Biochemical Diagnosis of Alzheimer Disease by Measuring the Cerebrospinal Fluid Ratio of Phosphorylated tau Protein to \(\beta\)-Amyloid Peptide\(_{42}\)

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**Background:** The antemortem diagnosis of Alzheimer disease (AD) requires time-consuming and costly procedures. Therefore, biochemical tests that can direct the physician rapidly to the correct diagnosis are highly desirable. Measurement of single biochemical markers in cerebrospinal fluid (CSF), such as total tau protein and \(\beta\)-amyloid peptide\(_{42}\) (A\(_{\beta}\)\(_{42}\)), shows robust alterations that highly correlate with the clinical diagnosis of AD but generally lack sufficient diagnostic accuracy.

**Objective:** To study the combination of CSF phosphorylated tau protein (phospho-tau) and A\(_{\beta}\)\(_{42}\) as biochemical markers for AD.

**Methods:** We combined CSF measurements of phospho-tau and A\(_{\beta}\)\(_{42}\) in 100 consecutive patients who underwent diagnostic workup for dementia and in 31 healthy control subjects.

**Results:** We found that the calculated ratio of phospho-tau to A\(_{\beta}\)\(_{42}\) was significantly increased in patients with AD and provided high diagnostic accuracy in distinguishing patients with AD from healthy control subjects (sensitivity, 86%; specificity, 97%), subjects with non-AD dementias (sensitivity, 80%; specificity, 73%), and subjects with other neurological disorders (sensitivity, 80%; specificity, 89%).

**Conclusion:** The diagnostic usefulness of the CSF ratio of phospho-tau to A\(_{\beta}\)\(_{42}\) is superior to either measure alone and can be recommended as an aid to evaluating individuals suspected of having dementia.

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**DIAGNOSING ALZHEIMER disease (AD) and distinguishing it from other dementias depends primarily on clinical evaluation, and, ultimately, on investigator judgment.** This procedure is time consuming and costly, requiring neurological examinations, neuropsychological testing, neuroimaging, and blood investigations. Despite such investigational efforts, diagnostic accuracy is less than 80% to 90%, in particular in early stages of the disease, as demonstrated by clinico-pathological comparisons. Therefore, the availability of biochemical markers that, at least in part, replace those clinical procedures, is highly desirable. Candidate diagnostic markers were identified by quantitating proteins associated with the characteristic histopathological hallmarks of AD: \(\beta\)-amyloid plaques and neurofibrillary tangles. Cerebrospinal fluid (CSF) levels of \(\beta\)-amyloid peptide\(_{42}\) (A\(_{\beta}\)\(_{42}\)) and total tau protein, as well as combinations of the two, corroborated the clinical diagnosis of AD but without appropriate diagnostic accuracy. The development of refined assays—for example, for phosphorylated tau protein (phospho-tau)—improved the separation of AD from other dementias but did not improve the sensitivity of detecting AD. Since measurement of total tau and A\(_{\beta}\)\(_{42}\) failed to reach sufficient diagnostic accuracy, we combined CSF measurements of phospho-tau and A\(_{\beta}\)\(_{42}\) in 100 consecutively recruited patients who underwent diagnostic workup for dementia and in 31 healthy control subjects. We found that calculation of the ratio of phospho-tau to A\(_{\beta}\)\(_{42}\) resulted in high diagnostic accuracy and may, therefore, constitute a diagnostic tool that is suitable for routine clinical use.

**METHODS**

**SUBJECTS**

We examined 100 outpatients (46 women, 54 men) who underwent diagnostic workup for...
dementia in our memory disorders unit after referral by the local general practitioner; community health services; or specialists in neurology, psychiatry, or geriatrics, as well as clinic-based neurological services. Patients underwent thorough clinical examination, including providing medical and family history; neurological, internal, and psychiatric examinations; routine laboratory testing; neuropsychological testing (eg, Consortium to Establish a Registry for Alzheimer Disease battery; selected neuropsychological tests); and computed tomographic or magnetic resonance imaging of the brain. The acceptance rate for lumbar puncture was 89%. The study covered 2 years—January 2000 to December 2001. Clinical diagnoses were made according to established international criteria.³⁴ Thirty-one healthy control subjects (11 women, 20 men; mean age ± SD, 64.2 ± 11.8 years; range, 41-84 years) were recruited among cognitively intact patients receiving spinal anesthetic before surgical intervention. Written informed consent was obtained from all patients and caregivers prior to inclusion. This study was approved by the local ethics committee for human studies.

