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## Increased Plasma TACE Activity in Subjects with Mild Cognitive Impairment and Patients with Alzheimer's Disease

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### Abstract

Evidence suggests that the tumor necrosis factor receptor (TNFR)-signaling pathway contributes to the pathogenesis of Alzheimer's disease (AD). TNF- $\alpha$  converting enzyme (TACE/ADAM-17) can cleave both pro-TNF- $\alpha$  and TNF receptors. Recently, we have shown that TACE activity in the cerebrospinal fluid (CSF) of subjects with mild cognitive impairment (MCI) and AD patients is significantly higher than that of cognitively healthy controls (HC). To date, it is not clear whether TACE activity could be detected in the human plasma and whether TACE activity in MCI and AD patients is different from that in HC. We analyze TACE expression and activity in a large clinical sample of 64 patients with AD, 88 subjects with MCI, and 50 age-matched HC recruited from two distinct academic centers. Plasma TACE protein levels did not differ significantly in the three study groups (AD, MCI, and HC). However, plasma TACE activity in subjects with MCI and AD patients was significantly higher than that in HC. Moreover, in MCI and AD groups, we found a significant correlation between plasma TACE activity and CSF t-tau and A $\beta_{42}$  levels and CSF A $\beta_{42}$ /tau ratios. In AD patients, the levels of plasma TACE activity correlated significantly

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and negatively with cognition. These findings further support the role of the TNF- $\alpha$  receptor complex in AD-related neuroinflammation and propose TACE plasma activity as a promising hypothesis-driven biomarker candidate for detection, diagnosis, and prognosis of prodromal and clinical AD.

### Keywords

Alzheimer's disease; biomarker; mild cognitive impairment; plasma; tumor necrosis factor converting enzyme

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## INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia worldwide, characterized by the presence of amyloid plaques composed of amyloid- $\beta$  (A $\beta$ ) peptide aggregates and neurofibrillary tangles consisting of hyperphosphorylated and aggregated tau protein in the gray matter structures of the brain [1]. Mild cognitive impairment (MCI) is a heterogeneous clinical condition with several underlying causes. However, the large proportion of MCI represents a transitional state between healthy aging and very mild AD [2–4]. Accordingly, studies suggest that MCI subjects tend to progress to clinically probable AD at a rate of approximately 10%–15% per year [4, 5].

The development of effective treatments of AD would be facilitated by easily accessible biomarkers. Although most biomarkers of AD are currently measured in the cerebrospinal fluid (CSF), the invasive nature of lumbar puncture has limited more widespread applicability [6, 7]. A major challenge therefore lies in the development of blood-based biomarkers which can reliably detect disease presence and predict disease risk. Blood-based biomarkers of AD could provide a cost- and time-effective way to enhance the utility of CSF and imaging biomarkers, such as the first step in a multi-stage screening and diagnostic process, which is common in medical practice [8–10]. This multistep screening process also has cost-savings potential for recruitment into clinical trials. Since sporadic AD is a genetically and etiologically complex and multifaceted disease characterized by various molecular mechanisms and signaling pathways pathophysiologically altered during chronic disease progression, we chose to explore hypothesis-driven candidate systems and derived biomarkers in blood (plasma and serum) to test their clinical utility and performance.

Neuroinflammation has been demonstrated in AD-affected brains by the presence of activated microglia, reactive astrocytes, and the complement system [11, 12], as well as increased cytokine, altered cytokine receptor complex expression [13], and elevated acute-phase proteins [14]. Extensive evidence supports the concept that neuroinflammation is an “on-off” phenomenon that contributes to neurodegeneration in AD [15]. Recently published genetic evidence provides further substantial support toward a significant role of neuroinflammation and involvement of the complement system in AD pathophysiology [16, 17].

Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) is one of the major inflammatory cytokines produced by many cell types, including macrophages, monocytes, lymphocytes, keratinocytes, and

fibroblasts, in response to inflammation, infection, injury, and other environmental challenges [18]. In the brain, TNF- $\alpha$  is expressed by neurons and glia and promotes inflammatory responses by recruiting microglia or astrocytes to lesion sites, leading to glial cell activation [18]. Interestingly, TNF- $\alpha$  may elicit the production of A $\beta$  *in vitro* [19]. Using double transgenic mice, we have previously shown that the inhibition of TNF-receptor (TNFR)-signaling in transgenic APP23 mice results in a significant reduction of brain amyloid plaques and A $\beta$  [20]. Furthermore, a recent clinical study has suggested that the inhibition of TNF- $\alpha$  expression may improve cognitive function in AD patients [21].

