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Title: Cerebrospinal fluid changes in the renin-angiotensin system in Alzheimer's disease

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Running title: RAS changes in CSF

Abstract (words 250)

Observations in autopsied brain tissue indicate that overactivation of the classical renin-angiotensin system (cRAS) and underactivity within regulatory RAS pathways (rRAS) are associated with pathology in Alzheimer's disease (AD). The primary aim of this study was to investigate whether cerebrospinal fluid (CSF) markers of RAS are altered in AD in relation to established CSF markers of disease pathology (lower Aβ42 and elevated tau) and CSF markers of capillary dysfunction. We studied 40 controls and 40 AD cases grouped according to a biomarker profile (i.e. AD cases t-tau >400 pg/mL, pTau >60 pg/mL and Aβ42 <550 pg/mL). ACE1 and ACE2 enzyme activity was measured using fluorogenic peptide substrates; sPDGFRβ and albumin level by sandwich ELISA and Angiotensin-I, -II, and 1-7 by direct ELISA. CSF Aβ$_{42}$, total and phosphorylated tau level was previously measured by INNOTEST sandwich ELISA. CSF ACE1 activity was significantly elevated in AD (p = 0.008) and positively correlated with ACE2 in AD (r = 0.420, p = 0.007). CSF ACE1 weakly correlated with t-tau (r = 0.294, p = 0.066) and p-tau (r = 0.329, p = 0.038) but not with Aβ42 in the controls but not in AD. ACE1 correlated positively with sPDGFRβ (r = 0.426, p = 0.007), a marker of pericyte injury, and ACE2 correlated positively with albumin (r = 0.422, p = 0.008), a marker of blood-brain barrier integrity. CSF angiotensin -I, -II and –(1-7) level was unchanged in AD. This cross-sectional CSF study indicates RAS dysfunction in relation to capillary damage in AD.

Keywords; renin-angiotensin system; cerebrospinal fluid; Alzheimer's disease; angiotensin-II converting enyme-1 (ACE1); angiotensin-II converting enyme-2 (ACE2); angiotensin-II; angiotensin-(1-7).

Introduction

Alzheimer's disease (AD) is characterised neuropathologically by the deposition of extracellular Aβ within senile plaques and the accumulation of intracellular tau within neurofibrillary tangles [1]. Recent imaging, CSF biomarker studies and disease modelling indicate that cerebrovascular dysfunction, including reduced cerebral blood flow, neurovascular uncoupling and blood-brain barrier (BBB) breakdown, are major contributors to cognitive decline and disease pathology in the early stages of Alzheimer's disease (AD) [2-4]. A recent study reported that BBB leakiness and a marker of pericyte injury, indicated by increased CSF levels of soluble platelet-derived growth factor receptor β (sPDGFRβ), are sensitive markers of cognitive decline in the very early stages of AD independently of CSF Aβ and tau [5]. Together, these data support a growing hypothesis that cerebrovascular dysfunction is a major and underestimated contributor to cognitive decline and disease pathology particularly in the early stages of AD [6].

The renin-angiotensin-system (RAS) is a hormonal system that regulates systemic blood pressure. The RAS is expressed and functions independently within the brain and is dysfunctional in Alzheimer's disease (AD) (reviewed in [7, 8]). Studies in human post-mortem brain tissue indicate that overactivation of the classical RAS (cRAS) pathway is associated with elevated Aβ and tau pathology in AD [9-12]. Angiotensin-converting enzyme-1 (ACE1) activity, the level of angiotensin-II (Ang-II), and the expression of angiotensin-II type 1 receptor (AT1R) are all increased in AD [9, 11-13]. Cerebroventricular infusion of Ang-II, the main effector peptide of the RAS, accelerates Aβ production (via proteolytic cleavage of APP) and promotes the accumulation of disease-modified tau in Sprague Dawley rats in association with cognitive decline [14, 15]. Alternative regulatory

RAS pathways exist within the brain that counteract the classical RAS axis [16-18]. We recently reported that ACE2 activity, which is primarily responsible for converting Ang-II to Ang(1-7) [19, 20], was reduced by almost 50% in the mid-frontal cortex in AD and was related to elevated parenchymal Aβ and tau load [10] and inversely related to elevated ACE1 activity.

Classical RAS-targeting agents, such as ACE1 inhibitors (ACE-Is) and angiotensin-II type 1 receptor (At1R) blockers (ARBs) have been shown to reduce the incidence [21-24], delay cognitive decline [25] and improve cognition in AD [26]. The protective effects of RAS-blockers in AD are associated with lower CSF-tau [27] and elevated CSF-Aβ (indicative of reduced parenchymal Aβ deposition) [28] and reduced Aβ and tau pathology [29, 30]. Together, these data highlight an imbalance in brain RAS in AD comprising classical RAS overactivation that is related to disease pathology that is, in part, associated with reduced regulatory RAS activity.

ACE1 and ACE2 are central effectors of the cRAS and rRAS pathways and are responsible for the production and catabolism of Ang-II respectively. ACE1 and ACE2 are single-span membrane-bound ectoenzymes [31] that are predominantly expressed in endothelial and smooth muscle cells throughout the body but are also localised within neuronal cells [32, 33] and non-neuronal glial cells within the CNS [34, 35]. Shedding and solubilisation of ACE1 [36] and ACE2 [37] results in the release of the active enzyme in various biological fluids including serum and CSF. ACE1 has previously been measured in CSF in AD but previous studies have provided inconsistent findings with some studies reporting increased CSF ACE1 [38, 39] others reporting reduced ACE1 [40-42] and one study showing

no change [43]. In contrast, ACE2 activity in CSF remains relatively uncharacterised with only a single study to-date showing unaltered CSF ACE2 in AD [41].

The major aim of this study was to investigate if CSF ACE1 and ACE2 activity are altered in AD, and if so, whether RAS CSF changes were associated with established CSF markers of disease pathology (i.e increased t-tau and p-tau and reduced Aβ42) and novel CSF markers of capillary damage including BBB breakdown (CSF albumin level) and pericyte injury (sPDGFRβ).

Methods

Study cohorts

We studied CSF from 40 control and 40 AD cases that were approximately matched for age (69.1 y ± 12.1 in controls and 76.3 y ± 6.1 in AD) and distribution of sex across cohorts (23M:15F in controls and 25:15F in AD) (Table 1). Measurements of CSF-Aβ$_{42}$, t-tau and p-tau using commercially available sandwich enzyme-linked immunosorbent assays (ELISAs) (INNOTEST, Fujirebio, Ghent, Belgium) had previously been determined. All AD patients had abnormal CSF levels of the core AD biomarkers (t-tau >400 ng/L, p-tau >60 ng/L and Aβ42 <550 ng/L), while controls had normal levels. The cut-off values used in this study are in-line with current clinical practice and closely resemble those outlined by Hansson et al. [44]. The present assays were performed on de-identified left-over aliquots from clinical diagnostic CSF samples and complied with the Swedish Biobank law (Biobanks in Medical Care Act). All procedures were previously approved by the Ethical Committee at the University of Gothenburg (Sweden). Information on cognitive assessment and medical

histories, including potential use of RAS-targeting or other classes of anti-hypertensives, were not collected for participants.

