BACE1 ACTIVITY IN CEREBROSPINAL FLUID AND ITS RELATION TO MARKERS OF AD PATHOLOGY

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ABSTRACT

Several studies have shown that reduced amyloid beta1-42 (Aβ1-42) and increased tau levels in cerebrospinal fluid (CSF) reflect increased Alzheimer’s disease (AD) pathology in the brain. Beta-site APP cleaving enzyme (BACE1) is thought to be the major β-secretase involved in Aβ production in the brain and therefore we investigated the relation between BACE1 activity and CSF markers Aβ40, Aβ42, total tau (t-tau) and tau phosphorylated at threonine 181 (p-tau) in CSF of control (n=12), mild cognitive impairment (n=18) and AD (n=17) subjects. Patients were classified according to their Aβ42, t-tau and p-tau CSF biomarker levels, with either an AD-like biomarker profile (two or three biomarkers abnormal: Aβ42 < 495 pg/ml in combination with t-tau > 356 pg/ml and/or p-tau > 54 pg/ml) or a normal biomarker profile (≤ one biomarker abnormal). This resulted in 19 subjects with an AD-like biomarker profile (66±6 years, 53% female and MMSE:23±5) and 28 subjects with a normal biomarker profile (62±11 years, 43% female and MMSE:27±4). Subjects with an AD-like biomarker profile had higher CSF BACE1 activity levels, compared to patients with a normal biomarker profile (20 pg/ml and 16 pg/ml respectively; p=0.01), when controlled for age and gender. In the whole sample, BACE1 activity correlated with CSF levels of Aβ40, t-tau and p-tau (r=0.38, r=0.63 and r=0.65; all p<0.05), but not with Aβ42. These data suggest that increased BACE1 activity in CSF relates to AD pathology in the brain.
INTRODUCTION

The main pathological hallmarks in Alzheimer’s Disease (AD) are the amyloid plaques and neurofibrillary tangles in the brain. The cerebrospinal fluid (CSF) is in direct contact with the extra-cellular space of the central nervous system, and therefore changes in the brain can potentially be reflected in CSF. Several studies have shown that lower CSF levels of amyloid beta 1-42 (Aβ42) and higher levels of tau correlate with increased amyloid plaque load and neurofibrillary changes in the brain 1-3. Furthermore, in positron emission tomography studies with the radiotracer Pittsburgh Compound-B, which is highly selective for insoluble (fibrillar) Aβ deposits in vivo 4, an inverse relation between the amount of PIB binding and levels of Aβ 42 in CSF was found 5-7.

Accumulation of Aβ in the brain is considered to be a main driving force for AD pathogenesis 8-9. Aβ42 and Aβ40 are both present in amyloid plaques in the brain and are generated from amyloid precursor protein (APP) following sequential cleavage by β- and γ-secretase enzymes. Knock-out of the beta-site APP cleaving enzyme 1 (BACE1) in APP transgenic mice completely abolishes the production and deposition of Aβ 10,11. Moreover, lowering BACE1 levels with small interfering RNA also reduced amyloid production and in addition was shown to substantially prevent the behavioural impairments seen in APP transgenic mice 12. In post-mortem brain samples of patients with AD, BACE1 activity is increased compared to controls 13-16, and in the CSF of incipient AD patients BACE1 activity was found to be higher than in CSF samples from healthy controls 17. Other studies have shown that patients with mild cognitive impairment (MCI), who are at high risk of developing AD 18,19, had higher levels of BACE1 activity compared to AD and control subjects, and that MCI patients who progressed to AD during follow-up had higher baseline BACE1 activity than subjects with MCI who remained cognitively stable 17,20. These studies suggest that BACE1 activity in CSF is elevated in the early stages of AD.

