Chapter 7

Angiotensin-converting enzyme and biomarkers for Alzheimer’s disease

H.M. Jochemsen, C.E. Teunissen, E.L. Ashby, W.M. van der Flier, R.E. Jones, M.I. Geerlings, Ph. Scheltens, P.G. Kehoe, M. Muller

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Abstract

Background
Lower angiotensin-converting enzyme (ACE) activity could increase the risk of Alzheimer’s disease (AD) as ACE functions to degrade amyloid-β (Aβ). Therefore, we investigated whether ACE protein and activity levels in cerebrospinal fluid (CSF) and serum were associated with CSF Aβ, total tau (tau) and tau phosphorylated at threonine 181 (ptau).

Methods
We included 118 subjects from our memory clinic based Amsterdam Dementia Cohort (mean age 66 ± 8 years) with subjective memory complaints (n=40) or AD (n=78), who did not use antihypertensive drugs. We measured ACE protein levels (ng/ml) and activity (RFU) in CSF and serum, and Aβ, tau and ptau (pg/ml) in CSF.

Results
ACE protein level and activity in CSF and serum were lower in patients with AD compared to controls. Lower CSF ACE protein level, and to a lesser extent serum ACE protein level and CSF ACE activity, were associated with lower CSF Aβ, indicating more brain Aβ pathology; adjusted B’s (95% CI) per SD increase were 0.09 (0.04; 0.15), 0.06 (-0.00; 0.12) and 0.05 (-0.00; 0.11), respectively. Further, lower CSF ACE protein level was associated with lower CSF tau and ptau levels; adjusted B’s (95% CI) per SD increase were 0.15 (0.06; 0.25) and 0.17 (0.10; 0.25), respectively.

Conclusion
These results strengthen the hypothesis that ACE degrades Aβ. This could suggest that lowering ACE levels by for example ACE-inhibitors might have adverse consequences for patients with, or at risk for AD.
Introduction

Alzheimer’s disease (AD) is characterized by the accumulation of extracellular amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles (tau pathology), which are reflected by lower Aβ, and higher total tau (tau) and phosphorylated tau (ptau) in CSF.\(^1\)

In the last decade, the role of the renin-angiotensin system (RAS) in the etiology of AD has received increasing attention. It was shown that inheritance of the I-allele - associated with lower plasma ACE levels\(^2\) - was related to increased risk of AD\(^3,4\) although these findings have not been supported by recent GWAS studies\(^5,6\) and large haplotype studies.\(^7\) Further, \textit{in-vitro} studies showed that ACE functions to degrade Aβ, and administration of ACE-inhibitors promoted the accumulation of Aβ,\(^8-10\) while \textit{in-vivo} studies on various mouse models of AD showed variable indirect evidence that ACE can degrade Aβ (reviewed by Kehoe et al.)\(^11\) Together, these data suggest that ACE is important for Aβ degradation, and hence low ACE activity can lead to increased Aβ mediated neuronal damage, plaque accumulation, and risk of AD.\(^12\) Few studies have examined the association between ACE and AD biomarkers in CSF.\(^13,14\) Results on the association between the I-allele, or haplotypes of the \textit{ACE} gene, which were associated with lower ACE levels, and CSF Aβ were inconclusive.\(^13,14\) Further, one small-scaled study found no association between an ACE-inhibitor and AD biomarkers in CSF.\(^15\) The current literature almost entirely comes from laboratory-based and genetic association studies. However, the laboratory-based studies are not comparable to the human disease-state and the findings from genetic-association studies may be explained by linkage-disequilibrium with the true risk factor.\(^3\) Therefore, examining direct measures of ACE (such as serum or CSF ACE) in relation to CSF AD biomarkers might be more clinically meaningful. Especially since the widely prescribed antihypertensive ACE-inhibitors strongly inhibit ACE activity,\(^16\) thereby possibly not only reducing the unfavorable angiotensin I to angiotensin II conversion, but also of the favorable Aβ degradation.