CSF ACE1 fluorogenic activity assay

ACE1 activity was measured in CSF using an ACE1 specific FRET peptide substrate (Abz-FRK(Dnp)-P) (Biomol International, Exeter, UK) as previously described [10, 11, 13, 45]. Recombinant ACE1 (1000-31.1 ng/ml) or CSF (5μl CSF diluted in 45ul assay buffer) was incubated with ACE1 substrate (10 μM diluted in assay buffer) for 2.5 h at 26°C. Fluorescence was measured with excitation at 320nm and emission at 405nm in a plate reader (FLUOstar, BMG Labtech, Aylesbury, UK). All cases were run in duplicate in the presence and absence of captopril for 10 minutes at 26°C prior to the addition of the FRET substrate – we previously showed that pre-incubation with captopril blocked ACE1 activity by over 90% [11, 13]. ACE1 activity was determined by subtracting the fluorescence in the captopril-inhibited from the untreated wells. A serial dilution of recombinant human ACE1 and measurements on carry-over samples across all plates was used to minimise variation between plates. ACE1 activity was expressed as relative fluorescence units (r.f.u).

CSF ACE2 fluorogenic activity assay

ACE2 activity was measured using the ACE2 specific FRET substrate (Mca-APK(Dnp) (Enzo Life Sciences, Exeter, UK) as previously described [10]. Recombinant ACE2 (440 – 6 ng/ml) (R&D systems, Cambridge, UK) or CSF (50 μl undiluted) was incubated with the FRET substrate (10 μM) diluted in assay buffer (75 mM Tris, 1M NaCl, pH7.5) for 3 h at 37 C. Recombinant ACE2 and CSF samples were assayed in duplicate in the presence or absence of an ACE2 specific inhibitor (MLN4760) (10 μM) (Merck, Nottingham, UK) diluted in distilled

water for 10 mins at 37°C prior to the addition of the fluorogenic substrate. ACE2 activity

was determined by subtracting the fluorescence in the inhibited from the untreated wells.

Cleavage of the ACE2 FRET peptide was measured using a microplate reader (BMG Labtech,

Aylesbury, UK) at excitation/emission wavelength 330/390nm. A serial dilution of

recombinant human ACE2 and measurements on carry-over samples across all plates was

used to minimise variation between plates. ACE2 activity was expressed in relative

fluorescence units (r.f.u).

CSF Angiotensin-I, -II and -(1-7) measurement by direct ELISA

Ang-I, -II and Ang-(1-7) concentrations were measured by direct ELISA as previously

described [9, 10]. Serial dilutions of recombinant human Ang-I, Ang-II and Ang-(1-7) (5000-

78.125 pg/ml) (Abcam, Cambridge, UK) or CSF samples (100ul undiluted) were incubated for

2 hours in clear high-binding capacity NUNC maxisorp plates (ThermoFisher Scientific,

Waltham, MA, USA) at 26°C with shaking. The wells were washed five-times in PBS with

0.05% tween-20 and blocked for 1 hour in 1 % PBS: BSA (Sigma Aldrich, Dorset, UK). After

another five washes, the wells were incubated with either biotinylated anti-human Ang-I

(diluted 1 in 100 in PBS), biotinylated anti-human Ang-II (diluted 1 in 500 in PBS), or

biotinylated anti-human Ang-(1-7) (diluted 1 in 100 in PBS) (all from Cloud-Clone, Wuhan,

China), for 2 hours at 26°C with shaking, followed by a further wash step. Plates were

incubated with streptavadin:HRP (1:200) in PBS:0.01% Tween-20 for 20 minutes at room

temperature in the dark and TMB substrate (R&D systems) was added after a further wash

and left to develop in the dark for 20 minutes. The reaction was stopped following the

addition of 2N sulphuric acid and the absorbance was read at 450nm using a FLUOstar

OPTIMA plate reader (BMG labtech, Aylesbury, BUCKS, UK). Concentrations were

interpolated for each sample measured in duplicate following interpolation from respective standard curves generated by serially diluting recombinant standards. We previously demonstrated specificity of each assay which showed specificity for the respective target with minimal cross reactivity with other closely-related angiotensin peptides [9, 10].

ELISA measurement of CSF sPDGFRβ

CSF soluble PDGFRβ (sPDGFRβ) level was measured using a commercially available sandwich ELISA (Invitrogen Cat no EHPDGFRB) (ThermoFisher Scientific, Loughborough, UK) following the manufacturer's protocol. CSF samples (100µl) were loaded without dilution. Standards, samples and blanks were added in duplicate. Absorbance was read at 450 nM following the addition of 2N sulfuric acid in a FLUOstar OPTIMA plate reader (BMG labtech, Aylesbury, UK). sPDGFRβ concentration in samples was interpolated from the standard curve for each case, derived from the serial dilution of recombinant PDGFRβ (18000-24 pg/ml). The average of duplicate measurements for each sample is presented.

ELISA measurement of CSF albumin

CSF albumin level was measured using a commercially available sandwich ELISA (Cat no 108788) (Abcam, Cambridge, UK) following the manufacturer's protocol. CSF samples were diluted 1 in 2000 in proprietary dilution buffer supplied with the kit. Standards, samples and blanks were added in duplicate. Absorbance was read at 450nM in a FLUOstar OPTIMA plate reader (BMG labtech, Aylesbury, UK). Albumin concentration in each sample was interpolated from the standard curve for each case, derived from the serial dilution of recombinant human albumin (200-3.125ng/ml).

Statistical analysis

Unpaired two-tailed t-tests or ANOVA with Bonferroni post-hoc analysis was used for comparisons between groups, and Pearson's test was used to assess linear correlation with the help of SPSS version 24 (SPSS, Chicago) and GraphPad Prism version 6 (GraphPad Software, La Jolla, CA). P-values < 0.05 were considered statistically significant.

Results

ACE1 enzyme activity is elevated in CSF from Alzheimer's disease patients and correlates positively with ACE2 activity

CSF ACE1 activity was significantly higher in AD (2148 rfu ± 153.8) compared to controls (1635 rfu ± 110.9) (p = 0.008) (Figure 1A). CSF ACE2 activity was unchanged in AD (360.1 rfu ± 24.8) compared to controls (318.1 rfu ± 16.3) (p = 0.169) (Figure 1B). We previously reported an inverse relationship between ACE1 and ACE2 in the mid-frontal cortex in AD [10]. We examined the relationship between ACE1 and ACE2 in CSF and found a positive correlation in AD (r = 0.420, p = 0.007) but not in controls (r = 0.190, p = 0.239) (Figure 1C).

CSF ACE1 activity did not vary according to age in either controls (r = 0.056, p = 0.732) or AD (r = 0.114, p = 0.489) (Figure 2A) however, CSF ACE2 activity correlated positively with age in controls (r = 0.423, p = 0.0065) but not in AD (p = 0.172, p = 0.290) (Figure 2B). ACE1 and ACE2 activity did not vary according to sex (Figure 2C-D).

