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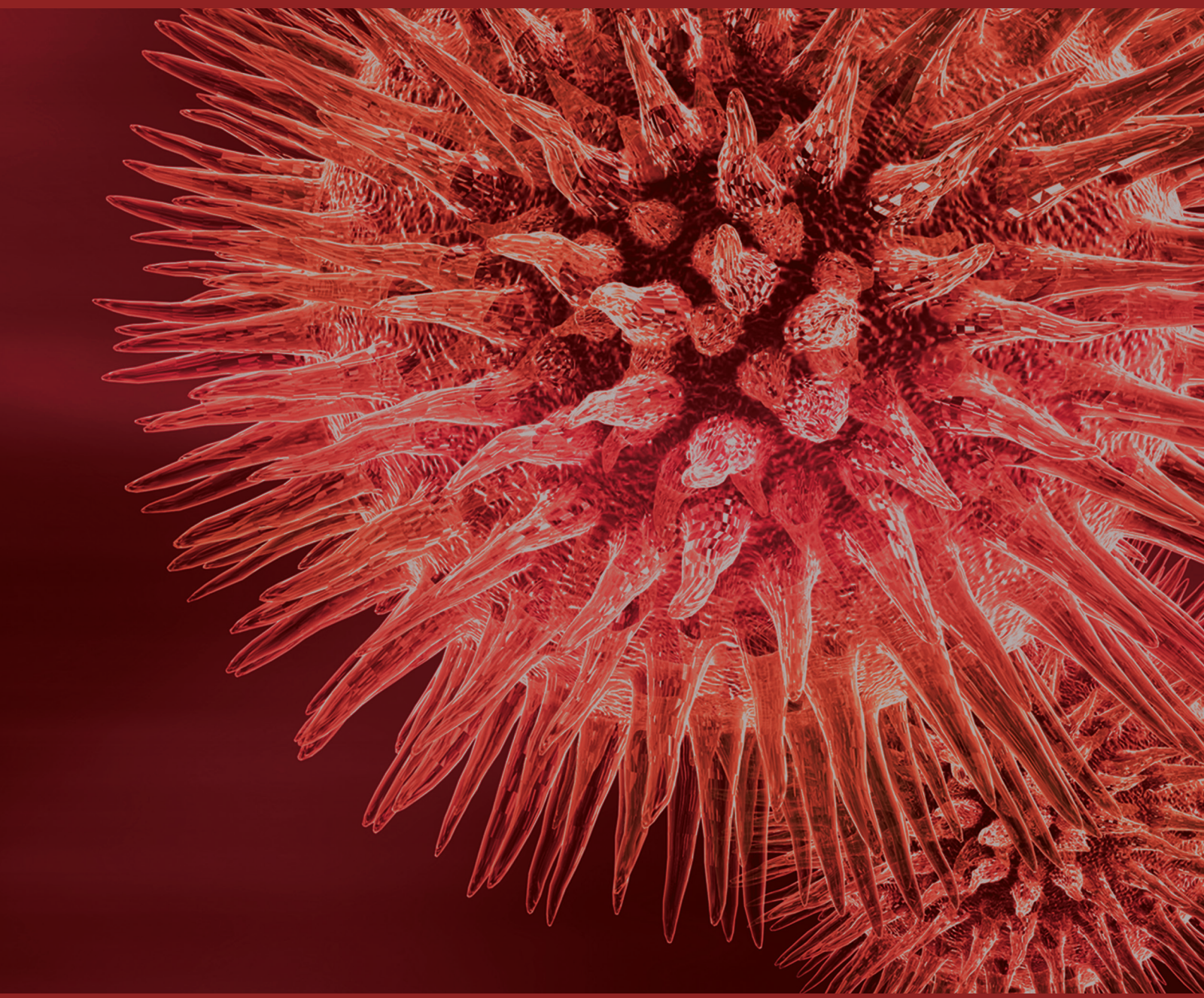
Guest Editors: Grazia D'Onofrio, Zhuowei Yu, and Orestes V. Forlenza



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Editorial

Alzheimer's Disease and Cognitive Frailty: Novel Therapeutic Technologies

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Worldwide, 46.8 million people have dementia, and every year there are over 9.9 million new diagnosed cases [1], with an increase of the economic impact and cost of the 35.4% from 2010 [1]. Alzheimer's disease (AD) is the most common form of dementia [2] and represents one of the major causes of disability, dependency, burden, and stress of caregivers increasing institutionalization among older people worldwide [3].

A precursor of neurodegenerative processes may be represented by Cognitive Frailty (CF) that includes reversible and potentially reversible subtypes [4].

Risk factors for CF and AD appear to change with age. Most very elderly individuals have beta-amyloid ($A\beta$) plaques within their brains, indicating that AD pathology may be present in asymptomatic elders and that these individuals will ultimately develop clinical symptoms of AD if they live long enough. According to the preclinical AD criteria, therefore, it is difficult to see how brain amyloidosis and brain aging could be considered separate entities arising through independent mechanisms.

In this special issue, investigators reported studies into the subject from all over the world (i.e., Switzerland, Germany, Egypt, China, Spain, Australia, Italy, Brazil, and USA).

They contributed to increasing the knowledge on to individualize genetic and clinic mechanisms of AD and CF considering age-related and multidimensional approaches to the purpose of appropriate and personalized treatment.

In a preclinical model, S. AbdAlla et al. subjected aged rats to chronic unpredictable mild stress, which is known to enhance the development of AD-related neuropathological features, and showed that four weeks of chronic mild stress induced a strong upregulation of the hippocampal angiotensin-converting enzyme (ACE), both at gene expression and at protein levels. The authors reported that ACE inhibition targets neurodegeneration triggered by environmental stress.

C. Ma et al. started from the assumption that advancing age, chronic inflammation, oxidative damage, and disorders of lipid metabolism are positively linked to the late-life cognitive impairment. This study demonstrated that age, levels of fundus atherosclerosis, serum biomarkers peroxidase, interleukin-6, serum levels of high-density lipoprotein cholesterol, ApoA2, and ApoC2 were significantly related to cognitive status. Moreover, ApoA1 and ApoA2 were found to be possible risk factors of cognitive impairment and late-life dementia.

J. A. Monge-Argilés et al. analyzed cerebrospinal fluid (CSF) biomarkers and tau/Aβ ratios in MCI patients and control subjects, using ELISA methodology. This study contributed to evaluation of the association between apolipoprotein E (ApoE) genotype and CSF levels of AD biomarkers and the influence of ApoE genotype on the development of AD in a Spanish population.

G. Lyons et al. described the "Deep Assessment," which is a novel multifaceted framework for delivering a more comprehensive and authentic assessment of the internal states of people with severe cognitive impairments who are unable to self-report. This paper suggested how Deep Assessment can be applied to people with advanced AD to develop others' understanding of their inner states and to help improve their quality of life. Moreover G. Lyons et al. discussed the potential utility and efficacy of this technique for this population and also proposed other human conditions that may benefit from research using a Deep Assessment approach.

F. Panza et al. reviewed tau-centric targets and drugs in clinical development for the treatment of AD and reported that methylene blue seems to be an inhibitor of the tau protein aggregation.

Finally, A. M. de Oliveira et al. reviewed studies of nonpharmacological interventions published in the last 10 years and reported that such approaches may help reduce behavioral and psychological symptoms of dementia (BPSD) such as agitation, psychotic symptoms, and apathy. This study highlighted the role of nonpharmacological interventions programs in the clinical management of BPSD, as an alternative to (or in combination with) conventional pharmacological treatments (e.g., antipsychotics and benzodiazepines) that may elicit undesired side effects.

The pharmacologic treatments, even if they are supported by several studies, deliver limited symptomatic benefits, so the provision of nonpharmacological treatments in addition to standard outpatient care is an asset of good clinical practice.

Therefore prognostic evaluation of AD patients plays a key role in the decision analyses of care processes including the organization of social health care system, the support to families, caregivers, and patients as well as the choice of appropriate treatment.

Grazia D'Onofrio
Zhuowei Yu
Orestes V. Forlenza

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Review Article

Tau-Centric Targets and Drugs in Clinical Development for the Treatment of Alzheimer's Disease

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The failure of several Phase II/III clinical trials in Alzheimer's disease (AD) with drugs targeting β-amyloid accumulation in the brain fuelled an increasing interest in alternative treatments against tau pathology, including approaches targeting tau phosphatases/kinases, active and passive immunization, and anti-tau aggregation. The most advanced tau aggregation inhibitor (TAI) is methylthioninium (MT), a drug existing in equilibrium between a reduced (leuco-methylthioninium) and oxidized form (MT^+). MT chloride (methylene blue) was investigated in a 24-week Phase II clinical trial in 321 patients with mild to moderate AD that failed to show significant positive effects in mild AD patients, although long-term observations (50 weeks) and biomarker studies suggested possible benefit. The dose of 138 mg/day showed potential benefits on cognitive performance of moderately affected AD patients and cerebral blood flow in mildly affected patients. Further clinical evidence will come from the large ongoing Phase III trials for the treatment of AD and the behavioral variant of frontotemporal dementia on a new form of this TAI, more bioavailable and less toxic at higher doses, called TRx0237. More recently, inhibitors of tau acetylation are being actively pursued based on impressive results in animal studies obtained by salsalate, a clinically used derivative of salicylic acid.

1. Introduction

The 2015 figures suggested that Alzheimer's disease (AD) may affect over 5.3 million people in the USA [1]. By 2050, the number of new cases of AD per year is expected to grow, resulting in nearly 1 million new cases per year, and the estimated prevalence is expected to range from 11 million to 16 million [1]. In the last three decades, notwithstanding considerable advances in the AD neurobiology and medicinal chemistry, no disease-modifying treatments have been introduced for this devastating and progressive neurodegenerative disease [2]. The neuropathological hallmarks of

AD are intracellular neurofibrillary tangles (NFTs) composed of paired helical filaments (PHFs) and straight filaments (SFs) mainly constituted of hyperphosphorylated tau protein, a microtubule associated protein (MAP), neuropil threads (NTs), dystrophic *neurites*, and extracellular deposits of β-amyloid (Aβ) as the major component of senile plaques (SPs) in the brain. These neuropathological hallmarks of AD strongly influenced recent therapeutic approaches, with a large portion of the many therapeutic approaches under development for AD treatment directed against the production and accumulation of Aβ [3]. However, several drugs targeting Aβ with different mechanisms of action have failed to demonstrate efficacy in randomized clinical trials or their development has been halted [4, 5]. For the amyloid-based approach, passive anti-Aβ immunization is the most advanced strategy for treating AD, and solanezumab, a monoclonal antibody directed at the mid-region of Aβ, also failed but suggested some beneficial cognitive effects in mildly affected patients [4]. A Phase III study with a planned size of 2100 mild AD patients is ongoing to confirm these potential benefits. Solanezumab is also being tested in a prevention study in asymptomatic older subjects, who have positive positron emission tomography (PET) scans for brain amyloid deposits [6]. Two other monoclonal antibodies, gantenerumab, which preferentially binds to fibrillar Aβ, and crenezumab, which preferentially binds to soluble, oligomeric and fibrillar Aβ deposits, are being tested in secondary prevention trials in presymptomatic subjects with autosomal dominant AD mutations [4, 6]. These ongoing secondary prevention trials will tell us if Aβ really plays a crucial role in the pathophysiology of AD. In fact, notwithstanding the preeminence assigned to Aβ, one crucial point was that large numbers of amyloid SPs can occur in the course of normal ageing without any evidence of clinical dementia. Given the repeated failures of trials targeting the Aβ pathway in mild or moderate AD [4], there is increasing interest in the possibility that tau-targeted compounds could have therapeutic utility in AD, particularly tau aggregation inhibitors (TAIs) [5, 7, 8]. The aim of this paper was to provide a comprehensive review of tau-directed drugs for the treatment of AD, with a particular focus on TAIs and the most clinically advanced of these compounds, that is, leucomethylthioninium (LMT, leucomethylene blue (MB), LMTX™, TRx0237, TauRx Therapeutics Ltd., Republic of Singapore), a second-generation TAI for the AD treatment. TRx0237 shares the same active ingredient and mode of action of another first-generation TAI, that is, methylthioninium (MT, Rember™, TRx-0014, TauRx Therapeutics Ltd., Republic of Singapore), of which is the reduced form, designed to have improved bioavailability and tolerability. The chloride salt of oxidized MT^+ is methylthioninium chloride (MTC or MB).

2. Pathophysiology of Tau Protein in Alzheimer's Disease

Among pathological hallmarks of AD, the intracellular NFTs contain two aggregated tau species, hyperphosphorylated PHFs of MAP tau (or tau) and SFs. Tau is a 50–75 kDa protein with six different splice variants, referred to as 0N3R, 1N3R, 2N3R, 0N4R, 1N4R, and 2N4R [9, 10]. A short segment of tau protein, referred to as the PHF core, from the repeat region of the molecule is an integral structural constituent of the PHF [11]. Abnormal phosphorylation/hyperphosphorylation occurs in tau protein in AD, beginning to pair up with other threads of tau into PHFs and tangle together, resulting in the movement of tau proteins from axons to the somatodendritic compartment of neurons, causing disintegration of microtubules, collapse of neuron's transport system, and formation of extremely insoluble aggregates. These changes are presumed to disrupt neuronal communication and lead to cell death [12]. NFTs are generated intracellularly, but when neurons die, the only NFTs remaining are "ghost tangles" which are localized outside of cells where the host neuron has died. Ghost tangles are a common finding in AD patients which can occur also in preclinical stages [13]. In this preclinical phase of AD, the earliest involved neurons are those in the locus coeruleus, and the subcortical tau lesions then reach the noradrenergic coeruleus neurons of the contralateral brainstem, so that the pathological process becomes symmetrical soon after its onset. Thereafter, additional nuclei with diffuse cortical projections become involved [14]. H. Braak and E. Braak demonstrated that appearance of tau pathology in AD occurs in a characteristic pattern of development in six stages, with NFTs and NTs appearing first in the entorhinal cortex (stages I and II), followed by hippocampal (stages III and IV), and neocortical areas (stages V and VI) [13–15]. A corresponding staging for Aβ deposition was compared with tau staging, with three levels of increasing Aβ deposits (stages A–C), in a large autopsy case series of subjects between the ages of 25 and 95 years [16]. These findings suggested that tau aggregation precedes Aβ deposits by about three decades [16], confirming earlier reports showing the same pattern [17, 18].

The exact mechanisms by which tau protein becomes a nonfunctional entity are under debate. Tau pathology in AD is principally characterized by abnormal phosphorylation/hyperphosphorylation of tau proteins, but also proteolytic cleavage (truncation), glycosylation, nitration, acetylation, O-GlcNAcylation, ubiquitination, and other abnormal posttranslational modifications are responsible for altered tau structure in this devastating neurodegenerative disease [11, 19–25]. All these molecular events are associated with the formation of PHFs and the appearance of NFTs. In particular, abnormal phosphorylation/hyperphosphorylation, acetylation, and truncation are further supported as pathological events by *in vitro* experiments [22, 26–29], demonstrating that these modifications increase fibrillization of tau and induce cell toxicity. Truncation/proteolytic cleavage of tau protein, as an alternative mechanism involving in the abnormal aggregation of tau, was proposed after extensive biochemical analysis of the PHF core [11, 21], with prion-like properties *in vitro*. Until today, identification of the enzyme that produces this abnormal posttranslational modification is uncertain. Caspases, which are apparently elevated in AD brain [30, 31], are likely involved in the proteolytic processing of tau protein. The repeat domain of tau is able to catalyse and propagate the conversion of normal soluble tau into accumulations of the aggregated and truncated oligomeric forms [5].

In fact, hyperphosphorylated tau proteins bind together and form oligomeric tau, from dimers to octamers [32]. Both hyperphosphorylated tau by itself and oligomeric tau are involved in synaptic loss, as observed in the wild-type human tau transgenic mouse [25, 33]. Indeed, protein aggregates may in general be protective in neurodegeneration by sequestering dispersed small aggregates, oligomers, or misfolded proteins, minimizing their toxicity and eventually facilitating their clearance by proteasomal activity or autophagy [34–36], a model that remains to be validated with respect to tau protein and AD [37]. However, proteolytically stable tau oligomers are able to propagate between neurons and initiate the cascade of self-propagating misfolded proteins from neuron to neuron [38, 39]. Therefore, the tau pathology of AD can be understood as a self-propagating "prionosis," reflecting degrees of spread of tau that may form an endopathogenic species transmitting neurodegeneration from one cell to the next throughout the brain [40]. On this basis, vaccination of mice in experimental models of tauopathy and synucleinopathy, involving intracellular proteins, has showed promising findings [41, 42].

3. Tau-Based Drugs for the Treatment of Alzheimer's Disease

In AD, given the confirmed link existing between NFT topography and clinical phenotype [43], and the abnormal posttranslational modifications of tau protein linked to the disease [11, 19–25], therapies targeting NFTs and tau protein may have potential application as drug targets against neurodegeneration [44–46], although their development has lagged behind drugs targeting Aβ. At present, therapies targeting tau aim to reduce, stabilize, or prevent aggregation or hyperphosphorylation of the protein [44–46]. In particular, several therapeutic approaches with a disease-modifying potential have been suggested: (1) inhibition of tau phosphorylation (with the inhibition of tau kinases or the activation of tau phosphatases); (2) increase of microtubule stabilization; (3) increase of tau clearance and (4) inhibition of tau aggregation. Some of these approaches have actually reached the clinic [7].

Abnormal phosphorylation of tau protein may play a critical role in the pathogenesis of NFT degeneration, with the balance between kinases and phosphatases disturbed in AD, leading tau protein to become detached from microtubules, secondarily to aggregate. There is approximately a four- to fivefold higher level of total tau in AD brain compared to that of age-matched healthy brains and this increase is all in the form of the abnormally hyperphosphorylated tau [47]. In AD, the abnormal phosphorylation of tau could be, but not mutually exclusive, the result of upregulation of tau kinases or downregulation of tau phosphatases [9]. In this scenario, a tau-based therapeutic approach would target a kinase particularly responsible for a pattern of phosphorylation causing reduced microtubule stability.

Tau protein kinases are grouped into three main classes: proline-directed protein kinases (PDPK), protein kinases non-PDPK, and tyrosine protein kinases (TPK) [48]. Among these enzymes, the kinases with the most important role in phosphorylation of tau protein in the brain include glycogen synthase kinase 3β (GSK-3β), cyclin-dependent kinase 5 (CDK5), cAMP-dependent protein kinase (PKA), and calcium/calmodulin-dependent kinase II (CaMKII) [49]. GSK-3β may play a major role in regulating tau phosphorylation in both physiological and pathological conditions. Interactions between GSK-3β and CDK5 also exist and will require further evaluation to optimize treatments aimed at these kinases [50, 51]. Despite the challenges faced by this approach with respect to toxicity and specificity, a number of efforts are underway to develop kinase inhibitors. In particular, *in addition to* a series of compounds directed at kinases of the PDPK and non-PDPK groups in preclinical development that should be tested in *in vivo* studies [48, 52], one GSK-3β inhibitor, tideglusib (NP031112, NP-12, Nypta®, Zentylor™, Noscira SA, Madrid, Spain), a drug which belongs to the thiadiazolidinone family, was in clinical trials for AD and progressive supranuclear palsy (PSP) [5, 7, 53]. In a previous Phase IIa trial, tideglusib was orally administered at escalated doses of 400 up to 1000 mg/day for 20 weeks to 30 patients with mild to moderate AD and the active group showed positive trends in four out of five clinical scales and had significantly better response on the Mini-Mental State Examination (MMSE), with asymptomatic elevation of transaminases, reversed with withdrawal of the drug [54]. The ARGO study, a subsequent six-month, Phase IIb trial, was conducted to assess safety and efficacy of tideglusib in mild to moderate AD patients with the 15-item modified version of the Alzheimer's Disease Assessment Scale (ADAS-cog$_{15}$) as the principal outcome measure. However, the results demonstrated no statistically significant findings, although the drug was well tolerated with diarrhea and asymptomatic transaminase elevations as the only side effects [55]. There are no current FDA approved trials ongoing for treating AD with tideglusib. Activation of phosphatases, in particular protein phosphatase 2A (PP2A), has also been proposed as a possible alternative strategy to kinase inhibition for reducing tau phosphorylation [44, 49, 56]. Multiple PP2As exist and inhibition of these phosphatases results in hyperphosphorylation of tau, formation of NFT-like structures, and memory impairment in animal models [57–59]. Drugs increasing the activity of PP2As, probably through the endogenous proteins that inhibit their activity, have the therapeutic potential for treating AD [60, 61], but no clinical trials with PP2A activators have been started yet.

Among tau-based anti-AD drugs, several microtubule-stabilizing agents have been tested and the studies carried out have provided proof of concept that compounds with the ability to stabilize microtubules may have therapeutic potential for the treatment of AD and other neurodegenerative diseases [62], given that tau detachment from microtubules results in loss of its normal microtubule-stabilizing function, probably leading to axonal transport impairment and eventually to synaptic dysfunction. Some antimitotic compounds such as paclitaxel (Taxol, Bristol-Myers Squibb Company, New York City, USA), epothilone D (Epo D, BMS-241027, Bristol-Myers Squibb Company, New York City, USA), or TPI-287 (Cortice Biosciences, New York City, USA, formerly Archer Biosciences) have been used in tau transgenic animals

for their microtubule-stabilizing activity [7, 63, 64], but at present, these compounds did not reach the clinic *due to* toxic side effects (paclitaxel) or have been discontinued for AD (epothilone D) or are in Phase I of clinical development (TPI-287) for mild to moderate AD [65] (Table 1) and primary four-repeat tauopathy, corticobasal degeneration (CBD), CBD syndrome, and PSP [66]. In particular, in a preventative study, epothilone D was administered weekly for 3 months to young PS19 tau Tg mice that initially lacked significant tau pathology, preventing the axonal microtubule loss and dystrophy, as well as spatial learning deficits, that manifested as these mice developed forebrain tau pathology with age [67]. In another preclinical study, in both young and old animals of the PS19 tauopathy model, in which tau pathology is developing or well established, respectively, epothilone D reversed behavioral and cognitive deficits, cleared tau pathology, and increased hippocampal neuronal integrity [63]. Based on these encouraging findings, in February 2012, Bristol-Myers Squibb started a Phase I trial to evaluate the tolerability and pharmacology of epothilone D in 40 patients with mild AD, comparing 0.003, 0.01, and 0.03 mg/kg infused once a week for nine weeks to placebo [68]. The study ended in October 2013, but evaluation of epothilone D for AD was subsequently discontinued.

Among microtubule-stabilizing agents, davunetide (NAPVSIPQ, NAP, AL-108, Allon Therapeutics Inc., Vancouver, Canada, Paladin Labs Inc., Montreal, Canada), an eight-amino acid peptide (with NAP representing the first three amino acids in the peptide) derived from the activity-dependent neuroprotective protein (ADNP), has demonstrated the potential to decrease tau phosphorylation and Aβ levels in animal models [69]. In particular, NAP stabilizes microtubules and reduces hyperphosphorylated tau levels [70] and in a mouse model of amyotrophic lateral sclerosis (ALS) it protected against impairments in axonal transport [71], suggesting that reduction of tau hyperphosphorylation, stabilization of microtubules, and neuroprotective effects may be beneficial to prevent disease progression. An intranasal formulation of davunetide was tested in Phase II clinical trials for both mild cognitive impairment (MCI) and PSP, given that intranasally administered NAP treatment can cross the blood-brain barrier (BBB). In 2007-2008, the Phase II trial in 144 subjects with MCI demonstrated a statistically significant improvement in memory performance compared with placebo at eight weeks and 16 weeks, but not 12 weeks, with well-tolerable side effects [72]. However, the results of the Phase II/III trial in the pure tauopathy PSP were unimpressive [73], suggesting intervention at early stages of the disease [62]. This result halted, for the time being, clinical development of davunetide. This decision also prompted a halt to recruitment into an ongoing safety and biomarker trial, begun in 2010, of davunetide in frontotemporal lobar degeneration (FTLD) with predicted tau pathology, CBD syndrome, or PSP [73]. An intravenous formulation of davunetide also exists (AL-208) and this version of the drug was tested between 2006 and 2008 in a Phase II trial of the safety and efficacy of a single 300 mg IV dose on cognitive impairment following coronary artery bypass surgery [74], with no published results.