The aim of the current study was to investigate the association of serum and CSF ACE protein levels and activity with CSF AD biomarkers - Aβ, tau and ptau - in a memory clinic cohort. It has increasingly been recognized that nearly all organs of the body have their own local paracrine-like RAS, with organ-specific functions.\(^17\) Also, the brain has its own RAS system, acting largely independent of the peripheral RAS.\(^17\) Therefore, we hypothesize that CSF and serum ACE reflect the activity in the brain RAS and peripheral RAS, respectively, and CSF ACE activity, and to a lesser extent serum ACE activity are associated with lower CSF Aβ, indicating worse Aβ-related pathology in the brain.
Methods

Study population

Patients were included from the memory clinic based Amsterdam Dementia Cohort of the Alzheimer center of the VU University Medical Center. They underwent a standard dementia screening including physical and neurological examination, as well as laboratory tests, EEG, brain MRI and comprehensive neuropsychological testing. The diagnosis of probable AD was made according to the National Institute of Neurological Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) criteria by consensus of a multidisciplinary team, without knowledge of CSF results and the APOE genotype. When the results of all examinations were normal, patients were considered to have subjective complaints (i.e., criteria for mild cognitive impairment not fulfilled). Diabetes mellitus and hypercholesterolemia were defined based on self-reported medical history and medication use. Blood pressure (BP) was measured manually in a standardized manner using a sphygmomanometer with the patient in sitting position after five minutes of rest. The level of education was classified using the 7-point rating scale of Verhage ranging from 1 (low, elementary school not completed) to 7 (high, university).

For the current study, patients using antihypertensive drugs were excluded, as some of these drugs strongly influence ACE levels. Of the patients aged 50 to 80 years old with available CSF, we included 40 persons with subjective complaints (control group), and 78 persons with AD (AD group). The ethical review board of the VU University Medical Center approved the study and all subjects gave written informed consent.

Sampling of blood and CSF

A blood sample was taken, and after half an hour of clotting the samples were spun at 1800 g for 10 min at 4°C, next aliquoted into Sarstedt polypropylene cryovials and immediately stored at -80°C until further analyses. CSF was obtained by lumbar puncture between the L3/L4 or L4/L5 intervertebral space, using a 25-gauge needle, and collected in 10 mL polypropylene tubes. Within two hours, 2 ml of CSF samples was centrifuged at 1800 g for 10 min at 4°C and stored at -20°C for analysis of CSF biomarkers within two months (see below). The remainder of CSF was aliquoted in polypropylene tubes of 0.5 or 1 mL, and stored at -80°C until further analysis.
APOE and AD CSF biomarkers

DNA was isolated from 10 mL EDTA blood and APOE genotype was determined using the Light Cycler APOE mutation detection method (Roche diagnostics GmbH, Mannheim, Germany). CSF Aβ\textsubscript{1-42}, total tau (tau), and tau phosphorylated at threonine-181 (ptau) were measured by Innotest sandwich enzyme-linked immunosorbent assay (ELISA). The performance of the assays was monitored with internal quality controls consisting of pools of surplus CSF specimens. In the study period multiple internal quality controls with various concentrations have been used. The inter-assay coefficient of variation (CV) (mean ± SD) was 11.3 ± 4.9% for Aβ, 9.3 ± 1.5% for tau, and 9.4 ± 2.5% for ptau.\textsuperscript{21}

ACE protein assays

A commercially available sandwich ELISA (R&D systems, Abingdon, UK) was used according to the manufacturer’s guidelines to measure ACE concentration in CSF and serum, as previously described.\textsuperscript{22} Absorbance was read at 450 nm on a FLUOstar OPTIMA plate reader (BMG Labtech, Buckinghamshire, UK). The samples were analyzed in duplicate, and ACE concentrations were interpolated from the standard curves of known concentrations of recombinant human ACE (929-ZN) (R&D systems, Abingdon, UK). The ACE inhibitor captopril was purchased from Enzo Life Sciences (Exeter, UK). The inter-assay CV (mean ± SD) was 8.7 ± 8.9% for CSF ACE and 13.0 ± 7.0% for serum ACE. The intra-assay CV was 7.9 ± 6.5% for CSF ACE and 7.6 ± 5.6% for serum ACE.

