Chapter 3: CSF measurements in AD: Clinical chemical properties

Chapter 3

CSF measurements in AD: Clinical chemical properties
3.1
Variability in longitudinal CSF Tau and P-Tau measurements

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Abstract

Background
The influence of assay variation and duration of storage on changes in CSF levels of Tau and P-Tau with time, is evaluated in 112 patients with various neurological disorders.

Material and Methods
These patients (age 66±9 years; 52% male), referred to our memory clinic, underwent two spinal taps (mean interval 19 months) and the baseline samples were assayed twice in a sandwich ELISA: once after the first spinal tap (A1) and once, in a separately stored aliquot (A2) simultaneous with the follow-up sample (B).

Results
Coefficients of variance (CV) of Tau and P-Tau levels determined in repeated spinal taps (ΔB-A2) measured in one assay (10.9% and 7.6%) were lower (p<0.01) than the CV’s observed in two different (ΔB-A1) assays (16.5% and 11.7%). The CV’s of Tau and P-Tau measurement of one CSF-sample repeated on two occasions (ΔA1-A2) were 12.3% and 8.6%. A difference in mean P-Tau level was found if the same CSF samples were repeatedly measured in two different ELISAs (A1-A2).

Conclusion
Longitudinal CSF Tau and P-Tau are best measured in one assay resulting in a lower variability compared to measurement in two different assays. The within person variability in levels of these markers currently limits the use of these ELISAs in a longitudinal clinical setting.
Introduction

Tau is a microtubule-associated protein in the neuronal axons. Phosphorylation of Tau leads to dissociation of Tau from the microtubuli. A pronounced increase in CSF levels of Tau and Tau protein phosphorylated at threonine 181 (P-Tau) is found in AD patients. For this reason measuring Tau and P-Tau has gained acceptance not only for discriminating AD patients from controls or patients with other types of dementia, but also to detect incipient AD in MCI patients. It is a current major challenge to use these biomarkers in a longitudinal fashion, to monitor disease progression and effects of treatment. Several studies described longitudinal changes of Tau and P-Tau level in CSF which showed contradictory results. These contradictory results are probably not only due to the biological variability in the CSF samples, but also to effects of CSF processing, complicating studies measuring longitudinal changes in these biomarkers. Surprisingly, only two of these studies report intra and inter assay variations. Recently, we demonstrated considerable variability in longitudinal measurements of Amyloid-beta 1-42. In the current study we evaluated changes in CSF levels of Tau and P-Tau with time in the same cohort. We assessed the influence of assay variation and duration of storage of specimen on the results of Tau and P-Tau assessment in patients with various cognitive and neurological disorders.

Material and Methods

Participants

One-hundred twelve patients with several neurological disorders were recruited at the Alzheimer Centre of the VU University Medical Centre (VUMC) between November 2000 and October 2004. Clinical diagnoses were made in a multidisciplinary meeting. The diagnosis of MCI (N=37) was made according to the Petersen criteria. The diagnosis of AD (N=50) was made using the criteria of National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA). When the outcomes of all clinical investigations were normal, patients were considered to have subjective complaints (N=16). Nine patients had other neurological diseases. (1 FTLD (Frontotemporal Lobar Degeneration) patient, 1 patient with a status after an AVM (Arteriovenous malformation), 1 patient with temporal epilepsy, 1 patient with coeliac disease with meningencephalitis, 1 patient with Pelizaeus-Merzbacher Disease and the diagnosis of four patients was postponed due to clinical disagreement. At follow up we diagnosed these four patients; two as AD and two as MCI.) All patients underwent two spinal taps. Mean follow-up time, defined as the time between the first and second lumbar puncture (LP), was 19 (SD = 8) months. The ethical review board of the VUMC approved the study and all subjects gave written informed consent.

CSF analysis

CSF was obtained by LP between the L3/L4 or L4/L5 intervertebral space, using a 25-gauge needle, collected in 12-mL polypropylene tubes. Within two hours, CSF samples were centrifuged at 2100g for 10 min at 4°C, aliquoted in polypropylene tubes of 0.5 or 1 ml, and stored at -80°C until further analysis. Tau and P-Tau were measured with a sandwich ELISA
Baseline samples were assayed twice, i.e. once shortly after the first spinal tap (A1) and once, in a separately stored aliquot (A2) concomitant with the follow-up sample (B). The measurements were performed by one experienced research analyst, the Innotest® protocol was followed and the samples were run without dilution. All samples were run in duplicate. The intra-assay coefficients of variation for the baseline Tau and P-Tau (A1) were 4.4% and 1.4%. The intra-assay coefficients of variation for the follow-up sample (B) and the repeated baseline sample (A2) were 4.0% and 1.8%. The inter-assay coefficients of variation for Tau and P-Tau were calculated using a CSF-pool sample. Between June and December 2004 these were 6.1% (N=6) and 9.0% (N=9) and between December 2004 and February 2006 they were 8.5% (N=9) and 9.3% (N=16).

**Statistical analysis**

For statistical analysis, SPSS version 12.0 (Chicago, IL) was used. Differences in CSF levels between the three assessments (A1, A2, B) were analysed with paired sample t-tests. The coefficients of variances (CV) were calculated according to the following formula: mean of (SD of the two measurements) / (mean of the two measurements). Variances were visualized in Bland-Altman plots and compared using Pitman’s test. Correlations between storage time and changes in CSF Tau and P-Tau levels were assessed using Pearson correlation coefficient. Significance was set at p<0.05.

