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Classification and characterization of Alzheimer's disease patients based on ATN biomarkers in the cerebrospinal fluid

조선대학교 대학원

생명과학과

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이 논문을 이학 석사학위 신청 논문으로 제출함.

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ABSTRACT

Classification and characterization of Alzheimer's disease patients based on ATN biomarkers in the cerebrospinal fluid

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Alzheimer's disease (AD) causes irreversible damage to the neuronal cells of the brain. It can be characterized by symptoms such as hippocampal volume atrophy and cognitive impairment, which have been reported to be decreased in the patients with AD. Based on previous studies, subjects are classified as 4 groups based on cognitive impairment and amyloid positron emission tomography (PET), which consisted of Healthy control (HC), asymptomatic AD (aAD), prodromal AD (pAD) and AD dementia (ADD). Multimodal data are compared across theses groups using structural magnetic resonance imaging (MRI), and neuropsychological full screening battery, cerebrospinal fluid (CSF).

Identification of biomarkers in the early stages of AD is important in disease modifying therapies. AD specific biomarkers can be found in CSF at very early stages. The AD biomakers in CSF consist of amyloid $\beta_{1\text{-}42}$ ($A\beta_{42}$), phosphorylated tau protein (pTau) and total tau protein (tTau), which are recently defined as ATN biomarkers.

In order to measure the accurate values of these CSF biomarkers and investigate the relationships between ATN biomarkers and multimodal data, CSF

ATN biomarkers from 211 subjects were measured by xMAP INNOBIA (Luminex) platform and INNOTEST (ELISA) platform. These subjects were assessed for the multimodal data including clinical diagnosis, MRI and neuropsychological test.

Two assay platforms were compared for the diagnostic accuracy. The diagnostic classification power of pTau level from INNOBIA was stronger than that from INNOTEST, whereas $A\beta_{42}$ and tTau levels showed no significant difference between two platforms. In addition, the ratio value of pTau/$A\beta_{42}$ from Luminex platform was most useful in classifying early stages of AD from HC. The AUC of diagnostic classification by pTau/$A\beta_{42}$ ratio was 0.98 for INNOBIA compared to 0.93 for INNOTEST.

The relationship among CSF biomarkers, neuroimaging and neuropsychological assessments were analysed. The ATN classification was assessed with CSF biomarkers. The overall A-/T-/N- subjects consisted of HC groups, whereas A+/T-/N- and A+/T+/N+ subjects consisted of AD groups. Interestingly, despite AD patients, some subjects showed low levels of Tau. In addition, A+/T-/N- AD group did not show significant decreases in subcortical volumes and showed cortical thickness atrophy in entorhinal cortex, precuneus and superior frontal cortex. Additionally, neuropsychological assessments of visuospatial memory tested using RCFT showed decline.

In summary, the comparison of two platforms suggests that the INNOBIA is more useful in diagnostic classification for early stage of AD with high AUC rather than INNOTEST. The relationship analysis suggest that the different profiles of tau levels showed the significantly different visuospatial memory loss. These results provide the possibility for early diagnosis of AD by the CSF biomarkers and for prediction of tau levels by neuropsychological tests.

Ⅰ. INTRODUCTION

Dementia is regarded as an umbrella term for a wide range of clinical syndromes characterized by continuous decline in two or more cognitive domains, including memory, personality and behavior, executive and visuospatial function, language, which interferes with the basic activities of daily life and loss of abilities to perform instrumental task. There is an overwhelming impact on the quantity and quality of life of the personal with dementia, subsequently, weighing down on their carefivers, friends and family, and the wider society with immense emotional, physical and economic burden that gradually intensify as the patient symptoms progress affecting the mobility communication and self-care. (Duong *et al*., 2017). The worldwide 50 million people suffered from dementia in 2018, a global community around the sized of Republic of Korea or Spain population, with this numbers increasing 152 million by 2050 (Patterson et al., 2018). The total estimated global cost of dementia is 818 billon U.S. Dollar in 2015. If global dementia care were a country. it ould be the 18th largest economy in the world exceeding the market values of companies such as Apple and google. By 2030, dementia will rise to 2 trillion U.S. Dollar (Prince *et al*., 2015).

According to world aging The prevalence was estimated to be approximately 11% among population over age 65 and 81% of people who have Alzheimer's disease (AD) are over age 75 (Alzheimer's Association, 2016). AD is the most frequent neurodegenerative form of dementia in the elderly population and can be characterized by symptoms slowly such as neurocognitive disorder, memory impairment and neuronal dysfunction. however It is difficult to tell the onset of AD from normal aging and proteins play important roles the development of the Alzheimer's disease (Petersen et al., 1999). Neuronal cell death caused irreversible damages in the brain. Neurodegenerative disorder is characterized by the complexity of the multifactor since they cannot be explained only by one reason. Although the exact way through which it contributes to onset and severity of

disease is still unknown. Until now, the majority of studies have focused on finding genetic links and understanding the molecular mechanisms leading to neuronal cell death. New approaches focusing on unveiling environmental clues, which may contribute for epigenetic modulation, may contribute to advancing the comprehending of the mechanisms associated with the etiology of these pathologies (Marques *et al*., 2010).

Many of candidates confirmed can be related to aspects of the disease pathology. However, apolipoprotein E (APOE) gene variant is most highly associated with AD (Stocker et al., 2018). Human APOE Gene is located on chromosome 19q13 and encodes three major APOE isoforms: APOE ε2, APOE ε3, and APOE ε4. The differences among these isoforms are limited to amino acid substitution at residues 112 and 158, which have a significant effect on the function and structure of APOE at the cellular and molecular levels are associated with neuropathological conditions (Zhong *et al*., 2009). Various tissues expressed APOE, with the highest expression in the human liver followed by the human brain. In the brain, The primary source of APOE have recognized astrocytes, although neurons and microglia synthesize this protein too (Xu *et al*., 2006). In human central nervous system (CNS), APOE embarks on lipids generated after redistributes and neuronal degeneration them to cells demanding lipids for remyelination, membrane repair, proliferation or of new axons (Hauser *et al*., 2011). The role of ApoE in late onset AD pathogenesis is not fully elucidated (Yu *et al*., 2014).

The common indicators of AD should know them; neuropsychological test and magnetic resonance imaging (MRI), before understanding the biology of ATN biomarkers. The neuropsychological test mainly included measures of functional abilities, complex memory, attention, learning, language, and visuospatial domains using the sub-score tests to predict AD. However, standards have not been defined on the best neuropsychological outcomes to be measured for AD until now (Salvatore *et al*., 2018). The brain atrophy can be evaluated by mean of anatomical brain volume measurements, or neuroimages can be scored by global

cortical atrophy (GCA) scale and medial temporal lobe atrophy (MTA) scale. Quantitative measurements of brain volume are mostly used in research, owing to their good sensitivity and relative reliability compared to visual scores. Despite these inaccuracies, volumetric MRI can predict alteration to AD in compliance with the MTA and the GCA (De Vis *et al*., 2016).

The National Institute of Neurological and communication Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) proposed guidelines in 1984 for the clinical diagnosis of the Alzheimer's disease. Diagnosis of AD based on clinical syndromes and neuropsychological assessments including Mini-Mental State Examination (MMSE), clinical dementia rating (CDR) or some similar assessments (McKhann *et al*., 1984). In 2011, Diagnostic classification standard as revised by the National Institute on Aging and the Alzheimer's Association (NIA-AA) charged workgroup (Jack *et al*., 2011). This guideline suggest such as hippocampal volumetric loss, persistent memory complaints, Aβ depostition detected by bio-fluid or neuroimaging based biomarkers are precise criteria used for early diagnosis of AD. Despite numerous studies, the findings that are identified are currently to inconsistent to reach firm and generalizable conclusions regarding underlying trends. Recently, NIA-AA new research framework refers to an aggregate of neuropathology changes. according to this definition, criteria does not base on clinical symptoms but in vivo A/T/N biomarkers and postmortem brain examination (Jack *et al*., 2018).

The ATN biomarkers are divided into 3 binary classes. 'A' refers to the value of an Aβ biomarker such as CSF $A\beta_{42}$ or amyloid PET; 'T', the value of a tau pathology biomarker such as CSF p-tau or tau PET; and 'N', neuronal injury or neurodegeneration biomarker such as CSF t-tau, FDG-PET, or structural MRI. In addition, the ATN system includes all individuals in any population and thus is suited to cognitive aging research where AD constitutes only part of the etiologic landscape (Jack *et al*., 2016). Based on the definitions of AD pathologic change and outlined earlier, the A/T/N biomarker system arranges individually to categorized three biomarkers: first, individuals with normal AD biomarkers; second,

those in the AD continuum (classified into pathological change and AD); and third, those with normal accumulation of amyloid biomarker but with abnormal Tau biomarker. This latter biomarker profile suggest evidence of more neuropathologic changes other than AD, which has been labeled SNAP. (Jack *et al*., 2018). The hallmark Cerebrospinal fluid (CSF) biomarker for AD should reflect the pathogenical changes in the brain such as the axonal and synaptic degeneration, the Aβ with deposition in plaques, and the hyperphosphorylated tau tangles (Yu *et al*., 2014). The tau protein aggregation and deposition, Aβ peptides over the course of decades produce neurofibrillary tangles and amyloid plaques, respectively. While amyloid plaques and tau tangles are characterized for AD, current evidence defines the Aβ and tau that soluble extracellular amyloid precursor protein (APP) and phosphorylated tau may be the critical cause of cognitive impairment and synaptic dysfunction in AD (Hu *et al*., 2018).

The colorless body fluid of CSF found in the spinal cord and brain. The ependymal cells produce CSF in the choroid plexuses, and the arachnoid granulations absorbed it (Sakka *et al*., 2011). CSF plays role as a buffer, providing immunological protection and metabolic function to the brain. Additionally, CSF particularly supports a vital function of blood flow in cerebral autoregulation. (Simon *et al*., 2016).

Finally, CSF $A\beta_{42}$ biomarker are still first to change, positioned slightly before amyloid PET imaging. Subsequently, CSF tau increase. MRI and (Fluoro-D-glucose) FDG PET are drawn correspondingly as the last biomarkers. Therefore, CSF biomarkers based diagnosis of AD offers great opportunities for early differential of AD faster than imaging results (Jack *et al*., 2013). Such information will be significant for patient care in the long term and currently for useful designs. Likewise, clinical trials of disease-modifying therapies will be useful resources for interpretation and translation (Fagan, 2014).

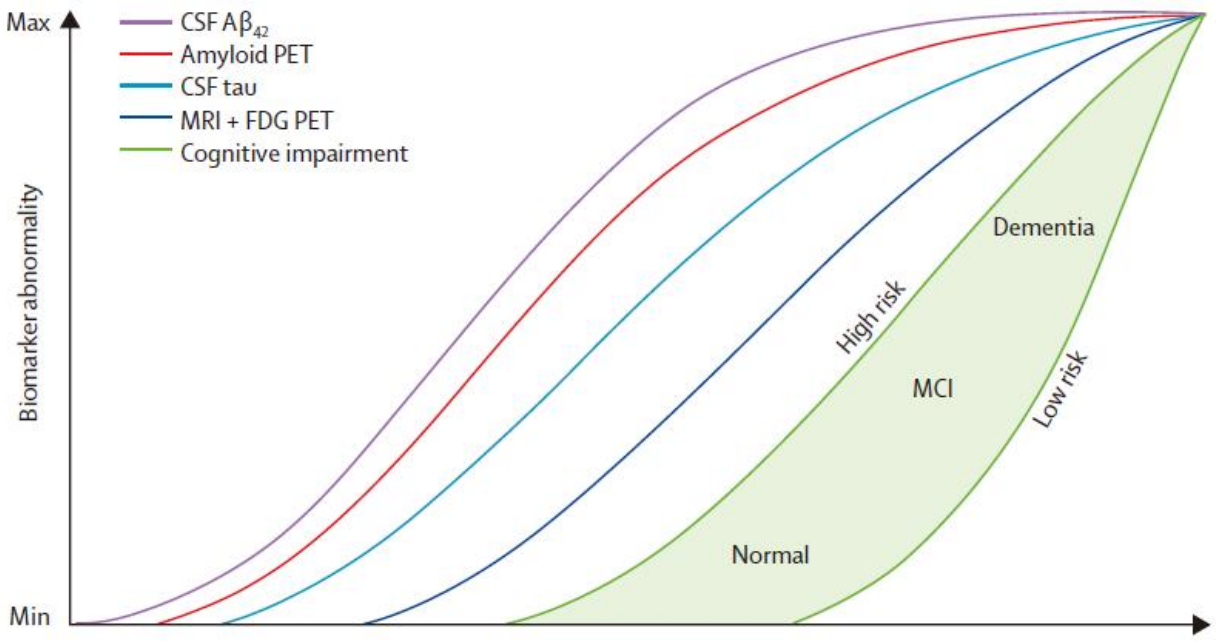

Fig. 1. The dynamic ATN biomarkers of AD pathological stages

*Adopted from Jack *et al*., 2013

Ⅱ. MATERIALS AND METHODS

Ⅱ-1. Materials

Ⅱ-1-1. CSF subjects

All subjects took informed consent, and all study processes were approved by the institutional review boards (IRB) at the Chosun university hospital and chonnam national university hospital. The 211 subjects were collected from September 2015 to December 2018. The majority of subjects were examined by medical doctors who were called a neurologist. All subjects were classified as healthy control and AD continuum groups who were called AD cascades. The classified AD cascade were grouped by the combination of the clinical diagnostic status and the amyloid burden for healthy control; cognitive normal and amyloid positron emission tomography (PET) negative, asymptomatic AD; cognitive normal and amyloid PET positive, prodromal AD; mild cognitive impairment and amyloid PET, and AD dementia; severe cognitive impairment and amyloid PET.