Recent efforts to develop safe and efficacious anti-Aβ immunotherapy using Aβ peptide as an immunogen in active vaccination approaches or anti-Aβ antibodies for passive vaccination may be translated to the development of a tau-based immunotherapy [45]. Clearance of extracellular misfolded tau protein may prevent the transmission and spreading of tau pathology throughout the brain. Active immunization of wild-type mice with recombinant unphosphorylated full-length human tau protein led to encephalomyelitis with neurological and behavioral deficits, axonal damage, and inflammation [75], suggesting a neurotoxic potential of tau immunization. However, the feasibility of this approach was later demonstrated with a 30-amino acid tau phosphopeptide spanning amino acids 379–408, including phospho-Ser at positions 396 and 404, in two different transgenic mouse models of disease, the JNPL3 (P301L) and htau/presenilin 1 (PS1) lines [41, 76], which both resulted in a specific antibody response, reduced tau burden, and attenuation in the severity of behavioral and cognitive phenotypes [77]. Among active vaccines in clinical trials, AADvac1 (Axon peptide 108 conjugated to KLH, Axon Neuroscience, Bratislava, Slovak Republic) was the first anti-tau vaccine to enter clinical trials and it is a synthetic peptide derived from amino acids 294 to 305 of the tau sequence, that is, KDNIKHVPGGGS, coupled to keyhole limpet hemocyanin (KLH) through an N-terminal cysteine, and administered with an Alhydrogel alum adjuvant. In transgenic tau rats, the vaccine reduced tau pathology and associated behavioral deficits [78]. AADvac1 was designed to target misfolded tau in AD, and its safety, tolerability, and efficacy have been evaluated in a first-in-man Phase I clinical trial conducted in three centers in Austria on 30 patients with mild to moderate AD, completed on March 2015 [79] (Table 1). Two withdrew due to adverse events, of which one (a viral infection followed by epileptic seizure) was considered to be possibly related to the vaccine. Unfortunately, the double-blind, placebo-controlled portion of the study lasted only 12 weeks and the study evaluated only one dose of the vaccine (40 μg). No data on cerebrospinal fluid (CSF) biomarkers were reported. These deficiencies limit the interpretability of the results both in terms of safety and on target engagement. The subsequent 12-week open label portion of the study is of limited information [80]. Patients completing this 24-week study had the option to enter a further 18-month open label extension (FUNDAMANT) [81] (Table 1). A separate 24-month Phase II study in 185 patients with mild AD and a magnetic resonance imaging (MRI) consistent with this diagnosis was planned to start on March 2016. This study will compare 8 subcutaneous injections of 40 μg of AADvac1 with the adjuvant aluminum hydroxide to placebo. The primary outcome will be safety, and secondary outcomes will include cognitive and clinical batteries as well a measure of immunogenicity. Fluorodeoxyglucose (FDG) PET, MRI volumetry, and CSF biochemistry were set as exploratory outcomes (ClinicalTrials.gov Identifier: NCT02579252, ADAMANT) [82] (Table 1).

The vaccine ACI-35 (AC Immune AG, Lausanne, Switzerland and Janssen Pharmaceuticals, Beerse, Belgium) is a liposomal-based 16-amino acid tetrapalmitoylated phospho-tau peptide with specific amino acid areas incorporated into

Table 1: Ongoing phase I–III randomized controlled trials (RCTs) of tau-directed drugs in clinical development for the treatment of Alzheimer's disease (AD).

Compound (company) Clinicaltrials.gov identifier	Mechanism of action	Estimated enrollment	Characteristics	Status
TRx0237 (LMTX) (TauRx Therapeutics Ltd.) NCT01626391	Tau aggregation inhibitor	9 patients already taking medications for probable mild to moderate AD (2012-2013)	TRx0237 tablets 250 mg/day (given as 125 mg bid) for 4 weeks	Phase II trial (completed)
TRx0237 (LMTX) (TauRx Therapeutics Ltd.) NCT01689233	Tau aggregation inhibitor	700 patients with probable mild AD (2012–2015)	TRx0237 100 mg tablets administered twice daily	Phase III trial (active not recruiting)
TRx0237 (LMTX) (TauRx Therapeutics Ltd.) NCT01689246	Tau aggregation inhibitor	833 patients with probable mild to moderate AD (2013–2016)	TRx0237 125 mg tablets administered twice daily	Phase III trial (active not recruiting)
TRx0237 (LMTX) (TauRx Therapeutics Ltd.) NCT01626378	Tau aggregation inhibitor	220 patients with behavioral variant of FTD (2013–2016)	TRx0237 100 mg tablet administered twice daily	Phase II trial (active not recruiting)
TRx0237 (LMTX) (TauRx Therapeutics Ltd.) NCT02245568	Tau aggregation inhibitor	Subjects who have completed participation in a Phase II or Phase III trial with TRx0237 continued access to therapy to evaluate the long-term safety of TRx0237 (2014–2017)	All subjects will initially be given 200 mg/day of TRx0237 administered twice daily. Thereafter, dosing is flexible (100 mg/day to 300 mg/day)	Open label Phase II trial (currently recruiting)
TPI-287 (University of California, San Francisco) NCT01966666	Microtubule-stabilizing agent	33 patients with mild to moderate AD (2013–2015)	The purpose of the study is to determine the highest dose of TPI-287 that is safe and tolerable when administered as an intravenous infusion	Phase I trial (currently recruiting)
AADvac1 (Axon Neuroscience SE) NCT01850238	Active tau-based immunotherapy	30 patients with mild to moderate AD (2013–2015)	Patients will receive 1 dose of AADvac1 per month over 3 months, for a total of 3 administrations	Phase I trial (completed)
AADvac1 (Axon Neuroscience SE) NCT02031198 FUNDAMANT	Active tau-based immunotherapy	This follow-up study continues to observe patients who have completed the Phase I trial of AADvac1, for another 18 months (2014–2017)	Patients who have received 6 doses in the previous trial will be given 1-2 booster doses of AADvac1 (2 if their antibody titers decline below those achieved in the previous trial). Patients who have received 3 doses in the previous trial will be given another 3 doses and then vaccinated with booster doses as above	18-month follow-up Phase I trial (active, not recruiting)
AADvac1 (Axon Neuroscience SE) NCT02579252 ADAMANT	Active tau-based immunotherapy	185 patients with mild AD (2016–2019)	Patients will receive 6 doses of AADvac1 in 4-week intervals and then 2 individual booster doses in 6-month intervals, for a total of 8 doses	Phase II trial (currently recruiting)
ACI-35 (AC Immune AG)	Active tau-based immunotherapy	Patients with mild to moderate AD (2013–2014)	This Phase I trial compared two doses of ACI-35 to investigate its safety, tolerability, and immunogenicity	Phase I trial (active, not recruiting)
RG7345 (RO6926496, MAb86) (Hoffmann-La Roche) NCT02281786	Passive tau-based immunotherapy	48 healthy subjects (January 2015–October 2015)	Single, ascending dose, intravenous administration	Phase I trial (active, not recruiting)

the vaccine including phosphorylated S396 and S404 residues that also provides active immunization. It elicits a rapid immune response against the immunogen in wild type and transgenic JNPL3 (P301L) mice, resulting in a mild reduction of hyperphosphorylated pathological tau and tau pathology by immunohistochemical characterization and increased IgG titers and motor function of vaccinated mice [83]. ACI-35 also demonstrated a good safety profile for human studies, with no adverse inflammatory response [83]. Currently, a Phase Ib trial is underway in mild to moderate AD to assess safety profile along with secondary outcomes including biomarkers, functional, and clinical change (Table 1), but details are not available and this trial is not listed in ClinicalTrials.gov or the World Health Organization's clinical trial registry.

For passive vaccination, anti-tau oligomer antibodies may be ideal candidates for treating AD [84], similar to the ones developed for Aβ [85], with exciting opportunities to validate anti-tau oligomer immunotherapeutic approaches in animal models. In the first program to demonstrate the efficacy of tau-based immunotherapy, this approach has been tested by injecting anti-phospho-tau antibody PHF1, which recognizes the pS396/pS404 epitope, intraperitoneally to JNPL3 (P301L) tau transgenic mice, with preliminary findings indicating that treated animals showed decreased tau pathology and functional impairment [86]. Similar effects were obtained also with other antibodies against the pS396/pS404 epitope [87, 88]. Several tau antibodies are currently in early clinical development as therapies for AD and other tauopathies [45]. Among these antibodies, RG7345 (RO6926496, MAb86, F. Hoffmann-La Roche Ltd., Basel, Switzerland) is a human monoclonal antibody targeting a specific tau phosphorylated epitope at site pS422, which is prominent in neuronal dendrites [89, 90] and linked to the relocalization of tau protein away from microtubules and toward the somatodendritic compartment of the neuron [89]. Furthermore, in a triple transgenic mouse model of AD, the passive administration of the antibody demonstrated a reduced accumulation of tau pathology with intracellular clearance of tau antibody complexes [90]. In January 2015, a Phase I, single-ascending-dose study in 48 healthy young men in the United Kingdom started, comparing the safety and pharmacokinetic measures of six different doses to placebo, all infused intravenously [91] (Table 1). Finally, BMS-986168 (IPN007, Bristol-Myers Squibb Company, New York City, USA), although not currently approved for AD but only for PSP [92], is a humanized monoclonal antibody directed toward extracellular, N-terminally fragmented forms of tau (eTau), which were originally isolated from familial AD patient-derived pluripotent stem cells. A recent study demonstrated a correlation between eTau and Aβ both *in vitro* and in two transgenic *in vivo* mice models, with a reduction in Aβ that occurs when eTau is targeted with an antibody [93]. Secreted forms of tau were reported to cause neuronal hyperactivity, which could, in turn, increase Aβ production, fueling a feed-forward cycle [93]. In December 2014, a Phase I, single-center, single-ascending-dose study in 48 healthy volunteers in Texas started. This first human trial will assess safety parameters for up to eight months after administration of a single infusion of BMS-986168 [94].

4. Covalent and Noncovalent Tau Aggregation Inhibitors for the Treatment of Alzheimer's Disease

Among several tau-directed approaches in AD, small molecular weight compounds developed to inhibit formation of tau oligomers and fibrils by blocking tau-tau aggregation have already been tested in humans [5, 7, 95, 96]. In cell-based and/or *in vitro* screening assays, several classes of agents that may act to prevent tau aggregation have been identified, including but not limited to polyphenols [80], porphyrins [80], phenothiazines [97], benzothiazoles/cyanines [98, 99], N-phenylamines [100], thioxothiazolidinones (rhodanines) [101], phenylthiazole-hydrazides [102], anthraquinones [103], and aminothienopyridazines (ATPZs) [104]. However, for many TAIs there is a lack of evidence of efficacy *in vivo* for inhibiting tau aggregation. Currently, TAIs fall into two broad mechanistic classes, with the first class corresponding to covalent TAIs, that is, agents that either covalently modify tau directly or foster formation of covalent bonds within or between tau proteins to yield aggregation-incompetent products [95]. Covalent TAIs can attack any or all species in an aggregation pathway but appear to be especially efficacious modifiers of tau monomers [95]. Among covalent TAIs, oleocanthal, a natural product aldehyde, reacts with epsilon amino groups of lysine residues [105, 106], including residues residing in the microtubule binding repeat region, to form imines. In addition, other natural polyphenols are covalent TAIs, such as oleuropein aglycone [107], abundant in the extra virgin olive oil, or green tea-derived (−)-epigallocatechin gallate (EGCG) [108]. Other redox-active compounds, including the nonneuroleptic phenothiazine MB, that is, MTC, can also modulate cysteine oxidation when incubated in the absence of exogenous reducing agents [109]. High concentrations of reduced sulfhydryl groups in the form of glutathione normally maintain a reducing intracellular environment [110], and therefore compounds acting solely through this mechanism could have low potency and efficacy *in vivo*. In general, covalent mechanisms of tau aggregation inhibition in AD are predicted to have low utility *in vivo* [111]. However, dimethylfumarate, an electrophile capable of reacting covalently with cysteine sulfhydryls, was approved for oral treatment of multiple sclerosis [112], suggesting that electrophilic compounds acting through covalent inhibitory mechanisms can be useful therapeutic agents.

The second broad class of TAIs interacts with tau species noncovalently, through multiple mechanisms, and with different structures [95, 113]. Among different mechanisms, small molecules can interact directly but transiently with natively unfolded tau protein monomer [95]. For example, curcumin has been reported to increase the reconfiguration rate (i.e., a rapid rate of interconversion between aggregation competent and incompetent conformations) of α-synuclein, such that occupancy of assembly competent conformations is minimized [114]. Because tau aggregation is sensitive to curcumin conjugates [115], this mechanism may be relevant also for tau protein. Noncovalent TAIs also may act by blocking formation of steric zipper structures common to cross-β-sheet forming peptides. Short segments of amyloidogenic

sequences have been crystallized in forms that exhibit similar properties as their full-length counterparts [116]. Furthermore, tau filament formation can be inhibited by sequestering tau in the form of stable off-aggregation pathway oligomers. For example, phthalocyanine tetrasulfonate, a cyclic tetrapyrrole, interacts directly with tau monomers to form SDS-stable oligomers [117]. Similarly, in a study of α-synuclein aggregation, polyphenol, phenothiazine, polyene macrolide, porphyrin, and Congo red derivatives were found to stabilize SDS- and Sarkosyl-insoluble oligomers [118]. SDS-stable oligomers composed of full-length tau also rapidly form at low micromolar concentrations in the presence of cyanine, triarylmethine, rhodanine, and phenothiazine TAIs [80, 111]. Since tau can coaggregate with other proteins, including microtubule associated proteins and alpha-synuclein [119], TAIs may work through binding to these proteins. Indeed, numerous polyphenols have been identified that inhibit aggregation of a wide variety of amyloidogenic peptides including tau and α-synuclein [113], but no studies with selective TAIs are currently available to support this hypothesis.

5. Tau Aggregation Inhibitors in Clinical Development for the Treatment of Alzheimer's Disease: Preclinical Studies of Methylthioninium and Derivatives

TAIs are divided into covalent and noncovalent molecules depending on their way to interact with tau protein. The chemical structure of noncovalent TAIs differs significantly in terms of scaffold [95]. Structure-activity relationships (SARs) were established within specific chemical series [120, 121]. Like common dyes, most TAIs absorb electromagnetic radiation in the visible spectrum, a property linked to the property of delocalizing π-electron distribution [122]. Ligand polarizability correlates with tau aggregation inhibitory potency within specific chemical series of cyanine, phenothiazine, arylmethine, and rhodanine derivatives [111]. MB or MTC (Rember) is and old dye repurposed as medical treatment of tau pathologies [123]. Chemically, MTC is a tricyclic phenothiazine derivative [124] and exists in equilibrium between reduced (LMT) and oxidized form (MT^+). Under physiological conditions, it is present as a cation (MT^+) and formulated as a chloride salt (commonly known as MB). MTC may be reduced by nicotinamide adenine dinucleotide phosphate (NADPH) or thioredoxin to give LMT (leuco-MB), an uncharged colorless compound (methylene white). MTC is excreted in the urine as a mixture of MTC, LMT, and demethylated metabolites, for example, azure B and azure A [125]. MTC has been used to treat malaria [126], methemoglobinemia [123], and depression [127]. MTC efficiently crosses the BBB [128] and selectively penetrates neurons after systemic administration, particularly hippocampal cells [129]. At present, MTC and its derivatives represent the most advanced TAIs in clinical development for the treatment of AD. MTC has been shown to interfere with the tau-tau binding necessary for aggregation [97]. In a cell-based model of inducible tau aggregation, the inhibitory constant of MTC was found to be 123 nM [5]. Other studies reported quite different *in vitro* inhibitory potency (IC_{50}) varying from 1.9 μM [80] to 3.5 μM [99]. The estimated trough brain concentration of MT (Rember) and its active metabolites in the human brain at the 138 mg/day dose was 0.18 μM [130]. This value appears to be in the range of the *in vitro* IC_{50} values for dissolution of PHFs (0.16 μM) and the calculated intracellular Ki for TAI activity (0.12 μM) [131] but not in the range of IC_{50}s of other *in vitro* [80] and cell-based [99] studies. In tau transgenic mouse models, MT levels in the brain followed a sigmoidal concentration-response relationship over a 10-fold range (0.13–1.38 μM) after oral administration of 5–75 mg/kg for 3–8 weeks [132]. Alternative mechanisms of action have been proposed for MT [5] including inhibition of microtubule assembly [104] that requires IC_{50} of 50 μM [5, 104]. However, the dose required to achieve inhibition of microtubule assembly with MTC would be about 50 g of MTC/day [5], exceeding the median lethal dose (LD_{50}) for MTC in several species. Similarly, it has been proposed that MTC may reduce endogenous production of tau protein [133], but EC_{50} for this effect is 10 μM, requiring a human clinical dose of 9 g of MTC/day, a dose that could not safely be administered in humans. It has been also proposed that MTC could affect tau phosphorylation via inhibition of Hsp70 ATPase [134], but again EC_{50} for this effect is 83 μM, with a theoretical dose in humans of 75 g MTC/day.

Recent *in vivo* and *in vitro* studies have suggested that MTC may reduce tau protein aggregates in AD through proteasomal [135] and macroautophagic [136, 137] degradation of the protein. Other potential effects of MTC are oxidation of cysteine sulfhydryl groups in the tau repeat domain preventing formation of disulphide bridges to keep tau monomeric [138], acetylcholinesterase inhibition [139], nitric oxide synthase inhibition [140], noradrenaline uptake inhibition [141], glutamatergic inhibition [142], monoamine oxidase B inhibition [143], guanylate cyclase inhibition [140], and inhibition of the aggregation of Aβ peptides [80, 97, 144], stimulation of Aβ clearance [145], improvement of brain metabolism [146–150], improvement of astrocyte cellular respiration [151], improvement of brain mitochondrial amyloid-binding alcohol dehydrogenase (ABAD) functions [150], improvement of mitochondrial antioxidant properties [152, 153], improvement of the Nrf2/antioxidant response element (ARE) [154–156], antagonism of α7-nicotinic acetylcholine receptors [157], inhibition of β-secretase activity [149], enhancement of mitochondrial oxidation [158], and inhibition of monoamine oxidase A [143]. However, the clinical relevance of these potential effects is doubtful. On the other hand, there are only a few reports on the effect of MTC on tau aggregation *in vivo* [135, 136, 159–161]. In one study, MTC did not alter abnormal tau phosphorylation and failed to inhibit tau-dependent neuronal cell toxicity in zebrafish [159]. In another study, MTC treatment reduced detergent-insoluble phosphorylated tau levels in the JNPL3 (P301L) tau transgenic mice [160]. Treatment of 3-month-old rTg4510 mice for 12 weeks with oral MTC prevented behavioral deficits and reduced soluble tau levels in the brain [135]. JNPL3 (P301L) mice treated with MTC for 2 weeks showed reduced soluble tau levels without affecting insoluble tau levels [136]. These studies indicate that MTC

treatment may reduce soluble tau levels and prevent cognitive decline when treatment begins at a time point before NFTs are present in the brain [135]. A recent study suggested that 6 weeks of oral treatment with MTC did not reverse established NFT pathology in the rTg4510 mouse model of tauopathy [161]. Some studies reported a generalized antiaggregation effect for MTC against aggregation-prone proteins, such as prion protein [162], α-synuclein, and transactivation response (TAR) DNA-binding protein of Mr 43 kDa (TDP-43) [163, 164]. This further activity of MTC has potential relevance for the treatment of ALS and FTLD [165].

6. Clinical Efficacy and Safety of Methylthioninium and Derivatives

A double-blind, randomized, placebo-controlled study evaluates the safety and explorative efficacy of MT (Rember) given doses of 69 mg, 138 mg, and 228 mg/day (equivalent to 30 mg, 60 mg, and 100 mg MTC) for 24 weeks to 321 mild to moderate AD patients who were not taking acetylcholinesterase inhibitors (AChEIs) or memantine (ClinicalTrials.gov Identifier: NCT00515333) (Table 1). The primary efficacy outcome of the study was the change in the ADAS-cog at 24 weeks relative to baseline. The effects of treatments on regional cerebral blood flow (rCBF) decline were determined in a subgroup of 135 patients using hexamethyl-propyl-amine-oxime single photon emission computed tomography (HMPAO-SPECT). At the end of the 24-week, double-blind, placebo-controlled treatment period, patients had the option to enter two consecutive open label extensions of 26 and 48 weeks, respectively [166]. At 24 weeks, there were not significant differences between treatment groups compared to placebo in any of the efficacy variables. *Post hoc* subgroup analyses revealed that in moderately affected patients there was significant treatment benefit of the intermediate dose of 138 mg/day compared to placebo on the ADAS-cog scale (5.42 points, $p = 0.047$). In mildly affected patients, there was a significant beneficial effect of the 138 mg/day compared placebo on the all regions other than the left frontal lobe (1.97%, $p < 0.001$) [166].

A total of 111 patients completed the first open label extension of 26 weeks (ClinicalTrials.gov Identifier: NCT00684944) [167] (Table 1). At 50 weeks, the mean change of ADAS-cog score of the 138 mg/day dose group was better than the mean change of patients initially receiving placebo for 24 weeks and then 152 mg/day for 26 weeks (2.8 and 5.2 points in mild and moderate patients, resp.). The most commonly reported adverse events (incidence $\geq$ 5%) in MTC-treated subjects included gastrointestinal disorders (primarily diarrhea), renal and urinary disorders (primarily dysuria and frequency), and falls [166]. No changes of clinical significance were observed in any routine clinical chemistry parameters in any treatment group. Treatment with MTC produced dose-dependent decreases in red cell count and hemoglobin and increases in methemoglobin. There were 4 cases (of 307 exposed to MTC) with methemoglobin greater than 3.5% (a threshold set for withdrawal of treatment) which resolved on cessation of treatment [166]. The authors of the study reported that the delivery of the highest dose was impaired due to dose-dependent dissolution and absorption factors of the 100 mg MTC gelatin capsule formulation [130]. At present, MTC (Rember) was discontinued for AD treatment.

7. Pharmacokinetic, Preclinical, and Clinical Studies with Leucomethylthioninium and Derivatives

To the light of this functional and clinical dissociation identified for MT for AD treatment, TauRx Therapeutics developed the synthesis of a novel chemical entity, TRx0237 (LMTX), a second-generation TAI that is a stabilized, reduced form of MTC, in which LMT is available in an anhydrous crystalline form as the dihydromesylate or the dihydrobromide that is stable in an oxygen atmosphere [131]. X-ray crystal structure determinations of TRx0237 demonstrated that the nitrogen atoms at positions 3 and 7 have tetrahedral geometry [131], distinguishing it from LMT, in which the corresponding nitrogen atoms are in a trigonal pyramidal geometry and not protonated. Synthesis of LMT has to be performed under an inert atmosphere because it rapidly oxidizes on exposure to air, while TRx0237 can be manufactured in bulk without the need for deoxygenation and remains stable for at least 2 years in air atmosphere. Thus, TRx0237 represents a new chemical entity that is distinct from both MTC and LMT, and it is highly soluble and exists as a single polymorph, in contrast to MTC, which is far less soluble and demonstrates heterogeneous polymorphism. TRx0237 remains stable for at least 2 years in air atmosphere, is highly soluble, and exists as a single polymorph [168]. An *in vitro* study showed the ability of TRx 0237 in disrupting PHFs isolated from AD brain tissues at the concentration at 0.16 μM [131]. The comparative *in vivo* pharmacological efficacy of MTC and LMT salts (TRx0237: 5–75 mg/kg with oral administration for 3–8 weeks) was assessed in these two novel transgenic tau mouse lines modeling cognitive and motor endophenotypes of AD and FTLD tauopathies [169], namely, impairment in spatial learning (L1) and motor learning (L66), respectively [132]. In this *in vivo* study, both MTC and TRx0237 dose-dependently rescued the learning impairment and restored behavioral flexibility in a spatial problem-solving water maze task in L1 (minimum effective dose: 35 mg MT/kg for MTC, 9 mg MT/kg for TRx0237) and corrected motor learning in L66 (effective doses: 4 mg MT/kg) [132]. Both compounds reduced the number of tau-reactive neurons, particularly in the hippocampus and entorhinal cortex in L1 and in a more widespread manner in L66. The relative superiority of TRx0237 compared with MTC appears to be therefore more likely due to factors related to absorption, metabolism, and distribution, rather than to inherent pharmacodynamic differences.

No direct information on Phase I trials is available. A 4-week Phase II safety study of 250 mg/day of TRx0237 in 9 patients with mild to moderate AD already taking AChEIs and/or memantine began in September 2012 but was terminated in April 2013, reportedly for administrative reasons (ClinicalTrials.gov Identifier: NCT01626391) [170] (Table 1). Currently, three Phase III trials with TRx0237 are

ongoing plus an open label extension study (Table 1). The first study compares a single 200 mg/day dose of the compound to placebo in 700 patients with a diagnosis of either all-cause dementia or AD mild enough to score above an MMSE of 20 (ClinicalTrials.gov Identifier: NCT01689233) [171] (Table 1). Started in November 2012, this trial is ramping up to involve more than 90 sites in North America and Europe, using as primary outcome measures of efficacy the ADAS-Cog 11 and the ADCS-CGIC scales. Temporal lobe brain metabolism is measured by 18F-fluorodeoxyglucose- (FDG-) PET imaging and safety parameters. The second Phase III trial compares 150 and 250 mg/day of TRx0237 to placebo in 833 patients with mild to moderate AD with an MMSE of 14 or higher (ClinicalTrials.gov Identifier: NCT01689246) [172] (Table 1). Begun in 2013, this trial is being conducted at more than 80 sites in North America, Australia, Europe, and Asia, using clinical (ADCS-CGIC), cognitive (ADAS-Cog 11), and safety measures as primary outcomes. The third Phase III trial is evaluating TRx0237 (200 mg/day) in 220 patients affected by the behavioral variant of frontotemporal dementia (bvFTD) and a MMSE above 20 (ClinicalTrials.gov Identifier: NCT01626378) [173] (Table 1). This trial adopted a modified version of the ADCS-CGIC scale as measure of clinical efficacy and the revised Addenbrooke's Cognitive Examination as cognitive measure. This trial was started in August 2013 and will involve 45 sites in North America, Europe, Australia, and Singapore. Finally, an open label extension study targets providing subjects who have completed participation in a Phase II or Phase III trials with TRx0237 continued access to therapy and to evaluate the long-term safety of the compound with an estimated study completion date of January 2017 (ClinicalTrials.gov Identifier: NCT02245568) [174] (Table 1). All three Phase III trials use "active placebo" tablets that include 4 mg of TRx0237 as a urinary and fecal colorant to help maintain blinding; therefore, the placebo group will receive a total of 8 mg/day of TRx0237. These Phase III trials are now fully recruited and results from these ongoing studies involving 250 centers in 22 countries around the world and 1,753 patients with mild to moderate AD or bvFTD are expected in early 2016.

