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Review

Oxidative Modification of Brain Proteins in Alzheimer's Disease: Perspective on Future Studies Based on Results of Redox Proteomics Studies

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Abstract. Aging is the major risk factor associated with neurodegenerative diseases, including Alzheimer's disease (AD). Until now no clear understanding of the mechanisms of initiation and progression of this dementing disorder exists. Based on the studies that have been conducted so far amyloid β-peptide (Aβ), a protein found in senile plaques, one of the key pathological hallmarks of AD, has been reported to be critical in the pathogenesis of AD. Studies from our laboratory and others showed that Aβ can induce oxidative stress, which leads to oxidative modification of biomolecules, thereby diminishing the normal functions of neuronal cells and eventually leading to loss of neurons and AD. In this review paper, we summarize evidence of oxidative stress in brains of AD and mild cognitive impairment patients, as well as the results from redox proteomics studies. The investigations have provided insights into the downstream effects of oxidative modification of key brain proteins in the pathogenesis of AD. Based on these redox proteomics results, we suggest future areas of research that could be considered to better understand this devastating dementing disorder.

Keywords: Alzheimer's disease, lipid peroxidation, mild cognitive impairment, oxidative stress, protein carbonylation, protein nitration, redox proteomics

INTRODUCTION

We appreciate being invited to share perspectives on our prior publications of highly cited papers on Alzheimer's disease (AD), many of which have dealt with oxidative stress and redox proteomics analysis in AD and amnestic mild cognitive impairment (MCI), and on some future studies these papers suggest. Accordingly, this review paper summarizes evidence of oxidative stress in AD and MCI brain and results using redox proteomics, followed by our view of some future directions in AD research. Aging is the major risk factor associated with neurodegenerative diseases, including AD, which is histopathologically characterized by the presence of senile plaques (SP), neurofibrillary tangles

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(NFTs), and loss of synapses [1]. However, until now the exact mechanism(s) of AD progression or pathogenesis largely remain unknown. Studies involving familial AD suggested that the mutations of *presenilin-1*, *presenilin-2*, and *amyloid-β protein precursor (AβPP)* genes cause familial AD (FAD), and implicate amyloid β-peptide (Aβ) as the underlying cause for the onset of pathology, clinical presentation, and biochemical alterations in this devastating disease. In addition, mutation in other genes, such as *apolipoprotein E* allele 4 (*APOE 4*), *endothelial nitric oxide synthase-3*, *phosphatidylinositol-binding clathrin assembly protein* (*PICALM*), *clusterin* (*CLU*, also called *apolipoprotein J*], and α*2-macroglobulin,* have been suggested as risk factors for AD [2–5].

The main component of the SP is a 40–42 amino acid peptide, Aβ, generated by the proteolytic cleavage of AβPP by the action of β- and γ-secretases [6]. The Aβ-peptide exists in both soluble and insouluble forms, and has been shown to be toxic. The toxicity induced by Aβ has been associated with the single methionine (Met) residue present at 35 position in Aβ peptide [7, 8]. Met can undergo one-electron oxidation to form sulfuranyl or hydroxysulfuranyl radical cations, which can abstract allylic hydrogen atoms from phospholipid acyl chains, thereby initiating the lipid peroxidation via chain reaction processes and consequently in the generation of highly reactive products such as 4-hydroxy-2-*trans* nonenal (HNE) and acrolein [7, 9]. Since the plasma membrane and organelle membranes have both lipid and protein components, the generation of reactive products like HNE by lipid peroxidation makes the membrane proteins in the membrane highly susceptible to oxidative modification via Michael addition, which affects the protein structure and eventually impairs cellular function as reported in AD [10, 11] (Fig. 1). Studies from our laboratory and others showed that lack of Met leads to significantly diminished oxidative stress [12–14].