TNF- $\alpha$  exerts its proinflammatory effects by binding to two transmembrane receptor subtypes termed TNFR1 and TNFR2 [22]. Soluble forms of TNF- $\alpha$  receptors 1 and 2 (sTNFR1 and sTNFR2) represent the circulating isoforms of the corresponding membrane-bound receptors [22]. These stable receptors render soluble TNF- $\alpha$  receptors more reliable markers of TNF- $\alpha$ -dependent inflammatory activity. Interestingly, TACE (tumor necrosis factor- $\alpha$ -converting enzyme) can cleave both TNFR1 and TNFR2 to the corresponding soluble forms [23]. TACE, also known as ADAM17, is a transmembrane disintegrin metalloprotease that cleaves precursor TNF- $\alpha$  to generate soluble, secreted TNF- $\alpha$  in macrophages and monocytes [24, 25]. Both the cell-associated and the released forms of TNF are biologically active, but full inflammatory responses require the soluble form in at least some situations [26].

Previous studies have shown that CSF levels of TNF- $\alpha$  are increased in individuals with AD and MCI [27]. Accordingly, elevated plasma TNF- $\alpha$  levels have been associated with incident AD in subjects with MCI [28]. Subjects with MCI who subsequently progressed to AD dementia were also characterized by higher plasma and CSF levels of sTNFR1 and sTNFR2 compared to MCI subjects who did not convert to AD dementia [29]. Recently, we have shown that TACE activity is significantly increased in the CSF of MCI subjects and AD patients [30]. In addition, the concentrations of both sTNFR1 and sTNFR2 were found to be significantly correlated with TACE activity [30]. Therefore, the TNF- $\alpha$  receptor complex is a mechanistically-linked promising candidate biomarker for both MCI and AD.

To date, however, data on TACE expression and activity in plasma during the different stages of dementia remain scarce. To answer these questions, the aim of this study was to analyze TACE expression and activity in a large clinical sample of MCI and AD subjects recruited from two distinct academic centers.

## MATERIALS AND METHODS

### Subjects

A total of 202 individuals were recruited from two independent international academic research centers specialized in AD. The clinical material included 64 patients with AD, 88 subjects with MCI, and 50 age-matched healthy controls (HC) recruited from the Alzheimer Memorial Center, Department of Psychiatry, Ludwig-Maximilian University, in Germany and the Department of Clinical Neuroscience, University of Goteborg, Sahlgren's University Hospital, in Sweden. As we have previously described [31, 32], the diagnosis of AD was performed according to the National Institute of Neurological and Communicative Disorders

and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria [33, 34], which included the Mini-Mental State Examination (MMSE). The diagnosis of MCI was performed according to the Petersen criteria [35]. MCI subjects performed 1.5 SD below the age-adjusted reference average in memory scales, as assessed using the CERAD cognitive battery [36] which included verbal learning, recognition, and recall tests. The global cognitive function and activities of daily living were unimpaired. Patients with other causes of cognitive impairment, including brain tumors, sub-dural hematomas, CNS infections, major depressive episodes, schizophrenias, and current alcohol abuse were excluded.

Controls were cognitively healthy individuals whose psychiatric comorbidity was excluded by means of medical history, clinical examination, and Composite International Diagnostic Interview [37]. The baseline demographic variables of these AD and MCI patients and controls are given in Table 1.

At baseline, non-fasting plasma samples were collected in the morning by venipuncture in EDTA-containing tubes. After centrifugation, plasma samples were aliquoted in polypropylene tubes and stored at  $-80^{\circ}\text{C}$  until biochemical analyses, without being thawed and re-frozen. All samples were processed and evaluated in a blinded fashion. Informed consent was obtained either by direct subject consent (controls) or by consent of both patient and caregiver (subjects with dementia).

#### **TACE enzymatic activity assay**

TACE activity assays were performed by using synthetic peptide substrates containing the TACE cleavage site (Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH<sub>2</sub>) (R&D System, Inc., Minneapolis, MN, USA) at a 10 mM concentration in reaction buffer (50 mM acetic acid, pH 4.5, 100 mM sodium chloride). The study has shown that this peptide is the main substrate for TACE [38]. Ten microliters of each sample was used to examine TACE activity. The fluorescence was measured using a fluorescent microplate reader with an excitation wave length at 320 nm and an emission wavelength at 405 nm. TACE activity results were normalized to total protein concentration of plasma samples.

