Novel Biomarkers of Alzheimer’s Disease: Based Upon N-methyl-D-aspartate Receptor Hypoactivation and Oxidative Stress

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Early detection and prevention of Alzheimer’s disease (AD) is important. The current treatment for early AD is acetylcholine esterase inhibitors (AChEIs); however, the efficacy is poor. Besides, AChEI did not show efficacy in mild cognitive impairment (MCI). Beta-amyloid (Aβ) deposits have been regarded to be highly related to the pathogenesis of AD. However, many clinical trials aiming at the clearance of Aβ deposits failed to improve the cognitive decline of AD, even at its early phase. There should be other important mechanisms unproven in the course of AD and MCI. Feasible biomarkers for the diagnosis and treatment response of AD are lacking to date. The N-methyl-D-aspartate receptor (NMDAR) activation plays an important role in learning and memory. On the other hand, oxidative stress has been regarded to contribute to aging with the assumption that free radicals damage cell constituents and connective tissues. Our recent study found that an NMDAR enhancer, sodium benzoate (the pivotal inhibitor of D-amino acid oxidase [DAAO]), improved the cognitive and global function of patients with early-phase AD. Further, we found that peripheral DAAO levels were higher in patients with MCI and AD than healthy controls. We also found that sodium benzoate was able to change the activity of antioxidant. These pieces of evidence suggest that the NMDAR function is associated with anti-oxidation, and have potential to be biomarkers for the diagnosis and treatment response of AD.

KEY WORDS: Alzheimer disease; Receptors, N-methyl-D-aspartate; Oxidative stress.

INTRODUCTION OF ALZHEIMER’S DISEASE (AD)

As advancement of technology and elevating medical standard, mankind can live longer life than our ancients. Accompanied with longevity, we face aging among all of the organs, including brain. AD, the most common type of dementia, is a disease highly correlated with aging which featured as insidious memory impairment as well as executive dysfunction [1]. While disease progressed gradually, neuropsychiatric symptoms such as delusions, hallucinations and aggression become more common. These symptoms lead to impaired daily function, decreased quality of life and higher economic burden [2]. According to G8 statement in 2013, dementia population worldwide in 2015 was around 47.47 million, reaching 75.63 million in 2030 and 135.46 million in 2050. In 2015, it cost over $ 600 billion per year for 35 million patients with dementia, equivalent to one percent of global Gross Domestic Product [3]. Caregivers of Alzheimer’s and other dementias patient provided unpaid assistance which was equivalent to eight times of McDonald’s total annual income in 2014. Huge care burden have adverse effect not only on economic aspects, but also emotional well-being and physical health among caregivers [4]. There are sev-
eral advantages of early detection, including leading to attempts at therapy (both pharmacological and non-pharmacological therapies), early access to appropriate agencies or support networks and may slow the disease progressive course [5,6]. Moreover, based on early detection, primary and secondary prevention strategies could be developed before irreversible neuronal dysfunction and loss occur. Postponing the onset of AD for a few years would have a huge impact on public health [7]. It is important to identify AD and provide effective treatment as early as possible, to improve patients’ wellbeing and relieve disease-related adverse loading. Therefore, searching for feasible biomarkers of AD is crucial.

In the past decades, accumulation of abnormally folded beta-amyloid (Aβ) and tau protein tangles are classical etiology of AD. As growing clinical and pathological studies being published, more and more evidences indicated that AD is a complex and multifactorial neurodegenerative disease [1]. Mutations at genes such as APP, PSEN1, or PSEN2 from familial studies provided the strongest evidence of Aβ disease [1]. Mutations at genes such as APP, PSEN1, or AD is a complex and multifactorial neurodegenerative being published, more and more evidences indicated that

Evidence of alterations in the expression of diabetes-related genes, insulin depletion, impaired insulin signaling, and mitochondrial dysfunction were found in AD brains [17].

