Amino-Truncated \(\beta\)-Amyloid\(_{42}\) Peptides in Cerebrospinal Fluid and Prediction of Progression of Mild Cognitive Impairment

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Background: Early identification of patients with mild cognitive impairment (MCI) progressing to Alzheimer disease (MCI-AD) by use of biomarkers in cerebrospinal fluid (CSF) is an essential step toward improving clinical diagnosis and drug development. We evaluated whether different \(\beta\)-amyloid\(_{42}\) (\(\beta_{42}\)) peptides can add further information to the combined use of tau and \(\beta_{42}\) for predicting risk of progression of MCI to AD.

Methods: We used xMAP\textsuperscript{\textregistered} technology to simultaneously quantify different \(\beta_{42}\) peptides modified at the amino terminus, tau, and phosphorylated tau (P-tau181P) in CSF. \(\beta_{42}\) peptide concentrations were measured by use of immunoreactivity toward \(\beta\) monoclonal antibodies \([3D6 (\beta_{42-3D6}), WO2 (\beta_{42-WO2}), 6E10 (\beta_{42-6E10}), \text{and } 4G8 ([\beta_{42-4G8}])\]. The discriminant ability of the markers was evaluated by ROC curve analysis.

Results: The areas under the curves for the separation of MCI-AD from nonprogressing MCI (MCI-N) was significantly higher when we used \(\beta_{42-3D6}/\beta_{42-WO2}\), \(\beta_{42-3D6}/\beta_{42-6E10}\), or \(\beta_{42-3D6}/\beta_{42-4G8}\) compared with \(\beta_{42-3D6}\). In addition, differentiation of MCI-N from MCI-AD was improved by quantification of full-length \(\beta_{1-42}\) (\(\beta_{42-3D6}\)) compared with \(\beta_{42-WO2}, \beta_{42-6E10}\), or \(\beta_{42-4G8}\). Several \(\beta_{42}\) peptides truncated at the amino terminus (\(\beta_{11-42}\) and \(\beta_{8-42}\)) were identified in CSF by surface-enhanced laser desorption/ionization time-of-flight technology.

Conclusion: The CSF markers tau, \(\beta_{42}\) forms, and P-tau181P, when used as adjuncts to clinical diagnosis, have the potential to help identify AD pathology and could be a valuable asset for early AD diagnosis.

Therapeutic strategies for Alzheimer disease (AD)\(^5\) should be based in part on the prevention of disease progression by initiating treatment as early as possible. It is therefore crucial to accurately identify incipient AD in patients with mild cognitive impairment (MCI). Autopsy data have revealed that AD pathology (e.g., hippocampal atrophy, neurofibrillary tangles in the hippocampus and entorhinal cortex, neuritic plaques in the neocortex, synaptic loss, and oxidative stress) can be present as long as 20 years before the clinical onset of dementia (1). However, MCI-diagnosed persons do not ultimately share the same fate; whereas some may develop AD, others progress to another dementia type [e.g., dementia with Lewy bodies (DLB), frontotemporal lobe dementia, or Parkinson disease (PD)], some will never progress to any significant extent. Individual who present at dementia clinics with MCI symptoms have a 50% probability of progressing to symptomatic AD within a 4-year period (2).

According to a published consensus report, an ideal biological marker should have a clinical sensitivity $\geq 80\%$ for detecting AD and a clinical specificity $\geq 80\%$ for distinguishing AD from control or other dementia types.
(3). These criteria can be achieved by use of protein determinations of cerebrospinal fluid (CSF), which is a continuum of the brain and the spinal cord interstitial fluid. CSF is an obvious source of markers reflecting central neuropathologic features of the disease [e.g., neuronal degeneration, the disturbance of β-amyloid (Aβ) metabolism with formation of plaques, and hyperphosphorylation of tau leading to formation of neurofibrillary tangles]. The most promising markers at present are total tau, phosphorylated tau, and β-amyloid42 (Aβ42). Different immunoassays for these proteins have been described (4).

Since the discovery of Aβ as the major constituent of neuritic plaques in AD (5), several amino (N)-terminally truncated as well as carboxy (C)-terminally elongated Aβ peptides have been identified in the brain, cell culture supernatant, and CSF by different methodologies (6–8). Truncated Aβ has been described in 35% of AD patients, based on immunoreactivity toward specific monoclonal antibodies (mAbs) (9); these truncated forms represent more than 60% of all Aβ species and function as a nucleus for Aβ oligomerization and deposition into neuritic plaques (8). The specific chemical nature and relative distributions of Aβ peptides at the very early stages of Aβ deposition, their presence in biological fluids in different stages of the disease process, and their discriminating properties are still unclear.