**Results**

The demographic characteristics of the study population are summarized in Table 1. The mean (SD) Tau of the baseline sample (A1), the repeated baseline measurement (A2) and the follow-up sample (B) were 634(473)ng/L, 627(501)ng/L and 672(544)ng/L. Paired t-tests showed a significant increase in CSF Tau level when the baseline sample and the follow-up sample were assessed in the same assay (ΔB-A2=45(158)ng/L; p<0.01), but not when they were assessed in different assays (ΔB-A1=38(248)ng/L; p=0.11). The mean CSF level of Tau did not change on repeated assessment of the baseline sample (ΔA1-A2=7.0(189)ng/L; p=0.69) (Table 2). CV’s for Tau, were 16.5% (ΔB-A1), 10.9% (ΔB-A2), and 12.3% (ΔA1-A2), this is visualized in Bland-Altman plots (Fig 1 and Fig 3). Analysis with the Pitman’s test revealed that the variance of baseline and repeat sample assessed in two different assays (ΔB-A1) was larger than the variance of assessment in the same assay (ΔB-A2) (p<0.01) and than the variance of the repeated assessment of the baseline sample in two assays (ΔA1-A2) (p=0.01). The variance of the repeated assessment of the baseline sample (ΔA1-A2), was larger than the baseline and the repeat sample assessed in the same assay (ΔB-A2) (p=0.02). The mean (SD) P-Tau of the baseline sample (A1), the repeated baseline measurement (A2) and the follow-up sample (B) were 77(39)ng/L, 73(35)ng/L, and 75(36)ng/L. Paired sample t-tests showed no change when the baseline sample and the follow-up sample assessed in the same assay (ΔB-A2=1.8(13.1)ng/L; p=0.15) nor when they were assessed in different assays (ΔB-A1=1.9(18.6)ng/L; p=0.29). However, significant differences in CSF P-Tau level were observed when the baseline sample was assessed in two different assays (ΔA1-A2= 3.7(13.2) ng/L; (p<0.01)). (See Table 2) The CVs for P-Tau, were 11.7% (ΔB-A1), 7.6% (ΔB-A2), and 8.6% (ΔA1-A2) (Fig 2 and Fig 3). Pitman’s test
revealed that the variance of baseline and repeat sample assessed in two different assays (ΔB-A1) was larger than assessment in the same assay (ΔB-A2) (p<0.01) and than variance of the baseline sample assessed in two assays (ΔA1-A2) (p<0.01). The variance of the repeated assessment of the baseline sample (ΔA1-A2) was not different from the variance for assessment in the same assay (ΔB-A2) (p=0.48). To determine whether there was an effect of CSF storage time, we calculated the correlation between storage time and differences in Tau and P-Tau concentrations between the repeated assessments of the baseline samples (Δ A1-A2). No correlations were found (Tau: r=0.05; p=0.58 and P-Tau: r=0.08; p=0.41).

Table 1

<table>
<thead>
<tr>
<th>Men/Women</th>
<th>60/52</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td>66 (9)</td>
</tr>
<tr>
<td>Baseline MMSE a</td>
<td>24 (5)</td>
</tr>
<tr>
<td>Follow up time in months</td>
<td>19 (8)</td>
</tr>
<tr>
<td>Diagnosis: AD, MCI, SC, OND (%)</td>
<td>50, 37, 16, 9</td>
</tr>
</tbody>
</table>

Demographic characteristics of subjects (n=112). MMSE=mini mental state examination; AD=Alzheimer’s Disease; MCI=Mild Cognitive Impairment; SC=subjective complaints; OND=other neurological diseases. Data are represented as mean (± SD), unless indicated otherwise; a available for n=110.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Tau</th>
<th>P-Tau</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (A1)</td>
<td>634 (473)</td>
<td>77 (39)</td>
</tr>
<tr>
<td>Baseline repeated (A2)</td>
<td>627 (501)</td>
<td>73 (35)</td>
</tr>
<tr>
<td>Follow up (B)</td>
<td>672 (544)</td>
<td>75 (36)</td>
</tr>
<tr>
<td>∆B-A2</td>
<td>45 (158)</td>
<td>1.8 (13.1)</td>
</tr>
<tr>
<td>p-value (B versus A2)</td>
<td>&lt;0.01</td>
<td>0.15</td>
</tr>
<tr>
<td>∆B-A1</td>
<td>38 (248)</td>
<td>-1.9 (18.6)</td>
</tr>
<tr>
<td>p-value (B versus A1)</td>
<td>0.11</td>
<td>0.29</td>
</tr>
<tr>
<td>∆A1-A2</td>
<td>7 (189)</td>
<td>3.7 (13.2)</td>
</tr>
<tr>
<td>p-value (A1 versus A2)</td>
<td>0.69</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Mean results of the Tau and P-Tau CSF measurements. A1=Baseline sample assayed shortly after collection; A2=Stored baseline sample assayed together with the follow-up sample; B=Follow-up sample. Tau and P-Tau levels are in ng/L. Data are represented as mean (± SD), unless indicated otherwise.

Discussion

The main finding of this study is the higher variability of Tau and P-Tau concentrations in baseline and follow up samples measured in different analytical runs (B-A1), compared with the assessment performed in the same analytical run (B-A2). Second, we found higher
variability in the repeated Tau measurement of the baseline sample in different assays (A1-A2), compared with Tau concentrations of the baseline and follow-up samples measured in one assay (B-A2). Finally, a significant difference was found between the repeated baseline P-Tau measurements (A1-A2). Even with acceptable within- and between-assay variation as judged from the results from the quality-control pools, high variances are found.