Currently, there are three cerebrospinal fluid (CSF) biomarkers with potential in identifying prodromal AD in the mild cognitive impairment (MCI) stage, including Aβ42, total tau (T-tau) and phosphorylated tau (P-tau) [18,19]. Reduced Aβ42 levels, reflecting brain amyloidosis, are characteristic of AD as well as prodromal AD [20]. Besides, the CSF Aβ42/Aβ40 ratio is better to identify AD than CSF Aβ42 alone [21,22]. CSF T-tau is a marker that can reflect the severity of acute brain damage and intensity of neurodegeneration [23]. As a potential biomarker for neurodegenerative diseases, CSF T-tau level is 10–20 times higher in Creutzfeldt–Jakob disease than in AD [24]. When both levels are increased in CSF, T-tau, and P-tau may indicate a more rapid disease progression [25]. However, to date, these biomarkers still have disadvantages including high cost, invasiveness assays, and lack of standardized cutoffs among different laboratories [1,26]. Other than the three aforementioned core CSF biomarkers, some novel synaptic biomarkers such as neurogranin, synaptosomal-associated protein 25, syntaxin1, and vesicle-associated membrane protein have been also under investigation [20]. In addition to CSF, various methods for detecting such biomarkers and their specific usage in clinical and research fields are under rapid development, including in vivo brain imaging with positron emission tomography and magnetic resonance imaging, biotechniques skin and blood cells [27]. To be less invasive, blood samples to acquire biomarkers is easier to collect than CSF. In many studies, highly heterogeneous blood profiles such as proteins, lipids and metabolites were used to differentiated AD patients from healthy individuals [28].
Nevertheless, these data are often overlapping between patients and controls, difficult to analyze and hardly to be replicated in different studies [29,30]. A recent systematic review and meta-analysis study about neurodegenerative CSF and blood biomarkers suggested that among blood biomarkers, only plasma T-tau had a large effect size to differentiate between AD patients and controls though its \( p \) value was only 0.02 [31].

**IMPORTANCE OF TREATING AD**

Current treatment of AD can be grossly divided into two categories, pharmacological and non-pharmacological. Among pharmacological treatment, cholinesterase inhibitors (ChEI) including donepezil, galantamine, and rivastigmine as well as N-methyl-D-aspartate receptor (NMDAR) antagonist, memantine are four Food and Drug Administration (FDA) approved medication for AD. ACh plays a key role in mediating memory and learning [32]. ChEI are now widely used in patients with mild to moderate AD, however, their effect is only modest [33]. Statistically significances were noted but with borderline clinically improvement while assessing global assessment of dementia and cognition function [34]. According to some comparative trials, hardly could they find consistently significant efficacy differences between the three ChEIs, the main differences were frequency and type of adverse events [35].

In a recent meta-analysis and meta-regression study, ChEI had a dissatisfied risk-benefit relationship and a higher than placebo all-cause discontinuation [36]. Memantine, though frequently prescribed in moderate to severe dementia patient, according to most recent Cochrane review in 2019, it has small clinical benefit versus placebo and no benefit in mild AD [37].

Other than FDA approved medical treatment, several putative therapies are under study. However, there is no inspiring result among hormone replace therapy (HRT), folate, vitamin B12, Ginkgo biloba and statins. No convincible evident suggested these putative therapies had benefit in cognition function in people with dementia and HRT is even harmful [38].

There is no new effective treatment of AD developed in recent decades. In recent years, several huge late-phase clinical trials failed to find positive clinical outcome. In 2013, bapineuzumab, a monoclonal antibody specific to the N-terminus of the A\( \beta \) was lack of efficacy in treating mild to moderate AD [39]. In 2016, solanezumab, another monoclonal antibody designed to increase the clearance from the brain of soluble A\( \beta \) didn’t significantly affect cognitive decline in mild AD patients [40]. Despite these disappointing results, a different approach by verubecestat, a \( \beta \)-site amyloid precursor protein-cleaving enzyme 1 inhibitor, can reduce levels of A\( \beta 40 \), A\( \beta 42 \), and sAPP\( \beta \) in plasma and CSF after administration to rats and monkeys [41]. However, further study in humans not only failed to reduce cognitive or functional decline in patients with mild-to-moderate AD but also brought treatment-related adverse events [42]. Moreover, in prodromal AD, patients under verubecestat even had worse cognition and daily function than control individuals in some measures [43]. In early 2019, a clinical trial of crenezumab (a immunoglobulin isotype G4 monoclonal antibody designed under the hypothesis of this antibody with reduced effector function would have a lower risk of inducing amyloid-related imaging abnormalities indicative of vasogenic edema or effusions and microhemorrhage and siderosis) in prodromal to mild AD patients was terminated under the recommendation of the Independent Data Monitoring Committee [44,45]. The committee suggested that crenezumab was unlikely to meet the primary endpoint of change from baseline in Clinical Dementia Rating-Sum of Boxes [45]. Besides, another trial of antibody that targets oligomers and fibrils of A\( \beta \), aducanumab, once the most promising candidate, was terminated in March, 2019, owing to unlikely to meet their primary endpoint upon completion [46]. Though determination of these late phase clinical trials is quite frustrated, hundreds of clinical trials are still undergoing. These trials focusing on domains including not only A\( \beta \) but also synaptic plasticity, metabolism and bioenergetics, vasculature, hormones, inflammation and oxidative stress [47]. As Selkoe [48] said, "we obviously have no choice but to redouble our efforts to understand more deeply all facets of the biological process of AD and to identify more effective therapeutic agents as quickly as possible."

