

# Combined assessment of tau and neuronal thread protein in Alzheimer's disease CSF

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**Article abstract**—*Objective:* Comparative study of CSF levels of tau and AD7C-neuronal thread protein (NTP) in patients with AD and control subjects. *Background:* AD is characterized by neurofibrillary tangles composed of the abnormally hyperphosphorylated microtubule-associated protein tau. AD7C-NTP is a proposed AD marker expressed at early stages of neurofibrillary degeneration. *Methods:* Enzyme-linked immunosorbent assays specific for tau and AD7C-NTP. CSF samples were obtained from 35 demented patients (25 with antemortem clinical diagnosis of probable AD, 5 with neuropathologic diagnosis of definite AD, 5 with Lewy body pathology), 29 nondemented patients with PD, and 16 elderly healthy control subjects. Receiver operating characteristics (ROC) and multivariate discriminant analysis for AD versus controls. Correlational analysis of CSF tau and AD7C-NTP and of each marker with Mini-Mental State Examination (MMSE) scores was performed. *Results:* Levels of both tau and AD7C-NTP were significantly elevated in the AD patients compared with control subjects. ROC analysis showed that CSF tau distinguished between patients with AD and nondemented control subjects with 63% sensitivity and 89% specificity, AD7C-NTP with 70% sensitivity and 87% specificity. Combined evaluation of both markers with discriminant analysis raised the specificity to 93% at a 63% sensitivity level. Both markers positively correlated with each other within the AD group, but not among control subjects. CSF levels of AD7C-NTP, but not of tau, showed a small but significant inverse correlation ( $r = -0.43$ ) with MMSE scores of AD patients. *Conclusions:* CSF levels of tau and AD7C-NTP may be useful biomarkers for AD. **Key words:** AD CSF—Tau—AD7C-NTP.

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AD brain is characterized by an excessive burden of senile plaques and neurofibrillary tangles.<sup>1</sup> Another leading cause of dementia in the elderly is characterized by Lewy bodies (LB), which can occur as the primary lesions (pure LB dementia), as well as in conjunction with AD pathology.<sup>2,3</sup> LBs are commonly found in PD regardless of mental state.<sup>3</sup> These neuropathologic hallmarks of dementia are well described, but, to date, no biologic marker has been established for the antemortem diagnosis of AD.<sup>4</sup> Moreover, there is a need for marker(s) that reflect the stage of the disease and provide prognostic information.

Neurofibrillary degeneration is characteristic of AD.<sup>5</sup> The main proteinaceous component of neurofibrillary tangles is the microtubule-associated protein tau. In healthy neurons, tau stabilizes axonal microtubules. When abnormally hyperphosphorylated, tau dissociates from microtubules and self-assembles

into paired helical filaments, eventually leading to the formation of a neurofibrillary tangle. Tau is believed to be liberated into the CSF from neurons in the process of neurofibrillary degeneration. The development of sensitive ELISAs has allowed the detection of elevated levels of tau in the CSF of patients with AD.<sup>6,7</sup> Increasing levels of CSF tau with progressive cognitive impairment have been reported in some studies without end-stage AD patients,<sup>8,9</sup> but other studies have not confirmed a correlation between CSF tau and cognition.<sup>7,10-13</sup> CSF tau levels increased after several months follow-up in some, but not all, AD patients with serial CSF samples.<sup>9,11,13</sup> CSF levels of tau are also elevated in some neurologic conditions other than AD.<sup>6,7,10,12-14</sup>

AD7C-neuronal thread protein (NTP) is a novel protein that may be involved in neuritic sprouting.<sup>15</sup> AD7C-NTP immunoreactivity has been found to be

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specifically expressed in brains of AD patients, and it was also found at elevated levels in CSF.<sup>15</sup> Interestingly, AD7C-NTP expression was found to be very high in neurons at early stages of neurofibrillary degeneration and in dystrophic neurites.<sup>16</sup> CSF levels of AD7C-NTP have been observed to correlate with cognitive impairment in one study.<sup>15</sup>

In the current study, we measured lumbar CSF levels of tau and AD7C-NTP with specific ELISAs in patients with AD, elderly healthy control subjects, and patients with PD. Both markers were found to be significantly elevated in patients with AD compared with control subjects, and they were moderately correlated with each other. CSF levels of AD7C-NTP, but not tau, correlated with cognitive impairment.

