Amyloid β peptide ratio 42/40 but not Aβ42 correlates with phospho-Tau in patients with low- and high-CSF Aβ40 load

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Abstract
Neurochemical dementia diagnostics (NDD) can significantly improve the clinically based categorization of patients with early dementia disorders, and the cerebrospinal fluid (CSF) concentrations of amyloid β peptides ending at the amino acid position of 42 (Aβx-42 and Aβ1-42) are widely accepted biomarkers of Alzheimer's disease (AD). However, in subjects with constitutively high- or low-CSF concentrations of total Aβ peptides (tAβ), the NDD interpretation might lead to erroneous conclusions as these biomarkers seem to correlate better with the total Aβ load than with the pathological status of a given patient in such cases. In this multicenter study, we found significantly increased CSF concentrations of phosphorylated Tau (pTau181) and total Tau in the group of subjects with high CSF Aβix-40 concentrations and decreased Aβix-42/x-40 concentration ratio compared with the group of subjects with low CSF Aβix-40 and normal Aβ ratio (p < 0.001 in both cases). Furthermore, we observed significantly decreased Aβ ratio (p < 0.01) in the group of subjects with APOE ε4 allele compared with the group of subjects without this allele. Surprisingly, patients with low-Aβx-40 and the decreased Aβ ratio characterized with decreased pTau181 (p < 0.05), and unaltered total Tau compared with the subjects with high Aβix-40 and the Aβ ratio in the normal range. We conclude that the amyloid β concentration ratio should replace the 'raw' concentrations of corresponding Aβ peptides to improve reliability of the neurochemical dementia diagnosis.

Keywords: amyloid β, biomarkers, cerebrospinal fluid, dementias.


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Abbreviations used: AD, Alzheimer’s disease; APP, β-amyloid precursor protein; CDR, Clinical Dementia Rating; CSF, cerebrospinal fluid; MCI, mild cognitive impairment; NDD, Neurochemical dementia diagnostics.
With increasing life expectancy of the population, Alzheimer’s disease (AD) becomes one of the most severe problems in the industrialized countries. Clinically based diagnosis of AD carries a severe uncertainty: although its sensitivity is relatively high (93%), specificity may be lower, e.g. reported as much as 55% in a multicenter clinical-autopsy controlled study (Mayeux 1998). In expert hands, the clinical diagnosis of moderate to severe 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. Although it is still considered incurable, the advent of new promising therapeutic avenues like amyloid β immunization (Hock et al. 2003) calls urgently for an improved early and differential diagnosis of AD.

Since cerebrospinal fluid (CSF) is in direct contact with the central nervous system, several potentially promising CSF biomarkers have been tested alone or in combinations (Vannmechelen et al. 2001a; Blennow and Hampel 2003; Hampel et al. 2004a; Wiltfang et al. 2005). Among them, amyloid β peptides (Aβ peptides) and Tau protein(s) fulfill the criteria for good AD diagnostic tests, as summarized by an expert review (The Working Group on: ‘Molecular and Biochemical Markers of Alzheimer’s Disease’ 2003), which 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.

Amyloid β peptides result from the enzymatic cut of β-amyloid precursor protein (APP) by two enzymes known as β- and γ-secretases, whereas enzymatic processing of APP by α-secretase leads to non-amyloidogenic pathway. The CSF concentration of one of the peptides resulting from this process, namely Aβ1-42 is regularly found decreased to about 50% of the normal value in patients with AD (Wiltfang et al. 2001, 2002; Lewczuk et al. 2004c). Sensitivity and specificity of Aβ1-42 alone to distinguish AD from elderly controls were 78% and 81%, respectively, in the study of Hulstaert et al. (1999), and Galasko et al. (1998) reported similar figures of 78% and 83% for sensitivity and specificity, respectively.

The disease-related alterations of either CSF total Aβ peptides (tAβ) content or the most abundant species, Aβ40 have not been reported (van Gool et al. 1995; Ida et al. 1996; Wiltfang et al. 2001, 2002; Lewczuk et al. 2003, 2004b). However, our preliminary observations suggested that alterations of Aβ1-42 and/or Aβx-42 concentrations might not only result from Alzheimer’s disease pathology, but may also be related to total Aβ peptides concentrations. In such case, healthy individuals with relatively low tAβ might be misdiagnosed as having ‘pathologically low’ Aβ1-42 and Aβx-42 concentrations, and vice versa, AD subjects with high tAβ might be misinterpreted as normal Aβ1-42 and/or Aβx-42-carriers. To test our working hypothesis that the Aβ ratio compensates for constitutively low or high CSF amyloid β load, we have measured corresponding biomarkers in carefully selected patients characterizing with high- or low-CSF tAβ, and correlated them to phosphorylated Tau protein as an AD biomarker independent from Aβ pathology.

