



Research Paper

Measurement of Blood Thiamine Metabolites for Alzheimer's Disease Diagnosis



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ABSTRACT

Background: Brain glucose hypometabolism is an invariant feature and has significant diagnostic value for Alzheimer's disease. Thiamine diphosphate (TDP) is a critical coenzyme for glucose metabolism and significantly reduced in brain and blood samples of patients with Alzheimer's disease (AD).

Aims: To explore the diagnostic value of the measurement of blood thiamine metabolites for AD.

Methods: Blood TDP, thiamine monophosphate, and thiamine levels were detected using high performance liquid chromatography (HPLC). The study included the exploration and validation phases. In the exploration phase, the samples of 338 control subjects and 43 AD patients were utilized to establish the models for AD diagnosis assayed by receiver operating characteristic (ROC) curve, including the variable γ that represents the best combination of thiamine metabolites and age to predict the possibility of AD. In the validation phase, the values of models were further tested for AD diagnosis using samples of 861 control subjects, 81 AD patients, 70 vascular dementia patients, and 13 frontotemporal dementia patients.

Results: TDP and the γ exhibited significant and consistent values for AD diagnosis in both exploration and validation phases. TDP had 0.843 and 0.837 of the areas under ROC curve (AUCs), 77.4% and 81.5% of sensitivities, and 78.1% and 77.2% of specificities respectively in the exploration and validation phases. The γ had 0.938 and 0.910 of AUCs, 81.4% and 80.2% of sensitivities, and 90.5% and 87.2% of specificities respectively in the exploration and validation phases. TDP and the γ can effectively distinguish AD from vascular dementia (64.3% for TDP, 67.1% for γ) and frontotemporal dementia (84.6% for TDP, 100.0% for γ).

Interpretation.

The measurement of blood thiamine metabolites by HPLC is an ideal diagnostic test for AD with inexpensive, easy to perform, noninvasive merits.

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1. Introduction

We still lack not only disease-modifying therapy but also ideal diagnostic tool for Alzheimer's disease (AD), a devastating neurodegenerative disease. The ideal diagnostic tool for AD should be inexpensive, easy to perform, and non-invasive, as defined by the Ronald and

Nancy Reagan Research Institute in 1998 (The Ronald and Nancy Reagan Research Institute of The Alzheimer's Association and National Institute on Aging Working Group's, 1998). Existing tests for AD diagnosis include the measurement of β -amyloid and tau in cerebrospinal fluid (CSF), the analysis of brain amyloid plaques and glucose metabolism by positron emission tomography, the evaluation of brain atrophy by magnetic resonance imaging (Palmert et al., 1990; Price et al., 2005; Rosenmann, 2012; Vigo-Pelfrey et al., 1995). Although they can increase the certainty of AD diagnosis, they have not been recommended for routine diagnosis in the new AD diagnosis guideline (McKhann et al., 2011),

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because they are either expensive or difficult to perform (Cummings, 2011).

Impaired cerebral glucose metabolism is an invariant feature of AD and precedes the onset of clinical symptoms (Chen and Zhong, 2013; Small et al., 1995; Reiman et al., 1996; Mosconi et al., 2008a; Chen et al., 2010). Thiamine diphosphate (TDP), the active form of thiamine, is critical for glucose metabolism as a key coenzyme of three rate-limiting enzymes of glucose catabolism (pyruvate dehydrogenase and α -ketoglutarate dehydrogenase in the Krebs cycle, and transketolase in pentose phosphate pathway) (Zhao et al., 2009). The reduction in TDP level and activities of TDP-dependent enzymes has been demonstrated both in blood and autopsied brain samples of a small number of AD patients (Gibson and Blass, 2007; Butterworth and Besnard, 1990; Gibson et al., 1988; Héroux et al., 1996; Gold et al., 1995; Mastrogiacoma et al., 1996; Bettendorff et al., 1996). However, the diagnostic value of the measurement of blood thiamine metabolites for AD has not been reported. Here, we found that the measurement of blood thiamine metabolites using high performance liquid chromatography (HPLC) fluoroscopy exhibited significant performance for AD diagnosis with over 80% of sensitivity and specificity, significant capacities of differentiating AD from vascular dementia (VaD) and frontotemporal dementia (FTD). It is an ideal test for AD diagnosis with inexpensive, easy to perform, noninvasive merits.

2. Methods

2.1. Study and Subjects

This study was approved by the Committee on Medical Ethics of Zhongshan Hospital, Fudan University. Informed consent was obtained from all participating subjects.

The study was designed into the exploration and validation phases. In the exploration phase, the diagnostic models were established and their diagnostic values for AD were preliminarily evaluated by receiver operating characteristic (ROC) curve using samples of 43 AD patients and 338 control subjects with cognitive ability in the normal range assayed by the Mini-Mental Status Evaluation (MMSE) scoring. These samples were collected from three centers (Shanghai Mental Health Center, Haiwan Hospital and Regional Health Service Center of Xujiahui) from January to May 2012. In the validation phase, the diagnostic values of the models for AD were further tested using samples of 81 AD patients, 70 VaD patients, 13 FTD patients, and 861 control subjects collected from other four hospitals (Zhongshan hospital, Fengcheng hospital, Huanhu hospital, and Brain Hospital affiliated to Nanjing medical university) as well as the three hospitals in the exploration phase (Shanghai Mental Health Center, Haiwan Hospital, and Regional Health Service Center of Xujiahui) from June 2012 to December 2014.

All subjects received the MMSE scoring. Subjects with reduced MMSE scores received further neurological examination and neuropsychological evaluation including the Activity of Daily Living (ADL) scoring, Clinical Dementia Rating (CDR), and Hamilton Depression Rating Scales (HDR) by enquiring patients and his/her healthcare givers. All patients had cranial MRI/CT scan and were diagnosed simultaneously by two neurologists and/or psychiatrists specialized in dementia (Chunjiu Zhong, Guoqiang Fei, Lirong Jin, Weihong Hu, Rong Ji, Jun Xu and Qunying Zhang) according to DSM-IV. All subjects were tested for blood hemoglobin, hepatic and renal functions. AD patients were further tested blood folate and vitamin B12 levels, and thyroid function.