A possible cause is higher variability at higher Tau and P-Tau concentrations compared with lower concentrations, as suggested in Fig. 1 and 2. However, after exclusion of the 5 highest Tau and P-Tau concentrations from analysis, no essential change in variability was found (results not shown). Probably these measurement errors are more likely to be caused by methodological limitations of the Tau and P-Tau ELISAs. These ELISA limitations might reflect the batch-to-batch differences of the monoclonal antibodies incorporated in the assays which are probably created during the monoclonal antibody production. The production involves different stages and all of them are crucial, for instance: differences in medium used and differences in conditions to culture hybridomas, concentration / purification / filtration of the antibody, and eventually the precise measurement of the coating and detecting antibody concentrations. Any difference in this production can lead to a different final concentration of the catching and detecting antibodies used in the different ELISAs, resulting in Tau and P-Tau variability. Other possibilities for batch-to-batch differences can be induced by differences in standards and/or plates used. The synthesis of peptide, the weighing and the differences in dissolving the standard may result in different Tau and P-Tau values, and small changes in the plates production can result in differences in the antibody coating efficiency. Contradictory results concerning longitudinal changes of Tau and P-Tau CSF have been reported 12-25.

In only two longitudinal studies intra- and an inter-assay variability are mentioned 13, 15 and not more than three studies explicitly reported that baseline and follow-up CSF samples were assayed in the same analytical run 13, 24, 25. A meta-analysis of cross-sectional studies and an international quality control survey demonstrated considerable variability in absolute concentrations of Tau among centres, even when using the same commercial assay 75, 80. This is in line with our results, since we found large variances in the levels of Tau and P-Tau, while all performances were performed in the same laboratory, using the same assay (with every time the same incubation steps) and by the same technician. Differences in follow-up time, and thus storage time, of CSF samples as a cause of variability was not likely because we found no association between follow-up time and differences in repeated Tau and P-Tau assessments measured in baseline samples. This confirms earlier data on the effects of processing and storage conditions on Tau and P-Tau concentrations 85. In our study, it seems that methodological errors exceed the biological variability. This implicates that repeated assessments of Tau and P-Tau in CSF are currently not useful in a clinical setting. The biological significance of repeated lumbar punctures in individual patients remains to be established, but if assessment of follow-up specimens is required or indicated, our results indicate that baseline and follow-up sample should be measured in the same assay.
Figure 1

Figures in boxes represent Bland-Altman plots of Tau concentrations in baseline and follow-up samples.

1A. Baseline and follow-up CSF samples [mean(SD) 38 (248)] measured in different analytical runs (ΔB-A1; CV=16.5%).

1B. Baseline and follow-up CSF samples [mean(SD) 45 (158)] measured in the same analytical runs (ΔB-A2; CV=10.9%).

1C. Baseline CSF samples [mean(SD) 7 (189)] measured in different runs (ΔA1-A2; CV=12.3%).

The lines in the figures represent the mean +/- 1.96SD.
Figure 2

Figures in boxes represent Bland-Altman plots of P-Tau concentrations in baseline and follow-up samples.

2A. Baseline and follow-up CSF samples [mean(SD) 1.9 (18.6)] measured in different analytical runs (ΔB-A1; CV=11.7%).

2B. Baseline and follow-up CSF samples [mean(SD) 1.8 (13.1)] measured in the same analytical runs (ΔB-A2; CV=7.6%).

3C. Baseline CSF samples [mean(SD) 3.7(13.2)] measured in different runs (ΔA1-A2; CV=8.6%).

The lines in the figures represent the mean +/- 1.96SD.
Figure 3

Summary of different inter-assay (=coefficients of variances) results. A1=Baseline sample assayed shortly after collection; A2=Stored baseline sample assayed together with the follow-up sample; B=Follow-up sample; ** For Tau and P-Tau p<0.01; * For Tau p=0.02 and for P-Tau p=0.48.
3.2
Aβ42, Tau and P-Tau as CSF biomarkers for the diagnosis of AD.


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Abstract

Background
To improve ante-mortem diagnostic accuracy of Alzheimer’s disease (AD) the use of biomarkers amyloid-beta(1-42) (Aβ42), total Tau (Tau) and hyperphosphorylated Tau181 (P-Tau) in cerebrospinal fluid (CSF) has been proposed. We have used these markers and evaluated their performance.

Material and Methods
From January 2001 till January 2007 Aβ42, Tau and P-Tau were assessed by commercial ELISAs in CSF from 248 consecutive AD patients and 131 patients with subjective memory complaints attending our outpatient memory clinic. Diagnoses were made blind to the results of the biomarker assays. Sensitivity and specificity were assessed and trends over time were analyzed.

Results
Inter-assay CV’s (mean±SD) as judged from results of pools of surplus CSF specimens were 11.3±4.9% for Aβ42; 9.3±1.5% for Tau and 9.4±2.5% for P-Tau respectively (n=7-18). To achieve 85% sensitivity, cut-off values were 550ng/L for Aβ42 (95%CI: 531-570); 375ng/L for Tau (95%CI: 325-405) and 52 ng/L for P-Tau (95%CI: 48-56). Corresponding specificities were 83% (95%CI:76-89) for Aβ42, 78% (95%CI:70-85) for Tau and 68% (95%CI: 60-77) for P-Tau. Logistic regression to investigate the simultaneous impact of the three CSF biomarkers on the diagnosis yielded a sensitivity of 93.5% and specificity of 82.7%, at a discrimination line: Aβ42=373+0.82∗Tau. Over time only the area under the ROC curve of P-Tau showed significant fluctuation.