**Methods.** *Study participants.* A total of 15 women and 20 men with antemortem clinical diagnosis of probable AD were included in the study. All were part of a longitudinal study of AD conducted at the Stanford/Veterans Affairs NIMH Clinical Research Center for the Study of Senile Dementia. Criteria for entry into the Center were a clinical diagnosis of probable AD by NINCDS-ADRDA criteria,<sup>17</sup> and an absence of active major medical problems. To determine the diagnosis of probable AD, all patients had a complete medical, psychiatric, neurologic, neuroimaging, and neuropsychological assessment. Based on these evaluations a consensus diagnosis was reached by an interdisciplinary team including one to three physicians. The degree of cognitive impairment was assessed using the Mini-Mental State Examination (MMSE).<sup>18</sup> The mean level of MMSE scores was  $16.1 \pm 6.9$ ; median 18, range 1 to 27. Six patients with a MMSE score  $<10$  were classified as severely demented, 14 patients with a MMSE score from 10 to 20 as moderately demented, and 10 patients with a score  $>20$  as mildly demented. MMSE scores were not available at the time of CSF collection for 5 patients.

Complete gross and microscopic neuropathologic evaluations were performed on 10 of the patients. In 5 patients, a diagnosis of definite AD was made using criteria similar to those of Khachaturian.<sup>19</sup> Three patients had neuropathologic changes sufficient for a diagnosis of AD as well as additional pathology: 2 had AD/LB (dementia but no clinical parkinsonism antemortem; LBs and AD changes at autopsy) and 1 had AD/PD (dementia and clinical signs of parkinsonism antemortem; AD changes and LBs at autopsy). Two patients with insufficient AD changes at autopsy to meet diagnostic criteria were classified as pure LB dementia (dementia antemortem and LBs at autopsy). Criteria for neuropathologic diagnosis of patients with LBs are described by Forno.<sup>3</sup>

Although our CSF sample included a number of individuals with Lewy body pathology, the proportion of patients with LB pathology in our Center autopsy cohort of 61 patients is similar to that reported by others (23%).<sup>2</sup> In the subpopulation of 11 patients for whom both CSF samples and neuropathologic examinations were available, the proportion of cases with LB pathology was greater than in the entire cohort of 61 patients. This difference is most likely attributable to chance.

As a non-AD neurologic control group, PD CSF was obtained from 29 volunteers who were patients at The

Parkinson's Institute. CAPIT criteria were used for diagnosis.<sup>20</sup> Sixteen healthy, nondemented older adults served as control subjects (8 men and 8 women). Most of the control subjects were recruited from a pool of healthy community volunteers enlisting for studies involving neuroimaging and biochemistry. One control was an AD caregiver; 2 were age-matched volunteers at The Parkinson's Institute with no symptoms or signs of neurologic disease. Healthy control subjects were screened for dementia using the MMSE. At the time of lumbar puncture, none of the control subjects had MMSE scores of less than 24.

*CSF collection.* This project had Institutional Review Board approval. After obtaining informed consent and within 1 week of the lumbar puncture, each subject underwent a medical evaluation including the following assessments: medical history, review of systems, physical examination, electrocardiogram, and laboratory tests (CBC, sedimentation rate, SMA-18, and urinalysis). The night before the lumbar puncture, subjects fasted and remained at bedrest on an inpatient research unit. Lumbar punctures were performed at 7 A.M., and 25 mL of CSF was withdrawn. A 500  $\mu$ L aliquot was obtained from a sample containing the 4th through the 10th mL of spinal fluid collected from each patient and was used for these studies. The appropriate assays were performed consistently on the same aliquots to guard against gradient artifacts. Cell counts, glucose, total protein, pH, and syphilis serology were assessed to rule out routine abnormalities. CSF samples were placed on ice at the time of lumbar puncture and then transported immediately to a  $-80$  °C freezer where they were stored until ready for ELISA.