Ⅱ-1-2. Magnetic resonance imaging (MRI)

For the experiment of subcortical volume and thickness measurements. Three tesla structural MRI data used from the National research center for Dementia (NRCD) with just one device. One hundred ninety-nine subjects of the AD cascades subjects were examined by brain MRI. Based on ATN biomarkers classification, A-/T-/N- HC were used 72 samples, A+/T-/N- AD were used 16 samples, and A+/T+/N+ AD were used 56 samples.

Ⅱ-1-3. Neuropsychological test

For the experiment of neuropsychological test; CDR, KMMSE, and Seoul neuropsychological screening battery (SNSB) data used from the NRCD. All subjects examined by KMMSE, SNSB test. However, Some of items were refused to answer questions by subjects. The two hundred of AD cascades subjects also were examined by CDR. According to test items; SVLT immediate recall scores of

A-/T-/N- HC were used 71 samples, A+/T-/N- AD were used 17 samples, and A+/T+/N+ AD were used 57 samples ; SVLT delayed recall scores of A-/T-/N- HC were used 71 samples, A+/T-/N- AD were used 17 samples, and A+/T+/N+ AD were used 56 samples; SVLT recognition scores of A-/T-/N- HC were used 70 samples, A+/T-/N- AD were used 17 samples, and A+/T+/N+ AD were used 57 samples; RCFT delayed recall scores of A-/T-/N- HC were used 71 samples, A+/T-/N- AD were used 17 samples, and A+/T+/N+ AD were used 56 samples ; RCFT delayed recall scores of A-/T-/N- HC were used 71 samples, A+/T-/N- AD were used 17 samples, and A+/T+/N+ AD were used 55 samples ; RCFT recognition scores of A-/T-/N- HC were used 71 samples, A+/T-/N- AD were used 17 samples, and A+/T+/N+ AD were used 57 samples.

Ⅱ-2. CSF collection

For the sample preparation of human CSF used from NRCD. The CSF samples were taken from Chonnam national university hospital and Chosun university hospital. Within 15 minutes, Bio-team centrifuged the collecting samples at 2,000rpm for 10 min at 4℃, aliquoted to 0.5 mL samples in polypropylene tubes, and then stored liquid nitrogen tanks and deep freezers until analysis.

Ⅱ-3. CSF biomarkers assessment

All of the CSF samples were analyzed performing the Luminex 200 xMAP system platform (INNOBIA AlzBio3 for research only reagents, Fujirebio, Ghent, Belgium) and ELISA platform (INNOTEST for diagnosis reagents, Fujirebio, Ghent, Belgium). Samples were taken from the deep freezer 4 hours ago before using and continuously kept on ice. After prewashing the 96 wells filter plate for vacuum with a 1/25 diluted wash buffer, 25uL of each microsphere binding the corresponding ATN biomarkers specific capture antibodies (4D7A3, AT270, and AT120 for $A\beta_{42}$, pTau, and tTau respectively) were mixed with dilution buffer and added 100uL of mixture beads to the plate. Two kinds of premade mixture with

biotinylated monoclonal antibodies were designed to bind specifically the capture antibodies (3D6 for $A\beta_{42}$, and HT7 for pTau, tTau), and 75uL of CSF, quality control, or standards samples were added to the 96 wells filter plate and incubated 18hr on a orbital shaker with aluminum foil at room temperature. Next, the 96 wells filter plate was washed and added a 100uL of detection conjugate buffer, including the phycoerythrin-labeled streptavidin and incubated for 1hr with aluminum foil at room temperature. Finally the 96 wells filter plate was washed and added a 100uL of reading solution, including phosphate buffer saline (PBS). The assay was performed with a Luminex® 200™ platform (Luminex, Austin, Tx, USA). The standard curves were constructed for ATN biomarkers using a sigmoidal curve-fitting (SCF) method, and fluorescence intensity of the mean values for the duplicate samples were used to obtain with the concentration of ATN biomarkers. The results of INNOTEST were kindly provided by Prof. Jung Sup Lee (Department of biomedical science, Chosun Univ.). The values of fluorescence for the triplicate samples were used to obtain with the concentration of ATN biomarkers.

Ⅱ-4. Classification of ATN biomarkers profiles

The cut-off values for ATN biomarkers were the values that were determined of the youden index methods. the values were considered to be positive in late stages of AD as follows (pg/mL): ‘A’ biomarker of $A\beta_{42}$ < 387.47, ‘T’ biomarker of pTau > 41.88, and ‘N’ biomarker of tTau > 79.00.

Ⅱ-5. Statistical analyses

For the statistics, GraphPad prism 5.00, IBM SPSS version 25.0 (IBM, Armonk, NY), and R statistical software program version 3.5.1 for Windows various packages were used. Additionally, PLINK 1.07, and Haploview for Linux were used. AD pathological stages of scatter plot were used GraphPad prism. In statistical analysis, *P*-value of less than 0.05 was included consideration significant. The continuous variables were tested using the Shapiro-Wilk test. For univariate

comparisons of variables, one-way ANCOVA, and ANOVA were conducted with performing post hoc tests. The post hoc analyses of Games-Howell were corrected for multiple comparisons. Chi square test was conducted for gender as categorical variable. Intra assays of analytical assays were compared with passing Bablok, Spearman correlations, Bland & Altman plots, and intra class correlation (ICC). Relationship between two platforms were compared with Spearman correlations and ICC, whereas relationship between memory domain assessments and tau biomarkers levels were compared with Pearson correlation.

Receiver operating characteristics (ROC) curves were depicted with the true positive rate for sensitivity and the false-positive rate for 1-specificity by R. The area under the curves (AUC) were estimated from ROC curve using the empirical method by SPSS. In addition, the cut-offs for ATN biomarkers were the values that were determined of the youden index.

The hierarchical multiple linear regressions were performed using visuospatial, verbal memory assessments and CSF tau biomarkers levels by SPSS. The demographic variables for sex, age and education were implemented by entering method at model 1, visuospatial and verbal memory assessments were added by stepwise method to predict the tau levels at model 2.

The follow up association analyses were peformed using visuospatial memory assessment, and CSF tau levels from the NRCD cognitive assessments dataset and CSF tau biomarkers.

Samples were excluded for gender inconsistency between analysis of x-chromosome SNPs and, examined sex. The SNPs quality control processing; call rate < 97%, Hardy-Weinberg equilibrium (HWE) test *P*-value < 10^{-6} and minor allele frequency (MAF) < 3% were excluded by Plink. The linear regression in GWAS implemented to detect the effect of SNPs by Plink. The manhattan plots were performed using SNPs QC list and linear regression results by Haploview. All output variables of linear regression were applied at the suggestive level with $P=10^{-3}$ as the significance threshold.

Ⅲ. RESULTS

Ⅲ-1. Demographics and CSF ATN biomarkers values of subjects grouped based on diagnosis

The demographics and characteristics data of 211 subjects are presented for comparing the means of pathological stage groups (Table 1). The mean age, education, and MMSE values of the AD dementia subjects were significantly different compared to the other stages ($P \leq 0.002$). However, sex was not significantly different among pathological stages ($P = 0.506$). The $A\beta_{42}$ was highest in the healthy control groups, whereas The pTau and tTau were highest in the AD dementia group compared to the other stages ($P < 0.001$).

Ⅲ-2. Reproducibility of intra assay and relationship of inter assay

The coefficient of variation of two analytic methods were 6.6 ± 6.9, and 4.5 ± 3.5 for $A\beta_{42}$, 3.3 ± 3.5, and 2.9 ± 2.1 for pTau, 5.3 ± 4.4, and 3.7 ± 2.4 for tTau, respectively (Table 2). The Passing-Bablok analysis for replicative intra-assay showed high reproducibility (Fig. 2). The Bland & Altman plot showed majority of residuals fitting the regression line and 95% confidence intervals (Fig. 3).

The inter-assay performance data of 211 subjects were represented with the Spearman's correlation and inter-class correlation coefficient analysis. Although the ranges of values for biomarker measurements obtained with two platforms were different, the spearman's correlation values for each biomarkers were 0.837, 0.756, and 0.898 for $A\beta_{42}$, pTau, and tTau respectively ($P < 0.001$) (Fig. 4). CSF $A\beta_{42}$ have been compared directly to the mean cortical standardized uptake value ratio (SUVR) score by the two platforms. The result showed negative correlation between SUVR score and CSF $A\beta_{42}$ measurements. When comparing SUVR and CSF $A\beta_{42}$ correlation, INNOBIA $A\beta_{42}$ was stronger than INNOTEST $A\beta_{42}$ ($P < 0.001$) (Fig. 5).

Table 1. Clinical characteristics and CSF biomarkers data of the different pathological stages

	Total	Healthy control	Asymptomatic AD	prodromal AD	AD dementia	*P*-value
Demographic information						
N	211	86	20	56	49	
Age, y	211	71.1 ± 5.7	72.7 ± 4.8	72.5± 7.4	67.7 ± 8.5†‡	.002
Education, y	211	9.9 ± 5.0	10.5 ± 5.7	9.8 ± 4.5	6.4 ± 3.6*†‡	< .001
Female (n, %)	211	46 (53.4)	10 (50.0)	25 (44.6)	29 (59.2)	.506
MMSE	211	26.7 ± 2.6	26.9 ± 2.5	25.1 ± 3.5*	18.7 ± 5.3*†‡	< .001
INNOBIA CSF biomarkers						
Aβ_{42} (pg/mL)	211	542.7 ± 108.5	291.7 ± 138.0*	254.0 ± 118.2*	247.1 ± 96.7*	< .001
pTau (pg/mL)	211	31.7 ± 8.5	45.1 ± 13.9*	48.7 ± 15.3*	59.4 ± 18.1*†‡	< .001
tTau (pg/mL)	211	56.2 ± 21.3	77.9 ± 36.0	86.9 ± 41.8*	111.6 ± 46.6*†‡	< .001
INNOTEST CSF biomarkers						
Aβ_{42} (pg/mL)	211	1013.1 ± 239.5	572.6 ± 227.1*	460.6 ± 216.0*	429.0 ± 174.1*	< .001
pTau (pg/mL)	211	46.3 ± 18.1	53.9 ± 18.7	61.9 ± 26.8*	75.2 ± 26.6*†	< .001
tTau (pg/mL)	211	237.6 ± 103.5	291.5 ± 114.0	378.4 ± 201.4*	509.6 ± 208.5*†‡	< .001

Data are presented as number (percentage) or mean ± standard deviation. Parametric statistics were used to compare the characteristics and CSF biomarker data (oneway ANOVA test with post-hoc Games-Howell tests for age, Education, MMSE, Aβ_{42}, pTau and tTau). A Chi Square test was used to test gender distribution. Significant differences are indicated with the following symbols: * The analysis between the indicated group and the Healthy control group; † The analysis between the indicated group and asymptomatic AD group; ‡ The analysis between the prodromal AD and AD dementia group. Abbreviations: AD, Alzheimer's disease; MMSE, Korean Mini-Mental State Examination; Aβ_{42}, amyloid $\beta_{(1\text{-}42)}$; pTau, phosphorylated tau; tTau, total tau.

Table 2. The Coefficient of variation of two analytic methods in CSF biomarkers

Analytic method	Analyte	CV(%) ± SD	Median (25th, 75th percentile)
INNOBIA	$A\beta_{42}$	6.6 ± 6.9	4.7 (1.9, 8.8)
	pTau	3.3 ± 3.5	2.2 (1.1, 4.4)
	tTau	5.3 ± 4.4	4.0 (1.9, 7.0)
INNOTEST	$A\beta_{42}$	4.5 ± 3.5	3.5 (1.6, 6.4)
	pTau	2.9 ± 2.1	2.4 (1.3, 4.0)
	tTau	3.7 ± 2.4	3.2 (1.7, 5.0)

Data are presented as CV ± standard deviation or Median (25-75th percentile) for INNOBIA and INNOTEST (n=211). Abbreviations: $A\beta_{42}$, amyloid $\beta_{(1-42)}$; pTau, phosphorylated tau; tTau, total tau; CV, coefficient of variability; CSF, cerebrospinal fluid.

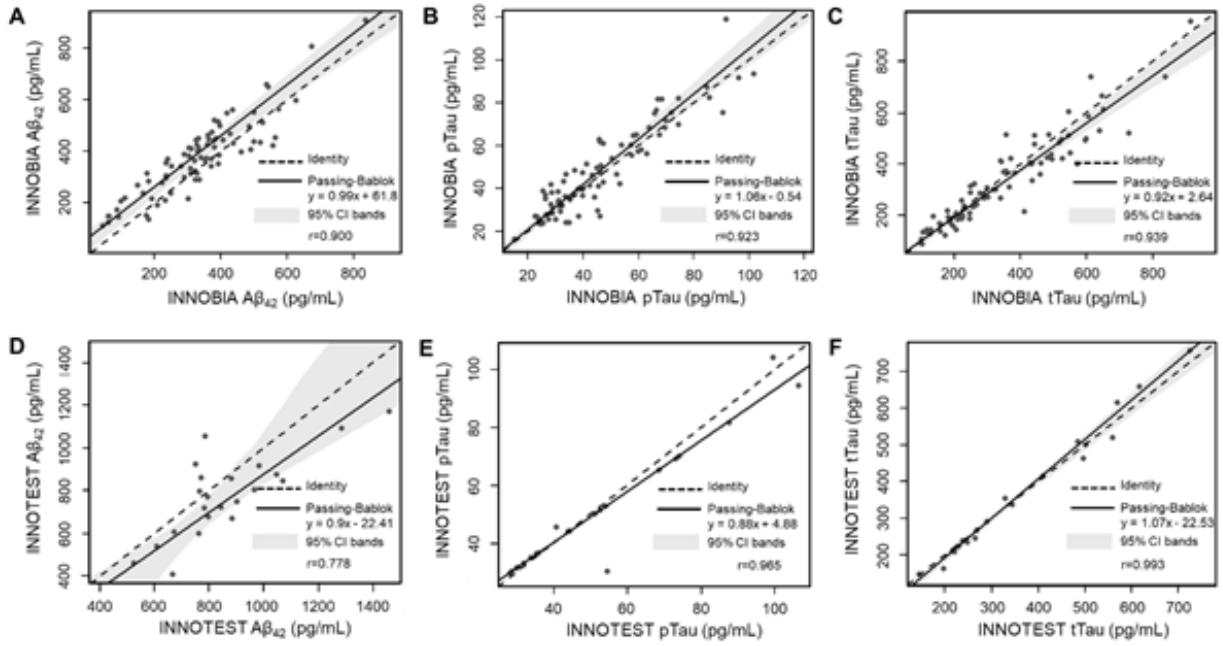

Fig. 2. Validation of analytical assays for INNOBIA and INNOTEST. The Passing-Bablok regression, Spearman correlation were used to evaluate systematic differences (A-C) The concentration of $A\beta_{42}$ (A), pTau (B), and tTau (C) measured by INNOBIA, (D-F) The concentration of $A\beta_{42}$ (D), pTau (E), and tTau (F) measured by INNOTEST with identical preanalytical protocols (A-C; n= 81, D-E; n=22, and F; n=21). Intra class correlation coefficients (ICC) were 0.947, 0.959, and 0.968 for $A\beta_{42}$, pTau, and tTau by INNOBIA and 0.873, 0.981, 0.995 by INNOTEST, respectively. The solid diagonal line represents the regression line and the gray area represents the 95% CI. Abbreviations: $A\beta_{42}$, amyloid $\beta_{(1\text{-}42)}$; pTau, phosphotylated tau; tTau, total tau; CI, confidence interval.

