in erythrocytes, which are considered as passive 'reporter cells' for the oxidative status of the whole organism, could be a useful marker in this field.[140] In the milieu of plasma enzymes with antioxidant capacity, one of the most thoroughly investigated is paraoxonase 1 (PON-1). The next paragraph will focus on this enzyme and recent findings suggesting the potential implication of this enzyme in AD pathogenesis.

PON-1: potential relevance in AD pathogenesis

PON-1 is synthesized in the mammalian liver and circulates in the blood, bound to the surface of high-density lipoprotein (HDL), apolipoprotein (apo) A-1, and apo J. PON-1 exerts a number of enzymatic activities, including paraoxonase, arylesterase, and lactonase, which are spectrophotometrically detected using three different substrates, organophosphates, aromatic esters, and lactones, respectively.[141 142] There are simple and fast spectrophotometric assays available for the measurement of the activities of serum PON-1.[141] However, despite the wide use and adequate standardization of these methods, normal reference intervals have not been established to date.

Although the natural physiological substrate and catalytic mechanisms of this enzyme remain unknown, it has been suggested that PON-1 endows HDL particles with antioxidant properties. In vitro, PON-1 scavenges hydrogen peroxide and lipid hydroperoxides either free or present in atherosclerotic lesions or in minimally oxidized LDL.[142] Further validation of the antioxidant properties of PON-1 has been generated through in vivo experiments in PON-1/apo E double knockout mice.[143] Moreover, data from large population-based studies have consistently demonstrated a role for PON-1 in protecting the human body from CVD through inhibiting oxidative and pro-atherogenic modifications in lipoproteins and decreasing systemic lipoperoxidative damage.[144]

The finding that PON-1 single nucleotide polymorphisms (particularly Q192R and L55M) affect enzymatic activity and influence the risk of OxS-related diseases suggests the potential involvement of this enzyme in AD pathogenesis.[145 146] Unfortunately, a recent meta-analysis showed inconsistent results regarding the association between PON-1 phenotypes (Q192R) and AD prevalence.[146] These results do not rule out the potential relationship between PON-1 and this disease, as multiple polymorphisms in the PON-1 gene influence the catalytic function of this enzyme.[147] Moreover, recent studies[148–150] have shown that subjects affected with AD have lower levels of serum PON-1 enzymatic activity compared with healthy controls.

While the mechanism(s) for PON-1-mediated systemic antioxidant effects remain elusive, there is clear evidence that this HDL-associated protein might impact systemic OxS in humans.[151] Thus, in the light of the prominent role of OxS as a 'primary progenitor of AD',[85] it is reasonable to speculate that the high level of PON-1 might confer protection from AD development, or at least might contrast the clinical progression of this disease. In support of this hypothesis, it has been demonstrated that high paraoxonase activity provides protection from CVD,[152] which in turn is a well-recognized risk factor for vascular dementia and AD development.[21 24] A recent study conducted with 596 older participants revealed a deficit in PON-1 activity in MCI[150]; consequently, this deficit might reflect an early feature of dementia.

Notably, since longitudinal studies employing PON-1 are lacking, conclusions concerning the cause-effect link between the levels of this enzyme and AD remain elusive. Furthermore, PON-1 might not have a high clinical specificity for AD, being a multifactorial enzyme widely associated with many different disorders.[150 152 153]

OXS AND AD: FROM THEORY TO PRACTICAL APPLICATIONS

Research needs and perspectives

Considering the published data discussed thus far, it is conceivable to ascribe OxS as a member of the proposed pathogenic mediators of AD. In addition, increasing evidence suggests that this condition might precociously affect both CNS and non-CNS compartments. Notably, the definitive proof of this 'simultaneity' is still lacking, reflecting the underscored methodological shortcomings concerning the in vivo assessment of OxS.

The association between OxS and AD represents the rationale of many cross-sectional and prospective population-based studies examining whether the intake of antioxidants in the diet or in nutritional supplements might be beneficial to disease prevention and/or course.[154–156] The results of these observational studies are mixed. Two prospective studies showed lower risks of dementia or AD in patients who consumed higher amounts of dietary antioxidants.[154 156] In contrast, a recent study has shown a lack of association between AD and antioxidant vitamin consumption (either in the diet or in supplements).[155] Farina *et al*[11] described similar disappointing results, obtained from double-blind randomized trials in which vitamin E treatment was compared with placebo in patients with AD or MCI. In particular, Petersen *et al* found that vitamin E supplementation failed to prevent the progression from MCI to AD during the 3 years of study.[12]