*Determination of CSF levels of tau, AD7C-NTP, and amyloid  $\beta$ -protein.* CSF tau levels were determined using a sandwich ELISA based on the original method of Vandermeeren et al.<sup>6</sup> (INNOTEST hTAU; Innogenetics, Zwijndrecht, Belgium). Samples were diluted 1:3 in sample buffer, or when  $<300$  pg/mL assayed undiluted. Tau concentrations were extrapolated from OD (450 nm) readings by sigmoidal curve fit (Microplate Manager; Bio-Rad, Hercules, CA) of recombinant human tau standards. Detection limit was typically 0.1 ng/mL. AD7C-NTP in CSF was measured with the sandwich ELISA described by Ghanbari and Ghanbari<sup>15,21</sup> (detection limit: 0.15 ng/mL). The 42 amino-acid form of amyloid  $\beta$ -protein was measured by sandwich ELISA<sup>22</sup> at Athena Diagnostics (Worcester, MA). Researchers performing assays were blinded to sample diagnosis.

*Statistical analysis.* Patient and control groups were compared in age using the Student's *t*-test and for gender distribution using the  $\chi^2$ -test. Group data are presented as means  $\pm$  SD. Nonparametric Kruskal-Wallis analysis of variance (ANOVA) was used to assess overall differences among groups. A significant overall effect was followed up by single pairwise comparisons between groups for the two markers using the nonparametric Mann-Whitney *U* test. For between-groups comparisons, the level of significance was set at  $p < 0.05/3$ , using the Bonferroni correction. Descriptive statistics and significance tests for group differences were computed with StatView v4.0 (Abacus Concepts, Berkeley, CA).

Diagnostic accuracy was assessed with receiver operating characteristics (ROC) curve analyses. Areas under the ROC curves and standard error were calculated and com-

pared according to the algorithms of Hanley and McNeil.<sup>23</sup> This approach takes into account correlations between two different variables measured on the same sample and thereby reduces the standard error of between area differences, resulting in an increased power of the test. The ROC analyses were performed to maximize the sum of sensitivity and specificity and were programmed with Visual Basic and Excel Software (Microsoft, Redmond, WA). Subsequently, both markers were introduced into a stepwise multivariate discriminant analysis,<sup>24</sup> using SAS software (SAS Institute, Cary, NC). Correlations between both markers and between both markers and MMSE and age were tested by the Spearman rank correlation.

**Results.** AD patients and healthy control subjects were matched for age (68.7 years  $\pm$  7.7 years and 66.8 years  $\pm$  7.5 years, respectively;  $p > 0.4$ ) and gender distribution (male to female = 1:1). Patients with PD were younger (61.5 years  $\pm$  8.3 years;  $p < 0.04$ ) and had a significantly lower proportion of female subjects (female to male = 6:23;  $p < 0.04$ ). Kruskal-Wallis ANOVA showed significant differences in tau and AD7C-NTP levels among the three groups ( $p < 0.001$ ). CSF levels of tau were elevated on average 2.5-fold in the AD cohort compared with control subjects (figure 1A). The mean tau level in 30 patients with AD was 0.84 ng/mL  $\pm$  0.56 ng/mL (25 with clinical diagnoses of AD and 5 with definite AD; the 5 patients with LB pathology were excluded for this calculation), which was significantly higher compared with the CSF tau in 16 healthy control subjects (0.34 ng/mL  $\pm$  0.23 ng/mL;  $p < 0.002$ ). The 5 patients with postmortem verified definite AD, with the exception of 1 severely demented patient (see below), exhibited very high tau levels ( $>1.4$  ng/mL). The non-AD neurologic control group of 29 patients with PD had a mean CSF tau level of 0.32 ng/mL  $\pm$  0.12 ng/mL. This was significantly lower than in the AD group ( $p < 0.001$ ) but not significantly different from healthy control subjects. The heterogeneous group of demented patients with LB pathology had a median CSF tau of 0.52 ng/mL.