8. Conclusion

In the last two decades, drug discovery and development efforts for AD research have been dominated by the "amyloid cascade hypothesis," focusing on targets defined by this hypothesis and proposing amyloid as the main cause of neural death and dementia. Decreasing the formation or removing Aβ from the brain should attenuate dementia symptoms. Unfortunately, several clinical trials with anti-Aβ agents failed, challenging the hypothesis that Aβ accumulation is the initiating event in the pathological AD cascade and underscoring the need for novel therapeutic approaches and targets. In recent years, tau-based treatments for AD have become a point of increasing focus and current and previous investigational therapies can be grouped into four categories including tau-centric active and passive immunotherapies, microtubule-stabilizing agents, tau protein kinase inhibitors, and TAIs. Among these different approaches, small molecular weight compounds developed to inhibit formation of tau oligomers and fibrils by blocking tau-tau aggregation have already reached the clinic. Among TAIs, MT belongs to a class of diaminophenothiazines that have TAI activity *in vitro* [97, 131]. MTC, in which MT is dosed as the oxidized form MT^+, was investigated in an exploratory Phase II dose-ranging double-blind clinical trial in 321 patients with mild to moderate AD [167]. The minimum effective dose was identified as 138 mg MT/day at both clinical and molecular imaging endpoints at 24 weeks. Treatment at this dose was found to prevent the decline in regional cerebral blood flow, particularly in medial temporal lobe structures and temporoparietal regions.

Given that the delivery of the highest dose of MT was impaired due to dose-dependent dissolution and absorption limitations, four Phase I studies [131] and two preclinical *in vitro* [132] and *in vivo* studies [133] were required to get to the bottom of the bioavailability limitations of the form of MT tested in the Phase II trial [167], setting out the basis for proceeding into Phase III trials with TRx0237 for AD treatment. Therefore, clinical development of MT for AD continues, along with a new form that is more bioavailable and less toxic at higher doses, called TRx0237, representing a new chemical entity that is distinct from both MTC and LMT. A broad-based approach to tau therapy appears favourable due to the numerous pathologic mechanisms for tau pathology. The potential contribution of tau conformation to inhibitory potency of TAIs suggests a route toward selectivity and an important target for future structural studies. In fact, identification of descriptors of inhibitory potency may provide a rational approach to compound optimization [95]. Therefore, the therapeutic benefit that has been reported for MT in Phase II stage and data from current Phase III trials will allow us to glean on the larger scale impact of TRx0237 and its therapeutic potential. However, the role of tau protein in AD pathogenesis should be better understood with future research including investigation of the mechanisms/pathways regulating the degradation of tau as determined by its post-translational state, studies on soluble, nonaggregated forms of tau as a primary AD agent, exploring the role of tau as an enhancer of Aβ-induced degeneration, and clarifying the mechanisms by which pathological forms of tau may negatively impact mitochondrial biology.

In this direction, the observation that acetylation of soluble tau has important effects on the properties of tau, including its stability and aggregation, and that tau acetylation is elevated in patients at early and moderate Braak stages of tauopathy [23] has opened new possibilities of tau-based pharmacological approaches. A recent study has proved that tau acetylated at lysine 174 is one of the toxic species [175]. Increases in levels of this species have been associated with toxicity and cognitive impairment in transgenic mice. Conversely, blocking this acetylation with salsalate, a nonsteroidal anti-inflammatory drug, preserved cognition and led to improvements in pathology. Two to three months of treatment preserved hippocampal volume and reduced the number of NFTs by up to two-thirds. Moreover, treated animals maintained their memories better than their untreated littermates [175]. Because salsalate is an approved

drug with a relatively good safety profile, it might be worth testing in AD patients.

Competing Interests

The authors declare no conflict of interests.

Authors' Contributions

Francesco Panza and Davide Seripa contributed equally to this work.

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Clinical Study

Influence of APOE Genotype on Alzheimer's Disease CSF Biomarkers in a Spanish Population

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Objectives. To evaluate the association between apolipoprotein E (APOE) genotype and cerebrospinal fluid (CSF) levels of Alzheimer's disease (AD) biomarkers and to study the influence of APOE genotype on the development of AD in a Spanish population. *Material and Methods*. The study comprised 29 amnestic mild cognitive impairment (MCI) patients and 27 control subjects. Using ELISA methodology, CSF biomarkers and tau/Aβ ratios were obtained. ANOVA and adjusted odds ratios were calculated. *Results*. We observed the effect of APOE genotype and age on CSF AD variables. The progression to AD was more clearly influenced by CSF AD variables than by age or APOE status. *Conclusions*. APOE status influences CSF AD variables. However, the presence of APOE ε4 does not appear to be a deterministic factor for the development of AD, because CSF variables have a greater influence on progression to the disease. These results confirm previous observations and, to our knowledge, are the first published in a Spanish population.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia in the elderly. Genetic, pathological, and functional studies have shown that an imbalance between production and clearance of amyloid-β (Aβ) peptides in the brain results in accumulation and aggregation of Aβ. Aggregates of toxic Aβ in the form of soluble oligomers, intraneuronal Aβ, and amyloid plaques injure synapses and ultimately cause degeneration and dementia [1].

The APOE gene regulates lipid homeostasis by mediating lipid transport from one tissue or cell to another. The human APOE gene exists as three polymorphic alleles (ε2, ε3, and ε4) and it is known to play an important role in Aβ metabolism [1].

In the National Institute of Aging-Alzheimer's Association (NIA-AA) criteria for the diagnosis of AD, CSF biomarkers have been accepted as evidence of the pathophysiological process, mostly for research purposes [2]. However, there is no consensus on what constitutes a "CSF Alzheimer profile" [3], probably because different factors influence the absolute values of these biomarkers. Although these factors include age [4], there is some controversy about the influence of APOE genotype on CSF biomarkers. Some authors find no association between them [5, 6] while others find a clear relationship [1, 7, 8].

When it has been proved, the relation was found in American and European populations, but the incidence and prevalence rates of AD varied between countries [9]. For example, incidence ranges from 0.04 per 1000 person-years in the UK (people aged 45–65 years) to 16.8 per 1000 person-years in the USA (people aged 65 years and over). Prevalence ranges from Spain (6.7% in those older than 75 years old) to USA (4.9% in those older than 70 years old) [9]. The reasons for this are not completely understood, but the APOE genotype should be a factor to exclude. We hypothesized that

the APOE genotype influences CSF AD biomarker levels in a Spanish population, as it is does in other populations. To our knowledge, this is the first publication on this matter.

2. Material and Methods

2.1. Study Design and Subjects. This longitudinal study included 29 consecutive amnestic MCI patients, according to the Petersen criteria 2006 [10], who attended the cognitive deterioration out-patient clinic of the General Hospital of Alicante in 2011. All participants underwent physical and neurological examination, neuropsychological studies, cerebral magnetic resonance imaging (MRI), blood tests, APOE genotyping, and lumbar puncture (LP) for assessment of CSF AD biomarkers. These patients were reviewed every six months for two years regarding the development of dementia, using both the NIA-AA criteria [2] and the Global Deterioration Scale (GDS) by Reisberg.

A control group comprising 27 consecutive subjects without subjective memory loss or known cognitive deterioration was included. These subjects were patients due to undergo spinal anaesthesia for orthopaedic or nonmalignant urological conditions. Clinical details, including blood test results, were collected. A neuropsychological study and APOE genotyping were performed a few days after the relevant surgical procedure. These subjects were then invited to attend the cognitive impairment out-patient clinic annually for follow-up over two years. Both groups were matched for age, gender, and education.

2.2. Inclusion Criteria. The inclusion criteria required that the patients be over the age of 55 with concordant clinical and neuropsychological diagnosis. In the control group, no patients had subjective memory loss, all mini mental state examination (MMSE) results were above 27, and scores on the informant questionnaire on cognitive decline in the elderly (IQCODE) were below 78. The neuropsychological criteria for the MCI group were MMSE scores between 23 and 26 and IQCODE above 78. Informed consent was obtained before inclusion and also before LP.

2.3. Exclusion Criteria. Exclusion criteria were the presence of dementia or other neurological, psychiatric, or medical disorders which could provoke cognitive deterioration, anticoagulant therapy, failure to obtain informed consent, or a score greater than five using the Depression Scale by Yesavage.

2.4. Procedures. The neurologist responsible for each MCI patient diagnosed single or multiple domain amnestic MCI according to Petersen's criteria [10]. Following this, a neuropsychological report enabled reclassification of the MCI patients. The neuropsychological examination included the following: MMSE, IQCODE, Rey Auditory Verbal Learning, Trail Making Test, and the Geriatric Depression Scale by Yesavage. With these tests we evaluated memory, language, executive function, attention, and visuoconstructive capacity. Alteration of a function was defined as a Z result of −1.5 or less, which was at least 1.5 standard deviations below the mean of the control subjects, in at least one of the tests used to evaluate that function. The same neuropsychological tests were performed in the control group and in the patient group. The GDS and the NIA-AA clinical criteria were used for the diagnosis of AD at the two-year follow-up.

2.5. Extraction and Analysis of CSF. The extraction of CSF was performed between January and December 2011. The samples were collected between 10:00 a.m. and 2:00 p.m. In patients with MCI, the LP was performed by their own neurologist with a 20 × 3.5 gauge needle. The CSF sample was collected in standard tubes and centrifuged, if minimally sanguinolent, before being frozen. Obvious sanguinolent CSF was discarded. The CSF (±1 mL) of control subjects was obtained in the operating theatre by the anaesthetist performing the spinal anaesthesia. After LP, all patients were advised to avoid Valsalva manoeuvres for at least three days.

2.6. Quantification of CSF Biomarker Levels. Quantification was performed using ELISA methodology and INNOTEST reagents from Innogenetics (Ghent, Belgium). The details of this reagent combination for immunoassay and analytic platform have been published previously [11]. All samples were analysed simultaneously after recruitment was completed and blindly regarding the clinical details.

2.7. Analysis of APOE Genotype. The APOE allele status was determined by genotyping with polymerase chain reaction and restriction fragment length polymorphism by gel electrophoresis, as described previously [12]. All serum samples were kept frozen at −70°C until assay.

2.8. Study Variables. Variables were levels of $A\beta_{1-42}$, T-tau, and p-tau_{181p} proteins in the CSF as well as the T-tau/$A\beta_{1-42}$ and p-tau/$A\beta_{1-42}$ ratios. These latter variables are frequently used by many authors and appear to reflect the relationship between the two pathophysiological mechanisms of the disease (amyloid and tau). We classified each subject by APOE genotype as either $\varepsilon 2$ or $\varepsilon 4$ (homozygous or heterozygous) and $\varepsilon 3$ (only homozygous).

2.9. Statistical Analysis. To study the association between APOE genotype and different biomarkers adjusted for age, the analysis of variance (ANOVA) was used for multiple variables. To quantify the association between APOE genotype and progression of Alzheimer's disease-adjusted levels of each biomarker and age, we used a multiple logistic regression model to calculate the adjusted odds ratios (OR) and their 95% confidence intervals. The level of statistical significance used in hypothesis testing was $p < 0.05$. Data analysis was performed with IBM-SPSS version 19.1 software (IBM Corp., Armonk, NY).

2.10. Ethical Criteria. The two pharmaceutical companies who contributed to this project had no role in the study design, data collection and interpretation, or drafting of the

Table 1: Demographic, clinical, and genetic characteristics of the study population.

	MCI patients	Control subjects	Significance level
Number of cases	29	27	n.s.
Gender, male (%)	38	44	n.s.
Age (mean ± SD)	70.34 ± 7.3	68.74 ± 7.5	n.s.
Medical history			
HTA	16	17	
DM	2	8	—
HPL	17	18	
Depression	7	6	
MMSE Folstein	24 ± 0.8	28.48 ± 2.5	0.05
IQCODE	82.89 ± 3.5	52.62 ± 14.9	0.01
Progression at 2 years			
Stable normal	—	22	
Stable MCI	17	—	—
Develop MCI	—	3	
Develop AD	12 ($\varepsilon4 = 8, \varepsilon3 = 4$)	2 ($\varepsilon3 = 1, \varepsilon2 = 1$)	
APOE genotype			
$\varepsilon2$	2	8	
$\varepsilon3$	14	18	
$\varepsilon4$	13	1	
Education (years)	5	6	n.s.

SD = standard deviation. HTA = hypertension. DM = diabetes mellitus. HPL = hyperlipidemia. MMSE = mini mental status examination. IQCODE = informant questionnaire on cognitive decline in the elderly. MCI = mild cognitive impairment. AD = Alzheimer's disease.

Table 2: APOE genotype influence on CSF AD biomarker variables.

CSF variables	APOE genotype	Number of subjects	Mean ± SD	Significance level	Adjusted significance level
Aβ protein (pg/mL)	$\varepsilon2$	10	1320.50 ± 489.51	0.0001	0.001
	$\varepsilon3$	31	1178.23 ± 428.40		
	$\varepsilon4$	15	656.80 ± 201.96		
T-tau protein (pg/mL)	$\varepsilon2$	10	207.00 ± 110.89	0.0001	0.004
	$\varepsilon3$	31	259.39 ± 130.60		
	$\varepsilon4$	15	488.27 ± 284.34		
			Median ($P25$–$P75$)		
p-tau protein (pg/mL)	$\varepsilon2$	10	38 (30.27–42.73)	0.06*	0.02
	$\varepsilon3$	31	44 (42.97–63.94)		
	$\varepsilon4$	15	71 (57.0–95.17)		
Ratio T-tau/Aβ	$\varepsilon2$	10	0.14 (0.08–0.26)	0.18*	0.0001
	$\varepsilon3$	31	0.18 (0.19–0.34)		
	$\varepsilon4$	15	0.74 (0.55–1.06)		
Ratio p-tau/Aβ	$\varepsilon2$	10	0.03 (0.02–0.03)	0.09*	0.0001
	$\varepsilon3$	31	0.03 (0.03–0.07)		
	$\varepsilon4$	15	0.11 (0.08–0.17)		

ANOVA 1 factor. * Chi-square. SD: standard deviation.

final report. This study was fully approved by the University General Hospital of Alicante Ethical Committee.

3. Results

The demographic, genetic, and clinical characteristics of the study population are shown in Table 1. Overall, 17.8% were genotype $\varepsilon2$, 55.3% were $\varepsilon3$, and 26.7% were $\varepsilon4$. At inclusion, there were significant differences in MMSE and IQCODE between both groups. No differences were found in age, medical history, or education level.

The APOE genotype had a clear influence on all CSF AD variables, after the exclusion of the influence of age (adjusted significance level), as indicated in Table 2. As expected, the $\varepsilon4$

Table 3: Influence of age on CSF AD variables.

CSF variables	Age	Number of patients	Mean ± SD	Significance level	Adjusted significance level
Aβ protein (pg/mL)	≥75	16	858.63 ± 457.41	0.03	0.06
	<75	40	1146.1 ± 444.34		
T-tau protein (pg/mL)	≥75	16	381.75 ± 172.46	0.11	0.38
	<75	40	283.18 ± 218.34		
			Median ($P25$–$P75$)		
p-tau protein (pg/mL)	≥75	16	59 (52.0–88.68)	0.01*	0.21
	<75	40	40 (42.1–60.15)		
Ratio T-tau/Aβ	≥75	16	0.42 (0.35–0.74)	0.007*	0.26
	<75	40	0.16 (0.21–0.45)		
Ratio p-tau/Aβ	≥75	16	0.07 (0.05–0.15)	0.004*	0.09
	<75	40	0.03 (0.04–0.07)		

Student's t-test. *Mann-Whitney U test.

Table 4: CSF AD variables after clinical diagnosis at two-year follow-up.

CSF variables	Diagnosis	Number of patients	Mean ± S.D.	Significance level
Aβ protein (pg/mL)	AD	14	743.36 ± 292.64	0.002
	No AD	42	1170.83 ± 462.0	
T-tau protein (pg/mL)	AD	14	511.21 ± 271.6	0.0001
	No AD	42	244.71 ± 131.75	
			Median ($P25$–$P75$)	
p-tau protein (pg/mL)	AD	14	76.0 (63.53–106.75)	0.0001*
	No AD	42	41.5 (40.3–53.94)	
Ratio T-tau/Aβ	AD	14	0.83 (0.52–1.08)	0.0001*
	No AD	42	0.16 (0.09–0.32)	
Ratio p-tau/Aβ	AD	14	0.11 (0.08–0.18)	0.0001*
	No AD	42	0.03 (0.03–0.05)	

Student's t-test. *Mann-Whitney U test.

subjects showed lower Aβ42 and higher protein levels of T-tau and p-tau and ratios of T-tau and p-tau to Aβ42 than the other genotypes. Table 3 shows that age influenced almost all the CSF variables, but this influence was lost after adjustment.

Table 4 shows the differences in CSF AD variables after the clinical diagnosis at the two-year follow-up. As expected, patients who had developed AD at the follow-up had lower Aβ42 levels and higher protein levels of T-tau and p-tau and ratios of T-tau and p-tau to Aβ42 at inclusion.

The influence of the APOE genotype and age on progression to AD at the two-year follow-up is shown in Table 5. Older patients and those with genotype ε4 had higher adjusted OR.

Table 6 shows the influence of CSF AD variables on progression to AD at the two-year follow-up, after adjusting for APOE genotype and age. T-tau protein levels and ratios had the highest adjusted OR for progression to AD, whereas the Aβ42 levels had the lowest.

Cerebral MRI excluded structural lesions. There were no differences in white matter hyperintensities or degree of cerebral atrophy between groups.

Table 5: Influence of APOE genotype and age over the progression to AD.

	Frequency of progression to AD	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Genotype			
ε2	10.0% (1/10)	1	1
ε3	12.9% (4/31)	1.3 (0.1–13.5)	0.9 (0.08–10.5)
ε4	60.0% (9/15)	13.5 (1.3–136.0)	4.7 (0.3–69.9)
Age			
≥75	43.8% (7/16)	3.7 (1.1–13.2)	2.9 (0.6–13.4)
<75	17.5% (7/40)	1	1

4. Discussion

In our results, the APOE ε4 status was associated with lower CSF Aβ42, as well as higher CSF T-tau and p-tau protein levels and tau/Aβ42 ratios, in the early prodromal stages of AD patients and in the control subjects, taking into account the influence of age. In the last few years, the APOE ε4

Table 6: Influence of CSF AD variables on progression to AD.

	Frequency of progression to AD	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Aβ protein levels			
≤710 (pg/mL)	60.0% (9/15)	10.8 (2.7–43.)	3.8 (0.6–22.1)
≥711 (pg/mL)	12.2% (5/41)	1	1
T-tau protein levels			
≤421 (pg/mL)	71.4% (10/14)	1	1
≥422 (pg/mL)	9.5% (4/42)	23.7 (5.0–112.0)	13.9 (2.2–87.0)
p-tau protein levels			
≤63 (pg/mL)	11.6% (5/43)	1	1
≥64 (pg/mL)	69.2% (9/13)	17.1 (3.8–76.8)	10.34 (1.84–58.14)
T-tau/Aβ ratio			
<0.54	9.5% (4/42)	1	1
≥0.54	71.4% (10/14)	23.75 (5.0–112.0)	19.83 (2.64–148.56)
p-tau/Aβ ratio			
<0.10	9.5% (4/42)	1	1
≥0.10	71.4% (10/14)	23.75 (5.0–112.0)	13.91 (2.18–88.74)

status has been accepted as important in CSF AD biomarker levels [4], as witnessed in our findings as well. However, the APOE genotype showed a clearer influence on all the variables obtained from the CSF AD analysis, especially on Aβ42 levels and ratios, as published previously [7, 8].

The influence of the APOE ε4 genotype on CSF AD biomarkers has been widely described [13–16], but there is no consensus as some authors find no association between them [5, 6]. Our results agree with the first group of publications. It is currently accepted that APOE isoforms differentially regulate Aβ aggregation and clearance in the brain and have distinct functions in regulating brain lipid transport, glucose metabolism, neuronal signalling, neuroinflammation, and mitochondrial function [1, 7]. The effect of the APOE genotype on amyloid deposition has been shown in middle-aged and older cognitively healthy adults [8], as well as in patients with MCI and AD [17–22].

All these basic, clinical, and laboratory data underscore the importance of considering the APOE genotype when evaluating CSF biomarkers, because it could be at least partially responsible for the observed disease heterogeneity [21]. A gene described recently as SUCLG2 (Succinyl-CoA Ligase) appears to determine CSF Aβ levels and attenuate cognitive decline in AD [23]. Moreover, other genes have been found to enhance the risk of sporadic AD, such as phosphatidylinositol-binding clathrin assembly protein (PICALM) [24], or the translocase of the outer mitochondrial membrane (TOMM40) [25, 26], but, to our knowledge, their influence on CSF AD biomarkers has not yet been studied.

Based on our results, the APOE genotype has less influence than every CSF AD variables in the development of AD in MCI patients and control subjects, except Aβ42. These data agree with recent publications showing that the APOE genotype did not significantly improve the prediction of AD in MCI patients [22, 27]. However, there is broad evidence of the ability of CSF AD biomarkers to improve the prediction of AD in MCI patients [11, 28–32] and elderly control subjects [33, 34], despite the difficulties in achieving global standardization measures [35] and the optimal method to evaluate the results [3].

In this study, the progression rate of control subjects to AD was near the expected rate (3.5% per year). However, the progression rate of MCI patients to AD was slightly higher than what would be expected (about 40% at two years) and highlights the importance of the utilisation of that clinical entity in these studies.

The present study has some limitations. First, even though our sample size was small, we nevertheless obtained results similar to those of previous studies. Second, we did not evaluate all known AD biomarkers nor did we use PIB-PET or advanced MRI techniques. Finally, we did not have pathological confirmation of the diagnosis of AD.

In conclusion, the APOE ε4 status is associated with lower CSF Aβ42 as well as higher CSF T-tau and p-tau protein levels and tau/Aβ ratios, in patients in the early prodromal stages of AD and in control subjects. However, the presence of APOE ε4 does not seem to be a deterministic factor for the development of AD. To the best of our knowledge, these are the first results described in a Spanish population.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Inhibition of ACE Retards Tau Hyperphosphorylation and Signs of Neuronal Degeneration in Aged Rats Subjected to Chronic Mild Stress

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With increasing life expectancy, Alzheimer's disease (AD) and other types of age-associated dementia are on the rise worldwide. Treatment approaches for dementia are insufficient and novel therapies are not readily available. In this context repurposing of established drugs appears attractive. A well-established class of cardiovascular drugs, which targets the angiotensin II system, is such a candidate, which currently undergoes a paradigm shift with regard to the potential benefit for treatment of neurodegenerative symptoms. In search for additional evidence, we subjected aged rats to chronic unpredictable mild stress, which is known to enhance the development of AD-related neuropathological features. We report here that four weeks of chronic mild stress induced a strong upregulation of the hippocampal angiotensin-converting enzyme (*Ace*) at gene expression and protein level. Concomitantly, tau protein hyperphosphorylation developed. Signs of neurodegeneration were detected by the significant downregulation of neuronal structure proteins such as microtubule-associated protein 2 (*Map2*) and synuclein-gamma (*Sncg*). *Ace* was involved in neurodegenerative symptoms because treatment with the brain-penetrating *ACE* inhibitor, captopril, retarded tau hyperphosphorylation and signs of neurodegeneration. Moreover, *ACE* inhibitor treatment could counteract glutamate neurotoxicity by preventing the downregulation of glutamate decarboxylase 2 (*Gad2*). Taken together, *ACE* inhibition targets neurodegeneration triggered by environmental stress.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia. While genetic factors are causally linked to familial AD, the pathogenesis of the predominant late-onset sporadic AD is diverse and less defined. Age is one of the best-documented risk factors for sporadic AD, which acts in concert with a wide array of brain insult-promoting vascular and metabolic factors such as hypertension, ischemia, diabetes, high cholesterol, and different forms of environmental stress and stress-related psychiatric symptoms, that is, depression and anxiety [1–3].

Experimental models of AD often reproduce genetic alterations whereas the impact of additional brain-damaging factors is more difficult to assess. The chronic unpredictable mild stress (CUMS) model imitates psychiatric risk factors such as psychological, psychosocial, and physical stress [4]. In agreement with the well-established impact of stress on neuronal viability, the model promotes signs of neuronal degeneration such as decreased synaptic transmission at

hippocampal CA1-CA3 synapses, impaired neurogenesis, and cognitive dysfunction [5–7]. The sensitivity to stress in this model increases with age, a major factor of AD pathogenesis [8]. The relationship to AD is further supported by several studies, which show that the CUMS procedure worsens disease progression in genetic AD mouse models [9–11].

