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Original Article

Assessment of plasma N-acetyl aspartic acid by Liquid chromatography-mass spectrometry in patients with Alzheimer's disease

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Abstract: Objective: Alzheimer's disease (AD) is a neural degenerative disease with progressive cognitive impairment. N-acetyl aspartic acid (NAA) is an important biomarker for assessing neuronal activity and brain cells metabolic abnormalities. This investigation is aimed to establish a liquid chromatography-mass spectrometry (LC-MS) method for measuring plasma NAA level, in order to make clear whether plasma NAA level can be used as a plasma biomarker in AD. Methods: A LC-MS method was established and validated for quantitative analysis of plasma NAA, and the plasma NAA concentrations of 23 AD patients and 36 healthy controls were analyzed and compared. In addition, the bilateral hippocampus NAA levels in 7 AD patients and 7 age-matched healthy controls were comparative analyzed by ^{1}H-MRS method, and corresponding correlation analyses were also performed. Results: An optimized LC-MS method was applied for measuring plasma NAA levels. Plasma NAA levels were significantly higher in AD patients (14.02±2.324 μM) than controls (11.12±2.67 μM) ($P<0.01$), and statistically different between different age group in both AD patients and healthy controls ($P<0.05$), with a significantly negative correlation with age in healthy controls ($r = -0.462$, $P = 0.008$). The ratios of NAA/Ch, NAA/Cr in both hippocampus measured by ^{1}H-MRS were significantly decreased in AD patients than in controls ($P<0.05$), and there were no statistical correlations between plasma NAA concentrations and NAA/Ch, NAA/Cr and Ch/Cr ratios in the selected 7 AD patients. Conclusions: LC-MS method can be used as a simple, rapid and accurate quantitative analysis method for plasma NAA measurement. As NAA level increased in plasma and decreased in the brain, the plasma NAA level could be used as an index for early clinical AD screening.

Keywords: Alzheimer's disease, plasma N-acetyl aspartic acid, high performance liquid chromatography-mass spectrometry, ^{1}H-MRS

Introduction

Alzheimer's disease (AD) is a complex cognitive dysfunction and memory impairment disease, characterized by an accumulation of β-amyloid (Aβ) plaques and neurofibrillary tangles [1, 2]. As the most common form of dementia, the incidence of AD increases sharply in worldwide, mainly driven by increased life expectancy, especially in large low-income and middle-income countries including China [3-5]. According to the 2010 World Alzheimer report, 35.6 million people living with dementia at a total cost of more than US$600 billion are estimated worldwide in 2010, and the incidence of AD is expected to double in the next 20 years [6]. And in China, the problem of AD will become more serious in think of the one-child policy and large internal migration in the next few decades [7]. There is no known effective medical treatment program for treatment or even slowing down the progression of AD currently, and the early clinical diagnosis of AD is especially important [8]. The early diagnosis of AD main relies on clinical manifestations, although many medical methods were designed to develop clinical, imaging, genetic, and biochemical biomarkers for early tracking of AD, it is still difficult to obtain a satisfactory result for early diagnosing of AD [9, 10]. There

is a pressing need to find biomarkers for both predicting future clinical declines and assisting in clinical treatments of AD [3].

N-acetyl aspartic acid (NAA), a specific metabolite derivative from aspartic acid for central nervous system, is suggested to be a very promising biomarker in clinical trials of neuronal diseases [11, 12]. NAA is synthesized by aspartic acid and acetyl coenzyme A primarily in neuronal mitochondria [13], and then output and maintained at a very high concentration level in central nervous system both in humans and other animals [14, 15]. NAA is updated daily through regional recycling of neural network between extracellular neurons liquid, astrocytes and oligodendrocytes [16]. Typically, NAA clearance is transferred from neurons to oligodendrocytes. When pathological changes occur, the release of NAA will increase in the injured neurons, NAA first flows into astrocytes and then enters the blood circulation. Besides, NAA expressed in a very high level in nerve cells, so it may be used as a biomarker for diagnosing and treatment of neuronal diseases [12], such as AD [17], Canavan's disease [18], Schizophrenia [19] and generalized anxiety disorder [16]. Many previous studies showed brain NAA levels declined in patients of Alzheimer's disease, especially in the early stage of Alzheimer's disease, suggesting brain NAA level measurement can be used as an effective method for early clinical AD screening [20, 21]. Some detection techniques and methods have been developed for brain NAA level measurement, such as whole-brain MRI, however, with expensive inspection equipment, high technical requirements or undesirable sensitivity and specificity, these techniques and methods have not yet used in clinical. In think of NAA flows from injured neurons to astrocytes and then enters the blood circulation, suggesting elevated plasma NAA may reflect the decline of brain NAA. Some studied focused on the relationship between brain NAA level, plasma NAA level and neuronal diseases have confirmed this speculation. For example, Tortorella *et al* showed plasma and CSF N-acetyl aspartate levels differ in multiple sclerosis and neuromyelitis optica, and there were no correlation of plasma NAA concentrations with age, sex in healthy people, finally suggested determination of plasma and CSF NAA levels may represent a suitable tool in the diagnostic laboratory workup to differentiate MS and NMO [22]. However, there are no studies on changes of plasma NAA concentrations in AD patients.