The reported failure of vitamin E supplementation in AD does not preclude that antioxidants might be beneficial for this disease. It has been suggested that combinations of antioxidants or combinations of antioxidants with aldehyde-trapping agents[157] might be worthy of clinical investigation. For example, it has been observed that the combined intake of a mixture of various tocopherols might be more neuroprotective than α-tocopherol alone.[158] Moreover, the use of other antioxidants in combination with vitamin E could improve the effects of therapy because antioxidants work synergistically against ROS.[48] Relevant to this notion, a study of a large cohort of elderly individuals demonstrated an association between the self-reported use of vitamin E supplement and the reduced incidence of AD but only in combination with ascorbic acid.[12] Unfortunately, to the best of our knowledge, large and well-designed double-blind studies on the use of a mixture of different antioxidant compounds in patients with AD and/or MCI are lacking. Although we propose that this approach might potentially be the most effective, we are also aware that it would be mandatory to identify the components of this 'cocktail' and determine the dosage that would decrease systemic OxS. Although the available experimental and epidemiological evidence is too sparse and disconnected to obtain a definitive solution to this issue, there are some interesting indications. Bonda *et al*[85] suggested that the most effective therapeutic antioxidants for AD should target the mitochondrial components because these organelles are the primary generators of ROS, and in AD, oxidative injury to mitochondrial structures occurs prior to that in any other cell components.[26 85] Thus, coenzyme Q10 and R-α-lipoic acid could be promising compounds, based on the results of preclinical studies.[85] Other antioxidant candidates for AD might be the variegated family of plant-derived polyphenols. Cumulating evidence in transgenic mice has shown that the administration of a single polyphenol (eg, quercetin or resveratrol) or a mixture of polyphenols increased the serum activity of PON-1,[159] which is depressed at the preclinical stage of AD.[150] In addition, phytochemicals are not good ROS-scavengers per se but rather positively modulate the expression of key cellular antioxidants and phase 2 enzymes involved in ROS-scavenging activity, such as SOD, CAT, glutathione, and Gpx.[160 161] Thus, the postulated modulation of PON-1 activity might be ascribed as part of the ROS-detoxifying mechanisms triggered by polyphenols.

The identification of the most suitable population sample that could be treated with antioxidants is a further important issue to consider. Since the herein reported converging evidence indicates that redox balance is already compromised in the preclinical stage of AD, it should be recommended to target cognitively healthy individuals (by using the most commonly used cognitive test), rather than MCI or AD, for possible antioxidant interventions. Indeed, it must be borne in mind that the antioxidants are effective in preventing OxS-related injuries while being unable to repair them.[24] In agreement with other authors,[74] we believe that,[74] a possible new research avenue for studying the response to antioxidants in AD might consist in including the assessments of antioxidants (such as PON-1, low-molecular non-enzymatic antioxidants) and markers of oxidative damage. To accomplish this aim, it becomes mandatory to overcome the challenging methodological shortcomings affecting the in vivo assessment of OxS.

CONCLUSIONS

AD is regarded as a heterogeneous and multifactorial CNS disease, with no single etiopathogenetic pathway leading to the typical dementia syndrome. OxS might be ascribed as one of the 'smoking guns' that accounts for the pathogenesis of the disease, in the light of the multiple roles of OxS in systemic and neurological abnormalities characterizing AD.