The following subjects were enrolled: 1. AD patients: (1) with main complaint of memory decline characterized by gradual onset and continuing deterioration over one year; (2) with decreased MMSE score (<26) and at least one other cognitive deficit beyond memory impairment; (3) with increased CDR score (≥ 0.5) and impairment in the performance of daily activities; (4) without a sign of damage in brain structure as assayed by cranial CT and/or MRI scans, except for brain atrophy; (5) without a diagnostic clue of other neurological disorders; (6) ≥ 65 years old. 2. VaD patients (Roman et al., 1993):

(1) with two or more cognitive deficits; (2) with acute or sudden onset; (3) with focal neurological sign(s) consistent with the evidence of stroke by cranial CT and/or MRI imaging. 3. Frontotemporal dementia patients included frontal variant frontotemporal dementia and progressive non-fluent aphasia (McKhann et al., 2001): (1) early and progressive change in personality or language; (2) causing significant impairments in social and occupational function; (3) with a gradual onset; (4) excluding other neurological conditions or systematic diseases.

The following subjects were excluded from this study: (1) Alzheimer's disease patients with reduced blood folate and vitamin B12 levels, or thyroid dysfunction; (2) subjects with major disorders of gastrointestinal tracts; (3) subjects with major depression (HDR over 7 scores); (4) subjects taking thiamine supplements within one month; (5) subjects with chronic alcohol abuse.

2.2. Blood TDP, Thiamine Monophosphate (TMP) and Thiamine Measurement

Blood TDP, TMP, and thiamine levels in all subjects were measured using HPLC fluoroscopy at a single center. The HPLC operators were blind to participant information. The HPLC method for measuring TDP, TMP and thiamine levels was essentially as previously described (Pan et al., 2010). Briefly, after fasting whole blood samples anticoagulated with heparin were collected, 150 μ l of the whole blood samples were mixed immediately with equal volume of 7.4% perchloric acid in a 1.5 ml of Eppendorf tube. The mixture was vibrated for 30 s for deproteinization. After centrifugation at 10,000 rpm at 4 °C for 6 min, the supernatant was collected and stored at -20 °C until assay within two weeks. Thiamine and its phosphate esters were derivatized into thiochromes using potassium ferricyanide and separated by gradient elution with a C18 reversed-phase analytical column (250 \times 4.6 mm). The derivatives were measured by HPLC fluoroscopy (Agilent 1100, Santa Clara, CA) with an excitation wavelength of 367 nm and an emission wavelength of 435 nm. Blood TDP, TMP and thiamine levels were quantified using standard samples of TDP, TMP, and thiamine (Sigma-Aldrich, St. Louis, MO).

2.3. Apolipoprotein E Genotypes Analysis

Apolipoprotein E (APOE) alleles were detected using an ABI real-time TaqMan SNP genotyping assay (ABI, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Purification of human genomic DNA was performed using a genome extraction kit (TIANGEN, Beijing, China). All subjects were divided into two groups, APOE $\epsilon 4$ -positive group with $\epsilon 2/\epsilon 4$, $\epsilon 3/\epsilon 4$, or $\epsilon 4/\epsilon 4$ genotypes and APOE $\epsilon 4$ -negative group with $\epsilon 2/\epsilon 2$, $\epsilon 2/\epsilon 3$, or $\epsilon 3/\epsilon 3$ genotypes.

2.4. Statistical Analysis

The results were expressed as mean \pm SEM except for demographic data (means \pm SD). Statistical analyses were performed using the Statistical Package for the Social Sciences software version 18.0 (SPSS Inc., Chicago, IL). Kruskal–Wallis test, Pearson chi-square test or Fisher exact probability test were used to compare demographic data among groups of control subjects and AD, VaD, FTD patients. Covariance analysis was used to compare TDP levels adjusted for age and hemoglobin levels. Mann–Whitney U test or Kruskal–Wallis test was utilized to compare TMP and thiamine levels among groups of control subjects and AD, VaD, FTD patients as well as AD subgroups divided by the disease severity. Spearman's rank correlation analysis was utilized between the levels of thiamine metabolites and age or hemoglobin levels.

ROC analysis was utilized to evaluate the values of different parameters for AD diagnosis by assessing the area under the ROC curve (AUC), sensitivity, and specificity. Cut-off point was determined by maximizing the sum of sensitivity and specificity. The parameters included TDP and a new variable γ predicted the probability of logic (P) value for AD

diagnosis from the multivariate logistic regression analysis of TDP, TMP, thiamine, and age. The variable γ was derived to identify the best combination of multiple diagnostic biomarkers based on the following equation: $\gamma = (1/\text{TDP}) * ((\text{TMP} + 1)^{-0.01}) * ((T + 1)^{1/6}) * \text{Age}^2$ by MATLAB R14 (Mathworks, Natick, MA).

3. Results

3.1. Study Subjects

The detailed demographic data were listed in Table 1 and Supplementary Fig. S1. Forty-three AD patients and 338 control subjects were enrolled in exploration phase. There were no significant differences in gender ratio between the two groups. AD patients were older than control subjects and had fewer years of education, decreased MMSE scores and hemoglobin levels (all $P < 0.001$). The presence of APOE $\epsilon 4$ allele in AD patients was significantly higher than that in control subjects. In validation phase, 81 AD patients, 861 control subjects, 70 VaD patients, and 13 FTD patients were recruited. FTD patients included two patients with progressive non-fluent aphasia and 11 patients with frontal variant FTD. There were no significant differences in gender ratio among AD, VaD, FTD, and control groups. Patients with AD and VaD were older than control subjects ($P < 0.001$) and FTD patients ($P < 0.05$). Decreased educational years and MMSE scores were observed in patients with AD, VaD, and FTD as compared with control subjects ($P < 0.001$). The presences of APOE $\epsilon 4$ alleles in AD and VaD patients were higher than that in control subjects ($P < 0.001$ in AD; $P < 0.05$ in VaD).