We hypothesize that patients with underlying AD pathology, will have higher levels of BACE1 activity in their brain, and consequently also in the CSF, compared to healthy controls. Because Aβ42, t-tau and p-tau are related to AD pathology 1-3, we examined the relation between BACE1 activity in CSF and AD pathology in control, MCI and AD patients that were classified according to their CSF biomarker profile, based on levels of Aβ42, t-tau and p-tau, either as having an AD-like biomarker profile or a normal biomarker profile.
METHODS

Subjects
Twelve controls, 18 patients with MCI and 17 patients with probable AD from our memory clinic were included in this study. All subjects underwent a standardized clinical investigation including medical history, physical and neurological examination, screening laboratory tests, and magnetic resonance imaging. Global cognition was measured using the Mini Mental State Examination (MMSE). The clinical diagnoses were made in conference by a multidisciplinary team based on accepted clinical diagnostic criteria, without knowledge of CSF data. Patients with normal clinical and neuropsychological investigations were considered to have subjective memory complaints and were included in this study as controls. This study was approved by the local ethical review board and all subjects gave written informed consent.

CSF analyses
CSF samples were obtained by lumbar puncture between the L3 and L4 or L4 and L5 intervertebral space, and collected in polypropylene tubes. A small amount of CSF was used for routine analysis, including erythrocyte count, total cell number and total protein determination. Within two hours, CSF samples were centrifuged at 1800 g (4 °C) for 10 minutes to remove cells. Immediately thereafter, CSF was stored in aliquotes of 0.5 or 1 ml in polypropylene tubes at -80 °C until analysis.

Aβ42, t-tau and p-tau-181 were determined by commercially available ELISAs (Inno- genetic, Ghent, Belgium) as described before. The team involved in the CSF analysis was not aware of the clinical diagnoses. As the manufacturer does not supply control data, performance of the assays was monitored using pools of surplus CSF specimens including multiple specimens with various concentrations in 7-18 runs. The inter-assay coefficient of variation was 11.3% ± 4.9% for Aβ42, 9.3% ± 1.5% for t-tau and 9.4% ± 2.5% for ptau-181. Cut-off values for CSF Aβ42, t-tau and p-tau were calculated in our laboratory from Receiver Operating Characteristics (ROC) curves based on a sensitivity of ≥85% as described before. These cut-off values were used to stratify patients as having a CSF biomarker profile characteristic for AD (AD-like biomarker profile: two or three biomarkers abnormal: Aβ42 < 495 pg/ml in combination with t-tau > 356 pg/ml and/or p-tau > 54 pg/ml) or a normal biomarker profile (≤1 abnormal biomarker). CSF Aβ40 concentrations were quantified using the Aβ40 ELISA kit, according to the manufacturer’s instructions (Genetics company, Schlieren, Switzerland).

BACE1 activity assay
BACE1 activity in CSF was determined with an immuno-capture assay as described before. In short, BACE1 in CSF was captured by a BACE1 specific immobilized antibody. After washing, functionally active BACE1 was allowed to splice the
modified procaspase-3, which serves as a substrate for BACE1 and upon splicing becomes activated. Next, a substrate for activated modified caspase 3 was added and the subsequent color formation was monitored kinetically. BACE1 activity is expressed as pg/ml active BACE1. The intra-assay coefficient of variation (CV) was 4% at 1μg/L and 8% at 0.1μg/L (n=5). The inter-assay CV was 15% at 1μg/L (n=7). This assay is commercially available as a kit (Sigma Aldrich CS1060 or QuickZyme biosciences www.quickzyme.com).

ApoE genotyping
ApoE genotyping was performed by isolating DNA from 10 ml EDTA blood by the QIAamp DNA blood isolation kit from Qiagen. The genotype was determined with the Light Cycler ApoE mutation detection kit (Roche Diagnostics GmbH, Mannheim, Germany). Subjects were classified as ApoE-ε4 carriers if they had one or two alleles with the ε4 variant, other patients were classified as ApoE-ε4 non-carriers.

Statistics
All analyses were performed using SPSS version 15.0 for Windows (SPSS Inc, Chicago, USA). Logarithmic transformations were made of the values for all CSF markers to obtain a symmetric distribution. Univariate analysis of variance (ANOVA) was used to analyze the differences in BACE1 activity between the three patient groups and also between the two CSF profile groups, followed by a Bonferroni posthoc test for multiple comparisons. Age and gender were considered covariates. Pearson’s correlation was applied to investigate the relations of BACE1 activity with \( A_42 \), \( A_40 \), t-tau and p-tau. The level of statistical significance was set at p<0.05.