The importance of Aβ in inducing oxidative stress and being a key player in AD pathogenesis is supported by the studies involving Down syndrome, individuals characterized by a trisomy of chromosome 21. The extra copy of AβPP gene in these individuals leads to increased levels of Aβ, and correlated with increase oxidative stress and AD-like pathology if they live long enough [15, 16]. However, until now it is not clear what the first step is that leads to increasing load of Aβ. Individuals with AD haves elevated

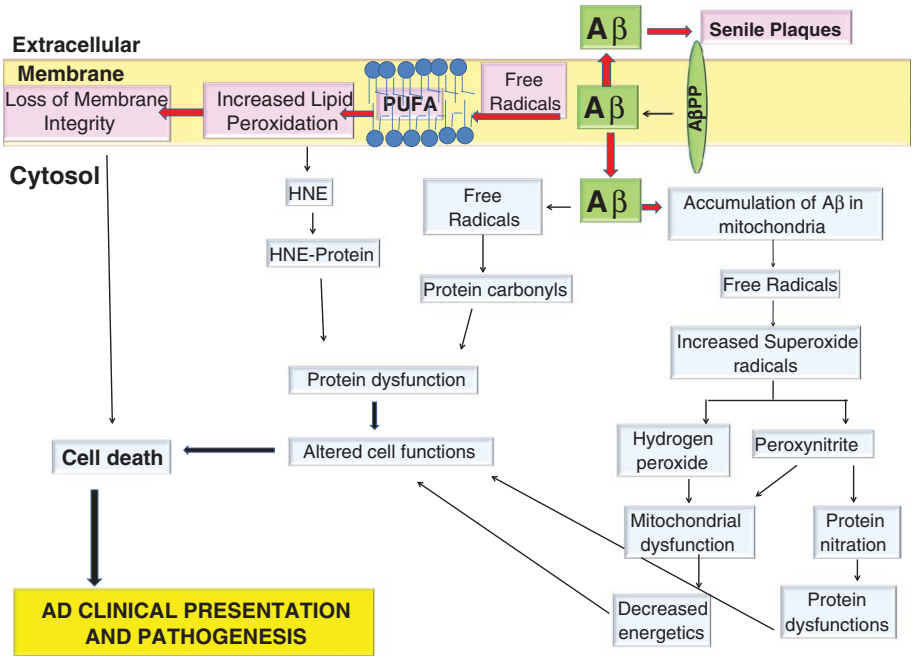

Fig. 1. Amyloid-β (Aβ) peptide generated by the proteolytic cleavage of amyloid-β protein precursor (AβPP) is important in inducing oxidative and nitrosative damage in AD and is key in the progression of this dementing disorder. Lipid peroxidation induced by bilayer-soluble oligomeric $A\beta_{1\text{-}42}$ and mitochondrial accumulation of this neurotoxic peptide lead to a cascade of events schematically depicted here. Other cellular changes are not indicated, but may include oxidation of nuclear and mitochondrial DNA.

levels of cerebral Aβ and increased markers of oxidative stress such as protein carbonylation, protein nitration, HNE, acrolein, 8-hydroxy guanosine, and advanced glycation end products. [17–28]. Aβ-induced oxidative stress is further supported by a number of *in vitro* and *in vivo* studies [29–33]. Further, elevated levels of oxidative stress have also been found in brains from subjects with MCI, arguably the earliest form of AD, and early AD [21, 26, 29, 34–36]. Individuals with preclinical AD (PCAD) have significant levels of Aβ deposition, but no significant increase in oxidative stress compared to age-matched control, which is consistent with the recent finding that small oligomers of Aβ are highly toxic in these individuals. Indeed, the oligomeric form of Aβ is of lower concentration in brains of PCAD subjects compared to AD and MCI [37, 38]. $A\beta_{1\text{-}42}$ has been shown to aggregate more quickly than $A\beta_{1\text{-}40}$ and is proposed to play a central role in AD pathogenesis. The evidence of $A\beta_{1\text{-}42}$ being involved in AD pathogenesis is largely derived from the observation that FAD cases have increased Aβ load and increased oxidative stress [39].

OXIDATIVELY-MODIFIED PROTEINS

Early approaches used to gain insight into the specific protein targets of oxidative modification involved immunoprecipitation techniques, in which a protein of interest was immunoprecipitated and then probed with the antibody of the type of modification that was to be tested. This technique is labor intensive, time consuming, and additionally requires the availability of the antibody of the protein that needed to be tested. To achieve the goal of identification of multiple oxidatively-modified brain proteins simultaneously, our laboratory pioneered a technique called redox proteomics [40–44]. One manifestation of redox proteomics couples two-dimensional gel electrophoresis (2D) with isoelectrofocusing (IEF) to separate the large number of brain proteins followed by protein transfer to a 2D Western blot on which proteins are probed for protein carbonyls, 3-NT, or bound HNE [41, 43, 45–47]. Sophisticated imaging analysis, coupled with trypsin digestion and use of software, identified spots of interest, and mass spectrometry allowed interrogation of protein databases that led to the identification of a large number of targets of oxidation.