Conclusions
CSF biomarkers Aβ42 and Tau can be used as a diagnostic aid in AD. P-Tau did not have additional value over these two markers. Cut-off values, sensitivities, specificities, and discrimination lines depend on the patient groups studied and laboratory experience.
Introduction

An ante-mortem diagnosis of “probable Alzheimer’s disease” (AD) is achieved by application of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) criteria using a standardized protocol including medical history, physical and neurological examination, screening laboratory tests, psychometric evaluation, electro encephalography (EEG), and brain magnetic resonance imaging (MRI) or computed tomography (CT). Although the NINCDS-ADRDA criteria have been reported to have a high accuracy rate of 80%-90% \(^{129, 130}\), studies of the diagnostic accuracy came from specialized centres, and most data are from patients in later stages of the disease who were studied for several years before death and autopsy. To improve the clinical diagnostic accuracy, assessment of biomarkers in cerebrospinal fluid (CSF) has been proposed \(^{131}\). Candidate biomarkers obviously are proteins that occur in senile plaques (SP) and neurofibrillary tangles (NFT). The principal component of the SP is the hydrophobic amyloid-beta (1-42) (A\(_\beta\)42), whereas hyperphosphorylated Tau (P-Tau), a fraction of the concentration of total Tau (Tau), is a characteristic component of NFT \(^{132-134}\). Previous studies showed that these biomarkers can discriminate AD patients from healthy controls with a good sensitivity and specificity but cut-off levels differ between laboratories \(^4, 7, 60, 135-138\). The aim of the study was to establish the sensitivity and specificity of assays for determination of A\(_\beta\)42, Tau, and P-Tau in CSF to distinguish patients with probable AD from patients with subjective complaints (SMC) in a memory clinic setting. This paper describes our experience with the use of CSF biomarkers during a six year period.

Materials and Methods

Patients

From January 2001 till January 2007, 379 patients, referred to the Alzheimer Center of the VU University Medical Center, were included. All patients underwent a standard clinical assessment, including medical history, physical and neurological examination, screening laboratory tests, psychometric evaluation, EEG, MRI. Diagnosis of “probable AD” was made according to the NINCDS-ADRDA criteria \(^1\) by consensus in a multidisciplinary team, which was blinded to the results of the CSF analyses. When all investigations yielded normal results, subjects were considered to have subjective complaints and were designated as controls. After evaluation, 131 subjects aged 61.4±10.1 years, 52% female, with mini mental state examination (MMSE) score 28.5±1.8 were classified as having subjective memory complaints and were designated as controls while 248 patients aged 66.7±9.2 years, 48.4% female, with MMSE score 20.8±5.1 were diagnosed as “probable AD”. Seventy three percent of the AD patients carried ApoE\(\varepsilon\)4, as compared to 30% of the controls.

CSF analysis

CSF was obtained by lumbar puncture between the L3/L4 or L4/L5 intervertebral space, and collected in 12 mL polypropylene tubes. The protocol has been approved by the ethical review board of our institution and all subjects have given written consent to undergo the lumbar puncture. Within two hours CSF samples were centrifuged at 2100g for 10 minutes at
4 °C. A small amount of CSF was used for routine analysis, including total cells, total protein, and erythrocytes. The remaining CSF was mixed and aliquoted into 0.5 or 1 mL polypropylene tubes, and stored at -80 °C until further analysis of the biomarkers which occurred within a month. Aβ42, Tau, and P-Tau concentrations were determined with sandwich ELISAs [Innotest® β-Amyloid1-42, Innotest® hTAU-Ag, and Innotest® Phosphotau181P; Innogenetics, Ghent, Belgium]. As the manufacturer does not supply control specimens, the performance of the assays was monitored with pools of surplus CSF specimens. In the study period multiple specimens with various concentrations which were included in 7-18 runs have been used for this purpose. The inter-assay CV’s obtained (mean±SD) were 11.3±4.9% for Aβ42; 9.3±1.5% for Tau and 9.4±2.5% for P-Tau respectively.

Table 1

<table>
<thead>
<tr>
<th>CSF Marker</th>
<th>Cut-off for 85% sensitivity [ng/L; (95%CI)]</th>
<th>Specificity [%; (95%CI)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ42</td>
<td>550 (531-570)</td>
<td>83 (76-89)</td>
</tr>
<tr>
<td>Tau</td>
<td>375 (325-405)</td>
<td>78 (70-85)</td>
</tr>
<tr>
<td>P-Tau</td>
<td>52 (48-56)</td>
<td>68 (60-77)</td>
</tr>
</tbody>
</table>


Data analysis
Cut-off values to achieve the 85% sensitivity as advocated in the Reagan Consensus Report, were determined and corresponding specificities were calculated. Receiver Operating Characteristic (ROC) curves were drawn by plotting the true-positive rate (sensitivity) against the false-positive rate (100-specificity). In addition, the areas under the ROC curve (AUC) and its standard error were calculated. The ROC curves were compared with the Hanley and McNeil method using Medcalc V 4.30 Software (Mariakerke, Belgium). Logistic regression analysis with backward stepwise selection was used to estimate the simultaneous impact of the continuous variables Aβ42, Tau, and P-Tau in CSF on the diagnosis “probable AD”. Correlations were calculated with the Spearman method.