ACE activity assays

Monoclonal anti-human ACE antibody (MAB929), recombinant human ACE (929-ZN) and the internally quenched fluorogenic peptide substrate (Mca-RPPGFSAFK(Dnp)-OH) (ES005) were purchased from R&D systems (Abingdon, UK) and used to optimize an immuno-capture based fluorogenic assay for measurement of ACE activity in CSF and serum (see also Supplementary Material ACE activity) based on the method previously used by Miners and colleagues.\textsuperscript{23} Fluorescence was measured by excitation at 320 nm and emission at 405 nm, in a FLUOstar OPTIMA plate reader. The mean fluorescence of the negative controls was subtracted from standard and sample readings, as was done for the fluorescence of the ACE-specific captopril-inhibited well. Captopril inhibited ACE-activity mediated fluorescence by more than 90%. Each sample was measured in duplicate and the mean fluorescence of ACE activity was calculated, based on the standard curve of recombinant ACE. The inter-assay
CV (mean ± SD) was 8.5 ± 8.4% for CSF ACE and 8.1 ± 8.7% for serum ACE. The intra-assay CV was 8.7 ± 5.9% for CSF ACE and 9.1 ± 8.5% for serum ACE.

Data analyses

We used multiple imputation (10 datasets) to address missing values (see below Table 1), and data were analyzed using SPSS version 20.0 (Chicago, Ill, USA), by pooling the 10 imputed datasets. First, patient characteristics were calculated for separate study groups (control group and AD group) and differences were evaluated by ANOVA for continuous variables, or Fisher's exact test for categorical variables. Second, Pearson's correlation coefficients between ACE protein and activity levels each of CSF and serum in turn were examined. Third, linear regression analysis was used to investigate the associations of ACE measures (continuous) with log-transformed CSF biomarker levels of Aβ, tau and ptau (continuous), unadjusted (Model 1) and adjusted for age, sex and study groups (Model 2). Since inheritance of the AD risk-associated APOE-ε4 allele, and vascular risk factors including BP measures could influence the association between ACE measures and CSF biomarkers, we additionally adjusted for APOE-ε4 (carrier vs. non-carrier), and vascular risk factors (SBP, DBP, BMI, smoking, history of hypercholesterolemia or diabetes mellitus) (Model 3). Finally, ANCOVA was used to estimate the adjusted mean log-transformed CSF Aβ, tau and ptau levels across tertiles of ACE measures within the separate study groups. For graphical purposes, the log-transformed values were back transformed.

Results

In the total population of 118 subjects, mean age was 66 (SD, range; 8, 50 - 79) years and 52% were female. Table 1 shows that patients in the AD group were older, more often female, and were more often APOE-ε4 carriers than patients in the control group. Also, they had less hyperlipidemia and higher BP levels. Further, all ACE measures were lower in patients with AD compared to patients in the control group (Table 1). The Pearson correlation coefficient between CSF ACE protein level and CSF ACE activity was 0.26 (p-value = 0.005), between serum ACE protein level and serum ACE activity was 0.28 (p-value = 0.002), between CSF and serum ACE protein levels was 0.54 (p-value < 0.001), and between CSF and serum ACE activity levels was 0.07 (p-value = 0.494).
Table 1. Baseline characteristics for the separate study groups.

