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Amyloid-β(1–42), Total Tau, and Phosphorylated Tau as Cerebrospinal Fluid Biomarkers for the Diagnosis of Alzheimer Disease

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BACKGROUND: To improve ante mortem diagnostic accuracy of Alzheimer disease (AD), measurement of the biomarkers amyloid-β(1–42) (Aβ42), total tau (Tau), and tau phosphorylated at threonine$_{181}$ (pTau) in cerebrospinal fluid (CSF) has been proposed. We have used these markers and evaluated their performance.

METHODS: From January 2001 to January 2007, we assessed Aβ42, Tau, and pTau by commercial ELISAs in CSF from 248 consecutive AD patients and 131 patients with subjective memory complaints attending our outpatient memory clinic. Diagnoses were made blind to the results of the biomarker assays. We assessed sensitivity and specificity and analyzed trends over time.

RESULTS: Interassay CVs from analysis of pools of surplus CSF specimens were mean 11.3% (SD 4.9%) for Aβ42; 9.3% (1.5%) for Tau, and 9.4% (2.5%) for pTau, respectively (n = 7–18). To achieve 85% sensitivity, cutoff values were 550 (95% CI 531–570) ng/L for Aβ42; 375 (325–405) ng/L for Tau, and 52 (48–56) ng/L for pTau. Corresponding specificities were 83% (95% CI 76%–89%) for Aβ42, 78% (70%–85%) for Tau, and 68% (60%–77%) for pTau. Logistic regression to investigate the simultaneous impact of the 3 CSF biomarkers on the diagnosis yielded a sensitivity of 93.5% and specificity of 82.7%, at a discrimination line of Aβ42 = 373 + 0.82 × Tau. The area under the ROC curves of Tau and pTau showed significant fluctuation over time.

CONCLUSIONS: CSF biomarkers Aβ42 and Tau can be used as a diagnostic aid in AD. pTau did not have additional value over these 2 markers. Cutoff values, sensitivities, specificities, and discrimination lines depend on the patient groups studied and laboratory experience.

An ante mortem diagnosis of "probable" Alzheimer disease (AD)[3] is achieved by application of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria *(1)* using a standardized protocol including medical history, physical and neurological examination, screening laboratory tests, psychometric evaluation, electroencephalography (EEG), and brain magnetic resonance imaging (MRI) or computed tomography (CT). Although the NINCDS-ADRDA criteria have been reported to have a high accuracy rate of 80%–90% *(2, 3)*, studies of the diagnostic accuracy came from specialized centers, and most data are from patients in later stages of the disease who were studied for several years before death and autopsy.

To improve the clinical diagnostic accuracy, assessment of biomarkers in cerebrospinal fluid (CSF) has been proposed *(4)*. Candidate biomarkers obviously are proteins that occur in senile plaques (SP) and neurofibrillary tangles (NFT). The principal component of the SP is the hydrophobic amyloid-β(1–42) (Aβ42), whereas hyperphosphorylated tau (pTau), a fraction of the concentration of total tau (Tau), is a characteristic component of NFT *(5–7)*. Previous studies showed that these biomarkers can discriminate AD patients from healthy controls with a good sensitivity and specificity, but cutoff levels differ between laboratories *(8–15)*.

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[3] Nonstandard abbreviations: AD, Alzheimer disease; NINCDS-ADRDA, National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association; EEG, electroencephalogram; MRI, magnetic resonance imaging; CT, computed tomography; CSF, cerebrospinal fluid; SP, senile plaques; NFT, neurofibrillary tangles; Aβ42, amyloid-β(1–42); pTau, hyperphosphorylated tau; Tau, total tau; SMC, subjective memory complaint; MMSE, Mini Mental State Examination; AUC, area under the ROC curve; MCI, mild cognitive impairment.

The aim of the study was to establish the sensitivity and specificity of assays for determination of Aβ42, Tau, and pTau in CSF to distinguish patients with probable AD from patients with subjective memory complaints (SMCs) in a memory clinic setting. This article describes our experience with the use of CSF biomarkers during a 6-year period.