#### **TACE western blot assay**

The plasma samples from each group were mixed with an equal volume of sodium dodecyl sulfate (SDS) sample buffer and separated using 10% SDS-PAGE gel. A total of 50  $\mu\text{g}$  of protein was loaded. Protein was then transferred to a nitrocellulose membrane (Bio-Rad, USA) by wet transfer according to the standard procedure. The membrane was stained with 1% Ponceau S as loading control and a brain lysate from an AD patient was used as positive control. The membrane was probed using an anti-TACE polyclonal antibody (Sigma, USA).

#### **Assays of levels of A $\beta$ <sub>42</sub> and tau in CSF**

CSF-A $\beta$ <sub>1-42</sub> ELISA concentration was determined by INNOTEST  $\beta$ -amyloid (1-42) (Innogenetics, Ghent, Belgium) as previously described [31]. CSF-tau concentration was quantified with Luminex xMAP technique as previously described [29].

## Statistical analysis

Separate one-way analyses of covariance were performed for the comparisons between diagnostic groups for continuous data and multiple comparisons adjusted for Bonferroni tests were used. The Pearson's  $\chi^2$ -test was employed for dichotomous variables. Spearman's correlation coefficient was used for correlation analyses. Multiple linear regression analysis was performed to analyze the associations of TACE activity with diagnosis, age, gender, and recruiting centers. The statistical analyses were accomplished with SPSS for Windows, version 17.0.

## RESULTS

### TACE protein expression in plasma samples

To investigate whether TACE protein could be identified in human plasma, western blot analysis was used. The membrane was stained with 1% Ponceau S as loading control and a brain lysate from an AD patient was used as positive control. Western blots revealed a prominent immunoreactive band at the expected molecular weight of the ~80-kD TACE protein in all of plasma samples and in AD brain lysate. No statistically significant differences were found in plasma TACE protein levels among the three study groups (AD patients, MCI subjects, and controls) (Fig. 1).

### Plasma TACE enzymatic activity is increased in samples of MCI subjects and AD patients

Plasma TACE activity assays were performed by using synthetic peptide substrates containing the TACE cleavage site (Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH<sub>2</sub>). We found that plasma TACE activity of HC, MCI, and AD subjects is  $6.52 \pm 2.14$ ,  $8.94 \pm 2.74$ , and  $10.64 \pm 2.93$  (mFU/min/ug), respectively. When comparing TACE activity in plasma to the HC group, TACE activity was found to be increased by 37.1% in MCI subjects ( $p < 0.001$ ) and by 63.2% in the AD subjects ( $p < 0.001$ ) (Fig. 2). In addition, TACE activity was significantly higher in AD patients than in MCI subjects ( $p < 0.05$ ) (Fig. 2).

### Multiple linear regression analysis for TACE activity

To study the association of plasma TACE enzymatic activity with diagnostic subgroups, age, gender, and recruiting centers, multiple linear regression analysis was performed. Plasma TACE enzymatic activity was significantly and positively associated with the presence of MCI and AD. No other associations were observed between TACE activity and age, gender, or recruiting centers (Table 2).

### Correlations of the baseline levels of plasma TACE activity with CSF tau and A $\beta$ <sub>42</sub> levels, and CSF A $\beta$ <sub>42</sub>/tau ratios

CSF A $\beta$ <sub>42</sub> and tau concentrations in the same cases were determined as previously described [29, 31]. To explore whether the measured plasma TACE activities were related to AD neuropathology, the correlation between the baseline levels of plasma TACE activity with CSF tau and A $\beta$ <sub>42</sub> levels and CSF A $\beta$ <sub>42</sub>/tau ratios in AD and MCI groups was performed. In MCI and AD subjects, the levels of plasma TACE activity showed a moderate positive

correlation with CSF tau levels ( $r_s = 0.347, 0.469, p < 0.05$ ) (Fig. 3A, D). In both MCI and AD groups, there were statistically negative correlations between the levels of plasma TACE activity and CSF A $\beta_{42}$  levels ( $r_s = -0.383, -0.351, p < 0.05$ ) (Fig. 3B, E). Furthermore, we found that in MCI and AD subjects, the levels of plasma TACE activity showed a negative correlation with CSF A $\beta_{42}$ /tau ratios ( $r_s = -0.575, -0.476, p < 0.05$ ) (Fig. 3C, F).

