ORIGINAL ARTICLE

Measurement of Phosphorylated Tau Epitopes in the Differential Diagnosis of Alzheimer Disease

A Comparative Cerebrospinal Fluid Study

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Background: Abnormal hyperphosphorylation of the microtubule-associated protein tau and its incorporation into neurofibrillary tangles are major hallmarks of the pathogenesis of Alzheimer disease (AD). Different tau phosphoepitopes can be sensitively detected in cerebrospinal fluid (CSF).

Objective: To compare the diagnostic accuracy of CSF concentrations of tau proteins phosphorylated at 3 pathophysiologically important epitopes (p-tau) to discriminate among patients with AD, nondemented control subjects, and patients with other dementias.

Design and Setting: Cross-sectional, bicenter, memory clinic–based studies.

Participants: One hundred sixty-one patients with a clinical diagnosis of AD, frontotemporal dementia, dementia with Lewy bodies, or vascular dementia and 45 nondemented controls (N=206).

Main Outcome Measures: Levels of tau protein phosphorylated at threonine 231 (p-tau$_{231}$), threonine 181 (p-tau$_{181}$), and serine 199 (p-tau$_{199}$). The CSF p-tau protein levels were measured using 3 different enzyme-linked immunosorbent assays.

Results: The mean CSF levels of the studied p-tau proteins were significantly elevated in patients with AD compared with the other groups. Applied as single markers, p-tau$_{231}$ and p-tau$_{181}$ reached specificity levels greater than 75% between AD and the combined non-AD group when sensitivity was set at 85% or greater. Statistical differences between the assay performances are presented. Particularly, discrimination between AD and dementia with Lewy bodies was maximized using p-tau$_{181}$ at a sensitivity of 94% and a specificity of 64%, and p-tau$_{231}$ maximized group separation between AD and frontotemporal dementia with a sensitivity of 88% and a specificity of 92%. Combinations of the 3 markers did not add discriminative power compared with the application as single markers.

Conclusions: The p-tau proteins in CSF come closest to fulfilling the criteria of a biological marker of AD. There is a tendency for p-tau proteins to perform differently in the discrimination of primary dementia disorders from AD.

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ABNORMAL HYPERPHOSphorylation of the microtubule-associated protein tau and its incorporation into neurofibrillary tangles are major components of the pathogenesis of Alzheimer disease (AD).

Using monoclonal antibodies specific for different phosphorylated epitopes of tau, enzyme-linked immunosorbent assays have been developed that sensitively measure concentrations of phosphorylated tau protein (p-tau) in cerebrospinal fluid (CSF). Statistically significant increases in CSF p-tau concentrations in patients with AD have recently been demonstrated in independent pilot studies, mainly using 3 different immunoassays specific for the phosphorylated epitopes threonine 231 (p-tau$_{231}$),[1,2] threonine 181 (p-tau$_{181}$),[3,4] and serine 199 (p-tau$_{199}$).[5] Evidence from these pilot studies indicates that quantification of tau phosphorylated at these specific sites may improve early detection, differential diagnosis, and tracking of disease progression in AD (for a review see Blennow et al[6,7]).

In a recent study,[1] CSF p-tau$_{231}$ distinguished between patients with AD and those with other neurologic disorders (ONDs) with a sensitivity of 85% and a specificity of 97%. Furthermore, p-tau$_{231}$ significantly improved differential diagnosis between AD and other non-AD groups, particularly frontotemporal dementia (FTD).[8] In AD vs FTD, p-tau$_{231}$ correctly allocated 91% of patients vs only 66% using total tau.[8] Itoh and colleagues[5] showed that CSF p-tau$_{199}$ discriminates between AD and the combined non-AD groups with a sensitivity and a specificity of 85%. The level of CSF p-tau$_{181}$ was elevated in pa-

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Table 1. Characteristics of Patients and Controls and CSF p-Tau Values

Group	Age, Mean ± SD (Range), y	Sex, F/M, No.	MMSE Score, Mean ± SD (Range)	CSF Level, Median (25th-75th Percentile)		
				p-Tau$_{231}$, pg/mL	p-Tau$_{181}$, pmol/L	p-Tau$_{199}$, fmol/mL
AD (n = 108)	72.6 ± 5.7 (54-84)	65/43	22.2 ± 5.4 (5-30)	667.5 (361.0-907.2)	20.5 (16.5-26.2)	1.7 (1.3-2.2)
DLB + FTD + VaD (n = 53)	71.3 ± 8.8 (46-87)	26/27	20.5 ± 5.0 (10-30)	140.0 (50.5-285.5)*	11.3 (9.4-15.7)*	1.1 (0.9-1.5)*
DLB (n = 22)	76.1 ± 6.1† (65-87)	7/15	21.2 ± 5.4 (10-29)	213.5 (92.8-374.5)*	11.3 (9.0-16.9)*	1.1 (0.8-1.4)*
FTD (n = 24)	66.0 ± 0.9* (46-79)	15/9	19.8 ± 4.8† (13-30)	86.5 (9.0-174.5)*	10.7 (9.3-14.2)*	1.1 (0.9-1.6)*
VaD (n = 7)	74.1 ± 4.3 (65-77)	4/3	21.3 ± 4.9 (12-25)	201.0 (44.0-612.0)†	13.6 (12.1-19.0)†	1.3 (0.9-1.9)
OND (n = 22)	61.1 ± 9.0* (49-81)	16/6	27.8 ± 3.2* (17-30)	54.0 (13.3-97.0)*	11.3 (8.6-12.6)*	0.8 (0.6-1.0)*
Control (n = 23)	60.0 ± 10.1* (44-77)	14/9	29.2 ± 0.7* (27-30)	35.0 (18.0-70.0)*	11.0 (9.0-13.4)*	0.8 (0.6-1.1)*