3.2. Blood TDP, TMP, Thiamine Levels and Their Diagnostic Value for AD

Blood TDP levels in AD patients were significantly lower as compared with that in control subjects in the exploration phase (85.02 ± 4.01 nmol/L, $n = 43$ in AD patients, vs. 117.70 ± 1.32 nmol/L, $n = 338$ in control subjects, $P < 0.001$). TMP and thiamine contents in AD patients were not significant different with that in control subjects (TMP: 4.83 ± 0.87 vs. 3.61 ± 0.14 nmol/L; thiamine: 4.88 ± 0.70 vs. 3.31 ± 0.16 nmol/L, both $P > 0.05$; Fig. 1A). Consistently, TDP contents in AD patients were significantly lower than that in control subjects, VaD patients, and FTD patients in the validation phases (86.50 ± 2.30 nmol/L, $n = 81$ in AD, 118.30 ± 0.92 nmol/L, $n = 861$ in control, 117.60 ± 4.56 nmol/L, $n = 70$ in VaD and 132.70 ± 12.64 nmol/L, $n = 13$ in FTD, $P < 0.001$). TMP levels had no significant differences among AD patients, control subjects, and FTD patients (AD: 10.84 ± 0.97 nmol/L, control: 9.85 ± 0.22 nmol/L, FTD: 9.60 ± 2.14 nmol/L, $P > 0.05$). VaD patients exhibited a significant reduction in TMP levels

(5.71 ± 0.51 nmol/L) compared to AD patients and control subjects (both $P < 0.001$). Thiamine levels had no significant differences among AD patients and control subjects, VaD and FTD patients (AD: 4.92 ± 1.04 nmol/L; control: 3.74 ± 0.13 nmol/L, VaD: 5.29 ± 0.99 nmol/L; FTD: 4.66 ± 1.23 nmol/L, $P > 0.05$, Fig. 1C).

According to data from the exploration phase, when TDP was used as a diagnostic biomarker, ROC analysis showed that AUC reached 0.843 (95% CI 0.774–0.911), with sensitivity of 77.4% and specificity of 78.1%, when the cut-off point was set to 99.48 nmol/L. Further, γ gave better diagnostic value, with AUC of 0.938 (95% CI 0.899–0.976), sensitivity of 81.4%, and specificity of 90.5%, when the cut-off point was set to 75.97 (Fig. 1B).

In the validation phase, both TDP level and γ exhibited consistent power for AD diagnosis as compared with that in the exploration phase, with AUCs of 0.837 (95% CI 0.790 to 0.883) and 0.910 (95% CI 0.880 to 0.941), sensitivities of 81.5% and 80.2%, specificities of 77.2 and 87.2% when the cut-off points of TDP level and γ were 99.48 nmol/L and 75.97, respectively (Fig. 1D).

VaD and FTD patients can be effectively distinguished from AD patients according the cut-off points of TDP and γ . TDP reached differentiating rates of 64.3% for VaD and 84.6% for FTD. γ reached differentiating rates of 67.1% for VaD and 100.0% for FTD.

3.3. Effect of the Disease Severity in AD Patients on Levels of Blood Thiamine Metabolites

The MMSE scores had no significant correlations with blood TDP levels in both control subjects ($r = 0.0518$, $P = 0.0731$, $n = 1199$) and AD patients ($r = 0.0706$, $P = 0.4359$, $n = 124$, Fig. 2A and B). To further exclude the effect of the severity of cognitive impairment on the levels of blood thiamine metabolites, we divided AD patients into three subgroups, including, severe moderate, and mild cognitive impairments, according to either their MMSE (MMSE < 10 in severe subgroup $n = 59$, MMSE of 10 - 17 in moderate subgroup $n = 42$, MMSE > 17 in mild subgroup $n = 23$) or CDR scores (CDR = 3 in severe subgroup $n = 55$, CDR = 2 in moderate subgroup $n = 29$, CDR ≤ 1 in mild subgroup $n = 40$). No significant differences in blood TDP and thiamine levels were observed among the three subgroups either divided by MMSE scores (TDP: in severe subgroup: 84.90 ± 3.23 , in moderate subgroup: 84.01 ± 2.89 , and in mild subgroup: 92.40 ± 4.69 nmol/L; thiamine: in severe subgroup: 4.45 ± 0.59 , in moderate subgroup: 3.36 ± 0.39 , in mild subgroup: 8.90 ± 3.45 nmol/L, all $P > 0.05$) or divided by CDR scores (TDP: in severe subgroup: 87.80 ± 3.10 , in moderate subgroup: 79.73 ± 3.34 , and in mild subgroup: 88.03 ± 3.94 nmol/L; thiamine: in severe subgroup: 4.54 ± 0.63 , in moderate

Table 1
Characteristics of study subjects.

Characteristics (mean \pm SE)		Gender		Age (years)	Education (years)	MMSE scores	Hemoglobin (g/L)	APOE $\epsilon 4$ (n, %)	Folate (ng/ml)	Vitamin B12 (pg/ml)
		Male (n, %)	Female (n, %)							
Exploration phase	Control (n = 338)	156 (46.15)	182 (53.85)	71.50 \pm 0.30 (63–89)	10.62 \pm 0.22 (0–16)	28.56 \pm 0.07 (26–30)	133.80 \pm 0.74 (83–169)	63 (18.64)	–	–
	AD (n = 43)	14 (32.56)	29 (67.44)	82.19 \pm 1.13 (65–95) ^a	5.09 \pm 0.71 (0–16) ^a	5.70 \pm 0.98 (0–20) ^a	122.80 \pm 1.55 (95–141) ^a	22 (51.16) ^a	6.88 \pm 0.62 (3.01–20)	363.30 \pm 25.77 (203.30–856.7)
Validation phase	Control (n = 861)	411 (47.74)	450 (52.26)	71.98 \pm 0.20 (63–91)	11.48 \pm 0.13 (0–16)	28.73 \pm 0.04 (26–30)	139.80 \pm 0.44 (89–193)	186 (21.60)	–	–
	AD (n = 81)	35 (43.21)	46 (56.79)	79.67 \pm 0.74 (66–101) ^{b,c}	7.16 \pm 0.59 (0–16) ^b	12.07 \pm 0.87 (0–25) ^b	127.1 \pm 1.79 (90–159) ^b	38 (46.91) ^b	7.79 \pm 0.50 (3.01–20)	516.50 \pm 42.80 (210.00–2000)
	VaD (n = 70)	37 (52.86)	33 (47.14)	77.23 \pm 0.90 (55–91) ^{b,c}	6.23 \pm 0.58 (0–16) ^b	8.15 \pm 0.93 (0–25) ^b	125.90 \pm 2.12 (87–170) ^b	23 (33.33) ^{d,s}	–	–
	FTD (n = 13)	5 (38.46)	8 (61.54)	59.31 \pm 1.81 (45–70) ^b	5.69 \pm 0.95 (0–12) ^b	7.08 \pm 2.15 (0–23) ^b	130.70 \pm 3.55 (118–148)	5 (41.66) [#]	–	–

a: compared with control in exploration phase, $P < 0.001$.

b: compared with control in validation phase, $P < 0.001$.

c: compared with FTD, $P < 0.001$.

d: compared with control, $P < 0.05$.

s: 23/69, 1 patient was unable to be determined; # 5/12, 1 patient was unable to be determined.

