

## RESEARCH ARTICLE

**Tau oligomers in cerebrospinal fluid in Alzheimer's disease**Urmi Sengupta<sup>1,2</sup>, Erik Portelius<sup>3</sup>, Oskar Hansson<sup>4,5</sup>, Kathleen Farmer<sup>1,2</sup>, Diana Castillo-Carranza<sup>1,2</sup>, Randall Woltjer<sup>7</sup>, Henrik Zetterberg<sup>3,6</sup>, Douglas Galasko<sup>8</sup>, Kaj Blennow<sup>3</sup> & Rakez Kaye<sup>1,2</sup><sup>1</sup>Mitchell Center for Neurodegenerative Diseases, University of Texas Medical Branch, Galveston, Texas<sup>2</sup>Department of Neurology, and Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, Texas<sup>3</sup>Clinical Neurochemistry Laboratory, Institute of Neuroscience and Physiology, the Sahlgrenska Academy at University of Gothenburg, Mölndal, Sweden<sup>4</sup>Clinical Memory Research Unit, Department of Clinical Sciences, Lund University, Lund, Sweden<sup>5</sup>Memory Clinic, Skåne University Hospital, Lund, Sweden<sup>6</sup>Department of Molecular Neuroscience, UCL Institute of Neurology, Queen Square, London WC1N 3BG, United Kingdom<sup>7</sup>Department of Department of Pathology, Oregon Health & Science University, Portland, Oregon<sup>8</sup>Department of Neuroscience, University of California San Diego, San Diego, California**Correspondence**

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**Abstract**

**Objective:** With an increasing incidence of Alzheimer's disease (AD) and neurodegenerative tauopathies, there is an urgent need to develop reliable biomarkers for the diagnosis and monitoring of the disease, such as the recently discovered toxic tau oligomers. Here, we aimed to demonstrate the presence of tau oligomers in the cerebrospinal fluid (CSF) of patients with cognitive deficits, and to determine whether tau oligomers could serve as a potential biomarker for AD. **Methods:** A multicentric collaborative study involving a double-blinded analysis with a total of 98 subjects with moderate to severe AD ( $N = 41$ ), mild AD ( $N = 31$ ), and nondemented control subjects ( $N = 26$ ), and two pilot studies of 33 total patients with AD ( $N = 19$ ) and control ( $N = 14$ ) subjects were performed. We carried out biochemical assays to measure oligomeric tau from CSF of these patients with various degrees of cognitive impairment as well as cognitively normal controls. **Results:** Using a highly reproducible indirect ELISA method, we found elevated levels of tau oligomers in AD patients compared to age-matched controls. Western blot analysis confirmed the presence of oligomeric forms of tau in CSF. In addition, the ratio of oligomeric to total tau increased in the order: moderate to severe AD, mild AD, and controls. **Conclusion:** These assays are suitable for the analysis of human CSF samples. These results here suggest that CSF tau oligomer measurements could be optimized and added to the panel of CSF biomarkers for the accurate and early detection of AD.

**Introduction**

Alzheimer's disease (AD), the most common cause of dementia in the elderly, imposes a tremendous socio-economic burden worldwide.<sup>1</sup> Increasing life span has augmented the incidence of AD in developed countries, where the patients are principally at risk of developing this disease with their increasing age. AD, like other neurodegenerative disorders, is caused by the abnormal accumulation of pathogenic protein aggregates. The well-established pathological hallmarks of AD include plaques, consisting mainly of amyloid- $\beta$  ( $A\beta$ ) peptides, and neurofibrillary tangles,

consisting of phosphorylated tau (p-tau) protein.<sup>2</sup> Prior to the manifestation of symptoms and clinical diagnosis, the neurodegenerative processes have already progressed to a relatively advanced stage with marked synaptic loss and neuronal damage.<sup>3</sup> A recent study by Rajan et al. has shown that impairment in cognition starts almost 18 years before AD dementia could be clinically diagnosed.<sup>4</sup> The distinction between memory decline due to the normal course of aging and early stage AD dementia is so ambiguous that the category of mild cognitive impairment (MCI) has been developed to better characterize the earliest signs of AD.<sup>5</sup> The MCI category was initially created to identify the early

stages of AD and was primarily based on impairment in memory, but with the advances in research made over time, it was noticeable that some patients with MCI remain stable while others progress to AD.<sup>6,7</sup> Identifying biomarkers at the preclinical stage of AD is essential in properly diagnosing and determining the probability of developing AD. At this point, cerebrospinal fluid (CSF) biomarkers are of great significance, as they reflect the neuropathology of the diseased brain.<sup>8</sup>