Moreover, the chronic mild stress protocol induces major neuropathological hallmarks of AD, that is, enhanced β-amyloid (Aβ) generation, tau hyperphosphorylation, and the appearance of various other AD markers [9, 11, 12]. In this respect, the chronic mild stress model could complement genetic AD models because the CUMS model solely relies on endogenously expressed proteins. In view of the recent failure of large clinical trials, which targeted β-amyloid plaques [13, 14], such alternative models, which imitate the disease process before the accumulation of Aβ plaques, could become of substantial value.

Inefficient Aβ plaque-targeting approaches also raise the necessity to identify players in AD pathogenesis, which could interrupt the disease process at an earlier stage [15]. For one player in AD pathogenesis a paradigm shift is currently on the way, which is the angiotensin system, notably the angiotensin-converting enzyme (*ACE*). While many studies considered *ACE* primarily as an enzyme that could promote proteolysis of β-amyloid in vitro [16–18], recent experimental and clinical studies provide strong evidence that inhibition of *ACE*-dependent angiotensin II generation in vivo could actually reduce signs of neurodegeneration in experimental AD models [19, 20] and slow the cognitive decline of patients with Alzheimer's disease [21–25].

In view of this emerging development, we aimed to further analyse the impact of *ACE* inhibition on early signs of neurodegeneration and applied the chronic unpredictable mild stress model in our study.

2. Materials and Methods

2.1. Chronic Unpredictable Mild Stress Model. Male Wistar rats (15 months of age) were subjected to the chronic unpredictable mild stress (CUMS) battery for four weeks. The CUMS protocol was performed essentially as described previously [8, 9]. After four weeks of stress, more than 90% of stressed rats showed signs of anhedonia as documented by a decrease in sucrose preference compared to nonstressed controls (i.e., <50% of sucrose consumption compared to nonstressed controls). The age-matched control group was housed under standard conditions and had free access to food and water. The captopril treatment group received captopril in drinking water (50 mg/kg/day, dissolved fresh every day) during the CUMS protocol. After 28 days of stress, at an age of 16 months, 4 h before the beginning of the dark phase, rats were anesthetized with ketamine and xylazine (100 mg/kg and 10 mg/kg, i.p.), and brains were removed and processed for immunohistology [9, 20]. For whole genome microarray gene expression profiling, biochemical analyses, and immunoblotting, the hippocampus was dissected out on ice and processed for further use as detailed below.

All animal experiments were performed in accordance with the NIH guidelines and were approved by the local committee on animal research (MRC, Ain Shams University Hospitals, Cairo, Egypt).

2.2. Whole Genome Microarray Gene Expression Profiling of Hippocampal Gene Expression. For whole genome microarray gene expression analysis, total hippocampal RNA was isolated from stressed rats and age-matched nonstressed control rats. Total RNA was processed for whole genome microarray gene expression profiling and hybridized to the GeneChip Rat Genome 230 2.0 Array (more than 31 000 probe sets, Affymetrix) as detailed previously [20]. The total RNA from 3 animals was pooled for one gene chip, and two gene chips are presented for each group. Microarrays were scanned with the Affymetrix GeneChip Scanner 7G, and signals were processed with a target value of 300 using GCOS (version 1.4, Affymetrix). Applied selection criteria for differently expressed genes (2-fold change requirement, just alpha, no false discovery correction, and $P < 0.05$) were validated specifically for drug treatment effects [20] and follow the guidelines of the Microarray Quality Control (MAQC) project for the identification of reproducible gene lists [26, 27]. Probe sets with significant difference ($P < 0.05$ and ≥2-fold difference, with call present and/or signal intensity ≥100) between stressed rats and nonstressed, age-matched control rats were used for GO classification. Microarray data are available at the NCBI GEO repository (GSE72062).

Gene expression of *Ace* and *Map2* in the hippocampus of stressed rats and nonstressed controls was determined by real-time quantitative (q) RT-PCR with a LightCycler 480 (Roche Diagnostics). Sequences of the forward and reverse primers were as follows: *Ace* forward 5′-GATTGCAGC-CGGGCAACTTTTC-3′; *Ace* reverse 5′-CGGATCCGA-TGATCCTTCGC-3′; *Map2* forward 5′-CACTGGAAGAAG-CCTCGAAGA-3′; *Map2* reverse 5′-CACGGGCATTTC-GATGAACC-3′.

2.3. Immunoblotting, Immunohistology, and Biochemical Methods. Immunoblot detection of hippocampal proteins was performed with hippocampal tissue extracts as described [9, 20]. For immunohistology and immunofluorescence analysis, we used paraffin-embedded brain sections obtained from stressed and nonstressed rats (8 μm, taken at 50 μm intervals, 10–15 sections per set). The following antibodies were used for immunoblotting, immunofluorescence, and immunohistology: anti-Ace antibodies raised in rabbit against an antigen corresponding to amino acids 720–750 of mouse Ace [20]; anti-angiotensin II antibodies raised in rabbit against synthetic angiotensin II [20]; anti-APP/Aβ antibodies raised in rabbit against a peptide corresponding to amino acids 672–714 of human APP [20]; anti-AT1R antibodies raised in rabbit against an antigen corresponding to amino acids 306–359 of the mouse Agtr1a sequence [20]; anti-Gad65 antibody (clone N-GAD65, developed in mouse against a synthetic peptide corresponding to amino acids 4–22 of human GAD65); anti-Grin3a antibodies raised in rabbit against a synthetic peptide derived from

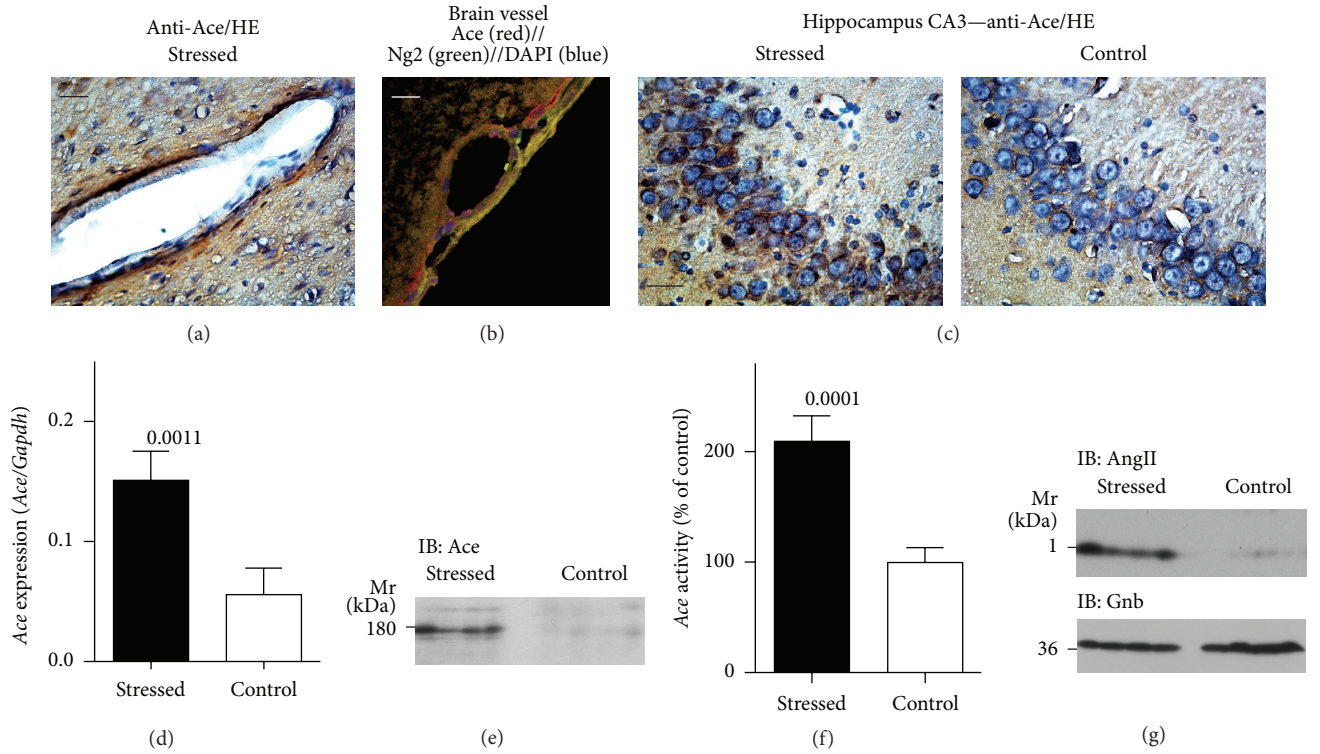

FIGURE 1: Chronic unpredictable mild stress induced upregulation of hippocampal Ace protein and gene expression. (a) Immunohistological localization of Ace in a brain vessel of a stressed rat. Nuclei were stained with hematoxylin (HE); bar: 20 μm. (b) Immunofluorescence localization of Ace (red) and the pericyte marker Ng2 (green) in a brain vessel of a stressed rat. Ace was detected with affinity-purified rabbit anti-Ace antibodies followed by $F(ab)_2$ fragments of Alexa Fluor 546-labeled (red) secondary antibodies, and Ng2 was detected with affinity-purified murine anti-NG2 antibody followed by $F(ab)_2$ fragments of Alexa Fluor 488-labeled (green) secondary antibodies. Nuclei were stained with DAPI (bar: 20 μm). (c) Immunohistological localization of Ace in hippocampal CA3 neurons of a stressed rat (left) relative to a nonstressed control (right). Nuclei were stained with hematoxylin (HE); bar: 20 μm. Histological experiments are representative of 4 rats/group (a–c). (d) Hippocampal *Ace* gene expression was determined by qRT-PCR and is presented as the ratio of *Ace*/*Gapdh* expression (±s.d.; $n = 4$; $P = 0.0011$). (e) Immunoblot detection of the hippocampal Ace protein with anti-Ace antibodies in stressed rats relative to nonstressed controls ($n = 4$/group). (f) Hippocampal *Ace* activity of stressed rats relative to nonstressed controls (i.e., 100%; ±s.d., $n = 8$; $P = 0.0001$). (g) The hippocampal angiotensin II content was determined by immunoblot in stressed rats relative to nonstressed controls (upper panel). The lower panel shows a control immunoblot, which detects Gnb ($n = 4$/group).

the extracellular domain of rat Grin3a (Abcam); anti-MAP2 mouse monoclonal antibody generated against bovine microtubule-associated protein, MAP2 (Clone AP-20); anti-chondroitin sulfate proteoglycan NG2 mouse monoclonal antibody generated against a truncated form of NG2 (MAB5384); anti-PHF-tau pSer202 and pThr 205 antibody (mouse monoclonal antibody, clone AT8; Pierce); and anti-Sncg antibodies raised in goat against an internal region of human SNCG (Santa Cruz Biotechnology).

Hippocampal *Ace* activity was assessed with a fluorogenic substrate (Abz-FRK(Dnp)-P; Biomol) as described [20], and determination of hippocampal glutamic acid decarboxylase (Gad) enzyme activity was performed as detailed previously [8].

2.4. Statistical Analysis. The results are presented as means ± s.d. We used unpaired two-tailed Student's t-test for comparisons between two groups. Statistical significance was set at a P value of <0.05, unless otherwise specified.

3. Results

3.1. Chronic Unpredictable Mild Stress Induced Upregulation of Hippocampal Ace Protein and Gene Expression. Chronic stress worsens the progression of AD in patients and transgenic disease models [9–11] but underlying mechanisms are incompletely understood. Because the angiotensin-converting enzyme (*ACE*) is upregulated in brains of AD patients and mice [20], we asked for an interrelationship between *Ace* and stress. We used the chronic unpredictable mild stress model to investigate the role of *Ace* in stress-induced signs of neurodegeneration. Initially, we analysed the localization of *Ace* in stressed and nonstressed rat brain. Complementary to previous data with AD mice [20], endothelial cells lining a brain vessel of a stressed rat showed strong immunostaining for *Ace* (Figure 1(a)). Immunofluorescence localization of the pericyte marker, chondroitin sulfate proteoglycan *Ng2*, indicated that *Ace* was predominantly present in vascular endothelial cells whereas adjacent pericytes showed weaker staining for *Ace* (Figure 1(b)).

We further investigated the localization of *Ace* by immunohistology and focused on the hippocampal area because chronic mild stress induces profound changes at hippocampal CA3-CA1 synapses [7], that is, the brain area, which is highly susceptible to AD-related neuronal damage. In agreement with previous results [20], immunohistology analysis detected Ace immunoreactivity in cell bodies of hippocampal CA3 neurons (Figure 1(c)). Moreover, immunohistology revealed a strong increase in Ace immunoreactivity in hippocampal CA3 neurons of a stressed rat relative to that of a nonstressed control (Figure 1(c)).

In agreement with the increased Ace immunoreactivity, the gene expression level of hippocampal *Ace* was also significantly increased in stressed rats, that is, the hippocampal *Ace* gene expression level was 2.72 ± 0.43-fold higher in stressed rats compared to that in nonstressed controls ($P = 0.0011$; Figure 1(d)). Concomitantly, with *Ace* gene expression, the increased hippocampal Ace protein level of stressed rats was detected in immunoblot (Figure 1(e)). The increase in Ace protein was accompanied by a significantly elevated hippocampal *Ace* activity; that is, the hippocampal *Ace* activity was 2.09 ± 0.24-fold higher ($P = 0.0001$) in stressed rats compared to that in nonstressed controls (Figure 1(f)). The elevated *Ace* activity was also reflected by an increased hippocampal angiotensin II content in stressed rats relative to nonstressed controls (Figure 1(g)). Taken together, chronic unpredictable mild stress led to a significant upregulation of the Ace protein in the hippocampal area of stressed rats. As a consequence of *Ace* upregulation, the hippocampal angiotensin II content in stressed rats was elevated compared to that in nonstressed controls.

3.2. Tau Hyperphosphorylation in the Hippocampus of Stressed Rats. The strong upregulation of *Ace* in the hippocampus of stressed rats prompted us to search for signs of hippocampal neurodegeneration because previous data showed a causal relationship between *Ace* upregulation and signs of neurodegeneration in a genetic model of AD [20]. In this context we determined the level of tau hyperphosphorylation as a hallmark of AD. Immunohistological analysis demonstrated that four weeks of chronic unpredictable mild stress induced a strong increase in the level of phosphorylated tau protein in the hippocampus of an aged rat, as detected in a hippocampal section with anti-PHF (AT8) antibody (Figure 2(a)). Immunohistology also revealed that the increased tau phosphorylation was predominant in neurons of the CA1-CA3 region (Figure 2(a)). In contrast, PHF antibody staining was negligible in hippocampal neurons of the nonstressed control (Figure 2(a)).

Immunoblot detection of phosphorylated tau in hippocampal tissue extracts confirmed the immunohistology data and showed an increased level of hyperphosphorylated tau protein in stressed rats compared to that in nonstressed controls (Figure 2(b)). As an additional control, the total level of hippocampal tau protein was not different between stressed and nonstressed rats (Figure 2(b), lower panel). Taken together, the upregulation of *Ace* and angiotensin II in hippocampal neurons of stressed rats was accompanied by tau hyperphosphorylation.

Ace-dependent angiotensin II generation promotes signs of hippocampal neurodegeneration by activation of the AT1 receptor (AT1R) in a genetic AD model [20]. Complementary to those data, immunofluorescence analysis of a stressed rat brain showed AT1R immunoreactivity in hippocampal CA1 neurons, which also displayed accumulation of hyperphosphorylated tau protein (Figure 2(c)). Concomitantly, neuronal loss of a highly PHF-positive CA1 neuron was detected (Figure 2(c)). Thus, stress promoted *Ace*-dependent release of the AT1R-stimulating angiotensin II peptide together with tau hyperphosphorylation in hippocampal AT1R-positive neurons.

3.3. Whole Genome Microarray Gene Expression Profiling Detected Stress-Induced Signs of Hippocampal Neurodegeneration. We further investigated the impact of stress on signs of neurodegeneration and performed whole genome microarray gene expression profiling of hippocampal gene expression. Gene ontology (GO) analysis searched for differently expressed neuron-specific genes by applying neuron-specific GO terms, that is, neuron, axon, dendrite, and synapse. Following established selection criteria for significantly different probe sets (≥2-fold difference and $P < 0.05$), the GO analysis documented that stress triggered the significant downregulation of neuron-specific genes, which were categorized according to the cellular localization of the respective proteins, that is, cytoplasm and membrane (Figures 3(a) and 3(b)).

The list of significantly downregulated genes encompasses major neuronal structure proteins such as microtubule-associated protein 2 (*Map2*) and synuclein-gamma, *Sncg* (Figure 3(a)). Because the loss of neuronal *Map2* is a characteristic feature of AD-related neurodegeneration, which is causally linked to stress-induced tau hyperphosphorylation [9, 28], we performed immunolocalization of the hippocampal *Map2*. Immunohistology analysis detected the decreased *Map2* content in hippocampal CA1 neurons of a stressed rat compared to that of a nonstressed control (Figure 3(c)). The stress-induced downregulation of the hippocampal Map2 protein was further confirmed by qRT-PCR (Figure 3(d)). Together these data indicate that chronic unpredictable mild stress led to tau hyperphosphorylation and decreased the level of neuron-specific genes such as *Map2*. The decreased expression of neuronal structure proteins complements previous data, which show that chronic mild stress triggers alterations of hippocampal synapses and promotes a decrease in dendritic spine density [7].

The expression of glutamic acid decarboxylase 2 (*Gad65/Gad2*) was also downregulated by chronic unpredictable mild stress (Figure 3(a)). The decreased *Gad65* expression could account for the significantly decreased hippocampal Gad enzyme activity, which was measured in hippocampal tissue extracts of stressed rats compared to that of nonstressed controls (Figure 3(e)). The ensuing decreased metabolism of the excitatory neurotransmitter, glutamate, could be partially responsible for the increased tau phosphorylation under chronic mild stress [8, 29]. In addition to the decreased glutamate degradation, the expression of the inhibitory and

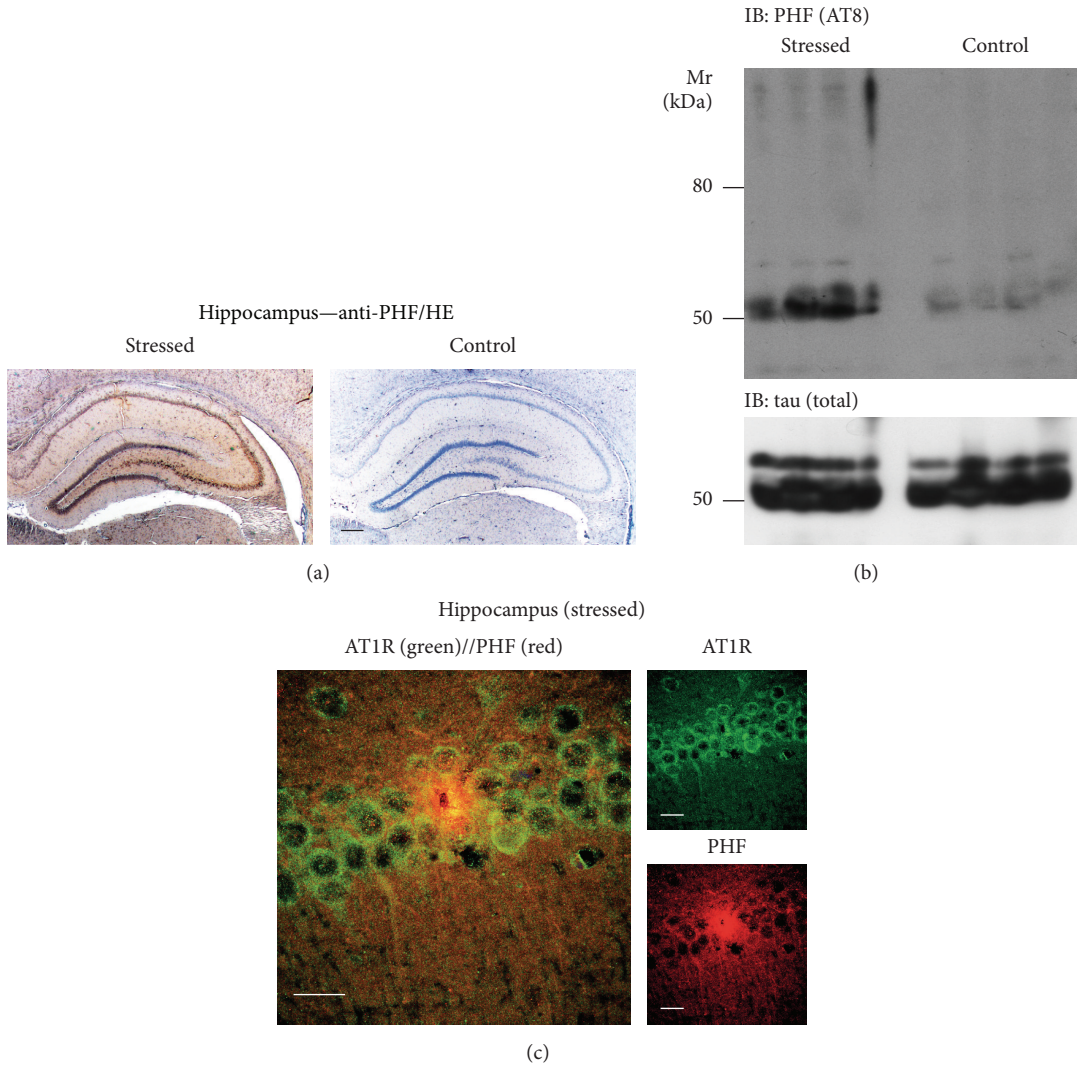

Figure 2: Tau hyperphosphorylation in the hippocampus of stressed rats. (a) Immunohistological detection of the hyperphosphorylated tau protein (anti-PHF) in the hippocampus of a stressed rat (left panel) relative to a nonstressed control (right panel). Nuclei were counterstained with hematoxylin (HE; bar: 200 μm). (b) Immunoblot detection of hyperphosphorylated tau in hippocampal extracts of stressed rats relative to nonstressed controls was performed with anti-PHF antibody (IB: PHF (AT8)). The lower panel is a control immunoblot detecting total tau protein (n = 4/group). (c) Immunofluorescence localization of AT1R (green) and PHF (red) in hippocampal CA1 neurons of a stressed rat (bar: 20 μm). The AT1R was detected with affinity-purified rabbit anti-AT1R antibodies followed by $F(ab)_2$ fragments of Alexa Fluor 488-labeled (green) secondary antibodies, and hyperphosphorylated tau was visualized with affinity-purified mouse anti-PHF antibody followed by $F(ab)_2$ fragments of Alexa Fluor 546-labeled secondary antibodies (red). Histological experiments are representative of 4 rats/group ((a) and (c)).

neuroprotective NMDA receptor subtype *Grin3a* (NR3A) [30, 31] was also downregulated by stress (Figure 3(b)). Immunohistology data complemented the microarray study and showed abundant membrane-localized Grin3a protein in hippocampal CA1 neurons of a nonstressed control whereas the neuronal Grin3a protein was barely detectable in the hippocampal neurons of a stressed rat (Figure 3(f)). As a consequence of stress-induced *Grin3a* (NR3A) downregulation, glutamate excitotoxicity, for example, triggered by decreased Gad enzyme activity, could be further augmented [30, 31]. Taken together, whole genome microarray gene expression profiling detected signs of neurodegeneration and markers of enhanced glutamate excitotoxicity in the hippocampus of stressed rats.

