Neurochemical diagnosis of Alzheimer’s dementia by CSF Aβ42, Aβ42/Aβ40 ratio and total tau

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Abstract

Cerebrospinal fluid (CSF) concentrations of amyloid β peptides ending at positions 42 and 40 (Aβ42 and Aβ40, respectively), and total tau (tTau) protein were measured by ELISA in order to compare their accuracy in discriminating patients with Alzheimer’s disease (AD, n = 22), non-Alzheimer dementia (nAD, n = 11) and control subjects (CON, n = 35). As compared to the other groups, the concentrations of Aβ42 and tTau were decreased (P < 0.001) and increased (P < 0.001) in AD, respectively, while Aβ40 did not differ significantly among the groups. Receiver operating characteristic (ROC) analysis was performed to define cut-off values for maximized sensitivity and specificity. For all groups compared the Aβ42 peptide ratio 42/40 classified more patients correctly, as compared to the concentration of Aβ42 alone: AD versus controls, 94 and 86.7%; AD versus nAD, 90 and 85% and AD versus nAD plus controls, 90.8 and 87%, respectively. The percentage of correctly classified patients was further improved when the Aβ ratio was combined with the analysis of the tTau concentration. Presence of the apolipoprotein E ε4 allele, age or degree of mental disability did not significantly influence the parameters studied.

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1. Introduction

Alzheimer’s disease (AD) is the major cause of memory impairment and dementia in the elderly, and is one of the most severe causes of health problems in this group. Meanwhile, the advent of new therapeutic avenues call for an improved early and differential diagnosis of AD. Since cerebrospinal fluid (CSF) is in direct contact with the central nervous system (CNS), several potentially promising CSF biomarkers have been tested alone or in combinations [4,24]. Among these biomarkers, amyloid β peptides (Aβ peptides) and tau protein(s) fulfill the criteria for good AD diagnostic tests, as recently summarized by an expert review [1]. This is not surprising since these factors are directly involved in pathologic events of the disease, namely deposition of senile plaques and formation of neurofibrillary tangles. CSF concentration of amyloid β peptide ending at amino acid position 42 (Aβ42) was reported to be decreased in AD [8,20,25,26], and reports have been published demonstrating that a combination of Aβ42 and tau protein can increase the accuracy of the neurochemical diagnosis of AD [3,6,8]. Interestingly, although the concentration of another amyloid β peptide species, Aβ40, was reported to be unaltered in AD, the ratio of Aβ42 to Aβ40 was suggested to be superior to the concentration of Aβ42 alone in discriminating patients with AD [21]. This effect was even more prominent for a recently published quantitative Aβ-SDS-PAGE/immunoblot [26].

In the present study, we used receiver operating characteristic (ROC) analysis to compare sensitivities, specificities
2. Materials and methods

2.1. Patients

The study was approved by the ethics committee of the University of Goettingen. All patients gave their informed consent. CSF was obtained from all patients by a lumbar puncture as a part of a routine diagnostic procedure. CSF was aliquoted, immediately frozen and stored at −80 °C until analysis. The demographic data of subjects are presented in Table 1. The following groups of patients were investigated:

- The group of AD patients (n = 22) consisted of patients diagnosed according to the criteria of ICD-10 and National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) [14]. The degree of mental impairment was assessed with the Mini Mental State Examination (MMSE) [5]. As presented in Table 1, the majority of patients of this group had a moderate to severe stage of mental impairment.
- The group of patients with non-Alzheimer dementia (nAD, n = 13) were diagnosed according to psychiatric and neurologic criteria (ICD-10). The diagnostic work up included either cranial CT or MRI. The following subjects were included in this group: vascular dementia (n = 11) were diagnosed according to psychiatric and neurologic criteria (ICD-10), dementia due to alcohol abuse (n = 1), subcortical arteriolar sclerotic encephalopathy (n = 1), senile dementia of vascular origin (n = 1), frontotemporal degeneration accompanied by Still-Richardson-Olszewski syndrome (n = 1), dementia due to alcohol abuse (n = 1) and dementia of unclear etiology (n = 1).
- The control group (CON, n = 35) comprised patients with neurologic or psychiatric diseases but without memory complaints. All subjects underwent extensive dementia investigation including careful clinical examination, routine blood, urine and CSF tests, magnetic resonance imaging or computed tomography and neuropsychological tests when applicable.
- Routine CSF/serum analysis was performed according to Reiber [19] including assessment of blood—CSF barrier function and symptoms indicating neuroinflammatory disorders.