We used the xMAP technology (Luminex) (10) to design and evaluate the ability of a new multiplexed bead-based assay format to discriminate Aβ peptides. The previously described bead immunoassay format (INNOBIA Alzbio3; for research use only) provided possibilities for simultaneous quantification of Aβ42-40, total tau, and tau phosphorylated at Thr181 (P-tau181P) in a small volume of sample with high precision (11). This report presents results obtained with a bead immunoassay for quantification of Aβ42 forms, tau, and P-tau181P. A research study was performed with CSF samples obtained from healthy controls, patients with AD, patients with MCI who progressed to AD (MCI-AD) within a period of 3 years, patients with MCI but who did not progress to AD (MCI-N), and patients with other neurologic disorders (PD or DLB).

**Materials and Methods**

**CHEMICALS**

All reagents were of analytical grade. Synthetic peptides were obtained from Bachem, Neosystems, or AnaSpec. The characteristics of the calibrators (recombiant tau, Aβ1–42, and a phosphorylated tau peptide) have been described in detail elsewhere (12, 13). mAbs 6E10 and 4G8 were obtained from Signet Laboratories, and WO2 was obtained from The Genetics Company. The INNOTEST® β-AMYLID(1–42), INNOTEST hTAU Antigen, and INNOTEST PHOSPHO-TAU (181P) were from Innogenetics NV.

**ANTIBODY CHARACTERISTICS AND COUPLING ON MICROSPHERES**

The characteristics of the mAbs generated against tau (H17 and BT2) and P-tau181P (AT270) have been described previously in detail (12). The analytical specificities of some of the N-terminal-specific mAbs (3D6, 6E10, 4G8, and WO2) used in the present study have been further evaluated by use of sandwich immunoassays (ELISA format). Plates were coated with the N-terminal–specific mAbs in phosphate-buffered saline (PBS) solution at a concentration of 5 mg/L. After a block-and-wash step, the Aβ peptides were incubated simultaneously with the biotinylated detector antibody (21F12) for 1 h at room temperature. After a wash step, peroxidase-labeled streptavidin (RDI) was used for quantification. The Aβ42-peptide concentration between assay formats was normalized with respect to immunoreactivity toward binding to 4G8. Aβ42 peptides binding to 3D6, 4G8, or 6E10 are further described in the text as Aβ42-3D6, Aβ42-WO2, Aβ42-6E10, or Aβ42-6E10.

**xMAP TECHNOLOGY**

The xMAP technology is a flow cytometric method involving covalent coupling of a capture mAb to spectrally specific fluorescent microspheres. Because different microsphere sets can be combined within one method and each bead number is linked with only one antibody, unequivocal identification of the analyte in the mixture is possible. Differences from classic immunoassays include multianalyte testing, lower sample volumes, fewer handling steps, less total hands-on time, and only one test protocol for all analytes. Carboxylated beads were chemically coupled with mAbs, as described by Olsson et al. (11). More technical details on selected assay components are described in Table 1.

**METHODS FOR BEAD-BASED xMAP TECHNOLOGY**

All CSF incubations were performed at room temperature (defined as 18–30 °C) and in the dark (plates covered with aluminum foil). A 96-well filter plate (Millipore Corporation) was prewetted with 225 μL of wash buffer. The wash buffer was removed from the plates by use of a vacuum manifold (Millipore). Beads were sonicated and vortex-mixed before addition to the filter plates (3000 beads/well of each bead region in 100 μL of solution), followed by removal of buffer. A mixture of 2 biotinylated detector mAbs (21F12 and HT7) was added to the wells of the filter plate (25 μL/well). Subsequently, calibrators, blanks, or CSF samples were added (75 μL/well) in duplicate and incubated overnight on a plate shaker. The plate was washed 3 times with 225 μL of wash buffer. Phycoerythrin-labeled streptavidin (Caltag Laboratories) was added (100 μL/well) and incubated for 1 h on a plate shaker, after which the plate was washed 3 times with 225 μL of wash buffer. Finally, 100 μL of PBS was added to each well. Samples were incubated for 2 min on a plate shaker and then analyzed on the Luminex 100 IS; 100
beads were analyzed for each bead region. Median fluorescence values were reported. Results were excluded if the counted number for a specific bead region was too low.