Abbreviations: AD, Alzheimer disease; CSF, cerebrospinal fluid; DLB, dementia with Lewy bodies; FTD, frontotemporal dementia; MMSE, Mini-Mental State Examination; OND, other neurologic disorder; p-tau, phosphorylated tau protein; VaD, vascular dementia.

*$P<.001$, AD group vs the other groups studied.

†$P<.05$, AD group vs the other groups studied.

tients with AD compared with patients with other dementias and controls,[9] and p-tau$_{181}$ has been proposed as a potential marker for discriminating patients with AD from those with dementia with Lewy bodies (DLB).[10] Furthermore, CSF p-tau$_{231}$ concentrations declined over time during the clinical progression of AD and correlated with cognitive performance at baseline.[11] Results of these first studies suggested that CSF p-tau proteins are promising biomarker candidates for AD.

Levels of CSF p-tau and the discriminative power of the 3 different p-tau assays among patients with AD, nondemented controls, and patients with other dementias, however, have not yet been assessed in the same set of patients. Investigation of the 3 p-tau assays would allow for direct comparison of the diagnostic performance of the 3 markers to differentiate AD from other relevant diseases. In the advent of large international multicenter trials (such as the National Institute on Aging Initiative on Neuroimaging in Alzheimer's Disease and the studies of the European Alzheimer's Disease Consortium) on the value of biological markers and neuroimaging in the diagnosis of AD, this is the first study to address the important clinical issue regarding the differential diagnostic performances of p-tau proteins as core biological marker candidates.

In particular, we tested the diagnostic accuracy of the 3 p-tau assays according to the recommendations of a consensus group[12] suggesting a sensitivity level of 85% or greater and a specificity level of at least 75% for useful biomarkers of AD. In addition, we asked whether use of a combination of the 3 markers might be superior to the application of single markers. To our knowledge, this is the first comparative study applying 3 developed and published immunoassays detecting different p-tau epitopes on the same set of controls and patients to compare individual and combined diagnostic accuracy. Concentrations of p-tau$_{231}$, p-tau$_{181}$, and p-tau$_{199}$ in the CSF were studied in the same group of patients with AD, DLB, FTD, or vascular dementia (VaD); patients with ONDs; and controls.

METHODS

PATIENT SELECTION

A total of 206 individuals were studied. One hundred eight patients had probable AD (National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association criteria)[13] and 53 had other dementia disorders (24 patients with FTD,[14] 22 with DLB,[15] and 7 with VaD).[16] Structural and functional imaging results were consistent with the diagnoses in these patients. Twenty-two patients with ONDs were diagnosed as having mild psychiatric (eg, depressed mood) or neurologic (eg, dizziness) symptoms. One patient with OND experienced a bulbar syndrome of unknown etiology. We also studied 23 controls. Study participants were recruited at 2 academic expert centers: the Dementia Research Section and Memory Clinic, Alzheimer Memorial Center and Geriatric Psychiatry Branch, Department of Psychiatry, Ludwig-Maximilian University (22 patients with AD, 6 with FTD, 7 with VaD, 9 with DLB, 1 with OND, and 13 controls), and the Department of Clinical Neuroscience, University of Göteborg (86 patients with AD, 18 with FTD, 13 with DLB, 21 with OND, and 10 controls). Participants from the former center had been studied previously with a different objective, and the results of this study have been published.[8] Characteristics of the patients and controls are given in **Table 1**. The protocol was approved by the local ethical committees and the institutional review boards of the 2 participating medical centers. Informed consent was obtained from all participants.

Examination of the controls included medical history, physical examination, routine blood tests (blood cell count; international normalized ratio; partial thromboplastin time; and sodium, potassium, creatinine, urea, and blood glucose levels), and a cognitive test using the Consortium to Establish a Registry for Alzheimer's Disease battery.[17] Ten of the 23 controls were volunteers without any medical, neurologic, or psychiatric disorders. Samples of CSF were collected from 13 controls while they underwent spinal anesthesia for surgery of the urinary tract or lower extremities. They were cognitively normal according to the Consortium to Establish a Registry for Alzheimer's Disease battery (results within ±1 SD in all subtests). Three of these control subjects had diabetes mellitus as a somatic comorbidity.