NAA is a pathognomonic hallmark of nervous diseases, and some quantification detection methods for it have been developed. For example, as a non-invasive and *in situ* measurement method, MR-spectroscopy (MRS) is widely used to determine parenchymal NAA concentrations in vivo, especially for estimation of regional brain concentrations of NAA [20, 21, 23, 24]. As aclassic detection method, LC-MS has been traditionally applied in measurement of NAA in body fluids in many nervous diseases [25, 26]. Nevertheless, with tedious sample preparation, long-time chromatographic analysis process and limited metabolic product detection range of LC-MS, improved LC-MS methods have been applied for measuring NAA in urine, and revealed a huge technological advantages, such as simple sample preparation, high recovery rate, good stability and short analysis time [25, 26]. These previous studies suggesting LC-MS based method may be able to use as a rapid, accurate and high-through method for measuring NAA in body fluids.

Brain NAA levels were found decreased in AD patients by the ^{1}H-MRS, suggesting the plasma NAA concentrations may be elevated through daily regional recycling, while there is no study confirmed. In this study, we intends to measure plasma NAA concentration by LC-MS method, in order to explore the relationship between plasma NAA concentration and AD, and to provide a basis for the establishment of a new AD screening and diagnostic methods.

Materials and methods

Subjects

All the 26 AD patients were consecutive selected from patients diagnosed and treated with AD in department of neurology, second affiliated hospital of Nanchang university, during January 1, 2013 to December 30, 2013, with the criteria as follows: (1) strictly conform to the 2011 U.S. NIA/AA Alzheimer's disease diagnostic criteria, (2) neuropsychological tests based on domestic rating criteria of mini-mentai state examination (MMSE), illiteracy <17 points, pri-

Table 1. Ages and gender between AD patients and controls

Characteristics	Controls	AD patients	P (*t*-test)
Average age (years)	63.67±9.11	67.19±6.72	>0.05
Gender (male/female)	10/26	11/15	>0.05

mary <20 points, above the middle school <24 points, Hachinski ischemia <4 points, Hamilton Depression table <8 points. Accordingly, 36 healthy people who had no history of any nervous diseases were enrolled as controls in this study. As shown in **Table 1**, there was no significant difference regarding age and gender between case and control groups (P> 0.05). This study was approved by the ethics committee of second affiliated hospital of Nanchang university, and the informed consent form was obtained by each subject.

Main reagents and instruments

N-acetyl aspartic acid (NAA), formic acid and acetonitrile were purchased from Sigma-Aldrich Corp. (MO, USA). Agilent 1100 Series LC/MSD system, chromatography sampling bottle and pipe liner were purchased from Agilent Technologies (CA, USA). 3.0T GE MR 750 system was purchased from GE Company (WI, USA). Rotanta 460R Centrifuge was purchased from HettichHolding GmbH & Co. oHG (Tuttlingen, Germany). Reacti-Therm evaporator was purchased from Thermo Fisher Scientific Inc. (Ill., USA).

Plasma collection

2 mL fasting venous blood was collected from AD patients and healthy controls in the next morning after fasting for 8 h, placed in EDTA anticoagulation tube and brought back to the laboratory under low temperature. Plasma was separated by centrifugation at 5000 rpm, 4°C for 10 min, then placed in a 2 mL tube and stored at -80°C for spare.