The amyloid β peptides measurements were performed in a blinded fashion, i.e. the investigator to perform the assays was not aware of the diagnosis of the patients until the assay was performed, and the results of these measurements did not influence the diagnosis. Aβ42 and tTau were measured in all patients investigated. Aβ40 and consequently Aβ peptides ratio were measured and evaluated in n = 21 cases of AD, n = 10 cases of nAD and n = 33 cases of CON. In the remaining four cases, Aβ40 was not assayed due to limited volume of the CSF obtained from the patients. ELISA kits to measure Aβ42 and Aβ40 were obtained from Abeta (Heidelberg, Germany), and ELISA kits to measure tTau were obtained from Innogenetics ( Zwijndrecht, Belgium). All samples were run in duplicates.

Briefly, to measure Aβ42 concentration in CSF, 50 μl of undiluted CSF from patients or standards included in the kit were applied to microtiter plates pre-coated with antibody, WO-2. This antibody (isotype, IgG2a, κ) specifically recognizes the N-terminal of Aβ peptides since its epitope is localized at the amino acid positions 4–10. Therefore, the antibody reacts with both Aβ40 and Aβ42 peptides as well as other Aβ species [9,11]. Following an overnight incubation at +4 °C and washing steps, detection antibody indirectly linked to an enzyme was applied. This antibody, G2-13 (isotype, IgG1, κ), has its epitope localized at the carboxy terminus of the Aβ42 peptide, and thus, specifically recognizes this peptide. After incubation and washing, chromogene was added to incubate for 15–20 min, and the reaction was stopped with sulfuric acid. The color reaction was measured with an automatic ELISA reader (Benchmark, Bio Rad, USA) with the wavelength set at 450 nm and a reference wavelength of 655 nm. The software (Microplate Manager, Bio Rad, USA) was used to create standard curves and to calculate the concentration of the samples.

To measure the Aβ40 concentration in CSF, a similar procedure was applied, except that before application to a microtiter plate, the samples were pre-diluted 1:5 with diluting buffer included in the kit. The capture antibody used in this kit is this same one as in the Aβ42 assay (WO-2),
and the detection antibody (G2-10, isotype, IgG2b, κ) recognizes specifically carboxyl terminus of the Aβ40 peptide (amino acid positions, 31–40) [9,11]. The percentage ratio of Aβ42/Aβ40 (the Aβ peptides ratio) of each patient was calculated as the concentration of Aβ42 divided by the concentration of Aβ40 and multiplied by 100. 

To measure tTau in CSF, undiluted CSF or standards were applied to each well of the microtiter plate simultaneously with conjugate. After an overnight incubation at room temperature followed by washing steps, second conjugate was applied to incubate for 30 min. The wells were then washed and substrate solution was applied. The reaction was stopped with sulfuric acid, and the color reaction was read at 450 nm with a correction wavelength set at 620 nm.

Apolipoprotein E (ApoE) genotyping was done in n = 21 subjects with AD. Briefly, genotyping was performed with Inno-LiPA ApoE kits (Innogenetics, Ghent, Belgium) on genomic DNA purified from blood with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The reaction was performed using thermocycler Biometra (Goettingen, Germany) according to a previously described protocol [16]. The results are presented with regard to the presence or absence of ε allele of ApoE gene.

2.3. Statistical analysis

The results are expressed as medians and percentile ranges (p25–p75). Since normal distribution of some parameters was rejected (Shapiro–Wilk’s test), further statistical comparison between groups was performed with the Mann–Whitney’s U test (Statistica 6.0, Statsoft, USA). The correlation between parameters was assessed with Spearman’s correlation rank. A P level less than 0.05 was considered significant. Sensitivity was calculated as the ratio of the number of patients with the positive result of the assay to the number of patients without the disease. Receiver operating characteristic analysis was performed (SPSS software, version 10) to optimize sensitivities and specificities of the assays tested. The Youden index was calculated for each cut-off value as corresponding ([sensitivity + specificity] − 1) to find the cut-off values which maximize discriminating accuracy of the tests. The equations of discriminatory lines (Fig. 3A and C) were calculated with projection pursuit of Fisher. Statistical power and the smallest sample size required were analyzed with SPSS Power module, version 2.

3. Results

3.1. CSF Aβ peptides and tTau

The concentrations of Aβ42, Aβ40 and tTau protein in the CSF as well as the percentage ratio of the two Aβ peptides are presented in Fig. 1. The concentration of Aβ42 was significantly lower in the AD group (370.5, 296–447 pg/ml) as compared to the controls (865, 599–1111 pg/ml, P < 0.001) as well as to nAD (650, 508–1223 pg/ml, P < 0.001). Similarly to Aβ42 concentration, the Aβ42/Aβ40 ratio was significantly decreased in AD (6.37, 5.29–8.45) compared to the controls (14.26, 11.71–15.98, P < 0.001) and to nAD (13.91, 11.55–17.92, P < 0.001).