RESULTS
Clinical characteristics of patients included in this study are summarized in Table 1. CSF levels of t-tau and p-tau were increased in the AD group compared to controls and MCI subjects (p<0.05), but did not differ between control and MCI cases. The CSF levels of \( A_42 \) as well as \( A_40 \) were lower in MCI patients compared to controls (p<0.05). Although \( A_42 \) levels were also lower in AD compared to controls, this was not seen for \( A_40 \). No significant differences in BACE1 activity levels were found between controls (16 pg/ml [9-31 pg/ml]), MCI (18 pg/ml [9-31 pg/ml]) and AD patients (19 pg/ml [10-34 pg/ml]; p=0.38) (Table 1).

Using previously determined cut-off values for CSF levels of \( A_42 \), t-tau and p-tau our cohort was divided into 19 subjects with an AD-like biomarker profile and 28 subjects with a normal biomarker profile (Table 2). Thirteen out of 17 (76%) AD patients, five out of 18 (28%) MCI patients, and one out of 12 (8%) controls were classified with an AD-like biomarker profile. There were no differences in age and gender distribution
Table 1. Demographics and biomarker concentrations of control, MCI and AD patients.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=12)</th>
<th>MCI (n=18)</th>
<th>AD (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years (mean ± SD)</td>
<td>63 ± 13</td>
<td>62 ± 11</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>Gender (%F)</td>
<td>42</td>
<td>50</td>
<td>47</td>
</tr>
<tr>
<td>MMSE (mean ± SD)</td>
<td>29 ± 1</td>
<td>27 ± 2</td>
<td>21 ± 5ab</td>
</tr>
<tr>
<td>ApoE-ε4 carrier (%)</td>
<td>27</td>
<td>60</td>
<td>80a</td>
</tr>
<tr>
<td>BACE1 activity (pg/ml)</td>
<td>16 (9 – 31)</td>
<td>18 (9 - 31)</td>
<td>19 (10 - 34)</td>
</tr>
<tr>
<td>AD-like biomarker profile (%)</td>
<td>8%</td>
<td>28%</td>
<td>76%</td>
</tr>
<tr>
<td>Aβ42 (pg/ml)</td>
<td>817 (339 – 1118)</td>
<td>593 (177 – 976)a</td>
<td>413 (273 – 807)a</td>
</tr>
<tr>
<td>t-tau (pg/ml)</td>
<td>269 (120 – 818)</td>
<td>263 (92 – 1156)</td>
<td>746 (382 - 2000)b</td>
</tr>
<tr>
<td>p-tau (pg/ml)</td>
<td>40 (27 - 109)</td>
<td>46 (16 – 154)</td>
<td>91 (47 - 205)b</td>
</tr>
<tr>
<td>Aβ40 (pg/ml)</td>
<td>4753 (3698 – 6455)</td>
<td>3247 (1280 – 5438)c</td>
<td>4970 (2229 – 9079)</td>
</tr>
<tr>
<td>t-tau/Aβ42</td>
<td>0.31 (0.15 – 2.41)</td>
<td>0.46 (0.13 – 5.23)</td>
<td>2.25 (0.91 – 3.70)b</td>
</tr>
<tr>
<td>p-tau/ Aβ42</td>
<td>0.08 (0.03 – 0.32)</td>
<td>0.08 (0.03 – 0.70)</td>
<td>0.22 (0.06 – 0.43)b</td>
</tr>
</tbody>
</table>

¥: control group: one missing value, MCI group: three missing values, AD group: two missing values

a vs controls, b vs MCI, c vs AD; p<0.05

All biomarker levels depicted as median (min – max).

Table 2. Demographics and biomarker concentrations of cases when stratified according to their biomarker profile, either having an AD-like, or a normal biomarker profile.