Sample preparation

Sample preparation of plasma NAA was achieved as the recommended method [25] with some modifications. Briefly, Experiments were performed on five aliquots (50 μL each) from each samples or prepared standard plasma NAA solutions (2.5, 5, 10, 20, 30, 40 and 50 μM) in 1.5 mL microcentrifuge tubes. After vortex-mixing (3 min) and centrifugation at 4°C, 10000 rpm for 10 min, all the supernatant were transferred into a new set of 1.5 mL microcentrifuge tubes and evaporated in a dry nitrogen blowing instrument at 50°C. The residue was reconstituted in 100 μL acetonitrile, after vortex-mixing (3 min) and centrifugation at 4°C, 20000 rpm for 10 min, 50 μL the supernatant were injected into chromatography pipe liner for LC-MS/MS analysis.

LC-MS/MS system and operating conditions

NAA detection and analysis was performed on Agilent 1100 Series LC coupled to a mass selective detector (MSD) Ion Trap XCT mass spectrometer. A Shim-pack VP-ODS C18 (150× 2.1 mm i.d., 5 μm) was used for analyzing NAA from other compounds in the sample. The mobile phase was a mixture of acetonitrile (HPLC grade, 100%) and water (1:9 v/v) containing 0.1% formic acid at a flow rate of 0.20 ml/min. The Ion Trap was operated with an orthogonal electrospray source in negative ion mode using a selective ion monitoring. For NAA, the ion transitions as 174Y88, 174Y58 and 174Y130 were monitored, with the CDL and heater block temperature was 250°C and 200°C, the CDL and detection voltage was 25 V and 185 kV, atomizing gas and drying gas flow rate was 1.5 L/min and 2.0 L/min.

^{1}H-MRS protocol

^{1}H-MRS measurement of all subjects was performed on a 3.0T GE MR 750 system with a commercially 8-channel high-density phased-array head coil. A trained imaging technician placed an 8 cm^3 (2×2×2 cm) voxel on a T2-weighted image over bilateral hippocampus. Examinations were performed using the FRFSE sequence with TR = 2000 ms, TE = 30 ms, 6 mm thickness, skip 2 mm, 1 stimulus. Inspection of ^{1}H-MRS was performed by using a perfusion and diffusion-sensitive stimulated-echo acquisition mode (STEAM) MRSI acquisition method with TR = 1000 ms, TE = 35 ms, 2 cm thickness, skip 2 cm, 2 stimulus, pressurized water from 96% to 99%, bandwidth = 2-6 Hz/pixel. The chemical frequency shift position of each metabolite as follows: N-acetyl aspartate (NAA) 2.0 ppm, creatine (Cr) 3.0 ppm, choline (Ch) 3.2 ppm. MRS metabolite ratios included NAA/Cr, choline (Ch)/Cr, ml/