### Correlations between baseline plasma TACE activity and MMSE scores

To study whether the measured plasma TACE activities were related to cognitive decline in patients, the correlation between the baseline levels of plasma TACE activity with MMSE scores in AD and MCI subjects was performed. In the AD subgroup, the levels of plasma TACE activity correlated significantly and negatively with clinical MMSE scores ( $r_s = -0.393, p < 0.05$ ) (Fig. 4A). However, we found no significant association between plasma TACE activity and MMSE scores in the MCI subgroup, possibly because these subjects all have high MMSE scores (Fig. 4B).

## DISCUSSION

Recently, we have shown that TACE activity is significantly increased in the CSF of both MCI and AD subjects as compared with healthy controls [30]. To date, there have been few reports about TACE expression and activity in peripheral plasma. The aim of the present study was to investigate TACE protein expression and enzymatic activity in a large clinical sample of patients with different stages of dementia recruited from two independent academic centers.

We found no significant statistical differences in plasma TACE protein levels between the three study groups. However, plasma TACE activity was significantly higher in both MCI and AD subjects compared with healthy controls. Moreover, in MCI and AD subgroups, the levels of plasma TACE activity showed significant associations with CSF T-tau and A $\beta_{42}$  levels and CSF A $\beta_{42}$ /tau ratios. We also identified a significant negative association between plasma TACE activity and MMSE scores in AD patients. Given the increase in TACE activity, with no change in TACE protein levels, one possible explanation is that there may be increased levels of endogenous TACE activators or decreased levels of TACE inhibitors in MCI and AD. The synthetic peptide we used for TACE enzymatic activity assay contains the TACE cleavage site (Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH<sub>2</sub>) is in widespread use for TACE enzymatic activity assay as the substrate is favorable for TACE (ADAM17) compared to ADAM10 [38, 39]. TACE activity and protein expression have been found in both blood cells and plasma and ADAM10 is so far only found in blood cells. Nonetheless, we cannot exclude the possibility that other enzymes may cleave the peptide in human plasma.

TNF- $\alpha$  functions through binding to its two transmembrane receptor subtypes, TNFR1 and TNFR2 [22]. Soluble forms of TNF- $\alpha$  receptors 1 and 2 (sTNFR1 and sTNFR2) represent the circulating isoforms of the membrane-bound receptors [22]. Interestingly, evidence suggests that both TNFR1 and TNFR2 can be shed as soluble isoforms from the cell surface through the activity of TACE [23]. TACE, also known as ADAM17, is a transmembrane disintegrin metalloprotease that cleaves the TNF- $\alpha$  precursor to generate soluble, secreted

TNF- $\alpha$  in macrophages and monocytes [24, 25]. Both the cell-associated and the released forms of TNF are biologically active, but the full inflammatory responses require the soluble form [26].

Growing evidence suggests that neuroinflammation is common in AD-affected brains as revealed by the presence of activated glial cells, complementary proteins, pro-inflammatory cytokines expression, and acute-phase proteins [11–15]. In addition to inflammation in the CNS, peripheral immune system activation has been reported in AD patients. This has been demonstrated by cross-sectional studies reporting elevated plasma levels of the inflammatory proteins  $\alpha$ -1-antichymotrypsin [40, 41], C-reactive protein [42], interleukin-1, and interleukin-6 [43] in AD patients compared with control subjects. TNF- $\alpha$  is one of the main pro-inflammatory cytokines and its role in the pathogenesis of AD has been proposed in a number of studies [18–21]. Plasma and CSF levels of TNF- $\alpha$  and soluble TNF receptors tend to increase in both MCI and AD subjects [27–29]. Since TACE is being shed from cell membranes after the cleavage of TNF- $\alpha$  and the two TNF receptors, TACE activity can be detected both in the plasma and CSF throughout different stages of AD.

In our study, plasma TACE activity was significantly higher in both MCI subjects and AD patients than in healthy controls. These results suggest that plasma TACE activity may increase progressively over the clinical course of AD, thus suggesting a substantial and dynamic (deviation from normal) inflammatory response during the progression from the prodromal to the mild-to-moderate dementia stages of AD. Another interesting finding of this study is that plasma TACE was significantly associated with the established CSF markers (CSF tau and CSF A $\beta_{42}$ ) in both the AD and MCI subgroups. These results suggest that plasma TACE is not only associated with the presence of either the MCI or the AD groups but may reflect the pathophysiology of the disease as emphasized by the activity and dynamic of established core CSF biomarkers during the progression from prodromal to the mild-to-moderate dementia stages of AD.