Several articles pointed out that brain stimulation techniques are developing as promising tools for neurodegenerative diseases. Non-invasive brain stimulation techniques provide a reliable method to improve cognitive decline in healthy older adults and AD patients [49]. On the other hand, invasive brain stimulation such as...
deep brain stimulation and vagus nerve stimulation yielded inconsistent results in AD patients [50,51]. Generally, brain stimulation techniques may shed light on novel treatment of improving specific cognitive function and memory; however, due to no standard guidelines and protocols, it is still an immature field [51].

Some rehabilitation approaches including music and exercise therapies may benefit cognitive, emotional and behavioral symptoms in AD patient. Due to relative small data, more researches are needed to be done to confirm the effectiveness and impact to the disease [52,53].

**GLUTAMATE THEORY, FOCUSING ON NMDAR**

In the past decades, researches have munificent evidence suggested that beta-amyloid (Aβ) and tau protein abnormally accumulation in amyloid plaques and intracellular neurofibrillary tangles respectively are hallmark pathologies of AD [54]. However, therapies aiming at the clearance of Aβ failed to date [39,40]. As growing studies indicated different hypothesis and possible pathophysiology of dementia, we now believe that dementia is a multifactorial disease with complex network [55,56].

Glutamate is a critical excitatory neurotransmitter in mammalian CNS. It regulates neurogenesis, neurite outgrowth, synaptogenesis, and neuron survival. Also, glutamate interacts with neurotrophic factors to modulate neuroplasticity [57]. Glutamine—glutamate cycle is also related to several psychiatric diseases such as major depressive disorder (MDD), schizophrenia, and AD. A recent study in elderly individuals with MDD showed that the ratio of glutamine to glutamate was significantly higher at baseline than in controls. Moreover, the ratio decreased over the 3-year follow-up, and the reduction was correlated with a decrease in the severity of depression [58]. A newly published nationwide longitudinal study in Taiwan also indicated that late-onset (age > 65 years) treatment-resistant depression was associated with an elevated risk of AD [59].

At most excitatory synapses, NMDAR, an ion-channel receptor, could be found and responds to the neurotransmitter glutamate [60]. It is an essential neurotransmitter receptor not only as an excitatory neurotransmission but also to the intricacy of memory and learning [61]. Besides, it owns special properties and plays an important role in synaptic plasticity. Impairment of NMDAR is associated with multiple pathologies of neuropsychiatric disease, for instance, schizophrenia and AD [62].

**NMDAR Hypoactivation and AD**

Under physiological condition, NMDAR is blocked by magnesium (Mg) ion. Upon glutamate binding to NMDAR, the Mg block ends and the receptor activates, calcium and sodium flow into cells via NMDA channels. Several mechanisms exist to protect this route to prevent hyperactivation; however, during some acute brain damages (e.g., ischemia, hypoxia) or chronic brain pathology (e.g., AD), NMDAR overacts, leading to excitotoxicity and cause neural death [63].

On the contrary, NMDAR hypoactivation also generates neurological disorders. Newcomer et al. [64] hypothesized that in AD patients, NMDAR follows a two-stage process leading to cognitive disturbances. In the first stage, Aβ interacts with NMDAR causing hyperactivation and disinhibition processes to several excitatory pathways which terminate in posterior cingulated and retrosplenial cortical regions and further result in NMDAR hypoactivation status. Then, glutamatergic activation is depressed and synaptic components are downregulated [65]. This hypothesis was supported by hippocampal dynamic model which shows hippocampal hyperactivation occurring before hypoactivation during MCI and both lead to cognitive impairment [66,67]. Although hypofunction of NMDAR appears in normal brain aging, in AD patients, this process interacts with other pathogenesis (e.g., amyloidopathy and oxidative stress) and increase NMDAR hypofunction loading [64]. Human study revealed significant decreased NMDAR1 messenger RNA levels in Alzheimer brains, comparing to age-controlled individuals, suggesting certain change is specific for the disease itself [68]. A study focusing on NMDAR synaptic plasticity among AD rat model found that blocking NMDAR in rats’ hippocampi resulted in electrophysiological and behavioral changes, thereby altering long-term potentiation and long-term depression. These researches pointed a possible way to treat AD from the aspect of receptor function rather than neurotransmitter or nerve cells apoptosis [69].