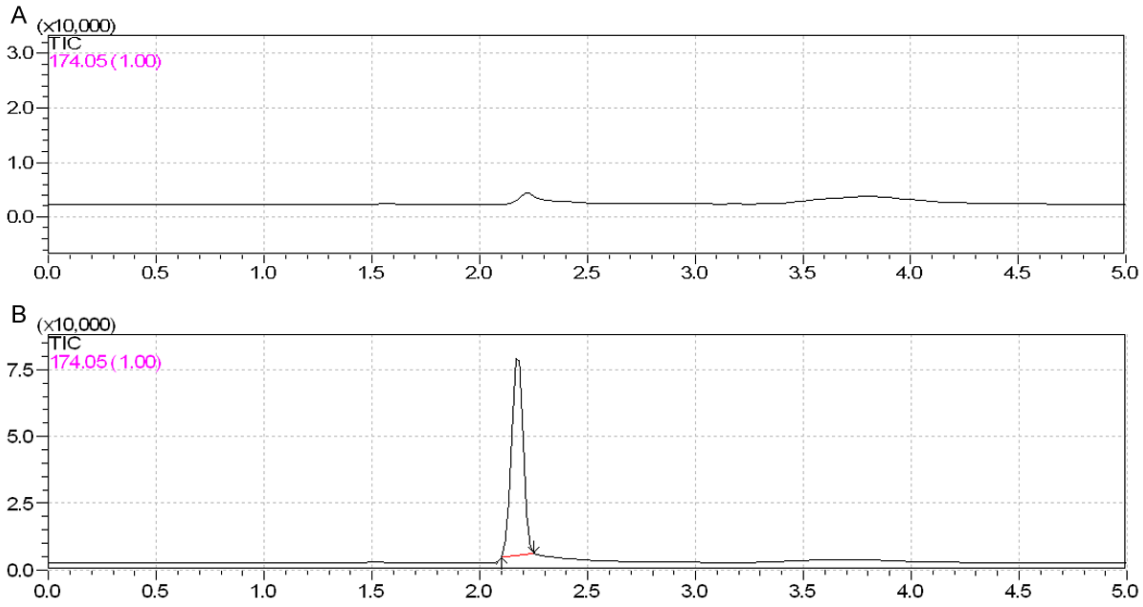

Figure 1. Typical liquid chromatograms obtained in selected chromatographic conditions for a blank plasma (A) and standard NAA plasma solution (B).

Cr and NAA/mI were automatic recognized and analyzed by the software provided by GE Company.

Statistical analysis

All statistical analyses were performed by using the Statistical Package for Social Sciences software (SPSS, Windows version release 18.0; SPSS Inc.; IL, USA). Each experiment were repeated in triplicate (n = 3). All data were presented as mean ± standard deviation ($\bar{x}\pm s$). All enumeration data were compared by chi-squared (χ^2) test or Fisher's exact test. All measurement data were analyzed with normality test first, then *t*-test was applied for 2 sets of measurement data analysis and variance analysis was applied for multiple sets of measurement data, a *P* value <0.05 was considered statistically significant. Spearman rank correlation test was applied to discern whether a set of two variables are correlated or not.

Results

Method validation

As verified by MS results, collision-induced three prominent fragments at m/z 88, m/z 58 and m/z 130, m/z 88 as a result of loss of CO_2 and acetyl groups, m/z 58 corresponds to the N-acetyl moiety, and m/z 130 corresponds to a decarboxylated $[M-H]^-$. As shown in **Figure 1**, under the selectedchromatographic conditions, the whole run time was about 5 min and NAA being eluted at 2.19 min, with no interference from other compounds in the biological matrix was observed.

By testing a series of concentration gradients NAA standard plasma solutions with blank plasma as negative control, results showed satisfactory linearity in the range 2.5-50 μM with a correlation coefficient R^2 = 0.9949 (y = 8740.3x-9134.7, R^2 = 0.9949, n = 7). Within-day (n = 5) and between-day (n = 5) variations determined at 2.5, 10 and 40 μM were less than or equal to 17.3%. The recovery rate for 2.5, 10 and 40 μM were 54.82±4.74%, 61.32±5.42% and 59.28±5.18% respectively (n = 5). The standard peak area ratio of NAA standard plasma solutions to NAA standard solutions ranged between 86 and 97%. For a comprehensive assessment, it is worth to mention that results obtained in this study were in excellent agreement.

Analysis of plasma NAA in AD patients and healthy controls

Plasma NAA levels between AD patients and healthy controls obtained by LC-MS detection

Table 2. Plasma NAA concentrations with different gender and age in AD patients and controls

	AD patients (n = 26)				Controls (n = 36)			
			t	p			t	p
Gender (Male/Female)	12.93±2.26 (n = 11)	14.83±2.46 (n = 15)	2.01	0.055	11.62±2.74 (n = 10)	10.94±2.67 (n = 26)	0.67	0.511
Age (<65/≥65 years)	10.57±2.97 (n = 7)	11.58±2.99 (n = 19)	1.16	0.027*	12.11±2.65 (n = 20)	9.87±2.19 (n = 16)	2.70	0.011*

*Represents P<0.05.

Table 3. Spearman rank correlations of NAA concentration with MMSE score, disease duration and age in AD patients and controls

	r	p
AD patients		
MMSE score	-0.036	0.861
Disease duration	-0.236	0.245
Age	-0.156	0.445
Controls		
Age	-0.462	0.008**

**Represents P<0.01.

method were compared in this study. The mean value of plasma NAA concentration in AD patients was 14.02±2.32 μM, about 1.26-fold higher than that in healthy controls (11.12±2.67 μM), and this differences were very significant at the 0.01 level (t = 4.33, P<0.01). Then plasma NAA concentrations in both AD patients and healthy controls with different genders and ages were compared. As shown in **Table 2**, there were no significant differences in plasma NAA concentrations between different genders in both AD patients and healthy controls, while the differences of plasma concentrations between different age group in both AD patients and healthy controls were statistically significant (P<0.05). For a further study, the possible correlations of plasma NAA concentration with MMSE score, disease duration and age in AD patients and healthy controls were analyzed. As shown in **Table 3**, plasma NAA concentration did not correlate with MMSE score (r = -0.036, P = 0.861), disease duration (r = -0.236, P = 0.245) or age (r = -0.156, P = 0.445) in AD patients, while had a significantly negative correlation with age (r = -0.462, P = 0.008) in controls.