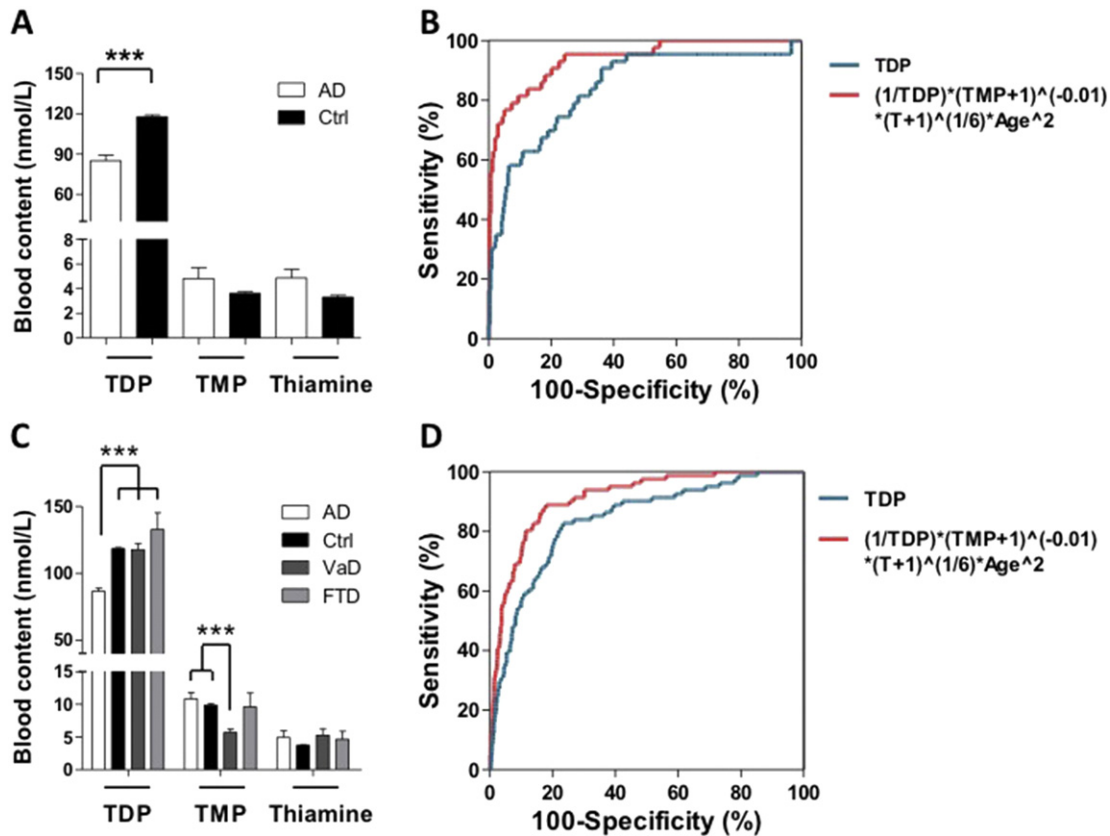


Fig. 1. Altered levels of blood thiamine metabolites in AD patients and their diagnostic values in the exploration and validation phases. **A.** Blood TDP levels in AD patients were significantly reduced as compared with that in control subjects ($P < 0.001$). There were no significant differences in TMP and thiamine contents between AD and control subjects (both $P > 0.05$). **B.** Diagnostic performances of blood TDP and the variable $\gamma = (1/TDP) \cdot (TMP + 1)^{-0.01} \cdot (T + 1)^{1/6} \cdot Age^2$. TDP: AUC was 0.843, the sensitivity was 77.4%, and the specificity was 78.1% when the cut-off point was set to 99.48 nmol/L; The variable γ : AUC was 0.938, the sensitivity was 81.4% and the specificity was 90.5% when the cut-off point was set to 75.97. **C.** In the validation phase, blood TDP levels in AD patients still were significantly reduced as compared with that in control subjects as well as that in VaD patients and FTD patients (all $P < 0.001$). Blood TMP levels in AD patients were not significantly different from that in control subjects and FTD patients ($P > 0.05$). TMP levels in VaD patients were significantly lower than that in AD and control subjects ($P < 0.001$). Thiamine levels in AD patients were not significantly different from that in control subjects, VaD patients, and FTD patients ($P > 0.05$). **D.** Diagnostic performances of blood TDP level and the variable γ in the validation phase. TDP: AUC was 0.837, the sensitivity was 81.5% and the specificity 77.2% when the cut-off point was 99.48 nmol/L; The variable γ : AUC was 0.910, the sensitivity was 80.2% and the specificity was 87.2% when the cut-off point was 75.97.

subgroup: 2.96 ± 0.35 , in mild subgroup: 6.83 ± 2.03 nmol/L, all $P > 0.05$). Importantly, TDP levels in the three subgroups still were significantly decreased as compared with that in control subjects (118.20 ± 0.76 nmol/L, $P < 0.001$, $n = 1199$). Blood TMP levels among AD patients with MMSE scores < 10 (6.39 ± 0.80 nmol/L) and of 10–17 (9.24 ± 1.52 nmol/L), as well as control subjects (8.09 ± 0.18 nmol/L) were not significantly different ($P > 0.05$). TMP levels in AD patients with MMSE scores > 17 (13.94 ± 1.64 nmol/L) were significantly increased as compared with that in control subjects ($P < 0.001$), and AD patients with MMSE scores < 10 ($P < 0.001$) and MMSE scores of 10–17 ($P < 0.01$). Blood TMP levels in AD subgroup with CDR score of ≤ 1 (12.56 ± 1.23 nmol/L) were significantly increased as compared with that in AD subgroups with CDR scores of 3 (6.00 ± 0.79 nmol/L, $P < 0.001$) and 2 (8.74 ± 2.02 nmol/L, $P < 0.01$), as well as control group ($P < 0.001$, Fig. 2C and D).