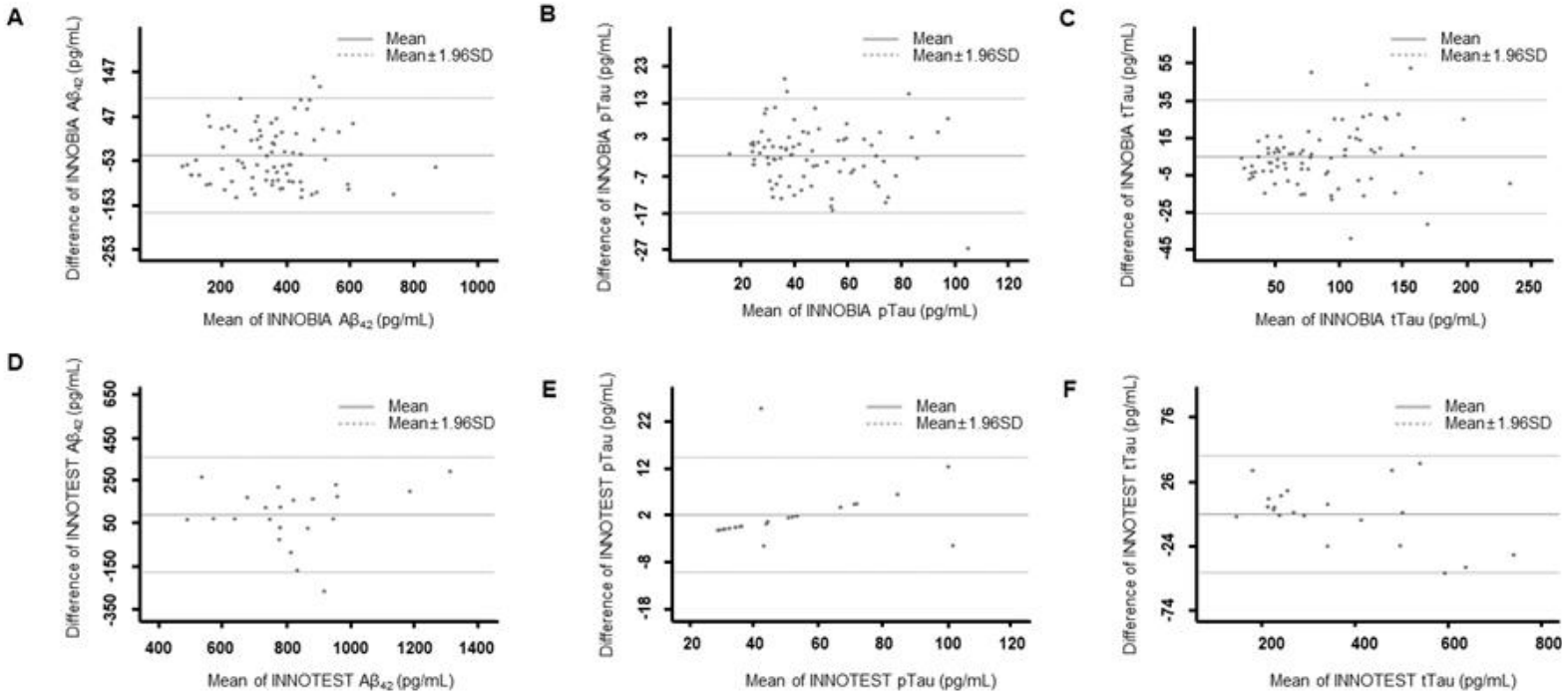

Fig. 3. Validation of analytical difference and mean values for INNOBIA and NNOTEST. The Bland & Altman plots show the mean of concentration (x-axis) and difference of concentration (y-axis), which are defined as the mean difference ± 1.96 times of the standard deviation of the difference. (A-C) The ATN biomarkers concentration of $A\beta_{42}$ (A), pTau (B), and tTau (C) measured by INNOBIA, (D-F) The mean biomarkers concentration of $A\beta_{42}$ (D), pTau (E), and tTau (F) measured by INNOTEST with identical preanalytical protocols (A-C; n= 81, D-E; n=22, and F; n=21). The dotted lines represent the 95% CI. Abbreviations: CSF, cerebrospinal fluid; $A\beta_{42}$, amyloid $\beta_{(1-42)}$; pTau, phosphorylated tau; tTau, total tau; CI, confidence interval; SD, standard deviation.

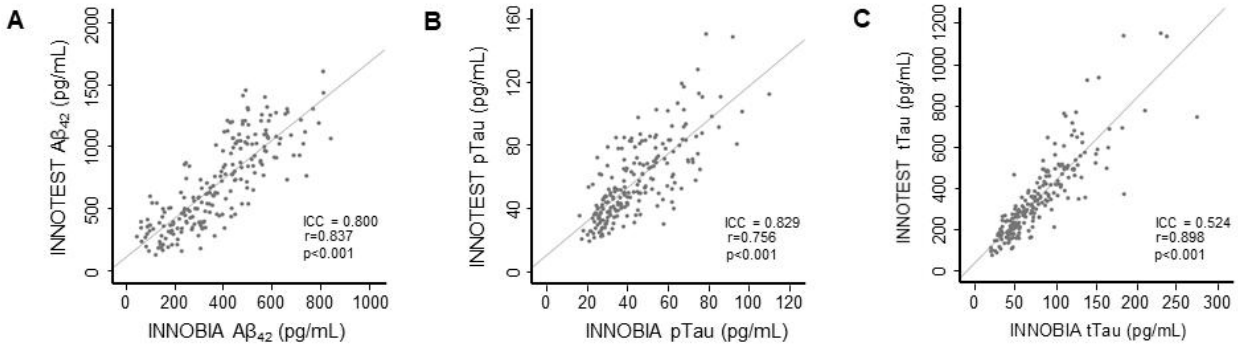

Fig. 4. Relationship between INNOBIA and INNOTEST in the analysis of CSF biomarkers. The scatterplots show the correlations between INNOBIA (x-axis) and INNOTEST (y-axis) for $A\beta_{42}$ (A), pTau (B), and tTau (C). The solid diagonal line represents the regression line. Inter class correlation coefficients (ICC) were 0.800, 0.829, and 0.524 for $A\beta_{42}$, pTau, and tTau, respectively. The correlation coefficient (rho) and *P*-values were from the spearman's correlation analysis (n=211). Abbreviations: CSF, cerebrospinal fluid; $A\beta_{42}$, amyloid $\beta_{(1\text{-}42)}$; pTau, phosphorylated tau; tTau, total tau.

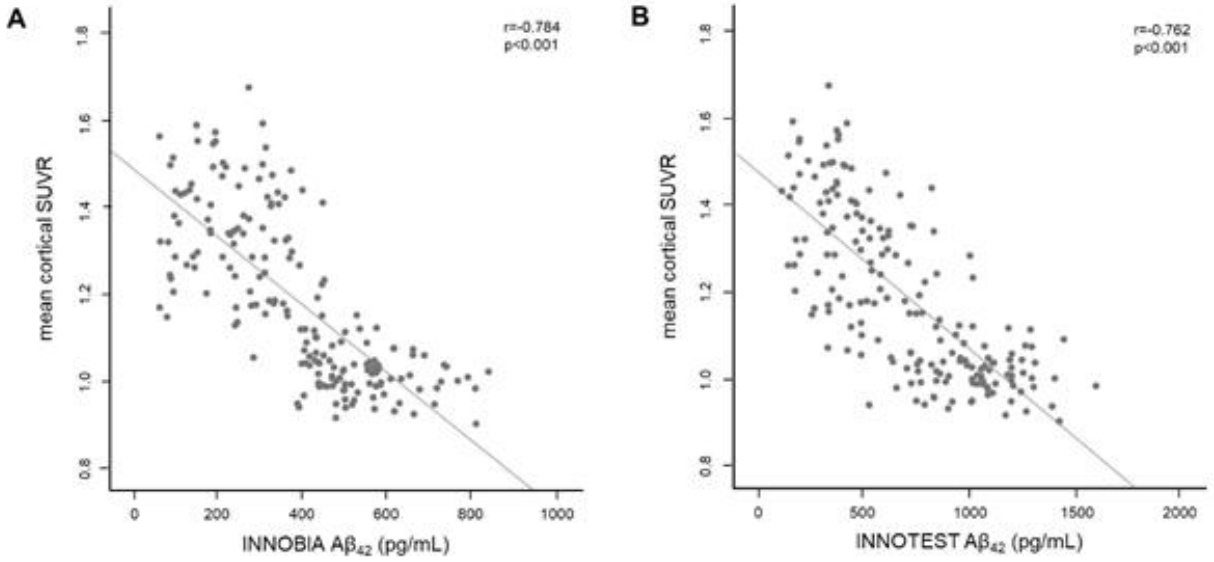

Fig. 5. Relationship between CSF Aβ$_{42}$ analyte values and cortical amyloid burden. The scatterplots showed the correlations between CSF Aβ$_{42}$ (x-axis) for INNOBIA (A), and INNOTEST (B) and mean cortical SUVR (y-axis). The solid diagonal line represents the regression line. The correlation coefficient (rho) and *P*-values were from the spearman's correlation analysis (n=185). Abbreviations : CSF, cerebrospinal fluid; Aβ$_{42}$, amyloid β$_{(1-42)}$; SUVR, standardized uptake value ratio.

Ⅲ-3. Classification of CSF ATN biomarkers in AD continuum

The CSF $A\beta_{42}$ levels were negatively correlated to the amyloid burden (Fig. 6). In other words, CSF $A\beta_{42}$ were highest in HC. Approximately 2-fold decrease between HC and aAD for INNOBIA and INNOTEST, respectively (Fig. 6A-B). The CSF pTau and tTau were highest in ADD and gradually decreased from ADD to HC (Fig. 6C-F). AD continuum were well classified by pTau from INNOBIA with statistical significance ($P < 0.05$ for aAD vs. HC; $P < 10^{-9}$ for pAD vs. HC; $P < 10^{-9}$ for ADD vs. HC), whereas for pTau from INNOTEST were not similar (n.s for aAD vs. HC; $P < 0.05$ for pAD vs. HC; $P < 10^{-6}$ for ADD vs. HC) (Fig. 6C-D). According to the ATN biomarkers hypothesis, subjects with cognitive decline, classified as pAD and ADD should show high levels of tau protein. However, approximately 20% of samples did not follow this patterns. (Fig. 6C-F).

Ⅲ-4. CSF ATN biomarkers diagnostic accuracy and cut-off values in AD continuum.

ROC curves for each ATN biomarkers and ratio values were used to best discriminate the pathological stages from HC. The values of tTau from INNOBIA and INNOTEST platforms showed similar trends of AUC in AD cascade. On the other hand, the values of the AUC were well classified by $A\beta_{42}$ and pTau from INNOBIA ($P < 0.001$). The INNOBIA ratios of pTau/$A\beta_{42}$ were the highest values among the CSF biomarkers in the early stages of AD (aAD and pAD) ($P < 0.001$) (Table 3). The cut-off values discriminated AD stages from HC based on AUC, sensitivity, specificity, diagnostic accuracy values. Both aAD vs. HC and pAD vs. HC. The cut-off values of AD continuum for the ATN biomarkers with the highest sum of sensitivity and specificity. INNOBIA pTau/$A\beta_{42}$ ratio were higher than INNOTEST, whereas ADD vs. HC were similar values of AUC. In other words, INNOBIA pTau/$A\beta_{42}$ ratio, best differentiated early stages of AD from HC (Table 4). Thereby, proving to be effective for early diagnosis of AD patients.