Results
Cut-off values to distinguish AD patients from controls with a sensitivity of 85% and the associated specificities are shown in Table 1. Clearly, Aβ42 at a cut-off value of 550ng/L demonstrates the highest discriminatory power. Both in AD and SMC patients concentrations of Tau and P-Tau were highly correlated (both groups r=0.89). To investigate whether the performance of the biomarker tests was reproducible over time, the study period was divided in four quarters of 18 months each and cut-off levels and specificities were calculated for each period (Fig. 1). Over time, the cut-off value for Aβ42 turned out to be the most stable (532±31ng/L) showing a coefficient of variation of only 5.7%.
over the four study periods. Cut-off values for Tau and P-Tau showed modest fluctuation (9.1% resp. 9.6%). Also with respect to the specificity at 85% sensitivity Aβ42 showed less fluctuation than the other two markers (9.1% vs. 13.8% and 28.9%). It appears from Figure 1 that the highest fluctuation was observed in periods 1 and 2, whereas in periods 3 and 4 a more stable performance was observed. We therefore considered the first two periods as learning or proficiency time and restricted further analysis to periods 3 and 4, covering the last three year period of our study in which 155 AD patients and 84 SMC patients were included. For 194 patients in this group (81.2%) the ApoE4 status was known. Table 2 shows the CSF biomarker levels according to the ApoE4 status. In controls CSF Aβ levels were significantly lower in ApoE4 carriers than in non carriers. ROC curves for the three CSF biomarkers are shown in Figure 2. Pair wise comparison of the ROC curves for Aβ42, Tau and P-Tau in CSF showed no significant difference for Aβ42 (AUC 0.928; 95%CI: 0.888-0.952) vs. Tau (AUC 0.911; 95%CI: 0.868-0.944; p=0.51) or Aβ42 vs. P-Tau (AUC 0.880; 95%CI: 0.832-0.918; p=0.082). By contrast, the AUC’s of Tau and P-Tau were found to be statistically different (p=0.017). Logistic regression analysis with diagnosis (probable AD and controls) as dependent variable and Aβ42, Tau, and P-Tau in CSF as independent continuous variables resulted in correct classification of 145/155 (93.5%) probable AD patients and 76/84 (90.5%) controls, with an overall correct percentage of 92.5%, at a cut-off line Aβ42=373+0.82∗Tau (Fig. 3). In this model P-Tau did not contribute to distinguish patients with “probable” AD from controls. We also investigated whether P-Tau would have additional diagnostic value in the non ApoE4 carriers. In a logistic regression model, no such value was observed. CSF Biomarker levels (ng/L; Mean±SD) according to ApoE4 status of the patients studied in periods 3 and 4. * P<0.01 vs ApoE4 non carriers.

Table 2

|        | ApoE4 - |    | ApoE4 + |    | AD ApoE4 - |    | AD ApoE4 + |    |
|--------|---------|    |---------|    |------------|    |------------|    |
| N (%)  | 54 (70) | 23 (30) | 32 (27) | 85 (73) |
| Aβ42   | 916±182 | 745±205* | 507±180 | 460±137 |
| Tau    | 276±116 | 300±161 | 804±404 | 792±484 |
| P-Tau  | 44±14   | 50±23   | 91±39   | 89±39   |

* P<0.01 vs ApoE4 non carriers

Discussion

An important lesson to be learnt from our experience with assays for CSF biomarkers is that a certain degree of experience appears to be required in order to have confidence in the results obtained. Results appear to reach stability only in the second half of our study period. Reasons for the lack of stability in the first half may include limited experience with the assays and differences in the patient groups referred for analysis, such as age, severity of symptoms etc. Also the mere fact that more patients were studied in the second half of the study may be of influence. Despite efforts to prevent pre-analytical variation such as time between lumbar puncture and storage, this may also have contributed.
Finally, analytical variation is a source of variation. This may have had several reasons, ranging from lot-to-lot variations in reagents and variation in technical skills. Although we have carefully reviewed all possibilities, we have not been able to identify the reason(s) for the higher fluctuation in the first half of our study. In order to overcome the issue of lot-to-lot variation, we nowadays purchase large numbers of kits from the same lot. Obviously, this is only possible since our request load has significantly increased recently. We anticipate to see a decrease in the inter-assay variation in the near future. The second finding of our study is that despite variation in time, the assays for CSF biomarkers Aβ42 and Tau appear to be sufficiently stable to be used to distinguish AD from controls. CSF P-Tau was ruled out in the logistic regression model and hence had no additional value in the separation of patients with AD from controls. As shown before, the proportion of ApoE4 carriers is considerably higher in AD patients than in control subjects. The significantly lower CSF Aβ levels in controls carrying the ε4 allele may be an indication of their increased risk to develop AD. Optimal sensitivity and specificity are obtained when the contrast between the AD patients and controls is optimal, i.e. the AD patients are correctly classified as AD patients and the controls as healthy subjects.

Figure 1

Performance of CSF biomarkers in AD over time (from 2001 to 2007). Cut-off values for Aβ42, Tau and P-Tau yielding 85% sensitivity and specificity at 85% sensitivity.
The accuracy of the clinical diagnosis for ‘probable’ AD has been reported to be relatively low, with a sensitivity of 81% and a specificity of 70% \(^4\). The power of biomarkers for separating AD patients from healthy controls has been demonstrated by Riemenschneider et al. \(^{135}\) who investigated 74 patients with AD and 40 cognitively healthy control subjects. These authors reported higher cut-off levels than we found. It is unclear whether this has to be attributed to differences in the patient groups or to experimental differences. It does illustrate the necessity for laboratories to establish their own reference values. The importance of the composition of the patient groups and co-morbidity has been shown in a multicenter study \(^{60}\) as well as in a meta-analysis \(^7\). Although patient groups differed in the various studies, for \(\beta\)-42 the sensitivity was always >75%, whereas the lowest value for Tau a broader range was reported, the lowest value being 30%.

**Figure 2**

ROC curves of \(\beta\)-42, Tau and P-Tau in CSF. Sensitivity vs. specificity was calculated for all subjects in period 3 plus 4. The graph was drawn with 84 controls and 155 probable AD patients studied in 2004-2007.