In addition, we also divided AD patients into two subgroups according to their ADL scores: the subgroup with ADL scores of ≥ 23 ($n = 45$) and subgroup with ADL scores of < 23 ($n = 79$). No statistical differences in TDP and thiamine levels were found between two subgroups (TDP: 85.73 ± 3.61 vs. 86.13 ± 2.47 nmol/L; thiamine: 4.50 ± 0.68 vs. 5.14 ± 1.07 nmol/L, both $P > 0.05$). Blood TDP levels in the two subgroups still were significantly reduced as compared with that in control group ($P < 0.001$). TMP levels in the subgroup with ADL < 23 (10.53 ± 1.03 nmol/L) were significantly increased as compared with that in the subgroup with ADL ≥ 23 (5.64 ± 0.80 nmol/L, $P < 0.001$) and control subjects ($P < 0.01$, Fig. 2E).

3.4. Effects of Age, the Presence of Apolipoprotein E $\epsilon 4$ Allele, and Hemoglobin Contents on the Levels of Thiamine Metabolites

There was a very weak negative correlation between age and blood TDP levels in control subjects ($r = -0.0823$, $P = 0.0022$) but not in AD patients ($r = -0.0001$, $P = 0.9988$, Fig. 3A and B). To exclude the potential effect of age on the levels of thiamine metabolites, we divided AD patients and control subjects into four subgroups: under 70 years (AD $n = 13$, control $n = 596$), 71–75 years (AD $n = 16$, control $n = 260$), 76–80 years (AD $n = 25$, control $n = 250$), and over 80 years (AD $n = 70$, control $n = 93$). TDP levels in all AD subgroups had no significant differences ($P > 0.05$) but were significantly reduced as compared with that in the age-matched control subgroups (≤ 70 years old subgroup: 87.31 ± 5.54 vs. 119.90 ± 1.07 nmol/L; 71–75 years old subgroup: 90.49 ± 6.28 vs. 118.10 ± 1.63 nmol/L; 76–80 years old subgroup: 81.27 ± 3.87 vs. 116.70 ± 1.72 nmol/L; over 80 years old subgroup: 86.40 ± 2.85 vs. 111.00 ± 2.60 nmol/L; all $P < 0.001$, Fig. 3C). No significant differences were found in TMP and thiamine levels between all AD subgroups and the age-matched control subgroups (TMP: ≤ 70 years old subgroup: 11.08 ± 3.59 vs. 8.29 ± 0.26 nmol/L, 71–75 years old subgroup: 11.58 ± 2.50 vs. 8.18 ± 0.42 nmol/L, 76–80 years old subgroup: 7.63 ± 1.48 vs. 7.36 ± 0.36 nmol/L, > 80 year subgroup: 8.08 ± 0.85 vs. 8.58 ± 0.74 nmol/L, all $P > 0.05$; thiamine: ≤ 70 years old subgroup: 8.28 ± 4.26 vs. 3.57 ± 0.14 nmol/L, 71–75 year subgroup: 8.09 ± 3.75 vs. 3.66 ± 0.22 nmol/L, 76–80 years old subgroup: 2.91 ± 0.41 vs. 3.84 ± 0.27 nmol/L,