CSF ACE1 and ACE2 enzyme activity are not strongly associated with established CSF markers of disease pathology in AD

We previously reported that increased ACE1 and reduced ACE2 activity are associated with parenchymal Aβ and tau load in mid-frontal cortex in AD [10, 11, 13]. . We examined the relationship between CSF ACE1 and ACE2 activity with established CSF markers of disease pathology (t-tau, p-tau and Aβ42). CSF ACE1 activity did not correlate with CSF t-tau in either control (r = 0.294, p = 0.066) or AD group (r = -0.01, p = 0.945) (Figure 3A) but was weakly positively correlated with CSF p-tau in controls (r = 0.329, p = 0.038) but not AD (r = 0.135, p = 0.413) (Figure 3B). CSF ACE1 did not correlate with $A\beta_{42}$ in either control (r = 0.216, p = 0.179) or AD groups (r = -0.05, p = 0.761) (Figure 3C). CSF ACE2 did not correlate with CSF-p-tau, CSF-t-tau or CSF-$A\beta_{42}$ in either the AD or control groups (Table 2)

CSF ACE1 and ACE2 activity correlates with markers of capillary damage

ACE1 is enriched in cerebral vessels [46] and there is evidence of vascular breakdown in AD [47] that together are suggestive of a link between RAS activation and vascular dysfunction in AD. Here we examined the relationship between CSF ACE1 and ACE2 with albumin, an established CSF marker of BBB leakiness, and with soluble PDGFRβ, a marker of pericyte injury that is elevated in AD [5]. A positive correlation was observed between CSF ACE1 enzyme activity and CSF sPDGFRβ level in AD cases alone (r = 0.426, p = 0.007) but not in controls (r = 0.112, p = 0.482) (Figure 4A). CSF ACE2 in contrast did not correlate with CSF sPDGRβ in either control (r = 0.047, p = 0.778) or AD (r = 0.121, p = 0.483) (Figure 4B) but did correlate positively with CSF albumin in AD (r = 0422, p = 0.008) but not in controls (r =

0.179; p = 0.282) (Figure 4D). CSF ACE1 activity did not correlate with CSF albumin in either controls (p = 0.038, p = 0.820) or AD (r = 0.129; p = 0.439) (Figure 4C).

CSF angiotensin -I, -II, and –(1-7) level was unaltered in AD

Angiotensin-I, II and (1-7) levels were unchanged between AD and controls in CSF (Figure 5) and did not correlate with Aβ, t-tau and p-tau level (data not shown).

Discussion

In this study, we explored disease-related changes in CSF markers of RAS in relation to established CSF markers of disease pathology (lower $A\beta_{42}$ and elevated t-tau and p-tau) and markers of capillary damage (sPDGFRβ and albumin) in clinical diagnostic CSF samples in AD. Our observational study indicates that CSF ACE1 activity is elevated in AD. CSF ACE1 was positively correlated with CSF ACE2 in AD. We also observed that CSF ACE2 activity was positively associated with age in the control cohort suggesting it is elevated as a result of normal ageing. In contrast to our recent findings in human autopsy brain tissue, there was little evidence of a relationship between CSF ACE1 and ACE2 with CSF t-tau,p-tau or $A\beta_{42}$. We did, however, find moderately strong associations between CSF ACE1 activity and sPDGFRβ, a marker of pericyte damage, and between CSF ACE2 activity and CSF albumin level, a marker of BBB leakiness, within the AD cohort only. Angiotensin peptide concentrations were expressed at low levels in CSF and were unaltered in AD. These novel findings indicate that disease-related changes in RAS are associated with markers of

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capillary dysfunction in AD. Together, this study provides further mechanistic insights and improves our current understanding of the role of RAS in AD.

An imbalance in RAS resulting in overactivation of the classical RAS (disease-associated) pathway and reduced activity in regulatory RAS, is observed in human brain tissue in association with disease pathology in AD [9-11, 13]. In this study, CSF ACE1 activity was increased in AD supporting findings from previous studies in post-mortem tissue [11, 13] and the majority of CSF studies [38, 39]. CSF ACE2 activity was unchanged in AD supporting a recent study [41], but it should be noted that CSF ACE2 activity was higher in AD albeit non-significantly in this study possibly due to the relatively small cohort size. Interestingly, ACE2 was increased in association with ageing in the controls but not in AD, potentially indicating that ACE2 changes precede changes associated with disease pathology. CSF ACE1 activity correlated positively with CSF ACE2 activity in CSF whereas we previously reported the opposite in brain tissue i.e. elevated ACE1 and reduced ACE2 [10]. ACE1 and ACE2 are predominantly membrane bound but both can be cleaved at their respective transmembrane domains and are released extracellularly (solubilised) in an active form [48, 49]. Shedding of membrane-bound ACE2 by ADAM-17, resulting in loss of membrane-bound ACE2 has previously been implicated in the pathogenesis of neurogenic hypertension [50] and could also account for the differences between brain activity and CSF activity in AD. ACE1 in contrast is elevated in both brain tissue and CSF, it is currently unclear whether over-production of ACE2 or post-translational modifications are responsible for elevated ACE1 in AD.

We previously reported that ACE1 correlated positively with Aβ in AD, and reduced ACE2 activity correlated inversely with Aβ and tau in human brain tissue [10, 11, 13]. An

association between CSF ACE1 and the ratio of p-tau$_{181}$:Aβ_{42} has previously been reported in two separate cohorts: the Knight Alzheimer's Disease Research Center (N = 311) and the Alzheimer's Disease Neuroimaging Initiative (N = 293) [51]. In contrast, we did not find strong evidence to support similar changes between CSF RAS and markers of disease pathology in CSF. This could be due to many factors and potential limitations in the current study including the small cohort size and/or the fact the groups were based on CSF biomarkers levels, rather than clinical or pathological diagnosis. We also have no information on hypertension status or use of RAS-targeting anti-hypertensives that may conceal a potential relationship. It is also unclear and difficult to interpret why we observed a positive correlation between CSF ACE1 and p-tau in controls but not AD. It should, however, also be noted that changes in CSF reflect global changes throughout the brain and do not necessarily reflect specific regional changes previously reported within the brain. This is also an observational cross-sectional study and does not provide any clues as to the temporal sequence of RAS activation in relation to disease pathology. Further studies are required to address these current limitations and confirm or refute our current findings.

In contrast, we have found stronger evidence of a relationship between RAS overactivation and capillary breakdown indicated by moderate associations between CSF ACE1 and ACE2 enzyme activities with CSF markers of capillary damage in AD, namely pericyte damage (elevated CSF sPDGFRβ) and BBB breakdown (elevated CSF albumin) respectively. The microvasculature has been shown to degenerate in AD [47] and ACE1 activity is highly enriched in brain microvessels [46] that together are suggestive of a possible involvement of RAS in vascular disruption in AD. Recent studies also indicate that vascular dysfunction, including BBB breakdown and pericyte loss, are major contributors to cognitive decline and disease pathology in AD [2, 52]. Increased solubilisation of PDGFRβ, a

marker of pericyte injury (although PDGFRβ is also expressed by sub-populations of reactive glia), has recently been reported in CSF and is associated with BBB leakiness in the ageing and diseased hippocampus [29], and has been shown to be a significant predictor of cognitive decline independently of Aβ and tau in the early stages of disease [5]. Our data indicate a relationship between RAS activation and both pericyte loss and BBB function. This potential link is supported by indirect evidence of a relationship between RAS and BBB permeability *in vitro* [53] and *in vivo* [54]. Ang-II is a potent vasoconstrictor and mediates pericyte contraction and vessel constriction in vessel extracts from rat retina via AT1R activation [55]. Pericyte contraction and subsequent vessel constriction may be a major contributor to cerebral hypoperfusion in AD [56] and is likely to also lead to pericyte injury and release of soluble PDGFRβ. Collectively our findings indicate a potential relationship between RAS activation and vascular dysfunction in AD that warrants further investigation.