There were no significant differences between the groups regarding the concentration of Aβ40 in CSF. The results obtained in AD (5810, 4334–7895 pg/ml) differ neither from the concentration of the controls (6345, 5323–7404 pg/ml, P = 0.61) nor from the concentration of nAD (7130, 5608–7780 pg/ml, P = 0.42).

The concentration of tTau was higher in the group of AD (507.5, 442–805 pg/ml). The results were significantly increased compared to the results of controls (214, 154–277 pg/ml, P < 0.001) and the results of nAD (282, 165–423 pg/ml, P < 0.001).

The optimal cut-off values were established according to a Youden index maximizing sensitivities and specificities of the evaluated assays. These results are presented in Table 2. For each comparison, the ratio of correctly classified patients

<table>
<thead>
<tr>
<th>Marker</th>
<th>AD vs. CON</th>
<th>AD vs. nAD</th>
<th>AD vs. Both groups (nAD and CDB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ42</td>
<td>Cut-off: 550 pg/ml; correct: 86.7%; S: 100%; Sp: 83%</td>
<td>Cut-off: 580 pg/ml; correct: 85%; S: 88%; Sp: 82%</td>
<td>Cut-off: 550 pg/ml; correct: 87%; S: 100%; Sp: 80%</td>
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<tr>
<td>Aβ40 ratio</td>
<td>Cut-off: 9.75; correct: 94%; S: 95.2%; Sp: 91%</td>
<td>Cut-off: 11.8; correct: 90%; S: 95.2%; Sp: 89%</td>
<td>Cut-off: 9.75; correct: 90.8%; S: 95.2%; Sp: 88.4%</td>
</tr>
<tr>
<td>Aβ42 and tTau</td>
<td>NE</td>
<td>NE</td>
<td>Cut-off: tTau = −23.118 × 0.758 × Aβ42; correct: 95%; S: 100%; Sp: 89%</td>
</tr>
<tr>
<td>Aβ40 ratio and tTau</td>
<td>NE</td>
<td>NE</td>
<td>Cut-off: (τατ = −72.98 × Aβ42) ratio; correct: 94%; S: 100%; Sp: 91%</td>
</tr>
</tbody>
</table>

*p Correct, the percentage of correctly classified patients.

*S Sensitivity

Sp Specificity

NE Not evaluated.
Fig. 1. Individual concentrations of Aβ42 (A), Aβ40 (B), Aβ peptides ratio (C) and total tau protein (D) in CSF of patients investigated. The horizontal bars represent medians.
turned out to be slightly higher when the Aβ42 peptide ratio was used instead of the Aβ42 concentration alone.

Statistical power analysis showed for a double-sided alpha of 0.001 only 0.35% ($z$-power: $-2.697$) for Aβ40, but 99.49% for tTau ($z$-power: 2.567) and approximately 100% for Aβ42 ($z$-power: 4.379) and Aβ ratio ($z$-power: 5.627). Therefore, we conclude that results leading to similar conclusions as these in our study are expected also in other studies with reasonably high probability. The calculations of the smallest sample size for alpha = 0.01 and the statistical power = 95% revealed that the smallest sample size were 22, 18 and 38 for Aβ42, Aβ ratio and tTau, respectively. Therefore, we assume that our sample size was large enough to draw statistical conclusions.

3.2. Comparison of accuracy of the Aβ42/Aβ40 ratio and Aβ42 to discriminate AD from other groups

To examine further the accuracy of Aβ42 concentration and Aβ peptides ratio to discriminate AD individuals from controls and from patients with nAD, we used ROC curve analysis. ROC curves are presented in Fig. 2 to compare accuracy of the two tests, namely the Aβ42/Aβ40 ratio and the Aβ42 concentration to discriminate AD from controls (Fig. 2A), nAD (Fig. 2B) and combined group of controls and nAD (Fig. 2C).

For each comparison, the area under the receiver operating characteristic curve (AUROCC) characterizing the Aβ peptide ratio turned out to be slightly larger than AUROCC of Aβ42, however, these differences did not reach statistical significance. To discriminate AD from controls, AUROCCs were 0.951 and 0.926 for the Aβ42/Aβ40 ratio and Aβ42, respectively. To discriminate AD from nAD, AUROCCs were 0.92 and 0.913 for the Aβ42/Aβ40 ratio and Aβ42, respectively. To discriminate AD from a combined group of controls and nAD, AUROCCs were 0.944 and 0.923 for the Aβ42/Aβ40 ratio and Aβ42, respectively.