Materials and Methods

PATIENTS

Our investigation included 379 patients referred to the Alzheimer Center of the VU University Medical Center from January 2001 to January 2007. All patients underwent a standard clinical assessment, including medical history, physical and neurological examination, screening laboratory tests, psychometric evaluation, EEG, and MRI. We made the diagnosis of probable AD according to the NINCDS-ADRDA criteria *(1)* by consensus in a multidisciplinary team, which was blinded to the results of the CSF analyses. When all investigations yielded normal results, we considered subjects to have subjective complaints and designated them as controls. After evaluation, we classified 131 subjects age mean 61.4 (SD 10.1) years, 52% female, with Mini Mental State Examination (MMSE) score 28.5 (1.8) as having SMCs (controls) and diagnosed 248 patients age 66.7 (9.2) years, 48.4% female, with MMSE score 20.8 (5.1) as having probable AD. Of the AD patients, 73% carried ApoE ε4, compared to 30% of the controls.

We collected CSF (obtained by lumbar puncture in the L3/L4 or L4/L5 intervertebral spaces) in 12-mL polypropylene tubes. The protocol has been approved by the ethical review board of our institution, and all subjects have given written consent to undergo the lumbar puncture.

CSF ANALYSIS

Within 2 h, CSF samples were centrifuged at 2100*g* for 10 min at 4 °C. We used a small amount of CSF for routine analysis, including total cells, total protein, and erythrocytes. We mixed the remaining CSF, divided it into 0.5- or 1-mL polypropylene tubes, and stored it at −80 °C until further analysis of the biomarkers, which occurred within a month.

We determined Aβ42, Tau, and pTau concentrations using sandwich ELISAs [Innotest™ β-Amyloid$_{(1–42)}$, Innotest hTAU-Ag, and Innotest Phosphotau$_{(181P)}$; Innogenetics]. As the manufacturer does not supply control specimens, we monitored the performance of the assays with pools of surplus CSF specimens. In the study period, multiple pools with various concentrations included in 7–18 runs were used for this purpose. The interassay CVs obtained were 11.3% (4.9%) for Aβ42, 9.3% (1.5%) for Tau, and 9.4% (2.5%) for pTau.

Table 1. Discrimination between 248 patients with probable Alzheimer disease and 131 controls by CSF biomarkers during 2001–2007.

CSF marker	Cutoff for 85% sensitivity, ng/L (95% CI)	Specificity, % (95% CI)
Aβ42	550 (531–570)	83 (76–89)
Tau	375 (325–405)	78 (70–85)
pTau	52 (48–56)	68 (60–77)

DATA ANALYSIS

We determined cutoff values to achieve the 85% sensitivity as advocated in the Reagan Consensus Report *(16)* and calculated corresponding specificities. ROC curves were drawn by plotting the true-positive rate (sensitivity) against the false-positive rate (100 − specificity). In addition, we calculated the areas under the ROC curve (AUCs) and corresponding standard errors. The ROC curves were compared with the Hanley and McNeil method *(17)* using Medcalc V 4.30 Software. We used logistic regression analysis with backward stepwise selection to estimate the simultaneous impact of the continuous variables Aβ42, Tau, and pTau in CSF on the diagnosis of probable AD. Correlations were calculated with the Spearman method.

Results

Cutoff values to distinguish AD patients from controls with a sensitivity of 85% and the associated specificities are shown in Table 1. Aβ42 at a cutoff value of 550 ng/L demonstrated the highest discriminatory power. In both AD and SMC patients, concentrations of Tau and pTau were highly correlated ($r = 0.89$ in both groups).