CSF levels of AD7C-NTP were elevated on average two-fold in the AD cohort compared with control subjects (figure 1B). The mean AD7C-NTP level in 30 patients with AD was 3.09 ng/mL  $\pm$  1.49 ng/mL. All patients with definite AD had AD7C-NTP levels  $>2.1$  ng/mL. The mean AD7C-NTP level for the 16 healthy control subjects was 2.09 ng/mL  $\pm$  1.84 ng/mL. The median CSF AD7C-NTP level in the 5 demented patients with LB pathology was 2.25 ng/mL. In the 29 patients with PD, a mean CSF AD7C-NTP value of 1.56 ng/mL  $\pm$  0.99 ng/mL was detected. The difference in AD7C-NTP levels between the AD cohort and the control subjects was statistically significant (AD versus healthy control subjects:  $p = 0.0061$ ; versus PD:  $p < 0.0001$ ), whereas the PD and healthy control cohorts did not differ significantly.

One individual with normal cognition (MMSE score of 30 at age 69 after a 6-year interval from the original testing) had AD7C-NTP levels between 8 ng/mL to 9 ng/mL in two independent duplicate measurements of the same CSF sample. This aberrant value was greater than 3 SDs above the mean for the entire control group and 8 SDs greater than the mean AD7C level for the remaining 15 control subjects. This extreme value for a control was 10 SDs above the mean for CSF AD7C-NTP in control subjects reported by de la Monte et al.<sup>15</sup> Further, the CSF tau level

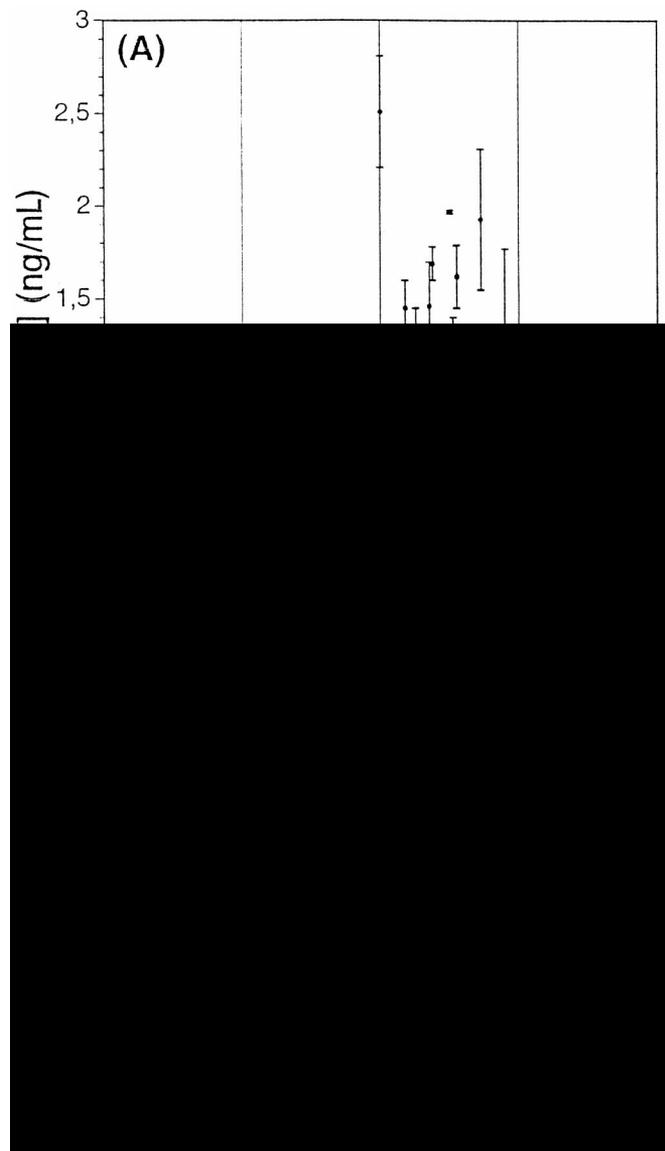


Figure 1. CSF tau (A) and AD7C-NTP (B) levels in healthy control subjects (HC) and patients with Lewy body (LB) dementia, AD, and PD. Note the two patients with AD/LB at the boundary between the LB and AD cohorts and the AD/PD patient at the boundary between the AD and PD cohorts. Each symbol represents the mean of triplicate (A) or duplicate (B) ELISA determinations; error bars in (A) denote SD.