PROTEIN OXIDATION: CARBONYLATION AND NITRATION

Beta-actin and creatine kinase BB have been identified as specifically oxidized proteins in AD brain using 2D electrophoresis and 2D Western blots [48]. These techniques form the basis of the methodology needed to further examine the role of oxidative modifications of specific brain proteins in AD pathogenesis and have led to the development and use of redox proteomic [44] techniques to identify carbonylated brain proteins in AD [41, 42, 49]. 2D gel electrophoresis coupled with mass spectrometry [44, 45] have allowed the discovery of increased carbonylation creatine kinase BB, glutamine synthase, ubiquitin carboxy-terminal hydrolase L-1 (UCH L-1), dihydropyrimidinase-related protein 2 (DRP-2), α-enolase and heat shock cognate 71 in AD inferior parietal lobule (IPL) compared to age-matched controls [41, 42, 50]. Subsequent studies of AD hippocampus demonstrate specific carbonylation of peptidyl prolyl cis-trans isomerase (Pin1), phosphoglycerate mutase 1, UCH L-1, DRP-2, carbonic anhydrase II, triose phosphate isomerase (TPI), α-enolase, and γ-SNAP compared to age-matched controls [45]. Consistent with the notion that oxidative modification of proteins leads to dysfunction of normal cellular processes in AD, the activities of Pin1, enolase, and carbonic anhydrase II were significantly lower in AD hippocampus compared to matched tissue samples from control subjects [45]. Alterations in enzymatic function in these systems could contribute to pathogenesis of AD through inhibition of cellular degradation machinery, alteration of protein conformation, decreased cerebrospinal fluid production, and impairment in cellular metabolic processes.

Others [51] using redox proteomics showed significant decreased protein carbonyls in malate dehydrogenase 1 (MDH), glutamate dehydrogenase, 14-3-3 protein zeta/delta, aldolases A and C, and increased oxidation of carbonic anhydrase 1. The sample processing in this study did not use detergents and may have led to identification of fewer oxidized proteins than that seen in other studies as a result of decreased exposure of protein carbonyls. More recent studies identified DJ-1 as a carbonylated protein in the frontal cotex of AD patients [52]. In the IPL of FAD subjects, increased carbonylation of UCH-L1, γ-enolase, actin, and dimethylarginine dimethylaminohydrolase 1 (DMDMAH-1) have been reported [35]. Others also reported oxidation and accumulation of proteins like UCH L1, ATP synthase, and Cu,Zn-superoxide

dismutase in AD brain [49, 53, 54], confirming our prior results.

Many of the proteins that are oxidatively modified in AD brain have roles in energy metabolism. This observation may contribute to the results of PET studies that demonstrate decreased glucose utilization in AD brain. The extent of oxidatively-modified brain proteins by proteomics correlates well with AD pathology, including both SP and NFT burden [41–43, 45]. Further, the identification of common targets of protein carbonylation between FAD, sporadic AD, and MCI is consistent with the idea that increased oxidative stress is invariate in respect to cause (genetic versus sporadic) or stage (amnestic MCI versus dementia) in the pathogenesis of AD [29].

Brains from subjects with MCI also demonstrates increased levels of protein carbonyls [26, 45, 55]. Redox proteomics studies in MCI hippocampus led to the identification of α-enolase, glutamine synthetase, pyruvate kinase M2, and Pin1 as specifically carbonylated proteins, recapitulating many of the findings seen in fulminate AD brain tissue [34]. Recent reports identified oxidatively modified carbonic anhydrase II, heat shock protein 70, mitogen activated protein kinase I, and syntaxin binding protein I in MCI indexed by elevated protein carbonyls [56].