**Oxidative Stress and AD**

Since 20th century, oxidative stress has been regarded as a crucial factor in differentiation and aging. The level of
oxidative stress is linked to the rate of aging and altered gene expression during the process [70]. Though there is doubt in regarding oxidative stress as a possible etiology causes AD and some researchers take it as a physiologically phenomenon, increasing biomarkers were found correlated to AD or MCI recently [71,72]. Two species, reactive nitrogen species and reactive oxygen species (ROS) can modify biological properties of the cell membrane and eventually generate new oxidized products that can be measured in peripheral fluids as an oxidative stress index [71].

Mitochondria are the energy source of most cellular reaction by undergoing aerobic metabolism in the brain. Mitochondrial dysfunction, leading to overproduction of ROS, is related to sporadic, late-onset AD [73]. Besides, early mitochondrial dysfunction was found in not only animal but also human AD individuals [74-78]. Imbalanced ROS production and antioxidant defense alter cellular interaction at early aging process, prior to detectable clinical symptoms and even Aβ pathology [79].

Brain tissue is abundant of polyunsaturated fatty acids which may interact with ROS [80]. Elevated unstable lipid hydroperoxides products such as β lipid hydroperoxides products such as malondialdehyde and 4-hydroxynonenal were detected in patients with AD and MCI [81-83]. Various animal experiments showed that lipid peroxidation exceeded Aβ pathology [84]. The possible mechanism may be associated with triggering hydroxynonenal or iron (Fe)-activated γ-secretase activity [85].

Other than mitochondrial dysfunction and lipid peroxidation, several bioactive metals like zinc (Zn), Mg, Fe, copper (Cu), aluminum, and manganese were also related to oxidative stress influencing Aβ and Tau aggregation [86,87]. In AD patients, abnormal levels of Zn, Cu, and Fe were observed within severe histopathological changes in amygdala, hippocampus, and other brain regions [88]. By different techniques, elevated Fe, Cu, and Zn levels were found in mouse models of AD and AD patients’ amyloid plaques brain tissue [89-92]. On the other hand, Fe interacts with homocysteine, a kind of non-protein amino acid that elevates in AD patients’ plasma and serum, resulting in increased protein carbonylation and leading to oxidative damage [84].

Interaction between NMDAR Hypoactivation and Oxidative Stress

Various studies have revealed relationship between NMDAR hypoactivation and oxidative stress. In normal physiological condition, NMDARs have redox sites [93]. Synaptic NMDAR function under neuronal antioxidant defense mechanisms is activated [94]. There are different redox state subunits on NMDAR, for instance, glutamate ionotropic NMDAR type subunit 1 (GRIN1) and GRIN2A. Especially among GRIN2A-containing NMDARs, the redox regulation is powerful. By reducing agents such as antioxidant glutathione (GSH) or dithiothreitol, a region of the N-terminus is sufficient to mediate the potentiation of currents [95,96]. Temporal GSH deficits are able to induce NMDAR hypofunction [97]. Importantly, the relationship between NMDAR hypofunction, oxidative stress, and GSH deficits is reciprocal [98,99]. Besides, GRIN1 was found with increment of ROS levels and down-regulation of the regulator in mitochondrial energy metabolism and antioxidant defenses [100] (Fig. 1). Other than these oxidative-sensitive site subunits, functional down regulation of NMDAR is also related to calcium ion/calmodulin-dependent protein kinase II (CaMKII), which is responsible for NMDAR redox sensitivity and trafficking of glutamate receptors at the synapse [101]. Age-related intracellular redox state difference, which is linked to NMDAR activity, can be regulated by CaMKII and rescued by intracellular GSH [102]. In MCI and AD hippocampi, active CaMKII immunoreactivity redistribution correlates with cognitive assessment scores [103].