for this individual was within the control range (0.38 ng/mL). Another individual in our control cohort displayed CSF values of AD7C-NTP (3.16 ng/mL) and tau (0.69 ng/mL) similar to those of patients with AD, as well as reduction of the 42 amino acid-long form of amyloid  $\beta$ -protein in CSF (0.587 ng/mL), all suggestive of preclinical AD. Unfortunately we were unable to contact the subject with elevated tau and AD7C-NTP for follow-up.

ROC curve analysis and stepwise multivariate discriminant analysis were used to explore the potential of tau protein and AD7C-NTP to discriminate patients with AD from healthy control subjects and patients with PD. The

**Table** Statistical assessment of AD diagnostic accuracy for CSF tau and AD7C-NTP

Marker	Technique	AD vs PD‡	AD vs HC‡	AD vs CON‡
AD7C-NTP	ROC*			
	AUC (SE)	0.805 (0.057)	0.748 (0.072)	0.784 (0.056)
	Cutoff	2.48–2.51 ng/mL	2.07–2.11 ng/mL	2.48–2.51 ng/mL
	Sensitivity	70	77	70
Tau	ROC*			
	AUC (SE)	0.771 (0.062)	0.782 (0.067)	0.775 (0.058)
	Cutoff	0.53–0.55 ng/mL	0.45–0.55 ng/mL	0.53–0.55 ng/mL
	Sensitivity (%)	63	63	63
Tau, AD7C-NTP	DISCRIM†			
	Sensitivity (%)	77	60	63
	Specificity	93	81	93

\* Receiver operating characteristics analysis: area under the curve (AUC) and standard error (SE), cutoff given for sum of sensitivity and specificity maximized.

† Multivariate stepwise discriminant analysis: a priori probability of group membership assumed equal; sensitivity and specificity estimated from all cases in the equation.

‡ Patients with AD (n = 30), patients with PD (n = 29), HC (healthy control subjects) (n = 16), CON (healthy control subjects and patients with PD combined) (n = 45).

table summarizes the results from these analyses. Areas under ROC curves (figure 2) ranged between 0.77 to 0.79 for tau and 0.75 to 0.80 for AD7C-NTP. There were no differences detectable between areas under tau and AD7C-NTP ROC curves for comparisons of AD patients with healthy control subjects, with PD subjects, or with the pooled non-AD data ( $|z| = 0.04$  to  $0.4$ ,  $p = 0.97$  to  $0.7$ ). This indicates that tau and AD7C-NTP did not differ in their accuracy of classification. Using ROC analysis under the constraint to maximize the sum of sensitivity and specificity, a cutoff value of  $>0.53$  ng/mL for tau discriminated AD patients with 63% sensitivity from healthy control subjects and PD patients at a specificity level ranging between 75% and 96% (see table). AD7C-NTP values identified patients with AD with a sensitivity of 70% to 77% and a specificity between 80% to 90% at a cutoff value of  $>2.48$  ng/mL (see table). These values were close to recently reported cutoff points.<sup>12,15</sup> Linear discriminant analysis between patients with AD and healthy control subjects using both tau and AD7C-NTP values showed a significant multivariate difference between the groups (Wilk's lambda = 0.77,  $F(2,43) = 6.3$ ,  $p < 0.004$ ). Classification of AD patients versus healthy control subjects with discriminant analysis resulted in a sensitivity of 60% and a specificity of 81% (see table). Discriminant analysis also classified patients with AD versus patients with PD with a sensitivity of 77% and a 93% specificity. For patients with AD versus all non-AD subjects, discriminant analysis yielded a sensitivity of 63%, a specificity of 93%, and an overall diagnostic accuracy (sum of true-positives plus true-negatives divided by total number of subjects) of 81%.