Materials and methods

Patients and lumbar punctures

The study was approved by the ethics committees of all the participating universities. All the patients and/or their relatives have given informed consent. Twelve German gerontopsychiatric university departments have participated in this study recruiting 312 patients with early dementias and mild cognitive impairment (MCI). Patients with early Alzheimer’s disease (D-AD) 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) (McKhann et al. 1984). Patients with other types of dementias (D-O) fulfilled the criteria of corresponding dementias. Cognitive dysfunction was assessed with CERAD neuropsychological test battery (Thalman et al. 1998), WMS-R Logical Memory (Häring 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 Clinical Dementia Rating (CDR) (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). Patients with MCI were categorized into MCI of Alzheimer’s disease (MCI-AD), and MCI of other dementia types (MCI-O) according to clinical judgement. The clinical data are presented in the Table 1.

Lumbar punctures have been performed in the sitting position according to the standardized procedure. After collection of the CSF for routine diagnosis, additional 4.5 mL of the material have been sampled into a polypropylene test tube for this study. The CSF has been centrifuged immediately after the tap (1600 g, 20°C, 15 min), aliquoted into 16 polypropylene test tubes, and frozen within 30–40 min after the puncture. The material has never been thawed/ refrozen.

Determination of biomarkers with ELISAs

Amyloid β peptides have been determined with commercially available ELISAs. Briefly, to measure the CSF concentration of

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Age (average, SD)</th>
<th>Sex (m/w)</th>
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<tbody>
<tr>
<td>D-AD</td>
<td>74</td>
<td>71.8 (8.0)</td>
<td>33/41</td>
</tr>
<tr>
<td>D-O</td>
<td>28</td>
<td>65.2 (12.1)</td>
<td>13/15</td>
</tr>
<tr>
<td>MCI-AD</td>
<td>141</td>
<td>67.1 (7.9)</td>
<td>84/57</td>
</tr>
<tr>
<td>MCI-O</td>
<td>69</td>
<td>60.4 (8.4)</td>
<td>46/23</td>
</tr>
</tbody>
</table>
amyloid β peptides family ending at the amino acid position of 42 (Aβx-42), 50 μL of undiluted CSF from patients or standards included in the kit (The Genetics Co., Zürich, Switzerland) have been applied to microtiter plates pre-coated with an antibody recognizing the N-terminal end of the peptide (WO-2). Following an overnight incubation at + 4°C and washing steps, detection antibody G2-13 (isotype, IgG1, κ) indirectly linked to an enzyme has been applied. After incubation and washing, chromogene has been added to incubate for 15–20 min, and the reaction has been stopped with sulfuric acid. To measure the concentration of Aβ peptides family ending at the amino acid position of 40 (Aβx-40) (assay from The Genetics Co), a similar procedure has been applied, except that before application to a microtiter plate, the samples were pre-diluted 1 : 5 with diluting buffer. This assay uses the same capturing antibody (WO-2), and the detection antibody (G2-10, isotype IgG2b, κ) recognizes specifically the amino acid position of 40.

As both assays are unspecific regarding the N-terminus of the Aβ peptides, we decided to use ‘x’ (Lewczuk et al. 2003). Hence, as a matter of fact, ‘Aβx-42’ consists of a family of peptides beginning at positions of 1, 2, 3, etc, and ending at the position of 42, and correspondingly ‘Aβx-40’ is a family of peptides beginning at positions of 1, 2, 3, etc, and ending at the position of 40.

Following the measurements of the Aβ peptides concentrations, the amyloid β peptides concentration ratio has been calculated (Aβx-42/Aβx-40).

The concentration of pTau181 has been measured with an ELISA (InnoGenetics, Ghent, Belgium) based on the monoclonal antibody specifically directed against the epitope in question. The phosphorylated molecules of Tau protein have been captured by the first antibody, HT7. After an overnight incubation at +4°C with a biotinylated antibody (AT270) followed by washing steps, the antigen–antibody complex has been detected by a peroxidase-linked streptavidin. The color reaction has been stopped with sulfuric acid after the incubation with the substrate solution. The optical density has been read at 450 nm without correction.