Much less is known regarding changes in angiotensin peptides in AD. We recently reported that Ang-II and Ang-III, despite short half-lives, were detectable in post mortem brain tissue, and were elevated in AD in relation to markers of disease pathology [9]. Despite changes in brain tissue, the levels of Ang-I, Ang-II and Ang-(1-7) were unchanged in CSF and did not correlate with CSF markers of disease or vascular function. The differences between brain tissue and CSF measures may reflect the relative instability and shorter half-life of angiotensin peptides in CSF compared to brain. Another possibility that cannot be addressed in this study is that CSF changes in angiotensin peptide level may first require acute or chronic and sustained changes in the activity of brain ACE1 and ACE2.

This study has several limitations which we have already been mentioned, including the criteria used to group AD cases, the small cohort sizes, and the lack of clinical details

relating to the de-identified AD cases, including cognitive assessment and disease status markers. This is also an observational cross-sectional study and does not provide clues as to the timing and regional changes in RAS in relation to disease and vascular markers and it is also unclear what percentage of the participants were prescribed RAS-targeting anti-hypertensives. Despite these limitations, which will need to be carefully addressed in future studies, this study still provides novel insights into the potential relationship between RAS activation and capillary damage in AD and provides further evidence of a link between RAS and the pathogenesis of AD.

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Conflict of Interest/Disclosure statement

JSM, NAM and PGK declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

KB has served as a consultant or at advisory boards for Alector, Alzheon, CogRx, Biogen, Lilly, Novartis and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg, all unrelated to the work presented in this paper.

HZ has served at scientific advisory boards for Wave, Samumed, CogRx and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU

Ventures-based platform company at the University of Gothenburg, all unrelated to the work presented in this paper.

Author contribution

JSM and PGK were responsible for the conception and design of experiments; HZ and KB provided the clinical CSF samples and previously measured of t-tau, p-tau, Aβ42 and CSF/serum albumin; JSM and NAM were responsible for acquisition of ACE1, ACE2 and angiotensin peptide data; JSM analysed and interpreted the data; JSM drafted the paper; JSM, PGK, HZ and KB revised and edited the final article for intellectual content and final approval.

References

[1]   Gorevic PD, Goni F, Pons-Estel B, Alvarez F, Peress NS, Frangione B (1986) Isolation and partial characterization of neurofibrillary tangles and amyloid plaque core in Alzheimer's disease: immunohistological studies. *J Neuropathol Exp Neurol* **45**, 647-664.

[2]   Iturria-Medina Y, Sotero RC, Toussaint PJ, Mateos-Perez JM, Evans AC, Alzheimer's Disease Neuroimaging I (2016) Early role of vascular dysregulation on late-onset Alzheimer's disease based on multifactorial data-driven analysis. *Nat Commun* **7**, 11934.

[3]   Montagne A, Barnes SR, Sweeney MD, Halliday MR, Sagare AP, Zhao Z, Toga AW, Jacobs RE, Liu CY, Amezcua L, Harrington MG, Chui HC, Law M, Zlokovic BV (2015) Blood-brain barrier breakdown in the aging human hippocampus. *Neuron* **85**, 296-302.

[4]   Sweeney MD, Kisler K, Montagne A, Toga AW, Zlokovic BV (2018) The role of brain

        vasculature in neurodegenerative disorders. *Nature Neuroscience* **21**, 1318-1331.

[5]   Nation DA, Sweeney MD, Montagne A, Sagare AP, D'Orazio LM, Pachicano M,

        Sepehrband F, Nelson AR, Buennagel DP, Harrington MG, Benzinger TLS, Fagan AM,

        Ringman JM, Schneider LS, Morris JC, Chui HC, Law M, Toga AW, Zlokovic BV (2019)

        Blood-brain barrier breakdown is an early biomarker of human cognitive

        dysfunction. *Nat Med*.

[6]   Sweeney MD, Montagne A, Sagare AP, Nation DA, Schneider LS, Chui HC, Harrington

        MG, Pa J, Law M, Wang DJJ, Jacobs RE, Doubal FN, Ramirez J, Black SE, Nedergaard

        M, Benveniste H, Dichgans M, Iadecola C, Love S, Bath PM, Markus HS, Salman RA,

        Allan SM, Quinn TJ, Kalaria RN, Werring DJ, Carare RO, Touyz RM, Williams SCR,

        Moskowitz MA, Katusic ZS, Lutz SE, Lazarov O, Minshall RD, Rehman J, Davis TP,

        Wellington CL, Gonzalez HM, Yuan C, Lockhart SN, Hughes TM, Chen CLH, Sachdev P,

        O'Brien JT, Skoog I, Pantoni L, Gustafson DR, Biessels GJ, Wallin A, Smith EE, Mok V,

        Wong A, Passmore P, Barkof F, Muller M, Breteler MMB, Roman GC, Hamel E,

        Seshadri S, Gottesman RF, van Buchem MA, Arvanitakis Z, Schneider JA, Drewes LR,

        Hachinski V, Finch CE, Toga AW, Wardlaw JM, Zlokovic BV (2019) Vascular

        dysfunction-The disregarded partner of Alzheimer's disease. *Alzheimers Dement* **15**,

        158-167.

[7]   Kehoe PG (2018) The Coming of Age of the Angiotensin Hypothesis in Alzheimer's

        Disease: Progress Toward Disease Prevention and Treatment? *J Alzheimers Dis* **62**,

        1443-1466.

[8]   Kehoe PG, Miners S, Love S (2009) Angiotensins in Alzheimer's disease - friend or

        foe? *Trends Neurosci* **32**, 619-628.

[9]   Kehoe PG, Hibbs E, Palmer LE, Miners JS (2017) Angiotensin-III is Increased in

        Alzheimer's Disease in Association with Amyloid-beta and Tau Pathology. *J*

        Alzheimers Dis **58**, 203-214.

[10]   Kehoe PG, Wong S, Al Mulhim N, Palmer LE, Miners JS (2016) Angiotensin-converting

        enzyme 2 is reduced in Alzheimer's disease in association with increasing amyloid-

        beta and tau pathology. *Alzheimers Res Ther* **8**, 50.

[11]   Miners JS, Ashby E, Van Helmond Z, Chalmers KA, Palmer LE, Love S, Kehoe PG

        (2008) Angiotensin-converting enzyme (ACE) levels and activity in Alzheimer's

        disease, and relationship of perivascular ACE-1 to cerebral amyloid angiopathy.

        Neuropathol Appl Neurobiol **34**, 181-193.

[12]   Savaskan E, Hock C, Olivieri G, Bruttel S, Rosenberg C, Hulette C, Muller-Spahn F

        (2001) Cortical alterations of angiotensin converting enzyme, angiotensin II and AT1

        receptor in Alzheimer's dementia. *Neurobiol Aging* **22**, 541-546.