Analysis of brain NAA in AD patients and healthy controls

With the hypothesis that plasma NAA levels were correlated with the brain NAA levels through daily regional recycling, NAA concentrations of brain bilateral hippocampus in 7 AD patients and 7 age-matched healthy controls were measured by ^{1}H-MRS method. As shown in **Table 4**, the NAA/Ch ratios of both left hippocampus and right hippocampus were significantly less in AD patients than in controls, and this differences were very significant at the 0.01 level both at left hippocampus (t = 4.58, P<0.01) and right hippocampus (t = 4.58, P<0.01), and the NAA/Cr were also less in AD patients than in controls, with the difference was significant at the 0.05 level at left hippocampus (t = 2.61, P = 0.025) and very significant at right hippocampus (t = 8.27, P< 0.01), while the Ch/Cr ratios between in AD patients and in controls had no statistical difference either at left hippocampus (t = 0.27, P = 0.792) or right hippocampus (t = 0.44, P = 0.662). Then the possible correlations of NAA/Ch, NAA/Cr and Ch/Cr ratios with MMSE score and age in AD patients were analyzed. As shown in **Table 5**, the NAA/Ch, NAA/Cr and Ch/Cr ratios of both left and right hippocampus did not correlate with MMSE score and age in AD patients.

Correlation analysis between plasma NAA levels and brain bilateral hippocampus NAA/Ch, NAA/Cr and Ch/Cr ratios

For further to explore the possible correlations between plasma NAA and brain NAA, Spearman rank correlations were performed on plasma NAA concentrations measured by LC-MS method and brain bilateral hippocampus NAA/Ch, NAA/Cr and Ch/Cr ratios measured by ^{1}H-MRS method. Results showed there were no statistical correlations between plasma NAA concentrations and NAA/Ch, NAA/Cr and Ch/Cr ratios in the selected 7 AD patients (**Table 6**).

Discussion

AD has become one of the leading causes of morbidity and mortality in the world, especially in low-income and middle-income countries, such as China. And with life expectancy steadily

Table 4. NAA concentrations of brain bilateral hippocampus in 7 AD patients and 7 controls

Metabolite ratios	Left hippocampus				Right hippocampus			
	AD patients	Controls	t	P	AD patients	Controls	t	P
NAA/Ch	0.99±0.33	1.67±0.80	4.58	0.000**	1.04±0.25	1.57±0.49	5.62	0.000**
NAA/Cr	1.00±0.26	1.34±0.73	2.61	0.025*	1.01±0.30	1.91±0.66	8.27	0.000**
Ch/Cr	1.10±0.33	1.08±0.31	0.27	0.792	1.02±0.26	0.98±0.31	0.44	0.662

*Represents P<0.05, and **represents P<0.01.

Table 5. Spearman rank correlations of NAA/Ch, NAA/Cr and Ch/Cr ratios of both left and right hippocampus with MMSE score and age in AD patients

Regions	NAA/Ch		NAA/Cr		Ch/Cr	
	r	p	r	p	r	p
MMSE score						
Left hippocampus	0.068	0.740	-0.035	0.865	-0.145	0.480
Right hippocampus	-0.144	0.484	0.121	0.555	-0.152	0.457
Age						
Left hippocampus	-0.169	0.410	-0.015	0.941	0.046	0.823
Right hippocampus	0.217	0.287	0.040	0.847	-0.156	0.447

Table 6. Spearman rank correlations of plasma NAA concentration with brain bilateral hippocampus NAA/Ch, NAA/Cr and Ch/Cr ratios in AD patients