To investigate whether the performance of the biomarker tests was reproducible over time, we divided the study period into 4 quarters of 18 months each and calculated cutoff values and specificities for each quarter (Fig. 1). Over time, the cutoff value for Aβ42 was the most stable [532 (31) ng/L], showing a CV of only 5.7% over the 4 study quarters. Cutoff values for Tau and pTau showed modest fluctuation (CVs of 9.1% and 9.6%, respectively). Additionally, the specificity of Aβ42 at 85% sensitivity showed less fluctuation than the other 2 markers (9.1% vs 13.8% and 28.9%). It appears from Fig. 1 that the highest variability was observed in quarters 1 and 2, whereas in quarters 3 and 4 more stable performance was observed. For Aβ42, the AUC under the ROC curve in quarter 2 (0.84; 95% CI 0.75–0.91) was marginally different from that in quarter 3 (0.94; 0.88–0.98) ($P = 0.074$). For Tau, the AUC in quarter 2 (0.77; 0.67–0.86) differed from that in quarters 3 (0.92; 0.85–0.96) ($P < 0.02$) and 4 (0.91;

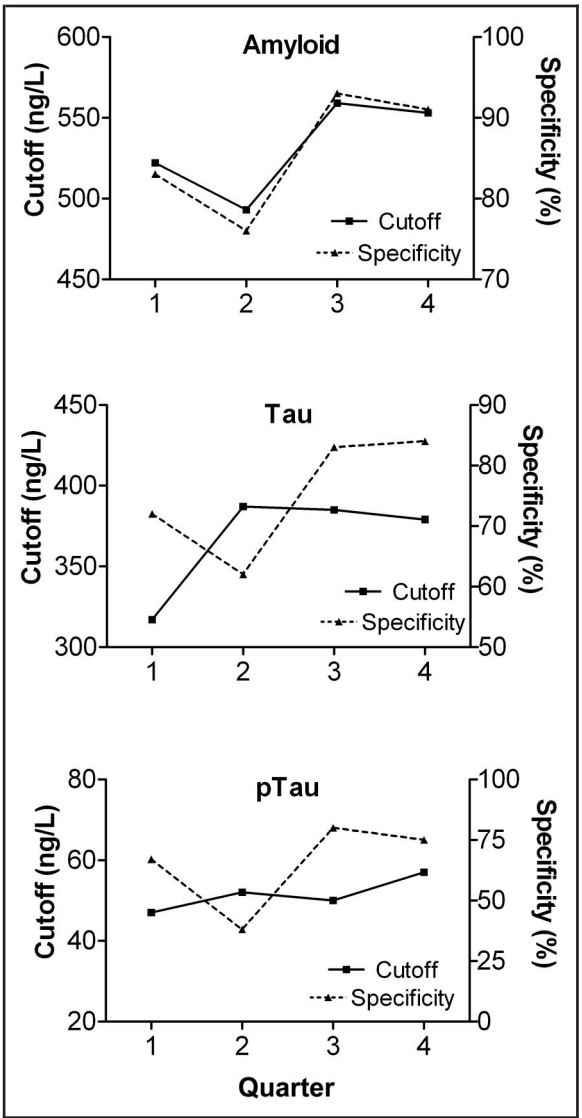

Fig. 1. Performance of CSF biomarkers in AD over time (2001–2007).

Cutoff values for Aβ42, Tau, and pTau yielding 85% sensitivity and specificity at 85% sensitivity.

0.85–0.95) ($P < 0.02$) and was not different from that in quarter 1 (0.88; 0.75–0.95) ($P = 0.14$). For pTau, the AUC in quarter 2 (0.72; 0.61–0.81) was also significantly different from the AUCs in quarters 3 (0.89; 0.81–0.94) ($P < 0.01$) and 4 (0.88; 0.81–0.93) ($P < 0.02$). The AUC in quarter 1 (0.86; 0.73–0.94) was not significantly different from the AUC observed in quarters 3 and 4 ($P = 0.64$ and 0.79, respectively). The difference between the AUCs in quarters 1 and 2 almost reached statistical significance ($P = 0.06$). We therefore considered the first 2 quarters as learning or proficiency time and restricted further analysis to quarters 3 and 4, covering the last 3-year period of our study, during which 155 AD patients and 84 SMC patients were included.