**ASSAY CALIBRATION**

The calibrators used in the present assay formats were identical to those used in the ELISA methods for the respective proteins [INNOTEST β-AMYLOID(1–42)\textsubscript{p}, INNOTEST hTAU Antigen, and INNOTEST PHOSPHO-TAU\textsubscript{(181P)}]. The preparation and characteristics of these calibrators have been described in detail previously (12–15); however, the 3 proteins are combined in one vial for the research multiplexed immunoassays. All biomarker data are presented as ng/L and do not take into account possible differences in concentrations observed between ELISA assay formats and multiplexed products as was described in Olsson et al. (11).

**SURFACE-ENHANCED LASER DESORPTION/IONIZATION TECHNOLOGY**

Surface-enhanced laser desorption/ionization (SELDI-TOF) technology was used to identify Aβ\textsubscript{42} species (16). mAb 4D7A3 (Innogenetics) or a control mouse IgG was covalently linked to the PS20 ProteinChip array. Briefly, 3 µg of 4D7A3 was applied to the array spot and incubated in a humidity chamber (3 h at room temperature) to allow covalent binding to the PS20 ProteinChip array. The arrays were washed twice with Dulbecco PBS (DPBS) containing 1 mL/L Triton X-100 and twice with PBS (pH 8.0). Unreacted sites were then blocked by incubating 3 µL of 10 g/L bovine serum albumin in DPBS for 2 h at room temperature. Excess bovine serum albumin was removed by 2 washes with DPBS containing 5 mL/L Triton X-100 followed by 3 washes with DPBS. Blocking was performed with 0.5 mol/L Tris (pH 8.0) for 2 h at room temperature, after which the wash procedure described above was repeated. CSF samples (100 µL) in 0.1 mol/L urea containing 1 g/L CHAPS were loaded on a spot by use of the ProteinChip bioprocessor and incubated overnight at 4 °C with constant shaking. Two wash steps, as described above, followed by 2 additional washes with 50 mmol/L HEPES (pH 8.0) completed the reaction. After the arrays had dried, 0.8 µL of a 20% saturated solution of α-cyano-4-hydroxycinnamic acid (Ciphergen Biosystems) in 5 mL/L trifluoroacetic acid–500 mL/L acetonitrile–495 mL/L water was applied to each spot. Mass analysis was performed on a ProteinChip reader (Model PBS II; Ciphergen). For calibration purposes, 7 fmol of Aβ\textsubscript{42} peptide (AnaSpec) and 6 fmol of bovine insulin (Ciphergen) were applied and used for data calibration.

**RESEARCH STUDY**

**Samples.** The study was performed on CSF samples archived at the Sahlgrenska University Hospital, Göteborg, Sweden. Lumbar puncture was performed in the morning under standard conditions. CSF samples were taken at baseline (first visit to the memory clinic). A volume of 12 mL of CSF was collected and gently mixed to avoid gradient effects. CSF samples with >500 erythrocytes/µL were excluded from the analysis. All CSF samples were centrifuged at 2000 g for 10 min to remove cells and debris. CSF was stored in polypropylene tubes (to avoid adsorption of proteins to the test tube walls) at −80 °C until analysis. All CSF samples (75 µL/sample) were analyzed on the same day. No specific training was required.

**Specimen collection.** The diagnostic performance of the new xMAP multianalyte bead-based immunoassay was evaluated with CSF samples from 66 AD patients, 29 healthy controls, 15 patients with DLB, 15 with PD, and 41 with MCI. Recruitment of the patients was based on admission to the hospital for evaluation of cognitive symptoms. The control material was collected from healthy volunteers. Of the MCI patients who were followed up for a period of 3 years, 15 progressed to AD (MCI-AD), whereas 26 patients did not (MCI-N). Clinical data for the patients are given in Table 2. The control group consisted of individuals without histories, symptoms, or signs of psychiatric or neurologic disease, malignant disease, or systemic disorders (e.g., rheumatoid arthritis or infectious diseases). All patients underwent a thorough clinical investigation, which included a medical history; physical, neurologic, and psychiatric examinations; screening laboratory tests; an electroencephalogram; and a computerized tomography scan of the brain. The diagnosis of probable AD was made according to the National Insti-