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REFERENCES

1 Ott A, Breteler MM, van Harskamp F, *et al*. Prevalence of Alzheimer's disease and vascular dementia: association with education. The Rotterdam study. *BMJ* 1995;310:970–3.
2 Parihar MS, Hemnani T. Alzheimer's disease pathogenesis and therapeutic interventions. *J Clin Neurosci* 2004;11:456–67.
3 Sies H. Oxidative stress: oxidants and antioxidants. *Exp Physiol* 1997;82:291–5.
4 Chen L, Na R, Gu M, *et al*. Lipid peroxidation up-regulates BACE1 expression in vivo: a possible early event of amyloidogenesis in Alzheimer's disease. *J Neurochem* 2008;107:197–207.
5 Praticò D, Uryu K, Leight S, *et al*. Increased lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer amyloidosis. *J Neurosci* 2001;21:4183–7.
6 Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. *Nat Rev Drug Discov* 2004;3:205–14.
7 Butterfield DA, Swomley AM, Sultana R. Amyloid β-peptide (1–42)-induced oxidative stress in Alzheimer disease: importance in disease pathogenesis and progression. *Antioxid Redox Signal* 2013;19:823–35.
8 Dalle-Donne I, Rossi R, Colombo R, *et al*. Biomarkers of oxidative damage in human disease. *Clin Chem* 2006;52:601–23.
9 Lesko LJ, Atkinson AJ. Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: criteria, validation, strategies. *Annu Rev Pharmacol Toxicol* 2001;41:347–66.
10 Di Domenico F, Coccia R, Butterfield DA, *et al*. Circulating biomarkers of protein oxidation for Alzheimer disease: expectations within limits. *Biochim Biophys Acta* 2011;1814:1785–95.
11 Farina N, Isaac MG, Clark AR, *et al*. Vitamin E for Alzheimer's dementia and mild cognitive impairment. *Cochrane Database Syst Rev* 2012;11: CD002854.
12 Petersen RC, Thomas RG, Grundman M, *et al*. Vitamin E and donepezil for the treatment of mild cognitive impairment. *N Engl J Med* 2005;352:2379–88.
13 Serrano-Pozo A, Frosch MP, Masliah E, *et al*. Neuropathological alterations in Alzheimer disease. *Cold Spring Harb Perspect Med* 2011;1:a006189.
14 Price JL, Morris JC. Tangles and plaques in nondemented aging and "preclinical" Alzheimer's disease. *Ann Neurol* 1999;45:358–68.
15 Jack CR, Knopman DS, Jagust WJ, *et al*. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol* 2010;9:119–28.
16 Sperling RA, Aisen PS, Beckett LA, *et al*. Toward defining the preclinical stages of Alzheimer's disease: Recommendations from the National Institute on Aging and the Alzheimer's Association workgroup. *Alzheimers Dement* 2011;7:280–92.
17 Dickerson BC, Goncharova I, Sullivan MP, *et al*. MRI-derived entorhinal and hippocampal atrophy in incipient and very mild Alzheimer's disease. *Neurobiol Aging* 2001;22:747–54.
18 Vemuri P, Wiste HJ, Weigand SD, *et al*. MRI and CSF biomarkers in normal, MCI, and AD subjects: predicting future clinical change. *Neurology* 2009;73:294–301.
19 Misra C, Fan Y, Davatzikos C. Baseline and longitudinal patterns of brain atrophy in MCI patients, and their use in prediction of short-term conversion to AD: results from ADNI. *Neuroimage* 2009;44:1415–22.
20 Petersen RC. Mild cognitive impairment as a diagnostic entity. *J Intern Med* 2004;256:183–94.
21 Markesbery WR, Schmitt FA, Kryscio RJ, *et al*. Neuropathologic substrate of mild cognitive impairment. *Arch Neurol* 2006;63:38–46.
22 Jack CR, Petersen RC, Xu Y, *et al*. Rates of hippocampal atrophy correlate with change in clinical status in aging and AD. *Neurology* 2000;55:484–9.
23 Hardy JA, Higgins GA. Alzheimer's disease: the amyloid cascade hypothesis. *Science* 1992;256:184–5.
24 Swerdlow RH. Pathogenesis of Alzheimer's disease. *Clin Interv Aging* 2007;2:347–59.
25 Maccioni RB, Farías G, Morales I, *et al*. The revitalized tau hypothesis on Alzheimer's disease. *Arch Med Res* 2010;41:226–31.
26 Swerdlow RH, Khan SM. A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease. *Med Hypotheses* 2004;63:8–20.
27 de la Torre JC. Pathophysiology of neuronal energy crisis in Alzheimer's disease. *Neurodegener Dis* 2008;5:126–32.
28 de la Torre JC. Is Alzheimer's disease a neurodegenerative or a vascular disorder? Data, dogma, and dialectics. *Lancet Neurol* 2004;3:184–90.