The redox proteomic studies of brain from subjects with AD and MCI identified proteins such as enolase, Pin1, and glutamine synthetase as targets of carbonylation common to both AD and MCI. The functions of these proteins are important not only in regulating energy metabolism, but have also been linked to tau hyperphosphorylation, alterations in protein conformation, AβPP processing, and glutamate regulation, all of which are thought to be relevant to neurodegenerative processes in AD [56]. Of interest, not all brain proteins that appear to be targets of protein carbonylation at an early stage (MCI) appear in advanced stage (AD). This suggests that the specific targets of oxidative stress vary with stage of disease and represent a specific rather than non-selective injurious process. Such findings may have implications for the use of specific disease modifying treatments in relation to stage of disease. It is possible that oxidatively induced alterations in specific cellular pathways contribute specifically to the disease process early on, altering transcription and translational mechanisms that may further damage neurons. Such transcriptional and translational alterations can further reduce the specific substrates for carbonylation, exacerbating the loss of function caused by oxidation early in the disease process. As neuronal injury ensues, the effects of oxidative

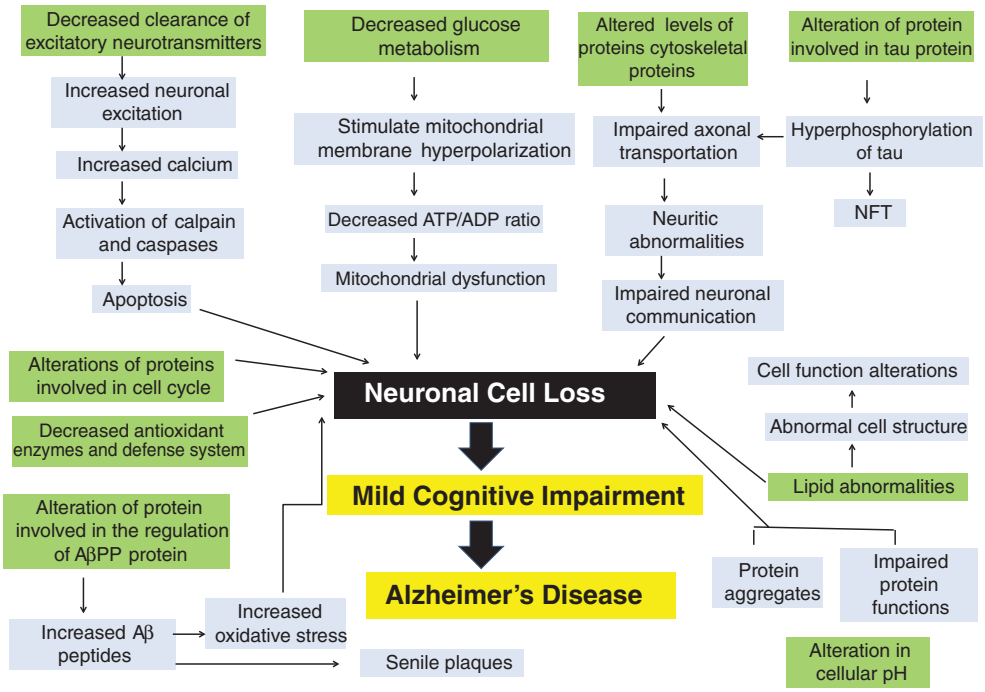

Fig. 2. The oxidative modification of proteins affects a number of pathways in AD and amnestic MCI, arguably the earliest form of AD. The fact that the same pathways are affected in MCI as in AD suggest that alterations in these pathways could be involved in the progression of amnestic MCI to AD. Depicted in this figure are some of these pathways implicated by oxidatively-modified proteins identified by redox proteomics.

damage become more widespread and non-selective as part of the end-stage process of AD.

Figure 2 presents potential pathways affected by protein oxidation in AD and amnestic MCI revealed by redox proteomics. Overlap of pathways in these two conditions involved in energy metabolism, neurotransmitter function, neuritic abnormalities, cell cycle, tau phosphorylation, Aβ production, pH regulation, and antioxidant system are consistent with the notion that these pathways could be involved in the progression of amnestic MCI (with memory loss) to AD (with dementia).