Fifty-one patients (mean age ± SD, 70.1 ± 8.7 years; range, 51-87 years) fulfilled the criteria for probably having AD. Thirty patients (mean age ± SD, 66.3 ± 11.2 years; range, 40-90 years) had non-AD dementias: vascular dementia, 8; cerebral amyloid angiopathy, 2; Lewy body dementia, 2; frontotemporal lobe dementia, 3; Parkinson dementia, 4; progressive supranuclear palsy, 1; corticobasal degeneration, 2; Creutzfeldt-Jakob disease, 3; Huntington disease, 2; cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, 2; neuroacanthocytosis, 1. Nineteen patients (mean age ± SD, 67.1 ± 8.7 years; range, 49-84 years) had other neurological disorders without dementia: amyotrophic lateral sclerosis, 13; vascular encephalopathy, 2; multisystem atrophy, 2; multiple sclerosis, 1. Dementia severity was mild to moderate, with Mini-Mental State Examination scores of 21 ± 3.5 in patients with AD, 21 ± 5.7 in subjects with non-AD dementias, and 28 ± 1.7 in subjects with other neurological disorders without dementia. Cerebrospinal fluid was obtained by means of lumbar puncture in all patients within 1 week after neuropsychological testing, and 0.25-mL aliquots were produced, frozen on dry ice immediately at withdrawal at the bedside, and stored at −85°C until analysis.

Aβ42 ENZYME-LINKED IMMUNOSORBENT ASSAY

We used a sandwich enzyme-linked immunosorbent assay (ELISA; INNOTEST β-Amyloid [1-42], Innogenetics, Gent, Belgium) with monoclonal antibody 21F12 specific for the free C-terminal end of Aβ42 (β-amylloid peptide,α1-42) as the capturing antibody. Monoclonal antibody 3D6 specific for the N-terminal end of Aβ42 (β-amylloid peptide,α1-42) was used as the detector antibody. After washing 5 times at room temperature, horseradish peroxidase-labeled streptavidin and subsequently 3,3',5,5'-tetramethylbenzidine were added. Absorbance was read at 450 nm on a microplate reader (Victor2 Multilabel; EG&G Wallac, Turku, Finland). The linear range of the assay was 30 pg/mL to 2 ng/mL. There was no cross-reactivity with β-amyloid peptide,α40. The CSF samples and the standards were assayed in duplicates.

PHOSPHO-TAU ELISA

Monoclonal antibody AT270, specific for tau proteins phosphorylated at threonine 181, was used in a sensitive sandwich ELISA (INNOTEST Phospho-tau [Thr214], Innogenetics). Microtiter plates precoated with HT7 (tau epitope 159-163) were incubated with 75-µL CSF samples. After washing, the biotinylated monoclonal antibody AT270 was used as the detector antibody. After incubation with peroxidase-conjugated strep-tavidin and a final washing step, 3,3',5,5'-tetramethylbenzidine was added as chromogen. Absorbance was read at 450 nm. The CSF samples and the standards were assayed in duplicates.

GENOTYPING

Leukocyte DNA was isolated according to standard protocols. Apolipoprotein E genotyping was performed as described by Nauck et al.¹³

STATISTICAL ANALYSIS

Diagnostic accuracy was assessed by using receiver operating characteristic (ROC) analysis. This method is described in detail elsewhere.¹⁴ It permits calculation of overall test performance by considering sensitivity/specificity pairs for every possible threshold of a test. The resulting ROC curve can be used for estimation of the optimal cutoff according to the costs of false-positive and false-negative results. In the present study, the optimal cutoff was defined as the point on the ROC curve where the product of the corresponding sensitivity/specificity pair reached a maximum. This cutoff implies that the costs for false-positive and false-negative rates are considered equal. The area under the ROC curve was used as an indicator of test performance and was calculated according to nonparametric methods.¹⁵ Analysis of variance with Bonferroni-corrected post hoc comparisons was used for the assessment of statistical differences in CSF Aβ42 and phospho-tau levels between diagnostic groups. The Pearson product moment correlation was used to assess the relationship between the Mini-Mental State Examination score and ratio of phospho-tau to Aβ42. Statistical significance was assumed at P ≤ .05.

Cerebrospinal fluid levels of Aβ42 were lower in patients with AD (0.42 ± 0.19 ng/mL), as compared with levels in healthy control subjects (0.73 ± 0.22 ng/mL; P < .001), subjects with non-AD dementias (0.64 ± 0.33 ng/mL; P < .01), and subjects with other neurological disorders (0.85 ± 0.33 ng/mL; P < .001) (Figure 1). Cerebrospinal fluid levels of phospho-tau were higher in patients with AD (52 ± 19 pg/mL), as compared with levels in healthy control subjects (27 ± 10 pg/mL; P < .001), subjects with non-AD dementias (37 ± 18 pg/mL; P < .01), and subjects with other neurological disorders (36 ± 15 pg/mL; P < .01). The CSF ratio of phospho-tau to Aβ42 was higher in patients with AD (147 ± 80), as compared with that in healthy control subjects (39 ± 23; P < .001), subjects with non-AD dementias (74 ± 60; P < .001), and subjects with other neurological disorders (48 ± 28; P < .001). Cerebrospinal fluid levels of both Aβ42 and phospho-tau, as well as the ratio of phospho-tau to Aβ42, were independent of the apolipoprotein E genotype.