<table>
<thead>
<tr>
<th></th>
<th>AD-like biomarker profile (n=19)</th>
<th>Normal biomarker profile (n=28)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years (mean ± SD)</td>
<td>66 ± 6</td>
<td>62 ± 11</td>
<td>0.16</td>
</tr>
<tr>
<td>Gender (%F)</td>
<td>53</td>
<td>43</td>
<td>0.52</td>
</tr>
<tr>
<td>MMSE (mean ± SD)</td>
<td>23 ± 5</td>
<td>27 ± 4</td>
<td>p=0.002</td>
</tr>
<tr>
<td>ApoE-ε4 carrier (%)</td>
<td>81¥</td>
<td>44¥</td>
<td>p=0.02</td>
</tr>
<tr>
<td>BACE1 activity (pg/ml)</td>
<td>20 (12 – 34)</td>
<td>16 (9 - 32)</td>
<td>p=0.01</td>
</tr>
<tr>
<td>Aβ42 (pg/ml)</td>
<td>392 (177 – 441)</td>
<td>733 (241 – 1118)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>t-tau (pg/ml)</td>
<td>818 (382 – 1505)</td>
<td>249 (92 - 2000)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>p-tau (pg/ml)</td>
<td>101 (57 - 176)</td>
<td>43 (16 - 205)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Aβ40 (pg/ml)</td>
<td>4673 (1735 – 9079)</td>
<td>4527 (1280 – 8465)</td>
<td>0.47</td>
</tr>
<tr>
<td>t-tau/Aβ42</td>
<td>2.31 (0.93 – 5.23)</td>
<td>0.32 (0.13 – 3.61)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>p-tau/ Aβ42</td>
<td>0.31 (0.14 – 0.70)</td>
<td>0.06 (0.03 – 0.37)</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

¥ p value does not change upon correction for age and gender; ¥: three missing values

All biomarker levels depicted as median (min – max).
between these two groups. Patients with an AD-like biomarker profile had a lower MMSE-score compared to patients with a normal biomarker profile ($p=0.002$). A higher proportion of ApoE-ε4 carriers was present in the AD-like biomarker profile group compared to the normal biomarker group ($p=0.02$). No differences in $A\beta_{40}$ levels were observed between cases with an AD-like biomarker profile and those with a normal biomarker profile ($p=0.47$).

BACE1 activity, with correction for gender and age, was elevated in subjects with an AD-like biomarker profile (20 pg/ml [12-34 pg/ml]) compared to subjects with a normal biomarker profile (16 pg/ml [9-32 pg/ml]; $p=0.01$) (Figure 1). BACE1 activity did not correlate with MMSE ($r=-0.17$; $p=0.25$). Furthermore, BACE1 activity levels did not differ between ApoE-ε4 carriers and ApoE-ε4 non-carriers (18 pg/ml [12-34 pg/ml] and 17 pg/ml [9-31 pg/ml] respectively, $p=0.28$) and no interaction was present between ApoE genotype and biomarker profile ($p=0.54$).

No statistical differences in BACE1 activity levels were found between MCI patients who remained stable (n=9; 17 pg/ml [9-24 pg/ml]) or converted to either AD (n=6; 18.5 pg/ml [13-31 pg/ml]) or FTD (n=2) ($p=0.49$; total mean follow up time 2.1 years, and from one patient no follow-up data was available).

Correlation analysis of BACE1 activity with $A\beta_{42}$, $A\beta_{40}$, t-tau and p-tau was performed and depicted in figure 2. No correlation between BACE1 activity and $A\beta_{42}$ was found.
Figure 2. Scatter plots showing the correlations of Aβ_{42} (A), Aβ_{40} (B), t-tau (C) and p-tau (D) as function of BACE1 activity in CSF of control (Ο), MCI (Δ) and AD subjects (□).

(r=0.18; p=0.23) (fig 2 A). However, BACE1 activity and Aβ_{40} levels significantly correlated (r=0.38, p=0.01) (fig. 2B). Moreover, both t-tau (fig. 2C) and p-tau (fig 2D) were strongly correlated with BACE1 activity (t-tau: r=0.63, p<0.01; p-tau r=0.65, p<0.01).