TACE has been shown to be responsible for the  $\alpha$ -secretase cleavage of the amyloid- $\beta$  protein precursor (A $\beta$ PP) [44, 45]. In cultured cells, TACE and  $\beta$ -secretase (BACE) compete for the cleavage of A $\beta$ PP. Importantly, the increased cleavage of A $\beta$ PP by TACE results in decreased generation of A $\beta$  [46]. In addition, the release of sA $\beta$ PP $\alpha$  precludes the formation of amyloidogenic peptides by BACE. The increase of TACE in the CSF of AD patients suggests that the high TACE activity may be paralleled by increased sA $\beta$ PP $\alpha$  and reduced A $\beta_{42}$  levels. However, the most abundant form of A $\beta$ , i.e., A $\beta_{40}$ , is not decreased in AD [7], which argues against a role of TACE activity in the modulation of CSF A $\beta$  levels. Indeed, several independent studies demonstrate that modifying TACE activity does not affect A $\beta$ PP cleavage by BACE and A $\beta$  production [47–50]. This would explain why increased TACE activity in AD does not alter the A $\beta$  production from A $\beta$ PP. Inhibition or knockdown of TACE also does not affect sA $\beta$ PP formation and A $\beta$  secretion under normal conditions [44, 51]. The most frequently named  $\alpha$ -secretases are three members of the ADAM (a disintegrin and metalloprotease domain) family: ADAM9, 10, and 17 [45, 46]. Two very recent studies have reported that ADAM10, but not ADAM9 or ADAM17, is the physiologically relevant, constitutive  $\alpha$ -secretase in primary neurons [52, 53]. Therefore, our analyses of TACE in CSF and in plasma suggest that TACE may predominantly cleave TNFRs rather than A $\beta$ PP,

and contribute to upregulation of BACE and consecutive increase of A $\beta$  production through the TNFR signaling pathway as discussed above.

In conclusion, our results demonstrated that plasma TACE activity is significantly increased in MCI and AD subjects compared with age-matched cognitively healthy controls. Notably, we found that TACE activity may increase progressively over the clinical course of AD. Taken together, these positive findings further support the role of the TNF- $\alpha$  receptor complex in the neuroinflammatory pathogenesis of AD. We conclude that plasma TACE activity may serve as a promising hypothesis-driven biomarker candidate for the detection, diagnosis, and prognosis of prodromal and clinical AD.