<table>
<thead>
<tr>
<th></th>
<th>Control group (n=40)</th>
<th>AD group (n=78)</th>
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<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years) ‡</td>
<td>63 (8)</td>
<td>68 (7) **</td>
</tr>
<tr>
<td>Female sex, %</td>
<td>47%</td>
<td>64% *</td>
</tr>
<tr>
<td>Education (range 1-7) †</td>
<td>6 (3 – 7)</td>
<td>5 (3 – 6)</td>
</tr>
<tr>
<td>APOE-ε4 carrier, %</td>
<td>23%</td>
<td>66% *</td>
</tr>
<tr>
<td>MMSE (range 0-30) †</td>
<td>28 (26 – 30)</td>
<td>22 (16 – 27) *</td>
</tr>
<tr>
<td><strong>Vascular risk factors/disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking, % current</td>
<td>23%</td>
<td>23%</td>
</tr>
<tr>
<td>Body mass index (kg/m²) ‡</td>
<td>25 (5)</td>
<td>24 (5)</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>14%</td>
<td>14%</td>
</tr>
<tr>
<td>Hypercholesterolemia, %</td>
<td>28%</td>
<td>22% *</td>
</tr>
<tr>
<td><strong>Blood pressure measures</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg) ‡</td>
<td>133 (14)</td>
<td>147 (22) **</td>
</tr>
<tr>
<td>Diastolic BP (mmHg) ‡</td>
<td>83 (9)</td>
<td>86 (13) **</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>30%</td>
<td>55% *</td>
</tr>
<tr>
<td><strong>ACE measures</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF ACE protein level (ng/ml) ‡</td>
<td>3.78 (1.30)</td>
<td>3.56 (1.16) **</td>
</tr>
<tr>
<td>CSF ACE activity (RFU) ‡</td>
<td>155 (61)</td>
<td>148 (58) *</td>
</tr>
<tr>
<td>Serum ACE protein level (ng/ml) ‡</td>
<td>4.22 (1.50)</td>
<td>3.72 (1.14) **</td>
</tr>
<tr>
<td>Serum ACE activity (RFU) ‡</td>
<td>255 (152)</td>
<td>205 (116) **</td>
</tr>
<tr>
<td><strong>CSF biomarkers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ42 (pg/mL) †</td>
<td>887 (622 – 1157)</td>
<td>475 (334 – 743) **</td>
</tr>
<tr>
<td>Total tau (pg/mL) †</td>
<td>246 (122 – 371)</td>
<td>619 (344 – 1102) **</td>
</tr>
<tr>
<td>Ptau (pg/mL) †</td>
<td>45 (29 – 74)</td>
<td>87 (54 – 137) **</td>
</tr>
</tbody>
</table>

‡ mean (SD), † median (10th – 90th percentile). % of missing values before imputation: education, 0.8%; APOE genotyping, serum ACE protein and activity levels, 1.7%; MMSE, 3.4%; smoking, 7.6%; diabetes mellitus and hypercholesterolemia, 14%; all other variables 0%. MMSE: mini-mental state examination; BP: blood pressure; Aβ42: amyloid-β₁₋₄₂; Ptau: tau phosphorylated at threonine 181. * p < 0.05 versus controls, ** p < 0.01 versus controls.
CSF ACE measures and CSF biomarkers

In the total population, higher CSF ACE protein level was independently associated with higher CSF Aβ, tau and ptau levels (Table 2). When stratified by study groups, the associations between higher CSF ACE protein levels and higher CSF Aβ, tau and ptau were present in the control group and AD group, although significance was lost for CSF tau in the demented group (Figure 1, 2 and 3).

Also, higher CSF ACE activity was marginally associated with higher CSF Aβ levels, but not with CSF tau or ptau levels (Table 2). When stratified by study groups, the association was particularly present in the AD group (Figure 1).

<table>
<thead>
<tr>
<th></th>
<th>Ln AB42 (B (95% CI))</th>
<th>Ln TAU (B (95% CI))</th>
<th>Ln PTAU (B (95% CI))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF ACE protein level a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.12 (0.05; 0.19)**</td>
<td>0.10 (-0.01; 0.22)</td>
<td>0.14 (0.05; 0.22)**</td>
</tr>
<tr>
<td>2</td>
<td>0.09 (0.04; 0.15)**</td>
<td>0.15 (0.06; 0.25)**</td>
<td>0.17 (0.10; 0.25)**</td>
</tr>
<tr>
<td>3</td>
<td>0.11 (0.05; 0.16)**</td>
<td>0.16 (0.05; 0.26)**</td>
<td>0.19 (0.10; 0.27)**</td>
</tr>
<tr>
<td>CSF ACE activity b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.07 (-0.00; 0.14)</td>
<td>-0.06 (-0.18; 0.06)</td>
<td>0.02 (-0.07; 0.11)</td>
</tr>
<tr>
<td>2</td>
<td>0.05 (-0.00; 0.11)</td>
<td>-0.03 (-0.13; 0.07)</td>
<td>0.04 (-0.04; 0.12)</td>
</tr>
<tr>
<td>3</td>
<td>0.06 (0.00; 0.11)*</td>
<td>-0.04 (-0.14; 0.06)</td>
<td>0.03 (-0.05; 0.11)</td>
</tr>
<tr>
<td>Serum ACE protein level c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.09 (0.02; 0.17)*</td>
<td>-0.09 (-0.21; 0.03)</td>
<td>-0.02 (-0.11; 0.07)</td>
</tr>
<tr>
<td>2</td>
<td>0.06 (-0.00; 0.12)</td>
<td>-0.03 (-0.13; 0.07)</td>
<td>0.02 (-0.06; 0.10)</td>
</tr>
<tr>
<td>3</td>
<td>0.04 (-0.02; 0.11)</td>
<td>-0.04 (-0.15; 0.06)</td>
<td>0.02 (-0.06; 0.11)</td>
</tr>
<tr>
<td>Serum ACE activity d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.06 (-0.01; 0.14)</td>
<td>-0.10 (-0.22; 0.01)</td>
<td>-0.02 (-0.10; 0.07)</td>
</tr>
<tr>
<td>2</td>
<td>0.03 (-0.03; 0.09)</td>
<td>-0.05 (-0.15; 0.05)</td>
<td>0.02 (-0.06; 0.10)</td>
</tr>
<tr>
<td>3</td>
<td>0.02 (-0.04; 0.08)</td>
<td>-0.06 (-0.17; 0.05)</td>
<td>0.01 (-0.08; 0.10)</td>
</tr>
</tbody>
</table>

