Multiplexed quantification of dementia biomarkers in the CSF of patients with early dementias and MCI: A multicenter study

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Abstract

In this report we evaluated the clinical performance of APOE genotyping and three protein biomarkers (total tau, $\beta$-amyloid$_{1-42}$, and tau phosphorylated at threonine 181) in a prospective multicenter study using the INNO-BIA AlzBio3 assay applied on Lumines platform. Concentration of biomarkers of Alzheimer’s disease in cerebrospinal fluid (CSF) was measured with multiplexing technology ($n=223$), and compared to the results of ELISA assays in patients with early dementias or mild cognitive impairment (MCI) collected at 12 gerontopsychiatric university departments, and APOE genotyping was performed.

Concentrations of A$\beta_{1-42}$ were statistically significantly lower in MCI-AD subjects compared to MCI-O, and significantly lower in D-AD patients compared to MCI-O. P-tau$_{181P}$ concentrations were significantly higher in MCI-AD patients compared to MCI-O, and significantly higher in D-AD patients compared to MCI-O. The total tau concentrations in MCI-AD patients were significantly higher compared to MCI-O, and higher in D-AD compared to MCI-O, moreover, the concentration of total tau was significantly higher in D-AD compared to MCI-AD patients. For the differential diagnosis between D-AD and D-O, the optimal cutoff concentration of A$\beta_{1-42}$ was 197.7 pg/mL, and that...
for P-tau181P was 47.9 pg/mL. These cutoff values were also applied to discriminate between MCI-AD and MCI-O subjects. Simultaneous measurement of the biomarkers significantly improves management of the samples and quality control of the assays’ performance.

Keywords: Alzheimer’s disease; Biomarkers; Amyloid; Tau; Cerebrospinal fluid; Multiplexing

1. Introduction

As the age of the general population increases, so does the incidence of dementia disorders, resulting in an increasing burden on the health care system. In the case of Alzheimer’s disease (AD), the increasing number of patients has not so far resulted in the achievement of accurate standards of during vita diagnosis. Although sensitivity of clinical diagnosis is relatively high (93%), specificity may be lower, being reported as 55% in a multicenter clinical-autopsy study (Mayeux, 1998). In expert hands the clinical diagnosis of AD is predictive of AD pathology in 80–90% of cases. Nevertheless, very early diagnosis of AD, and differential diagnosis of unusual presentations of patients with dementia remains difficult on clinical grounds.

With the introduction of potentially successful treatments for dementias that were previously considered to be irreversible, such as acetylcholinesterase inhibitors in AD (recently reviewed in Bullock, 2002; Knopman, 2001), the need for an early and differential diagnosis of dementia becomes even more urgent (The Working Group, 1998; Wiltfang et al., 2005).

Since cerebrospinal fluid (CSF) is in direct contact with the central nervous system, it is obvious that any changes in biochemical composition of brain parenchyma should be predominantly reflected in CSF. Lumbar puncture is an easy procedure, with a low incidence of complications. In a large study by Andreassen et al. (2001), only 4.1% of all patients experienced post-lumbar headache, and an even smaller incidence of 2% was reported in a study by Blennow et al. (1993). It is therefore reasonable to postulate that lumbar puncture is a feasible, moderately invasive procedure, and CSF analysis could possibly improve current clinical and neuroimaging-based approaches to diagnosis. Nevertheless, the low and limited amount of the material obtained with a lumbar puncture requires very precise planning of the laboratory tests to be performed. The growing number of possibly important biomarkers to be analyzed in a limited volume of the CSF makes techniques of a simultaneous analysis of several parameters in a single small-volume CSF sample (multiplexing) a method of choice in the future.

The flow cytometric-based Luminex xMAP® technology (Luminex Corp., Austin, TX) involves coupling of specific monoclonal capturing antibodies to the surface of microsphere sets uniquely identified with a combination of two fluorescence dyes (Carson and Vignali, 1999; Vignali, 2000). This allows a simultaneous reaction with up to one hundred (theoretical limit) antigens in a single sample assuming no cross-reactivity of the particular antibodies. Recent studies by Olsson et al. (2005) and Vanderstichele et al. (2005) reported a successful application of this technology for analysis of the three crucial AD biomarkers: β-amyloid 1–42 (Aβ1-42), total tau, and the phosphorylated form of tau (P-tau181P) with a research version of the INNO-BIA AlzBio3 assay. The present study describes the first application of this multiplexed technique to measure AD biomarkers in the CSF of patients recruited in a multicenter study involving 12 German gerontopsychiatric university departments that cooperate with the German Competence Net Dementias (http://www.kompetenznetz-demenzen.de/).