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**Table 1. Assay format.**

<table>
<thead>
<tr>
<th>Assay*</th>
<th>Protein</th>
<th>Coating</th>
<th>Capture</th>
<th>Calibrator</th>
</tr>
</thead>
<tbody>
<tr>
<td>INNO-BIA</td>
<td>Tau</td>
<td>BT2</td>
<td>HT7</td>
<td>Recombinant tau</td>
</tr>
<tr>
<td>Research format</td>
<td>P-Tau(_{181P})</td>
<td>AT270</td>
<td>Phosphorylated peptide</td>
<td></td>
</tr>
<tr>
<td>Aβ(_{42})</td>
<td>3D6</td>
<td>Aβ peptide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ(_{42})</td>
<td>6E10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ(_{42})</td>
<td>4G8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Immuonoassays were developed with the xMAP-technology.
Table 2. Demographic data for the patients enrolled in the present study.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. in group (M/F)</th>
<th>Age, years</th>
<th>MMSE(^{a,b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>66 (19/47)</td>
<td>77.5 (73.3–82)</td>
<td>20.5 (5–28)</td>
</tr>
<tr>
<td>Controls</td>
<td>29 (10/19)</td>
<td>66 (63–71)</td>
<td>30 (28–30)</td>
</tr>
<tr>
<td>MCI-AD</td>
<td>15 (7/8)</td>
<td>77 (74.3–82.8)</td>
<td>29 (28–30)</td>
</tr>
<tr>
<td>MCI-N</td>
<td>26 (15/11)</td>
<td>63 (56–69.8)</td>
<td>29.5 (25–30)</td>
</tr>
<tr>
<td>DLB</td>
<td>15 (9/6)</td>
<td>79 (76–80)</td>
<td>23 (17–27)</td>
</tr>
<tr>
<td>PD</td>
<td>15 (11/4)</td>
<td>71 (63–76.5)</td>
<td>29 (25–30)</td>
</tr>
</tbody>
</table>

\(^{a}\)Median (range).
\(^{b}\)Median (25th–75th percentiles).
\(^{c}\)MMSE, Mini Mental State Examination.

Ethics
The Ethics Committees of the Universities of Göteborg and Umeå approved the study. All patients or their relatives gave informed consent to participate in the study.

Statistics
For the different analytes, the concentrations were compared among the treatment groups by all possible pairwise \(t\)-tests. The normality assumptions were verified by use of QQ plots. For each analyte, a Bonferroni adjustment was made to adjust for multiple testing. Analyses were performed in SAS (Ver. 9.1). Correlations are reported in terms of the Spearman rank correlation coefficient together with a test for difference from 0. The relationship between sensitivity and specificity for the biomarkers was described by ROC curve analysis. The area under the curve (AUC) was calculated by use of the Medcalc Program (Medcalc Software).

Results
Antibody characterization
Epitope mapping of different N-terminal–specific mAbs (3D6, 6E10, 4G8, and WO2) against the \(A\beta_{42}\) peptide was done with sandwich immunoassays with biotinylated 21F12 as detection antibody; the results are shown in Fig. 1. In contrast to 3D6, all other assay formats for \(A\beta_{42}\) detection were not specific for \(A\beta_{1–42}\). mAbs 4G8, 6E10, and WO2 recognized not only the full-length \(A\beta_{1–42}\) but also \(A\beta_{42}\) peptides truncated at the NH\(_2\) terminus. The antibody pair 3D6/21F12 preferably quantified full-length \(A\beta_{1–42}\), whereas all other combinations (21F12/6E10, 21F12/4G8, and 21F12/WO2) quantified \(A\beta_{N–42}\). The 6E10/21F12 antibody pair was able to detect \(A\beta_{42}\) peptides from \(A\beta_{1–42}\) to \(A\beta_{5–42}\), but \(A\beta_{8–42}\) and \(A\beta_{9–42}\) were not detected. The WO2/21F12 pair detected only \(A\beta_{1–42}\) and \(A\beta_{2–42}\). The 4G8/21F12 combination detected all peptides used in the present study, independent of the NH\(_2\) terminus.