Table 2. CSF biomarker concentrations according to ApoE status of the patients studied in quarters 3 and 4.[a]

	Control, ng/L		AD, ng/L	
	ApoE ε4 negative	ApoE ε4 positive	ApoE ε4 negative	ApoE ε4 positive
n (%)	54 (70)	23 (30)	32 (27)	85 (73)
Aβ42	916 (182)	745 (205)[b]	507 (180)	460 (137)
Tau	276 (116)	300 (161)	804 (404)	792 (484)
pTau	44 (14)	50 (23)	91 (39)	89 (39)

[a] Data are mean (SD) unless noted otherwise.
[b] $P < 0.01$ vs ApoEε4 noncarriers.

For 194 patients in this group (81.2%), the ApoE status was known. Table 2 shows CSF biomarker concentrations according to ApoE status. In controls, CSF Aβ levels were significantly lower in ApoE ε4 carriers than in noncarriers.

ROC curves for the 3 CSF biomarkers are shown in Fig. 2. Pairwise comparisons of the ROC curves for Aβ42, Tau, and pTau in CSF showed no significant

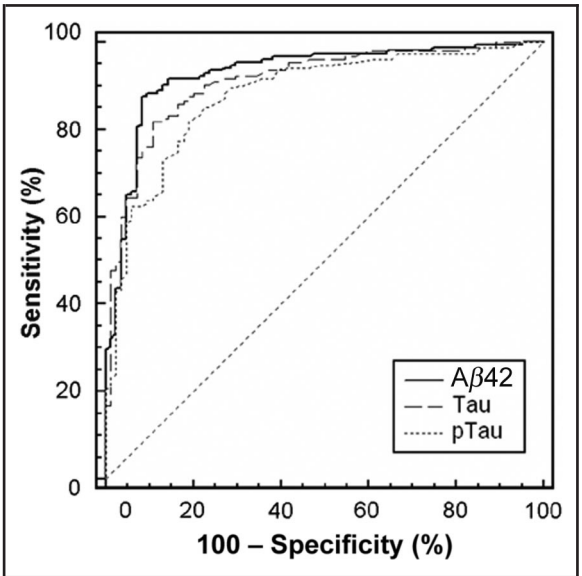

Fig. 2. ROC curves of Aβ42, Tau, and pTau in CSF.

Sensitivity vs specificity was calculated for all subjects in quarters 3 and 4. The graph was drawn with 84 controls and 155 probable AD patients studied in 2004–2007.

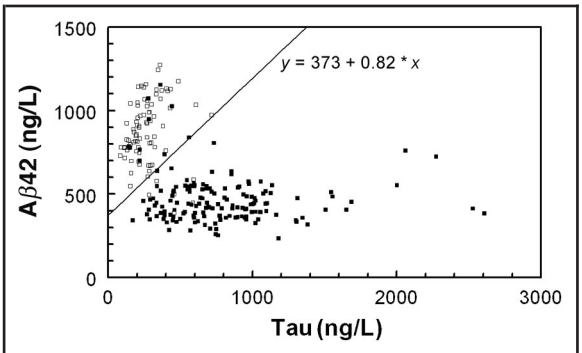

Fig. 3. Scatterplot of CSF Aβ42 vs Tau in 155 patients with probable AD (■) and 84 controls (□).

The equation of the line for optimal separation is Aβ42 = 373 + 0.82 × Tau.

difference for Aβ42 (AUC 0.928; 95% CI 0.888–0.952) vs Tau (0.911; 0.868–0.944) ($P = 0.51$) or Aβ42 vs pTau (0.880; 0.832–0.918) ($P = 0.082$). By contrast, the AUCs of Tau and pTau were found to be statistically different ($P = 0.017$).