Model 1: unadjusted. Model 2: adjusted for age, sex and study groups. Model 3: additionally adjusted for APOE-ε4 genotype, SBP, DBP, BMI, smoking, history of hypercholesterolemia and diabetes mellitus. a (superscript) per SD increase (1.32 ng/ml), b (superscript) per SD increase (59 RFU), c (superscript) per SD increase (1.30 ng/ml), d (superscript) per SD increase (133 RFU). * p < 0.05, ** p < 0.01.

Serum ACE measures and CSF biomarkers

In the total population, higher serum ACE protein level was associated with higher CSF Aβ (borderline significance), but not with CSF tau or ptau levels (Table 2). When stratified by study groups, the association was more evident in the control group and not the AD group: mean differences (95% CI) between the highest and lowest serum ACE protein tertiles were 193 (47; 340) and 44 (-62; 151), respectively (Model 2).

Serum ACE activity was not associated with higher CSF Aβ, tau or ptau levels (Table 2).
Figure 1. Angiotensin-converting enzyme (ACE) measures and cerebrospinal fluid (CSF) amyloid-β$_{1-42}$.

Means (standard error) in CSF amyloid-β$_{1-42}$ across tertiles of CSF ACE activity and CSF ACE protein level, in the control group (A) and demented group (B). Analyses were adjusted for age and sex. Tertiles CSF ACE activity: <121.66; 121.66 - 177.56; >177.56 RFU. Tertiles CSF ACE protein level: <2.95; 2.95 - 3.92; >3.92 ng/ml. * p < 0.05.
Figure 2. Angiotensin-converting enzyme (ACE) measures and cerebrospinal fluid (CSF) total tau.

Means (standard error) in total tau across tertiles of CSF ACE activity and CSF ACE protein level, in the control group (A) and demented group (B). Analyses were adjusted for age and sex. Tertiles CSF ACE activity: <121.66; 121.66 - 177.56; >177.56 RFU. Tertiles CSF ACE protein level: <2.95; 2.95 - 3.92; >3.92 ng/ml. ** p < 0.01.
Figure 3. Angiotensin-converting enzyme (ACE) measures and cerebrospinal fluid (CSF) phosphorylated tau.

Means (standard error) in phosphorylated tau across tertiles of CSF ACE activity and CSF ACE protein level, in the control group (A) and demented group (B). Analyses were adjusted for age and sex. Tertiles CSF ACE activity: <121.66; 121.66 - 177.56; >177.56 RFU. Tertiles CSF ACE protein level: <2.95; 2.95 - 3.92; >3.92 ng/ml. * p < 0.05, ** p < 0.01.
Discussion

The main findings of this study are that lower CSF ACE protein levels, and to a lesser extent serum ACE protein and CSF ACE activity levels, were associated with lower CSF Aβ levels, indicating increased Aβ accumulation in the brain. Surprisingly, lower CSF ACE protein levels were associated with lower CSF tau and ptau levels, indicating less brain tau pathology. These associations were found in both control and AD patients and were independent of age, sex, APOE-genotype, and vascular risk factors. Further, CSF and serum ACE protein and activity levels were lower in AD patients compared to controls.