3.3. Combination of analysis of Aβ peptides and total tau in CSF

Results of the simultaneous analysis of two parameters, namely tTau and one of the following: Aβ42 concentration, Aβ40 concentration or Aβ peptides ratio are presented in Fig. 3. In case of combined analysis of tTau plus Aβ42 and tTau plus Aβ peptides ratio, the discriminatory lines

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**Fig. 2.** Receiver operating characteristic curve analysis of two assays. Aβ42 concentration and Aβ peptides ratio for discriminating patients with Alzheimer’s disease from patients of the control group (A), patients of non-Alzheimer’s dementia group (B) and patients of both groups taken together (C).
Fig. 3. Results of combined analysis of concentration of total tau (tTau) protein and concentration of Aβ42 (A), concentration of Aβ40 (B) and Aβ peptides ratio (C). Within AD group, cases with and without ApoE ε4 allele are marked with discrete symbols. In case of combined analysis of tTau and Aβ42 and tTau and Aβ peptides ratio, linear discriminatory functions are shown distinguishing subjects with AD from other patients. These functions have the following equations: (Fig. 1A, tTau = -23.118 + 0.758 × Aβ42), (Fig. 1C, tTau = -329.77 + 72.98 × Aβ ratio).
are presented to distinguish AD patients from other cases investigated in this study. The results of the combined analysis of the tTau and the Aβ42 are presented in Table 2. The percentage of the correctly discriminated patients, the sensitivity and the specificity in case of the combination of tTau plus the Aβ42 concentration. As an interesting outcome, we observed that only 2 out of 64 patients (3%) were misclassified by all three assays, i.e. by Aβ42 concentration, Aβ peptides ratio and tTau, and the remaining 62 cases were classified correctly by at least one of the three assays discussed. This would mean that 97% of subjects are discriminated correctly.

3.4. Influence of ApoE ε4 allele presence on Aβ42, Aβ40 and tTau concentration and Aβ ratio in the AD group

Genotyping of AD patients revealed the presence of ApoE ε4 allele in 10 out of 21 cases investigated (47.6%). The concentrations of Aβ42, Aβ40 and tTau, as well as Aβ peptides ratio were similar in AD patients with and without the allele (P = 0.51, 0.81, 0.1 and 0.7, respectively). This is also quite clearly shown in Fig. 3 where AD cases with and without ApoE ε4 allele overlap extensively.

3.5. Influence of dementia severity (MMSE) and age on other parameters tested

In AD group, we observed no significant correlation between MMSE score and Aβ42 (P = 0.24), Aβ40 (P = 0.07), Aβ peptides ratio (P = 0.18), tTau (P = 0.56) or the presence of ApoE ε4 allele (P = 0.49). Similarly, in the two other groups investigated (i.e. nAD and controls) we did not observe statistically significant correlation between age and the CSF parameters investigated (data not shown).

4. Discussion

In this study, we compared the power of the CSF Aβ42 concentration and the Aβ peptide ratio to distinguish patients with AD from subjects with other dementias and controls. Our results indicate that the value of Aβ peptide ratio is slightly increased as compared to the Aβ42 alone for the neurochemical diagnosis of AD, however, this effect fails the level of significance. The accuracy of neurochemical dementia diagnosis was further improved when we combined the two Aβ biomarkers and tTau.

The vast majority of studies performed so far have reported a decreased concentration of Aβ42 in CSF of patients with AD (recently reviewed by [4,24]), and our results are in agreement with these reports. Median values of Aβ42 in AD and controls reported in our study are very close to those obtained by Otto et al. [17] and Spjøgren et al. [23] when other ELISA assays were used. A satisfying explanation of decreased Aβ42 in CSF of AD is still lacking. The suggested mechanism of decreased Aβ42 concentration in CSF due to a simple accumulation in plaques is not sufficient, since we found decreased Aβ42 concentrations in CSF of patients with Creutzfeldt-Jakob disease with no apparent plaques at all [18]. This points to other mechanisms, such as disturbances in amyloid peptides formation and breakdown in AD. Interestingly, Jensen et al. reported recently increased concentrations of Aβ42 in CSF of patients with early stage of AD followed by a steady decrease of the concentrations [10]. The discrepancies between the results obtained by Jensen et al. and the results of this study, results of our group reported earlier [26] as well as the results of other investigators who similarly like us found decreased CSF concentrations of Aβ42 in AD [3,6,8] might be explained by different sets of antibodies and protocols used by Jensen et al. as well as by differences in the stage of the disease when the patients were included.