[13]   Miners S, Ashby E, Baig S, Harrison R, Tayler H, Speedy E, Prince JA, Love S, Kehoe PG

        (2009) Angiotensin-converting enzyme levels and activity in Alzheimer's disease:

        differences in brain and CSF ACE and association with ACE1 genotypes. *Am J Transl*

        Res **1**, 163-177.

[14]   Tian M, Zhu D, Xie W, Shi J (2012) Central angiotensin II-induced Alzheimer-like tau

        phosphorylation in normal rat brains. *FEBS Lett* **586**, 3737-3745.

[15]   Zhu D, Shi J, Zhang Y, Wang B, Liu W, Chen Z, Tong Q (2011) Central angiotensin II

        stimulation promotes beta amyloid production in Sprague Dawley rats. *PLoS One* **6**,

        e16037.

[16] Ferrario CM, Trask AJ, Jessup JA (2005) Advances in biochemical and functional roles of angiotensin-converting enzyme 2 and angiotensin-(1-7) in regulation of cardiovascular function. *Am J Physiol Heart Circ Physiol* **289**, H2281-2290.

[17] Santos RA, Campagnole-Santos MJ, Andrade SP (2000) Angiotensin-(1-7): an update. *Regul Pept* **91**, 45-62.

[18] Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T (2003) Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A* **100**, 8258-8263.

[19] Elased KM, Cunha TS, Marcondes FK, Morris M (2008) Brain angiotensin-converting enzymes: role of angiotensin-converting enzyme 2 in processing angiotensin II in mice. *Exp Physiol* **93**, 665-675.

[20] Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsons T, Baronas E, Hsieh F, Acton S, Patane M, Nichols A, Tummino P (2002) Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem* **277**, 14838-14843.

[21] Davies NM, Kehoe PG, Ben-Shlomo Y, Martin RM (2011) Associations of anti-hypertensive treatments with Alzheimer's disease, vascular dementia, and other dementias. *J Alzheimers Dis* **26**, 699-708.

[22] Li NC, Lee A, Whitmer RA, Kivipelto M, Lawler E, Kazis LE, Wolozin B (2010) Use of angiotensin receptor blockers and risk of dementia in a predominantly male population: prospective cohort analysis. *BMJ* **340**, b5465.

[23]   Yasar S, Xia J, Yao W, Furberg CD, Xue QL, Mercado CI, Fitzpatrick AL, Fried LP, Kawas CH, Sink KM, Williamson JD, DeKosky ST, Carlson MC, Ginkgo Evaluation of Memory Study I (2013) Antihypertensive drugs decrease risk of Alzheimer disease: Ginkgo Evaluation of Memory Study. *Neurology* **81**, 896-903.

[24]   Wharton W, Zhao L, Steenland K, Goldstein FC, Schneider JA, Barnes LL, Gearing M, Yasar S (2019) Neurofibrillary Tangles and Conversion to Mild Cognitive Impairment with Certain Antihypertensives. *J Alzheimers Dis*.

[25]   Wharton W, Goldstein FC, Zhao L, Steenland K, Levey AI, Hajjar I (2015) Modulation of Renin-Angiotensin System May Slow Conversion from Mild Cognitive Impairment to Alzheimer's Disease. *J Am Geriatr Soc* **63**, 1749-1756.

[26]   Ohrui T, Tomita N, Sato-Nakagawa T, Matsui T, Maruyama M, Niwa K, Arai H, Sasaki H (2004) Effects of brain-penetrating ACE inhibitors on Alzheimer disease progression. *Neurology* **63**, 1324-1325.

[27]   Hajjar I, Levey A (2015) Association Between Angiotensin Receptor Blockers and Longitudinal Decline in Tau in Mild Cognitive Impairment. *JAMA Neurol* **72**, 1069-1070.

[28]   Nation DA, Ho J, Yew B, Alzheimer's Disease Neuroimaging I (2016) Older Adults Taking AT1-Receptor Blockers Exhibit Reduced Cerebral Amyloid Retention. *J Alzheimers Dis* **50**, 779-789.

[29]   Wharton W, Zhao L, Steenland K, Goldstein FC, Schneider JA, Barnes LL, Gearing M, Yasar S (2019) Neurofibrillary Tangles and Conversion to Mild Cognitive Impairment with Certain Antihypertensives. *J Alzheimers Dis* **70**, 153-161.

[30]   Hajjar I, Brown L, Mack WJ, Chui H (2012) Impact of Angiotensin receptor blockers on

Alzheimer disease neuropathology in a large brain autopsy series. *Arch Neurol* **69**,

1632-1638.

[31]   Erdos EG, Skidgel RA (1987) The angiotensin I-converting enzyme. *Lab Invest* **56**, 345-

348.

[32]   Doobay MF, Talman LS, Obr TD, Tian X, Davisson RL, Lazartigues E (2007) Differential

expression of neuronal ACE2 in transgenic mice with overexpression of the brain

renin-angiotensin system. *Am J Physiol Regul Integr Comp Physiol* **292**, R373-381.

[33]   Lin Z, Chen Y, Zhang W, Chen AF, Lin S, Morris M (2008) RNA interference shows

interactions between mouse brainstem angiotensin AT1 receptors and angiotensin-

converting enzyme 2. *Exp Physiol* **93**, 676-684.

[34]   Turner AJ, Hooper NM (2002) The angiotensin-converting enzyme gene family:

genomics and pharmacology. *Trends Pharmacol Sci* **23**, 177-183.

[35]   Gallagher PE, Chappell MC, Ferrario CM, Tallant EA (2006) Distinct roles for ANG II

and ANG-(1-7) in the regulation of angiotensin-converting enzyme 2 in rat

astrocytes. *Am J Physiol Cell Physiol* **290**, C420-426.

[36]   Beldent V, Michaud A, Wei L, Chauvet MT, Corvol P (1993) Proteolytic release of

human angiotensin-converting enzyme. Localization of the cleavage site. *J Biol Chem*

268, 26428-26434.

[37]   Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ (2000) A human

homolog of angiotensin-converting enzyme. Cloning and functional expression as a

captopril-insensitive carboxypeptidase. *J Biol Chem* **275**, 33238-33243.

[38]   He M, Ohrui T, Maruyama M, Tomita N, Nakayama K, Higuchi M, Furukawa K, Arai H (2006) ACE activity in CSF of patients with mild cognitive impairment and Alzheimer disease. *Neurology* **67**, 1309-1310.

[39]   Mateos L, Ismail MA, Gil-Bea FJ, Leoni V, Winblad B, Bjorkhem I, Cedazo-Minguez A (2011) Upregulation of brain renin angiotensin system by 27-hydroxycholesterol in Alzheimer's disease. *J Alzheimers Dis* **24**, 669-679.

[40]   Jochemsen HM, Teunissen CE, Ashby EL, van der Flier WM, Jones RE, Geerlings MI, Scheltens P, Kehoe PG, Muller M (2014) The association of angiotensin-converting enzyme with biomarkers for Alzheimer's disease. *Alzheimers Res Ther* **6**, 27.