To our knowledge, this is the first study that linked direct measures of ACE-activity to the CSF AD biomarkers Aβ, tau, and ptau. Our results on the inverse association of CSF ACE protein level and CSF Aβ are in line with studies that showed an association between the ACE gene and Aβ. Haplotypes of the ACE gene that were associated with lower ACE levels were related to lower CSF Aβ, and another study showed that the I-allele (indicating lower ACE levels) was associated with slightly more Aβ load in the brain at autopsy. The Rotterdam study examined ACE genotype in relation to brain atrophy (which is associated with increased brain Aβ plaques), and found that female I/I genotype-carriers had smaller hippocampal and amygdalar volumes. Further, we showed in the SMART-MR study that lower serum ACE levels were associated with more progression of cortical brain atrophy. Together, these data suggest that lower ACE could lead to more Aβ accumulation in the brain, reflected by lower CSF Aβ it supports previous reports that higher ACE levels, through its degradation of Aβ, may contribute to less accumulation of Aβ into senile plaques in the brain and subsequently be beneficial for the occurrence of brain atrophy.

Another novel finding was our observation that lower CSF ACE protein level was associated with lower CSF tau and ptau. A previous study relating ACE genotype to tau load in the brain found no association. The mechanisms explaining the relation between lower ACE and lower CSF tau levels remain largely unknown, although a role for angiotensin II (ANGII) could be suggested. A recent study showed that central infusion of ANGII in normal rat brains induced tau phosphorylation in a dose dependent manner via activating glycogen synthase kinase 3beta (GSK3β). Furthermore, this was reversed by co-administration of the ANG II type 1 receptor (AT1R) antagonist, Losartan, which is commonly prescribed for the treatment of hypertension. Our findings suggest that higher ACE levels which lead to higher ANGII levels, can induce tau pathology in the brain, and in turn result in higher tau levels in
the CSF. Coincidentally and perhaps ironically, while such elevations in ACE may exacerbate tau-related pathology, these same elevations may also have a slightly beneficial mitigating effect in terms of reducing Aβ pathology. Yet, no association was seen between CSF tau and ptau with CSF ACE activity, which we might expect to be a more biologically meaningful measure of the likely production of ANGII from its precursor ANGI.17

CSF ACE levels were more strongly associated with AD biomarkers than serum ACE levels. This indicates that CSF ACE, as might be expected, could reflect the activity of the brain RAS, thereby showing stronger associations with brain pathology. Further, ACE protein levels were more strongly associated with AD biomarkers than ACE activity levels. Whereas protein levels indicate the concentration of ACE in body fluid, activity levels particularly indicate the ANGI to ANGII conversion capacity of the enzyme.22 Thus one might have expected that ACE activity levels in particular would be more indicative and relevant to Aβ degradation, and would therefore be more strongly associated with CSF Aβ. At present we cannot explain this apparent inconsistency, it could be partly related to ACE genotype although the genetic contribution to ACE levels has only been shown to be relatively modest12 and unfortunately, we did not have information on ACE genotype in our population, so we could not explore this.