For the 30 patients with AD, 16 healthy control subjects, and 29 PD patients, there was a significant correlation between tau and AD7C-NTP levels (figure 3;  $r = 0.376$ ,  $z = +3.238$ ;  $p = 0.0012$ ). Importantly, there was a signi-

ficant correlation between the two markers among the 30 patients with AD ( $r = 0.362$ ,  $z = +1.949$ ,  $p < 0.05$ ). In contrast, no significant correlation was observed for either the control cohort or the PD patient when these subsamples were considered alone ( $r = 0.028$  and  $0.141$ , respectively).

In 27 patients with AD with MMSE assessments near the time of lumbar puncture, no significant correlation with severity of dementia was found for tau (figure 4A;  $z = +0.292$ ;  $p = 0.7704$ ). This was partly owing to very low ( $<0.4$  ng/mL) CSF tau levels in three severely demented patients with AD (MMSE  $<5$ ). In contrast, there was an inverse correlation of CSF AD7C-NTP with MMSE scores (figure 4B;  $r = -0.43$ ,  $z = -2.159$ ;  $p = 0.0309$ ). Neither CSF tau nor AD7C-NTP correlated with gender. Likewise, no correlation with the age at lumbar puncture was found in the patients with AD and control subjects. A direct comparison of CSF tau and AD7C-NTP levels in 4 patients with AD who had two lumbar punctures approximately 2 to 3.5 years apart did not show significant changes in either marker.

We were able to contact 10 of 16 control subjects for cognitive retesting. The interval between the original cognitive testing and follow-up testing ranged from 6 to 13 years. On 8 of the 10 healthy control subjects who were recontacted we obtained a follow-up MMSE score. Of these 8, 1 individual had declined into the mild dementia range (MMSE = 21), 1 had declined but was still in the nondemented range (MMSE = 25), 4 had shown no change in MMSE, and 2 showed an increase in MMSE score. The control individual with an aberrant AD7C-NTP value had an MMSE score of 30 at age 69 after a 6-year interval from the original testing, indicating normal cognition. Two of the 10 healthy control subjects who we recontacted could not come to clinic for MMSE testing. These two subjects

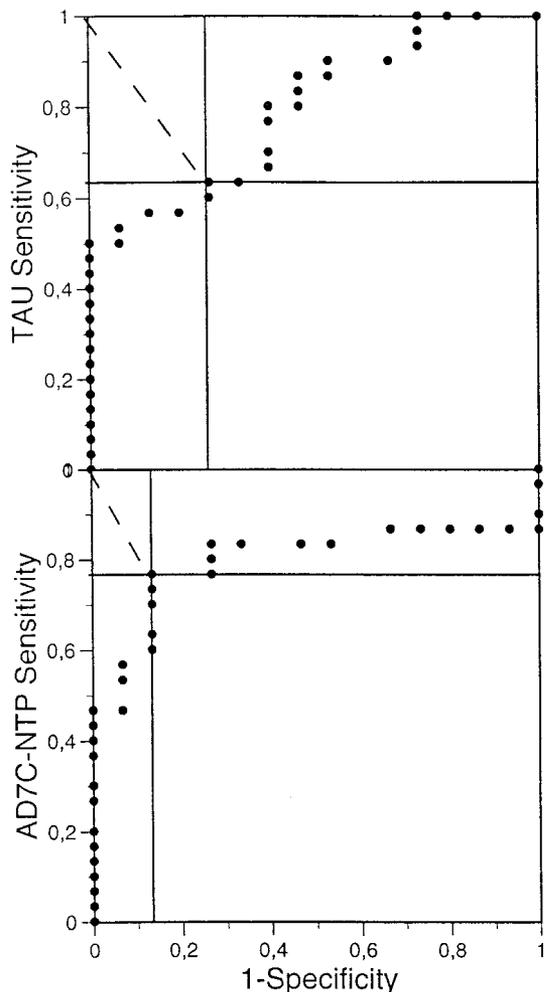


Figure 2. Receiver operating characteristics (ROC) curve depicts sensitivity and 1.0 minus specificity levels for discrimination between patients with AD and healthy control subjects for decreasing values of tau (A) and AD7C-NTP (B). At the chosen cutoff the distance  $d$  (dotted line) is minimized.

were given the Telephone Interview for Cognitive Status,<sup>25</sup> and both scored in the nondemented range. Spearman rank correlation coefficients were calculated for AD7C-NTP levels versus change in MMSE score ( $r = -0.27$ ;  $n = 8$ ) and for tau level versus change in MMSE score ( $r = 0.13$ ;  $n = 8$ ). Neither of these correlations reached statistical significance.