Comparison of formats
The concentrations of \(A\beta_{1–42}\), tau, and P-tau\(_{181P}\) measured by a research version of the INNO-BIA AlzBio3 (11) and those obtained by the present assay format were correlated: \(r = 0.824 \quad (P < 0.01; n = 159) \quad \text{for} \quad A\beta_{1–42}; 0.922 \quad (P < 0.01; n = 159) \quad \text{for} \quad \text{tau}; \quad 0.921 \quad (P < 0.01; n = 143) \quad \text{for} \quad \text{P-tau}_{181P}. \) In addition, the correlations \((r)\) between results of the present assay format and the classic ELISA immunoassays (INNOTEST) for the same analytes were 0.863 \((P < 0.01; n = 165) \quad \text{for} \quad A\beta_{1–42}; 0.865 \quad (P < 0.01; n = 165) \quad \text{for} \quad \text{tau}; \quad 0.900 \quad (P < 0.01 \text{ for values} < 200 \text{ ng/L in the INNOTEST}; \quad n = 162) \quad \text{for} \quad \text{P-tau}_{181P}. \)

Research study
We used the xMAP-based assay format to compare the concentration of each biomarker in CSF for different patient groups. \(A\beta_{3–42D6}\) was significantly decreased in the AD \((P < 0.01), \text{DLB} \quad (P < 0.01), \text{PD} \quad (P = 0.0072), \text{MCI-N} \quad (P < 0.01), \text{and} \text{MCI-AD} \quad (P < 0.01)\) groups compared with controls, and was significantly different in the MCI-N \((P < 0.01), \text{MCI-AD} \quad (P < 0.01), \text{and} \text{PD} \quad (P < 0.01)\) groups compared with the AD group.

\(A\beta_{42,WO2}\) was significantly decreased in AD \((P < 0.01), \text{DLB} \quad (P < 0.01), \text{MCI-N} \quad (P < 0.01), \text{and} \text{PD} \quad (P < 0.01)\) patients compared with controls, and was significantly higher in MCI-N \((P = 0.018), \text{MCI-AD} \quad (P < 0.01), \) and PD \((P < 0.01)\) patients compared those with AD. \(A\beta_{42,4G8}\) was significantly decreased in the AD \((P < 0.01), \text{DLB} \quad (P < 0.01), \text{PD} \quad (P = 0.033), \text{and} \text{MCI-N} \quad (P < 0.01)\) groups, but not the MCI-AD group \((P = 0.431)\), compared with controls and was significantly higher in patients with MCI-N \((P = 0.0458), \text{MCI-AD} \quad (P < 0.01), \) or PD \((P < 0.01)\) compared with those with AD. \(A\beta_{42,6E10}\) was significantly
decreased in the patients with AD ($P < 0.01$), DLB ($P < 0.01$), PD ($P < 0.01$), MCI-N ($P < 0.01$), or MCI-AD ($P = 0.039$) compared with controls, and was significantly higher in patients with MCI-N ($P = 0.028$), MCI-AD ($P < 0.01$), or PD ($P = 0.021$) compared with those with AD.

For tau, concentrations were significantly increased in the AD ($P < 0.01$) and MCI-AD ($P < 0.01$) groups compared with controls, and was significantly lower in patients with DLB ($P < 0.01$), MCI-N ($P < 0.01$), or PD ($P < 0.01$). No difference was observed for AD vs MCI-AD ($P = 0.574$).

P-tau$_{181P}$ concentrations were significantly increased in patients with AD ($P < 0.01$) or MCI-AD ($P < 0.01$) compared with controls and was significantly lower in patients with DLB ($P < 0.01$), MCI-N ($P < 0.01$), or PD ($P < 0.01$) compared with those with AD (Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue9/), whereas concentrations in MCI-AD patients were not statistically different ($P = 0.704$).

In subsequent analyses, we compared CSF from MCI-AD patients with CSF from MCI-N patients. In contrast to tau ($P < 0.01$), P-tau$_{181P}$ ($P < 0.01$), or a minor difference for A$eta_{42-4G8}$ ($P = 0.029$), there was no evidence of a difference between MCI-AD and MCI-N patients for the concentrations of A$eta_{42-3D6}$ ($P = 0.364$), A$eta_{42-6E10}$ ($P = 0.239$), or A$eta_{42-6E10}$ ($P = 0.106$; Fig. 2). However, when we used the ratios A$eta_{42-3D6}$/$Aeta_{42-6E10}$ ($P < 0.01$), A$eta_{42-3D6}$/$Aeta_{42-4G8}$ ($P < 0.01$), and A$eta_{42-3D6}$/$Aeta_{42-WO2}$ ($P < 0.01$), we observed a difference among the groups (Fig. 3). This

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**Fig. 1.** Epitope mapping of N-terminal–specific amyloid mAbs by ELISA. A$eta_{42}$ peptides: ■, A$eta_{1-42}$; ○, A$eta_{2-42}$; +, A$eta_{3-42}$; ×, A$eta_{4-42}$; ●, A$eta_{5-42}$; △, A$eta_{6-42}$; †, A$eta_{9-42}$. Experimental details are described in the Materials and Methods. Results are the mean of 2 measurements.
A noteworthy result needs to be confirmed in further studies with MCI patients, including an age-matched contrast group. In the present study, the MCI-N group was younger than the MCI-AD group.