Another reason might be differential levels of post-translational modification in the various physiological compartments the samples were derived from. It has been previously shown that ACE activity, and its proposed conversion of Aβ can be modulated by post-translational modification.30 Thus it may be possible that in both CSF and serum there are differential levels of glycosylation that can explain the lower than expected correlations of activity with some of our outcome measures. Miners and colleagues previously showed a trend of divergence between ACE levels and activity in post-mortem CSF, however, this was not significant and the patterns observed may have been an artifact of post-mortem delay where additional changes to the ACE may have occurred post-mortem.22 Yet, here remains the possibility that in the brain, from which the levels of Aβ, tau and p-tau emanate, levels of ACE activity are influenced in a more disease specific manner by advancing pathology, than occurs in the more peripheral compartments we have tested. Indeed Miners and colleagues have shown in post-mortem brain tissue from AD patients that their ACE activity and not levels are more markedly different than in the control group.22 Similarly according to our data, there is some support that disease-specific modifications of ACE may be involved since the apparent discrepant findings
between ACE levels and activity with respect to CSF Aβ were less marked and seemed to correlate more in the AD group (Figure 1B). Among the strengths of the current study is the use of CSF ACE measures and CSF biomarkers, which gives the opportunity to examine during life the association between ACE and AD pathology, without potential confounding from post-mortem delays. Second, we had paired CSF and serum samples of all the patients. Finally, all patients were assessed in a standardized way and diagnosed according to commonly used criteria. One of the limitations of our study is the relatively small study population. Yet, the interesting results recommend replication in a larger study sample. Another limitation is the cross-sectional design that prohibits conclusions on cause or effect. Similarly, we cannot ascertain whether lower CSF ACE protein and activity levels cause brain Aβ pathology. However, as we found that the associations were also present in the control group, it is reasonable to assume that the down-regulation of CSF ACE is not pathology-driven, and that lower ACE levels might precede the pathology, or alternatively, is a general phenomenon associated with neurodegeneration. This however, remains to be further investigated in a larger, longitudinal study.

This study has important clinical implications. If ACE in the brain does prevent Aβ accumulation in living humans, our data supports the suggestions that taking ACE inhibitors as antihypertensive drugs, particularly those that can cross the blood-brain barrier, might compromise Aβ degradation. Fortunately, while uncertainty remains on this issue, angiotensin-receptor blockers, which solely target ANG II effects whilst not interfering directly with ACE, could provide an immediate and readily available potential alternative anti-hypertensive therapy for patients at risk for AD.

To conclude, in a memory clinic cohort, we found that lower CSF ACE levels were associated with lower CSF Aβ levels, which suggests more accumulation of Aβ in the brain. This lends support to the hypothesis that ACE can degrade Aβ, thereby contributing to limiting its accumulation and possibly the development or rate of progression of AD.
### References


Supplementary material ACE activity assays

Monoclonal anti-human ACE antibody (MAB929), recombinant human ACE (929-ZN) and the internally quenched fluorogenic peptide substrate (Mca-RPPGFSAFK(Dnp)-OH) (ES005) were purchased from R&D systems (Abingdon, UK). Captopril was purchased from Enzo Life Sciences (Exeter, UK). Wells of a NuncMaxiSorp 96-well polystyrene immunoplate (Fisher Scientific, Loughborough, UK) were coated with capture monoclonal anti-human ACE antibody (1 µg/ml) diluted in PBS (pH 7.4), the plate sealed and incubated for 18 hours at room temperature. Wells were washed five times in PBS (pH 7.4) containing 0.05% tween-20 (Sigma Aldrich, Dorset, UK) and non-specific binding of antibody blocked by incubating with 300 µl of PBS (pH 7.4) containing 1% bovine serum albumin (BSA) (Fisher Scientific) for 2 hours at room temperature. Serial dilutions of recombinant human ACE (2500 - 40 ng/ml) were prepared in PBS (pH 7.4) to create a standard curve of enzyme activity. For sample wells, 10 µl of CSF or 15 µl of serum was diluted in PBS (pH 7.4) (100 µl/well), prepared in triplicate. After washing the plate five times, diluted recombinant human ACE and diluted samples were briefly vortexed and added to required wells. Control wells were included on each plate and contained 100 µl PBS alone. The plate was sealed and incubated for 2 hours at room temperature with continuous shaking. After a further five washes, an optimal concentration of a specific inhibitor peptide, captopril (1 mM) prepared in distilled water was added to required wells (50 µl/well) and distilled water was added to all uninhibited wells (50 µl/well). The plate was sealed and incubated for 10 minutes at room temperature prior to the addition of fluorogenic substrate (10 µM) prepared in 100 mM Tris-HCl pH 7.5 containing 50 mM NaCl and 10 µM ZnCl$_2$ to all wells (50 µl/well). The plate was sealed and incubated in the dark for 2 hours with continual shaking at 37°C, followed by incubation at 4°C for 16 hours. Fluorescence was measured with excitation at 320 nm and emission at 405 nm, in a FLUOstar OPTIMA plate reader (BMG Labtech, UK) after 18 hours incubation.