Similarly to our previous findings [26] as well as those of others [15,21], we did not observe differences in CSF Aβ40 concentration between the groups investigated. For further evaluation of the data, we applied ROC [7] analysis to test whether a combination of these two biomarkers could improve discriminating accuracy. Using this approach, we showed that correct classification of patients was slightly better when Aβ peptide ratio was compared to the concentration of Aβ42 alone, however, this effect was not significant statistically. This finding is in agreement with the results of Shoji et al. [21]. The reader is kindly asked to note that these investigators applied the inverse ratio (i.e. Aβ40/Aβ42). However, the type of statistical analysis used by Shoji et al. did not calculate specificity and sensitivity of some parameters. In our study, application of ROC analysis to evaluate cut-off values enabled us to maximize the discriminatory capacity of the assays. This was followed by a calculation of sensitivities, specificities and percentage of patients correctly classified of all assays tested. From this part of the study we conclude that Aβ peptides ratio is a slightly more accurate test for discriminating AD patients than Aβ42 concentration alone. As an explanation of this finding, we hypothesize that there may exist a group of AD patients which constitutively express increased levels of all Aβ peptides. As we have recently shown, absolute concentration of Aβ peptides in patients with AD possessing ApoE ε4 allele did not differ from the levels of the controls possessing this allele. However, when the ratio of the Aβ42 to all Aβ peptides was considered instead of the absolute concentration of the Aβ42, these two groups of patients were discriminated without any overlap [26]. These results may be explained by the finding that in cell culture experiments variation of the β-secretase activity strongly influences the total amount of Aβ40 and Aβ42, whereas variation of the γ-secretase activity modulates their relative abundances. Accordingly,
the percentage of CSF Aβ peptides ratio might reflect disease-associated changes in γ-secretase activity [26].

The increase in sensitivity, specificity and correct classification of patients when Aβ peptides ratio is applied instead of Aβ42 concentration alone is only a moderate one (e.g. correct discrimination of AD and controls increases from 86.7 to 94% when Aβ42 is replaced with Aβ peptides ratio) and does not reach statistical significance. In our understanding, this might be explained by the fact that the sensitivities and specificities discussed here are relatively high compared to those reported in other studies [6,8,21], and it is hard to expect a dramatic increase in assays’ accuracy in the range of these values. This holds true especially since studies based on patients without neuropathologically confirmed diagnosis of AD face uncertainty of such diagnosis [13], and absolute exactness of discrimination cannot be expected from such studies. The findings of our study, therefore, will have to be confirmed in an approach involving neuropathologic examination. However, assuming the prevalence of dementia in the European population over 65 years to be around 6.4% [12], even slightly increased accuracy of intra vitam diagnosis of AD would account for a correct diagnosis of thousands of patients yearly.

Further improvement of the discrimination between the groups was observed when analysis of Aβ peptides was accompanied by concentration of tTau protein in CSF. This is consistent with results reported by others [2,8]. However, interestingly, Shoji et al. [21] did not observe any improvement in discrimination accuracy between AD and other groups when Aβ peptides ratio was combined with tTau concentration. We did not observe a significant influence of ApoE ε4 allele presence on other parameters investigated, as has been reported in other studies [8]. This might be partially explained by the fact that our AD group was smaller than the groups in the studies where such a correlation was observed. Similarly to the results of a large multicenter study of Hulstaert et al. [8], decline in cognitive performance of patients in our study did not correlate significantly with CSF concentration of Aβ42 or tTau. However, in some reports such a correlation was observed, in particular patients with an early stage of the disease had a significantly higher concentration of ApoE4 compared to patients with a more advanced stage [10,20]. In our group, the majority of AD subjects were characterized by a moderate and severe mental disability with median MMSE score of 14, and further studies are required to compare the assays’ accuracy in a group of patients with a milder cognitive impairment.

We did not observe an influence of age on the parameters investigated, and published results regarding this question are discrepant. In a recent report, Shoji [22] observed a third order age-dependent alteration of CSF concentration of Aβ42 and Aβ40 with a moderate increase in older controls. On the other hand, Jensen et al. [10] observed an age-related decrease of Aβ42 in the healthy control group.

Concluding, we present decreased concentrations of CSF Aβ42, and Aβ42/Aβ40 ratios in patients with AD together with increased concentrations of CSF tTau protein. Comparison of Aβ42 concentration and Aβ peptides ratio to discriminate AD patients from nAD and non-demented controls shows slightly increased accuracy of Aβ peptides ratio, however, this effect was not statistically significant. This finding has to be further confirmed in larger groups of patients, in which a higher percentage of diagnoses would be confirmed by a neuropathologic examination.

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References