After completion of our study it became clear that in non-demented individual subjects \(\beta\)-42 levels may show significant diurnal variation, the lowest values being observed in the morning \(^{141}\). It is presently unclear whether this also applies to patients with AD. We could not evaluate this phenomenon in our material due to a lack of serial samples over the day, but it highlights the fact that diurnal variation of CSF biomarkers requires further investigation. Until the issue is settled, time of collection of CSF should be taken into account and lumbar punctures should preferably be performed at the same time of the day per centre. Many studies have demonstrated an association of low \(\beta\)-42 and high Tau levels with AD \(^{136-138}\). Calculation of a separation line, e.g. by logistic regression, may be helpful in the classification of the subjects with low Tau/low \(\beta\)-42 and high Tau/high \(\beta\)-42, which otherwise would be difficult to classify. We found that the best discrimination between 84
patients with mild cognitive impairment and 155 patients with probable AD was achieved by the line $A_\beta^{42}=373+0.82\times$Tau, leading to an overall correct classification of 92.5%. Other discrimination lines have been published, e.g. by Hulstaert et al. who reported that the line $A_\beta^{42}=240+1.18\times$Tau discriminated AD patients from a group of healthy volunteers and patients with other neurological disorders with 85% sensitivity and 86% specificity. As this was a multicenter study and the control group differed from ours, a direct comparison is not possible. Using the same discrimination line, Andreasen et al. reported a sensitivity of 94% for probable AD, 88% for possible AD, and 75% for mild cognitive impairment, whereas specificity was 100% for discrimination from psychiatric disorders and 89% for non demented individuals. Riemschneider et al. found a 92% sensitivity and a 95% specificity for separation of AD vs. controls with the line $A_\beta^{42}=644+0.25\times$Tau. The differences in the equations of the discrimination lines, illustrate considerable variation between institutions with respect to experimental procedures, composition of patient group and urgently calls for standardization.

One approach to this is the establishment of international quality control schemes for the assessment of the biomarkers. Our group has recently reported on such an initiative.

One other result of the logistic regression was the elimination of P-Tau as a valuable marker to distinguish patients with AD from controls both in the entire group and in the ApoE4 carriers. CSF P-Tau is claimed to reflect the pathology of microtubules and was, therefore, expected to contribute significantly. Nevertheless, this parameter was removed as a variable in the equation of the logistic model ($p=0.64$). The high correlation coefficient between P-Tau and Tau ($r=0.93; p<0.001$) is probably the reason for this. Despite this, P-Tau is considered to be of importance in the differential diagnosis of AD.

There are two important diagnostic issues, e.g.: the discrimination between AD and non AD on one hand and the conversion of MCI to AD on the other. In the daily practice of our memory clinic, the number one issue is to distinguish between AD and Non AD. Conversion of MCI to AD is a separate issue which requires longitudinal follow up of the patients. Previous work of our group has shown that for the three CSF biomarkers used serial measurement is of limited value. When the concentration of one analyte is increased and that of the other is decreased, a ratio might be more informative. When results of biomarker measurements are clearly method dependent, as is the case with CSF biomarkers, calculation of a ratio has only local value and is not useful for comparison with other studies. We have calculated the sensitivity and specificity of the Tau/Amyloid beta ratio. We found a ratio of 0.59 to yield a 91.2% sensitivity (95%CI: 87-96) and a 91.7% specificity (95%CI: 84-97). The high Tau level observed in some patients (Fig. 3) might raise the question whether these patients may be suffering from Creutzfeldt-Jakob disease. As implied in the methods section, such a diagnosis was not made in any of our patients. We have re-evaluated the records of patients in whom a CSF Tau level higher than 1300 ng/L was reported. No reason for the high Tau concentration became apparent.

The value of CSF biomarker analysis in the diagnosis of AD has been addressed in several recent papers. Mattsson et al. report that incipient AD is more accurately identified in single-centre studies than in their multi-centre study. They conclude that there is a need...
for standardization in analytical as well as in clinical procedures. In another multi-centre study, Buerger et al. found that analysis of specimens from all participating centres in a single laboratory increases the diagnostic accuracy. This also emphasizes the need for standardization. Welge et al. found that inclusion of other amyloid species, i.e., Aβ1-38 in the CSF biomarker panel increased sensitivity for the detection of AD and also the specificity for excluding non-Alzheimer dementia’s. Vemuri and colleagues showed that combination of CSF biomarker analysis and MRI is superior for the prediction of progression of MCI to AD to either procedure alone. In conclusion, CSF Aβ42 and Tau are useful as biomarkers to identify patients with probable AD from controls in a memory clinic setting. Cut-off values, sensitivities, specificities, and discrimination lines are dependent on the subjects referred and laboratory experience. International standardization and collaboration are expected to contribute to the further dissemination of CSF biomarker analysis in clinical practice.

**Figure 3**

Scatterplot of CSF Aβ42 vs. Tau in 155 patients with “probable” AD and 84 controls. The equation of the line for optimal separation is: [Aβ42] = 373 + 0.82 * [Tau].
3.3
A worldwide multicenter comparison of assays for CSF biomarkers in AD


*Ann Clin Biochem, 2009 May;46(Pt 3):235-40*
Abstract

Background
Different CSF Amyloid-beta 1-42 (Aβ42), total Tau (Tau) and Tau phosphorylated at threonine 181 (P-Tau) levels are reported, but quality control programs are currently lacking. The aim of this study was to compare the measurements of these CSF biomarkers, between and within centres.

Material and Methods
Three CSF-pool samples were distributed to 13 laboratories in 2004 and the same samples were again distributed to 18 laboratories in 2008. In 2004 six laboratories measured Aβ42, Tau and P-Tau and seven laboratories measured 1 or 2 of these marker(s) by ELISAs. In 2008 twelve laboratories measured all 3 markers, three laboratories measured 1 or 2 marker(s) by ELISAs, and three laboratories measured the markers by Luminex.

Results
In 2004 the ELISA inter-centre coefficients of variance (interCV) were 31%, 21% and 13% for Aβ42, Tau and P-Tau. These were 37%, 16% and 15% in 2008. When we restricted the analysis to the Innotest® (N=13) for Aβ1-42, lower interCV were calculated (22%). The centres that participated in both years (N=9) showed in 2008 interCV's of 21%, 15% and 9% and intra-centre coefficients (intraCV) of variance of 25%, 18% and 7%.