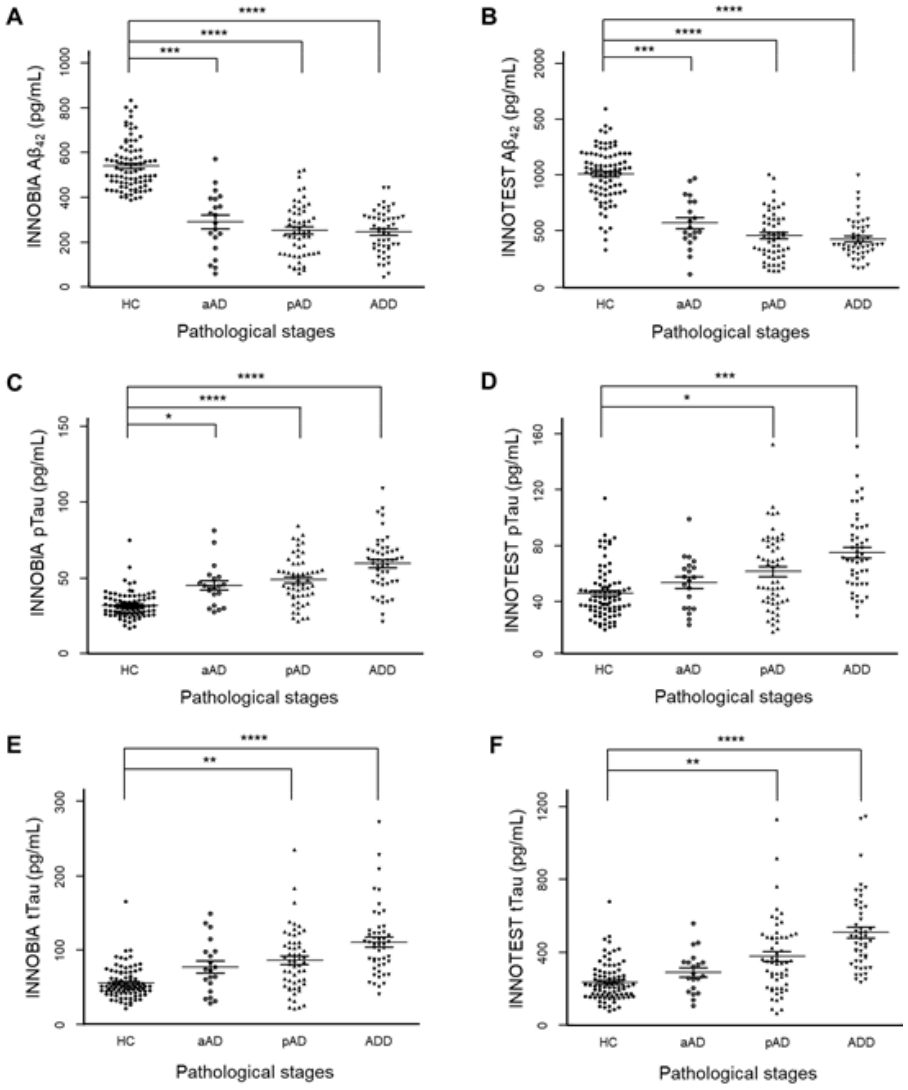

Fig. 6. The concentration of the CSF analyte values measured by INNOBIA and INNOTEST on pathological stages. The scatter plot showed levels of CSF biomarkers in the different diagnostic groups. Error bars represent the mean and 95% CI. *P*-values were assessed by ANOVA. the ANOVA was performed to evaluate the significance with post-hoc Games-Howell tests. $^{*}P < 0.05$, $^{**}P < 10^{-4}$, $^{***}P < 10^{-6}$, $^{****}P < 10^{-9}$. Abbreviations: CSF, cerebrospinal fluid; HC, healthy control; aAD, asymptomatic AD; pAD, prodromal AD; ADD, AD-dementia; $A\beta_{42}$, amyloid $\beta_{(1-42)}$; pTau, phosphorylated tau; tTau, total tau.

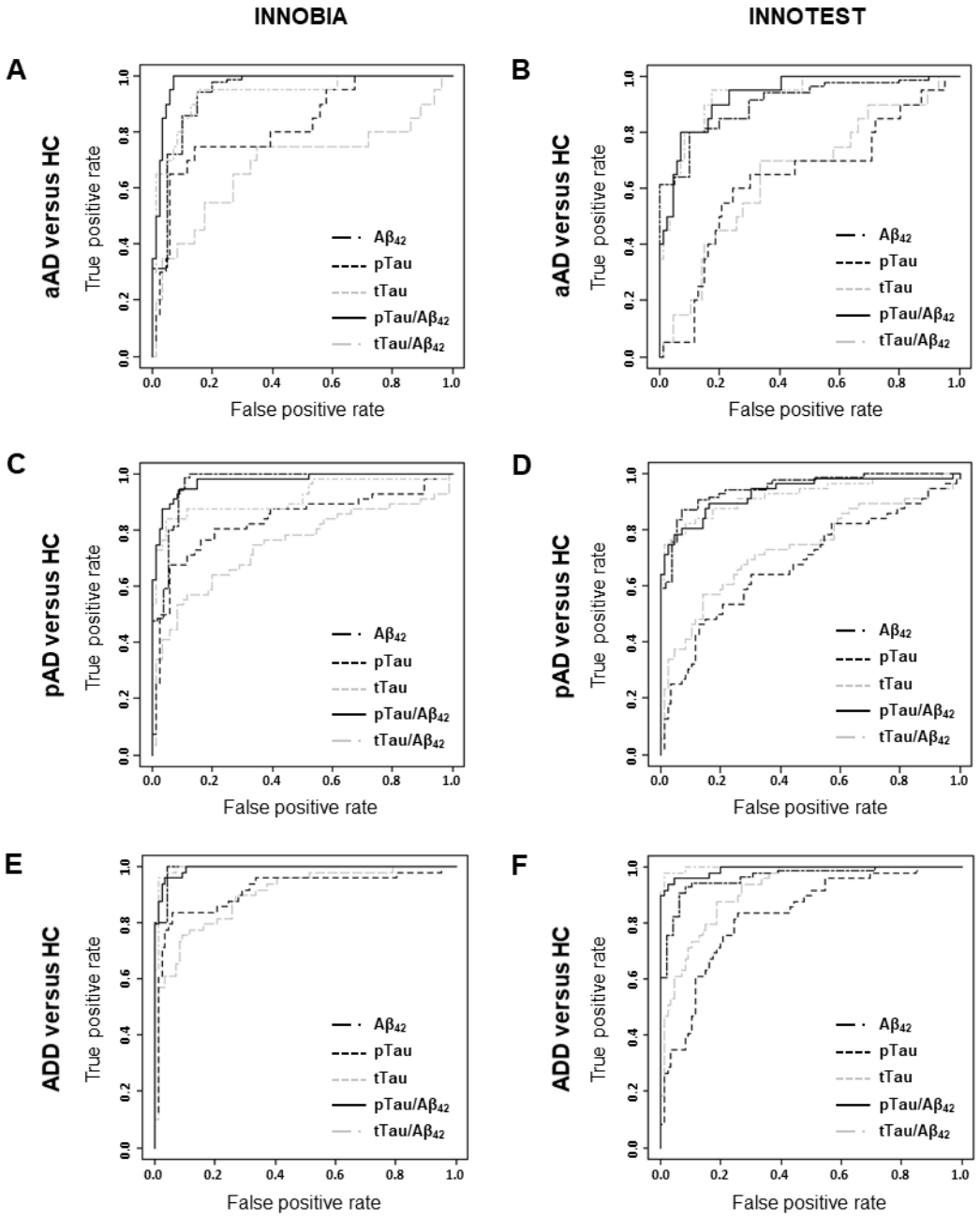

Fig. 7. ROC curves of two analytic methods representing CSF biomarkers for corresponding diagnostic performance. ROC curves for aAD versus HC (n=106, A-B), pAD versus HC (n=142, C-D), and ADD versus HC (n=135, G-I). Panels of A, C, and E are for INNOBIA, and panels B, D, and F are for INNOTEST. Abbreviations : HC, healthy control; aAD, asymptomatic AD; pAD, prodromal AD; ADD, AD-dementia; CSF, cerebrospinal fluid; $A\beta_{42}$, amyloid $\beta_{(1-42)}$; pTau, phosphorylated tau; tTau, total tau; ROC, Receiver operating characteristic.

Table 3. AUC values of two analytic methods representing CSF biomarkers for corresponding diagnostic performance

Analyte	aAD versus HC		pAD versus HC		ADD versus HC	
	INNOBIA	INNOTEST	INNOBIA	INNOTEST	INNOBIA	INNOTEST
$A\beta_{42}$	0.940	0.907	0.966	0.950	0.992	0.963
pTau	0.826	0.634	0.836	0.681	0.916	0.830
tTau	0.684	0.654	0.749	0.736	0.901	0.918
pTau/$A\beta_{42}$	0.980	0.933	0.975	0.933	0.993	0.991
tTau/$A\beta_{42}$	0.936	0.931	0.917	0.923	0.994	0.998

Data are presented as AUC values for aAD versus HC (n=106), pAD versus HC (n=142), and ADD versus HC (n=135). Abbreviations: HC, healthy control; aAD, asymptomatic AD; pAD, prodromal AD; ADD, AD-dementia; CSF, cerebrospinal fluid; $A\beta_{42}$, amyloid $\beta_{(1\text{-}42)}$; pTau, phosphorylated tau; tTau, total tau; AUC, area under the curve.

Table 4. Cut-off values of CSF biomarkers for corresponding diagnosis by two analytic methods

Analytic method	Analyte	AUC (95% CI)	*P*-value	Cutoff value (pg/mL)	Sen	Spec	DA
Asymptomatic AD versus healthy Control							
INNOBIA	$A\beta_{42}$	0.940 (0.869-1.000)	<0.001	< 409.92	0.85	0.94	0.92
	pTau	0.826 (0.719-0.932)	<0.001	> 38.89	0.75	0.86	0.84
	pTau/$A\beta_{42}$	0.980 (0.957-1.000)	<0.001	> 0.08069	1.00	0.93	0.94
INNOTEST	$A\beta_{42}$	0.907(0.845-0.968)	<0.001	< 832.95	0.90	0.80	0.82
	pTau	0.634 (0.491-0.777)	0.062	> 52.56	0.60	0.76	0.73
	pTau/$A\beta_{42}$	0.933 (0.880-0.985)	<0.001	> 0.07058	0.80	0.93	0.91
Prodromal AD versus healthy control							
INNOBIA	$A\beta_{42}$	0.966 (0.935-0.997)	<0.001	< 393.35	0.89	0.99	0.95
	pTau	0.836 (0.759-0.912)	<0.001	> 41.88	0.68	0.94	0.84
	pTau/$A\beta_{42}$	0.975 (0.952-0.998)	<0.001	> 0.07708	0.95	0.91	0.92
INNOTEST	$A\beta_{42}$	0.950 (0.918-0.983)	<0.001	< 751.94	0.93	0.87	0.89
	pTau	0.681 (0.587-0.775)	<0.001	> 49.80	0.64	0.70	0.68
	pTau/$A\beta_{42}$	0.933 (0.887-0.979)	<0.001	> 0.07687	0.79	0.95	0.89
AD dementia versus healthy control							
INNOBIA	$A\beta_{42}$	0.992 (0.980-1.000)	<0.001	< 384.75	0.96	1.00	0.99
	pTau	0.916 (0.859-0.974)	<0.001	> 43.47	0.84	0.94	0.90
	pTau/$A\beta_{42}$	0.993 (0.984-1.000)	<0.001	> 0.10049	0.96	0.97	0.96
INNOTEST	$A\beta_{42}$	0.963 (0.935-0.992)	<0.001	< 654.78	0.92	0.93	0.93
	pTau	0.830 (0.759-0.901)	<0.001	> 52.19	0.84	0.74	0.78
	pTau/$A\beta_{42}$	0.991 (0.980-1.000)	<0.001	> 0.09034	0.94	0.98	0.96
Early stages of AD versus healthy control							
INNOBIA	$A\beta_{42}$	0.959 (0.929-0.989)	<0.001	< 399.25	0.87	0.98	0.93
	pTau	0.833 (0.767-0.899)	<0.001	> 41.81	0.67	0.94	0.81
	pTau/$A\beta_{42}$	0.976 (0.956-0.996)	<0.001	> 0.07708	0.96	0.91	0.93
INNOTEST	$A\beta_{42}$	0.939 (0.904-0.973)	<0.001	< 783.24	0.91	0.84	0.87
	pTau	0.669 (0.584-0.754)	<0.001	> 49.80	0.64	0.70	0.67
	pTau/$A\beta_{42}$	0.933 (0.894-0.971)	<0.001	> 0.07058	0.80	0.93	0.84
Late stages of AD versus healthy control							
INNOBIA	$A\beta_{42}$	0.978 (0.960-0.996)	<0.001	< 387.47	0.91	1.00	0.95
	pTau	0.873 (0.821-0.926)	<0.001	> 41.88	0.75	0.94	0.84
	pTau/$A\beta_{42}$	0.983 (0.969-0.997)	<0.001	> 0.09696	0.91	0.97	0.94
INNOTEST	$A\beta_{42}$	0.956 (0.929-0.983)	<0.001	< 722.52	0.91	0.91	0.91
	pTau	0.751 (0.681-0.820)	<0.001	> 49.80	0.73	0.70	0.72
	pTau/$A\beta_{42}$	0.960 (0.934-0.986)	<0.001	> 0.07687	0.87	0.95	0.91

The analysis for AUC, *P*-value, cut-off value, sensitivity, specificity, diagnostic accuracy assessed by INNOBIA and INNOTEST in the different diagnostic groups. Abbreviations: AD, Alzheimer's disease; $A\beta_{42}$, amyloid $\beta_{(1-42)}$; pTau, phosphorylated tau; tTau, total tau; 95% CI, 95% confidence interval; AUC, area under the curve; Sen, sensitivity; Spec, specificity; DA, diagnostic accuracy. Early stages of AD groups included asymptomatic AD and prodromal AD, late stages of AD groups included prodromal AD and AD dementia.

Ⅲ-5. ATN biomarker and cognitive status based profiling of subjects

The CSF ATN biomarker profiling of subjects to confirm the tau biomarkers were performed based on INNOBIA cut-off values in late stages of AD (pAD and ADD) (Table 4). The subjects were grouped into A-/T-/N- HC, A+/T-/N- AD, and A+/T+/N+ AD groups. The demographics and characteristics data of 156 subjects and the statistical analysis of variance are shown (Table 5). Age, sex and level of education were not significantly different among ATN classification groups. However, MMSE and CDR values were significantly different among the groups ($P < 0.001$). The ATN biomarkers were significantly different among the groups ($P < 0.001$).