29 Winkler EA, Nishida Y, Sagare AP, *et al*. GLUT1 reductions exacerbate Alzheimer's disease vasculo-neuronal dysfunction and degeneration. *Nat Neurosci* 2015;18:521–30.
30 Kosenko EA, Solomadin IN, Tikhonova LA, *et al*. Pathogenesis of Alzheimer disease: role of oxidative stress, amyloid-β peptides, systemic ammonia and erythrocyte energy metabolism. *CNS Neurol Disord Drug Targets* 2014;13:112–19.
31 Valko M, Leibfritz D, Moncol J, *et al*. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007;39:44–84.
32 Aliev G, Priyadarshini M, Reddy VP, *et al*. Oxidative stress mediated mitochondrial and vascular lesions as markers in the pathogenesis of Alzheimer disease. *Curr Med Chem* 2014;21:2208–17.
33 Aliev G, Obrenovich ME, Tabrez S, *et al*. Link between cancer and Alzheimer disease via oxidative stress induced by nitric oxide-dependent mitochondrial DNA overproliferation and deletion. *Oxid Med Cell Longev* 2013;2013:962984.
34 Keisari Y, Braun L, Flescher E. The oxidative burst and related phenomena in mouse macrophages elicited by different sterile inflammatory stimuli. *Immunobiology* 1983;165:78–89.
35 Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1005–28.
36 Dhalla NS, Temsah RM, Netticadan T. Role of oxidative stress in cardiovascular diseases. *J Hypertens* 2000;18:655–73.
37 Bergamini CM, Gambetti S, Dondi A, *et al*. Oxygen, reactive oxygen species and tissue damage. *Curr Pharm Des* 2004;10:1611–26.
38 Grimm S, Hoehn A, Davies KJ, *et al*. Protein oxidative modifications in the ageing brain: consequence for the onset of neurodegenerative disease. *Free Radic Res* 2011;45:73–88.
39 Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. *Curr Med Chem* 2005;12:1161–208.
40 Borges F, Fernandes E, Roleira F. Progress towards the discovery of xanthine oxidase inhibitors. *Curr Med Chem* 2002;9:195–217.
41 Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* 2000;29:222–30.
42 Carr AC, McCall MR, Frei B. Oxidation of LDL by myeloperoxidase and reactive nitrogen species: reaction pathways and antioxidant protection. *Arterioscler Thromb Vasc Biol* 2000;20:1716–23.
43 Carreras MC, Pargament GA, Catz SD, *et al*. Kinetics of nitric oxide and hydrogen peroxide production and formation of peroxynitrite during the respiratory burst of human neutrophils. *FEBS Lett* 1994;341:65–8.
44 McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* 1969;244:6049–55.
45 Cadenas E. Basic mechanisms of antioxidant activity. *Biofactors* 1997;6:391–7.
46 Pastor N, Weinstein H, Jamison E, *et al*. A detailed interpretation of OH radical footprints in a TBP-DNA complex reveals the role of dynamics in the mechanism of sequence-specific binding. *J Mol Biol* 2000;304:55–68.
47 Marnett LJ. Oxyradicals and DNA damage. *Carcinogenesis* 2000;21:361–70.
48 Forman HJ, Davies KJ, Ursini F. How do nutritional antioxidants really work: nucleophilic tone and para-hormesis versus free radical scavenging in vivo. *Free Radic Biol Med* 2014;66:24–35.
49 Swartz HM, Khan N, Khramtsov VV. Use of electron paramagnetic resonance spectroscopy to evaluate the redox state in vivo. *Antioxid Redox Signal* 2007;9:1757–71.
50 Kohen R, Nyska A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol* 2002;30:620–50.
51 Kadiiska MB, Gladen BC, Baird DD, *et al*. Biomarkers of oxidative stress study II: are oxidation products of lipids, proteins, and DNA markers of CCl4 poisoning? *Free Radic Biol Med* 2005;38:698–710.
52 Kadiiska MB, Peddada S, Herbert RA, *et al*. Biomarkers of oxidative stress study VI. Endogenous plasma antioxidants fail as useful biomarkers of endotoxin-induced oxidative stress. *Free Radic Biol Med* 2015;81:100–6.
53 Pryor WA. Oxidative stress status: OSS, BOSS, and "Wild Bill" Donovan. *Free Radic Biol Med* 1999;27:1135–6.
54 Block G, Dietrich M, Norkus EP, *et al*. Factors associated with oxidative stress in human populations. *Am J Epidemiol* 2002;156:274–85.
55 Keaney JF, Larson MG, Vasan RS, *et al*. Obesity and systemic oxidative stress: Clinical correlates of oxidative stress in the Framingham study. *Arterioscler Thromb Vasc Biol* 2003;23:434–9.