Conclusions
The highest variability was found for Aβ42. Variability for Tau and P-Tau were lower in both years. The centres that participated in both years showed a high intraCV comparable to their interCV, indicating that there is not only a high variation between, but also within centres. Besides a uniform standardization of (pre)analytical procedures, the use of the same assay is needed to decrease the inter/intra-centre variation.
Chapter 3: CSF measurements in AD: Clinical chemical properties

Introduction

A pronounced increase in the CSF levels of Tau and P-Tau proteins, and a significant decrease in the CSF level of Aβ42, have been found in AD patients compared to controls. For this reason measuring Aβ42, Tau and P-Tau in CSF has gained wide recognition, not only to discriminate AD patients from controls or patients with other types of dementia, but also to detect incipient AD in MCI patients. The number of labs that measure these parameters has increased substantially over recent years. Different labs report different concentrations, resulting in differences in reference ranges and reference values. Variations are partly due to differences in ELISA systems, methods, and protocols used. Even if the same ELISA is used, inter-technician variations, differences in performing intra-centre validations, and differences in production of the commercially available ELISAs over time, can induce variability. Finally, pre-analytical factors as differences in sampling, processing and storage of CSF can contribute to intra- and inter-centre variations. External quality control measurements showing acceptable intra/inter centre variations are needed to compare inter-centre biomarkers results in multi-centre studies.

However, until now only one study performed a quality control program between 14 centres in Europe and reported inter-centre variations that varied from 26% to 29% for Aβ1-42, Tau and P-Tau. In this study we report a quality survey performed in 20 labs all over the world involving two time points one in 2004 and one in 2008.

Material and Methods

Participants

Participants were asked to collaborate and to measure Aβ42, Tau and P-Tau in three different CSF-pool samples. They were again contacted before sending the samples. Only after confirmation, the packages were shipped on dry ice with a 24 hour shipping service. The labs were asked to contact the Clinical Chemistry laboratory of the VUmc on arrival of the packages, to report the status of the arrived samples. If the samples were not frozen on arrival, new samples were sent.

Samples

In 2003 three different CSF-pool samples were provided by the Neurochemistry lab of Möldal Hospital Sweden, to the Clinical Chemistry laboratory of the VUmc. At least 150 aliquots of each sample were sent in dry ice and subsequently stored at -80°C. Sample 1 had high Tau and P-Tau levels, sample 2 had low Aβ42 level and sample 3 had normal biomarker profile. These three CSF-pool samples were distributed to 13 labs in 2004 and the same samples were again distributed to 18 labs in 2008. Before shipping, the samples were selected, screened and labelled by two technicians at the lab of the VUmc. The samples were blinded for the participating lab and were sent worldwide on dry ice with the instruction to store the samples at -80°C.

CSF analysis

In 2004 thirteen centres participated in the quality control. Aβ42, Tau and P-Tau were measured by 7 laboratories and 6 labs measured 1 or 2 parameter(s) by ELISA. Tau and P-
Tau levels were all measured by the Innotest®. Aβ42 was measured by eight centres with the Innotest®, 2 labs used the Genetics Company® test and one lab ran an in-house assay. One laboratory showed the opposite pattern of the expected biomarker results, probably due to sample switching. These results were excluded from analysis. In 2008 eighteen centres participated in the quality survey. Aβ42 and P-Tau were measured by 15 laboratories and 3 labs measured 1 or 2 biomarker(s) (see Table 1). To measure Aβ1-42, thirteen centres used the Innotest®, three laboratories used the Luminex (AlzBio®), 1 lab ran an in-house assay and one lab performed the Biosource® assay. Of the 11 centres that participated in both years, nine labs performed all measurements with the same assay. These nine centres used the Innotest® for Tau and P-Tau measurements. Aβ42 was measured by eight centres with the Innotest® and one lab used an in-house test. According to the data provided by the manufacturers of the Innotest®, the intra-assay variations are less than 6%, and the inter-centre variations are less than 10 %, for the three biomarkers.

Statistics
Data were analyzed with the SPSS software package (version 15.0 for Windows SPSS, Chicago, IL). Mann Whitney U test was used to compare CSF levels between 2004 and 2008. The inter-centre coefficients of variation (interCV) were calculated according to the following formula: (SD) / (mean of the measurements) for each biomarker and each sample. The intra-centre coefficients of variances (intraCV) were calculated according to the following formula: mean of (SD of the two measurements) / (mean of the two measurements). Subsequently, we calculated for each biomarker the mean CV of the three samples.

Results
Table 1 shows the median (min-max) values for the three biomarkers of the three samples measured in 2004 and 2008. There were no significant differences comparing the ELISA results of 2004 and 2008, except for Tau levels of sample 3 that showed a decrease (p<0.05). Figure 1 shows the individual biomarker values of the three samples in both years. Luminex (AlzBio®) results are shown with squares and ELISA measurements are shown as dots. Besides large differences in absolute CSF levels between these two methods (Table 1), a lack of linearity was seen, indicating that the differences cannot be attributed solely to standardization, rendering comparison of these two methods not useful. The ELISA InterCV’s for Aβ42, Tau and P-Tau were relatively high, with comparable variability in both years (Table 1). When only the Innotest® was used to measure Aβ1-42 (N=13), the mean interCV of 2008 (22%) was substantially lower. (Note that all centres used Innotest® to measure Tau and P-Tau). Luminex® mean interCV’s were comparable to Innotest® interCV’s for all three biomarkers. The centres that participated in both years (N=9) showed an ELISA interCV of 30%, 21% and 13% in 2004, and 21% 15% and 9% in 2008. IntraCV’s were 25%, 18% and 7% for Aβ42, Tau and P-Tau (individual values are shown in figure 1 by connecting lines).
Discussion