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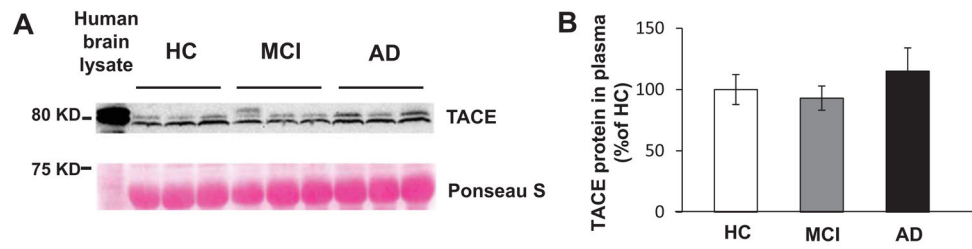
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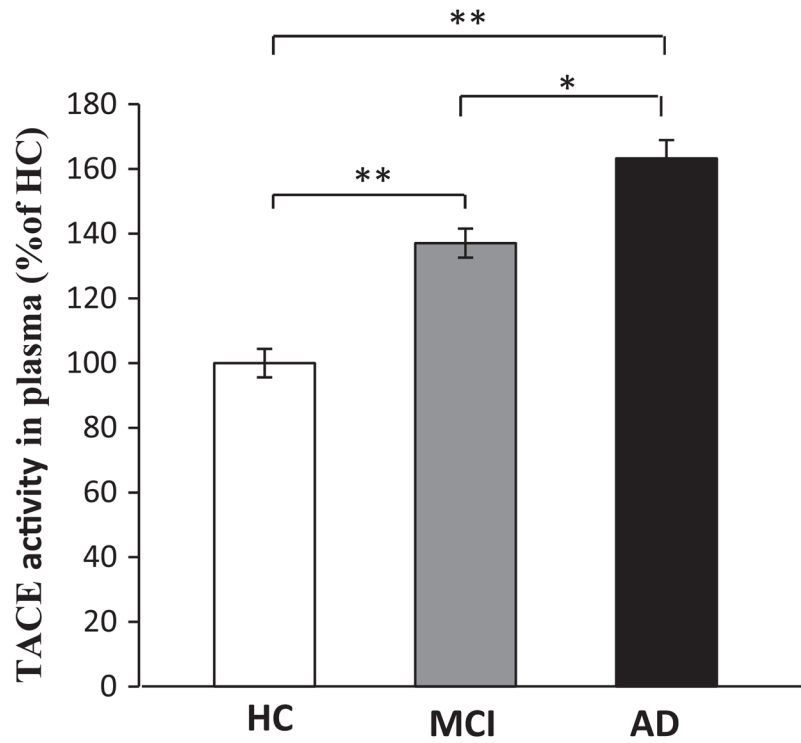
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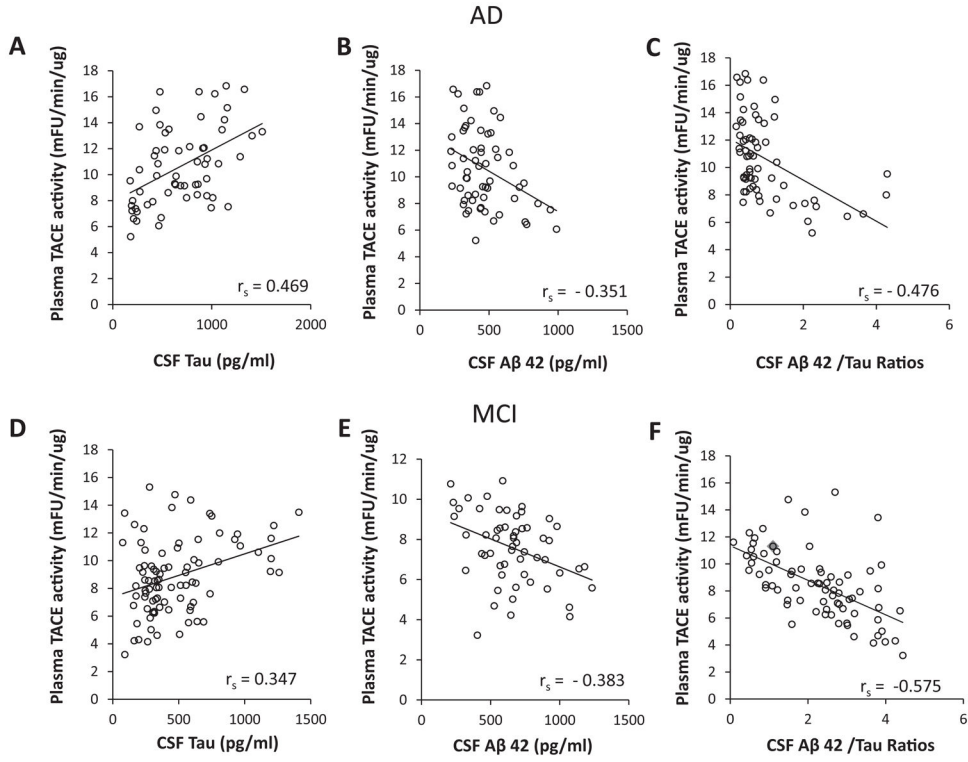
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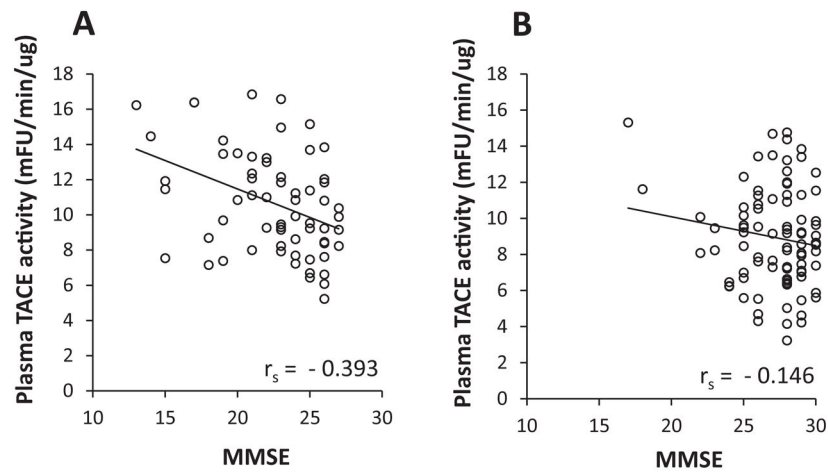
**Fig. 1.** TACE protein expression in human plasma samples. A) Western blot analysis of TACE protein expression in human plasma samples from AD, MCI, and HC groups ( $n = 10$ , separately). The membrane was stained with 1% Ponceau S as loading control and a brain lysate from an AD patient was used as positive control. B) represents the quantitative data of TACE protein expression in plasma samples. No statistically significant differences were found among the three study groups.