There was no evidence of a difference in P-tau181P ($P = 0.704$), tau ($P = 0.574$), Aβ$_{42}$-3D6/Aβ$_{42}$-6E10 ($P = 0.103$), Aβ$_{42}$-3D6/Aβ$_{42}$-4G8 ($P = 0.212$), or Aβ$_{42}$-3D6/Aβ$_{42}$-4G8 ($P = 0.142$) between AD and MCI-AD patients (Fig. 3), confirming the progression of the MCI-AD group to AD pathology.

ROC analysis was performed on samples for which all Aβ values were available (AD, n = 59; MCI-AD, n = 14; MCI-N, n = 24). This analysis revealed that the AUC for the separation of MCI-AD and MCI-N patients was significantly higher when the ratios Aβ$_{42}$-3D6/Aβ$_{42}$-6E10 [0.876; 95% confidence interval (CI), 0.729–0.960; $P < 0.01$], Aβ$_{42}$-3D6/Aβ$_{42}$-4G8 [0.943 (0.817–0.991); $P < 0.01$], and Aβ$_{42}$-3D6/Aβ$_{42}$-WO2 [0.943 (0.817–0.991); $P < 0.01$] compared with Aβ$_{42}$-3D6 alone [0.576 (0.405–0.734); Fig. 3]. At optimal cutoffs (maximum sensitivity and specificity), the sensitivity/specificity values were 71.4%/91.7%, 92.9%/79.2%, and 78.6%/100% for the ratios Aβ$_{42}$-3D6/Aβ$_{42}$-6E10, Aβ$_{42}$-3D6/Aβ$_{42}$-4G8, and Aβ$_{42}$-3D6/Aβ$_{42}$-WO2, respectively. In addition, differentiation of MCI-N from AD was improved by quantification of the full-length Aβ$_{42}$-3D6 [AUC = 0.850 (95% CI, 0.755–0.919)] compared with Aβ$_{42}$-6E10 [0.655 (0.543–0.756); $P < 0.01$], Aβ$_{42}$-4G8 [0.661 (0.549–0.762); $P < 0.01$], or Aβ$_{42}$-WO2 [0.656 (0.544–0.757); $P < 0.01$]. Identical results were obtained for the comparison between the control and AD groups.

**Identification of Aβ$_{42}$ Peptides by SELDI-TOF Technology**

We further investigated the presence of truncated Aβ$_{42}$ peptides, using SELDI-TOF technology. For this purpose, CSF samples from controls and AD patients were used. Application of the mAb 4D7A3 on the ProteinChip array revealed 3 distinct Aβ$_{42}$ peptide peaks (Fig. 4), which could be assigned on the basis of their molecular masses as Aβ$_{1-42}$, Aβ$_{11-42}$, and Aβ$_{8-42}$ (Table 3). The mass accuracy was calculated as the mean value for each peptide in 30 CSF samples.

**Discussion**

There is a growing need to shorten the lead times for development of new AD drugs. The increasing costs of research and development have encouraged pharmaceutical companies to consider the use of biomarkers in phase 1 clinical trials with the ultimate goal of finding surrogate markers that are influenced by the drug treatment protocol. A surrogate endpoint or marker is defined as a laboratory measurement or physical sign that is used in therapeutic trials as a substitute for a clinically meaningful endpoint that is a direct measure of how a patient feels, functions, or survives and is expected to predict the effect of the therapy (22). Diagnostic approaches are aimed at (a) the identification of incipient AD in patients with MCI or age-associated memory impairment (early diagnosis), (b) the differential diagnosis of AD from other types of dementia (DLB, frontotemporal lobe dementia, and PD; differential diagnosis), or (c) the establishment of
surrogate markers for patient management or therapy follow-up. At present, no single biomarker provides all of the necessary information, but the diagnostic accuracy is significantly improved if the results of measurement of several different proteins (tau, P-tau, and Aβ1–42) can be combined (4).