The main finding of this study is that inter-centre variability in three widely used CSF biomarkers for AD was high. The highest inter-centre variability was found for Aβ42. Variability’s for Tau and P-Tau were lower. Further, the intra-centre variability for the three biomarkers, calculated for the centres that participated in both years, was comparable with inter-centre variability, indicating that there is not only a lot of variation between, but also within centres. Considering the fact that for CSF markers external quality assessment schemes (EQAS) are not yet common, this result is nonetheless encouraging. There are several potential reasons for the high variation of the Aβ1-42 measurement found in 2004 and 2008. Different antibodies, incorporated in the different ELISA assays, catch/detect different kinds and different amounts for Aβ42, resulting in different calculated values of this peptide in CSF. In this study several assays (Biosource®, Genetics Company®, Innotest®, in-house assays) have been used, resulting in different values and thus higher variation. When we restricted the analysis to only the Innotest® or the Luminex® (AlzBio3®) results, lower variations were observed. However, inter-centre variability for all three biomarkers still remains relatively high in comparison with the data provided by the Innotest® manufacturer, indicating that there are other factors that cause variability. Several publications have shown that pre-analytical factors as sampling in different tubes, contamination with blood, processing-time after withdrawal, time of lumbar puncture and different storage conditions, influence the measured concentration of amyloid in CSF. However, these conditions as a cause of variability were not likely to be the case, since all samples were processed/collected/ aliquoted in the same manner and all aliquots were directly stored at -80 centigrade in polypropylene tubes. Variability can also be induced by analytical factors as differences in (room) temperature during running the assay, differences in incubation time, the use of manual or mechanical pipetting systems, inter-technician-variations in adherence to the assay procedure and differences in detecting machinery. However, few effects in the analytical results by these factors are reported in the literature. In addition, in this study, the measurements were performed in experienced laboratories, making it less probable that these factors, were responsible for much of the variation. Finally, variability can also be induced by assay lot-to-lot differences. This has been shown by our group, which ran the same assay using the same CSF in two different time points. These studies have shown a high degree of variability in the results of measurements of these three biomarkers, indicating that there are probably differences in the assays prepared by the same manufacturer over time (differences in production of antibodies, in standard solutions and in plates used). Unfortunately, we do not know which batch number the different labs used in this study, but this may be an important cause of variability between and maybe even more important also within centres. Large differences in absolute biomarker levels between centres have also been reported in a meta-analysis. In addition, the high ELISA inter-centre variability found for Aβ1-42 is in line with the results of Lewczuk et al. However, the inter-centre variations for Tau and P-Tau are considerable lower in our study than in this former multi-centre comparison, showing that lower variability can be reached and thus encouraging further studies. In summary, the high variability found, especially for Aβ42 (>22%), has clinical implications and lower variability (<10%) is required to reliably distinguish a difference in groups. For commonly used immunoassays EQAS with over 100
participants, inter-centre variability of 9-15% (TSH and Prolactin) are reported (data reported by the Dutch National External Quality Assessment Scheme for Ligand-Assays of Hormones, Tumour markers and Vitamins). In this perspective, together with the information from the assay-manufacturers and the within laboratory variations reported in the literature 13, 81, lower inter-centre variability for Aβ42, Tau and P-Tau should be obtained. This is needed to make multi-centre biomarker comparison possible and eventually to obtain an inter-centre reference range for AD.

Figure 1

The individual biomarker level for three different CSF samples measured by the different centres in both years.
Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>2004</th>
<th>2008</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean Levels</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ42 pg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>11</td>
<td>491 (345-702)</td>
</tr>
<tr>
<td>(min-max)</td>
<td></td>
<td>327 (237-882)</td>
</tr>
<tr>
<td>Mean</td>
<td>11</td>
<td>693 (509-1179)</td>
</tr>
<tr>
<td>CV</td>
<td></td>
<td>31 %</td>
</tr>
<tr>
<td>Median</td>
<td>2</td>
<td>987 (609-1091)</td>
</tr>
<tr>
<td>(min-max)</td>
<td></td>
<td>329 (263-616)</td>
</tr>
<tr>
<td>Mean</td>
<td>3</td>
<td>191 (138-649)</td>
</tr>
<tr>
<td>CV</td>
<td></td>
<td>21 %</td>
</tr>
<tr>
<td>Tau pg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>1</td>
<td>899 (609-1091)</td>
</tr>
<tr>
<td>(min-max)</td>
<td></td>
<td>329 (263-616)</td>
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<tr>
<td>Mean</td>
<td>2</td>
<td>191 (138-649)</td>
</tr>
<tr>
<td>CV</td>
<td></td>
<td>21 %</td>
</tr>
<tr>
<td>Median</td>
<td>3</td>
<td>108 (76-127)</td>
</tr>
<tr>
<td>(min-max)</td>
<td></td>
<td>51 (41-166)</td>
</tr>
<tr>
<td>Mean</td>
<td>3</td>
<td>38 (27-43)</td>
</tr>
<tr>
<td>CV</td>
<td></td>
<td>13 %</td>
</tr>
</tbody>
</table>

Results of the three biomarkers measured at two time points. Sample 1: high Tau and P-Tau profile; Sample 2: low Aβ42 profile; Sample 3: normal biomarker profile. Tau and P-Tau levels were all measured by the Innotest® in both years. In 2004, nine centres measured Aβ42 with the Innotest®, 2 labs with the Genetics Company® and one lab performed an in-house assay. In 2008, 13 centres measured Aβ42 with the Innotest®, 1 lab with an in-house assay and 1 lab performed the Biosource® assay. Three laboratories measured all 3 markers by Luminex (AlzBio3®).