Logistic regression analysis with diagnosis (probable AD and controls) as dependent variable and Aβ42, Tau, and pTau in CSF as independent continuous variables resulted in correct classification of 145 of 155 (93.5%; 95% CI 89.7%–97.4%) probable AD patients and 76 of 84 (90.5%; 84.2%–96.8%) controls, with an overall correct percentage of 92.5%, at a cutoff line of Aβ42 = 373 + 0.82 × Tau (Fig. 3). In this model, pTau did not contribute significantly to the discrimination of patients with probable AD from controls. We also investigated the additional diagnostic value of pTau in the non–ApoE ε4 carriers. In a logistic regression model, no significant diagnostic value of pTau was observed.

Discussion

An important lesson from our experience with assays for CSF biomarkers is that a certain degree of experience is required to have confidence in the results obtained. Our results appeared to reach stability only in the second half of the study period. Reasons for the lack of stability in the first half may include limited experience with the assays and differences in the patient groups referred for analysis, such as age or severity of symptoms. Also, the mere fact that more patients were studied in the second half of the study may have contributed to the improved accuracy and stability we observed in our results. Despite efforts to prevent preanalytical variation in factors such as time between lumbar puncture and storage, such variation may also have contributed. Analytical variation was also a potential source of variation, with multiple causes, ranging from lot-to-lot variations in reagents to variation in technical skills. Although we carefully reviewed all possibilities, we were not able to identify the reasons for the higher fluctuation in the first half of our study. To overcome the issue of lot-to-lot variation, we currently purchase large numbers of kits from the same lot; this is possible only because of the recent increases in our request load. We anticipate seeing a decrease in interassay variation in the near future.

Despite variation over time, the assays for CSF biomarkers Aβ42 and Tau appeared to be sufficiently stable to be used to distinguish AD from controls. CSF pTau was excluded as an important variable in the multivariate logistic regression model and hence appeared to confer no additional value in the separation of patients with AD from controls.

As shown before *(18)*, the proportion of ApoE ε4 carriers was considerably higher in AD patients than in control subjects. The significantly lower CSF Aβ levels in controls carrying the ε4 allele may be an indication of their increased risk for developing AD.

Optimal estimates of sensitivity and specificity can be obtained only when the contrast between the AD patients and controls is optimal, i.e., the AD patients are correctly classified as AD patients and the controls as healthy subjects. The accuracy of the clinical diagnosis for probable AD has been reported to be relatively low, with a sensitivity of 81% and a specificity of 70% *(9)*. The power of biomarkers for separating AD patients from healthy controls has been demonstrated by Riemenschneider et al. *(10)*, who investigated 74 patients with AD and 40 cognitively healthy control subjects. These authors reported higher cutoff concentrations than we found. It is unclear whether this difference in cutoffs can be attributed to differences in the patient groups or to experimental differences. It does illustrate the necessity for laboratories to establish their own reference values. The importance of the composition of the patient groups and comorbidity has been shown in a multicenter study *(11)* as well as in a metaanalysis *(8)*. Although patient groups differed in the various studies, for Aβ42 the sensitivity was always >75%, whereas a broader range was reported for the lowest Tau value, the lowest value being 30%.

After completion of our study, it became clear that in nondemented individual subjects Aβ42 levels may show significant diurnal variation, the lowest values being observed in the morning *(19)*. It is presently unclear whether such variation also applies to patients with AD. We could not evaluate this phenomenon in our patient population due to a lack of serial samples over the day, but diurnal variation of

CSF biomarkers will require further investigation. Until this issue is settled, time of collection of CSF should be taken into account, and lumbar punctures should be performed at the same time of the day in each center.