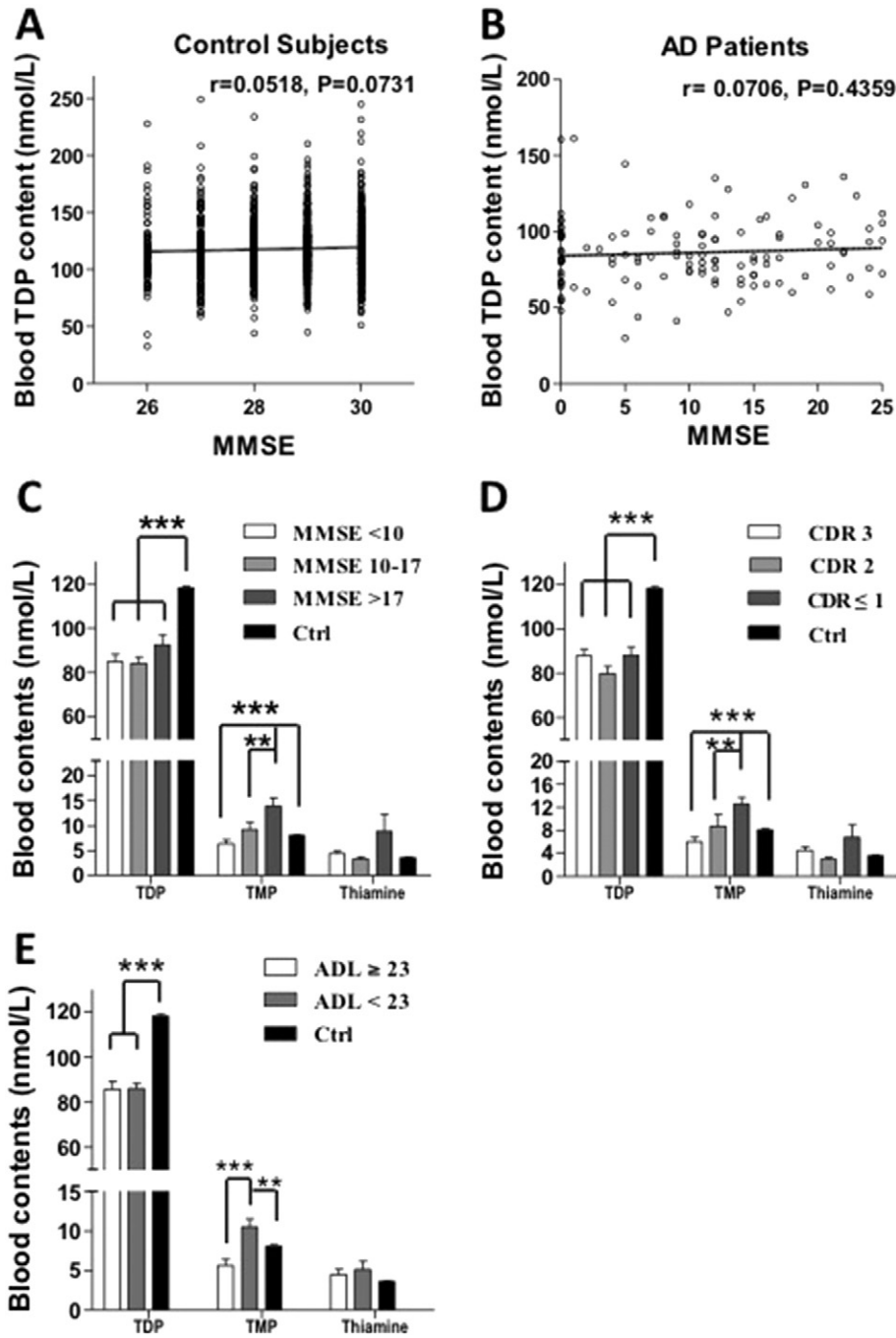


Fig. 2. Effect of the disease severity on the levels of blood thiamine metabolites. A. There were no significant correlations between blood TDP levels and MMSE scores in control subjects ($r = 0.0518$, $P = 0.0731$, $n = 1199$). B. There were no significant correlations between blood TDP levels and MMSE scores in AD patients ($r = 0.0706$, $P = 0.4359$, $n = 124$). C. Effects of cognitive impairment evaluated by MMSE scores on the levels of thiamine metabolites. D. Effect of cognitive impairment evaluated by CDR scores on the levels of thiamine metabolites. E. Effect of ADL scores on the measurement of thiamine metabolites.

>80 years old subgroup: 4.27 ± 0.50 vs. 3.23 ± 0.37 nmol/L, all $P > 0.05$; all $P > 0.05$, Fig. 3D and E).

The frequency of APOE $\epsilon 4$ allele in AD patients was 48.39% (60/124). This value was significantly higher than that of 20.77% in control subjects (249/1199, $P < 0.001$). However, the presence of APOE $\epsilon 4$ allele did not affect the levels of TDP, TMP, and thiamine in either AD patients (TDP: 83.37 ± 2.95 vs. 88.44 ± 2.80 nmol/L, TMP: 9.08 ± 1.15 vs. 8.45 ± 0.97 nmol/L, thiamine: 3.85 ± 0.48 vs. 5.90 ± 1.32 nmol/L, all $P > 0.05$, APOE $\epsilon 4$ -positive AD patients $n = 60$, APOE $\epsilon 4$ -negative AD patients $n = 64$; Fig. 3F) or control subjects (TDP: 116.00 ± 1.65 vs.

118.70 ± 0.86 nmol/L, TMP: 7.93 ± 0.39 vs. 8.13 ± 0.21 nmol/L, Thiamine: 3.41 ± 0.23 vs. 3.68 ± 0.12 nmol/L, all $P > 0.05$, $\epsilon 4$ -positive subjects $n = 249$, $\epsilon 4$ -negative subjects $n = 950$, Fig. 3G).

Blood hemoglobin levels significantly correlated with blood TDP levels in control subjects ($r = 0.2133$, $P < 0.0001$, Fig. 3H) and AD patients ($r = 0.3233$, $P < 0.001$, Fig. 3I). To minimize the potential effect of hemoglobin levels on the levels of blood thiamine metabolites, we divided AD patients and control subjects into two subgroups: those with hemoglobin levels < 120 g/L (AD patients $n = 39$, control subjects $n = 78$) and with hemoglobin levels ≥ 120 g/L (AD patients $n = 85$,