CSF SAMPLING AND ANALYSES OF p-TAU PROTEINS

Samples of CSF were acquired via lumbar puncture between 9 and 11 AM according to a routine protocol (established by the 2 participating medical centers). Samples of CSF were collected in polypropylene tubes on ice in 0.5-mL aliquots. For this study, a total of 1 mL was taken. Aliquots were centrifuged at 4°C at 10 000*g* for 10 minutes and stored at –80°C until analysis. The same procedures were performed at the 2 sites involved in the study. There was no effect of medical centers

on the variance of measured protein levels and no protein gradient in the CSF column for the markers (K.B., unpublished data, 2002).

Assay operators were masked to the diagnostic category of the samples. Levels of p-tau$_{231}$ were measured using an enzyme-linked immunosorbent assay (Applied NeuroSolutions Inc).[1] This assay uses a combination of CP27 (which recognizes amino acids 130-150 in normal tau and p-tau), Tau-1 (which recognizes amino acids 196-205 in nonphosphorylated tau), and CP9 (which recognizes phosphothreonine 231). Experiments describing the specificity of the detection antibody CP9 for phosphothreonine 231 have been reported previously.[1] Full-length recombinant tau (441 amino acids) phosphorylated at threonine 231 was used to produce a standard curve. Levels of p-tau$_{231}$ in our patients were calculated from the standard curve and expressed as CSF p-tau$_{231}$ in picograms per milliliter.

Levels of p-tau$_{181}$ were measured using a sandwich enzyme-linked immunosorbent assay method (prototype version of Innotest Phospho-Tau [181p]; Innogenetics), using a combination of monoclonal antibody HT7 (which recognizes amino acids 159-163 in normal tau and p-tau) and biotinylated monoclonal antibody AT270 (which recognizes p-tau containing the phosphorylated threonine 181 residue).[3] A synthetic phosphopeptide was used for standardization.

Levels of p-tau$_{199}$ were measured using a previously reported sandwich enzyme-linked immunosorbent assay method (Mitsubishi Chemical Corp, Shinagawa, Japan), using a combination of monoclonal antibody HT7 and polyclonal antibody anti-PS199 (specific for tau phosphorylated at serine 199).[5]

STATISTICAL ANALYSES

Differences among groups regarding age were assessed using the Mann-Whitney (M-W) test and regarding sex distribution using the χ^2 test.

Distributions of p-tau values differed statistically significantly from normal as revealed by the Kolmogorov-Smirnov test. Differences in mean CSF levels of the 3 p-tau subtypes among all groups were assessed using the Kruskal-Wallis test. Pairwise comparisons between patients with AD and the other groups were performed using the M-W test. Correlations between p-tau subtypes were assessed using the Spearman rank correlation.

Cutoff values for p-tau proteins were determined such that 85% of the patients with AD were correctly identified according to the recommendations of the consensus conference[12] of 85% sensitivity for an "excellent" biomarker. To determine differences in diagnostic accuracy among markers, the specificity levels that correspond to the 85% sensitivity cutoff level for each marker in the comparison groups were compared between all possible pairs of markers using the McNemar test.

To develop predictive cutoff values that optimized the combined use of the different p-tau markers, we used classification tree analysis with SYSTAT 7.0 (SPSS Inc, Chicago, Ill). Classification tree analysis uses recursive partitioning to consider all possible binary splits of the data in pursuit of optimal classification.[18] The analysis considered all 3 p-tau markers simultaneously to create cutoff values that maximize separation among groups, resulting in a decision tree. The number of branches depends on the separation that has been achieved at the first split. To avoid overfitting of the data, generation of further branches was interrupted if sensitivity or specificity declined below 80%. A similar technique has been presented in earlier CSF studies in AD.[19]

To show sensitivity and specificity levels over the entire range of cutoff levels, we determined receiver operating characteristic curves.