3.4. Treatment with the Brain-Penetrating ACE Inhibitor, Captopril, Retarded Signs of Neurodegeneration Induced by Chronic Mild Stress. In view of the strongly upregulated Ace protein, we asked whether treatment with the brain-penetrating *ACE* inhibitor, captopril, could affect the process of neurodegeneration triggered by stress. Aged rats were treated with captopril during the chronic unpredictable mild stress procedure. As a sign of neurodegeneration, we

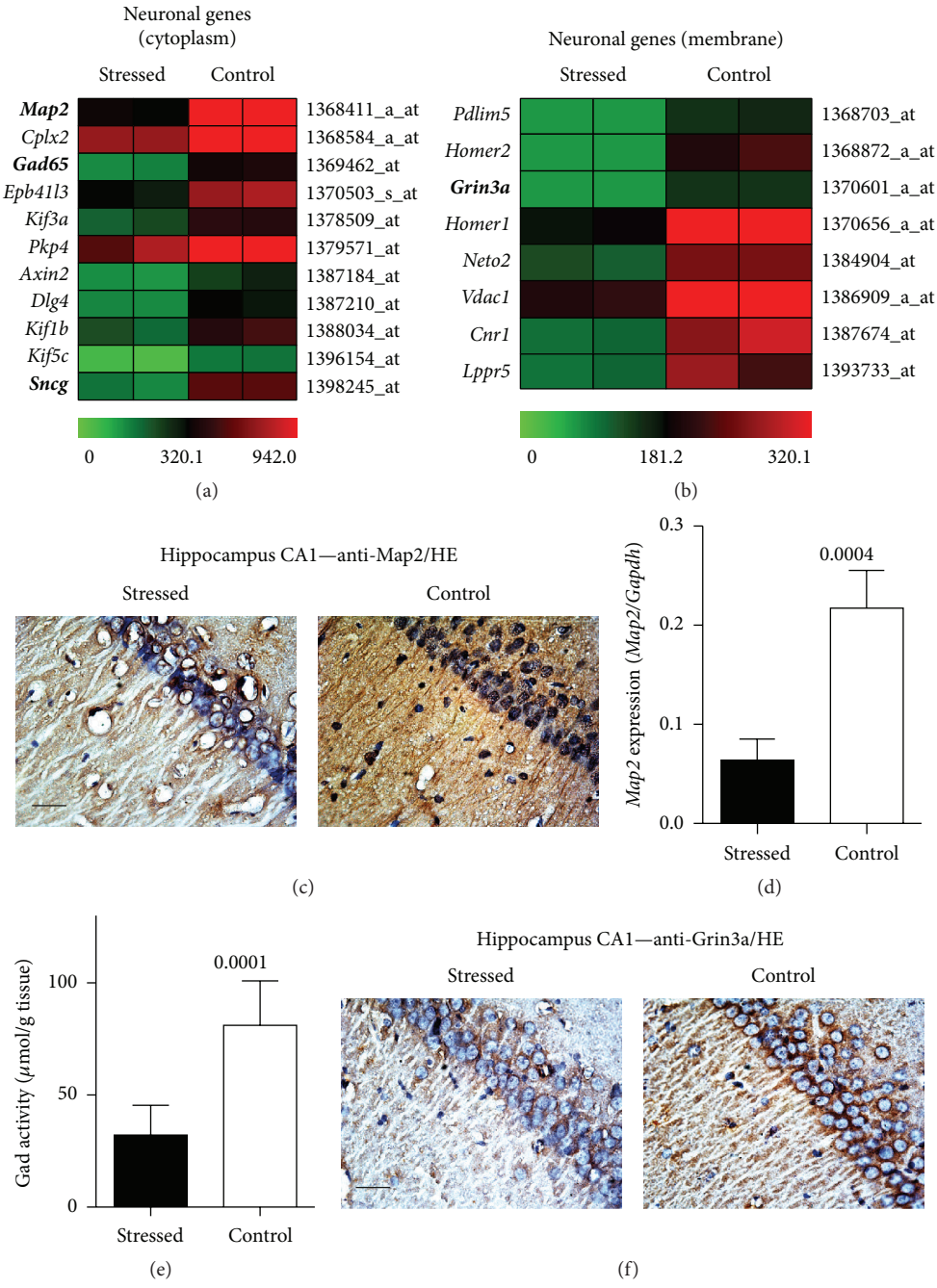

Figure 3: Whole genome microarray gene expression profiling detected stress-induced signs of hippocampal neurodegeneration. (a) and (b) Whole genome microarray gene expression profiling of hippocampal gene expression documents that stress promoted the significant downregulation of probe sets detecting neuron-specific genes with cytosolic localization (a) or membrane localization (b) according to GO analysis. All probe sets were significantly downregulated in the hippocampus of stressed rats relative to nonstressed controls ($P < 0.05$ and ≥2-fold difference). The heat map visualizes signal intensities (centered to the median value). (c) Immunohistological detection of Map2 in hippocampal CA1 neurons of a stressed rat (left panel) relative to a nonstressed control (right panel; bar: 20 μm). (d) Hippocampal *Map2* expression level was determined by qRT-PCR and is presented as the ratio of *Map2*/*Gapdh* expression (±s.d.; $n = 4$; $P = 0.0004$). (e) Hippocampal Gad activity of stressed rats relative to nonstressed controls (±s.d.; $n = 8$; $P = 0.0001$). (f) The decreased hippocampal Grin3a protein level of a stressed rat (left panel) relative to that of a nonstressed control (right panel) was detected by immunohistology in hippocampal CA1 neurons; bar: 20 μm. Immunohistology data are representative of 4 rats/group ((c) and (f)).

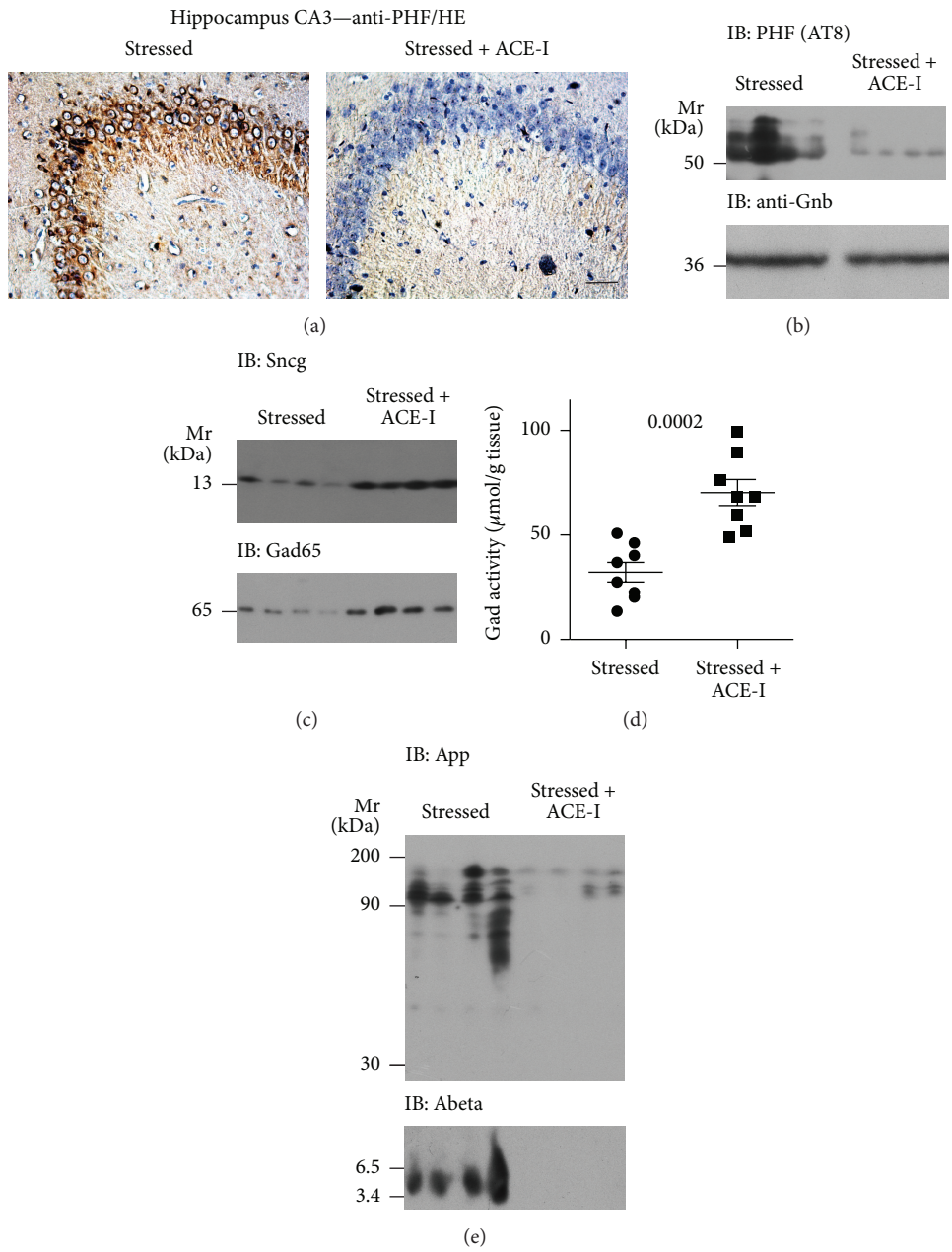

Figure 4: Treatment with the brain-penetrating *ACE* inhibitor captopril retarded signs of neurodegeneration induced by chronic mild stress. (a) Immunohistological detection of hyperphosphorylated tau protein with anti-PHF antibody in hippocampal CA3 neurons of a stressed rat without treatment (left panel) relative to a stressed rat treated with the *ACE* inhibitor captopril (Stressed + ACE-I) during the stress protocol (right panel); bar: 40 μm. Immunohistological experiments are representative of 4 rats/group. (b) Immunoblot detection of hyperphosphorylated tau protein (IB: PHF (AT8)) in hippocampal extracts of stressed rats without treatment relative to stressed rats treated with the *ACE* inhibitor captopril (Stressed + ACE-I) during the stress protocol (n = 4/group). (c) Immunoblot detection of Sncg (upper panel) and Gad65 (lower panel) in hippocampal extracts of stressed rats without treatment relative to stressed rats with *ACE* inhibitor (Stressed + ACE-I) treatment (n = 4/group). (d) Hippocampal Gad activity of stressed rats without treatment relative to stressed rats treated with the *ACE* inhibitor (Stressed + ACE-I) captopril (n = 8/group; P = 0.0002). (e) Immunoblot detection of the amyloid beta (A4) precursor protein, App (IB: App; upper panel), and β-amyloid (IB: Abeta; lower panel) in hippocampal extracts of stressed rats without treatment relative to stressed rats treated with the *ACE* inhibitor captopril (Stressed + ACE-I) during the stress protocol (n = 4/group).

determined the hippocampal tau phosphorylation. Immunohistology analysis revealed a substantially decreased level of hyperphosphorylated tau protein in hippocampal CA3 neurons of a stressed rat with captopril treatment relative to that of a stressed control without *ACE* inhibitor treatment (Figure 4(a)). Immunoblot detection of hyperphosphorylated tau confirmed immunohistology data and demonstrated the decreased hyperphosphorylated tau protein content in

the hippocampus of stressed rats with captopril treatment relative to that of stressed rats without treatment (Figure 4(b)). Concomitantly, with the decreased tau phosphorylation, the stress-induced loss of synuclein-gamma (*Sncg*) was also retarded by *ACE* inhibition with captopril (Figure 4(c), upper panel).

We next assessed the hippocampal Gad65 protein level and activity. Immunoblot analysis showed that captopril treatment counteracted the stress-induced decrease in hippocampal Gad65 protein (Figure 4(c), lower panel). The preserved Gad65 protein was accompanied by a significantly higher hippocampal Gad activity in stressed rats with captopril treatment compared to that in stressed rats without treatment (Figure 4(d)). Taken together, our data indicate that inhibition of *ACE* with a brain-permeable *ACE* inhibitor retarded the stress-induced tau hyperphosphorylation and counteracted the downregulation of Gad activity triggered by chronic mild stress.

Previous studies indicate that chronic mild stress enhances the generation of Aβ as a major neuropathological feature of AD [12] while *ACE* inhibition decreases the hippocampal Aβ level, as documented in a genetic AD model [20]. The increased Aβ generation could be a consequence of the stress-induced increase in the steady-state level of the amyloid beta (A4) precursor protein (App) [32]. Complementary to those data, immunoblot analysis detected the high hippocampal App load in stressed rats without treatment whereas the hippocampal App content was substantially decreased in stressed rats with *ACE* inhibitor treatment (Figure 4(e), upper panel). Concomitantly, immunoblot analysis revealed that the hippocampal Aβ level was also decreased in stressed rats with ACE inhibitor treatment compared to that in stressed rats without treatment (Figure 4(e), lower panel).

4. Discussion

4.1. Chronic Unpredictable Mild Stress Triggered Signs of Hippocampal Neurodegeneration. Our study applied the chronic unpredictable mild stress model, which imitates major features of sporadic AD such as tau hyperphosphorylation, enhanced Aβ generation, and other signs of dendritic and synaptic degeneration [7, 12]. In agreement with those previous studies, we also found that chronic mild stress triggered signs of hippocampal neurodegeneration, which was documented by hippocampal tau hyperphosphorylation, decreased levels of major neuronal proteins such as *Map2* and *Sncg*, and an increased level of Aβ-generating App. Concomitantly, with AD-related signs of neurodegeneration, glutamate excitotoxicity-enhancing features appeared such as decreased Gad65 protein level and enzyme activity and decreased hippocampal expression of the glutamate-inhibitory NMDA receptor subtype, *Grin3a* (NR3A). The ensuing sensitization of glutamate excitotoxicity could contribute to stress-induced signs of neurodegeneration because glutamate excitotoxicity is a major factor, which contributes to neurodegeneration and tau hyperphosphorylation [29].

4.2. Chronic Mild Stress Induced Upregulation of Hippocampal Ace Protein and Activity. In search for additional stimuli, which could account for the development of stress-induced signs of neurodegeneration, our study detected a significantly increased hippocampal protein level of the angiotensin II-generating enzyme, *Ace*. Immunohistology analysis localized the upregulated Ace protein not only in brain vessels but also in neuronal cell bodies of the hippocampus. Concomitantly, an increased hippocampal *Ace* activity and angiotensin II peptide level were detected. The upregulation of neuronal *Ace* expression and protein level could be a direct consequence of the stress-induced activation of the hypothalamic pituitary adrenocortical (HPA) axis because several studies found that *ACE* expression was triggered by glucocorticoids and glucocorticoid receptor activation [33–35].

4.3. ACE Inhibition Retarded Signs of Neurodegeneration Induced by Chronic Mild Stress. The upregulated Ace protein could be causally involved in signs of neurodegeneration triggered by chronic mild stress because treatment with the brain-penetrating *ACE* inhibitor, captopril, retarded the development of signs of stress-induced neurodegeneration, that is, tau hyperphosphorylation, downregulation of neuronal proteins, decreased Gad enzyme activity, and increased hippocampal App level.

The mechanism underlying the neuroprotective activity of central *ACE* inhibition is not entirely understood. Several studies indicated that the neuroprotective effect of central *ACE* inhibition is largely due to a decrease in angiotensin II generation and subsequently blunted activation of the angiotensin II AT1 receptor, AT1R [20, 36–40]. Such conclusion is supported by data, which show the involvement of angiotensin II-AT1R in major neuropathological features such as tau hyperphosphorylation [37], enhanced glutamate release [38], and increased Aβ formation [20, 39, 40]. In addition to direct neuroprotection, inhibition of the *ACE*-AT1R axis could also counteract the stress response by blunting the release of ACTH [41]. In agreement with a role of the *ACE*-angiotensin II-AT1R axis in stress-induced neurodegeneration, we found that the hippocampal angiotensin II content was increased by stress, and hippocampal neurons with hyperphosphorylated tau protein and overt neurodegeneration were AT1R-positive.

In view of the stress-induced upregulation of the hippocampal *Ace* and the neuroprotective activity of *ACE* inhibition in the chronic mild stress model, our data support a causative role of *ACE* in stress-induced neurodegeneration. Because the model reproduces major features of sporadic AD (i.e., tau hyperphosphorylation, Aβ generation, and glutamate excitotoxicity), our study could provide a rationale for the documented efficacy of centrally active *ACE* inhibitors in several clinical studies, which showed retardation of AD-related cognitive decline as a major sign of neurodegeneration [21–25]. In this context, the suggested repurposing of *ACE* inhibitors for clinical conditions related to AD and other types of dementia [42] could be a potential option, which deserves further study.

5. Conclusion

Inhibition of *ACE* with the centrally acting captopril retarded the development of hippocampal tau phosphorylation, signs of neurodegeneration, and amyloid beta (A4) precursor protein (App) upregulation in rats subjected to chronic unpredictable mild stress, as a model, which reproduces major pathological features of sporadic AD.

Conflict of Interests

The authors have no conflict of interests.

Acknowledgment

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Review Article

Nonpharmacological Interventions to Reduce Behavioral and Psychological Symptoms of Dementia: A Systematic Review

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Introduction. Behavioral and psychological symptoms of dementia (BPSD) are defined as a group of symptoms of disturbed perceptive thought content, mood, or behavior that include agitation, depression, apathy, repetitive questioning, psychosis, aggression, sleep problems, and wandering. Care of patients with BPSD involves pharmacological and nonpharmacological interventions. We reviewed studies of nonpharmacological interventions published in the last 10 years. *Methods*. We performed a systematic review in Medline and Embase databases, in the last 10 years, until June 2015. Key words used were (1) nonpharmacological interventions, (2) behavioral symptoms, (3) psychological symptoms, and (4) dementia. *Results*. We included 20 studies published in this period. Among these studies, program activities were more frequent (five studies) and the symptoms more responsive to the interventions were agitation. *Discussion*. Studies are heterogeneous in many aspects, including size sample, intervention, and instruments of measures. *Conclusion*. Nonpharmacological interventions are able to provide positive results in reducing symptoms of BPSD. Most studies have shown that these interventions have important and significant efficacy.

1. Introduction

The term BPSD stands for "behavioral and psychological symptoms of dementia" and has been used to describe a group of heterogeneous symptoms that arise in the course of dementia and are distressing and difficult to manage, both for caregivers and health professionals [1]. One or more symptoms of BPSD will affect up to 90% of patients with dementia during the disease course [2–4]. BPSD include many different behaviors such as screaming (disruptive vocalization), restlessness, repetitive questions, wandering, and apathy. As dementia is a progressive disease, BPSD worsen over time, requiring higher support and increasing cost of care [5]. BPSD have been associated with a poorer prognosis, a more rapid rate of cognitive decline, and illness progression [6], greater impairment in activities of daily living (ADLs) [6], and increased institutionalization at hospitals or residential care facilities [2, 7]. The frequency and severity of these symptoms have been strongly correlated with caregiver burden, reducing quality of life of patients and their caregivers [8].

Psychotropic medications have modest efficacy and can lead to undesired side effects [9–11] but they are frequently used to treat BPSD [12–15]. An increasing number of medical organizations and specialist groups such as the American Geriatric Society and the American Association for Geriatric Psychiatry currently consider nonpharmacological interventions to be first line Clinical Practice, except for emergency situations, referring mostly to situations in which the patient's

behavior is harmful to him/her or other persons [16–18]. According to Cohen-Mansfield [19] most professionals have some training in medication prescription for BPSD, but few are instructed about nonpharmacological interventions or receive information about their effectiveness. As a consequence, antipsychotics drugs are frequently prescribed before alternative nonpharmacological approaches are attempted, and patients are maintained in medication for long periods, which leads to increasing morbidity and mortality. This scenario may be improved if professionals involved in dementia care are better apprised of the indications and limitations for the several existing nonpharmacological therapies for BPSD.

Some studies have shown that nonpharmacological treatments pose fewer side effects, which render them as safer options [20]. Nonpharmacological alternatives, including music therapy, aromatherapy, art therapy, behavioral therapy, reality orientation, tailored activities, and physical exercises, have shown promising results for the management of BPSD [21–23]. The aim of the present review was to identify and summarize the main nonpharmacological interventions for BPSD in the treatment of patients with dementia published in the last ten years.

2. Methods

2.1. Literature Review. We systematically searched the Medline and Embase databases using the following keywords: (1) non-pharmacological interventions, (2) behavioral symptoms, (3) psychological symptoms, and (4) dementia. These terms were selected even in the absence of specific Mesh terms as to increase sensitivity. The date limits ranged from the first paper published in 2005 to June 2015. We also looked for reviews to identify relevant articles about the issue.

2.2. Eligibility Criteria. To be included in the review, papers had to be written in English, Spanish, or Portuguese and have appropriate description of the study design (e.g., clinical trials, interventional studies, or clinical studies). Systematic reviews, meta-analyses, case reports, and editorial letters were not included in our review.

After selection and analysis of papers according to the above-mentioned inclusion criteria, the following variables were extracted and organized: (a) Overview: study design, authors, and year of publication; (b) Demographic: total sample (number of participants) and location; and (c) Assessment of BPSD. A critical analysis was performed in order to investigate the response of patients presenting diverse symptoms of BPSD to different nonpharmacological approaches.

3. Results

Our initial search returned 33 references. Of these, 20 studies met the inclusion criteria and were included in our review: five on activities, four on music therapy, three on aromatherapy, three on exercises, two on light therapy, one on touch therapy, one on combination of activities, and one on cognitive rehabilitation (Table 1). A brief comment and critical overview on selected studies is presented as follows.

3.1. Occupational Activities. The use of activities as nonpharmacological intervention for people with dementia has shown potential benefits in quality of life and in reducing agitation and depression [37–39]. Five studies investigated the effect of activities in BPSD in patients with dementia, with three addressing the "Tailored Activities Program" (TAP). TAP is an occupational therapy intervention program that focuses on reducing undesirable behaviors associated with dementia [28]. The principle of TAP is the selection of activities that are specifically tailored to the patient according to his/her abilities, interests, and roles. The program also offers training for caregivers in order to simplify activities and to adapt them for future functional declines of the patient as well as to generalize strategies to other contexts, thus helping caregivers to develop an increased sense of self-efficacy. In the US-TAP study, Gitlin et al. [28] demonstrated reduction in the overall incidence of BPSD and specific behaviors such as shadowing, agitation, argumentation, and repetitive questioning in a sample with 60 dyads. The US-TAP interventions proved to be effective in reducing shadowing ($p = 0.003$) and behavioral occurrences ($p = 0.009$). The Australian TAP was published as "protocol-only paper" and results are not yet available [11].

3.2. Music Therapy. Music therapy is one of the nonpharmacological methods used to reduce BPSD [26, 40]. We found four studies about the effectiveness of music therapy for the management of BPSD. One investigated the effects of two interventions, simulated family presence and preferred music, where participants were exposed to 15-minute audiotape sessions. One group of participants heard audiotapes with a conversation about positive experiences from the past and the other was exposed to a selection of songs that the individuals used to enjoy in their youth. Both interventions proved to be effective in reducing agitation [26].

Holmes et al. [24] compared two methods of presenting music, live or prerecorded, in the treatment of apathy. Music sessions comprised three different activities of 30 minutes each. One 30-minute session consisted of silence alone, another 30-minute session consisted of background prerecorded songs, and the last one consisted of watching live music sessions. Music played during the live interactive and prerecorded sessions was the same and consisted of a mixture of favorite songs according to the age of the group. Live interactive music proved to be more effective than prerecorded music in reducing apathy in moderate and severe dementia in the short term ($p < 0.0001$). Prerecorded music did not show any efficacy in improving apathy.

Sung et al. [33] investigated the effects of group music intervention on anxiety and agitation in institutionalized elders with dementia who actively participated in a music group session of 30 minutes, twice a week, for six weeks. The 30 minutes of intervention consisted of a five-minute warm-up session with movements and breathing and a 20-minute session of active participation using percussion instruments and the last five minutes were a cool-down session with soft music. Previously the participants, caregivers, and family members were asked about patients' musical preferences and

Table 1: Summary of nonpharmacological interventions studies to reduce behavioral and psychological symptoms of dementia (BPSD).

Author	Country	Intervention	n	Place	Assessment	Symptoms treated	Comments
(1) Woods and Dimond (2002) [5]	(Canada) USA	Touch therapy	57	Long term care facilities	(i) Agitated Behaviors Rating Scale-modified (ii) Memory and Behavior Checklist	Manual manipulation (restlessness) and vocalization	
(2) Holmes et al. (2006) [24]	UK	Music therapy	32	Home care or nursing home facility	DCM	Apathy	
(3) Svansdottir and Snaedal (2006) [25]	Iceland	Music therapy	38	Nursing homes and geriatric wards	BEHAVE-AD	Agitation, aggressiveness, and anxiety	Positive effects mostly disappeared 4 weeks after the end of intervention
(4) Garland et al. (2007) [26]	Australia	Simulated family presence/preferred music	30	Nursing home		Shadowing and repetitive questioning	
(5) Lin et al. (2007) [27]	China	Aromatherapy	140	Care and attention homes	(i) Cohen-Mansfield Inventory (ii) NPI	Physically agitated behaviors and verbally agitated behaviors	
(6) Gitlin et al. (2008) [28]	USA	Activities program	60	Community	(i) Agitated Behaviors in Dementia Scale (ii) Revised Memory and Behavior Problem Checklist	Shadowing and repetitive questioning Agitation Argumentative behaviors	
(7) Burns et al. (2009) [29]	UK	Light therapy	48	Nursing care setting	Cohen-Mansfield Inventory	Physically agitated behaviors Sleep disturbance	
(8) Cerga-Pashoja et al. (2010) [22]	UK	Physical exercises	146	Community-dwelling individuals	NPI	BPSD in general	
(9) van der Ploeg et al. (2010) [30]	Australia	Activities		Aged care facilities	Cohen-Mansfield Inventory	Physically agitated behaviors	
(10) Burns et al. (2011) [31]	UK	Aromatherapy	81	Care homes	(i) Pittsburgh Agitation Scale (ii) NPI	Agitation	No statistical difference between melissa aromatherapy × medication × placebo
(11) Kolanowski et al. (2011) [32]	USA	Activities program	128	Nursing homes	(i) Cohen-Mansfield Inventory (ii) Passivity in Dementia Scale	Agitation and anxiety	Findings indicate that activities customized may help reduce agitation and passivity throughout the day and not just during the treatment
(12) Sung et al. (2012) [33]	Taiwan	Music therapy	60	Home care facility	(i) Cohen-Mansfield Inventory (ii) Rating of Anxiety in Dementia	Anxiety and wellbeing	

Table 1: Continued.