56 Cervellati C, Bonaccorsi G, Cremonini E, *et al*. Accumulation of central fat correlates with an adverse oxidative balance in non-obese postmenopausal women. *Gynecol Endocrinol* 2013;29:1063–6.
57 Trevisan M, Browne R, Ram M, *et al*. Correlates of markers of oxidative status in the general population. *Am J Epidemiol* 2001;154:348–56.
58 Pou KM, Massaro JM, Hoffmann U, *et al*. Visceral and subcutaneous adipose tissue volumes are cross-sectionally related to markers of inflammation and oxidative stress: The Framingham Heart Study. *Circulation* 2007;116:1234–41.
59 Cervellati C, Bonaccorsi G, Cremonini E, *et al*. Waist circumference and dual-energy X-ray absorptiometry measures of overall and central obesity are similarly associated with systemic oxidative stress in women. *Scand J Clin Lab Investig* 2014;74:102–7.
60 Skoumalová A, Hort J. Blood markers of oxidative stress in Alzheimer's disease. *J Cell Mol Med* 2012;16:2291–300.
61 Teunissen CE, de Vente J, Steinbusch HW, *et al*. Biochemical markers related to Alzheimer's dementia in serum and cerebrospinal fluid. *Neurobiol Aging* 2002;23:485–508.
62 de Zwart LL, Meerman JH, Commandeur JN, *et al*. Biomarkers of free radical damage applications in experimental animals and in humans. *Free Radic Biol Med* 1999;26:202–26.
63 Liu J, Yeo HC, Doniger SJ, *et al*. Assay of aldehydes from lipid peroxidation: gas chromatography-mass spectrometry compared to thiobarbituric acid. *Anal Biochem* 1997;245:161–6.
64 Moselhy HF, Reid RG, Yousef S, *et al*. A specific, accurate, and sensitive measure of total plasma malondialdehyde by HPLC. *J Lipid Res* 2013;54:852–8.
65 Carini M, Aldini G, Facino RM. Mass spectrometry for detection of 4-hydroxy-trans-2-nonenal (HNE) adducts with peptides and proteins. *Mass Spectrom Rev* 2004;23:281–305.
66 Weber D, Milkovic L, Bennett SJ, *et al*. Measurement of HNE-protein adducts in human plasma and serum by ELISA-Comparison of two primary antibodies. *Redox Biol* 2013;1:226–33.
67 Milne GL, Gao B, Terry ES, *et al*. Measurement of F2-isoprostanes and isofurans using gas chromatography-mass spectrometry. *Free Radic Biol Med* 2013;59:36–44.
68 Forman HJ, Augusto O, Brigelius-Flohe R, *et al*. Even free radicals should follow some rules: a guide to free radical research terminology and methodology. *Free Radic Biol Med* 2015;78:233–5.
69 Ho E, Karimi Galougahi K, Liu CC, *et al*. Biological markers of oxidative stress: applications to cardiovascular research and practice. *Redox Biol* 2013;1:483–91.
70 Davies MJ, Fu S, Wang H, *et al*. Stable markers of oxidant damage to proteins and their application in the study of human disease. *Free Radic Biol Med* 1999;27:1151–63.
71 Alamdari DH, Kostidou E, Paletas K, *et al*. High sensitivity enzyme-linked immunosorbent assay (ELISA) method for measuring protein carbonyl in samples with low amounts of protein. *Free Radic Biol Med* 2005;39:1362–7.
72 Cheng KC, Cahill DS, Kasai H, *et al*. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G----T and A----C substitutions. *J Biol Chem* 1992;267:166–72.
73 Evans MD, Olinski R, Loft S, *et al*. Toward consensus in the analysis of urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine as a noninvasive biomarker of oxidative stress. *FASEB J* 2010;24:1249–60.
74 Cuajungco MP, Goldstein LE, Nunomura A, *et al*. Evidence that the beta-amyloid plaques of Alzheimer's disease represent the redox-silencing and entombment of abeta by zinc. *J Biol Chem* 2000;275:19439–42.
75 Du Y, Wooten MC, Gearing M, *et al*. Age-associated oxidative damage to the p62 promoter: implications for Alzheimer disease. *Free Radic Biol Med* 2009;46:492–501.
76 Mangialasche F, Polidori MC, Monastero R, *et al*. Biomarkers of oxidative and nitrosative damage in Alzheimer's disease and mild cognitive impairment. *Ageing Res Rev* 2009;8:285–305.
77 Butterfield DA, Reed TT, Perluigi M, *et al*. Elevated levels of 3-nitrotyrosine in brain from subjects with amnestic mild cognitive impairment: implications for the role of nitration in the progression of Alzheimer's disease. *Brain Res* 2007;1148:243–8.
78 Butterfield DA, Poon HF, St Clair D, *et al*. Redox proteomics identification of oxidatively modified hippocampal proteins in mild cognitive impairment: Insights into the development of Alzheimer's disease. *Neurobiol Dis* 2006;22:223–32.
79 Markesbery WR, Kryscio RJ, Lovell MA, *et al*. Lipid peroxidation is an early event in the brain in amnestic mild cognitive impairment. *Ann Neurol* 2005;58:730–5.