PROTEIN NITRATION IN AD AND MCI

Redox proteomics studies have identified a large number of proteins that have specifically nitrated Tyr residues in AD hippocampus and IPL compared to control brain, including α- and γ-enolase, lactate dehydrogenase, neuropolypeptide h3, TPI, and α-actin in AD IPL [43], and α-enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ATP synthase α-chain, carbonic anhydrase-II, and voltage-dependent anion channel protein in AD hippocampus [19]. These nitrated proteins are involved in various cellular functions such as energy metabolism, structural maintenance, pH regulation, and mitochondrial function. Oxidative modification (i.e., nitration, carbonylation, etc.) may alter protein functionality [19]. Our redox proteomics finding of excess nitration of TPI was recently confirmed by Guix et al. [57] in hippocampus and frontal cortex of AD subjects, suggesting a link among decreased glucose metabolism via an impaired glycolytic pathway, nitrosylation of TPI, and the formation of Aβ and paired helical filaments. However, it is not clear why, in spite of oxidative modification, its activity remains unchanged in AD brain. In contrast, Reyes et al. demonstrated nitration of Tyr 18 followed by Tyr 29 of tau, which is mostly associated with or in close proximity to amyloid plaques [58]. Hence, nitration of proteins may reflect underlying posttranslational modification of proteins in AD.

Consistent with this notion, increased levels of 3-NT in MCI hippocampus and IPL using immunochemistry were reported [59]. There is also evidence for AD-specific nitration of MDH, α-enolase, glucose regulated protein precursor, aldolase, glutathione-S-transferase Mu, multidrug resistant protein-3, and 14-3-3 protein γ in MCI IPL [46]. In MCI hippocampus, α-enolase, MDH, peroxiredoxin 6 (PR VI), DRP-2, fascin 1, and heat shock protein A8 were identified as specifically nitrated compared to age-matched controls [46]. These redox proteomics-identified nitrated proteins in MCI are involved in the regulation of a number of important cellular functions including: energy metabolism, cellular signaling, antioxidant, and detoxification, in addition to regulating structural functions of brain cells. The identification of some of the brain protein targets of nitration in common between amnestic MCI and AD is consistent with the notion that these brain proteins might contribute to the progression of and increased synapse and functional loss in AD [19, 43, 46]. We showed increased nitration of p53 protein in MCI IPL compared to age-matched control and suggested that the oxidation of p53 may be involved in neuronal loss [60].

A recent study by Reiderer et al. [61] reported S-nitrosyl-cysteine modification of DRP-2, α-internexin, glutamate dehydrogenase 1, α-enolase, GFAP, MDH, ProSAAS precursor protein, proopiomelanocortin, proenkephalin, and septin in the entorhinal cortex of AD, and suggested that Aβ activation of glial cells surrounding the SP might have led to increased nitrosylation of GFAP contributing to the pathogenesis of AD. Protein disulfide isomerase, an enzyme that catalyzes thiol–disulphide exchange, has been reported to be S-nitrosylated in AD brain [62]. Increased nitrosylation and decreased activity of this protein in AD may lead to alteration in its ability to facilitate disulfide bond formation and rearrangement reactions, increased accumulation of polyubiquitinated proteins, and activation of the endoplamic reticulum-resident unfolded protein response. Recently Cho and colleagues [63] reported increased levels of S-nitrosylation of dynamin-related protein 1 in brains of subjects with AD and suggested that S-nitrosylation of this protein may trigger mitochondrial fission, consequently adding to known mitochondrial damage in AD, which could contribute to synapse loss and neuronal damage in this disorder.

Taken together, these studies suggest that oxidation of proteins is an integral part of the progression and pathophysiology of AD [64]. The appearance of common targets of oxidation of proteins between MCI and AD implies their important roles in loss of cellular energetics, alterations in neurotransmission and cell signaling pathways, as well as SP and NFT formation (Table 2).

Enolase, an oxidatively-modified protein in AD and MCI brain, is important for regulating glucose metabolism. However, a number of recent studies showed that enolase also plays important roles in cell

signaling, Aβ clearance, and activation of cell survival pathways [65]. This result demonstrated that oxidative dysfunction of one protein may alter several cellular pathways implicated in the pathogenesis of AD. This point is further illustrated by GAPDH, which is also selectively oxidized in AD [19]. GAPDH is a key enzyme in the glycolytic pathway; however, recent studies suggest that it may also play key roles in transcription regulation, cell signaling, and vesicular transportation [66, 67], in addition to binding to other small molecules such as nitric oxide, glutathione, and tumor necrosis factor-α [12, 68, 69]. GAPDH also interacts with AβPP [70]. Hence, oxidative dysfunction of enolase and GAPDH can lead to multiple changes consistent with pathology, biochemistry, and clinical presentations of AD and MCI. Modulation of the cellular pathways altered by the selective oxidation of both GADPH and enolase could prove to be fertile ground for the development of novel therapeutic agents for AD [12, 65].