Many studies have demonstrated an association of low Aβ42 and high Tau levels with AD *(12–15)*. Calculation of a separation line, e.g., by logistic regression, may be helpful in the classification of the subjects with low Tau/low Aβ42 and high Tau/high Aβ42, which otherwise would be difficult to classify. We found that the best discrimination between 84 patients with mild cognitive impairment (MCI) and 155 patients with probable AD was achieved by the line Aβ42 = 373 + 0.82 × Tau, leading to an overall correct classification of 92.5%. Other discrimination lines have been published, e.g., by Hulstaert et al. *(11)*, who reported that the line Aβ42 = 240 + 1.18 × Tau discriminated AD patients from a group of healthy volunteers and patients with other neurological disorders with 85% sensitivity and 86% specificity. As theirs was a multicenter study and the control group differed from ours, a direct comparison is not possible. Using the same discrimination line, Andreasen et al. *(20)* reported a sensitivity of 94% for probable AD, 88% for possible AD, and 75% for mild cognitive impairment, whereas specificity was 100% for discrimination from psychiatric disorders and 89% for nondemented individuals. Riemenschneider et al. *(10)* found a 92% sensitivity and a 95% specificity for separation of AD vs controls with the line Aβ42 = 644 + 0.25 × Tau. The differences in the equations of the discrimination lines illustrate considerable variation between institutions with respect to experimental procedures and composition of patient groups and stress the importance of standardization. One approach to improve standardization is the establishment of international quality control schemes for the assessment of the biomarkers. Our group *(21)* has recently reported on such an initiative.

One other result of our logistic regression analysis was the elimination of pTau as a valuable marker to distinguish patients with AD from controls, both in the entire group and in the ApoE ε4 carriers. CSF pTau is claimed to reflect the pathology of microtubules and was, therefore, expected to contribute importantly. Nevertheless, this parameter was removed in the stepwise selection process as a variable in the equation of the logistic model ($P = 0.64$). The high correlation coefficient between pTau and Tau ($r_s = 0.93$; $P < 0.001$) is probably the reason pTau failed to provide additional discriminatory value. Despite this, pTau is considered to be of importance in the differential diagnosis of AD.

In the evaluation of neurodegenerative diseases, discrimination between AD and non-AD and the conversion of MCI to AD are important diagnostic issues *(22, 23)*. In the daily practice of our Alzheimer Center, the number 1 issue is to distinguish between AD and non-AD. Conversion of MCI to AD is a separate issue which requires longitudinal follow-up of the patients. Previous work of our group has shown that for the 3 CSF biomarkers used, serial measurement appears to have limited value *(24, 25)*.

When the concentration of 1 analyte is increased and that of the other is decreased, a ratio might be more informative *(12, 26, 27)*. When results of biomarker measurements are clearly method dependent, as is the case with CSF biomarkers, calculation of a ratio has only local value and is not useful for comparison with other studies. We have calculated the sensitivity and specificity of the Tau/amyloid β ratio. We found a ratio of 0.59 to yield a 91.2% sensitivity (95% CI 87%–96%) and a 91.7% specificity (84%–97%).

The high Tau level observed in some patients (Fig. 3) raises the question whether these patients may be suffering from Creutzfeldt-Jakob disease *(28)*. As implied in "Materials and Methods," such a diagnosis was not made in any of our patients. We have reevaluated the records of patients in whom a CSF Tau level >1300 ng/L was reported. No reasons for the high Tau concentration became apparent.

The value of CSF biomarker analysis in the diagnosis of AD has been addressed in several recent articles *(29–32)*. Mattsson et al. *(29)* reported that incipient AD is more accurately identified in single-center studies than in their multicenter study. They conclude that there is a need for standardization in analytical as well as in clinical procedures. In another multicenter study, Buerger et al. *(30)* found that analysis of specimens from all participating centers in a single laboratory increases the diagnostic accuracy. This also emphasizes the need for standardization.

Welge et al. *(31)* found that inclusion of other amyloid species, i.e., Aβ1–38, in the CSF biomarker panel increased sensitivity for the detection of AD and also the specificity for excluding non-Alzheimer dementias. Vemuri et al. *(32)* showed that combination of CSF biomarker analysis and MRI is superior to either procedure alone for the prediction of progression of MCI to AD.

In conclusion, CSF Aβ42 and Tau are useful as biomarkers to identify patients with probable AD from controls in a memory clinic setting. Cutoff values, sensitivities, specificities, and discrimination lines dependent on the subjects referred and laboratory experience. International standardization and collaboration are expected to contribute to the further dissemination of CSF biomarker analysis in clinical practice.

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