80 Schrag M, Mueller C, Zabel M, *et al*. Oxidative stress in blood in Alzheimer's disease and mild cognitive impairment: a meta-analysis. *Neurobiol Dis* 2013;59:100–10.
81 Cervellati C, Cremonini E, Bosi C, *et al*. Systemic oxidative stress in older patients with mild cognitive impairment or late onset Alzheimer's disease. *Curr Alzheimer Res* 2013;10:365–72.
82 Montine TJ, Quinn JF, Milatovic D, *et al*. Peripheral F2-isoprostanes and F4-neuroprostanes are not increased in Alzheimer's disease. *Ann Neurol* 2002;52:175–9.
83 Sinem F, Dildar K, Gökhan E, *et al*. The serum protein and lipid oxidation marker levels in Alzheimer's disease and effects of cholinesterase inhibitors and antipsychotic drugs therapy. *Curr Alzheimer Res* 2010;7:463–9.
84 Mufson EJ, Leurgans S. Inability of plasma and urine F2A-isoprostane levels to differentiate mild cognitive impairment from Alzheimer's disease. *Neurodegener Dis* 2010;7:139–42.
85 Bonda DJ, Wang X, Perry G, *et al*. Oxidative stress in Alzheimer disease: a possibility for prevention. *Neuropharmacology* 2010;59:290–4.
86 Smith MA, Richey Harris PL, Sayre LM, *et al*. Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J Neurosci* 1997;17:2653–7.
87 Praticò D. Evidence of oxidative stress in Alzheimer's disease brain and antioxidant therapy: lights and shadows. *Ann N Y Acad Sci* 2008;1147:70–8.
88 Cai Z, Zhao B, Ratka A. Oxidative stress and β-amyloid protein in Alzheimer's disease. *Neuromolecular Med* 2011;13:223–50.
89 Bading JR, Yamada S, Mackic JB, *et al*. Brain clearance of Alzheimer's amyloid-beta40 in the squirrel monkey: a SPECT study in a primate model of cerebral amyloid angiopathy. *J Drug Target* 2002;10:359–68.
90 Lehner C, Gehwolf R, Tempfer H, *et al*. Oxidative stress and blood-brain barrier dysfunction under particular consideration of matrix metalloproteinases. *Antioxid Redox Signal* 2011;15:1305–23.
91 Golden TR, Hinerfeld DA, Melov S. Oxidative stress and aging: beyond correlation. *Aging Cell* 2002;1:117–23.
92 Richardson JR, Roy A, Shalat SL, *et al*. Elevated serum pesticide levels and risk for Alzheimer disease. *JAMA Neurol* 2014;71:284–90.
93 Wiener HW, Perry RT, Chen Z, *et al*. A polymorphism in SOD2 is associated with development of Alzheimer's disease. *Genes Brain Behav* 2007;6:770–5.
94 Morris JK, Honea RA, Vidoni ED, *et al*. Is Alzheimer's disease a systemic disease? *Biochim Biophys Acta* 2014;1842:1340–9.
95 Kaminsky YG, Reddy VP, Ashraf GM, *et al*. Age-related defects in erythrocyte 2,3-diphosphoglycerate metabolism in dementia. *Aging Dis* 2013;4:244–55.
96 Dias-Santagata D, Fulga TA, Duttaroy A, *et al*. Oxidative stress mediates tau-induced neurodegeneration in Drosophila. *J Clin Invest* 2007;117:236–45.
97 Wilkinson BL, Landreth GE. The microglial NADPH oxidase complex as a source of oxidative stress in Alzheimer's disease. *J Neuroinflammation* 2006;3:30.
98 Abramov AY, Canevari L, Duchen MR. Beta-amyloid peptides induce mitochondrial dysfunction and oxidative stress in astrocytes and death of neurons through activation of NADPH oxidase. *J Neurosci* 2004;24:565–75.
99 Yan SD, Stern DM. Mitochondrial dysfunction and Alzheimer's disease: role of amyloid-beta peptide alcohol dehydrogenase (ABAD). *Int J Exp Pathol* 2005;86:161–71.
100 Mao P, Reddy PH. Aging and amyloid beta-induced oxidative DNA damage and mitochondrial dysfunction in Alzheimer's disease: implications for early intervention and therapeutics. *Biochim Biophys Acta* 2011;1812:1359–70.
101 Zhao Y, Zhao B. Oxidative stress and the pathogenesis of Alzheimer's disease. *Oxid Med Cell Longev* 2013;2013:316523.
102 Markesbery WR. Oxidative stress hypothesis in Alzheimer's disease. *Free Radic Biol Med* 1997;23:134–47.
103 Keller JN, Schmitt FA, Scheff SW, *et al*. Evidence of increased oxidative damage in subjects with mild cognitive impairment. *Neurology* 2005;64:1152–6.
104 Bermejo P, Martín-Aragón S, Benedí J, *et al*. Peripheral levels of glutathione and protein oxidation as markers in the development of Alzheimer's disease from Mild Cognitive Impairment. *Free Radic Res* 2008;42:162–70.
105 Calabrese V, Sultana R, Scapagnini G, *et al*. Nitrosative stress, cellular stress response, and thiol homeostasis in patients with Alzheimer's disease. *Antioxid Redox Signal* 2006;8:1975–86.
106 Aldred S, Bennett S, Mecocci P. Increased low-density lipoprotein oxidation, but not total plasma protein oxidation, in Alzheimer's disease. *Clin Biochem* 2010;43:267–71.