2. Methods and materials

2.1. Patients and lumbar punctures

The study was approved by the ethics committees of all the participating universities, and all patients and/or their relatives gave their informed consent. Before starting the study, a training workshop for all participants was organized in order to introduce standard operating procedures (SOPs) for collection, storage, and shipment of the CSF samples.

Twelve German gerontopsychiatric university departments participated in the project, recruiting a total of 223 patients with early Alzheimer’s disease (D-AD), early other dementias (D-O), mild cognitive impairment (MCI) of AD-type (MCI-AD), or MCI of other dementias-type (MCI-O) between December 2002 and October 2004.

Patients with early AD (n = 53) were diagnosed according to the criteria of ICD-10, and the National Institute of Neurological and Communicative Disorders and the Stroke–Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) (McKhan et al., 1984). Patients with D-O (n = 15) fulfilled the criteria of corresponding disorders (vascular dementia, n = 4; frontotemporal degeneration, n = 6; Huntington’s disease, n = 1, other and unclassified dementia, n = 4). Cognitive dysfunction was assessed with CERAD neuropsychological test battery (Thalman et al., 1998), WMS-R Logical Memory (Harting et al., 2000), Trail Making Test (Reitan, 1979), and Clock Drawing Test (Shulman et al., 1986). Functional decline was assessed using informant questionnaires B-ADL (Hindmarch et al., 1998), and IQCODE (Jorm, 1994). Distinction of MCI and dementia was based on CDR rating (Morris, 1993). A diagnosis of MCI corresponded to a CDR score of 0.5, a diagnosis of mild dementia corresponded to a CDR score of 1.0. Severity of dementia was graded according to the Mini Mental State Examination (Folstein et al., 1975), showing scores charac-
Patients with MCI fulfilled the criteria of Petersen et al. (2001), and were subdivided into two groups: those with clinical, neuropsychological, and neuroradiological signs suggesting development of AD (MCI-AD, n = 106), and those with the signs suggesting development of other dementias (MCI-O; n = 49). The minimum condition for MCI was that there were deficits in at least one cognitive domain, one standard deviation below mean, and that caregivers attested at least mild decline in everyday competences recently. We asked staff members to do their best possible clinical judgement regarding etiological causes of cognitive impairment.

Since neuropsychological tests must be very carefully optimized before application in multicenter studies, in our project there was a highly standardized protocol regarding all psychometric scales and questionnaires applied to the patients and their caregivers. Moreover, before the beginning of the study, and then every 2 years, rater-trainings were organized by expert neuropsychologist. We applied the overall CDR score of 0.5 as the most reliable measure for defining ‘mild impairment’, and a CDR score of 1 or more was needed for a diagnosis of dementia according to data from the literature (Li et al., 2006; Perneczky et al., 2006; Storandt et al., 2006). The demographic data of the patients are shown in Table 1.

Lumbar punctures were performed with the patients in the sitting position according to the standardized procedure. After collection of the CSF for routine diagnosis (2–5 mL), an additional 4.5 mL of the CSF for this study was sampled into a polypropylene test tube, and proceeded according to the standardized protocol (Lewczuk et al., 2006): the CSF was centrifuged immediately after collection (1600 g, room temperature, 15 min), aliquoted into 16 polypropylene test tubes (each aliquote, 250 μL), and frozen within 30–40 min after the puncture. The CSF was at no time thawed/refrozen. The multiplexing analysis was performed in one center (University of Erlangen) to which all the aliquots of frozen CSF were shipped on dry ice.

### 2.2. Repeatability and reproducibility of the multiplex measurements

Repeatability and reproducibility were tested according to ISO 5725-2 guidelines (ISO, 1994) for low-, medium-, and high-reactive control samples. The samples were selected in the normal working concentration range for the calibrators of the assay and contained calibrator peptides/proteins dissolved in phosphate buffer solution. To assess repeatability, one performer tested the samples in duplicate using one calibrator series on kit components from one production run. To evaluate reproducibility, one performer tested the samples in duplicate in seven different experimental runs. The results are expressed as %CV where %CVr is defined as repeatability standard deviation/general mean and %CVR as reproducibility standard deviation/general mean.