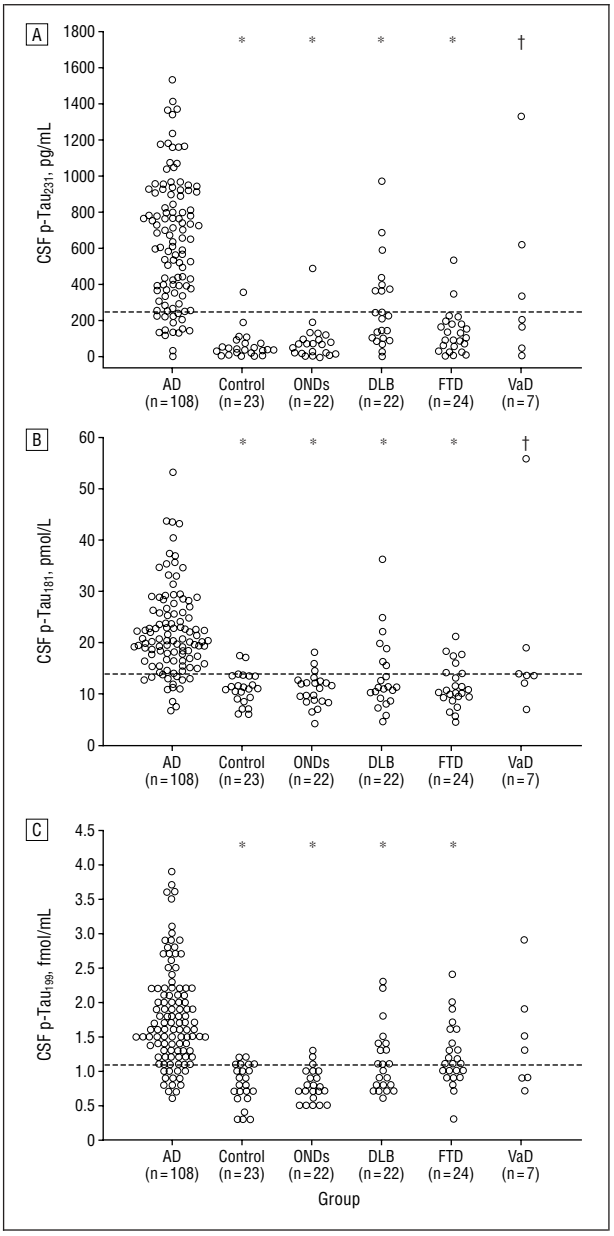

Figure 1. Levels of cerebrospinal fluid (CSF) phosphorylated tau protein (p-tau)$_{231}$ (A), p-tau$_{181}$ (B), and p-tau$_{199}$ (C) in patients and controls. Dashed lines represent the cutoff level when sensitivity was set at 85% or higher. Asterisk indicates differences from AD at $P<.001$; dagger, differences from AD at $P<.05$; AD, Alzheimer disease; ONDs, other neurologic disorders; DLB, dementia with Lewy bodies; FTD, frontotemporal dementia; and VaD, vascular dementia.

RESULTS

CSF p-TAU LEVELS

As illustrated in **Figure 1** and Table 1, levels of all p-tau subtypes were significantly increased in patients with AD compared with the other groups studied. Because the controls were significantly younger than the patients with AD, we investigated correlations between p-tau subtypes and age. In patients with AD, there was no correlation between the 3 p-tau subtypes and age ($\rho=-0.050$ to 0.002; $P=.61$-$.93$). In controls, p-tau$_{181}$ ($\rho=0.61$; $P=.002$) and p-tau$_{231}$ ($\rho=0.41$; $P=.05$) correlated with age,

Table 2. Correlations Between p-Tau Epitopes*

Group	p-Tau$_{231}$ × p-Tau$_{181}$	p-Tau$_{231}$ × p-Tau$_{199}$	p-Tau$_{181}$ × p-Tau$_{199}$
AD	0.807†	0.726†	0.734†
DLB	0.832†	0.800†	0.871†
FTD	0.571‡	0.193	0.360
VaD	0.857§	0.775§	0.847§
ONDs	0.433§	0.377	0.568‡
Control	0.632†	−0.069	0.384

Abbreviations: AD, Alzheimer disease; DLB, dementia with Lewy bodies; FTD, frontotemporal dementia; ONDs, other neurologic disorders; p-tau, phosphorylated tau protein; VaD, vascular dementia.
*Values are given as Spearman rho coefficients.
†$P<.001$.
‡$P<.01$.
§$P<.05$.

but p-tau$_{199}$ did not ($\rho=0.05$; $P=.80$). Therefore, we repeated the analyses in a group of 23 patients with AD and 23 controls who were matched for age (M-W$_1$=208; $P=.21$) and sex ($\chi^2_1<0.001$; $P>.99$). Differences between the AD group and the control group remained unchanged and statistically significant for all 3 p-tau subtypes (p-tau$_{231}$: M-W$_1$=32; $P<.001$; p-tau$_{181}$: M-W$_1$=52.5; $P<.001$; p-tau$_{199}$: M-W$_1$=46; $P<.001$). Consequently, we included all of the patients with AD in our analyses. Correlations among the 3 p-tau subtypes are given in **Table 2**.

SENSITIVITY AND SPECIFICITY OF THE SINGLE MARKERS

Specificity levels of single markers using p-tau proteins when sensitivity was set at 85% or higher, as recommended by a consensus report, are given in **Table 3**.[12] In the case of p-tau$_{199}$, several individuals in each comparison group had a value equal to the 85% sensitivity cutoff value. Therefore, for p-tau$_{199}$, a lower and an upper limit is given for the specificity, corresponding to 2 alternatives: (1) all patients with this specific value were allocated to the AD group and (2) all patients with this specific value were allocated to the comparison group. In the differentiation of patients with AD from those in the non-AD group, p-tau$_{231}$ (85%) and p-tau$_{181}$ (81%) reached specificity levels of 75% or higher, but p-tau$_{199}$ did not (61%-72%). All 3 p-tau proteins showed excellent specificity levels when patients with AD were compared with those with ONDs and controls.