Ⅲ-6. Patterns of brain atrophy of subjects profiled based on CSF ATN biomarkers

The variations in the cortical thickness and subcortical volumes of the brain among the groups profiled based on CSF ATN were analyzed using Analysis of covariance (ANCOVA). The majority of subcortical volumes were significantly different between A-/T-/N- HC and A+/T+/N+ AD ($P < 0.001$) (Fig. 8 and Table 6). Hippocampal and amygdala volumes were significantly decreased between A+/T-/N- AD and A+/T+/N+ AD (Fig. 8A-B). However, The majority of subcortical volumes were similar values between A+/T-/N- AD and A+/T+/N+ AD (Fig. 8C-H). The majority of cortical thickness were significantly different between A-/T-/N- HC and A+/T+/N+ AD ($P < 0.001$) (Fig. 9).

The cortical thickness atrophies between the two hemispheres were asymmetrical. The left hemisphere cortical thickness values showed more severe atrophy than the right hemisphere cortical thickness values (Table 7). However, entorhinal, superior frontal, and precuneus thickness values of left-right hemispheres did not show such pattern. The thickness values of these three regions showed significant decreased between A+/T-/N- AD and A+/T+/N+ AD (Fig. 9A-C). The results showed that the tau biomarker levels have effect on patterns of brain atrophy.

Table 5. Clinical characteristics and CSF biomarkers data of the different ATN biomarkers profiles

	Total	A-/T-/N- HC	A+/T-/N- AD	A+/T+/N+ AD	*P*-value
Demographic information					
N	156	74	18	64	
Age, y	156	70.8 ± 6.0	72.6 ± 5.3	69.3 ± 9.0	0.200
Female (n, %)	156	39 (52.7)	9 (50.0)	33 (51.6)	0.651
Education, y	156	9.8 ± 4.9	8.4 ± 5.9	8.0 ± 3.8	0.074
MMSE	156	26.6 ± 2.6	23.6 ± 4.7*	21.5 ± 5.5*	<0.001
CDR-SOB	147	0.6 ± 0.7	1.6 ± 1.0*	2.9 ± 2.2*†	<0.001
Hippocampus (mm^3)	144	8227.1 ± 954.8	7283 ± 1203.7*	6459.2 ± 941.6*†	<0.001
INNOBIA CSF biomarkers					
$A\beta_{42}$ (pg/mL)	156	533.9 ± 104.8	230.0 ± 103.0*	234.0 ± 91.0*	<0.001
pTau (pg/mL)	156	29.5 ± 5.5	32.1 ± 6.4	62.6 ± 13.9*†	<0.001
tTau (pg/mL)	156	50.4 ± 13.2	50.6 ± 16.3	121.5 ± 40.3*†	<0.001

Data are presented as number (percentage) or mean ± standard deviation. Abbreviations: HC, healthy control; AD, Alzheimer's disease; MMSE, Korean Mini-Mental State Examination; CDR-SOB, clinical dementia rating sum of boxes; $A\beta_{42}$, amyloid $\beta_{(1-42)}$; pTau, phosphorylated tau; tTau, total tau. Significant differences are indicated with the following symbols: * The analysis between the indicated group and the A-/T-/N- HC group; † The analysis between the indicated group and A+/T-/N- AD group.

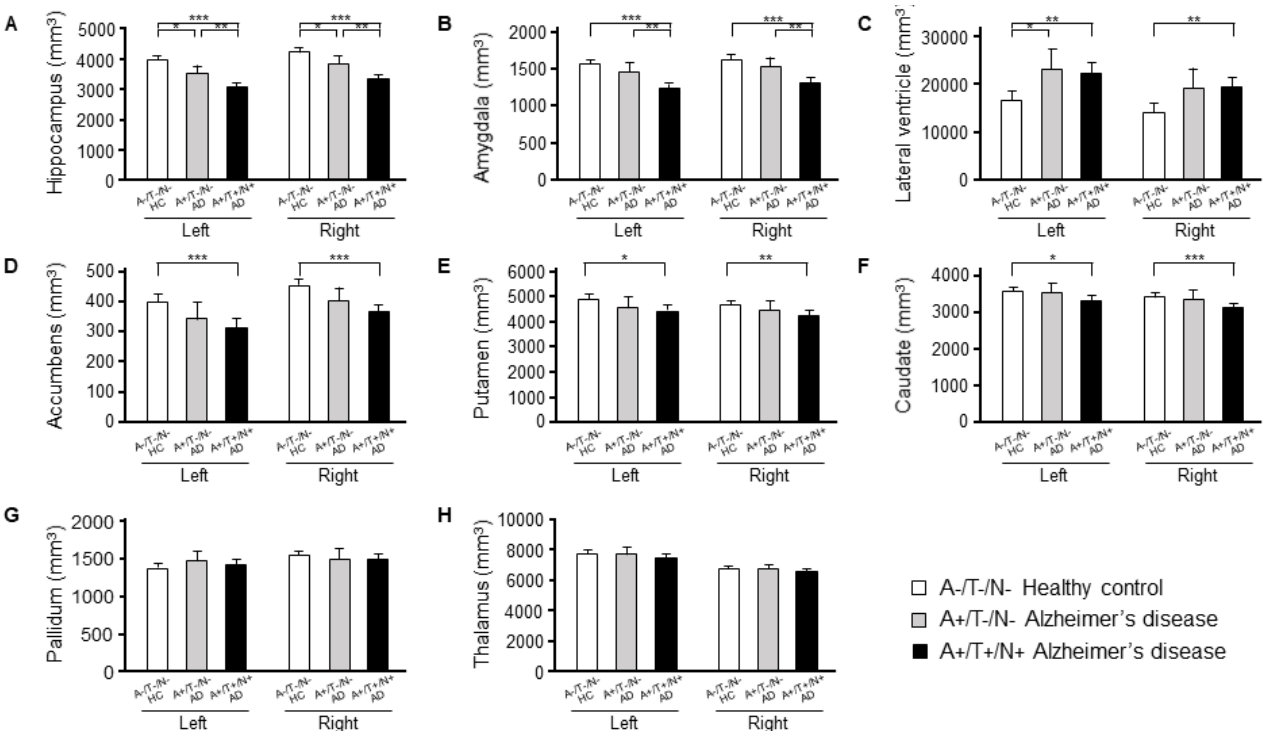

Fig. 8. The different atrophy of subcortical structures from CSF ATN biomarkers profiles in AD. The subcortical volume of AD patients and healthy controls were compared with precisely classified samples based on CSF ATN biomarkers. The ANCOVA were analyzed with covariates of age, sex, levels of education, and ICV. $^{*}P < 0.05$, $^{**}P < 10^{-2}$, $^{***}P < 10^{-3}$. Abbreviations: CSF, cerebrospinal fluid; HC, healthy control; AD, Alzheimer's disease; ICV, intracranial volume.

Table 6. Comparison of subcortical structures from CSF ATN biomarkers profiles in AD

Subcortical region	CSF ATN criteria & clinical and pathological diagnosis				
	A-/T-/N- HC[a]	A+/T-/N- AD[b]	A+/T+/N+ AD[c]	*P*-value	post-hoc
Left hippocampus	4000 ± 54	3536 ± 114	3075 ± 61	5.74E-20	a > b > c
Right hippocampus	4247 ± 60	3843 ± 126	3335 ± 68	8.89E-17	a > b > c
Left amygdala	1554 ± 26	1462 ± 56	1234 ± 30	5.74E-12	a, b > c
Right amygdala	1639 ± 25	1541 ± 53	1322 ± 29	1.07E-12	a, b > c
Left lateral ventricle	16704 ± 998	23238 ± 2099	22262 ± 1132	4.20E-4	a < b, c
Right lateral ventricle	14201 ± 930	19363 ± 1958	19398 ± 1056	7.77E-4	a < c
Left accumbens	399 ± 13	343 ± 28	313 ± 15	2.52E-4	a > c
Right accumbens	453 ± 10	404 ± 20	369 ± 11	5.02E-7	a > c
Left putamen	4875 ± 97	4567 ± 205	4432 ± 110	0.01	a > c
Right putamen	4655 ± 80	4479 ± 167	4247 ± 90	4.71E-3	a > c
Left caudate	3570 ± 53	3566 ± 112	3327 ± 61	9.71E-3	a > c
Right caudate	3437 ± 55	3365 ± 115	3125 ± 62	1.13E-3	a > c
Left pallidum	1380 ± 30	1478 ± 62	1434 ± 34	0.27	n.s
Right pallidum	1548 ± 30	1506 ± 63	1504 ± 34	0.60	n.s
Left thalamus	7766 ± 109	7757 ± 229	7479 ± 124	0.21	n.s
Right thalamus	6823 ± 72	6750 ± 150	6614 ± 81	0.16	n.s

Values are shown as mean ± standard error. ANCOVA test with post-hoc bonferroni correction adjusted age, sex, levels of education and ICV as covariates. Abbreviations: AD, Alzheimer's disease; HC, healthy control; ICV, intracranial volume; a, A-/T-/N- HC; b, A+/T-/N- AD; c, A+/T+/N+ AD. n.s; no significant difference.

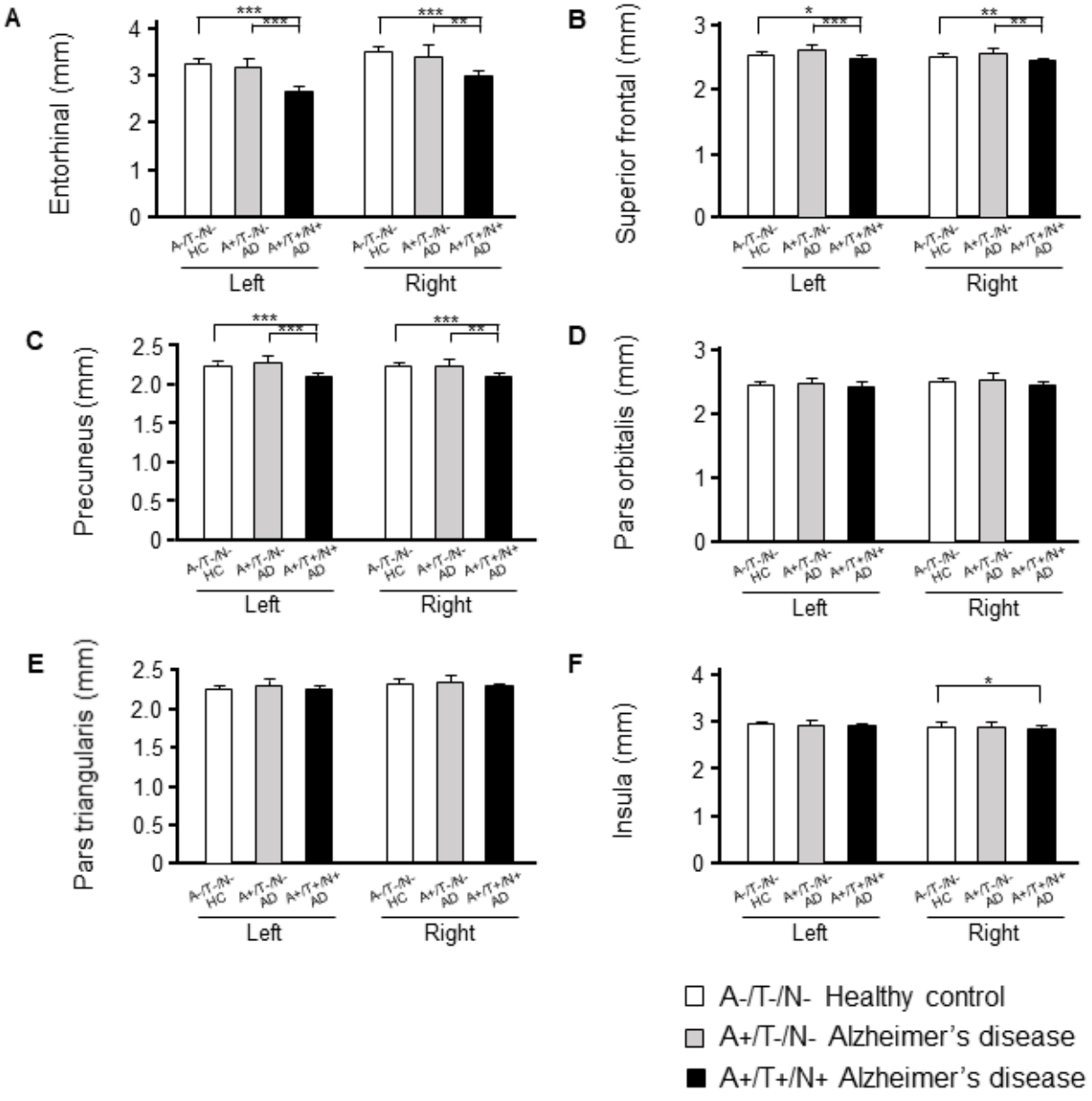

Fig. 9. The different atrophy of cortical structures from CSF ATN biomarkers profiles in AD. The cortical thickness of AD patients and healthy controls were compared with precisely classified samples based on CSF ATN biomarkers. The ANCOVA were analyzed with covariates of age, sex, and levels of education. $^{*}P < 0.05$, $^{**}P < 10^{-2}$, $^{***}P < 10^{-3}$. Abbreviations: CSF, cerebrospinal fluid; HC, healthy control; AD, Alzheimer's disease.