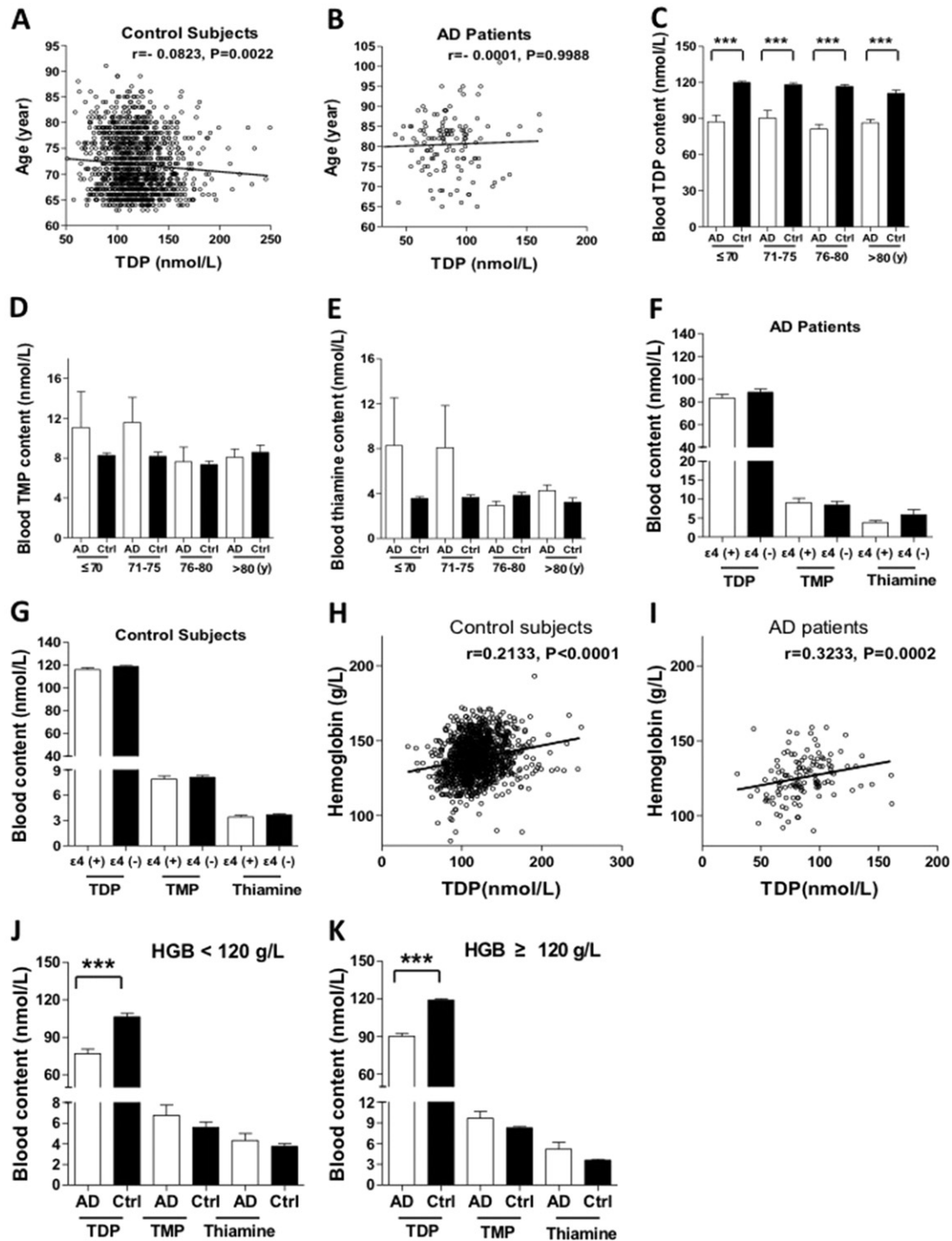


Fig. 3. The effects of age, APOE $\epsilon 4$ allele, blood hemoglobin levels on the levels of blood thiamine metabolites. **A.** There was a weakly negative correlation between blood TDP levels and age in control subjects ($r = -0.0823$, $P = 0.0022$, $n = 1199$). **B.** There were no significant correlations between TDP levels and age in AD patients ($r = -0.0001$, $P = 0.9988$, $n = 124$). **C.** Blood TDP levels were significantly reduced in all AD subgroups as compared with that in age-matched control subgroups. **D.** There were no significant differences in blood TMP levels between AD subgroups and age-matched control subgroups. **E.** There were no significant differences in blood thiamine levels between AD subgroups and age-matched control subgroups. **F.** There were no statistical differences in blood TDP, TMP and thiamine levels between APOE $\epsilon 4$ -positive and negative AD patients. **G.** No statistical differences were observed in blood TDP, TMP and thiamine levels between APOE $\epsilon 4$ -positive and APOE $\epsilon 4$ -negative control subjects. **H.** Blood hemoglobin levels weakly correlated with blood TDP levels in control subjects ($r = 0.2133$, $P < 0.0001$, $n = 1199$). **I.** Blood hemoglobin levels also showed a weakly correlation with blood TDP levels in AD patients ($r = 0.3233$, $P = 0.0002$, $n = 124$). **J.** Comparison of blood TDP, TMP, and thiamine levels between AD patients and control subjects with hemoglobin levels under 120 g/L. **K.** Comparison of blood TDP, TMP, and thiamine levels between AD patients and control subjects with hemoglobin levels over 120 g/L.

control subjects $n = 1121$). AD patients with hemoglobin levels < 120 g/L still exhibited a significant reduction in blood TDP levels as compared to control subjects (76.85 ± 3.63 vs. 106.30 ± 2.96 nmol/L, $P < 0.001$). No significant differences in blood TMP and thiamine levels

were observed between the two subgroups (TMP: 6.78 ± 0.99 vs. 5.60 ± 0.52 nmol/L; thiamine: 4.32 ± 0.68 vs. 3.76 ± 0.26 nmol/L, both $P > 0.05$; Fig. 3J). Consistently, the similar results were observed in subjects with hemoglobin levels ≥ 120 g/L (TDP: 90.18 ± 2.34 vs.

119.00 ± 0.78 nmol/L, $P < 0.001$; TMP: 9.67 ± 0.98 vs. 8.27 ± 0.19 nmol/L, $P > 0.05$; thiamine: 5.18 ± 1.01 vs. 3.61 ± 0.11 nmol/L, $P > 0.05$; Fig. 3K).

4. Discussion

In this work, we demonstrated that the alteration in the levels of blood thiamine metabolites serves as a promising biomarker for AD diagnosis, with high sensitivity and specificity. In both exploration and validation phases of our study, we found that AD patients had significantly reduced blood TDP levels as compared to control subjects (Fig. 1A,C). Blood TDP level exhibited a significant diagnostic value for AD using ROC analysis in the exploration phase, with AUC of 0.843, sensitivity of 77.4%, and specificity of 78.1% (Fig. 1B). Moreover, γ that represents the best combination of the levels of thiamine metabolites and age to predict the possibility of AD had an even higher diagnostic performance, with AUC of 0.938, sensitivity of 81.4%, and specificity of 90.5% (Fig. 1B). Because the measurement of blood thiamine metabolites by HPLC is noninvasive, inexpensive, and easy to perform and be standardized, these findings meet the criteria of an ideal biomarker for AD diagnosis, defined as sensitivity and specificity over 80% by the Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and the National Institute on Aging Working Group in 1998 (The Ronald and Nancy Reagan Research Institute of The Alzheimer's Association and National Institute on Aging Working Group's, 1998). Importantly, these findings can be further verified using a large scale of samples in the validation phase (Fig. 1D). Our study indicates the effectiveness and reliability of the measurement of blood thiamine metabolites by HPLC as an ideal tool for AD diagnosis.

Another important feature of good diagnostic biomarker is the ability to distinguish other diseases with similar symptomatology from the targeted disease (Langa et al., 2004). VaD and FTD are common diseases of non-AD-type dementia with many overlapping characteristics with AD. A previous study in autopsied brains showed that 55% of patients clinically diagnosed as VaD had significant AD-type pathological alterations (Victoroff et al., 1995). Our study showed that the alteration of the levels in blood thiamine metabolites was an efficient biomarker in differentiating AD from VaD. Blood TDP levels in VaD patients were in the same range as compared with that in control subjects but were significantly increased as compared with that in AD patients (Fig. 1C). Over 64% of VaD patients (64.3% for TDP cut-off point and 67.1% for γ cut-off point) could be differentiated from AD patients. Considering the high co-morbidity of VaD and AD, this is a significant result. In addition, the measurement of blood thiamine metabolites exhibited an excellent capacity of differentiating FTD from AD, with differentiation rates reaching 84.6% for TDP and 100.0% for γ value. Combining the above results with that of a previous study showing no changes in blood TDP levels in Parkinson's disease patients, as compared with control subjects (Gold et al., 1998), the altered levels of blood thiamine metabolites can serve as a specific biomarker for AD diagnosis.