### 2.3. Multiplexed analysis of biomarkers

The simultaneous analysis of total tau, Aβ1-42, P-tau181P in CSF was performed with a research version of the INNO-BIA AlzBio3 assay according to the manufacturer’s instructions (Innogenetics, Gent, Belgium, for research use only). Briefly, after pre-wetting of the filter plate with a wash buffer, 75 μL CSF or standard were incubated with a suspension of microspheres carrying corresponding capturing antibodies (t-Tau: AT120, pTau181: AT270, Aβ1–42: 4D7A3), 3000 microspheres per biomarker, and a mixture of the biotinylated detection antibodies (3D6 and HT7). Following overnight incubation in the dark and washing of the plate, phycoerythrin coupled to streptavidin was added to each well (100 μL), and the plate was incubated for 1 h at room temperature. The plate was then washed again and a reading solution applied. The resulting fluorescence signal was read with a Luminex 100 IS analyzer (50–100 microspheres per biomarker and per well). All analyses were done in duplicate.

### 2.4. ELISAs

In 233 cases the concentration of the biomarkers were measured on the Luminex platform and with corresponding with ELISAs (Innogenetics). ELISA assays were performed in duplicate and according to the manufacturer’s instructions.

### 2.5. Genotyping

Apolipoprotein E genotyping (APOE) was performed in 123 subjects of this study after additional informed consent from patients and/or their relatives had been obtained. Briefly, leukocyte DNA was isolated with the Qiagen blood isolation kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). The APOE genotype was studied as described by Hixson and Vernier (1990). The whole product was amplified using the following primers:

- **5′-ACA GAA TTC GCC CCG GCC TGG TAC AC-3′** (forward),
- **5′-TAA GCT TGG CAC GGC TGT CCA AGG A-3′** (reverse).

The amplification product was digested with Hha I (New England Biolabs, Frankfurt am Main, Germany) at 40 °C for 6 h. Samples were separated on a 3% agarose gel and visualized with SYBR Gold Nucleic Acid Gel Stain (Invitrogen).
rogen, Karlsruhe, Germany). The results are presented with regard to the presence or absence of e4 allele of the APOE gene.

2.6. Statistical analysis

Differences between groups were analyzed using the Mann–Whitney test with Bonferroni correction for multiple comparisons (Statistica 7.0, Statsoft, Tulsa, USA). Comparison of both methods (ELISA versus multiplexing) was performed according to Pearson’s test, and presented as scatter-plots. In addition, Petersen’s et al. (1997) method was applied to estimate the concentration-dependency of the correlations. Cutoff values were calculated with receiver operating characteristic curve (ROC) analysis, and optimized according to Youden’s index. The differences were considered statistically significant if \( p < 0.05 \).

3. Results

3.1. Performance of xMAP multiplexing and comparison of the methods

Run-validation samples have been prepared by spiking of pre-defined concentrations of the individual parameters (total tau, \( \text{A} \beta_{1-42} \), P-tau181P) in assay buffer in order to obtain low, medium, and high concentrations for each parameter. Repeatability and reproducibility of the xMAP measurements is presented in Table 2. Fig. 1 shows the correlations of the results obtained with the multiplexing with those obtained with the corresponding ELISA assays. The direct comparison of the concentrations revealed correlations: moderate, in case of \( \text{A} \beta_{1-42} \) (Fig. 1a, \( R = 0.47, p < 0.01 \)), and good in case of tau (Fig. 1b, \( R = 0.96, p < 0.01 \)), and P-tau181P (Fig. 1c, \( R = 0.87, p < 0.01 \)). The analysis of the slope of the regression line

Table 2
Repeatability (r) and reproducibility (R) of the assays

<table>
<thead>
<tr>
<th>Sample</th>
<th>P-tau181P</th>
<th>Total tau</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (pg/mL)</td>
<td>%CVr</td>
<td>%CVr</td>
</tr>
<tr>
<td>1</td>
<td>52</td>
<td>3.4</td>
</tr>
<tr>
<td>2</td>
<td>109.7</td>
<td>3.1</td>
</tr>
<tr>
<td>3</td>
<td>149</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Fig. 1. Correlation of biomarker concentration as measured by multiplexing and corresponding ELISAs: (a) \( \text{A} \beta_{1-42} \); (b) Tau; (c) P-tau181P.
Table 3