Table 7. Comparison of cortical structures from CSF ATN biomarkers profiles in AD

Thickness region	CSF ATN criteria & clinical and pathological diagnosis					
	A-/T-/N- HC[a]	A+/T-/N- AD[b]	A+/T+/N+ AD[c]	*P*-value	post-hoc	
Left entorhinal	3.287 ± 0.043	3.183 ± 0.090	2.678 ± 0.049	1.484E-15	a, b > c	
Right entorhinal	3.505 ± 0.049	3.431 ± 0.103	3.006 ± 0.056	2.280E-9	a, b > c	
Left superior frontal	2.583 ± 0.014	2.654 ± 0.030	2.519 ± 0.016	1.490E-4	a, b > c	
Right superior frontal	2.555 ± 0.015	2.600 ± 0.031	2.481 ± 0.017	4.340E-4	a, b > c	
Left precuneus	2.268 ± 0.017	2.308 ± 0.037	2.120 ± 0.020	4.442E-8	a, b > c	
Right precuneus	2.252 ± 0.016	2.268 ± 0.034	2.128 ± 0.018	1.000E-6	a, b > c	
Left lateral occipital	2.140 ± 0.015	2.135 ± 0.033	2.026 ± 0.018	1.100E-5	a, b > c	
Right lateral occipital	2.149 ± 0.015	2.195 ± 0.033	2.045 ± 0.018	5.000E-6	a, b > c	
Left superior parietal	2.152 ± 0.014	2.202 ± 0.030	2.083 ± 0.016	5.540E-4	a, b > c	
Left fusiform	2.659 ± 0.020	2.636 ± 0.043	2.489 ± 0.023	7.555E-7	a, b > c	
Right fusiform	2.628 ± 0.020	2.647 ± 0.041	2.520 ± 0.022	6.770E-4	a, b > c	
Left middle temporal	2.747 ± 0.021	2.737 ± 0.045	2.556 ± 0.024	8.780E-8	a, b > c	
Right middle temporal	2.751 ± 0.019	2.734 ± 0.039	2.607 ± 0.021	5.000E-6	a, b > c	
Left inferior parietal	2.353 ± 0.018	2.343 ± 0.037	2.198 ± 0.020	2.088E-7	a, b > c	
Right inferior parietal	2.333 ± 0.015	2.345 ± 0.033	2.212 ± 0.018	1.000E-6	a, b > c	
Left caudal middle frontal	2.449 ± 0.017	2.508 ± 0.035	2.370 ± 0.019	5.840E-4	a, b > c	
Left inferior temporal	2.886 ± 0.020	2.869 ± 0.043	2.695 ± 0.023	2.850E-8	a, b > c	
Right inferior temporal	2.827 ± 0.019	2.796 ± 0.041	2.687 ± 0.022	2.700E-5	a > c	
Left lingual	1.888 ± 0.013	1.917 ± 0.028	1.822 ± 0.015	8.840E-4	a, b > c	
Left superior temporal	2.742 ± 0.018	2.733 ± 0.038	2.612 ± 0.021	2.000E-5	a, b > c	
Right superior temporal	2.777 ± 0.017	2.766 ± 0.035	2.680 ± 0.019	7.750E-4	a > c	
Left supramarginal	2.421 ± 0.016	2.419 ± 0.034	2.320 ± 0.019	2.420E-4	a, b > c	

Values are shown as mean ± standard error. ANCOVA test with post-hoc bonferroni correction adjusted age, sex, and levels of education as covariates. Abbreviations: AD, Alzheimer's disease; HC, healthy control; a, A-/T-/N- HC; b, A+/T-/N- AD; c, A+/T+/N+ AD. n.s; no significant difference.

Ⅲ-7. Full scales neuropsychological screening battery assessments of subjects profiled by CSF ATN biomarkers

The variation in neuropsychological assessments among the groups profiled based on CSF ATN were analyzed using ANCOVA. Memory, visuospatial function, language, attention and executive function were the five domains assessed statistically. Four domain assessments showed significant decrease between A-/T-/N- HC and A+/T+/N+ AD ($P < 0.001$), except attention domain (Fig. 10). Memory and visuospatial domains were significantly decreased between A+/T-/N- AD and A+/T+/N+ AD (Fig. 10A-B), whereas the remaining domain assessments showed similar values between A+/T-/N- AD and A+/T+/N+ AD (Fig. 10C-E).

Different statistical models were analyzed adjusting covariates to obtain an unbiased classification of the subjects (Table 8). Memory domain of two model showed significant decrease between A+/T-/N- AD and A+/T+/N+ AD. The memory domain consisted of two sub-domains: visuospatial memory assessed with RCFT tests and verbal memory assessed with SVLT tests. (Fig. 11 and Table 9) RCFT and SVLT tests further were scored based on three individual sub-tests. RCFT and SVLT tests were significantly decreased between A-/T-/N- HC and A+/T+/N+ AD. Two out of three SVLT tests showed significant decrease between A+/T-/N- AD and A+/T+/N+ AD (Fig. 11A-C and Table 9). However, All RCFT sub-tests showed significant decrease between A+/T-/N- AD and A+/T+/N+ AD (Fig. 11D-F and Table 9). Additionally, Tau categorized AD groups were well classified by scores from RCFT with statistical significance ($P < 10^{-5}$ for immediate recall test; $P < 10^{-5}$ for delayed recall test vs. HC; $P = 10^{-4}$ for recognition test), whereas for scores from SVLT were not similar (n.s for immediate recall test; $P = 0.03$ for delayed recall test vs. HC; $P = 0.01$ for recognition test). The visuospatial domain consisted of RCFT copy and drawing test that showed significant decrease between A-/T-/N- HC and A+/T+/N+ AD (Fig. 10G-H and Table 9). RCFT copy test was significantly decreased between A+/T-/N- AD and A+/T+/N+ AD, whereas drawing test showed similar values between A+/T-/N- AD and A+/T+/N+ AD. RCFT tests showed significant decrease in tau positive AD groups than tau negative AD groups.

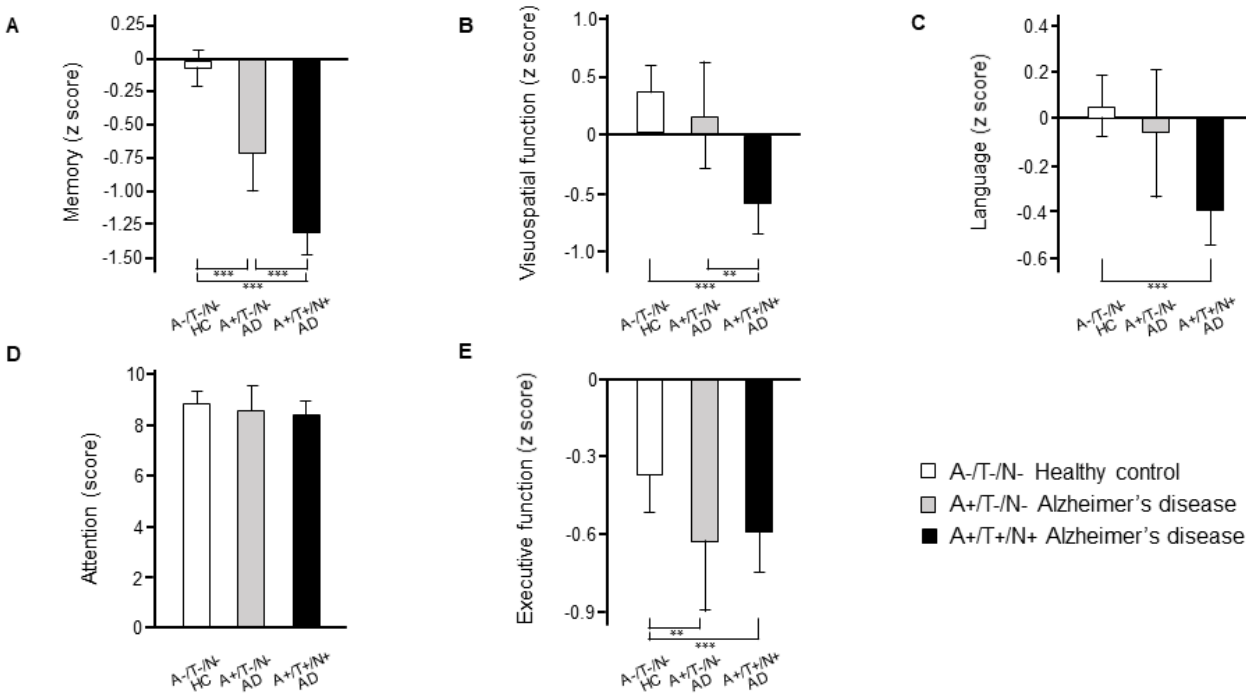

Fig. 10. The different patterns of cognitive domain decline from CSF ATN biomarkers profiles in AD. The neuropsychological assessments of AD patients and healthy controls were compared with precisely classified samples based on CSF ATN biomarkers. The ANCOVA were analyzed with covariates of age, sex, and levels of education. $^{*}P < 0.05$, $^{**}P < 10^{-2}$, $^{***}P < 10^{-3}$. Abbreviations: CSF, cerebrospinal fluid; HC, healthy control; AD, Alzheimer's disease.

Table 8. Comparison of full scale screening battery assessments with different covariates from CSF ATN biomarkers profiles

Cognitive domains	CSF ATN criteria &clinical and pathological diagnosis				
	A-/T-/N- HC[a]	A+/T-/N- AD[b]	A+/T+/N+ AD[c]	*P*-value	Post-hoc
Sex, age, education covariates					
Attention	8.87 ± 0.23	8.64 ± 0.48	8.42 ± 0.26	0.45	n.s
Language	0.05 ± 0.07	-0.07 ± 0.14	-0.40 ± 0.08	1.76E-4	a > c
Visuospatial	0.36 ± 0.11	0.23 ± 0.23	-0.59 ± 0.13	5.25E-7	a, b > c
Memory	-0.07 ± 0.07	-0.62 ± 0.14	-1.38 ± 0.08	3.31E-24	a > b > c
Executive	-0.12 ± 0.09	-0.60 ± 0.18	-0.89 ± 0.10	1.61E-7	a > b, c
CDR, sex, age, education covariates					
Attention	8.34 ± 0.24	8.73 ± 0.45	9.05 ± 0.27	0.21	n.s
Language	-0.11 ± 0.07	-0.04 ± 0.13	-0.20 ± 0.08	0.54	n.s
Visuospatial	-0.05 ± 0.10	0.32 ± 0.18	-0.10 ± 0.11	0.12	n.s
Memory	-0.23 ± 0.07	-0.59 ± 0.13	-1.18 ± 0.08	1.32E-12	a > b > c
Executive	-0.42 ± 0.08	-0.55 ± 0.14	-0.53 ± 0.09	0.64	n.s

Values are shown as mean ± standard error. ANCOVA test with post-hoc bonferroni correction adjusted age, sex and levels of education or adjusted age, sex, levels of education and CDR-SOB as covariates. Abbreviations: CDR-SOB, clinical dementia rating sum of boxes; AD, Alzheimer's disease; HC, healthy control; a, A-/T-/N- HC; b, A+/T-/N- AD; c, A+/T+/N+ AD. n.s; no significant difference.

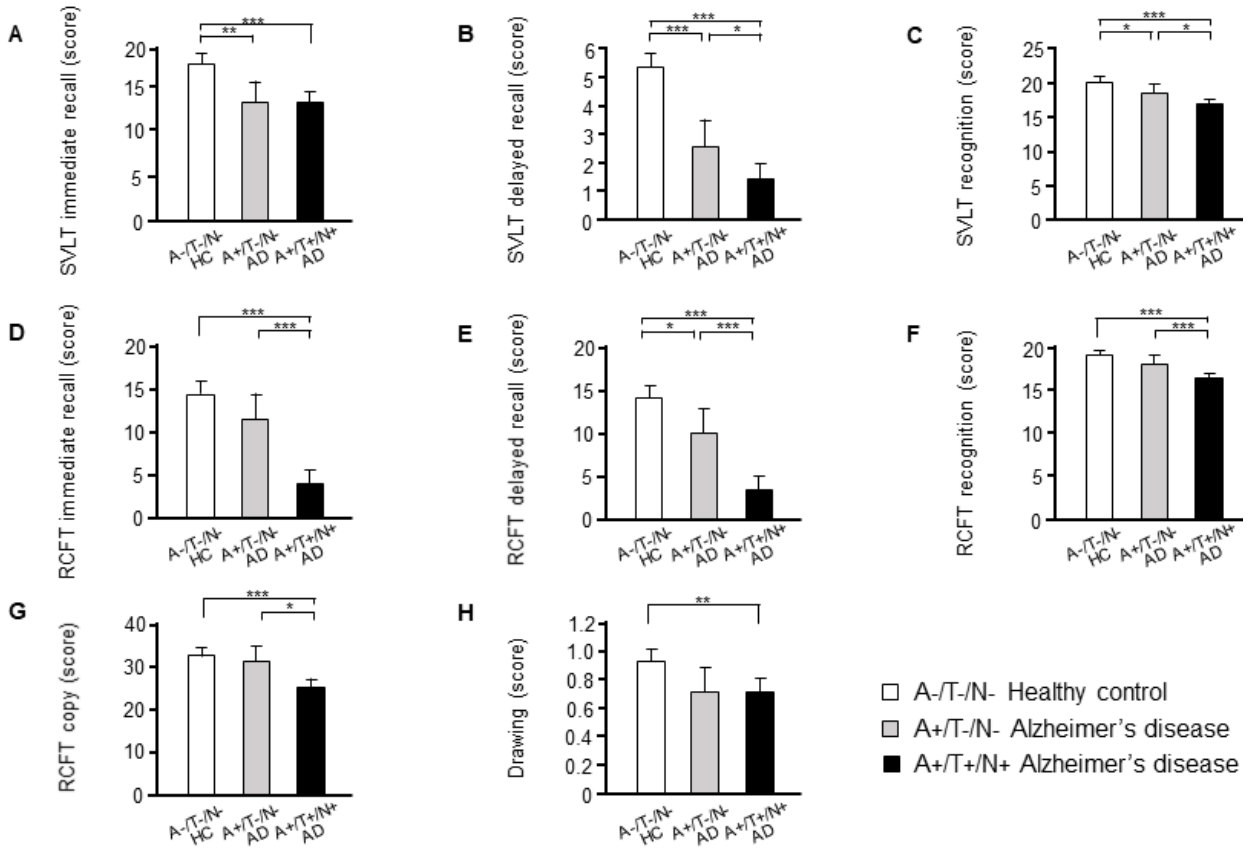

Fig. 11. The different patterns of memory and visuospatial domain decline from CSF ATN biomarkers profiles in AD. The visuospatial memory tests of A+/T+/N+ AD and A+/T-/N- AD were compared with precisely classified samples based on CSF ATN biomarkers. The ANCOVA were analyzed with covariates of age, sex, and levels of education. $^{*}P < 0.05$, $^{**}P < 10^{-2}$, $^{***}P < 10^{-3}$. Abbreviations: CSF, cerebrospinal fluid; HC, healthy control; AD, Alzheimer's disease; SVLT, Seoul verbal learning test-elderly's version; RCFT, Rey complex figure test.