[41]   Rocha NP, Toledo A, Corgosinho LTS, de Souza LC, Guimaraes HC, Resende EPF, Braz NFT, Gomes KB, Simoes ESAC, Caramelli P, Teixeira AL (2018) Cerebrospinal Fluid Levels of Angiotensin-Converting Enzyme Are Associated with Amyloid-beta42 Burden in Alzheimer's Disease. *J Alzheimers Dis* **64**, 1085-1090.

[42]   Zubenko GS, Volicer L, Direnfeld LK, Freeman M, Langlais PJ, Nixon RA (1985) Cerebrospinal fluid levels of angiotensin-converting enzyme in Alzheimer's disease, Parkinson's disease and progressive supranuclear palsy. *Brain Res* **328**, 215-221.

[43]   Konings CH, Kuiper MA, Scheltens P, Grijpma AM, van Pelt W, Wolters EC (1993) Re-evaluation of cerebrospinal fluid angiotensin-converting enzyme activity in patients with 'probable' Alzheimer's disease. *Eur J Clin Chem Clin Biochem* **31**, 495-497.

[44]   Hansson O, Zetterberg H, Buchhave P, Londos E, Blennow K, Minthon L (2006) Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study. *Lancet Neurol* **5**, 228-234.

[45]   Miners JS, van Helmond Z, Raiker M, Love S, Kehoe PG (2010) ACE variants and association with brain Abeta levels in Alzheimer's disease. *Am J Transl Res* **3**, 73-80.

[46]   Kalaria RN, Harik SI (1988) Adenosine receptors and the nucleoside transporter in human brain vasculature. *J Cereb Blood Flow Metab* **8**, 32-39.

[47]   Hase Y, Ding R, Harrison G, Hawthorne E, King A, Gettings S, Platten C, Stevenson W, Craggs LJL, Kalaria RN (2019) White matter capillaries in vascular and neurodegenerative dementias. *Acta Neuropathol Commun* **7**, 16.

[48]   Lambert DW, Yarski M, Warner FJ, Thornhill P, Parkin ET, Smith AI, Hooper NM, Turner AJ (2005) Tumor necrosis factor-alpha convertase (ADAM17) mediates regulated ectodomain shedding of the severe-acute respiratory syndrome-coronavirus (SARS-CoV) receptor, angiotensin-converting enzyme-2 (ACE2). *J Biol Chem* **280**, 30113-30119.

[49]   Sadhukhan R, Sen GC, Ramchandran R, Sen I (1998) The distal ectodomain of angiotensin-converting enzyme regulates its cleavage-secretion from the cell surface. *Proc Natl Acad Sci U S A* **95**, 138-143.

[50]   Xia H, Sriramula S, Chhabra KH, Lazartigues E (2013) Brain angiotensin-converting enzyme type 2 shedding contributes to the development of neurogenic hypertension. *Circ Res* **113**, 1087-1096.

[51]   Harari O, Cruchaga C, Kauwe JS, Ainscough BJ, Bales K, Pickering EH, Bertelsen S, Fagan AM, Holtzman DM, Morris JC, Goate AM, Alzheimer's Disease Neuroimaging I (2014) Phosphorylated tau-Abeta42 ratio as a continuous trait for biomarker discovery for early-stage Alzheimer's disease in multiplex immunoassay panels of cerebrospinal fluid. *Biol Psychiatry* **75**, 723-731.

[52]   Nation DA, Sweeney MD, Montagne A, Sagare AP, D'Orazio LM, Pachicano M, Sepehrband F, Nelson AR, Buennagel DP, Harrington MG, Benzinger TLS, Fagan AM, Ringman JM, Schneider LS, Morris JC, Chui HC, Law M, Toga AW, Zlokovic BV (2019)

Blood-brain barrier breakdown is an early biomarker of human cognitive dysfunction. *Nat Med* **25**, 270-276.

[53]   Matsugi T, Chen Q, Anderson DR (1997) Contractile responses of cultured bovine retinal pericytes to angiotensin II. *Arch Ophthalmol* **115**, 1281-1285.

[54]   Fuchtbauer L, Toft-Hansen H, Khorooshi R, Owens T (2010) Expression of astrocytic type 2 angiotensin receptor in central nervous system inflammation correlates with blood-brain barrier breakdown. *J Mol Neurosci* **42**, 89-98.

[55]   Kawamura H, Kobayashi M, Li Q, Yamanishi S, Katsumura K, Minami M, Wu DM, Puro DG (2004) Effects of angiotensin II on the pericyte-containing microvasculature of the rat retina. *J Physiol* **561**, 671-683.