DISCUSSION

In this study we examined the relation between BACE1 activity and AD pathology using a combination of Aβ_{42}, Aβ_{40}, t-tau and p-tau levels in CSF as surrogate marker for the pathological changes in the brain. BACE1 activity levels in CSF were found to be higher in patients with an AD-like biomarker profile compared to patients with a normal biomarker profile. When tested for its relation to the individual biomarkers, BACE1 activity was found to correlate with CSF levels of Aβ_{40}, t-tau and p-tau, but not with Aβ_{42}.

In this study we did not observe differences in BACE1 activity levels in CSF between controls, MCI and AD patients. This could relate to the low number of subjects in each
group, since a trend is visible with increased BACE1 activity levels in AD patients, which would confirm previous studies 17-29. Remarkably, Aβ_40 levels were reduced by roughly 30% in MCI patients compared to control and AD subjects, however no differences were found between AD and control subjects. An explanation could be that the increased BACE1 activity observed in AD brain 13-15, to some extent restores the CSF levels of Aβ_40, however no significant increase in BACE1 activity in CSF of AD cases was found.

To further investigate the relation between BACE1 activity and AD pathology, we stratified the patients according to their biomarker profile based on Aβ_42 and tau CSF levels. Several studies have shown a correlation between AD pathology in the brain and levels of Aβ_42 and tau in CSF 1-3, 5, 6, and that a combination of CSF levels of Aβ_42, p-tau and t-tau can not only distinguish AD patients from controls with a high sensitivity and specificity, but also can identify MCI patients with incipient AD with good accuracy 30-33. Based on these biomarkers, we constructed a biomarker profile, based on earlier established cut-offs 26-28. An AD-like biomarker profile was mainly found in MCI and AD patients. The diagnosis was solely based on clinical findings and not on CSF data. This explains why the number of AD patients with an AD-like biomarker profile is somewhat lower, but comparable to the 80-90% sensitivity for AD described for these three biomarkers by us 26 and others 30. Twenty-eight percent of the MCI patients had an AD-like biomarker profile, which was as expected since MCI can be a pre-clinical stage of AD 22, 31-33. Indeed, in our study four out of five MCI patients with an AD-like biomarker profile had converted to AD (follow up time 2.2 years). This is in contrast to MCI patients with a normal biomarker profile, where only two out of 12 subjects converted to AD and two subjects converted to FTLD (follow up time 2.1 years; follow-up data available from 12 out of 13 subjects). The one control patient with an AD-like biomarker profile, did not convert to MCI or AD upon follow-up (follow up time 6.2 years), however this patient might have some underlying AD pathology without apparent clinical decline and may represent the earliest stage of AD 34.