Author	Country	Intervention	n	Place	Assessment	Symptoms treated	Comments
(13) O'Connor et al. (2014) [11]	Australia	Activities program	160	Community	NPI-C	—	No results (protocol study)
(14) Brunelle-Hamann et al. (2015) [34]	Canada	Cognitive rehabilitation	15	—	NPI-12	Delusions	Small sample might have limited the power of the study and increased the likelihood of type 1 errors
(15) Chen et al. (2014) [23]	Taiwan	Combination of nonpharmacological interventions	92	Residential care facility	NPI	Hallucinations, delusion, and agitation	Not randomized controlled design The increased interaction between participants and the research staff might have caused a confounding effect in the "therapeutic" effects
(16) Lowery et al. (2014) [15]	UK	Physical exercises	131	Community mental health or primary clinical service	NPI	BPSD, except hallucinations and delusions	Physical exercise tailored to participant-carer dyads had no significant effect in BPSD but had statistical difference in caregiver burden
(17) Neville et al. (2014) [35]	Australia	Physical exercises (aquatic exercises)	11	Residential aged care facilities	(i) The Psychological Wellbeing in Cognitively Impaired Persons Scale (ii) Revised Memory and Behavior Problem Checklist	Psychological wellbeing	Small sample size No control group
(18) O'Connor et al. (2014) [11]	Australia	Activities	180	Community	NPI-C	—	No results (protocol study)
(19) Dowling et al. (2007) [36]	USA	Light therapy	70	Nursing homes	NPI	Agitation/aggression, depression/dysphoria, aberrant motor behavior, and appetite/eating disorders	
(20) Yang et al. (2015) [21]	Taiwan	Aromatherapy	189	Retirement homes for veterans and long term care facilities	Cohen-Mansfield Inventory	Agitation	

DCM: Dementia Care Mapping; NPI: Neuropsychiatric Inventory; NPI-C: Neuropsychiatric Inventory Clinician; BEHAVE-AD: Behavioral Pathology in Alzheimer's Disease Rating Scale.

a selection of familiar songs was used in each session. Participants in the control group received routine care: activities of daily living, basic nursing care, meal provision, and some social activities (TV watching, family visiting, etc.). Results indicated that music intervention had a significant effect in reducing anxiety ($p = 0.004$).

Svansdottir and Snaedal [25] investigated the effect of music therapy in a case-control study in a sample of 38 patients and reported significant improvement in aggressiveness and anxiety. In this intervention patients and therapist sang songs chosen by the group: each song was sung twice, accompanied by a guitar and other instruments of their choice. The therapy group received 18 sessions of music therapy, each lasting 30 minutes, three times a week for 6 weeks, while the control group did not change their daily care routine. The authors concluded that music therapy significantly reduced agitation and anxiety ($p = 0.02$) in moderate and severe dementia.

3.3. Aromatherapy. Three studies examined the use of aromatherapy to treat BPSD. Aromatherapy involves the diffusion of aromatic oil into the environment. Two oils were used to treat agitation: lavender and melissa oil (lemon balm). Lin et al. [27] conducted a crossover randomized study, comparing lavender inhalation (A), considered as the experimental group, and sunflower inhalation (B), considered as the placebo group. Lavender is a holistic relaxant that is regarded as having carminative, antiflatulent, and anticolic properties [27]. Sunflower preparation was selected as the placebo agent as it is odorless and does not possess any known therapeutic effect. Diffusers were placed on each side of the pillow of the participant during sleep at night for at least one hour. Each participant received both treatments (A and B) for three weeks with a washout period (two weeks) between each of the treatments. In this study, lavender was effective as an adjunctive therapy in alleviating agitation in patients with dementia ($p < 0.001$).

Burns et al. [31] assessed the efficacy of melissa aromatherapy in the treatment of agitation in dementia. This study was a randomized controlled trial, and the authors reported that there were no significant differences between aromatherapy, medication (donepezil), and placebo, with the three participant groups showing improvement in the NPI and in the Pittsburgh Agitation Scale (PAS).

Yang et al. [21] investigated aromatherapy and aroma acupressure (where acupuncture points were used in the aroma acupressure protocols to treat agitation). The procedures for the acupressure consisted of the following: each acupoint was pressed for two minutes with lavender oil and warm-up exercises were performed for five minutes, no longer than 15 minutes, once per day, five days a week, for four weeks in total. In the aromatherapy group, the lavender oil was applied at five acupoints with the same procedure. The aromatherapy and control groups did not receive any other interventions. Results showed that aroma-acupressure and aromatherapy had significant effect in reducing agitation ($p < 0.01$ and $p = 0.01$, resp.) when compared to the control group.

3.4. Physical Exercise. There were three articles on the effect of physical exercises on BPSD. However, one of them is just a "study protocol" without results [22]. From the remaining two studies, one addresses aquatic exercises, [35] consisting of a 45-minute group session (five to seven patients who performed exercises to strengthen agility, flexibility, and balance), followed by a relaxation session. Sessions were delivered twice a week over a 12-week period with a trained instructor and some assistants. Each participant performed the exercises accompanied by an assistant. This study identified a significant decrease in the number of BPSD ($p = 0.001$), improvement in psychological wellbeing, and reduction in staff distress associated with BPSD ($p = 0.001$). The second study focused on the effect of physical exercises on BPSD, considering individually tailored walking regimen designed to become progressively intensive and to last between 20 and 30 minutes at least five times a week. Results showed that exercises did not improve BPSD but were effective in attenuating caregiver burden [15].

3.5. Bright Light Therapy. Bright light therapy (BLT) has been used with different results in patients with dementia, showing benefits in the management of agitation [35]. The most often reported positive results are improved night-time sleep, reduction in agitation, and improvement in cognitive performance. The study by Burns et al. [29] assessed the effect of BLT on BPSD and found that sleep quality was particularly improved in 48 patients with dementia. Their experimental group consisted of patients exposed to light with an intensity of 10,000 lux and the control group consisted of patients exposed to standard fluorescent tube light at 100 lux during two weeks for two hours per day. Agitation improved but there was no statistically significant effect.

Dowling et al. [36] tested the effects of BLT in a randomized trial, with the BLT being administered for one hour daily (Monday to Friday) for 11 weeks. One group was exposed to light in the morning period; the second group was exposed to light in the afternoon, and the third group was exposed to indoor light. There were statistically significant differences between morning light exposure and afternoon light exposure in agitation/aggression scores ($p = 0.032$) and between morning light and indoor light in aberrant motor behavior (AMB) scores at the end of intervention ($p = 0.021$).

3.6. Touch Therapy. Touch therapies can include massage, craniosacral techniques, or therapeutic touch. Woods and Dimond [5] investigated the effect of touch therapy on BPSD in a double-blind three-group experimental study (one group with therapeutic touch, one group with placebo therapeutic touch, and the third group without any touch intervention). The interventions consisted of two daily sessions of 5–7 minutes for three days. The experimental group (therapeutic touch) experienced a statistically significant effect in reducing behavioral symptoms when compared to the group without any touch intervention ($p = 0.036$).

3.7. Combined Activities. One study investigated the efficacy of a combination of nonpharmacological interventions on

BPSD among older Chinese men in Taiwan [23] in a prospective study with residents in dementia care units. The combination included music therapy, orientation training, physical exercise, and art cognitive activities. All the interventions were delivered by trained occupational therapists and the frequency of interventions for the study group was twice a week for 12 weeks. Music therapy consisted of activities encouraging participants to sing, to move their arms to the rhythm of the songs, and to use simple percussion instruments. Exercise included ball games and other recreational activities designed to increase the inhabitants' activity level. Art cognitive activities included various painting activities; for example, participants were asked to color a drawing of a beach scene and at the same time a conversation about the characteristics of summer was introduced to increase orientation. The intervention group had more significant reduction than the reference group in the NPI score ($p = 0.046$), including delusion ($p = 0.018$), hallucination ($p = 0.004$), and agitation ($p = 0.038$) [23].

3.8. Cognitive Rehabilitation. Only one study addressed cognitive rehabilitation for BPSD [34, 41]. The impact of cognitive interventions on the BPSD is still not well known because most of studies have focused on improving global or specific cognitive functions [41]. Considering the importance of the relationship between cognitive interventions and BPSD, Brunelle-Hamann et al. [34] evaluated a cognitive program in patients with mild and moderate AD. This cognitive program consisted in a four-week home-based intervention of 45–60-minute sessions twice a week for four weeks and involved memory techniques to relearn an instrumental activity of the daily living chosen by patients and caregivers (e.g., origami, computer, and TV remote control). The level of assistance was provided according to the necessity of each participant. After interventions, there was a significant reduction of delusional symptoms with a large effect size; however, aberrant motor behavior increased significantly in the treatment condition when compared to the control group [34].

4. Discussion

This paper provides an overview of current evidence on the efficacy of nonpharmacological interventions to reduce BPSD published in the last 10 years. All the interventions discussed in this review were dedicated to patients and caregivers. Studies are heterogeneous regarding intervention protocols, instruments of clinical assessment, and evaluation of outcome.

Five studies investigated the effectiveness of activity programs and demonstrated positive results. However, the intervention methods varied across studies. These studies used different theoretical backgrounds and investigated the effect of personalized activities. The largest effect size was found when the treatments were tailored to participants' interests and skills [30].

Four studies assessed the impact of music therapy, using different interventions, showing positive effects. The use of familiar songs reduced anxiety [24, 33]. According to Gerdner [42, 43], music can change the focus of attention and provide an interpretable stimulus that elicits positive memories from an earlier period in the person's life, which would prevent, or alleviate, anxiety or agitation. Live interactive music presents efficacy in the short term management of apathy in patients with moderate and severe dementia, whereas prerecorded music produces a more limited effect [24].

Researchers have investigated therapeutic touch with experimental and longitudinal study designs. Woods and Dimond [5] found that therapeutic touch can be used to decrease behavioral symptoms of dementia, specifically restlessness and vocalization. The mechanism of action of therapeutic touch is still unknown.

Bright light therapy (BLT) has been increasingly studied and regarded as appropriate method to improve fluctuations in diurnal rhythms that may account for night-time disturbances and the "sundown syndrome" (confusion or agitation in the late afternoon or early evening). The BLT studies included in this review revealed significant positive effects of this intervention in BPSD, especially in agitated behavior and sleep disturbance. The most likely explanation for these effects is the influence of BLT on the melatonin system, which is implicated in the regulation of abnormal motor behavior during sleep [31].

Aromatherapy may be beneficial to agitated patients with dementia [21, 27, 31]. However, varying degrees of anosmia have been reported in people with dementia [27], which might lead to analytical bias.

Most studies included in our review focused on and reported behavioral abnormalities such as agitation. However, one study showed that an organized nonpharmacological intervention program was effective in managing both outward and intrinsic symptoms, including hallucination and delusion [23].

Studies focusing on the implementation of physical exercises programs have demonstrated reduction in BPSD and improvement of psychological wellbeing in patients with dementia. However, most studies were based on small samples, and further studies are warranted.

In our review, ten of the twenty studies indicated that nonpharmacological interventions are effective in reducing agitation. Agitation is a very common, persistent, and distressing symptom among people with moderate and severe dementia, affecting 30% of those living at home [44]. According to Livingston et al. [45], agitation in dementia is associated with poorer quality of life and impairs the engagement in daily activities and relationships. In addition to causing distress in family members and caregivers, it may precipitate institutionalization at nursing homes.

Currently, in clinical practice, pharmacological treatment of agitation is usually performed using antipsychotic drugs. However, clinical outcomes are poor and undesired side effects (including cognitive worsening, confusion, and extrapyramidal signs) are frequent, even with the use of the newer atypical drugs [14]. Therefore, nonpharmacological interventions seem to provide safer and effective alternatives for treating agitation in patients with dementia.

Regarding clinical settings, the majority of the studies ($n = 15$) included in this review focused on interventions

on patients with dementia residing at long term care facilities, and their application to home-based support remains uncertain [46, 47]. According to Trivedi et al. [1], two-thirds of patients with dementia live at home and yet there is limited evidence on which methods are the most effective in this setting.

Interestingly, one study reported significant worsening on BPSD. Brunelle-Hamann et al. [34], in a single blind, block-randomized and crossover-controlled study, investigated the impact of cognitive rehabilitation program on BPSD in AD patients. The results revealed that aberrant motor behaviors increased significantly in the treatment condition when compared to the control condition. The proposed hypothesis was that, during the rehabilitation intervention, as the dementia progresses, AD patients gradually lose their coping abilities and perceive their environment as more stressful.

Some limitations of the included studies need to be addressed. In terms of BPSD measures, most scales rely on information provided by caregivers, being thus subjected to the interference of variables such as caregiver's burden, personality, and even his/her ability to perceive changes in patients' behavior. However, the studies addressed in our review employed instruments that are validated and widely used in dementia research. Our review encompassed single-blind, double-blind, case-control, and prospective studies. Although these studies are heterogeneous in terms of design, intervention methods, and measures of outcome, bias can be reduced using statistical analysis strategies.

Some studies included in our review address tailored interventions [11, 26, 39]. According to Cohen-Mansfield [19], dementia patients become agitated when their needs are not perceived or addressed by caregivers. These needs can be addressed by a "person-centered care model." Tailored interventions are currently being considered as more effective than standardized interventions. Garland et al. [26] reported that audiotapes containing a family member's voice were more effective than audiotapes with songs to reduce agitation in patients with BPSD. Gitlin et al. [48] and O'Connor et al. [11] described a home-based occupational therapy program based on personal capabilities and individual preferences. This tailored program promoted a significantly greater reduction in agitation. In a randomized controlled trial, Gitlin et al. [48] found that nonpharmacological interventions based on Tailored Activities Program are cost-effective and should be considered as part of the clinical management of dementia.

5. Conclusion

Studies focusing on alternative approaches have disclosed that different nonpharmacological interventions are able to provide positive results in reducing symptoms of BPSD. Most studies have demonstrated that these interventions have important and significant efficacy improving BPSD such as agitation, psychotic symptoms, and apathy. Undesired side effects of pharmacological treatments, as antipsychotics and benzodiazepines, have promoted a search for alternative treatments for BPSD. Therefore, nonpharmacological interventions programs should be considered as first-option interventions to treat BPSD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Deep Assessment: A Novel Framework for Improving the Care of People with Very Advanced Alzheimer's Disease

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Best practice in understanding and caring for people with advanced Alzheimer's disease presents extraordinary challenges. Their severe and deteriorating cognitive impairments are such that carers find progressive difficulty in authentically ascertaining and responding to interests, preferences, and needs. Deep assessment, a novel multifaceted framework drawn from research into the experiences of others with severe cognitive impairments, has potential to empower carers and other support professionals to develop an enhanced understanding of people with advanced Alzheimer's disease and so deliver better calibrated care in attempts to maximize quality of life. Deep assessment uses a combination of techniques, namely, Behaviour State Observation, Triangulated Proxy Reporting, and Startle Reflex Modulation Measurement, to deliver a comprehensive and deep assessment of the inner states (awareness, preferences, likes, and dislikes) of people who cannot reliably self-report. This paper explains deep assessment and its current applications. It then suggests how it can be applied to people with advanced Alzheimer's disease to develop others' understanding of their inner states and to help improve their quality of life. An illustrative hypothetical vignette is used to amplify this framework. We discuss the potential utility and efficacy of this technique for this population and we also propose other human conditions that may benefit from research using a deep assessment approach.

1. Introduction

According to the World Health Organization the number of people with dementia worldwide was estimated in 2010 to be 35.6 million and is projected to double every 20 years [1]. Global costs for treating and caring for people with dementia already exceed 600 billion US$ per year. Alzheimer's disease is the most common form of dementia and has no available treatment. It is therefore highly important to focus on advancing effective methods of caring.

Caring for and supporting people with advanced Alzheimer's disease (AAd) present extraordinary challenges to their family, carers, and other support professionals (hereafter referred to as "carers") [2]. This is because, in addition to physical health decline, as their severe cognitive impairments impact functioning, patients often become increasingly difficult to communicate with and understand [3]. Therefore it becomes progressively more difficult for others to confidently ascertain and respond to changing interests, preferences, and needs [4].

Similar challenges face carers for people with comparable severe cognitive impairments like acquired brain injury and individuals with profound and multiple disabilities. For people with severe acquired brain injury there is generally an expectation that intervention will result in improved communication and understanding over time [5]. For people with profound multiple disabilities the challenges are life-long, but these people too can generally be expected to learn and develop improved communication skills over time [6]. Individuals with AAd, however, face a prognosis that includes an inevitable decline in communication skills and quality of life and eventually death. Carers struggle daily with this reality, as do people with AAd themselves [7].

The development of evidence-based practices in the care of people with Alzheimer's disease is the subject of considerable research activity [8]. Two of the most prominent

and established approaches, Dementia Care Mapping [9] and Validation Theory [10], are good examples of evidence-based practices that have contributed to improved care and quality of life for those with Alzheimer's disease. These tools have informed personalized planning and the refinement of tailored supports in care programs. The goal of both techniques is to refine an understanding about the individual experience of Alzheimer's disease informed by the principles of ecological complexity in the provision of appropriate supports. Nevertheless, people with AAd present even more complex challenges to carers and continue to experience, by any measure, a comparatively poor quality of life [8].

By comparison, the lives of a significant proportion of people with severe acquired brain injury have improved due to technological improvements in medical treatment and therapy [5]. Furthermore, the lives of many people with profound multiple disabilities have improved in direct relationship to policy, practice, and attitudinal shifts amongst carers specifically [11] and the wider community in general [12]. The techniques of Behaviour State Observation [13], Triangulated Proxy Reporting [14], and Startle Reflex Modulation Measurement [15] are amongst the evidence-based techniques that have contributed to improved practices in these fields. Used in a harmonious way, we contend that these three techniques, collectively referred to as "deep assessment," hold much promise for enhanced care and support for individuals with AAd.

Deep assessment is a novel multifaceted framework for delivering a more comprehensive and authentic assessment of the internal states of people with severe cognitive impairments who are unable to self-report. The term "severe cognitive impairments" arises from diverse sources including profound multiple disability, severe acquired brain injury, and advanced dementia. There is a history of using proxy reporters for people who are unable to self-report [21] but a continuing research agenda generally supports the view that simple proxy reporting lacks validity and accuracy [22]. This view is though contested [17] and research and policy around supported decision-making and substitute judgement are relevant here [23].

The next section describes the three components of deep assessment. The exposition is contextualized around current applications to people with profound multiple disabilities who, like their AAd counterparts, have severe cognitive impairments. They face significant challenges in communication and understanding of their inner states. This section is followed by a discussion about how deep assessment might be applied to persons with AAd, a group of persons who experience pervasive limitations in functioning due to severe cognitive impairments and communicative challenges. To illustrate this novel application, a hypothetical vignette is used to demonstrate in practical terms how deep assessment has, in the authors' opinions, the potential to inform carers about how to improve levels of understanding into various aspects of the internal states of people with AAd and consequently to improve both their quality of care and quality of life.

2. Methods

2.1. Deep Assessment. Generally, when we desire to know what other people think, the most reliable information comes from what they report or do. This includes overt behaviours such as facial expressions, body language, and verbal cues. However, this kind of information is limited if people cannot effectively or reliably provide this feedback, as is often the case with AAd patients [24]. This is also the case for infants and young children and people with severe communicative impairments allied with physical, cognitive, and/or emotional impairments. Sometimes it is necessary to ask others who know them well to provide their interpretation of this limited information.

People's inner states and how they are expressed are essential indicators of personal well-being, satisfaction, and subjective judgements on quality of life [25]. Understanding of these inner states informs daily and critical decision-making for carers [26]. Without this understanding, decisions about the care and well-being of others can only be based on secondary (proxy) reports informed mostly by notions of best interest [27] or by philosophical and moral judgement about what is judged to be right in any given situation [28].

At the heart of these debates is the question of veracity: how can we confidently know what a person wants if their wishes must be guessed by others? The deep assessment framework involves the strategic use of three complementary and synergistic assessment techniques that collectively deliver more robust data that is data less informed by "guessing." Table 1 provides a summary of the techniques and their unique advantages.

These techniques are combined in an individualized manner to suit the needs of persons with specific severe cognitive impairments. The approach typically comprises a sequence of observations, reporting, and Startle Reflex Modulation Measurement, repeated frequently enough to maintain currency and authenticity in the light of any presumed or evidenced changes in internal states demonstrated by that individual. The techniques have significant application histories in the fields of special education for students with profound multiple disabilities [13], speech and communication therapy for people with severe communicative and cognitive impairments [29], and neuroscience/psychiatry [30].

By strategically combining these three techniques into the more comprehensive deep assessment framework, we hope to strengthen the veracity (rigor, validity, and reliability) of AAd caring protocols. Although these are not new techniques, their collective application to the populations represented in aged care, diversional therapy, and geriatrics is completely novel. A brief overview of each technique follows.

2.2. Behaviour State Observation. Carers need to communicate with those they support in order to best respond to their needs [31]. In most cases direct verbal dyadic interactions will suffice. In other situations where there are barriers to verbal communication (i.e., when a person cannot or will not interact directly with another in a verbal manner) direct observation is the next best source of information, followed

Table 1: Summary of the three techniques comprising deep assessment.

Technique	Description	Advantages	Key refs.
Behaviour State Observation	An observational protocol that uses fine-grained category codes to allow judgements about a person and their social and communicative contexts.	A detailed and systematic means of connecting individual levels of alertness and engagement with a range of relevant contextual variables, including sociocommunicative elements.	[15, 16]
Triangulated Proxy Reporting	A systematic consultative process drawing on the close personal knowledge of individual needs as displayed in a range of natural settings.	It allows for the authentic and powerful input of those who know the person best. It allows the direct translation of carer knowledge into planning and support for the person with participation challenges.	[17, 18]
Startle Reflex Modulation	An electrophysiological measurement of emotion. The amplitude of a person's reflexive eye blink in response to a startling stimulus indicates whether the individual is experiencing a more unpleasant or a more pleasant inner state.	It allows unbiased and implicit measurement, not requiring verbal responses. A reliable indicator of basic emotional preference even for a person in cognitive decline.	[19, 20]

by, rather than relying only on, proxy reports. Behaviour State Observation sits at the base of observational protocols and seeks to ascertain individual "readiness" or capacity to engage with environmental stimuli. Using finely grained categorical codes, judgements are made regarding both contextual variables such as sociocommunicative opportunities for interaction and the judged level of alertness in the target individual.

Behaviour State Observation originated in the pioneering work of Brazelton and Nugent [32] on infant behaviours and refers to various procedures for systematically coding levels of alertness and engagement in individuals, including those with profound and multiple disability [33]. The validity and reliability of these procedures, when used with people with severe cognitive impairments, continue to improve as ongoing research informs practice. Data is collected using paper and pencil techniques as well as video recording, supported by systematic interobserver checks. Variables observed in real time in addition to behaviour states include communication indicators and physical positioning.

Behaviour State Observation techniques span more than twenty years of research history, moving from descriptive data to sequential analyses and transitional probabilities, involving individuals with profound and multiple disability in a range of educational settings [33]. Critically, if carers can ascertain the "right moment" to engage with people with severe cognitive impairments, they are most likely to experience meaningful communications and consequently make informed care or educational decisions [34]. Generally, behaviour states are coded on a continuum from nonalert to most alert. Partial interval recording techniques, sometimes retrospectively analysed using video recordings, deliver a measure of the changes in levels of alertness and contextual variables. As communication partners become more skilled at reading changes in levels of alertness, they can strategically focus on those moments when the individual is in a most facilitative behaviour state to communicate with them and thence engage them in experiences which might bring them the most benefit or satisfaction. These judgements are informed by the social, communicative, and other contextual data collected simultaneously.

2.3. Triangulated Proxy Reporting. Triangulated Proxy Reporting involves the triangulation of data, investigators, and methodological approaches to analysis (see Denzin and Lincoln [35] for an extended explanation). The use of triangulation to strengthen data collection, analysis, and interpretation has a long history in both qualitative and quantitative research [35, 36]. There is less research on its formal application in authenticating and validating interpretations of attempts at communication made by people with severe communicative impairments in dyadic interactions (see Money [18] for an early application). The work of Lyons [17] in generating the Life Satisfaction Matrix, a procedure for ascertaining and improving the life satisfaction of people with profound multiple disabilities, is principally informed by Triangulated Proxy Reporting and serves as an example here.

In Lyons' approach two colleagues (who are familiar communication partners) observe the behaviours displayed (albeit usually idiosyncratically but generally consistently) by a focus person with severe cognitive impairments to express various inner states. They are then independently interviewed by a third (unfamiliar) colleague who records their observations.

A preliminary Behavioral Communication Profile is drafted, informed solely by observable discernable behaviours. Although the two colleagues may deliver a similar profile, consistency at this point is encouraging but is not a necessity. The behavioral descriptions offered by the familiar colleagues can often be clarified by analyzing naturalistic video recordings of those behaviours. Put simply, the Behavioral Communication Profile shows what the person does to show his/her range of feelings. Importantly, at this stage, the third colleague has not been told which daily experiences elicit these expressions of internal state.