Another protein that exemplifies how oxidative modification of one protein can significantly affect function of AD-relevant pathways and be an important therapeutic target is Pin1. By its isomerization of proline on the carboxyl side of phosphorylated serine or threonine residues of target proteins, the regulatory protein Pin1 has been shown to play an important role in tau phosphorylation/dephosphorylation, AβPP regulation and processing, and synapse loss [71–73]. Hence, oxidatively dysfunctional Pin1 conceivably could be related to the major pathologies of AD: SP, NFT, and synapse loss [45]. Consequently, targeting Pin1 to treat AD might be a promising approach to treat or delay the onset of AD pathogenesis.

In addition, antioxidant enzymes were also found to be oxidized in common between AD and MCI brain, and loss of their function would have severe effects on cell survival [74]. As discussed further below, redox proteomic discoveries suggest several possible therapeutic strategies that may modulate AD progression and pathogenesis: 1) upregulate the endogenous levels of key oxidatively-modified proteins; 2) restore function in key oxidatively-modified proteins; or 3) augment cellular antioxidant systems.

HNE ADDUCTED BRAIN PROTEINS IN AD AND MCI

Proteomics studies identified regionally specific HNE modification of proteins, i.e., ATP synthase, GS, MnSOD, and DRP-2 in AD hippocampus and α-enolase, aconitase, aldolase, peroxiredoxin 6, and α-tubulin in AD cortex [75]. Some of these proteins were previously found to be either nitrated or carbonylated in AD [19, 33, 41–43, 45]. The appearance of different oxidative indices in target proteins modified by both protein carbonyls and 3-NT supports the role of oxidative stress in AD and is consistent with the notion that these specific proteins are highly vulnerable to oxidative modification and may be involved in AD.

In MCI hippocampus and cortex, increased levels of protein-bound HNE in neuropolypeptide h3, carbonyl reductase (NADPH), α-enolase, lactate dehydrogenase B, phosphoglycerate kinase, heat shock protein 70, ATP synthase α chain, pyruvate kinase, actin, elongation factor Tu, and translation initiation factor α were identified by proteomics [47]. Since most of the proteins that undergo HNE modification are dysfunctional, these proteomic results in amnestic MCI suggest that these HNE-bound proteins may be key players in the development of AD. Overlap of pathways involved in energy metabolism, neuritic abnormalities, cell cycle, tau phosphorylation, Aβ production, transcription and translation, mitochondrial abnormalities, and antioxidant system is consistent with the notion that these pathways could be involved in the progression of amnestic MCI to AD.

Increased lipid peroxidation in AD and MCI brain and a role for Aβ in this process were further supported by studies that showed loss of phospholipid asymmetry in AD and MCI brain, changes that are associated with apoptosis [76]. Noting that the high reactivity of free radicals requires that the initiator of lipid peroxidation must reside in the lipids, the findings above suggest that, in AD and MCI brain, oligomeric and hydrophobic Aβ inserts into the membrane of brain cells to cause lipid peroxidation and that such changes are an early event in the pathogenesis and progression of AD.

In conclusion, redox proteomics studies provided insights into key molecular pathways that, when oxidatively dysfunctional, play a significant role in the pathophysiology of AD [29]. Development of reliable and unique biomarkers to detect and diagnose MCI (or even earlier preclinical AD) and monitor drug efficacy in these disorders prior to cognitive decline will be of great importance for our rapidly aging population and may greatly accelerate the development of therapeutics and preventative approaches. In the future, redox proteomics of easily accessible bodily fluids or peripheral tissue in combination of other techniques, such as PET, MRI, and cognitive testing, may serve

as diagnostic tools to aid in monitoring the state of AD and therapeutic efficacy, and potentially provide a unique biomarker signature for AD. Other future studies in AD and MCI suggested by our redox proteomics results are therapeutic targeting (either pharmacologically or genetically) of key brain proteins that directly influence multiple AD-relevant pathways.

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