Comparing AD to FTD, p-tau$_{231}$ and p-tau$_{181}$ showed good discriminative power, with specificity levels of 92% and 79%, respectively. For p-tau$_{199}$, specificity ranged from 42% to 54% in the discrimination of AD from FTD. When AD was compared with DLB, p-tau$_{181}$ showed a specificity of 68%, whereas for p-tau$_{231}$ the specificity was 64%. For p-tau$_{199}$, specificity ranged between 50% and 64%. Values for AD vs VaD are not separately given owing to the small sample size of patients with VaD (n=7).

We tested for differences in diagnostic accuracy among p-tau proteins (Table 3). Discriminative power between AD and the combined non-AD groups was significantly higher for p-tau$_{231}$ (McNemar test $P=.004$ to $P<.001$) and p-tau$_{181}$ (McNemar test $P=.10$ to $P<.001$) compared with p-tau$_{199}$. Diagnostic accuracy was also significantly higher using p-tau$_{231}$ and p-tau$_{181}$ compared with p-tau$_{199}$ in differentiating AD from FTD (p-tau$_{231}$ vs p-tau$_{199}$: McNemar test $P=.004$ to $P<.001$; p-tau$_{181}$ vs p-tau$_{199}$: McNemar test $P=.07$ to $P=.01$). There was no statistically significant difference in diagnostic accuracy among p-tau proteins for discrimination between patients with AD vs ONDs and DLB. Receiver operating characteristic curves for pairwise comparisons of marker levels between patients with AD and comparison groups are shown in **Figure 2**.

COMBINATION OF THE MARKERS

We used classification tree analysis to investigate whether a combination of p-tau markers would improve group discrimination (**Table 4**). For AD vs the combined non-AD groups, AD vs controls, AD vs ONDs, and AD vs FTD, we found that p-tau$_{231}$ accounted for maximal group discrimination, whereas the other p-tau subtypes added no additional discriminatory power. Similarly, p-tau$_{181}$ alone maximized group separation between patients with AD and those with DLB and between the AD and VaD groups (data not shown). For discrimination between AD and non-AD dementias, a combination of p-tau$_{231}$ and p-tau$_{181}$ resulted in a slight increase in sensitivity from 86% to 94% at the cost of decreased specificity from 75% to 66%, resulting in only a slight increase in correct classification accuracy (from 83% to 85%) (**Figure 3**).

EFFECT OF AGE, SEX, MINI-MENTAL STATE EXAMINATION SCORE, AND CENTER ON CSF p-TAU LEVELS IN THE AD GROUP

Levels of CSF p-tau$_{231}$ ($\rho=-0.18$; $P=.06$) and p-tau$_{181}$ ($\rho=0.002$; $P=.99$) did not correlate with the Mini-Mental State Examination score in patients with AD. Only for p-tau$_{199}$ did we found a correlation with the Mini-Mental State Examination score ($\rho=-0.25$; $P=.01$). There was no significant effect of sex (p-tau$_{231}$: M-W$_{107}$=1302; $P=.55$; p-tau$_{181}$: M-W$_{107}$=1375; $P=.89$; p-tau$_{199}$: M-W$_{107}$=1203; $P=.22$) or age (p-tau$_{231}$: $\rho=0.002$; $P=.98$; p-tau$_{181}$: $\rho=-0.05$; $P=.61$; p-tau$_{199}$: $\rho=-0.013$; $P=.89$) on levels of p-tau proteins. Levels of p-tau did not differ significantly between participating centers (p-tau$_{231}$: M-W$_{107}$=903; $P=.74$; p-tau$_{181}$: M-W$_{107}$=822; $P=.34$; p-tau$_{199}$: M-W$_{107}$=813; $P=.31$).

COMMENT

In the present study, we investigated the diagnostic performance of 3 different pathophysiologically important CSF tau phosphorylation epitopes (p-tau$_{231}$, p-tau$_{181}$, and p-tau$_{199}$)[20-22] to discriminate patients with AD from those with other clinically important causes of dementia, those with ONDs, and nondemented controls. All 3 phosphorylation sites were studied using 3 recently developed and reported immunoassays.[1,3,5] So far, independent early studies of the single phosphorylation sites have indicated statistically significant discriminative power between the AD and non-AD study groups. Subsequently, CSF p-tau proteins in general were suggested as promising biological marker candidates for AD. In none of these pilot stud-

Table 3. Specificity of Single Markers With Sensitivity Set at 85% or Higher and Differences in Diagnostic Accuracy Between the Single Markers*

	CSF Marker			*P* Value‡		
AD Group	p-Tau$_{231}$	p-Tau$_{181}$	p-Tau$_{199}$†	p-Tau$_{231}$ vs p-Tau$_{181}$	p-Tau$_{231}$ vs p-Tau$_{199}$	p-Tau$_{181}$ vs p-Tau$_{199}$
Non-AD (n = 98)	83 (85)	79 (81)	60-71 (61-72)	.39	<.01 to <.001	.10 to <.001
Control (n = 23)	22 (96)	21 (91)	17-21 (74-91)	.99	.99	.99
ONDs (n = 22)	21 (95)	19 (86)	19-20 (86-91)	.50	.99	.99
DLB (n = 22)	14 (64)	15 (68)	11-14 (50-64)	.99	.99	.99
FTD (n = 24)	22 (92)	19 (79)	10-13 (42-54)	.38	<.01 to <.001	.07 to <.05

Abbreviations: AD, Alzheimer disease; CSF, cerebrospinal fluid; DLB, dementia with Lewy bodies; FTD, frontotemporal dementia; ONDs, other neurologic disorders; p-tau, phosphorylated tau protein.