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Aβ1-42 (pg/mL)</th>
<th>Total tau (pg/mL)</th>
<th>P-tau181P (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCI-AD (106)</td>
<td>172.6 (123.3–230.4)</td>
<td>74.2 (51.2–120.2)</td>
<td>48.6 (37.1–84.4)</td>
</tr>
<tr>
<td>MCI-O (49)</td>
<td>228.0 (189.7–264.4)</td>
<td>45.1 (40.0–59.9)</td>
<td>38.5 (30.1–47.0)</td>
</tr>
<tr>
<td>D-AD (53)</td>
<td>147.5 (120.5–194.6)</td>
<td>112.9 (72.0–152.1)</td>
<td>71.4 (53.2–88.1)</td>
</tr>
<tr>
<td>D-O (15)</td>
<td>207.6 (135.6–255.1)</td>
<td>72.8 (45.1–85.6)</td>
<td>33.2 (23.4–105.4)</td>
</tr>
</tbody>
</table>

Significant differences: aMCI-AD vs. MCI-O; bMCI-O vs. D-AD; cMCI-AD vs. D-AD.

Table 4

<table>
<thead>
<tr>
<th>Biomarker (cutoff, pg/mL)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-AD vs. D-O</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ1-42 (197.7)</td>
<td>75.5</td>
<td>60.0</td>
<td>87.0</td>
<td>40.9</td>
</tr>
<tr>
<td>P-tau181P (47.9)</td>
<td>77.4</td>
<td>73.3</td>
<td>91.1</td>
<td>47.8</td>
</tr>
<tr>
<td>tTau (86.0)</td>
<td>66.0</td>
<td>80.0</td>
<td>92.1</td>
<td>40.0</td>
</tr>
<tr>
<td>MCI-AD vs. MCI-O</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ1-42*</td>
<td>58.5</td>
<td>63.3</td>
<td>77.5</td>
<td>41.3</td>
</tr>
<tr>
<td>P-tau181P*</td>
<td>50.9</td>
<td>79.2</td>
<td>84.4</td>
<td>42.2</td>
</tr>
<tr>
<td>tTau*</td>
<td>39.6</td>
<td>91.8</td>
<td>91.3</td>
<td>41.3</td>
</tr>
</tbody>
</table>

* Cutoff values to separate MCI-AD and MCI-O are derived from the separation of D-AD and D-O.

Fig. 2. Simultaneous analysis of Aβ1-42 and P-tau181P in the CSF of patients subdivided according to their APOE genotype. In both figures, cutoff values best distinguishing D-AD from D-O subjects are shown: (a) early dementias; (b) MCI.

revealed changed difference between the two methods (slope different from 0) regarding Aβ1-42 and P-tau181P, and constant difference between the two methods regarding total tau (slope = 0).

3.2. Multiplexing quantification of biomarkers in different patient groups

For the analysis of the multiplex data in the different patient groups, results were accepted only if all three range-to-average ratios of duplicate measurements were lower than 20%. The concentrations of the three biomarkers in the diagnostic groups are presented in Table 3. Concentrations of Aβ1-42 were statistically significantly lower in MCI-AD subjects compared to MCI-O, and significantly lower in D-AD patients compared to MCI-O. There were no statistically significant differences between D-AD and MCI-AD with regard to Aβ1-42. P-tau181P concentrations were significantly higher in MCI-AD patients compared to MCI-O, and significantly higher in D-AD patients compared to MCI-O. Again, there were no differences between D-AD and MCI-AD. The total tau concentrations in MCI-AD patients were significantly higher compared to MCI-O, and higher in D-AD compared to MCI-O, moreover, the concentration of total tau was significantly higher in D-AD compared to MCI-AD patients.

For the differential diagnosis between D-AD and D-O, the optimal cutoff concentration of Aβ1-42 was 197.7 pg/mL, and for P-tau181P was 47.9 pg/mL. These cutoff values were also applied to discriminate between MCI-AD and MCI-O subjects. Fig. 2a presents the combined results of Aβ1-42 and
P-tau181P levels in early dementia subjects: D-AD and D-O further subdivided into carriers of least one ε4 allele of the APOE gene and non-carriers of the ε4 allele. Fig. 2b shows the subjects with MCI-AD and MCI-O subdivided according to their APOE genotype. In both cases, the APOE ε4+ patients cluster above P-tau181P and below Aβ1-42 cutoff values, and the difference of the percentage of ε4 positive subjects in the group of MCI-AD and MCI-O is highly statistically significant (Chi-square test, p < 0.01). We present statistical evaluation of the biomarkers in Table 4.