[56]   Nortley R, Korte N, Izquierdo P, Hirunpattarasilp C, Mishra A, Jaunmuktane Z, Kyrargyri V, Pfeiffer T, Khennouf L, Madry C, Gong H, Richard-Loendt A, Huang W, Saito T, Saido TC, Brandner S, Sethi H, Attwell D (2019) Amyloid beta oligomers constrict human capillaries in Alzheimer's disease via signaling to pericytes. *Science*.
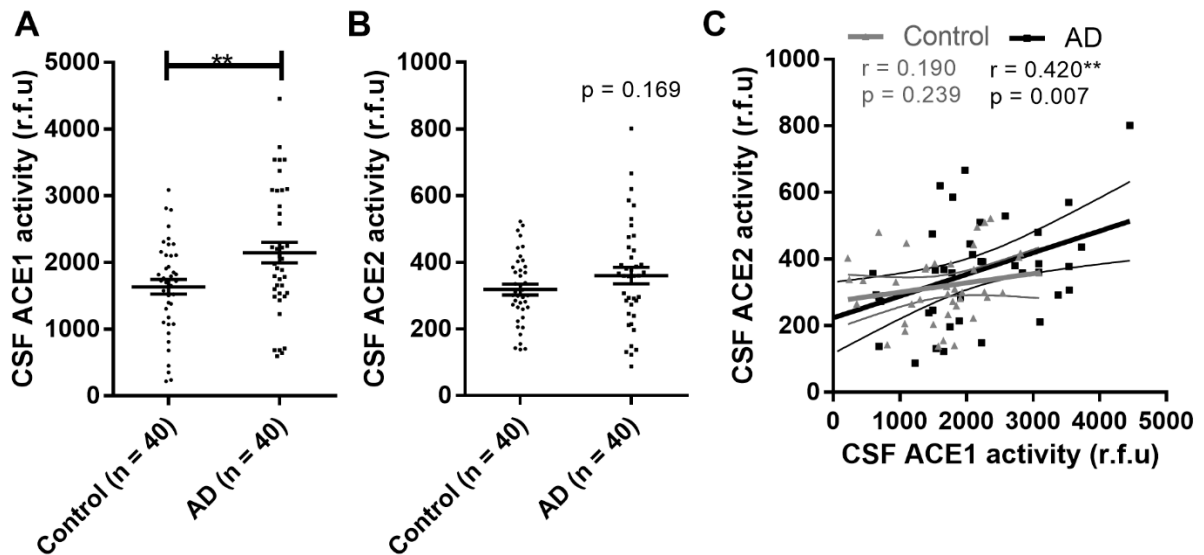
Figure 1. ACE1 and ACE2 activity in cerebrospinal fluid (CSF) in Alzheimer's disease (AD). A-B Bar charts showing ACE1 and ACE2 enzyme activity in CSF in Alzheimer's disease (AD) compared to age-matched controls. Bars represent the mean level and standard error of the mean. ** P < 0.01 **C** Scatterplot showing relationship between CSF ACE1 and ACE2 grouped into AD and controls. The best-fit linear regression lines and 95% confidence intervals are superimposed. Each point in the scatterplot indicates a single individual. ** P < 0.01 *** P < 0.001
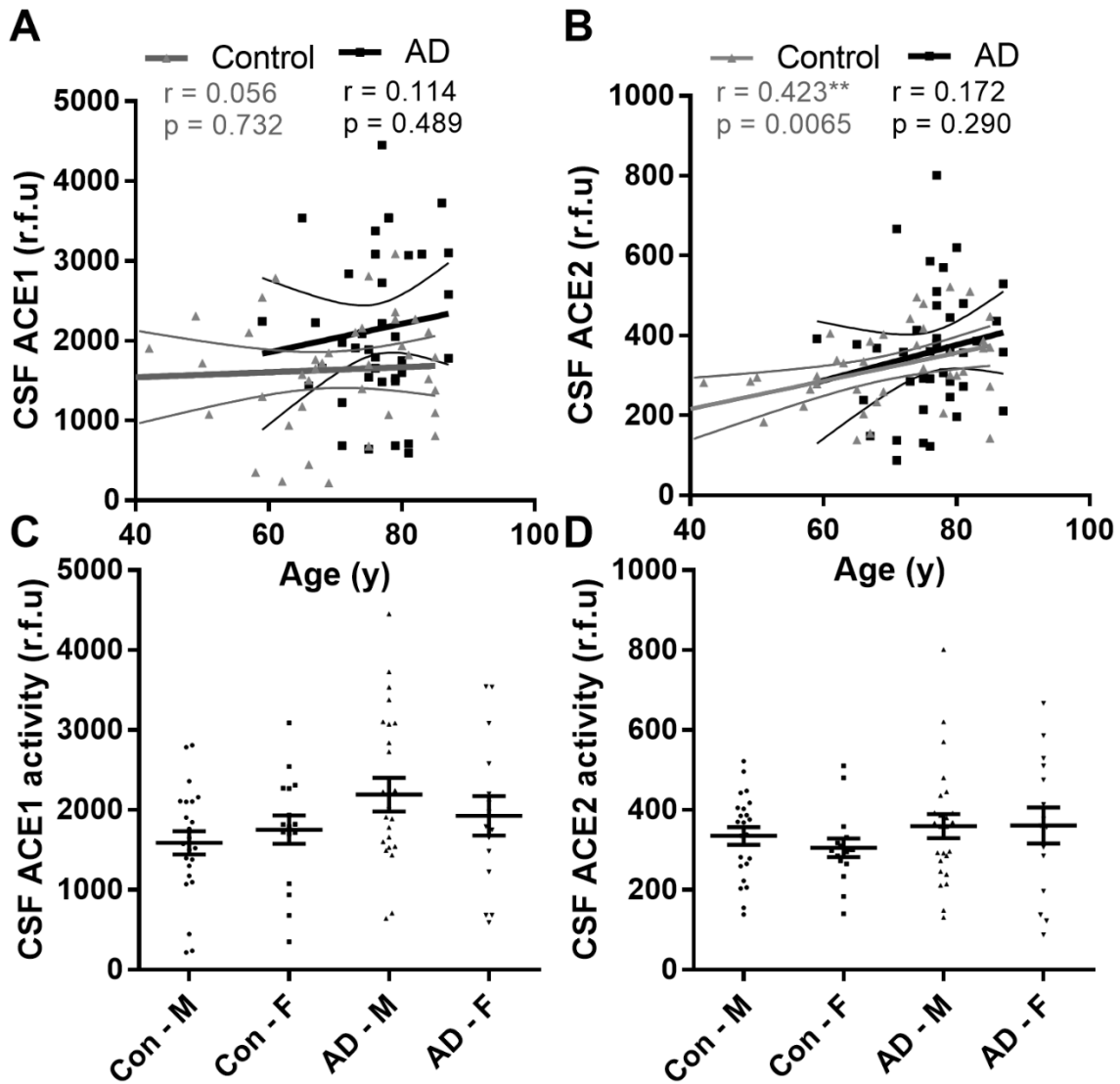
Figure 2: Relationship between CSF ACE1 and ACE2 enzyme activity in relation to age and sex in CSF in normal ageing and Alzheimer's disease (AD). A-B Scatterplots showing the relationship between ACE1 and ACE2 in normal ageing (blue) and AD (red). ACE1 did not correlate with age in either cohort whereas a positive correlation (r = 0.423, p = 0.0065) was observed between ACE2 and age in controls only. Each point in the scatterplot indicates a single individual. The best-fit linear regression lines and 95% confidence intervals are superimposed. **C-D** Bar charts show CSF ACE1 and ACE2 activity in control and AD groups according to gender. Bars represent the mean level and standard error of the mean.
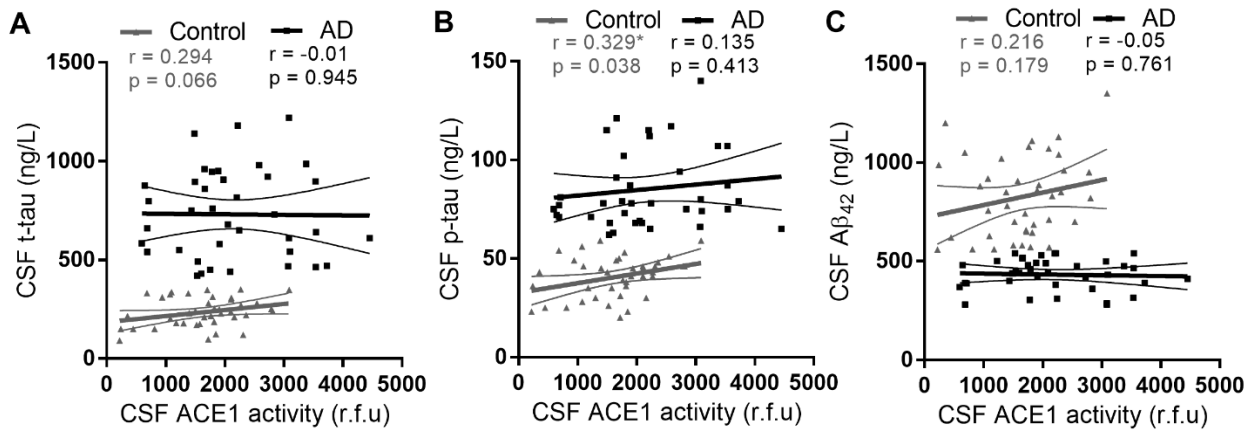