**Discussion.** Combined assessment of lumbar spinal CSF levels of tau and AD7C-NTP demonstrated that both markers were significantly (approximately twofold) and specifically elevated in patients with AD compared with healthy and PD control subjects, despite previously reported overlap between these groups.<sup>6,15</sup> Specificities for both markers assessed separately were between 80 and 90%, as determined by ROC analysis. Combined evaluation of both markers by discriminant analysis resulted in a slight im-

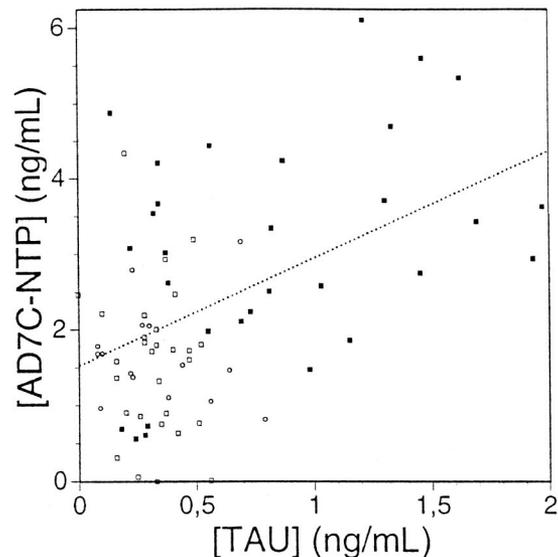


Figure 3. CSF tau versus AD7C-NTP plot. Open circles = healthy control subjects; closed squares = AD patients; open squares = PD patients. Correlational analysis was performed with data points from all individuals of the three cohorts.

provement in specificity between AD patients and non-AD subjects to 93% with a sensitivity of 63%.

One healthy control subject had an aberrantly high AD7C-NTP level. As additional data on AD markers in elderly control subjects are accumulated, it will be important to determine the frequency of aberrant CSF levels. Further, as 25 of our demented patients carry diagnoses of probable AD, estimation of true mean values for tau and AD7C-NTP in this sample must await neuropathologic confirmation. Importantly, ROC analysis did not show statistically significant differences between tau and AD7C-NTP in correctly predicting group membership. CSF AD7C-NTP may be as useful a CSF marker as the widely studied tau protein in the discrimination of patients with AD from non-AD control subjects if results of additional independent studies verify the findings of the current study.

The relationship between AD and neurodegenerative disorders with LBs remains controversial.<sup>2,3,26</sup> In this study, LBs were found in five demented patients who formed a heterogeneous group in terms of concurrent AD pathology (see Study Participants). Therefore, we presented data on the patients with "pure" LB dementia separately from patients who had widespread AD pathologic changes (see figure 1). It is noteworthy that elevated CSF tau levels have been reported for LB dementia cases,<sup>12,14</sup> whereas six patients with LB disorder in another study exhibited  $<2.5$  ng/mL AD7C-NTP in CSF.<sup>26</sup> Our data showed that one patient with autopsy-confirmed LB dementia had moderately elevated tau levels, and one patient had moderately elevated AD7C-NTP. Additional larger clinical studies will be required be-