In the present study, the multiplexed bead-based xMAP technology was used to develop assays for simultaneous quantification of Aβ42 peptides, tau, and P-tau181P in human CSF. The release of tau is considered a reflection of the intensity of neuronal damage and degeneration; P-tau concentrations reflect the phosphorylation state of tau, together with the formation of tangles; Aβ42 is a marker of plaque formation and reflects the stage of the disease (23); and identification of truncated Aβ42 points to the initiation of plaque formation (8).

We used different assay formats to determine the concentrations of biomarkers in CSF samples obtained from several patient groups. Notwithstanding the overlap in concentrations when different diagnostic groups were compared, the outcomes of this research study suggest that the combined use of tau, P-tau, and Aβ1–42 concentrations can lead to efficient diagnosis of AD. The concentrations of these 3 CSF biomarkers may change very early in the disease process and may, after further validation, be of value to differentiate MCI-AD cases from benign MCI cases (4). In the present study, we extended the panel of probable useful biomarkers by incorporating the ratios between different Aβ42 peptides. In a multiprotein assay format, it was shown that MCI patients with progression to AD could be identified some years before the onset of clinical dementia by incorporation of the ratios among different Aβ42 peptides (modified at the NH2 terminus) as outcome variables.

The observation that quantification of full-length Aβ1–42 provides a higher discriminating ability (for differentiation of AD from controls) than do shorter forms of the Aβ42 peptide explains some of the differences among
published results obtained with different Aβ assay formats. Efficient standardization of CSF assays should improve interpretation when data derived from different clinical centers are compared (4).

The combination of tau, P-tau, and the Aβ42 peptides provides a panel of biomarkers reflecting different pathologic hallmarks, relevant in different phases of the disease process, that can be used for diagnostic purposes as well as for patient management. It is important to perform an evaluation study with a large number of patient samples in which all biomarkers are quantified, to design algorithms for efficient diagnosis or patient follow-up. The discriminating ability of each protein should be compared with that of a panel of markers analyzed in clinical routine. In addition, the added value for each biomarker in the diagnostic work-up should be validated in detail.

The different Aβ42 peptides were measured by use of mAbs to different epitopes located at the NH2 terminus of Aβ. To our knowledge, this is the first study in which 4 different mAbs (3D6, 6E10, 4G8, and WO2) were compared for performance. mAb 3D6 (in contrast to 6E10, 4G8, or WO2) selectively identified Aβ peptides with a free amino group at Asp1, which confirmed previously published data obtained by peptide mapping experiments (13) or 2-dimensional gel electrophoresis (8). mAbs 6E10 and 4G8 are able to capture shorter peptides as well as Aβ1–42. According to Clarke and Shearman (24), residues Gly8 and Tyr9 of the Aβ sequence are considered as an essential part of the epitope of mAb 6E10 (24); the absence of reactivity against Aβ8–42 and Aβ9–42 points to a broadening of the epitope of mAb 6E10 toward position 6 and 7. Assays developed with mAb WO2 also have an epitope directed to the NH2 terminus of Aβ (positions 3–8) (25).

Because the differentiation of MCI-AD from MCI-N was comparable when mAb WO2, 6E10, or 4G8 was as the capture mAb, we expect no assay bias caused by potential interference by soluble amyloid precursor protein. For mAb 3D6, an absence of reactivity toward sAPP has been described previously (13). In addition, ROC analysis revealed no statistical difference for diagnosis of AD vs MCI-N for Aβ42-3D6 [AUC = 0.826 (95% CI, 0.732–0.908); n = 64] compared with Aβ42-3D6 quantified with a research version of the INNO-BIA AlzBio3 (11) [0.838 (0.745–0.907); n = 26; P = 0.804]; our multianalyte approach thus does not appear to introduce any assay bias. Furthermore, MCI-AD can also be differentiated by single-analyte (ELISA) analysis (our unpublished observations).

The mAb combinations WO2/21F12, 4G8/21F12, and 6E10/21F12 identified a greater number of different N-
modified forms than did the combination 3D6/21F12, which detected only Aβ1–42. Discrimination of MCI-AD patients from patients with MCI-N was improved by use of the ratio Aβ1–42/AβN–42 instead of Aβ1–42 concentrations alone. The relevance of the mAb approach was validated with SELDI-TOF technology, which confirmed the presence of truncated Aβ42 peptides. Apart from Aβ1–42, several other peptides, modified at the NH2 terminus, were identified: Aβ11–42, Aβ16–42, and Aβ25–42. In their study using mAb 1E8, Wiltfang et al. (9) found that there is some diagnostic value for quantification of CSF Aβ2–42, although no epitope mapping results were reported. However, when we used the SELDI technology with mAb 4D7A3, we could not confirm the presence of Aβ2–42 in CSF. In addition, there was no evidence of secretion of p3 (Aβ17–42) in CSF. Gouras et al. (26) also failed to detect Aβ17–42 in rodent primary neuronal cell culture media derived from day-17 embryonic rats, but the presence of Aβ11–42 is in line with our data.