Figure 3: Relationship between CSF ACE1 and established CSF markers of disease pathology in Alzheimer's disease. A-C Scatterplots showing the relationship between ACE1 activity and total tau (t-tau), phosphorylated tau (p-tau) and Aβ$_{42}$ in CSF in AD and age-matched controls. ACE1 enzyme activity weakly correlated with p-tau (r = 0.329, p = 0.038) in controls but not AD but all other correlations were not statistically significant. Each point in the scatterplot indicates a single individual. The best-fit linear regression lines and 95% confidence intervals are superimposed.
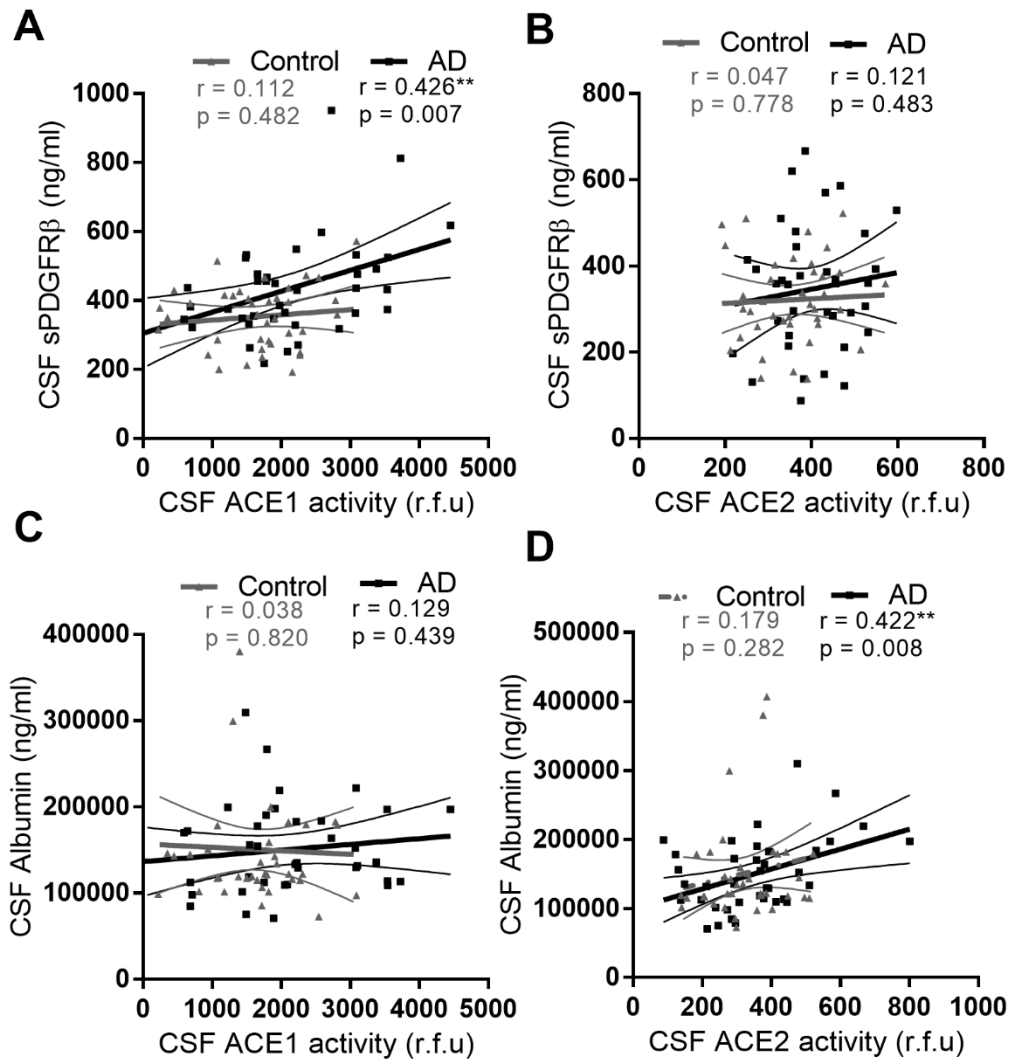
Figure 4. Relationship between CSF ACE1 and ACE2 with CSF markers of vascular dysfunction in Alzheimer's disease. A-D Scatterplots showing relationship between CSF ACE1 and ACE2 enzyme activity with markers of pericyte loss (elevated sPDGFRβ) and BBB breakdown (elevated albumin) in Alzheimer's disease (AD). ACE1 positively correlated with sPDGFRβ (r = 0.426, p = 0.007) and ACE2 positively correlated with CSF albumin (r = 0.422, p = 0.008) in AD. Each point in the scatterplot indicates a single individual. The best-fit linear regression lines and 95% confidence intervals are superimposed.
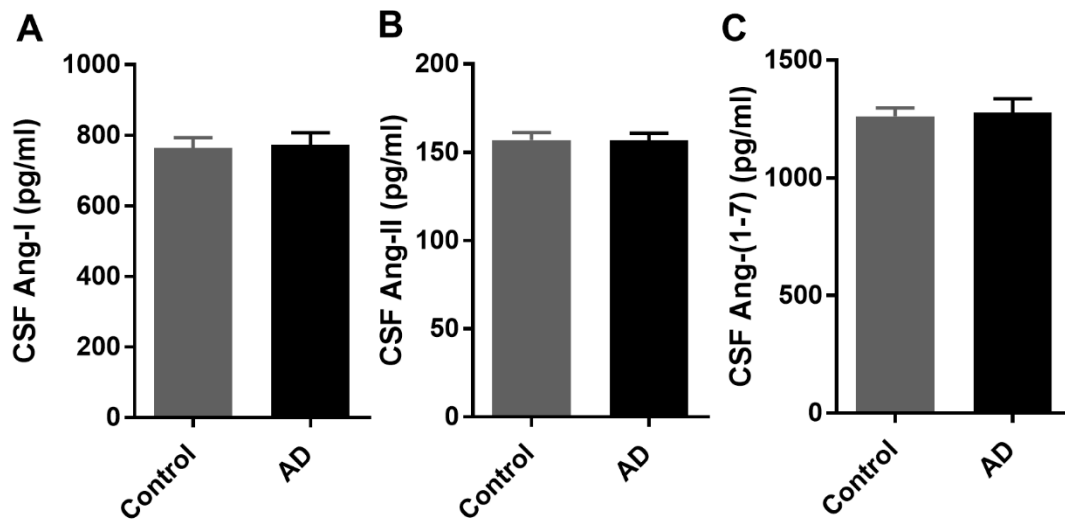
Figure 5. Angiotensin peptides are unchanged in CSF in Alzheimer's disease. Bar charts

showing angiotensin-I, -II and –(1-7) level in a subset of CSF samples (20 AD Vs 20 controls)

in Alzheimer's disease (AD). Bars represent the mean level and standard error of the mean.

Table 1: Demographics of the CSF cohort

	n =	Age (y) ± SD	Sex (M:F)	Aβ42 (ng/L)	t-tau (ng/L)	p-tau (ng/L)
Control	40	69.1 ± 12.1	23:16	825.3 ± 32.4	235.4 ± 11.6	40.6 ± 1.6
AD	40	76.3 ± 6.1	25:15	431.3 ± 12.2	724.1 ± 35.4	84.6 ± 3.1

Table 2: A relationship between CSF ACE2 activity and markers of disease pathology in CSF was not observed in Alzheimer's disease.

	t-tau (ng/L)	p-tau (ng/L)	Aβ$_{42}$ (ng/L)
AD alone (N = 40)	r = 0.113 p = 0.487	r = 0.076 P = 0.643	r = -0.063 P = 0.700
Control alone (N = 40)	r = 0.245 p = 0.128	r = 0.183 P = 0.259	r = 0.140 P = 0.390