107 Baldeiras I, Santana I, Proença MT, *et al*. Peripheral oxidative damage in mild cognitive impairment and mild Alzheimer's disease. *J Alzheimers Dis* 2008;15:117–28.
108 Dorszewska J, Kempisty B, Jaroszewska-Kolecka J, *et al*. Expression and polymorphisms of gene 8-oxoguanine glycosylase 1 and the level of oxidative DNA damage in peripheral blood lymphocytes of patients with Alzheimer's disease. *DNA Cell Biol* 2009;28:579–88.
109 Mecocci P, Polidori MC, Cherubini A, *et al*. Lymphocyte oxidative DNA damage and plasma antioxidants in Alzheimer disease. *Arch Neurol* 2002;59:794–8.
110 Dezor M, Dorszewska J, Florczak J, *et al*. Expression of 8-oxoguanine DNA glycosylase 1 (OGG1) and the level of p53 and TNF-αlpha proteins in peripheral lymphocytes of patients with Alzheimer's disease. *Folia Neuropathol* 2011;49:123–31.
111 Abe T, Tohgi H, Isobe C, *et al*. Remarkable increase in the concentration of 8-hydroxyguanosine in cerebrospinal fluid from patients with Alzheimer's disease. *J Neurosci Res* 2002;70:447–50.
112 Mecocci P, MacGarvey U, Beal MF. Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Ann Neurol* 1994;36:747–51.
113 Wang J, Markesbery WR, Lovell MA. Increased oxidative damage in nuclear and mitochondrial DNA in mild cognitive impairment. *J Neurochem* 2006;96:825–32.
114 Yao Y, Zhukareva V, Sung S, *et al*. Enhanced brain levels of 8,12-iso-iPF2alpha-VI differentiate AD from frontotemporal dementia. *Neurology* 2003;61:475–8.
115 Praticò D, MY Lee V, Trojanowski JQ, *et al*. Increased F2-isoprostanes in Alzheimer's disease: evidence for enhanced lipid peroxidation in vivo. *FASEB J* 1998;12:1777–83.
116 Montine TJ, Peskind ER, Quinn JF, *et al*. Increased cerebrospinal fluid F2-isoprostanes are associated with aging and latent Alzheimer's disease as identified by biomarkers. *Neuromolecular Med* 2011;13:37–43.
117 Montine KS, Quinn JF, Zhang J, *et al*. Isoprostanes and related products of lipid peroxidation in neurodegenerative diseases. *Chem Phys Lipids* 2004;128:117–24.
118 Praticò D, Clark CM, Lee VM, *et al*. Increased 8,12-iso-iPF2alpha-VI in Alzheimer's disease: correlation of a noninvasive index of lipid peroxidation with disease severity. *Ann Neurol* 2000;48:809–12.
119 Roberts LJ, Montine TJ, Markesbery WR, *et al*. Formation of isoprostane-like compounds (neuroprostanes) in vivo from docosahexaenoic acid. *J Biol Chem* 1998;273:13605–12.
120 Aybek H, Ercan F, Aslan D, *et al*. Determination of malondialdehyde, reduced glutathione levels and APOE4 allele frequency in late-onset Alzheimer's disease in Denizli, Turkey. *Clin Biochem* 2007;40:172–6.
121 Puertas MC, Martínez-Martos JM, Cobo MP, *et al*. Plasma oxidative stress parameters in men and women with early stage Alzheimer type dementia. *Exp Gerontol* 2012;47:625–30.
122 Martín-Aragón S, Bermejo-Bescós P, Benedí J, *et al*. Metalloproteinase's activity and oxidative stress in mild cognitive impairment and Alzheimer's disease. *Neurochem Res* 2009;34:373–8.
123 Torres LL, Quaglio NB, de Souza GT, *et al*. Peripheral oxidative stress biomarkers in mild cognitive impairment and Alzheimer's disease. *J Alzheimers Dis* 2011;26:59–68.
124 Cervellati C, Romani A, Seripa D, *et al*. Oxidative balance, homocysteine, and uric acid levels in older patients with Late Onset Alzheimer's Disease or Vascular Dementia. *J Neurol Sci* 2014;337:156–61.
125 Serra JA, Domínguez RO, Marschoff ER, *et al*. Systemic oxidative stress associated with the neurological diseases of aging. *Neurochem Res* 2009;34:2122–32.
126 Lovell MA, Ehmann WD, Butler SM, *et al*. Elevated thiobarbituric acid-reactive substances and antioxidant enzyme activity in the brain in Alzheimer's disease. *Neurology* 1995;45:1594–601.
127 McGrath LT, McGleenon BM, Brennan S, *et al*. Increased oxidative stress in Alzheimer's disease as assessed with 4-hydroxynonenal but not malondialdehyde. *QJM* 2001;94:485–90.
128 Sultana R, Perluigi M, Allan Butterfield D. Lipid peroxidation triggers neurodegeneration: a redox proteomics view into the Alzheimer disease brain. *Free Radic Biol Med* 2013;62:157–69.
129 Butterfield DA, Reed T, Perluigi M, *et al*. Elevated protein-bound levels of the lipid peroxidation product, 4-hydroxy-2-nonenal, in brain from persons with mild cognitive impairment. *Neurosci Lett* 2006;397:170–3.
130 Markesbery WR, Lovell MA. Four-hydroxynonenal, a product of lipid peroxidation, is increased in the brain in Alzheimer's disease. *Neurobiol Aging* 1998;19:33–6.
131 Cervellati C, Romani A, Seripa D, *et al*. Systemic oxidative stress and conversion to dementia of elderly patients with mild cognitive impairment. *Biomed Res Int* 2014;2014:309507.
132 Montine TJ, Markesbery WR, Morrow JD, *et al*. Cerebrospinal fluid F2-isoprostane levels are increased in Alzheimer's disease. *Ann Neurol* 1998;44:410–13.