*Data are given as number (percentage) of patients. The cutoff levels for the different p-tau assays were 250 pg/mL for p-tau$_{231}$, 14.3 pmol/L for p-tau$_{181}$, and 1.1 fmol/mL for p-tau$_{199}$.

†Since several patients in each group had identical p-tau$_{199}$ values, 2 specificity figures are given for this assay.

‡Calculated using the McNemar test.

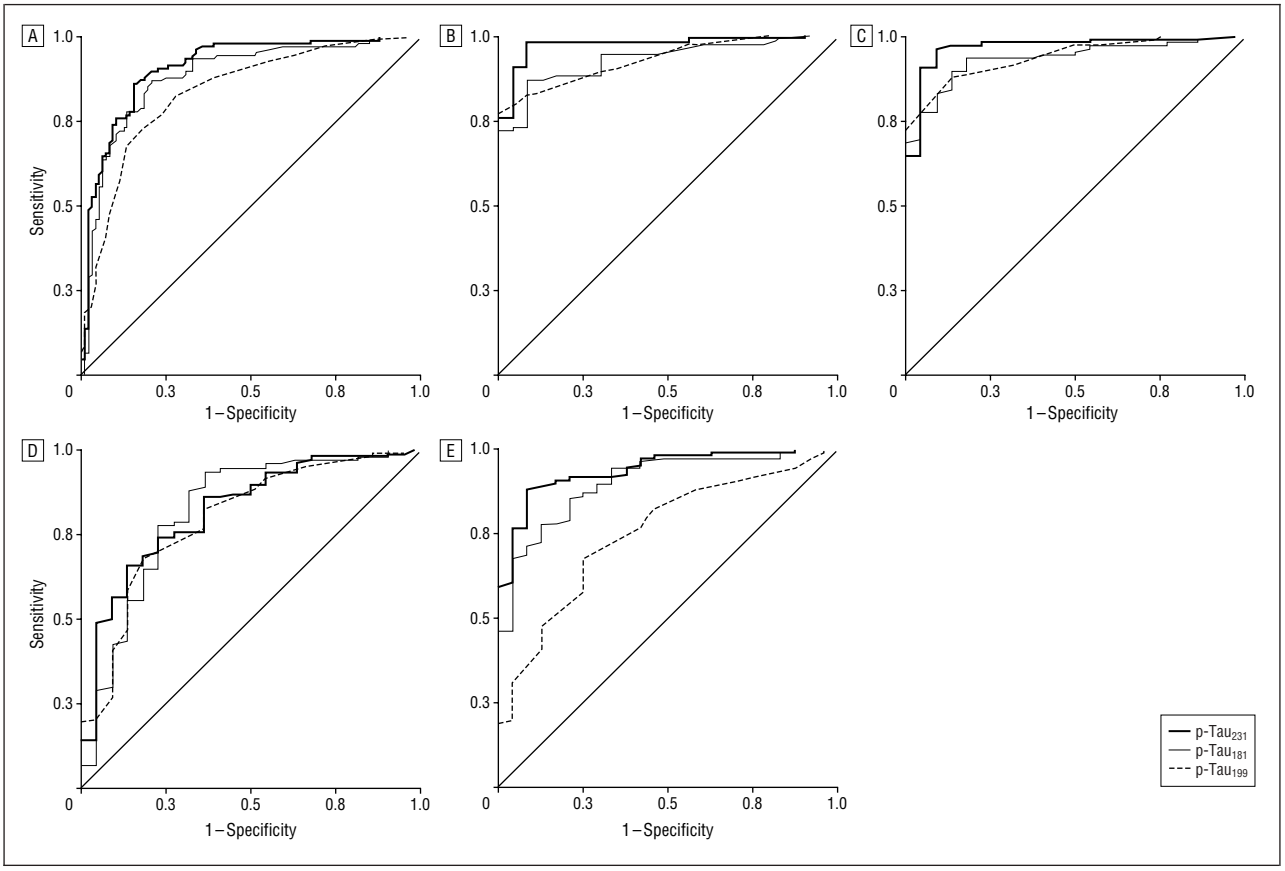

Figure 2. Receiver operating characteristic curves for cerebrospinal fluid phosphorylated tau protein (p-tau)$_{231}$, CSF p-tau$_{181}$, and CSF p-tau$_{199}$ when patients with Alzheimer disease were compared with the combined non–Alzheimer disease group (A), the control group (B), patients with other neurologic disorders (C), patients with dementia with Lewy bodies (D), and patients with frontotemporal dementia (E). Diagonal lines indicate an area of 50%, indicating no difference in marker levels between groups.

ies, however, were all 3 p-tau protein assays applied in the same set of subjects and patients to look at differential diagnostic assay accuracy. With the advent of international large-scale multicenter trials on putative biomarkers and neuroimaging in AD, the important issue has not yet been addressed of how p-tau assays perform in general and whether there are potential relevant differences in assay performance, particularly in diagnostic sensitivity and specificity. Moreover, it is not yet known whether a combination of different p-tau epitopes or assays might improve diagnostic accuracy.