**Fig. 2.** TACE enzymatic activity in human plasma samples. TACE enzymatic activity analysis was performed by using synthetic peptide substrates containing the TACE cleavage site in human plasma samples from AD (64 cases), MCI (88 cases), and HC (50 cases). TACE activity was found to be increased by 37.1% in MCI subjects ( $p < 0.001$ ) and by 63.2% in the AD subjects ( $p < 0.001$ ). In addition, TACE activity was significantly higher in AD patients than in MCI subjects ( $p < 0.05$ ). Error bars indicate SEM. \* $p < 0.05$ , \*\* $p < 0.01$ .

**Fig. 3.**

Correlations of the baseline levels of plasma TACE activity with CSF tau (A, D), A $\beta_{42}$  levels (B, E), and CSF A $\beta_{42}$ /tau ratios (C, F) in AD and MCI groups. A, D) The levels of plasma TACE activity showed a moderate positive correlation with CSF tau levels ( $r_s = 0.347, 0.469, p < 0.05$ ) in MCI and AD subjects. B, E) There were statistically negative correlations between the levels of plasma TACE activity and CSF A $\beta_{42}$  levels ( $r_s = -0.383, -0.351, p < 0.05$ ) in MCI and AD groups. The levels of plasma TACE activity showed a negative correlation with CSF A $\beta_{42}$ /tau ratios ( $r_s = -0.575, -0.476, p < 0.05$ ) in MCI and AD groups.



**Fig. 4.** Correlations of the baseline levels of plasma TACE activity and MMSE scores in AD and MCI groups. A) The levels of plasma TACE activity correlated significantly and negatively with clinical MMSE scores ( $r_s = -0.393$ ,  $p < 0.05$ ) in the AD subgroup. B) No significant association between plasma TACE activity and MMSE scores in the MCI subgroup.

**Table 1**

General demographics of subject populations in plasma TACE study

Variables	HC	MCI	AD
Group size	50	88	64
Age (years)	68.9 ± 6.8	69.6 ± 7.6	70.1 ± 8.9
Gender (M/F)	23/27	33/55	18/46
APOE ε4 carrier (%)	28.0	42.0	59.4 <sup>a,b</sup>
MMSE at baseline	29.6 ± 0.6	27.2 ± 2.5 <sup>a</sup>	22.6 ± 3.6 <sup>a,c</sup>
Plasma TACE activity (mFU/min/μg)	6.52 ± 2.14	8.94 ± 2.74 <sup>a</sup>	10.64 ± 2.93 <sup>a,b</sup>

Values are means ± S.D., except as noted otherwise. HC, healthy controls, with unimpaired cognition after at least 3 years follow-up; MCI, mild cognitive impairment patients; AD, Alzheimer's disease patients; MMSE, Mini-Mental State Examination; APOE ε4 carrier, at least one apolipoprotein E ε4 allele; TACE, tumor necrosis factor-α-converting enzyme.

<sup>a</sup>  $p < 0.001$  versus Controls;

<sup>b</sup>  $p < 0.05$  versus MCI;

<sup>c</sup>  $p < 0.001$  versus MCI.



**Table 2**

Multiple linear regression model for TACE enzymatic activity

<b>Model</b>	<b><math>\beta</math></b>	<b><i>p</i></b>
TACE enzymatic activity		
Subgroups		
MCI <sup>a</sup>	0.340	0.001*
AD <sup>a</sup>	0.591	0.001*
Age	-0.027	0.678
Gender	0.037	0.563
Center	0.079	0.215

<sup>a</sup>HC was set as the control subgroup.\*  $p < 0.05$ .