To measure tTau in CSF, undiluted CSF or standards were applied to each well of microtiter plate (Innogenetics) simultaneously with capture antibody, HT7. After an overnight incubation at +4°C, the material was captured by a second antibody, washed and horseradish peroxidase-coupled streptavidin has been added for 1 h. After the final wash, the chemiluminescence has been visualized with ECLPlus solution (Amersham Pharmacia, Uppsala, Sweden) according to the protocol of the manufacturer, using the CCD camera system (FluorSMax Multi-Imager; Bio-Rad). To separate and quantify amyloid β peptides in plasma (n = 28), a similar protocol was applied with the exception that immunoprecipitation step was performed before applying the material onto the gels (Lewczuk et al. 2004d).

Statistical analysis
Normality of the distribution of Aβx-40 CSF concentrations has been confirmed by Kolmogorov–Smirnov test. Correlations between methods have been assessed with Pearson’s correlation rank. A p < 0.05 has been considered significant. Differences between the groups have been statistically analyzed with Kruskal-Wallis test.

Results
Correlation between Aβx-40 and the total Aβ peptides concentration
Figure 1a presents the correlation between CSF concentrations of Aβx-40 as measured with ELISA and tAβ as measured with Aβ-SDS-PAGE/immunoblot (n = 20, R = 0.8, p < 0.001).

Distribution of Aβx-40 CSF concentration; high- and low-Aβ carriers
Figure 1b shows the normal distribution of Aβx-40 in the population of all the patients included into this study (average ± SD, 9426 ± 2713 pg/mL, n = 312). Correspondingly, high Aβx-40 CSF load was defined as Aβx-40 concentration higher than average + 1 SD (12 139 pg/mL, n = 50), and low Aβ CSF load has been defined as Aβx-40 lower than average – 1 SD (6713 pg/mL, n = 50).

Comparison of pTau181 concentrations between groups of patients with high- and low-Aβx-40
For further characterization of the groups, we divided patients with high- and low-Aβx-40 load into these with Aβx-42/x-40 ratio characteristic for AD, and these with the ratio non-characteristic for AD, using the cutoff from our previously published study optimized for separation of AD and non-AD dementia patients (0.11) (Lewczuk et al. 2004c). Comparison of pTau181 and total Tau concentrations between patients with low Aβx-40 and Aβx-42/x-40 concentration ratio higher than the cutoff, i.e. untypical for the diagnosis of AD (n = 31), and the patients with high Aβx-40 and Aβx-42/x-40 concentration ratio lower than the cutoff, i.e. typical for AD (n = 28) are presented in the Fig. 2. This shows that patients with Aβ ratio characteristic for AD have significantly higher pTau181 and total Tau (in both cases, p < 0.001) concentration compared with these with Aβ ratio untypical for AD.
Interestingly, patients with low-Aβx-40 and the decreased Aβ ratio \((n = 19)\) characterized with decreased pTau181 \((p < 0.05)\), and unaltered total Tau compared with the subjects with high Aβx-40 and the Aβ ratio in the normal range \((n = 22)\) (Fig. 3).

Only weak \((R < 0.3)\) correlation was observed between age of the patients and the biomarkers considered in this study. There were no statistically significant differences between biomarkers in the CSF of women and men (data not shown).

**Correlation of the biomarkers with the APOE genotype of the patients**

The correlation between the biomarkers studied and the APOE genotype is presented in the Fig. 4. We observed significant decrease of Aβ ratio \((p < 0.01)\) in patients with ε4 allele of the APOE gene compared with those without ε4 allele.

**Correlation between Aβ peptides concentrations in the CSF and plasma**

In 28 patients, we did not observe statistically significant correlation between CSF and plasma concentrations of any Aβ peptides of the ‘peptide quintet’ studied here \((\text{Aβ}1-37/38/39/40/42)\) (data not shown); however, weak correlation has been observed in the case of Aβ1-42/1-40 concentration ratio \((R = 0.56, p < 0.01)\).

**Discussion**

In this paper, we present the limitations of neurochemical dementia diagnostics in patients with either high- or low-concentration of amyloid βx-40 peptide in the CSF based on a multicenter study.

Although there have been several reports showing that the total concentration of Aβ-peptides in human CSF is not altered in pathological conditions [(Wiltfang et al. 2001, 2002; Lewczuk et al. 2004c), and reviewed in (Ida et al. 39/40/42)].
the issue how the total CSF Aβ load influence the reliability of the neurochemical dementia diagnosis has not been tested so far. Therefore, we addressed this question by measuring corresponding Aβ biomarkers, and relating them to the concentration of Aβx-40, i.e. the most abundant peptide in human CSF, hypothesized to represent the total CSF Aβ peptides load. Currently, to our best knowledge, no high throughput method is available to measure the total Aβ CSF load, and since we observed a good correlation between Aβx-40 (the most predominant Aβ peptide species in human CSF), and the total Aβ load as measured with a low throughput Aβ-SDS-PAGE/immunoblot, we hypothesize that Aβx-40 concentration describes well the total Aβ CSF concentration.