The third colleague then observes the focus person during their routine day, building a familiarity with the

Behavioral Communication Profile and looking opportunistically to record matches between these behaviours and daily experiences. This process should occur during regular daily activities, rather than during exceptional or irregular activities. Interviews are then repeated, this time reporting which daily experiences elicited which behaviours. If the third colleague is able to reasonably match the colleagues' reports, then, via the cross-referencing of the triangulation, the Behavioral Communication Profile is authenticated. Various daily experiences can then be confidently ranked in terms of their preferential appeal to the focus person. Different carers often engage with focus individuals in different daily experiences so ranking is unlikely to be highly similar across carers. However, observed dissimilarity in rankings does not detract from the evidence for the authenticity of the Behavioral Communication Profile itself. Furthermore, differences in views about the preferential appeal of different daily experiences can often be explained by conducting an activity or preference assessment similar to a task analysis (see Alberto and Troutman [37] for a detailed explanation) wherein differential preferences may be assigned to different components of a longer experience.

Triangulated Proxy Reporting functions to cross-check the knowledge and understandings of carers. It aims to produce a stronger collective confidence about the nature and purpose of observable behaviours displayed by individuals with severe cognitive impairments in the context of specific regular, naturalistic, and frequent daily experiences. Triangulated Proxy Reporting techniques have an extensive research history (see Lyons [17]). If carers can authenticate their knowledge about how people with severe cognitive impairments express their inner states and rank the relative preferential appeal of daily activities, then the carers can make informed care decisions. Like Behaviour State Observations, these techniques function as a secondary source of data that relies on intensive collaborative consultations and observations. Therefore, deep assessment proposes the inclusion of a third technique to enhance its rigor: Startle Reflex Modulation Measurements.

2.4. Startle Reflex Modulation Measurement. Thus far, the paper has described two techniques that have a substantial subjective element. In contrast, Startle Reflex Modulation Measurement (SRM) provides an objective measurement of individuals' inner states. In neuroscientific research, psychophysiological measures such as SRM, heart beat rate, breathing rate, and skin conductance have long been considered objective measures of basic emotional states because they provide direct and fast indications of emotional reactions that are typically obscured by higher cognitive processes [38]. In particular, SRM is considered the only objective physiological measure of emotion that is sensitive to emotional valence; that is, it can tell apart negative and positive internal emotional states [19]. When used within the deep assessment framework, SRM can complement and enhance assessments made using Behaviour State Observations and Triangulated Proxy Reporting. More importantly, as a measure of internal emotional states, SRM can provide authentic personalized information to empower carers of people with severe cognitive impairments, allowing them to make more informed decisions.

An advantage of SRM is its strong statistical properties. Psychophysiological measures face fewer criticisms around validity and reliability than measures based on behavioral observations and self-reporting and proxy reporting. The early work of Freeman et al. [39] demonstrated that physiological measures enhance understanding of the life experiences of people with severe cognitive impairments. More recent work by Vos et al. [40] showed that changes in some physiological measures correlated with changes in the perceived emotional states of people with profound multiple disabilities.

Most psychophysiological measures can indicate changes in the levels of arousal and can augment Behaviour State Observation. However, such measures are by and large not indicative of changes in emotional valence [19] because preferred (pleasant) and nonpreferred (unpleasant) experiences can deliver very similar measures of arousal. The SRM measurement technique, on the other hand, is unique due to its sensitivity to emotional valence (or affect) and has been argued to be even more sensitive to emotional states relative to self-reporting or proxy reporting [20]. SRM therefore has the potential to deliver a reliable measure of emotional valence (degree of experienced pleasantness) in terms of simple motivational preferences without requiring explicit responses. For people unable to communicate due to declining cognitive functioning, such as people who suffer AAd, SRM may be a window (perhaps the only known reliable window) into inner emotional states and preferences.

SRM is based on electromyographic data derived from differential eye blink amplitudes to distinguish between positive (pleasant) and negative (unpleasant) emotional internal states precipitated out of a diversity of sensory experiences [30]. SRM is a product of the startle reflex, an evolved motivational-based adaptation common to all complex living organisms that involves a spontaneous and involuntary full-body muscle contraction in response to a sudden and unexpected stimulus, allegedly intended to quickly and reflexively withdraw the organism from potential harm. The startle reflex may be elicited by tactile or visual sensory stimulations, but most commonly by a sudden, fast-rising auditory stimulus. The strength of the startle response and more specifically the strength of the associated eye blink provide an indication of the organism's current inner emotional state.

The robustness of the startle-associated eye blink provides a reliable index of relative emotional valence such that a stronger eye blink indicates a more negative or unpleasant current inner state, whereas a weaker eye blink indicates a more positive or pleasant inner state [41]. Figure 1 illustrates the simple apparatus used to collect the SRM data streams. The testing apparatus and procedure are only minimally intrusive. In addition to the headphones, two wired adhesive pads attached to the individual's face and a discreet grounding pad are the only attachments. Once the calibration of the individual's readings is completed, attachment time is minimal for each stimulus test.

Figure 1: The SRM apparatus attachments.

SRM is an objective measure that requires no conscious appraisal or language-related responses and is independent of cognitive processing and arousal. This is because the primary neural circuits involved in the eye blink component of the reflex occur in primitive subcortical regions of the brain that process emotional information at a basic motivational level, where simple approach or avoidance behaviours are generated, largely independent of higher cognitive function [38].

In terms of feasibility of the approach, measuring the startle eye blink reflex has been demonstrated in people with Alzheimer's disease [42] primarily for the purpose of understanding changes in brain structure and function related to the disease. SRM protocols have also been used across diverse clinical populations in neuroscientific and psychological research to cross-check the validity and reliability of self-reported emotional responses and behaviours (for reviews see [43, 44]). Current recommendations are that caregivers should develop nonverbal approaches to communicating with individuals with pervasive cognitive impairments in anticipation of losing the ability to communicate using language [45]. SRM measurement is ideal for this purpose, particularly when conducted in the broader context of a multifaceted deep assessment framework that allows for the utilization and comparison of multiple patient and caregiver data inputs.

3. Discussion

The techniques of Behaviour State Observation, Triangulated Proxy Reporting, and Startle Reflex Modulation have substantial independent histories in other fields of research and practice, but only the latter has found some traction in the field of gerontological research [45]. Deep assessment combines the three techniques into a novel, comprehensive assessment framework that benefits from two major considerations.

First, researchers and practitioners in the fields of biomedical research, aged care nursing, gerontology, and diversional therapy have not, to the best of our knowledge, connected these techniques. The research and practice fields of special education, intellectual disability education, aged care, and neuropsychology rarely cross paths so it is not surprising that these techniques have lacked collaborative investigations. Multidisciplinary engagement amongst researchers and practitioners is lauded and encouraged at a systemic level [46] but does not occur so often in practice because pilot funding for encouraging basic and applied action research around untried and untested (novel) theory and practice is hard to attract, and, in the social sciences, ethics hurdles may be insurmountable.

Second, care and support planning for other populations with severe cognitive impairments is generally developmental and intervention focused. That is, for people with severe acquired brain injury, planning is usually focused on partial recovery, rehabilitation, and restoration of functionality. For people with profound multiple disabilities, education and therapy are usually focused on identifying and developing skill and ability potentials. In something of a contrast, for people with AAd, care and support planning is generally focused on minimizing the unavoidable deleterious effects of advancing cognitive and physical decline.

Furthermore, this decline in people with AAd is predictable only in that it is unavoidable. On any one day and at any one time, people with AAd can present with very inconsistent apparent interests and preferences, although there are some predictability and patterns to prevailing behavioral problems [47, 48]. Substitute decision-making is fraught with challenges. On the one hand many people with AAd have significant histories of interests and preferences. These can change gradually, as they do for most people, or very quickly and unpredictably, and these changes can be temporary and/or fluctuating or sustained. Any assessments of behaviour state and inner state need to be repeated as often as possible, to maximize the probability that substitute decision-making enhances quality of care and improves the quality of life.

The following vignette illustrates how deep assessment might be operationalized to benefit May (pseudonym), a person with AAd. The vignette illustrates how the approach might be applied in a typical care scenario to maximize May's quality of care and quality of life.

4. A Hypothetical Vignette

May is an 86-year-old widow. She has lived in an aged care facility for three years. She is visited usually twice a week by Mary, her daughter, monthly by Bill, her son, and very occasionally by various grandchildren. May has had Alzheimer's disease for at least six years. At the time of moving into the facility she was physically frail, unable to ambulate, and incapable of caring for herself safely. She was also showing clear signs of midstage dementia with near-daily episodes of disorientation, confusion, and agitation.

May was previously a happy, active, and socially engaging person. Yet her Alzheimer's disease had now progressed to

the stage wherein she was most often nonalert, "dozing," or asleep, incommunicative, and primarily unresponsive to verbal and even physical prompts by her family and care staff. Her quality of life, by any measure, was poor and was deteriorating, and both the familiar care staff and her children were now unable to consistently or confidently determine what May wanted or did not want, preferred or did not enjoy, or even needed (both socially and emotionally). With a continuing trajectory of physical, intellectual, and emotional decline the only certainty for those supporting May was to address her high physical care needs. There had been familial discussions around the apparent futility and "senselessness" of May's life but Mary and Bill and the care staff still tried to bring some enjoyment to Mary by attempting to engage her in reminiscent conversation and sensory experiences that she had historically enjoyed.

After some discussions between Mary, Bill, and key care staff, it was decided to conduct a deep assessment. Diminishing quality of life was clearly the primary concern and it was hoped that deep assessment might offer more informed and authentic insights into May's difficult-to-discern interests and preferences and into her levels of alertness so that needs and desires might be better responded to.

The facility's diversional therapist first conducted a set of Behaviour State Observations on May. These suggested a "topography" or profile of alertness indicators and changes that were not evident through informal observations or indicated through historical proxy knowledge. At the same time codes were recorded about communication partners, interactions, and social grouping observed at any one time. This information helped key staff and family to identify and target key periods and moments in May's day when she was more likely to be receptive to communicative and potentially enjoyable engagements orchestrated by others. For example, though May was consistently unresponsive to background noise, when someone played a piano nearby her entire observed state altered and she was generally more responsive to the communication cues of those around her.

Around the same time key care staff and family "catalogued" and ranked those activities and experiences that had historically and most recently brought a positive emotional response from May. Using the Triangulated Proxy Reporting technique a reasonably confident consensus was reached concerning May's preferred and less preferred experiences. This analysis was complemented by a list of "new" experiences that care staff and family brainstormed to explore using Startle Reflex Modulation Measurement. The diversional therapist conducted two sessions of SRM measurements: first to initially calibrate the affect measurements and second to determine the affect potential of some of the "new" experiences put forward by care staff and family.

A "quality of life enhancement plan" was then drafted from a collaborative analysis of the data provided by the deep assessment. The gist of this plan was shared amongst the care staff and family members. May's overall care taking and daily schedule were modified to reflect and respond to the plan. Although the measures taken are not likely to extend May's life expectancy, they will improve the quality of her life, as well as that of a wider circle of people who surround her, such as family members and the facility's caregivers.

How might this approach differ from more traditional approaches to assessing May's support priorities? First, it involves potential input from a larger number of key participants in May's social ecology as well as May herself, though not via explicit verbal or motor directions. Second, data collected across time and modes enhances the internal validity of any conclusions drawn for planning purposes. Lastly, such a comprehensive approach avoids the dyadic "clinician and May" approach that still dominates traditional models of support.

5. Conclusions

Deep assessment is a new and innovative framework for empowering families, carers, and other support professionals to better understand and make improved care decisions for people with advanced Alzheimer's disease. Its component parts (Behaviour State Observation, Triangulated Proxy Reporting, and Startle Reflex Modulation) are also novel to this specific field of Alzheimer's disease but have substantial research histories in other areas of research.

The techniques within deep assessment have already demonstrated potential to inform and empower family and carers to enhance the quality of care and ultimately the quality of life of persons with profound multiple disabilities, who historically experienced a relatively low quality of life. It has been argued that it also has potential to inform and empower the family and carers of other persons with severe cognitive impairments, particularly those with advanced Alzheimer's disease. Persons with Alzheimer's disease constitute an expanding population in the world's aging society, and as advances in biomedical research and care practices continue to extend the average life expectancy, governments and society need to respond in a range of ways that will include moral, philosophical, and fiscal dimensions. We argue that there is a clear social justice imperative to find better ways to improve the diminishing quality of life of an increasing number of people who live with advanced Alzheimer's disease. In this context the authors have a commitment to drive a research agenda that aims to explore the potential of deep assessment for people with advanced Alzheimer's disease. Deep assessment represents an exciting opportunity in multidisciplinary collaborative research, generally for people with severe cognitive impairments and specifically for people with advanced Alzheimer's disease.

Notwithstanding challenges with respect to funding and ethical aspects in such a complicated but important area, the authors conclude by calling for interdisciplinary collaborations with colleagues in neuroscience, biomedicine, aged (nursing) care, geriatrics, and/or diversional therapeutics to launch this neophyte research agenda. As a starting point, pilot trials utilizing deep assessment with a small and relatively homogeneous sample may serve to inform practical and methodological considerations. These can then serve as a basis for larger scale investigations utilizing control groups and stratified participant groups as a means of testing its efficacy in a robust manner. In effect, this comprehensive

approach also holds promise for other subgroups in the aging population, including individuals with generalized dementia and stroke-induced communication challenges.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Serum Levels of ApoA1 and ApoA2 Are Associated with Cognitive Status in Older Men

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Background. Advancing age, chronic inflammation, oxidative damage, and disorders of lipid metabolism are positively linked to the late-life cognitive impairment. Serum biomarkers may be associated with the cognitive status in older men. *Methods.* 440 old male subjects with different cognitive functions were recruited to investigate probable serum markers. Pearson Chi-Squared test, univariate analysis, and multivariate logistic regression analysis were performed to evaluate biomarkers which may be associated with cognitive status. *Results.* Levels of fundus atherosclerosis (AS) ($P < 0.001$), age ($P < 0.001$), serum biomarkers peroxidase (POD) ($P = 0.026$) and interleukin-6 (IL-6) ($P = 0.001$), serum levels of high-density lipoprotein cholesterol (HDL-C) ($P < 0.001$), apolipoprotein A2 (ApoA2) ($P = 0.001$), and ApoC2 ($P = 0.005$) showed significant differences. Compared to group 3, ApoA1 in group 1 (OR = 1.30, 95% CI 1.01–1.67) and group 2 (OR = 1.47, 95% CI 1.11–1.94) were higher, while ApoA2 were lower (group 1: OR = 0.43, 95% CI 0.18–1.02; group 2: OR = 0.21, 95% CI 0.08–0.54) after adjusting for control variables. *Conclusion.* The results demonstrated that age, AS levels, POD, IL-6, HDL-C, ApoA2, and ApoC2 were significantly related to cognitive status. Moreover, ApoA1 and ApoA2 were independently associated with cognitive impairment and late-life dementia.

1. Introduction

The decline of cognitive function across the life span is referred to as age-related cognitive decline [1, 2]. In addition to age, many age-related health problems, such as sex, general medical health, heart disease, vascular diseases, hypertension, stroke, diabetes, high plasma cholesterol, and a high number comorbidities, are recognized as risk factors of cognitive decline [3–7]. Other susceptibility factors, including antioxidant status, inflammatory status, and apolipoproteins, result in a substantial variation in the rate of cognitive decline among older individuals in the population [8–10]. The pathology of neurodegeneration in cognitive function impairment, Alzheimer's disease (AD), and vascular dementia (VaD) involves oxidative stress and the accumulation of free radicals [8, 11]. The antioxidants selective monoamine oxidase inhibitor selegiline and alpha-tocopherol (vitamin E) can slow cognitive decline in patients with moderately severe impairment from AD [11]. The polymorphism of antioxidant genes had been demonstrated to be a risk factor for late-onset AD [12, 13]. The antioxidant status can predict neurodegeneration, such as medial temporal lobe atrophy in AD. A small drop in the antioxidant concentration increases the risk of medial temporal lobe atrophy in AD [8]. However, some clinical trials with antioxidants had largely been disappointing. The long-term use of *Ginkgo biloba* extract did not reduce the risk of progression to AD [14]. The use of vitamin E could maintain the cognitive status in some AD individuals and, meanwhile, accelerate the cognitive decline in other AD individuals [15]. Both chronic low-grade inflammation and immune activation are playing important roles in the pathogenesis of age-associated diseases, including cognitive decline, AD, and vascular dementia. Serum or plasma biomarkers of inflammation, especially IL-6 and C-reactive protein (CRP), were prospectively associated with cognitive decline in multiethnic cohorts of older populations

before the clinical onset of dementia, AD, and vascular dementia [16–20]. The level of IL-6 was negatively associated with the performance on the Minimental Status Examination (MMSE) in a stroke-free population-based multiethnic cohort, including Hispanic and black subjects; after adjusting for vascular disease and subclinical atherosclerosis (AS), the association did not attenuate [21]. The IL-6-174 G/C polymorphism (C allele caused increase of IL-6) was associated with worse cognitive function and steeper cognitive decline in participants in the Prospective Study of Pravastatin in the Elderly at Risk (PROSPER) [22]. Increased plasma IL-6 level also represented a biomarker for the risk of future cognitive decline in a relatively healthy midlife (age from 30 to 54) community sample [23]. In another large-scale, prospective occupational cohort study over 10 years, increased levels of inflammatory markers, both CRP and IL-6 at midlife, were moderately associated with a lower cognitive status but were only slightly associated with cognitive decline [24]. Systemic inflammation was associated with mild cognitive impairment (MCI) and its subtypes. The level of plasma TNF-α was higher in participants with MCI compared to individuals with normal cognitive function [25]. Nonamnestic multiple domains MCI was associated with higher levels of IL-1β, IL-12, and TNF-α compared with normal cognition, amnestic MCI (single and multiple domains), and nonamnestic single-domain MCI [25]. However, the systemic inflammation markers CRP and IL-6 were not associated with dynamic cognitive decline in amnestic MCI after a follow-up over 12 months [26].

Accumulating evidence suggests that serum lipid levels are associated with human longevity, cognitive decline, and dementia [27, 28]. Serum high-density lipoprotein cholesterol (HDL-C) level was associated with better survival in the frail community-living elderly [29]. Higher level of HDL-C indicated better functional status in individuals who were over 85 years old [30] and less use of prescribed drugs also appeared in nonagenarians [31]. Low-density lipoprotein cholesterol (LDL-C) level was related to male longevity, while triglycerides (TG) level was related to female in offspring of nonagenarian siblings (n = 1,664, mean age: 59 years) when compared to offspring of control population (n = 711, mean age: 60 years) [32]. In a longitudinal study (n = 1,449) with average follow-up of 21 years, the results showed that the association between serum total cholesterol (TC) levels and dementia might be bidirectional. High midlife serum TC represented a risk factor for subsequent dementia, and low serum TC after midlife was a risk factor for late-life cognitive impairment [33]. Low serum cholesterol and LDL-C levels were closely related to cognitive decline in elderly patients with Alzheimer's disease (AD) [28]. Subjects with probable mild cognitive impairment (MCI) and AD had significantly lower levels of TG, TC, or LDL-C in elderly people [34, 35].

Apolipoproteins are important components of plasma lipoproteins which are synthesized in liver and have been proved to play a significant role in the lipid metabolism and the formation of lipoproteins [36]. Some recent studies showed that apolipoproteins are potential plasma biomarkers of cognitive decline and then AD in older adults [37]. ApoA1 and ApoA2, in charge of transporting cholesterol to the liver, are critical components in the formation of HDL [38] and overexpression of ApoA1 may effectively inhibit the age-related decline in memory and learning ability [39]. Moreover, Kawano et al. found that the levels of both ApoA1 and ApoA2 were strikingly lower in a group of Japanese patients who suffered from late-onset nonfamilial AD [40]. Serum ApoB level had been demonstrated to be much higher in the patients with AD [41, 42], while little less is known about its function in some preclinical stages of dementia such as MCI. Kamino et al. found that there was no specific genetic association between ApoC2 and Senile dementia of Alzheimer's type [43]. The high concentration of ApoC3 in serum might lead to lipoprotein disorders and some vascular diseases such as atherosclerosis and hypertriglyceridemia; the relationship between ApoC3 and longevity is not clear yet [44, 45]. Some studies showed that the plasma level of ApoE in AD patients was much higher than the control group [46, 47]; however, others indicated that patients with AD had significantly lower level of ApoE [48, 49]. Studies about ApoJ showed that it could accelerate the progress of AD and other cognitive disorders [50]. The association between ApoH and dementia is not that clear until now.

However, most known risk factors are poor at individually discriminating risk in individuals with cognitive decline [51]. The goals of the present cross-sectional study were to examine the exact correlations of age, atherosclerosis levels, comorbidities, oxidative and inflammatory statuses, serum and plasma levels of lipid, and apolipoproteins with MMSE scores and to find out the main risk factors associated with cognitive impairment.

2. Material and Methods

2.1. Study Population. A total of 549 Han Chinese patients from Geriatrics Outpatient Clinic in Shanghai were invited to participate in a physical examination at Shanghai Huadong Hospital from June 1, 2012, to July 31, 2012. Each participant recruited was with a complete medical history. After excluding the female subjects because of the insufficient sample number (n = 60) and 49 older men who failed to finish the MMSE test due to either medical or psychological conditions, such as severe hearing loss, acute inflammation, and psychoneurosis, 440 male subjects (65 to 97 years old) were included in this cohort study. This study was approved by the ethics committee of Huadong Hospital of Shanghai Medical College, Fudan University, and written informed consent was obtained from all participants. Subjects in the experiment had more than 12 years of education and similar lifestyles (with no addiction of cigarettes and alcohol).

2.2. Assessments and Measurements

2.2.1. Cognition. Cognitive function was measured by means of the MMSE, which is frequently used in screening global cognitive dysfunction and dementia [1]. The scores range from 0 to 30 and cognitive impairment is defined as a difference in score of more than one standard deviation on the MMSE (≥3 points). The subjects for the study were divided into three groups according to MMSE scores: group 1 (28 ≤

MMSE ≤ 30), group 2 (24 ≤ MMSE ≤ 27), and group 3 (MMSE ≤ 23).

2.3. Comorbidity Profile and Levels of Fundus Arteriosclerosis. Participants in this study were asked whether they had a physician's diagnosis of 18 chronic diseases according to the International Classification of Diseases (ICD): hypertension, dyslipidemia, obesity, diabetes, coronary heart disease, other heart diseases, venous insufficiency, stroke, epilepsy, hypothyroidism, hyperthyroidism, chronic renal disease, anemia, chronic pulmonary obstructive disease, liver disease, arthrosis, prostatic disease, and cancer. Each disease from electronic records of medical history was added to a score ranging from 0 to 18, with a higher score indicating more chronic diseases. The degree of fundus atherosclerosis (AS) was graded as levels 0, I, and II from fundus photographs according to Scheie's classification [52].

2.4. Serum Lipids Profiles. The serum levels of TC, TG, HDL-C, and LDL-C were determined by the standard enzymatic colorimetric technique, using Cholesterol CHOD-PAP (CHOL) and Triglyceride GPO-PAP (TG) Kits (Roche Diagnostics GmbH, Mannheim, Germany) and L-Type HDL-C and L-Type LDL-C Kits (Wako Life Sciences, Inc.), respectively.

2.5. Multiplex Fluorescent Immunoassay for Cytokines. Serum from 2 mL fasting peripheral blood samples was collected at 2000 ×g for 10 minutes in the morning and rapidly stored at −80°C. Serum concentrations of cytokines were assayed using Bio-Plex Human 6-Plex (IL-1β, IL-6, IL-4, IL-10, TNF-α, and RANTES) Kits and Bio-Plex Human 1-Plex (TGF-β) Kits (Laboratories, Hercules, California, USA). The coupled beads, serum samples, antibodies, and streptavidin-PE (each 50 μL) were prepared and added prewetted to the wells of a 96-well filter plate one after another after two washing procedures. Samples were run in duplicate using Bio-Plex MAGPIX (Bio-Rad Laboratories, Inc., USA). The plate was read using Bio-Plex Manager software version 6.0. As the objective concentration of most samples was too low to read in pg/mL levels, the corresponding fluorescence intensity was used as the relative concentration. (The standard curves were presented in Supplementary Figures 1(a)–1(f), in Supplementary Material available online at http://dx.doi.org/10.1155/2015/481621; the quantitative range and the intra- and interassay CV% were shown in Supplementary Table 1).

2.6. Lipid Peroxidation and the Activity of Antioxidant Enzymes. The lipid peroxide (LPO) concentration in serum was determined quantitatively by using an LPO Assay Kit (Nanjing Jiancheng Bio, Jiangsu, China) according to the protocol of the manufacturer. The product absorbance of a molecule of LPO and two molecules of chromogenic agent was read at a wavelength of 586 nm with TECAN Sunrise immediately after incubation at 45°C for 60 min. The concentration of LPO (μmol/L) was calculated according to the following formula:

$$\text{LPO}\left(\mu\text{mol/L}\right) = \frac{\text{measured OD} - \text{blanked OD}}{\text{standard OD} - \text{blanked OD}} \times \text{standard sample}\left(10\,\mu\text{mol/L}\right). \quad (1)$$

Superoxide dismutase (SOD) activity was determined using an SOD Water-Soluble Tetrazolium Salt (WST-1) Assay Kit (Nanjing Jiancheng Bio, Jiangsu, China). The corresponding SOD of the 50% inhibition ratio in the response system of 20 μL of SOD solution, 20 μL of serum samples, and 200 μL of substrate was defined as 1 activity unit (U). The change of SOD activity was determined by measuring the absorbance at 450 nm using TECAN Sunrise.