The ROC analyses were performed for CSF levels of Aβ42 and phospho-tau and the ratio of phospho-tau to Aβ42 (Table, Figure 2). The ROC analysis of the CSF ratio of phospho-tau to Aβ42 revealed good separation of patients with AD from healthy control subjects (area under the ROC curve, 0.934; P < .001); similar separation was achieved when comparing patients with AD and subjects with other neurological disorders without dementia (area under the ROC curve, 0.906; P < .001). The sepa-
We found that measurement of the CSF ratio of phospho-tau to Aβ42 separated with excellent diagnostic accuracy patients with AD from healthy control subjects, as well as from subjects with other dementias and neurological disorders. Sensitivity and specificity were markedly higher, as compared with CSF measurement of total tau,16,17 phospho-tau,18 or total tau and Aβ42 combined (analyzed both by classification tree16,17 and by linear functions),17 as well as with the three parameters total tau, β-amyloid peptide40, and Aβ42 combined.21 The advantage of the present marker combination may lie in the use of phospho-tau instead of total tau and the use of Aβ42 to calculate the ratio.

The separation of patients with AD from elderly healthy control subjects, with a specificity of 97% and a sensitivity of 86%, may be attractive for general medical practice, provided that further testing of the CSF ratio of phospho-tau to Aβ42 consistently demonstrates added value to the usually brief clinical evaluation. The usefulness of clinical evaluation in diagnosing memory disorders should not be underestimated. According to the consensus report of the Working Group on Molecular and Biochemical Markers of Alzheimer’s Disease,24 the ideal biochemical marker for AD should have a sensitivity of more than 80% for detecting AD and a specificity of more than 80% for distinguishing other dementias. Measurement of the CSF ratio of phospho-tau to Aβ42 meets the guideline for sensitivity and comes close to meeting the guideline for specificity. Particularly, specificity close to 100% in distinguishing patients with AD from healthy control subjects is highly desirable to minimize false-positive AD diagnoses. The lesser sensitivity is acceptable because there are no true preventive or disease-modifying treatments available, so missing a few AD diagnoses may deprive patients of treatment for their symptoms but not of treatment for AD itself. There was no clear correlation between the CSF ratio of phospho-tau to Aβ42 and dementia severity, which suggests that the diagnostic potential of this measure is applicable to a broad spectrum of mild to moderate and advanced stages of the disease. The reliability and general application of the cutoff values determined here require further studies using independent groups of patients. In addition, the present measurements may be further evaluated in patient populations with a higher number of individuals aged 80 years or older.

Biochemical marker measurements in most previous studies were performed in residual CSF samples frozen for research purposes.4 In contrast, our study design was prospective and not biased by specific research criteria because we examined consecutive patients who were seen for diagnostic purposes. Similarly, Andreasen et al20 added CSF investigations to a population-based study to approach the clinical practice setting. Ideally, the patients should be monitored until the clinical diagnosis can be confirmed post mortem. Therefore, a histopathological confirmation study is under way to test the extent to which diagnosis of AD on the basis of measuring the CSF ratio of phospho-tau to Aβ42 correlates with the neuropathological diagnosis.
Patients were examined consecutively in our memory disorders unit after referral by the local general practitioner; community health services; or specialists in neurology, psychiatry, or geriatrics, as well as clinic-based neurological services, without specific preselection except that the patients were suspected of having memory impairment. However, because of this referral system, selection biases may have occurred, and the recruited patients may not have been entirely representative of the primary care practice. Since lumbar puncture is usually not performed in the primary care setting, we may have to consider this issue as an inherent limitation of CSF studies.

Measurement of the CSF ratio of phospho-tau to Aβ42 provides a biochemical diagnostic aid that may replace some of the current clinical investigational efforts and thereby speed up the diagnostic procedure and reduce its cost. Measurement of the CSF ratio of phospho-tau to Aβ42 was used to calculate the area under the receiver operating characteristic curve (ROC) for each group compared to the AD group.