133 Bucossi S, Ventriglia M, Panetta V, *et al*. Copper in Alzheimer's disease: a meta-analysis of serum, plasma, and cerebrospinal fluid studies. *J Alzheimers Dis* 2011;24:175–85.
134 Squitti R, Lupoi D, Pasqualetti P, *et al*. Elevation of serum copper levels in Alzheimer's disease. *Neurology* 2002;59:1153–61.
135 Gilca M, Lixandru D, Gaman L, *et al*. Erythrocyte membrane stability to hydrogen peroxide is decreased in Alzheimer disease. *Alzheimer Dis Assoc Disord* 2014;28:358–63.
136 Zuliani G, Ranzini M, Guerra G, *et al*. Plasma cytokines profile in older subjects with late onset Alzheimer's disease or vascular dementia. *J Psychiatr Res* 2007;41:686–93.
137 Polidori MC, Mecocci P. Plasma susceptibility to free radical-induced antioxidant consumption and lipid peroxidation is increased in very old subjects with Alzheimer disease. *J Alzheimers Dis* 2002;4:517–22.
138 Sticozzi C, Cervellati F, Muresan XM, *et al*. Resveratrol prevents cigarette smoke-induced keratinocytes damage. *Food Funct* 2014;5:2348–56.
139 Li Y, Cao Z, Zhu H. Upregulation of endogenous antioxidants and phase 2 enzymes by the red wine polyphenol, resveratrol in cultured aortic smooth muscle cells leads to cytoprotection against oxidative and electrophilic stress. *Pharmacol Res* 2006;53:6–15.
140 Kosenko EA, Aliev G, Tikhonova LA, *et al*. Antioxidant status and energy state of erythrocytes in Alzheimer dementia: probing for markers. *CNS Neurol Disord Drug Targets* 2012;11:926–32.
141 Charlton-Menys V, Liu Y, Durrington PN. Semiautomated method for determination of serum paraoxonase activity using paraoxon as substrate. *Clin Chem* 2006;52:453–7.
142 Aviram M, Rosenblat M, Bisgaier CL, *et al*. Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase. *J Clin Invest* 1998;101:1581–90.
143 Shih DM, Xia YR, Wang XP, *et al*. Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. *J Biol Chem* 2000;275:17527–35.
144 Bhattacharyya T, Nicholls SJ, Topol EJ, *et al*. Relationship of paraoxonase 1 (PON1) gene polymorphisms and functional activity with systemic oxidative stress and cardiovascular risk. *JAMA* 2008;299:1265–76.
145 Bednarska-Makaruk ME, Krzywkowski T, Graban A, *et al*. Paraoxonase 1 (PON1) gene-108C>T and p.Q192R polymorphisms and arylesterase activity of the enzyme in patients with dementia. *Folia Neuropathol* 2013;51:111–19.
146 Pi Y, Zhang L, Chang K, *et al*. Lack of an association between Paraoxonase 1 gene polymorphisms (Q192R, L55M) and Alzheimer's disease: a meta-analysis. *Neurosci Lett* 2012;523:174–9.
147 La Du BN, Eckerson HW. The polymorphic paraoxonase/arylesterase isozymes of human serum. *Fed Proc* 1984;43:2338–41.
148 Wehr H, Bednarska-Makaruk M, Graban A, *et al*. Paraoxonase activity and dementia. *J Neurol Sci* 2009;283:107–8.
149 Zengi O, Karakas A, Ergun U, *et al*. Urinary 8-hydroxy-2′-deoxyguanosine level and plasma paraoxonase 1 activity with Alzheimer's disease. *Clin Chem Lab Med* 2012;50:529–34.
150 Cervellati C, Romani A, Bergamini CM, *et al*. PON-1 and ferroxidase activities in older patients with mild cognitive impairment, late onset Alzheimer's disease or vascular dementia. *Clin Chem Lab Med* 2015;53:1049–56.
151 Sultana R, Butterfield DA. Oxidatively modified, mitochondria-relevant brain proteins in subjects with Alzheimer disease and mild cognitive impairment. *J Bioenerg Biomembr* 2009;41:441–6.
152 Martinelli N, Girelli D, Olivieri O, *et al*. Novel serum paraoxonase activity assays are associated with coronary artery disease. *Clin Chem Lab Med* 2009;47:432–40.
153 Draganov D, Teiber J, Watson C, *et al*. PON1 and oxidative stress in human sepsis and an animal model of sepsis. *Adv Exp Med Biol* 2010;660:89–97.
154 Commenges D, Scotet V, Renaud S, *et al*. Intake of flavonoids and risk of dementia. *Eur J Epidemiol* 2000;16:357–63.
155 Luchsinger JA, Tang MX, Shea S, *et al*. Antioxidant vitamin intake and risk of Alzheimer disease. *Arch Neurol* 2003;60:203–8.

156 Engelhart MJ, Geerlings MI, Ruitenberg A, *et al*. Dietary intake of antioxidants and risk of Alzheimer disease. *JAMA* 2002;287:3223–9.
157 Wood PL, Khan MA, Kulow SR, *et al*. Neurotoxicity of reactive aldehydes: The concept of "aldehyde load" as demonstrated by neuroprotection with hydroxylamines. *Brain Res* 2006;1095:190–9.
158 Morris MC, Evans DA, Tangney CC, *et al*. Relation of the tocopherol forms to incident Alzheimer disease and to cognitive change. *Am J Clin Nutr* 2005;81:508–14.
159 Costa LG, Giordano G, Furlong CE. Pharmacological and dietary modulators of paraoxonase 1 (PON1) activity and expression: the hunt goes on. *Biochem Pharmacol* 2011;81:337–44.
160 Bocci V, Valacchi G. Nrf2 activation as target to implement therapeutic treatments. *Front Chem* 2015;3:4.
161 Zhu H, Itoh K, Yamamoto M, *et al*. Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: Protection against reactive oxygen and nitrogen species-induced cell injury. *FEBS Lett* 2005;579:3029–36.