Our group showed that concentrations of all 3 p-tau proteins were equally significantly increased in patients with AD compared with the other groups studied. This finding is in strong agreement with all previously reported results.[1,3,5,8-10]

In a next step, discriminative power of the individual p-tau proteins was studied. To interpret the clini-

Table 4. Sensitivity, Specificity, and Correctly Allocated Cases (CACs) for Group Comparisons Derived From Tree Analysis*

AD Group vs	Marker Step I	Sensitivity	Specificity	CACs	Marker Step II	Sensitivity	Specificity	CACs
Non-AD	p-Tau$_{231}$	0.87	0.85	0.86	p-Tau$_{181}$	0.95	0.77	0.86
Controls	p-Tau$_{231}$	0.98	0.91	0.97				
ONDs	p-Tau$_{231}$	0.96	0.91	0.95				
DLB/FTD/VaD	p-Tau$_{231}$	0.86	0.75	0.83	p-Tau$_{181}$	0.94	0.66	0.85
DLB	p-Tau$_{181}$	0.94	0.64	0.88				
FTD	p-Tau$_{231}$	0.88	0.92	0.89				

Abbreviations: AD, Alzheimer disease; DLB, dementia with Lewy bodies; FTD, frontotemporal dementia; Non-AD, all demented and nondemented non-AD groups studied; ONDs, other neurologic disorders; p-tau, phosphorylated tau protein; VaD, vascular dementia.

*The analysis considered all 3 p-tau markers simultaneously to create cutoff values that maximize separation between groups, resulting in a decision tree. The number of branches depends on the separation that has been achieved at the first split. To avoid overfittng of the data, generation of further branches was interrupted if sensitivity or specificity fell below 80% (eg, see AD vs non-AD and AD vs DLB/FTD/VaD).

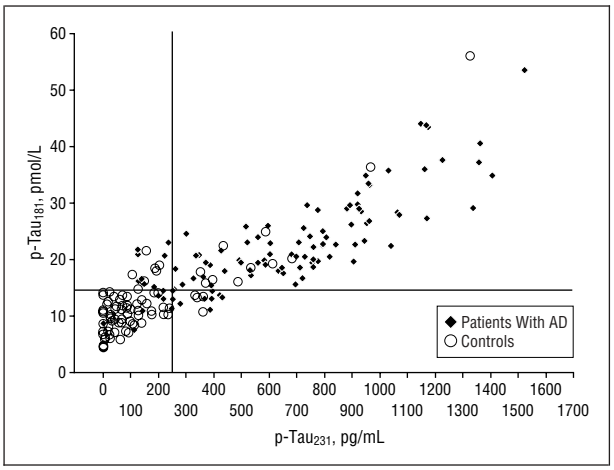

Figure 3. Group discrimination for Alzheimer disease (AD) vs non-AD using phosphorylated tau protein (p-tau)$_{231}$ and p-tau$_{181}$ derived from tree analysis. The vertical and horizontal lines indicate the cutoffs for cerebrospinal fluid p-tau$_{231}$ and p-tau$_{181}$, respectively, derived from tree analysis.

cal significance of the determined diagnostic accuracy, we followed the recommendations of a consensus report[12] for useful (ideal) biomarkers of AD, determining specificity levels after the sensitivity level is 85% or higher. According to a consensus report on molecular and biochemical markers of AD, a useful (ideal) biomarker should yield a specificity level of at least 75% to 85%. In the differentiation of AD from non-AD, p-tau$_{231}$ and p-tau$_{181}$, but not p-tau$_{199}$, reached the recommended specificity level. All 3 p-tau proteins, however, showed excellent specificity levels when patients with AD were compared with nondemented controls.

For the clinically relevant differential diagnosis of AD, it is essential to have a marker or a set of markers that discriminates AD from other clinically relevant dementias. Therefore, we included patients with FTD, DLB, and VaD as well. In the discrimination between AD and FTD, p-tau$_{231}$ and p-tau$_{181}$ fulfilled the proposed biomarker criteria for sensitivity and specificity. Comparison of the markers revealed that p-tau$_{231}$ and p-tau$_{181}$ discriminated better than p-tau$_{199}$ between the AD group and the combined non-AD group and between AD and FTD.