Regions	NAA/Ch		NAA/Cr		Ch/Cr	
	r	p	r	p	r	p
Left hippocampus	0.047	0.819	0.379	0.056	-0.221	0.277
Right hippocampus	0.100	0.626	0.242	0.233	0.052	0.801

raised, the percentage of aged population will increased accordingly, AD continues to be a more and more important health concern. According to previous study by Barnes & Yaffe, AD ranked as the 5th leading cause of death among people ages 65-85, the number of people aged over 65 with AD doubled every 5 years, and the prevalence of AD will triple from 5 minllion in 2013 to 14 million by 2050 if without any effective intervention [27]. While in China, this trend is even more obvious. China had more cases of AD in 2010 than any other country in the world, and the number people aged over 60 and 80 are expected to reach 397 million and 100 million, in think of the prevalence of AD was 5% in aged over 65 population, 10% in aged over 70 population and 30% in aged over 80 population, China should give more attention to take effective intervention of AD [7]. The early diagnosis of the disease is still very difficult, and the possible methods including functional imaging and molecular genetic test, while with expensive inspection equipments, high technical requirements or undesirable sensitivity and specificity, it is difficult to apply these techniques and methods in early clinical screening. It is urgent to discover much more suitable biomarkers for early clinical AD screening and to establish corresponding high sensitivity, simple, rapid and economic screening methods.

As a specific metabolite derivative from aspartic acid for central nervous system, NAA is suggested to be a very promising biomarker in clinical trials of neuronal diseases [11, 12]. Many previous studies showed NAA levels declined in the early stage of AD patients, and suggested brain NAA level detection can be used as an effective method for early diagnosing of AD [20, 21]. Although plasma NAA level was suggested to correlate with brain NAA level through daily regional recycling, there was no study focus on whether plasma could be used as an effective method for early diagnosing of AD yet. NAA works as one of the most important biomarkers of neuronal density and integrity, the concentration of NAA in brain is mainly determined by ^{1}H-MRS in clinical [15, 17]. However, although ^{1}H-MRS becomes to a common method in the clinical study of nervous system diseases, but it also has its limitations, such as no matter single or multiplex voxel collection was applied, ^{1}H-MRS belong to the localized MRS, and the results can not reflect changes of NAA levels in the whole brain, besides, with expensive inspection equipments, high technical requirements or undesirable sensitivity and specificity, it is

difficult to apply these techniques and methods in plasma NAA concentration measurement [28]. Previous study reported that concentrations of NAA in body fluids and urine were successfully measured by GC-MS and LC-MS [25, 26, 29], suggesting LC-MS may work as a rapid, accurate and high-through method for measuring NAA in plasma.

In this study, we established an optimized LC-MS condition for plasma NAA concentration measurement, and applied this LC-MS system in plasma NAA levels detection in 26 AD patients and 36 matched healthy controls. Results showed the plasma NAA concentrations were significant higher in AD patients than healthy controls ($t = 4.33$, $P<0.01$), and were statistically different between different age group in both AD patients and healthy controls ($P<0.05$), with a a significantly negative correlation with age in healthy controls ($r = -0.462$, $P = 0.008$). These results reveal LC-MS is a simple, rapid and accurate quantitative analysis method for plasma NAA measurement. NAA concentrations of brain bilateral hippocampus in 7 AD patients and 7 age-matched healthy controls were further measured by ^{1}H-MRS, results showed the NAA/Ch, NAA/Cr ratios of both left hippocampus and right hippocampus were significantly less in AD patients than in controls ($P<0.05$), while there were no statistical correlations between plasma NAA concentrations and NAA/Ch, NAA/Cr and Ch/Cr ratios in the selected 7 AD patients, suggesting as NAA levels were significantly elevated in plasma and decreased in brain in AD patients, which may contributed to the early clinical AD screening by measuring plasma NAA level. Our results were consistent with previous studies of ALS patients by Isabella *et al*, they found that the NAA levels were significantly elevated in serum and decreased in brain motor cortex area in ALS patients [30]. Of course, there are also many deficiencies in this study, such as with limited number of AD cases, we could not determine the referenced normal value and diagnosed abnormal value of plasma NAA levels for early clinical AD screening, and although there were no statistical correlations between plasma NAA levels and brain bilateral hippocampus NAA levels in the selected 7 AD patients, we could not verify to deny the correlations of plasma and brain NAA NAA occur through through daily regional recycling. Therefore, larger prospective studies are needed to confirm that measurement plasma NAA concentration by LC-MS method is a rapid, accurate and high-through method for early clinical AD screening and to elucidate the underlying mechanisms of correlations between plasma NAA and brain NAA in pathogenesis of AD.

Disclosure of conflict of interest

None.

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