Peroxidase (POD) activity was determined using a POD Assay Kit (Nanjing Jiancheng Bio, Jiangsu, China). The corresponding POD of catalysis of 1 μg of substrate H_2O_2 using 1 mL of serum at 37°C for 1 min was defined as 1 activity unit (U). The change of POD activity was determined by measuring the absorbance at 420 nm using TECAN Sunrise.

Catalase (CAT) activity was assessed by using a CAT Assay Kit (Nanjing Jiancheng Bio, Jiangsu, China). Dissociation of 1 μmol of substrate H_2O_2 using 1 mL of serum at 37°C for 1 min was defined as 1 activity unit (U). The chromogenic agent ammonium molybdate, combined with surplus substrate H_2O_2 in the response system, produced a yellow-colored product that was measured at 405 nm using TECAN Sunrise.

Glutathione peroxidase (GSH-px) activity was assessed by using a GSH-px Assay Kit (Nanjing Jiancheng Bio, Jiangsu, China). When 4 μL of whole blood reacts with the substrate H_2O_2 at 37°C for 5 min after deducting the effect of nonenzymatic response, a GSH concentration decline of 1 μmol/L in response system was defined as 1 activity unit (U). GSH combined with the chromogenic agent dithiobisnitrobenzoic acid produced stable yellow five-glucosinolate two-nitro benzoic acid anion that was measured at 412 nm using TECAN Sunrise.

2.7. Serum Apolipoproteins. The levels of different apolipoproteins were detected by Bio-Plex MAGPIX platform or ELISA. ApoA1, ApoA2, ApoB, ApoC2, and ApoC3 were assayed using Human Apolipoprotein MAGNETIC BEAD PANEL Kit (Millipore, MA, US). ApoE and ApoH were assayed using Apolipoprotein E and Apolipoprotein H Human ELISA Kits (Abcam, Cambridge, UK), respectively, and ApoJ was assayed using Human Clusterin Quantikine ELISA Kit (R&D Systems China Co. Ltd.). For ELISA, serum samples were diluted 1 : 400 into diluent. Apolipoprotein standards or samples (50 μL) were added per well of human apolipoprotein in a microplate, and the assay was run according to the manufacturer's protocol. After finishing the experiments, the absorbance was immediately read on a microplate reader at a wavelength of 450 nm. The mean value of the duplicate or triplicate readings for each standard and sample was calculated, and a standard curve using 8 standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis was generated. The best-fit

Table 1: Demographic information of participants in different cognitive statuses (total sample size: $N = 440$).

		MMSE scores			Total N	P
		28 ≤ MMSE ≤ 30[#]	24 ≤ MMSE ≤ 27	MMSE ≤ 23		
Age, years, n		76 ± 9.5, 316	83.9 ± 8.3, 93	89.1 ± 5.0, 31	440	<0.001
BMI, n		24.6 (22.7–26.5), 316	24.0 (22.5–27.1), 93	22.7 (21.2–25.9), 31	440	0.096[#]
AS levels[*], n, %	0	23 (7.3%)	1 (1.1%)	0 (0%)	24 (5.5%)	<0.001
	I	228 (72.2%)	60 (64.5%)	14 (45.2%)	302 (68.6%)	
	II	65 (20.6%)	32 (34.4%)	17 (54.8%)	114 (25.9%)	
Hs-CRP (mg/L)	<0.3	99 (32.8%)	21 (25.3%)	10 (38.5%)	130 (31.6%)	0.128
	0.3~1	149 (49.3%)	36 (43.4%)	9 (34.6%)	194 (47.2%)	
	1~3	43 (14.2%)	19 (22.9%)	6 (23.1%)	68 (16.5%)	
	≥3	11 (3.6%)	7 (8.4%)	1 (3.8%)	19 (4.6%)	
Comorbidities[*], n, %	1	2 (0.6%)	2 (2.2%)	0 (0%)	4 (0.9%)	0.13
	2	5 (1.6%)	1 (1.1%)	0 (0%)	6 (1.4%)	
	3	17 (5.4%)	1 (1.1%)	2 (6.5%)	20 (4.5%)	
	4	30 (9.5%)	4 (4.3%)	2 (6.5%)	36 (8.2%)	
	5	43 (13.6%)	10 (10.8%)	3 (9.7%)	56 (12.7%)	
	6	62 (19.6%)	18 (19.4%)	8 (25.8%)	88 (20.0%)	
	7	61 (19.3%)	24 (25.8%)	8 (25.8%)	93 (21.1%)	
	8	60 (19.0%)	11 (11.8%)	7 (22.6%)	78 (17.7%)	
	9	29 (9.2%)	17 (18.3%)	1 (3.2%)	47 (10.7%)	
	10	7 (2.2%)	5 (5.4%)	0 (0%)	12 (2.7%)	

One-way ANOVA was used to test the differences of age and BMI in different groups.
Pearson Chi-Squared test was used for categorical variables.
[*]Fisher's Exact Test was used for examining AS levels and comorbidities.
[#]In AS levels, compared to 24 ≤ MMSE ≤ 27, $P = 0.004$; compared to MMSE ≤ 23, $P < 0.001$.

line was determined by regression analysis using log-log or four-parameter logistic curve fit (the representative standard curve shown in Supplementary Figure 2). The unknown sample concentration from the standard curve was multiplied by the dilution factor. The average intra- and interassay CV% were 4.6% and 7.4%, respectively (Supplementary Table 1). The minimum detectable dose of apolipoprotein was typically ~0.03 μg/mL. The standard added value was 0.05–0.5 μg/mL. For the Milliplex 5-plex apolipoprotein assay, similar to the Bio-Plex Human 6-Plex assay with Bio-Plex MAGPIX, the coupled beads, serum samples (5 μL), and antibodies were prepared according to the manufacturer's instructions. Samples were run in duplicate using the Luminex 200 System (Luminex, Austin, USA). The objective concentration unit was ng/mL. The average intra- and interassay CV% were shown in Supplementary Table 1.

2.8. High-Sensitivity C-Reactive Protein Measurements. The serum high-sensitivity CRP (HsCRP) concentration was determined with an HsCRP Kit (Jun Shi Bioscientific, Shanghai, China) for study subjects using an immunonephelometric assay improved to provide greater sensitivity, which has been described in detail in the literature [38]. The World Health Organization CRP reference standard was used. The intra- and interassay CV% for this assay were ≤4.0% and ≤5.0%, respectively. The technicians were blinded to the case-control status of the samples. The normal value of Hs-CRP is <0.3 mg/L. The study subjects with Hs-CRP ≥ 10 were considered as suffering from acute infection and were excluded from the study.

2.9. Statistical Analysis. Body mass index (BMI) was used to show weight change. Hs-CRP levels were stratified into four degrees: (1) Hs-CRP < 0.3; (2) 0.3 ≤ Hs-CRP < 1; (3) 1 ≤ Hs-CRP < 3; (4) Hs-CRP ≥ 3. All statistical analyses were performed using SPSS Version 19.0 (SPSS Inc., Chicago, IL). Pearson Chi-Squared test was used to investigate the categorical variables including Hs-CRP, fundus atherosclerosis (AS) levels, and comorbidities. For the analyses of continuous variables, all of them were tested and, if necessary, transformed to more likely approximate the normal distribution. As a consequence, the levels of ApoC2, ApoC3, ApoE, and ApoH were log transformed, and one-way ANOVA was used for examining differences on age, ApoC2, ApoC3, ApoE, and ApoH among these groups. Kruskal-Wallis test was used to analyze the other continuous variables for they could not be transformed to normal distribution successfully under any transformations. To assess whether the factors which showed significant differences among different cognitive statuses could be associated with cognitive impairment, multivariate logistic regression analysis was performed. A P value <0.05 was considered statistically significant.

3. Results

3.1. Demographic Information. The total sample size of this cross-sectional study is 440. Table 1 showed the demographic

Table 2: Serum probable biomarkers of lipid, apolipoprotein, and oxidative and inflammatory status in participants with different cognitive statuses.

	MMSE scores						Total N	P
	$28 \leq$ MMSE ≤ 30	n	$24 \leq$ MMSE ≤ 27	n	MMSE ≤ 23	n		
TC (mmol/L)	4.6 (4.0–5.2)	316	4.5 (3.9–5.1)	93	4.8 (4.1–5.5)	31	440	0.273
TG (mmol/L)	1.4 (1.0–1.9)	316	1.3 (1.0–1.8)	93	1.4 (1.1–1.8)	31	440	0.268
HDL-C (mmol/L)	0.95 (0.82–1.12)	316	0.99 (0.88–1.12)	93	1.13 (0.99–1.38)	31	440	0.002
LDL-C (mmol/L)	2.78 (2.32–3.39)	316	2.73 (2.24–3.23)	93	2.89 (1.95–3.49)	31	440	0.793
LPO (μmol/L)	0.70 (0.46–0.98)	298	0.64 (0.47–0.97)	89	0.71 (0.50–0.96)	31	418	0.723
POD (U/mL)	7.81 (5.59–11.41)	304	9.04 (6.42–15.40)	89	9.57 (6.39–13.27)	31	424	0.026
SOD (U/mL)	10.44 (8.79–11.80)	275	10.84 (9.42–12.14)	84	10.73 (9.73–11.71)	29	388	0.134
CAT (U/mL)	51.82 (42.98–59.56)	297	52.25 (43.72–58.05)	84	51.92 (43.36–60.11)	30	411	0.962
GSH-px (μmol/L)	70.91 (59.18–79.87)	288	70.95 (48.07–79.57)	83	67.95 (44.43–82.19)	30	401	0.840
IL-1β**	9.0 (8.0–10.0)	301	9.0 (8.0–10.0)	85	9.0 (8.0–9.8)	29	415	0.708
IL-4**	8.8 (8.0–9.0)	302	8.0 (8.0–9.0)	89	8.0 (7.5–9.5)	31	422	0.462
IL-6**	21.0 (16.0–29.0)	299	26.0 (18.0–38.5)	87	27.0 (19.0–35.0)	29	415	0.001
IL-10**	21.0 (17.0–25.5)	299	21.0 (16.3–25.0)	84	20.0 (16.8–23.3)	30	413	0.961
TNF-α**	8.0 (7.0–9.0)	302	8.0 (7.0–9.0)	88	8.0 (7.0–9.0)	31	421	0.224
TGF-β**	8087.0 (601.0–15318.0)	303	9093.5 (1732.5–14060.8)	88	6455.5 (2442.2–11118.0)	31	422	0.565
RANTES**	585.3 (435.5–830.9)	302	589.0 (426.5–870.8)	89	670.5 (457.5–997.0)	31	422	0.699
ApoA1 (mg/mL)	1.14 (0.97–1.28)	289	1.10 (0.97–1.27)	81	1.05 (0.68–1.37)	28	398	0.545
ApoA2 (μg/mL)	292.20 (247.00–358.70)	295	262.30 (199.40–313.70)	85	271.60 (217.7–364.3)	28	408	0.001
ApoB (μg/mL)	60.20 (41.20–76.70)	295	60.20 (35.88–83.05)	93	47.50 (21.21–61.80)	31	419	0.079#
ApoC2 (ng/mL)	4.66 ± 0.25*	299	4.57 ± 0.27	85	4.58 ± 0.22	28	412	0.005
ApoC3 (ng/mL)	5.17 ± 0.28	298	5.12 ± 0.25	86	5.10 ± 0.32	29	413	0.147
ApoE (μg/mL)	1.74 ± 0.21	305	1.74 ± 0.22	89	1.79 ± 0.18	30	424	0.421
ApoJ (μg/mL)	159.4 (2.56–201.7)	305	161.79 (3.58–199.88)	89	163.40 (131.74–194.13)	30	424	0.682
ApoH (ng/mL)	5.58 ± 0.11	305	5.57 ± 0.15	89	5.62 ± 0.075	31	425	0.204

Statistics details: one-way ANOVA for ApoC2, ApoC3, ApoE, and ApoH; Kruskal-Wallis test for TC, TG, HDL-C, LDL-C, LPO, POD, SOD, CAT, GSH-px, IL-1β, IL-4, IL-6, IL-10, TNF-α, TGF-β, RANTES, ApoA1, ApoA2, ApoB, and ApoJ.

Data of ApoA2, ApoC3, ApoE, and ApoH are presented as mean ± SD; data of TC, TG, HDL-C, LDL-C, LPO, POD, SOD, CAT, GSH-px, IL-1β, IL-4, IL-6, IL-10, TNF-α, TGF-β, RANTES, ApoA1, ApoA2, ApoB, and ApoJ are presented as median (first quartile–third quartile).

*Compared to $24 \leq$ MMSE ≤ 27, $P = 0.007$.

#Borderline significance.

**As the objective concentration was too low, the fluorescence intensity was used as the relative concentration.

information of participants in different cognitive statuses, including age, BMI, Hs-CRP, AS levels, and comorbidities. Among three categorical variables, statistics analysis showed that age changed strikingly in three groups ($P < 0.001$) and the levels of AS are associated with cognitive status ($P < 0.001$). According to our study, participants with higher MMSE score, for example, subjects in group 1 ($28 \leq$ MMSE ≤ 30), shared lower levels of fundus AS when compared with those in group 2 ($24 \leq$ MMSE ≤ 27) ($P = 0.004$) and group 3 (MMSE ≤ 23) ($P < 0.001$) (Table 1), which probably suggested that the AS degree may be aggravated with cognitive impairment. However, there were no significant disparities in Hs-CRP and comorbidities among three cognitive statuses (Table 1).

3.2. Changes of Serum Biomarkers in Different Cognitive Statuses. We have examined differences of serum biomarkers among three groups including 5 markers of redox homeostasis (LPO, POD, SOD, CAT, and GSH-px), 7 markers of inflammatory status (IL-1β, IL-4, IL-6, IL-10, TNF-α, TGF-β, and RANTES), 4 serum lipoproteins (TC, TG, HDL-C, and LDL-C), and 8 serum apolipoproteins (ApoA1, ApoA2, ApoB, ApoC2, ApoC3, ApoE, ApoJ, and ApoH) (Table 2). The result showed significant differences of antioxidant enzyme POD ($P = 0.026$), proinflammatory cytokine IL-6 ($P = 0.001$), HDL-C ($P = 0.002$), ApoA2 ($P = 0.001$), and ApoC2 ($P = 0.005$) among three groups (Table 2). Subjects in group 3 (MMSE ≤ 23) and group 2 ($24 \leq$ MMSE ≤ 27) had higher levels of HDL-C, POD, and IL-6 than in group 1 ($28 \leq$ MMSE ≤ 30), and an obvious increase in the average age could also be observed from the higher scores of MMSE to the lower scores of MMSE, indicating that age, HDL-C, POD, IL-6, ApoA2, and ApoC2 might be closely related to the changes in cognitive status. There were no significant differences in other continuous variables under different MMSE scores according to our study (Table 2).

3.3. The Probable Serum Markers of Cognitive Impairment. To assess which factors could be associated with cognitive impairment, we performed multivariate logistic regression

Table 3: Logistic regression analyses for biomarkers associated with cognitive status.

	28 ≤ MMSE ≤ 30 versus *			24 ≤ MMSE ≤ 27 versus *		
	OR	95% CI	*P*	OR	95% CI	*P*
POD	1.01	0.93–1.10	0.744	1.04	0.95–1.13	0.385
IL-6	1.01	0.98–1.05	0.352	1.01	0.98–1.04	0.420
HDL-C	0.56	0.08–3.77	0.553	0.19	0.03–1.44	0.107
ApoA1	1.30	1.01–1.67	0.045	1.47	1.11–1.94	0.008
ApoA2	0.43	0.18–1.02	0.056	0.21	0.08–0.54	0.001
ApoC2	0.99	0.98–1.02	0.815	0.99	0.97–1.02	0.496

* Control group: MMSE ≤ 23.
Control variables: age, BMI, AS level, and comorbidities.

analysis with the factors above. In this analysis, age, BMI, AS levels, and comorbidities were set as control variables, and group 3 (MMSE ≤ 23) was regarded as the control group. The result presented that ApoA1 and ApoA2 might actually have impacts on cognitive impairment (Table 3). ApoA1 (28 ≤ MMSE ≤ 30: OR: 1.30, 95% CI 1.01–1.67; 24 ≤ MMSE ≤ 27: OR: 1.47, 95% CI 1.11–1.94) and ApoA2 (28 ≤ MMSE ≤ 30: OR: 0.43, 95% CI 0.18–1.02; 24 ≤ MMSE ≤ 27: OR: 0.21, 95% CI 0.08–0.54) were significantly associated with cognitive function (Table 3). Participants with a higher level of ApoA1 might have a lower risk of cognitive impairment, indicating that ApoA1 might be a protective factor in the process of cognitive impairment; on the contrary, ApoA2 might be an adverse factor of cognitive performance; that is, participants with a higher level of ApoA2 had a higher risk of undergoing cognitive impairment.

4. Discussion

We evaluated 32 factors which were known or unknown to be risks for cognitive impairment and found out that age, levels of fundus AS, POD, IL-6, HDL-C, ApoA2, and ApoC2 were significantly changed among different cognitive statuses, and ApoA1 and ApoA2 might be independently associated with cognitive impairment after adjusting for age, BMI, levels of fundus AS, and comorbidities. Subjects with older age, a lower level of ApoA1, and a higher level of ApoA2 might have more possibilities of undergoing cognitive impairment.

Vascular factors and related diseases were the risk factors in the development of the dementia syndrome [5, 6]. Endothelium-mediated mechanisms underlay vascular dysfunction in AD pathogenesis. Retinal microvascular changes may reflect similar pathophysiological processes in the brains of patients with AD [53]. A systemic review showed that retinal microvascular changes were significantly associated with dementia, cognitive impairment, and brain imaging abnormalities in cross-sectional studies [54]. Although the method to measure retinal microvascular changes in the present study was different, our results indicated that the severity of fundus AS was a significant risk factor of cognitive impairment. Moreover, the levels of fundus AS were the main independent factors that influenced aging [52].

Oxidative stress is strongly linked to brain aging. Although the results of clinical trials with antioxidant treatments were controversial [11, 14, 15], oxidative and antioxidant statuses were closely associated with neurodegeneration in AD patients [8, 12, 13]. In the present study, although the activity of POD was significantly different among three cognitive statuses, it did not remain of statistical significance in different cognitive statuses after adjusting for other factors (Tables 2 and 3). Numerous previous studies of antioxidant enzymes, including the activity of Cu/Zn superoxide dismutase 1, GSH, and CAT in the blood of AD or MCI patients, indicated no changes [55–58]. However, the analysis of total antioxidant status in serum samples of major studies previously revealed an overall decrease in MCI or AD, even if antioxidant enzymes were found to be unchanged [8, 59–61]. The results of assays of all antioxidant enzymes obviously reflected that the antioxidant statuses were much better than the evaluation of antioxidant enzyme activities. The antioxidant status in serum includes enzymatic and nonenzymatic antioxidants, such as soluble uric acid and ascorbic acid, lipid soluble vitamin A, vitamin E, and α- and β-carotene, dietary antioxidant nutrients, and metal proteins [8].

Chronic low-grade inflammation in older adults was closely linked to cognitive decline and dementia. A long-term study (over 20 years) indicated that repeated high or increasing IL-6 was associated with cognitive impairment; CRP alterations were inconsistent with cognition, which may involve impacts of statin medications and survival effects [62]. In our study, only a higher level of IL-6 was found in groups with lower MMSE scores, while other proinflammatory and anti-inflammatory cytokines, including systemic Hs-CRP levels, were not found to be related to cognitive status (Tables 1 and 2). The majority of previous studies demonstrated that both IL-6 and CRP were significantly elevated before the clinical onset of symptoms [16–20]. In combination with our present study, the measurements of these biomarkers might have a sensitive relationship with the asymptomatic high-risk individuals in the preclinical stage. Improving the sample size of the study is necessary to confirm the relationship between IL-6, Hs-CRP, and preclinical AD, such as memory impairment and MCI in middle-aged subjects and dementia in subjects with higher age.

Although there were contradictory results, some lipid and apolipoprotein patterns had been revealed as potential determinants of dementia and predementia syndrome [63–65]. Based on a wide range of functions, such as antioxidation, anti-inflammation, and proendothelial functions, HDL-C plays an important role in preserving cognitive function under normal and age-related and progressive neurodegenerative disorders [64]. Most studies suggested high level of cholesterol as risk factor of AD and cognitive impairment [66, 67] and later dementia [68, 69]. Moreover, high level of LDL and TC was not associated with increased risk in the individuals with one or more ApoEε4 alleles [66]. However, Mielke et al. conducted an 18-year longitudinal study on 70-year-old nonsmokers and found that the increased TC level in late life was associated with the decreased dementia risk [70]. This contradictory finding might be due to the different timings in TC measurements and the clinical onset

of dementia [63]. Accumulating lines of evidence suggested that serum lipid levels were bidirectionally associated with cognitive impairment, including dementia and predementia syndrome. High midlife serum TC represented a risk factor for subsequent dementia, and low serum TC and LDL-C after midlife were a risk factor for late-life cognitive impairment [28, 33]. Our cross-sectional data indicated that only HDL-C showed a statistical increase in the progress of cognitive impairment (Table 2); however, the statistical significance disappeared after adjusting for other factors (Table 3).

Regarding the apolipoproteins, a previous study demonstrated that MCI subjects demonstrated lower levels of ApoA1, ApoA2, and ApoH, higher levels of ApoE and ApoJ, and a higher ApoB/ApoA1 ratio [37]. The result in the literature on plasma or serum level of ApoE in AD was inconclusive. A study indicated that the level of ApoE was significantly higher in the MCI group [37], while several studies demonstrated no association of plasma ApoE level with AD [71, 72]. A cohort study conducted on patients with AD, patients with MCI, and healthy controls showed that the plasma level of ApoE was the lowest in those who were undergoing progress from MCI to dementia [73]. Recently, a meta-analysis revealed that the level of peripheral blood ApoE was lowered in AD patients, and ApoE might be a potential risk factor for AD [65]. Our cross-sectional data indicated that only ApoA2 and ApoC2 greatly changed in different cognitive statuses (Table 2). The result was in accordance with previous studies [40]. After adjusting for other factors, ApoA1 and ApoA2 were found to be associated with cognitive impairment (Table 3). However, there was no relationship between serum ApoE level and cognitive status. The negative result should be explained carefully because we neglect the effects of ApoE genotype on serum ApoE concentration. The ApoE genotype was related to a unique biochemical profile, such as ApoE, ApoB, and C-reactive protein levels, irrespective of AD or MCI diagnosis [74]. Some studies showed that the lower ApoE levels in individuals with AD [75] and the high plasma ApoE levels in old age with cognitive impairment [76] were not accounted for by the ApoEε4 allele. Other studies demonstrated that ApoE genotype significantly influenced serum ApoE concentration [37, 73, 77]. A significant trend in reduction of the levels of ApoE from ε2 to ε4 carriers or from ε4 noncarriers and ε4 heterozygote carriers to ε4 homozygous individuals was observed among healthy centenarians and adults, MCI, AD, and age-matched controls [37, 46, 73, 77, 78]. Actually, no consistent association of blood ApoE levels with cognitive impairment was seen when controlling for ApoE genotype [63]. For example, ApoE levels were significantly higher in the MCI group [37] and significantly lower in patients with AD [46], and no difference in ApoE levels was seen between AD and age-related control group [73, 77, 78].

Other potential limitations of this study include the following several aspects. Firstly, the present study is the cross-sectional design of the main analyses. Without a direction of causality, the association between serum apolipoprotein patterns and cognition could not have a predictive impact. Secondly, only MMSE was used as a single measure of cognitive performance. Given that the MMSE scores of group 3 are ≤ 23, this would potentially represent mild/moderate dementia or possibly other conditions affecting memory, and group 2 have MMSE range which could be consistent with mild cognitive impairment. However, to identify these conditions would require clinical diagnosis using DSM IV for AD, Petersen's criteria for MCI, and/or other clinical tools. Therefore, the three groups used in this study were likely making it hard to interpret what variation in blood biomarker levels would mean that subjects suffered from mild cognitive impairment or dementia due to AD. Thirdly, the constitution of subjects in our study was limited to elderly male individuals only, with an average education level above high school and similar lifestyles, which might bring about biases for lacking female subjects. Only male subjects were included due to the inadequate sample size ($n = 60$) of the females, which made the results apply to men only. Thus, sex failed to be a factor in the study of relationship between cognition and biomarkers. In addition, the lack of potential confounding factors, such as social and psychological factors, a sedentary lifestyle, and frailty have been reported to increase the risk of cognitive impairment [79–82].All these might result in the inconsistency between our results and the published literature on apolipoprotein levels in different cognitive statuses. Some borderline significance occurred in our study, such as BMI and ApoB (Tables 1 and 2), possibly because of these limitations.

5. Conclusions

In conclusion, age, fundus AS levels, HDL-C, POD, IL-6, ApoA2, and ApoC2 showed significant disparities in different cognitive statuses. However, after adjusting for age, AS levels, BMI, and comorbidity numbers, only ApoA1 and ApoA2 were found to be possible risk factors of cognitive impairment in older men.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Cheng Ma and Jin Li contributed equally to this paper.

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