### Measures of Diagnostic Accuracy of CSF Levels of Aβ42 and Phospho-tau and the Ratio of Phospho-tau to Aβ42

<table>
<thead>
<tr>
<th>Group and Measure*</th>
<th>Aβ42 Level</th>
<th>Phospho-tau Level</th>
<th>Ratio of Phospho-tau to Aβ42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control subjects (n = 31)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area under the ROC curve</td>
<td>0.872</td>
<td>0.887</td>
<td>0.934</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.792-0.951</td>
<td>0.815-0.969</td>
<td>0.879-0.990</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Cutoff</td>
<td>0.49 ng/mL</td>
<td>33 pg/mL</td>
<td>86</td>
</tr>
<tr>
<td>Sensitivity, %</td>
<td>78</td>
<td>84</td>
<td>97</td>
</tr>
<tr>
<td>Specificity, %</td>
<td>90</td>
<td>84</td>
<td>98</td>
</tr>
<tr>
<td>Positive predictive value, %</td>
<td>93</td>
<td>89</td>
<td>98</td>
</tr>
<tr>
<td>Negative predictive value, %</td>
<td>72</td>
<td>74</td>
<td>81</td>
</tr>
<tr>
<td>Subjects with non-AD dementias (n = 30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area under the ROC curve</td>
<td>0.731</td>
<td>0.710</td>
<td>0.801</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.606-0.857</td>
<td>0.591-0.830</td>
<td>0.706-0.903</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Cutoff</td>
<td>0.49 ng/mL</td>
<td>35 pg/mL</td>
<td>83</td>
</tr>
<tr>
<td>Sensitivity, %</td>
<td>78</td>
<td>73</td>
<td>80</td>
</tr>
<tr>
<td>Specificity, %</td>
<td>70</td>
<td>63</td>
<td>73</td>
</tr>
<tr>
<td>Positive predictive value, %</td>
<td>82</td>
<td>75</td>
<td>84</td>
</tr>
<tr>
<td>Negative predictive value, %</td>
<td>66</td>
<td>59</td>
<td>69</td>
</tr>
<tr>
<td>Subjects with other neurological disorders without dementia (n = 19)</td>
<td></td>
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<td></td>
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<tr>
<td>Area under the ROC curve</td>
<td>0.845</td>
<td>0.725</td>
<td>0.906</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.714-0.977</td>
<td>0.592-0.858</td>
<td>0.835-0.977</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;.001</td>
<td>&lt;.004</td>
<td>&lt;.001</td>
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<tr>
<td>Cutoff</td>
<td>0.58 ng/mL</td>
<td>39 pg/mL</td>
<td>84</td>
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<tr>
<td>Sensitivity, %</td>
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<td>67</td>
<td>80</td>
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<tr>
<td>Specificity, %</td>
<td>84</td>
<td>63</td>
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</tr>
<tr>
<td>Positive predictive value, %</td>
<td>93</td>
<td>81</td>
<td>95</td>
</tr>
<tr>
<td>Negative predictive value, %</td>
<td>67</td>
<td>41</td>
<td>63</td>
</tr>
</tbody>
</table>

Abbreviations: Aβ42, β-amyloid peptide42; AD, Alzheimer disease; CI, confidence interval; CSF, cerebrospinal fluid; phospho-tau, phosphorylated tau protein; ROC, receiver operating characteristic.

*Each group is compared vs the group of patients with AD (n = 51).
to Aβ42 may also constitute a tool for monitoring disease progression, which has to be investigated within a longitudinal design. Riemenschneider et al.23 recently suggested that CSF levels of total tau and Aβ42 could be used to predict if mild cognitive impairment would become AD. However, the reported 80% to 90% diagnostic accuracy achieved with conventional antemortem diagnosis of AD was achieved at highly specialized centers; this percentage is most likely much lower outside institutions dedicated to patients with dementia.

A potential limitation for the widespread use of CSF biochemical markers in general practice lies in collecting CSF at lumbar puncture. However, the technique of lumbar puncture has considerably improved recently with the use of atraumatic needles. As a consequence, incidence of headache after lumbar puncture in elderly patients in general, including those with dementia, is 2% or less.24

By what means can both sensitivity and specificity be set at a level higher than 90%? It is probably unrealistic to expect that this goal can be reached by measuring β-amyloid peptides and tau proteins alone, because postmortem analyses of brains with AD revealed a variety of additional lesions, such as infarcts, gliosis, argyrophilic grains, and Levy bodies. In addition, other dementing conditions, such as frontotemporal lobar degeneration, progressive supra-nuclear palsy, or corticobasal degeneration, display at least some neuropathological features that overlap AD, such as tau-positive filamentous lesions. In the future, a biochemical marker pattern reflecting the whole spectrum of abnormal proteins deposited in the brain will most likely provide a more accurate diagnosis of AD, comparable with the current criteria for the neuropathological classification.25

In summary, measurement of the CSF ratio of phosphorylated tau to Aβ42 may provide a promising tool for the biochemical antemortem diagnosis of AD, and its practical usefulness may be further evaluated in routine clinical settings.

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