The high level of discrimination between AD and FTD may originate in distinct differences in the biochemical and molecular signatures of tau-related pathophysiological changes between the 2 diseases.[23] There was an increase in the p-tau protein level in some patients with DLB and VaD. Concomitant AD-type neuropathological changes in the brain, including neurofibrillary tangles, has been described for many patients with VaD[24] and DLB[25] who are clinically indistinguishable from those with "pure" VaD and DLB, respectively. In a clinical setting, it has to be assumed that patients with VaD and DLB are heterogeneous regarding underlying AD characteristic neuropathological changes in the brain, resulting in an increase in the p-tau protein level in at least some patients with VaD and DLB.

Using marker combinations did not add discriminative power compared with applying single markers. This might be a consequence of the high intercorrelation of the markers and the accurate discrimination between groups applied as single markers. Group separation was maximized between AD and FTD using p-tau$_{231}$ and between AD and DLB using p-tau$_{181}$.

In addition, we considered the effect of potentially confounding factors on CSF p-tau levels to assess the clinical applicability of p-tau proteins. Mini-Mental State Examination score accounted for approximately 5% of the variance in p-tau levels, being significant only for p-tau$_{199}$. This effect was not reported in previous studies,[5,8] and it should be followed in independent samples. There was no effect of age and sex on levels of p-tau. In addition, different diagnostic centers did not affect variance of p-tau levels. These findings indicate that p-tau proteins may be valuable markers for the clinical diagnosis of AD irrespective of age, sex, and diagnostic center.

To our knowledge, this is the first comparative study applying 3 developed and published immunoassays detecting different p-tau epitopes on the same set of subjects and patients to compare the individual and combined diagnostic accuracy. The results of this study indicate that all 3 p-tau assays perform nearly equally well in discriminating patients with AD from nondemented controls. Both p-tau$_{231}$ and p-tau$_{181}$ fulfill the proposed criteria for useful biomarkers in the differentiation of AD and non-AD and particularly of AD and FTD.

Although there is no doubt that tau phosphorylation differs in AD, it is hard to speculate why. There have been few studies of phosphoserine 199 and phosphothreonine 181 in the human brain. Except for one study,[22]

all we really know about these sites is that they are phosphorylated in advanced AD neuropathological changes. The antibodies used in the 181 and 199 assays have not been investigated to the same extent as the antibodies to phosphothreonine 231. It is well established that phosphorylation of threonine 231 is a very early event in AD, occurring before the formation of paired helical filaments in neurons of the hippocampus.[21] According to Augustinack and collegues,[22] phosphorylation at threonine 181 and serine 199 occurs later, and these are only found to any appreciable extent in intracellular tangles. Reactivity to TG3, an antibody that recognizes phosphothreonine 231, is found in pretangles, intracellular tangles, and extracellular tangles and so is present at all stages of the disease. Augustinack and colleagues also suggest that several kinases can phosphorylate 199 and 231, but only extracellular regulated protein kinase 2 phosphorylates 181. We suggest further investigations of the temporal sequence of phosphorylations of the 3 sites, using the same antibodies as used in the CSF assays. Moreover, other potentially pathophysiologically relevant p-tau epitopes, such as serine 396 and serine 404, need to be further explored in their ability to differentiate between relevant dementia disorders.[26]

Another relevant issue is to distinguish patients with mild cognitive impairment (MCI) from controls and particularly to predict AD in MCI. We showed that CSF p-tau$_{231}$ levels are elevated in patients with MCI compared with controls.[27] In this longitudinal study, high p-tau$_{231}$ levels at baseline correlated with the rate of cognitive decline in Mini-Mental State Examination scores in patients with MCI. A subgroup of patients with MCI converted to AD. In agreement with the analysis of rates of cognitive decline, increased levels of p-tau$_{231}$ correlated with conversion to AD. De Leon and colleagues[28] showed a longitudinal increase in p-tau$_{231}$ levels in patients with MCI. Elevated levels of CSF p-tau$_{181}$ in patients with MCI compared with controls have also been shown.[29] Future studies are warranted to further explore CSF p-tau proteins in MCI and to compare their diagnostic and prognostic value.

This study was conducted in an academic clinical setting. Diagnoses were performed by experienced dementia experts according to National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association criteria,[13] with an estimated positive predictive value of 89% to 100%.[30] Part of our sample is enrolled in an ongoing neuropathological program designed to provide autopsy-confirmed diagnoses. In addition to autopsy-confirmed determination of assay performance, population-based studies are warranted to establish CSF p-tau proteins as potential biomarkers for routine diagnostic use. These studies are currently under way in international large-scale multicenter approaches. One large network, the National Institute on Aging Initiative on Neuroimaging in Alzheimer's Disease, will potentially start to evaluate neuroimaging this year, as well as an array of potential biomarkers in a 5-year longitudinal approach in 650 individuals (patients with MCI, patients with AD, and controls). In their recent proceedings, a subconsortium, the Biological Marker Working Group, has determined that measurement of CSF p-tau levels is a "feasible core marker" within the National Institute on Aging initiative.[31]

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