The improved discrimination of MCI-AD from MCI-N obtained with the ratio Aβ1–42/AβN–42 can be explained by the different amounts of N-truncated Aβ42 peptides present in CSF from the different diagnostic groups or by the onset of oligomerization/protofibril formation, a process identified by the use of mAbs. At present, we have not enough experimental evidence to make claims about differences in the detection of oligomers, protofibrils, or small aggregates by these mAbs. Aβ peptides have many structural and biological complexities. The loss of the NH2 terminus of Aβ can have important implications for the progression of plaque morphology or interaction with binding proteins (apolipoprotein E, complement component C1q, heparin, proteoglycans) and Zn2+ ions present and is implicated in plaque formation (27). N-Terminal degradation has been observed in Aβ peptide deposits from AD patients (28) and is possibly one of the first steps in the disease process toward amyloid oligomerization and deposition in neuritic plaques (8). Soluble Aβ peptides beginning at residues Glu3 and Glu11, with cyclization of the N-terminal Glu, have been detected in AD and Down syndrome, but not in control brains (29,30). Wiltfang et al. (9) detected Aβ peptides truncated at position 2 in 35% of AD patients. N-Truncated forms of Aβ42 are also present in biological fluids. Studies have shown that N-terminally modified Aβ42 is selectively increased in CSF, whereas Aβ2–42 is increased in the brains and CSF of some AD patients (9,31). Furthermore, low CSF Aβ42 concentrations, high CSF T-tau concentrations, or altered Aβ40/Aβ42 ratios are already present several years before the onset of clinical dementia (4,32). The diagnostic relevance of C-terminally elongated peptides, identified in CSF and brain tissue from AD patients, needs to be further validated (6,7).

Other possible types of Aβ modifications that could explain the observed results are the formation of oligomers or protofibrils, which are involved in the pathology of the disease. Oligomers are increased in the brains of persons with AD (33). The evidence for secretion of oligomers in CSF or blood or both is still unclear. Using fluorescence correlation spectroscopy with labeled Aβ protein as a probe, Pitschke et al. (34) showed that Aβ aggregates might be present in CSF samples from AD patients, but not in CSF from non-AD controls, although this result has not been confirmed by other research groups. Small oligomers accumulate in conditioned medium of cell cultures and increase after transfection with mutant familial AD presenilins (35). Protofibrils, first discovered as an intermediate in Aβ40 amyloidogenesis, can cause oxidative stress and neuronal cell death in cell cultures (36). Larner (37) suggested that subtle disturbances in the formation of Aβ peptides (N-truncation, oligomer formation, APP mismetabolism, C-terminal forms) may lead to a shift from neuroprotection to neurodegeneration, possibly through disruption of negative feedback pathways. It is not clear at present whether these observations can be extrapolated to plasma or serum and, if so, whether they are a reflection of pathophysiological changes in AD brains. More studies are needed to select the best mAb to design an assay for quantification of AβN–42.

As in other areas of medicine, CSF markers for AD should not be used as isolated tests, but should be added to the information from the clinical examination. Results on the diagnostic capacity of CSF markers come from studies with clinically diagnosed patients. As a consequence, the diagnostic performance of the selected markers cannot be higher than the accuracy of the clinical diagnostic criteria used. In most of the cases, there was an overlap between results for each variable. The selected biomarkers described in the present study are related to different hallmarks of AD. Tau proteins are considered “stage” markers, whereas Aβ42 proteins can be used as “stage” markers (4). Because of the diagnostic uncertainties and the heterogeneity of the disease process, it will be necessary to validate and implement an algorithm based on a combination of biomarkers to reduce the overlap among diagnostic groups (38). Validation of such a model will require a larger number of samples.

The presence of truncated Aβ42 peptides in CSF samples from patients with early AD points to a role in the acceleration of Aβ deposition. The multianalyte assay format that we describe, which allows quantification of tau, P-tau, and Aβ42 peptides, could be a useful adjunct in the clinical diagnosis of AD pathology and could become a valuable asset for early AD diagnosis.

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