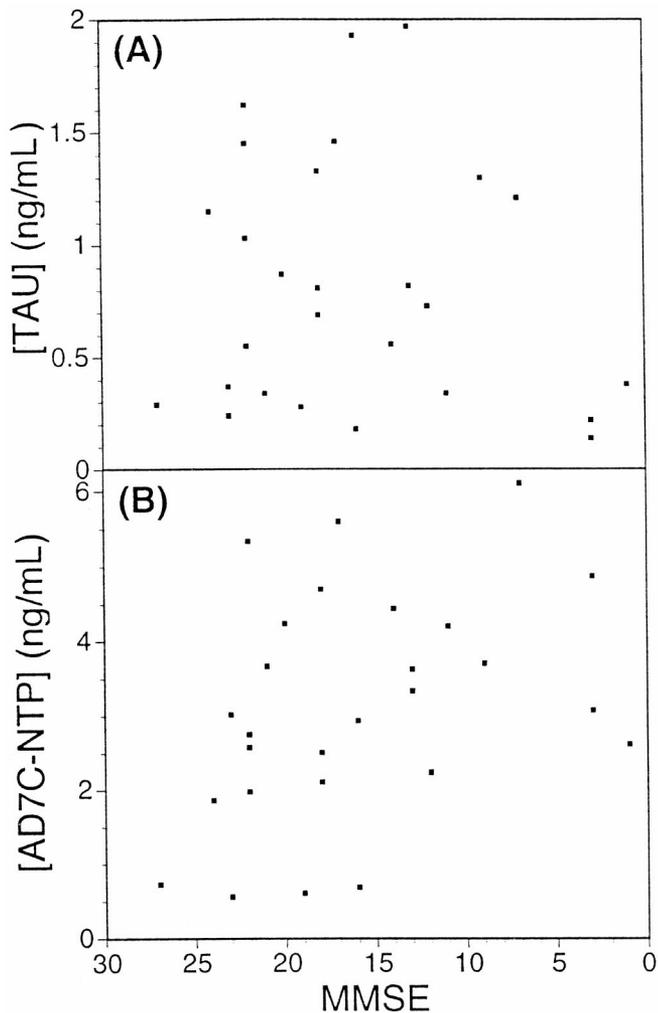


Figure 4. CSF levels of tau (A) and AD7C-NTP (B) from patients with AD as a function of Mini-Mental State Examination (MMSE) scores.

fore these markers can be used in the antemortem differential diagnosis of dementia.<sup>3</sup>

In 27 patients with AD, CSF levels of AD7C-NTP correlated with severity of dementia as assessed by MMSE, consistent with the previous study using the Blessed dementia scale.<sup>15</sup> In contrast, a correlation of CSF tau with MMSE scores was not observed, consistent with some previous reports.<sup>7,11-13</sup> Our results demonstrating no significant correlations between CSF AD7C-NTP or tau levels and change in cognition over time in control subjects suggest that these markers have limited utility in predicting cognitive change in healthy older adults. Additional control samples with longitudinal clinical evaluations will be required to verify these findings.

In a recent study of severely demented patients with AD, a wide distribution of CSF tau levels was found with a surprisingly high number of patients showing low values.<sup>27</sup> One longitudinal study with serial CSF sampling showed a decrease of CSF tau in patients with AD advancing from middle to late

stage.<sup>28</sup> Here we have found relatively low CSF tau levels in the three severely demented patients with AD (MMSE: <5). Possibly, neuronal death in severely demented patients with AD has depleted the source for CSF tau. Thus, severely affected AD patients might account for false-negatives in the CSF tau assay, limiting its sensitivity. However, in most clinical settings, CSF diagnostic studies would be infrequently ordered in patients with severe dementia.

It is unclear whether tau and AD7C-NTP are independently elevated during AD, or if these markers are released into CSF through a common neuropathologic phenomenon, such as neurofibrillary degeneration. AD7C-NTP immunostaining has been observed in neurons bearing traits of neurofibrillary degeneration.<sup>16</sup> When such double-immunopositive neurons die early in the course of the disease, they could become a source of extracellular tau and AD7C-NTP. Our data showed a modest correlation between CSF levels of the two markers that was strongest among the AD patients. Combined use of the two markers in multivariate discriminant analysis resulted in differentiation of patients with AD and non-AD subjects with a sensitivity of 63%, a specificity of 93%, and an overall diagnostic accuracy of 81%. However, these values were only slightly greater than those obtained for each marker alone. Future studies should examine the use of combined determination of CSF tau and AD7C-NTP in the early differential diagnosis of AD in additional patient, comparison, and control samples.

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