Table 9. Comparison of memory and visuospatial domain assessments from CSF ATN biomarkers profiles in AD

Function	CSF ATN criteria &clinical and pathological diagnosis				
	A-/T-/N- HC[a]	A+/T-/N- AD[b]	A+/T+/N+ AD[c]	*P*-value	Post-hoc
Verbal memory (SVLT)					
Immediate recall	18.45 ± 0.57	14.19 ± 1.15	13.94 ± 0.64	1.00E-6	a > b, c
Delayed recall	5.46 ± 0.22	2.73 ± 0.46	1.37 ± 0.25	2.69E-22	a > b > c
Recognition	20.15 ± 0.30	18.78 ± 0.60	16.74 ± 0.33	5.42E-11	a > b > c
Visuospatial memory (RCFT)					
Immediate recall	14.57 ± 0.68	11.99 ± 1.39	3.71 ± 0.77	3.09E-18	a, b > c
Delayed recall	14.24 ± 0.68	10.72 ± 1.39	2.88 ± 0.78	3.30E-19	a, b > c
Recognition	19.37 ± 0.26	18.95 ± 0.52	16.40 ± 0.29	1.37E-11	a, b > c
Visuospatial function					
RCFT copy	32.88 ± 0.89	31.94 ± 1.80	25.63 ± 1.00	1.00E-6	a, b > c
Drawing	0.96 ± 0.04	0.71 ± 0.09	0.70 ± 0.05	1.57E-4	a > b, c

Values are shown as mean ± standard error. ANCOVA test with post-hoc bonferroni correction adjusted age, sex, and levels of education as covariates. Abbreviations: SVLT, Seoul verbal learning test-elderly's version; RCFT, Rey complex figure test; AD, Alzheimer's disease; HC, healthy control; a, A-/T-/N- HC; b, A+/T-/N- AD; c, A+/T+/N+ AD. n.s; no significant difference.

Ⅲ-8. Correlation between memory domain and CSF ATN biomarkers for AD groups classified based on tau levels

AD groups were classified based on tau levels and their relationship with cognitive assessment values were determined. Among the CSF ATN biomarkers, A β_{42} was positively correlated with cognitive assessment, whereas pTau and tTau were negatively correlated. The visuospatial memory assessments were tested using three sub-tests: immediately recall, delayed recall and recognition tests. The correlation values were 0.269, 0.273, and 0.152 for $A\beta_{42}$, -0.447, -0.477, and -0.267 for pTau, -0.452, -0.436, and -0.321 for tTau, respectively. Similarly, the verbal memory assessments were tested using three sub-tests: immediately recall, delayed recall and recognition tests. The correlation values were 0.115, 0.151, and 0.198 for $A\beta_{42}$, 0.043, -0.320, and -0.218 for pTau, -0.013, -0.345, and -0.238 for tTau, respectively. Immediately recall and delayed recall assessment correlation with tau biomarkers were higher than recognition assessments. Likewise, the partial correlation of immediately recall and delayed recall assessments were higher than recognition assessments (Table 11). Overall, majority of visuospatial memory assessments were more significantly correlated than verbal memory.

A two-step hierarchical multiple linear regression correlations were conducted in order to explore the relationship between visuospatial memory assessments and tau levels. Two different models were used in this analysis. The first one using the demographic : age, sex and level of education as covariates and the second using the cognitive assessment values along with the aforementioned demographics. Among the cognitive tests, only RCFT delayed recall, SVLT delayed recall, and SVLT immediately recall showed statistically significant regression values with the tau levels. Both the delayed recall tests were negatively associated with tau levels, whereas SVLT immediate recall tests were positively associated. Among the various independent variables, the RCFT delayed recall assessment values were much stronger than other tests in the best fit model. The values of RCFT delayed recall predicted pTau and tTau levels in AD (Table 12).

Table 10. Relationship of CSF ATN biomarkers with visuospatial and verbal memory

Memory test	Aß$_{42}$		pTau		tTau	
	r	*P*-value	r	*P*-value	r	*P*-value
Verbal memory (SVLT)						
Immediate recall	0.115	0.327	0.043	0.717	-0.013	0.915
Delayed recall	0.151	0.201	-0.320	0.006	-0.345	0.003
Recognition	0.198	0.091	-0.218	0.062	-0.238	0.041
Visuospatial memory (RCFT)						
Immediate recall	0.269	0.021	-0.447	7.290E-5	-0.452	6.064E-5
Delayed recall	0.273	0.020	-0.477	2.253E-5	-0.436	1.268E-4
Recognition	0.152	0.195	-0.267	0.021	-0.321	0.005

Data are presented as correlation coefficient (rho) and *P*-values were from the pearson's correlation analysis included A+/T-/N- AD, and A+/T+/N+ AD (n=71). Abbreviations: CSF, cerebrospinal fluid; Aβ_{42}, amyloid $\beta_{(1-42)}$; pTau, phosphorylated tau; tTau, total tau; SVLT, Seoul verbal learning test-elderly's version; RCFT, Rey complex figure test; AD, Alzheimer's disease.

Table 11. Partial correlation of CSF ATN biomarkers with visuospatial and verbal memory

Memory test	Aß$_{42}$		pTau		tTau	
	r	*P*-value	r	*P*-value	r	*P*-value
Verbal memory (SVLT)						
Immediate recall	-0.036	0.850	0.053	0.783	0.100	0.599
Delayed recall	0.048	0.800	-0.034	0.858	-0.081	0.671
Recognition	0.084	0.660	-0.436	0.016	-0.307	0.098
Visuospatial memory (RCFT)						
Immediate recall	0.535	0.002	-0.588	6.250E-4	-0.375	0.041
Delayed recall	0.480	0.007	-0.618	2.740E-4	-0.456	0.011
Recognition	0.082	0.666	-0.182	0.336	-0.281	0.133

Data are presented as correlation coefficient (rho) and *P*-values were from the pearson's correlation analysis included A+/T-/N- AD, and A+/T+/N+ AD (n=71), controlling for age, sex, levels of education, and CDR-SOB as covariates. Abbreviations: CSF, cerebrospinal fluid; Aβ_{42}, amyloid $\beta_{(1\text{-}42)}$; pTau, phosphorylated tau; tTau, total tau; SVLT, Seoul verbal learning test-elderly's version; RCFT, Rey complex figure test; AD, Alzheimer's disease; CDR-SOB, clinical dementia rating sum of boxes.

Table 12. Hierarchical multiple regression analysis for tau biomarkers of visuospatial and verbal memory tests

Dependent variable	Independent variables	Multiple model 1		Multiple model 2	
		B	*P*-value	B	*P*-value
pTau	**Constant**	88.786	<0.001	78.436	<0.001
	Demographic data				
	Sex	-3.573	0.248	-3.813	0.146
	Age	-0.481	0.017	-0.363	0.025
	Education	-0.749	0.021	0.209	0.454
	Memory domain				
	RCFT delayed recall			-0.854	<0.001
	SVLT delayed recall			-2.964	<0.001
	SVLT immediate recall			0.761	0.012
	Adj. R^2	0.049		0.412	
	ΔF	3.593		6.456	
	P-value	0.015		0.012	
tTau	**Constant**	113.544	0.007	133.108	0.001
	Demographic data				
	Sex	-4.602	0.553	-3.190	0.635
	Age	-0.190	0.705	0.188	0.645
	Education	-1.620	0.046	0.823	0.249
	Memory domain				
	RCFT delayed recall			-1.499	0.008
	SVLT delayed recall			-6.831	<0.001
	SVLT immediate recall			2.178	0.005
	RCFT recognition			-3.713	0.013
	Adj. R^2	0.007		0.370	
	ΔF	1.374		6.320	
	P-value	0.253		0.013	

Data are presented as predictive of tau biomarkers from visuospatial and verbal memory tests. *P*-values were from the multiple regression analysis included A-/T-/N- HC, A+/T-/N- AD, and A+/T+/N+ AD (n=151), controlling for sex, age, and levels of education as covariates. Abbreviations: pTau, phosphorylated tau; tTau, total tau; SVLT, Seoul verbal learning test-elderly's version; RCFT, Rey complex figure test; HC, healthy control; AD, Alzheimer's disease.

Ⅲ-9. Identification of SNPs associated with CSF tau biomarkers and RCFT delayed recall

Individual GWAS analyses were performed for RCFT delayed recall, pTau and tTau. Linear regression involving 3,126 samples for RCFT delayed recall and 287 samples for pTau and tTau levels adjusting for age, sex, levels of education as covariates (Fig. 12).

A total of 827,784 SNPs were used in the chip. After the initial quality control and preprocessing, 351,221 SNPs were used for further analyses. The SNPs of significance were short listed based on the commonly used suggestive threshold of $1x10^{-3}$ and the significant threshold of $1x10^{-7}$. The most significant SNPs were found in chromosome 19 for the RCFT delayed recall GWAS and pTau GWAS (Fig. 12A-B), whereas for the tTau GWAS the most significant associated SNP was found in chromosome 4 (Fig. 12C).

The gene annotated to the significant SNPs of the RCFT delayed recall GWAS, pTau GWAS and tTau GWAS were APOC1, APOE and GALNT7, respectively. The significant SNPs from the three GWAS results were annotated to their respective genes. A total of 336 genes were obtained from the GWAS results of RCFT delayed recall, 226 genes from the pTau and 492 genes from the tTau ($P < 0.001$), totalling up to 1,054 genes which were used further. Overlapping genes from the three GWAS results were used for further biological validation.

The Venn diagrams illustrates the findings from the GWAS results (Fig. 13). A total of 313 genes were obtained based on significantly SNPs of the RCFT delayed recall GWAS, 154 genes were obtained with the pTau GWAS and 413 genes were obtained with the tTau GWAS. A total of 5 genes were seen to overlap between the RCFT delayed recall and pTau GWAS results, 12 genes were seen to overlap between the RCFT delayed recall and tTau GWAS results and 61 genes were seen to overlap between the pTau and tTau GWAS results, whereas only 6 genes overlapped among all three GWAS results. The results of this analysis supported the idea that these genes could be associated with AD.

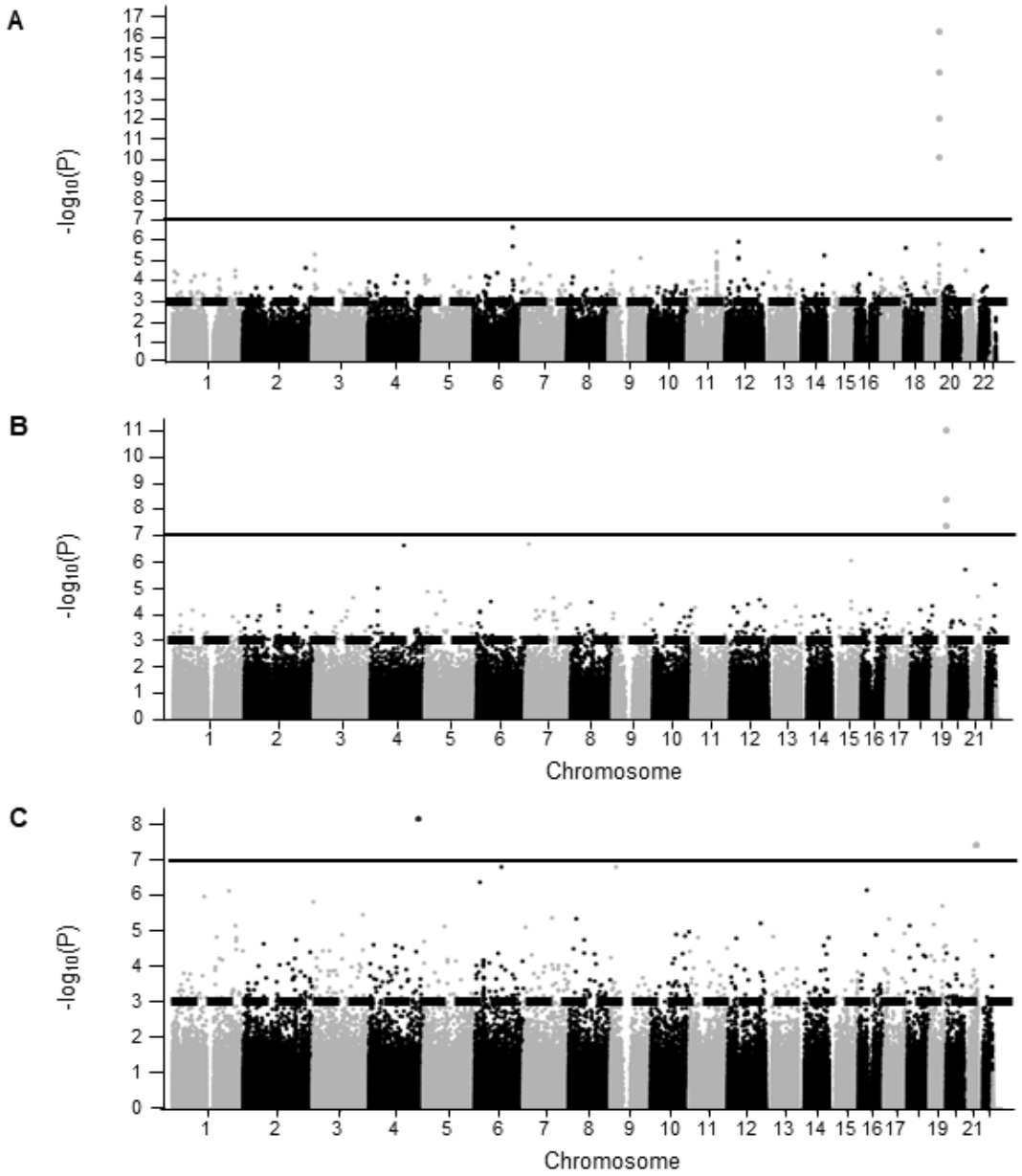

Fig. 12. The Manhattan plot for biomarker levels in CSF tau and visuospatial memory. The manhattan plot of tau and visuospatial memory test for genome wide *P*-values against base pair positions of 351,221 SNPs with biomarker levels after adjusting for age, sex, and levels of education. Panel A were for RCFT delayed recall tests, panel B were for pTau and panel C were for tTau. The threshold for genome-wide significance ($P < 10^{-7}$) is indicated by the full line, and genome-wide suggestive level ($P < 10^{-3}$) is indicated by the dotted line. Abbreviation: SNPs, single nucleotide polymorphisms.