An interesting question is, to what extent the biomarker profiles may help to identify the differences in BACE1 activity observed between controls, AD and MCI patients as described before 17, 20, 29. In this study, MCI patients with an AD-like biomarker profile (n=5) showed increased levels of BACE1 activity compared to MCI patients with a normal biomarker profile (n=13) (23 pg/ml [13-31 pg/ml] and 16 pg/ml [9-22 pg/ml] respectively; p=0.03). A trend towards increased BACE1 activity is also seen in AD patients with an AD-like biomarker profile (n=13; 20 pg/ml [12-34 pg/ml]) compared to AD patients with a normal biomarker profile (n=4; 14.5 pg/ml [10-32 pg/ml]), however this does not reach significance (p=0.29). Although the group size is rather small and further study is encouraged, it might indicate that an increase of BACE1 activity in CSF might not solely be related to diagnosis, but more likely to the presence of an AD-like biomarker profile in the investigated subjects.
Our data indicate that patients with an AD-like biomarker profile, presumably reflecting more pronounced AD pathology in their brains, have higher levels of BACE1 activity in CSF than patients with a normal biomarker profile. When instead of divided according to CSF biomarker profile, the MCI group was divided into MCI patients that either had progressed to AD or had remained stable, BACE1 activity was not found to be different across these groups. However, this could be due to low numbers of MCI patients in each group, since a different study showed that BACE1 activity was found to be higher in the MCI-AD group than in the other MCI groups 17, which could indicate that elevated levels of BACE1 activity may contribute to the AD related amyloidogenic process. Indeed, several post-mortem studies showed an increase in BACE1 activity in the brain of AD patients 13-16, however only a few examined the relation between BACE1 activity in the brain and amyloid plaques. The study of Li et al. showed that in brains of sporadic AD patients the number of plaques are positively correlated with BACE1 activity measured in the brain and that this BACE activity also correlated positively with \( \text{A}\beta_{1-42} \) and \( \text{A}\beta_{42} \) in the brain 14, although other studies did not confirm a correlation between BACE1 activity and either \( \text{A}\beta_{42} \) or formic-acid extractable \( \text{A}\beta \) 13, 35. Furthermore, \( \text{A}\beta \)-pyroglutamate 3-42, which is predominantly located in amyloid plaques and possibly precedes deposition of other \( \text{A}\beta \) species in plaques 36, correlated positively with BACE1 activity in brain 15. Remarkably, in the present study no correlation was found between BACE1 activity and \( \text{A}\beta_{40} \), whereas BACE1 activity in CSF positively correlated with \( \text{A}\beta_{40} \) levels, which confirms earlier findings 17. Next to \( \text{A}\beta_{40} \), total \( \text{A}\beta \) levels were also found to correlate with BACE1 activity in CSF 20, which might be due to a difference in solubility between \( \text{A}\beta_{42} \) and other \( \text{A}\beta \) species, since \( \text{A}\beta_{42} \) is more prone to aggregate in CSF and to deposit in the brain. Together, these findings clearly emphasize an important relation between BACE1 activity and \( \text{A}\beta \) in the brain.

Only one study examined the relation between brain \( \beta \)-secretase activity and tau tangles, however they did not find any correlation in post-mortem brain samples 15. In our study, strong correlations were observed between CSF levels of BACE1 activity and both t-tau and p-tau, this is in line with an earlier study in which a strong correlation between t-tau and BACE1 activity was reported by Zetterberg et al. 17. Elevated levels of p-tau in CSF may reflect the phosphorylation state of tau in the brain. The strong association between CSF levels of p-tau and BACE1 activity, might indicate an, although yet unknown, role of BACE1 activity in the phosphorylation of the tau protein and concomitantly in the formation of tangles in AD brain. On the other hand, the strong correlations between t-tau and BACE1 activity seen by others 17 and in our study, hints to a neurodegenerative process. The intensity of neuronal damage and degeneration in the brain is reflected by high levels of tau in CSF of AD patients. Both tau and BACE1 are primarily located in neurons and the strong correlation between BACE1 activity and both t-tau and p-tau in our study could indicate that increased BACE1 activity in CSF is related to the release of proteins by degenerating neurons, a phenomenon
that has been reported before. Interestingly, Zong et al. did not observe a correlation between BACE1 protein and t-tau in CSF, suggesting that cell damage might not contribute to the release of BACE1 protein in the CSF. Since AD related processes, such as inflammation and oxidative stress, can cause an increase in BACE1 activity in the brain and shedding of BACE1 into the extracellular space, increased BACE1 activity might mirror the pathological state of the brain regardless of neuronal damage. In order to address this hypothesis in further detail, more information is needed related to the neurodegenerative and inflammatory processes in the brain. Measurement of neuronal atrophy with MRI to study neurodegeneration and use of the PET radiotracer PK11195 to label activated microglia to study inflammation, could give more insight into whether BACE1 activity in CSF is elevated due to neurodegenerative and/or inflammatory processes in the brain.

Although in this study, no correlation between BACE1 activity and Aβ was found, which is probably related to the low solubility of the Aβ protein, the strong associations between BACE1 activity and Aβ, t-tau and p-tau indicate the ability of these proteins to readily diffuse from the brain into the CSF and thus to reflect the processes occurring in the brain.

In conclusion, this study shows that increased BACE1 activity in CSF is related to markers of Alzheimer pathology, suggesting a direct link between BACE1 activity and amyloid plaques and tangles in the brain. Future studies should reveal whether elevations of BACE1 in CSF are a cause or consequence of AD.

ACKNOWLEDGEMENTS

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