As independent biomarker describing the other pathological pathway of AD (i.e. hyperphosphorylation of Tau and formation of neurofibrillary tangles), we have used the CSF concentration of Tau phosphorylated at threonine 181 as this biomarker has been widely accepted as a AD-specific one (Vanmechelen et al. 2000, 2001b; Itoh et al. 2001; Schonknecht et al. 2003; Hampel et al. 2004b; Lewczuk et al. 2004a). We found significant increase of pTau181 and tTau in patients with high Aβx-40 and pathologic [<0.11, (Lewczuk et al. 2004c)] Aβ ratio as compared to the group of patients with low Aβx-40 and normal Aβ ratio. This points to the fact, that in these groups the Aβ ratio corresponds to the expected alterations of AD biomarkers of the ‘Tau family’, i.e. total, and hyperphosphorylated Tau.

This in turn could mean that the concentrations of Aβx-42 are not only influenced by the presence or absence of AD pathology but are also determined by the rate of APP metabolism of yet unknown background. Currently pathomechanisms of such differences in APP processing rate can only be speculated, but there are evidences that a genetic background could be responsible for it (Lammich et al. 2004). The observed significant decrease of Aβx-42 concentration, and Aβ ratio in the group of subjects with APOE ε4 allele, as well as significant increase of total Tau concentration in this group is in line with the observation of higher percentage of ε4-positive patients among AD patients compared with other dementias (Holmes 2002).

Interestingly, in a recent study a cluster of patients was identified that do not show increased CSF tau levels, while another clusters had most markedly decreased Aβ42 (Iqbal et al. 2005). Moreover, in vitro studies currently discussed
on the AlzForum (http://www.alzforum.org; Gain or Loss of Function) confirm that the Aβ peptides concentration ratio is a better biomarker of AD pathology than either of these peptides alone. Our finding are in line with these studies, and we believe that these finding might potentially have a strong impact on the interpretation of NDD outcome, since without knowing the concentration of total Aβ peptides (or Aβx-40), the ‘normal’ and ‘decreased’ Aβ peptides ending at the position of 42 may be easily misinterpreted.

Unexpectedly, we found increased pTau181 and unaltered total Tau concentration in patients with high-Aβx-40 concentration and normal Aβ ratio compared with these with low Aβx-40 concentration and normal ratio; however, it must be stressed that in majority of cases of both groups pTau181 was in the normal range or only slightly increased (Lewczuk et al. 2004a). This finding, which certainly requires further studies, might be explained to some degree by either extremely high- or extremely low-Aβx-42 concentrations in both groups, respectively (considering the mathematical definition of the Aβx-42/x-40 concentration ratio).

To our best knowledge, this report presents the distribution of Aβx-40 CSF concentration based on the biggest number of cases but not healthy controls. Explicit data of the distribution of Aβx-40 in healthy individuals are, to our knowledge, not available in the literature, however, from the inspection of the Fig. 4 in the report by Ida et al. (1996), it can be expected that this distribution is normal, too.

A satisfying explanation of decreased Aβ42 species 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 (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 increased concentration of the phospho-Tau is due to increased activity of kinase(s) or decreased activity of phosphatase(s) (Buee et al. 2000). Interestingly, some mutations of the amyloid precursor protein found in an early onset AD lead to a marked increase in synthesis of N-terminally truncated Aβ peptides ending at the position of 42, whereas Aβx-40 is obviously not affected (Ancolio et al. 1999).

We did not find correlations between the concentrations of Aβ peptides in the CSF and blood. This might point to the fact that the two sets of amyloid β peptides (in the CSF and blood, respectively) come from different metabolic mechanisms, like e.g. release from independent populations of cells in addition to diffusion through the blood-CSF barrier. To clarify this point, certainly further studies are necessary.

Concluding, the analysis of the CSF concentration of amyloid β species ending at the amino acid position of 42 might lead to misinterpretation of NDD outcome in subjects with constitutively high- or low concentration of total Aβ peptides. We postulate that the amyloid β concentration ratio (Aβx-42/x-40) can possibly improve reliability of the neurochemical dementia diagnosis.

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