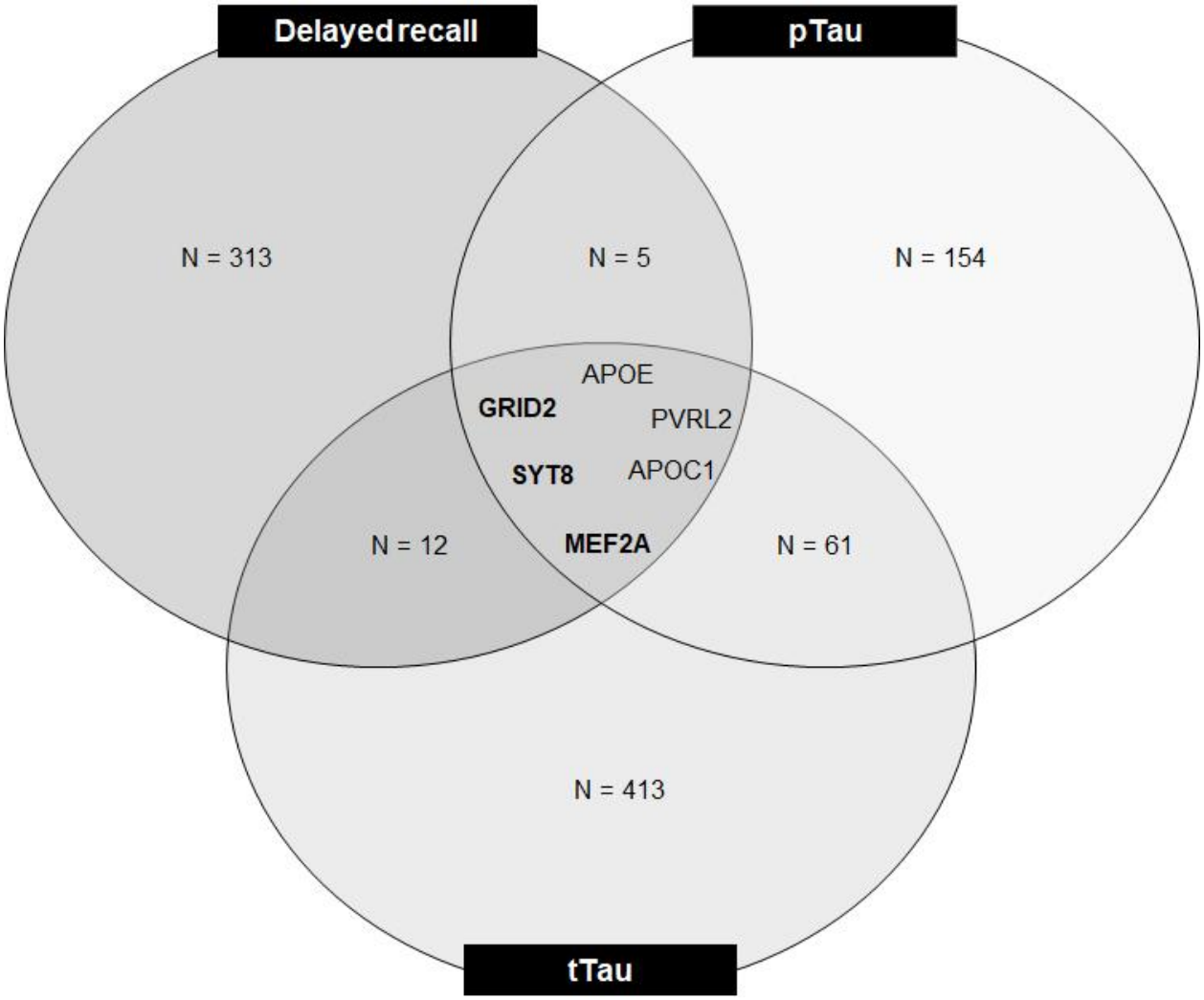

Fig. 13. Overview of similar genetic association with biomarker levels identified in CSF tau and visuospatial memory test levels. The venn diagram representing the number of similar genetic association in the CSF pTau, tTau and visuospatial memory test levels. Most of the genetic association were observed in the pTau and tTau, with only modest overlap with the three kinds of biomarkers.

Ⅳ. DISCUSSION

The biological definition of AD cascade can be classified by CSF ATN biomarkers (Jack *et al*., 2018). However, methodological variability and inconsistency of biomarkers cut-off values have an adverse effect on AD diagnosis (Pan *et al*., 2015). According to previous studies, they represents the comparison of different platforms or assays verification in Caucasian AD patients. Most studies of this analysis between two platforms showed that tau/Aβ_{42} ratios have been shown to predict AD (Fagan *et al*., 2011). However, the ratio value of pTau/Aβ_{42} was the highest AUC value in early stages of AD, whereas the reported of tTau/Aβ_{42} was similar AUC value with pTau/Aβ_{42} in AD dementia groups (Table 3). Such markers can be useful in the diagnosis of early AD (Table 4). One reason of this difference would be from the different classification quality of samples. The samples in other study were clinically diagnosed, whereas the samples in this study were diagnosed with clinical information and amyloid PET imaging (Table 1). Hence, the early stages of AD in this study could be well separated from HC, whereas the samples in other study had a possibility that normal group would include the asymptomatic AD, resulting in the inaccurate pathological classification.

According to the biological definition of AD cascade, ATN classification scheme presented cognitive decline along with increased biomarkers levels (Jack *et al*., 2018). However, some of the AD patients were not following the biological definition of tau levels (Fig. 6). The criteria of tau levels were separated from AD without asymptomatic AD group, which is called A+/T-/N- AD, and A+/T+/N+ AD. Comparison of two tau criteria groups showed significant decrease in subcortical volume, cortical thickness, and cognitive assessments. The hippocampus, and amygdala showed significant decrease in subcortical volume (Fig. 8 and Table 6). Several cortical thickness were significantly decrease in cortical thickness, but entorhinal cortex, superior frontal, and precuneus were apparently decrease both left and right (Fig. 9 and Table 7).

ATN biomarker scheme should be interpreted and applied together with brain atrophy information (Ekman *et al*., 2018). The CSF ATN biomarkers levels seem to be associated and affected with gradual loss of the cognitive function (Erik *et al*., 2010). Cognitive function showed significant decrease in visuospatial memory domain (Fig. 10). One reason of this difference would be from the neurodegenerative process, the progression of cognitive decline might be affected by ATN biomarkers change in AD cascade. Hence, changes in visuospatial delayed recall levels can be predicted in individuals CSF tau levels in AD cascade (Table 12).

APOE is well-known highest genetic risk factor for AD, but the role of APOE is not fully elucidated (Yu *et al*., 2014). Based on previous studies, the prevalence of APOE ε4 allele has been suggested to be increased among AD (Zhong *et al*., 2009). However, some APOE ε4 carrier subjects remain unaffected. Reason for this difference could probably be due to the complexity of genetic effects. Some genes might play a protective role whereas few others might have deleterious effects. APOE has been shown to have strong influence on tau levels and cognitive assessments related to visuospatial memory. Hence, genetic association was performed to check the role of APOE on visuospatial delayed recall and tau levels in our dataset. Similar genetic association were observed in these groups (Fig. 12). Except for those with chromosome 19, utilizing *in vivo* tau knock down for biological validation model will be critical of behavioral tests (Fig. 13).

Limitations to this study include lack of low levels of tau AD patients. however, These strategic approach of ATN profiles show promising studies for increased accuracy in predictive and the diagnostic on pathological stages of AD.

Ⅴ. 초 록

뇌척수액 ATN 바이오마커 기반 알츠하이머병 환자 분류체계 세분화 및 특성 연구

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알츠하이머병은 뇌의 신경세포에 비가역적인 손상을 야기하여 해마의 위축과 인지기능의 손상으로 특징지어 지는데, 이러한 현상은 알츠하이머병 환자의 임상결과를 통해 지속적으로 보고가 되고 있다. 기 보고된 연구 결과에 따르면, 알츠하이머병은 크게 인지장애 정도와 아밀로이드 이미지 판독 결과를 이용하여 건강한 대조군, 무증상 알츠하이머병, 전조증상 알츠하이머병, 알츠하이머성 치매 총 4개의 그룹으로 분류할 수 있는데, 최근에는 이 그룹간의 차이를 좀 더 세밀하게 분석하기 위해서 MRI 뇌영상, 신경심리검사, 뇌척수액 분석 등 다양한 데이터를 사용하여 비교하고 있다.

알츠하이머병은 비가역적 손상을 야기하기 때문에 기 확립되어 있는 생물표지자를 이용하여, 초기 단계의 알츠하이머병을 대상으로 한 질병의 진단 및 치료가 매우 중요하다. 질병의 특성상 알츠하이머병의 특이적인 생물표지자는 뇌척수액에서 측정이 가능하며, 초기단계에서 아밀로이드 베타 42 단백질(A), 인산화된 타우 단백질(T) 그리고 전체 타우 단백질(N)형태로 발견되고 있다. 최근 미국 노화학회에서는 이러한 뇌척수액에서 발견되는 3가지 단백질의 특징을 ATN이라는 이름을 명명하여 사용하고 있다.

본 연구는 3개의 뇌척수액 생물표지자를 정확히 측정하는 플랫폼과 ATN 생물표지자와 다양한 데이터들 간의 관계를 조사하기 위해 실시하였으며, 211명의 피험자로부터 xMAP Luminex 기술을 이용한 INNOBIA 플랫폼과 ELISA 기술을 이용한 INNOTEST

두 가지의 플랫폼을 사용하였다. 더불어, 측정된 뇌척수액의 ATN 생물표지자의 발현을 피험자의 진단정보, MRI 뇌영상, 신경심리검사 등의 다양한 데이터를 이용하여 두 가지 플랫폼의 뇌척수액 측정결과를 평가해 보고자 했다.

앞서 언급한 두 가지의 플랫폼의 진단의 정확도를 비교를 통해 INNOBIA의 인산화된 타우 단백질을 이용한 진단의 분류 정확도가 INNOTEST 보다 높다는 것을 발견하였지만, 아밀로이드 베타 42 단백질, 전체 타우 단백질을 이용한 진단의 정확도는 큰 차이가 없다는 것 또한 발견했다. 추가적으로 ATN 생물표지자를 단일마커로 사용하기보단 비율을 이용한 인산화된 타우 단백질 / 아밀로이드 베타 42 단백질의 INNOBIA 결과가 0.98로 0.93인 INNOTEST 결과보다 높고 진단의 정확도가 높아지는 것이 확인되었다. 특히, 이러한 분석을 통해 Luminex 플랫폼을 이용하여 정상 그룹을 기준으로 하면, 초기 알츠하이머병을 분류해 내는데 유용하다는 것을 비교 분석하여 확인할 수 있었다.

다음으로 측정한 뇌척수액 ATN 생물표지자 결과, 뇌영상, 신경심리검사 사이의 관계를 분석했다. ATN 생물표지자를 기준으로 하는 분류는 측정된 농도를 이용했으며, 전반적인 A-/T-/N- 피험자는 건강한 대조군이며, 반면에 A+/T-/N- 와 A+/T+/N+ 피험자는 알츠하이머병으로 분류하였다. 이러한 분류체계를 통해 알츠하이머병 환자임에도 불구하고 T와 N이 낮은 피험자들이 일부 있다는 흥미로운 점 또한 확인하였다. 이 집단은 기존 가설과는 달리 피질 하부의 부피와 내후각 뇌피질, 쐐기앞소엽, 상측전두엽 등 겉피질의 위축이 있으며, 또한 신경심리 검사 중 RCFT 테스트를 이용한 시공간 기억 점수가 감소하는 것을 확인하였다.

결과적으로, 본 연구 결과는 뇌척수액 생물표지자의 측정에 사용되는 두 개의 플랫폼의 비교 및 분석 결과 INNOBIA를 이용해 알츠하이머병을 초기에 분류해 내는 것이 INNOTEST보다 더 높은 정확도와 질병의 진단에 유용하다는 것을 제시하였으며, 높은 타우 단백질 농도로 분류된 알츠하이머병 환자에서 시공간 기억 손실이 발생하는 것을 보고하였다. 추가적으로, 뇌척수액을 이용한 알츠하이머병 조기 진단의 가능성과 신경심리검사를 이용한 타우 단백질 수준을 예측하는 가능성 또한 제시하였다. 이러한 연구결과를 토대로 비가역적인 알츠하이머병 초기 환자군을 조기에 선별하고, 저비용, 비침습적인 신경심리검사를 토대로 타우단백질의 발현을 예측하여 알츠하이머병의 진행을 관리 한다면, 최근 급속도로 증가하는 치매 유병률 억제에 기여 할 수 있을 것이라 생각된다.

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