Parvalbumin interneurons (PVI) are GABAergic neurons that form inhibitory synapses to pyramidal neurons [104]. Studies from Jiang et al. [100] indicate that redox dysregulation impairs maturation of PVI induced by NMDAR hypofunction. Some studies in mice suggest that NMDAR hypofunction may weaken antioxidant defenses, further yielding a redox imbalance and altering cell maturation [100,105,106]. In developing mouse model, relatively mild transient NMDAR hypofunction may hamper PVI function permanently [106,107]. The underlying mechanism eventually generate H2O2 [107]. Both NMDAR hypofunction and oxidative stress contribute to selective PVI functional disturbance as well as cognitive and behavioral impairment [108]. Combination of cellular-level and circuit effects of NMDAR hypofunction can exacerbate oxidative stress [108].
NMDAR Enhancer and Its Role in AD

Based upon the aforementioned studies, it is evident that NMDARs play important roles not only in synaptic plasticity, learning, memory, cognition, but also in the aging brain. Some mood and behavior symptoms of AD are similar to negative symptoms of schizophrenia, for instance, social withdrawal and apathy. In schizophrenia patients, the positive symptoms such as delusion, hallucination, disorganized speech and disturbing behavior, resemble some clinical manifestations in middle-stage AD. Previous studies showed that improvement of behavior and memory symptoms could be observed with administration of enhancers of NMDA neurotransmission [109,110]. However, there are controversial findings about whether D-cycloserine (a partial agonist of the NMDAR-glycine site) could improve dementia patients’ cognitive function [111-114]. Compared to D-cycloserine, D-serine is a more potent NMDAR co-agonist. Several clinical trials suggest that, with large amount, D-serine and D-cycloserine as an adjunctive therapy in schizophrenia patients may be able to improve positive, negative, or cognitive symptoms [115, 116]. There are abundant researches exploring the relationship between AD and D-serine level in serum or CSF. The results were controversial. Earlier research with smaller sample size showed that serum levels of D-serine in AD patients were slightly lower than those of normal controls [117]. In more recent studies, elevated D-serine levels were found in post-mortem AD brains as well as CSF of probable AD patients, however, the results failed to be confirmed in other studies [118,119]. Different from previous studies which recruited medicated AD patients, a newly published cohort study which enrolled whole clinical spectrum of drug-free AD patients with bigger sample size revealed indistinguishable CSF and serum D-serine levels and D-serine/total serine ratio compared to controls [120].

D-amino acid oxidase (DAAO), an enzyme, found in mammals’ brain, kidney, and liver, is responsible for degrading D-serine and other D-amino acids [121,122]. Sodium benzoate, a DAAO inhibitor, can increase synaptic concentrations of D-serine and then enhance NMDAR neurotransmission [123]. A 6-week, randomized, double-blind, placebo-controlled clinical trial revealed that sodium benzoate (1 g/day) as an adjunctive therapy in chronic schizophrenia patients significantly improved not only in positive and negative symptoms, but also cogni-
tive functions, such as processing speed and visual memory [110]. Furthermore, a 24-week randomized, double-blind, placebo-controlled trial of sodium benzoate substantially improved cognitive and overall functions in early-phase AD. Besides, these patients tolerated sodium benzoate 250–1,500 mg/day well without obvious side effects [124].

**SUMMARY**

As aging society is coming, growing numbers of older adults suffer from AD. Gradually declining memory and cognitive function lead to significant impairment in their daily life and result in huge burden upon caregivers as well as national finance. The efficacy of current FDA approved four medications including donepezil, galantamine, rivastigmine, and memantine for AD is not satisfied. Therefore, new effective treatment is urgently needed. AD, as an age-related progressively neurodegenerative disease displays complex pathogenesis leading to abnormal accumulation of Aβ and tau protein; however, numerous clinical trials have failed, suggesting there are more delicate interaction between neurotransmitters, synapse activity and many other factors.

In this article, we reviewed novel biomarkers of AD, especially based on the aspect from NMDAR hypoactivation and oxidative stress. Studies in both mouse and human models suggest that there is significant interaction between NMDAR hypoactivation and oxidative stress, further related to cognitive disintegration. These biomarkers may be considered as potential keys to develop new diagnostic tools or treatments for AD.

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**Conflicts of Interest**

No potential conflict of interest relevant to this article was reported.

**Author Contributions**

Hsien-Yuan Lane and Chieh-Hsin Lin conceived and designed the manuscript, drafted the manuscript, and approved the final version of manuscript. Ting-I Chiang and Yi-Hsiang Yu analyzed and interpreted data and drafted the manuscript.

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