To exclude the effect of the disease severity on the levels of blood thiamine metabolites by changes in life style in AD patients, we further assayed whether the disease severity correlated with the levels of thiamine metabolites. We found no differences in blood TDP, TMP and thiamine levels among mild, moderate and severe AD patients based on MMSE and CDR scores. Similarly, there were no significant differences in the levels of thiamine metabolites between two subgroups divided by ADL scores. TDP levels in all subgroups of AD patients were still significantly reduced as compared with that in control subjects (Fig. 2C–E), suggesting that the alteration of blood thiamine metabolites is an excellent marker of AD diagnosis, independent of the degree of disease severity.

APOE $\epsilon 4$ allele is a risk factor for sporadic AD. However, our study showed no significant differences in the levels of thiamine metabolites between APOE $\epsilon 4$ -positive and $\epsilon 4$ -negative AD patients and control

subjects (Fig. 3F and G). The results indicate that the presence of APOE $\epsilon 4$ allele does not affect the levels of blood thiamine metabolites.

We further examined the relationship between the levels of thiamine metabolites and age and blood hemoglobin levels. Blood TDP levels exhibited a mildly gradual decrease with increased age in control subjects (Fig. 3A). However, in all age subgroups examined, AD patients still had significantly lower TDP levels as compared to control subjects (Fig. 3C–E). In addition, blood TDP levels significantly correlated with hemoglobin contents both in control subjects and AD patients (Fig. 3H and I), consistent with it being mostly present in red blood cells. When subjects were sub-grouped according to hemoglobin level, AD patients still exhibited significantly lower TDP levels in both groups (Fig. 3J and K). Together, these results demonstrated that the measurement of blood thiamine metabolites is an effective and reliable biomarker for AD, independent of age and blood hemoglobin level.

Currently, the alterations of CSF A β 42, tau, and phosphor-tau are already accepted as biomarkers for AD diagnosis. CSF A β 42 has 0.913 of AUC, 96.4% of sensitivity, and 76.9% of specificity. Tau has 0.831 of AUC, 69.6% of sensitivity, and 92.3% of specificity. Phosphor-tau has 0.753 of AUC, 67.9% of sensitivity, and 73.1% of specificity (Shaw et al., 2009). The diagnostic value CSF A β 42 for AD is superior to that of blood TDP whereas the values of CSF tau and phosphor-tau are similar to that in blood TDP. The combination of APOE $\epsilon 4$, CSF A β 42 and tau can further improve the value for AD diagnosis, with 0.942 of AUC, 98.2% of sensitivity, and 79.5% of specificity (Shaw et al., 2009). This phenomenon also is similar to our results, which the γ variable reflecting the combination of age, TDP, TMP, and thiamine has better efficiency for AD diagnosis.

Brain glucose metabolism closely correlates with cognitive impairment in AD patients (Chen et al., 2010; Mosconi et al., 2008b). The improvement of brain glucose metabolism by the application of nasal insulin has the effects of enhancing cognitive function and neuroprotection on AD patients (Reger et al., 2008; Craft et al., 2012; Hölscher, 2014). The pathogenic factor(s) of cerebral glucose hypometabolism in AD may be modifiable targets. Previous studies from our and other laboratories have demonstrated that thiamine deficiency enhances brain amyloid deposit and cognitive impairment in the mouse models by multiple pathophysiological pathways, including oxidative stress (Karuppagounder et al., 2009) and elevated activities of β -secretase (Zhang et al., 2011) and glycogen synthase kinase-3 (Zhao et al., 2011). Thus, altered thiamine metabolism may contribute to cognitive impairment and neurodegeneration by perturbing brain glucose metabolism and inducing multiple pathogenic factors in AD, and is a potential target for AD therapy. The previous studies have demonstrated the beneficial effects of benfotiamine, a liposoluble derivative of thiamine, against AD through multiple pathways (Pan et al., 2010; Bozic et al., 2015; Gibson et al., 2013). Thus, the novel therapeutic strategy for AD that simultaneously targets at abnormal thiamine metabolism and multiple AD disease-causing mechanisms may offer new hope for AD therapy.

In summary, our results showed that the alteration in the levels of blood thiamine metabolites serves as a promising biomarker for AD diagnosis with sensitivity and specificity over 80%, and can effectively allow distinction of VaD and FTD from AD. Importantly, the detection of blood thiamine metabolites by HPLC is noninvasive, simple to perform and be standardized, reliable, and inexpensive. The measurement of blood thiamine metabolites fulfills the criteria of an ideal tool for AD diagnosis and is suitable for studies in large populations.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2015.11.039>.

Author contributions

Fei G, Jin L, Hu W, Ji Y, Xu J, Zhang Q and Zhong C were responsible for the diagnoses for patients with Alzheimer's disease, vascular dementia, and frontotemporal dementia. Pan X, Fei G, Lu J, Pan S, Chen

Z, and Wang C were responsible for evaluations for control subjects with normal cognition. Pan X, Fei G, Lu J, Jin L, Pan S, Chen Z, Wang C, Sang S, Liu H, Hu W, Zhang H, Wang H, Wang Z, Zhang Q, Xie X, Cui D, and Gu X were responsible for collecting the blood samples. Qin Y and Tan Q were responsible for HPLC detection. Pan X and Lu J were responsible for detecting apolipoprotein E genotypes. Yu Y was responsible for calculating the variable γ . Pan X, Fei G, and Jin L were responsible for the verification and statistic analyses of results. Zhong C designed the whole study and wrote the paper.

Disclosure

Chunjiu Zhong holds shares of Shanghai Rixin Biotech Company that focuses on developing new drugs against Alzheimer's disease. Xiaoli Pan, Guoqiang Fei, Jingwen Lu, Lirong Jin, Shumei Pan, Zhichun Chen, Changpeng Wang, Shaoming Sang, Huimin Liu, Weihong Hu, Hua Zhang, Hui Wang, Zhiliang Wang, Qiong Tan, Yan Qin, Qunying Zhang, Xueping Xie, Yong Ji, Donghong Cui, Xiaohua Gu, Jun. Xu, and Yuguo Yu report no disclosures relevant to this manuscript.

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