4. Discussion

In this study, a multiplex-based method was applied for the first time in a multicenter setting to simultaneously measure the cerebrospinal fluid concentrations of three crucial biomarkers of Alzheimer’s disease (β-amyloid 1–42, total tau, and phospho-tau). The results were compared to those of ‘classic’ ELISAs, and further analysis was performed taking the apolipoprotein E genotype of the patients into account.

Two reports have recently been published on the application of this technology to measure the biomarkers in the CSF in patients with dementia (Olsson et al., 2005; Vanderstichele et al., 2005), and our results fully support their conclusion that the xMAP-based method could potentially replace the single-biomarker ELISAs in the future. Note that in comparison to these two reports, different calibrators were used in our study, resulting in the ranges of concentrations quite different compared to one expect from usual ELISAs of Innogenetics. The multiplexing results of total tau and P-tau181P correlate very well with the results obtained with the corresponding ELISAs, whereas the correlation for Aβ1-42 is clearly lower. This might be, at least partially, attributed to different capturing antibodies used in both applications (21F12 in ELISA and 4D7A3 in xMAP).

In the present study, we observed significant differences in the pattern of biomarkers between MCI-AD and MCI-O patients, which fully supports the recently published report of Zetterberg et al. (2003). They demonstrated pathologic alterations of AD biomarkers at the stage of MCI in subjects who subsequently developed AD within 2–3 years, but not in the subjects who developed other dementias. In this context, it is also interesting to note that the cutoff values of Aβ1-42 and P-tau181P derived from the analysis of dementia patients (D-AD versus D-O) may correctly separate subjects with MCI-AD from MCI-O. Similarly, significant differences of total tau concentration (but neither Aβ1-42 nor P-tau181P) observed between MCI-AD and early AD might be explained by a progression of the neurodegenerative process. Moreover, although we did not observe significant differences between AD and non-AD patients (D-O) when single biomarkers were taken into consideration (which could be attributed to the relatively small group of D-O patients as well as the problems of a precise clinical differential diagnosis at a very early stage of dementia), a simultaneous analysis of two biomarkers (Aβ1-42, and P-tau181P) allowed differential diagnosis between these groups.

Clinically-based categorization of MCI/early dementia patients has an inherent risk of misclassification (Mayeux, 1998; The Working Group, 1998; Wiltfang et al., 2005), and neuropathological diagnosis might also be misleading if performed several years after the spinal tap. We therefore used APOE genotyping as an additional independent and objective parameter, hypothesizing that the MCI-AD and D-AD groups should be enriched in ε4 allele carriers. This was based on the assumption that the ε4 allele is known to occur three to four times more frequently in AD patients than in the general population (Mulder et al., 2000). The tendency of increased percentage of ε4 positive subjects in D-AD group versus D-O is indeed the case in our study, and in the case of MCI-AD the difference of APOE ε4 allele distribution is highly significant compared to MCI-O. Certainly, for the future it will be interesting to analyze APOE genotype-dependent concentrations of the biomarkers.

The majority of studies performed so far have reported a decreased concentration of Aβ42, and increased concentrations of tau and phospho-tau in the CSF of patients with Alzheimer’s disease (Hampel et al., 2004; Lewczuk et al., 2004a,b) (recently reviewed by Blennow and Vanmechelen (2003), and our results are in agreement with these reports. A satisfying explanation for the decreased levels of Aβ42, in CSF of patients with AD is still lacking. The suggested mechanism that this is due to a simple accumulation in plaques is not sufficient, since we found decreased Aβ1-42 concentrations in CSF of patients with Creutzfeldt–Jakob disease without apparent plaque formation (Wiltfang et al., 2003). This points to other mechanisms, such as epitope masking and chaperones dysfunction, and certainly requires further studies. Similarly, it is still not clear if an increased concentration of phospho-tau is due to increased activity of kinase(s) or decreased activity of phosphatase(s) (Buee et al., 2000).

To conclude, xMAP technology offers a possibility to increase the number of biomarkers that can be simultaneously and reliably quantified in a minute volume of CSF. Moreover, simultaneous analysis of more than one biomarker seems to be superior compared to a single-biomarker approach as offered by non-multiplexing techniques, while it requires substantially less material and working time. Further studies, including follow-up of the patients, are now being run to confirm and validate the results of the current report.

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