In 2011, the 1984 diagnostic criteria for AD were revised by a work group from the National Institute on Aging and the Alzheimer's Association. In this modified version, imaging and CSF biomarkers have been integrated into the criteria for pathophysiological processes occurring in AD dementia.<sup>9</sup> A $\beta$ 42, total-tau (t-tau), and phosphorylated tau (p-tau) are now considered the major CSF biomarkers in AD pathogenesis, and they have been included as research criteria for the diagnosis of AD since 2007.<sup>10</sup> Apart from these core CSF biomarkers, neurofilament light protein (NFL) and plasma t-tau have also been reported to be associated with AD.<sup>11</sup> CSF surrounds the brain, acting as a protective cushion for the brain and spine, and is in direct contact with the interstitial fluid of the brain. Thus, studying CSF protein profiles may increase the understanding of the pathological changes occurring in the brain. The t-tau levels are elevated in the CSF of AD and MCI patients as compared to normal controls.<sup>12,13</sup> In contrast, decreased levels of CSF A $\beta$ 42, reflecting plaque pathology, have been repeatedly and consistently reported in AD patients.<sup>14,15</sup> Soon after these reports, increased levels of p-tau were reported in MCI and AD cases compared to healthy controls.<sup>16–19</sup> It has been shown that by measuring the three core CSF AD biomarkers, t-tau, p-tau (threonine 181), and A $\beta$ 42, a 96.4% detection sensitivity for autopsy-confirmed AD can be achieved.<sup>20</sup> Routine clinical use in a central laboratory found high sensitivity and specificity for AD in over 5000 CSF samples.<sup>21</sup> This CSF signature appears to predict conversion from MCI to AD, but did not meet the criteria for correctly diagnosing MCI or predicting preclinical AD cases.<sup>20,22,23</sup> Elevated levels of total and p-tau (Thr 181) were also found in the CSF of familial Alzheimer disease (FAD) mutation carriers (presenilin-1 and APP); these levels were reported as sensitive indicators for presymptomatic AD.<sup>24</sup>

Despite, the availability of core CSF biomarkers, identification of new biomarkers is highly needed to better

understand the molecular mechanism underlying AD pathogenesis,<sup>25,26</sup> which will aid in AD diagnosis in its preclinical stage.<sup>27</sup> An evolving concept in AD research is that the intermediate species in the protein aggregation process, oligomers, are the most noxious entity that causes neuronal toxicity. We have previously demonstrated elevated levels of tau oligomers in the soluble fraction of brain homogenates from patients with AD, Parkinson's disease (PD), and dementia with Lewy body (DLB), using two anti-tau oligomer-specific antibodies, T22 (rabbit polyclonal), and TOMA (mouse monoclonal).<sup>28,29</sup> In this study, we aimed to measure the level of CSF tau oligomers in moderate to severe AD, mild AD, and nondemented controls to test the specific hypothesis that the CSF tau oligomer levels are increased in AD compared to controls.

## Methods

### Pilot studies

CSF samples (AD;  $N = 19$ , Co;  $N = 14$ ) used for indirect enzyme-linked immunosorbent assay (ELISA) and Western blot analyses were obtained from the Institute for Brain Aging and Dementia (UC Irvine), Prof. John Ringman (Mary S. Easton Center for Alzheimer's Disease Research, UCLA), Prof. Randall Woltjer (Oregon Health and Science University), Prof. Douglas Galasko (Shiley-Marcos Alzheimer's Disease Research Center, UCSD) (Table 1). MMSE scores are available for all patients. CASI scores are available for the presymptomatic patients (UCLA samples).<sup>24</sup> Prior to analyses, CSF samples were thawed on ice. Unused samples were aliquoted and refrozen at  $-80^{\circ}\text{C}$  until further analysis.

### Subjects included in clinical study

Subjects in the clinical study were recruited from the CSF samples from the Memory clinic, Skåne University Hospital, Sweden, and included moderate to severe AD ( $N = 41$ ), mild AD ( $N = 31$ ), and nondemented controls ( $N = 26$ ). See Table 2 for demographic and biomarker characteristics of the patients included in the study. The individuals were assessed by a medical doctor specialized in dementia disorders and they had undergone brain imaging and routine laboratory testing as well as neurological,

**Table 1.** Demographics and cerebrospinal fluid characteristics of the subjects from pilot studies.

Diagnosis	No. of patients		Age (Years)	MMSE Score	Total tau (pg/mL)	pThr <sub>181</sub> -tau (pg/mL)	A $\beta$ <sub>42</sub> (pg/mL)
	Male/Female						
Nondemented control	6/8		79.87 $\pm$ 5.15	27.33 $\pm$ 2.51	401 $\pm$ 181	NA	NA
AD	7/12		83.85 $\pm$ 8.53	13.87 $\pm$ 10.56*	852 $\pm$ 124	NA	NA

Statistical significance of AD and non-demented control was calculated using Mann-Whitney test, \* $P < 0.05$ .

**Table 2.** Demographics and cerebrospinal fluid characteristics of the subjects from clinical study.

Diagnosis	No. of patients	Age (Years)	MMSE score	Total tau (pg/mL)	pThr <sub>181</sub> -tau (pg/mL)	A $\beta$ <sub>42</sub> (pg/mL)
	Male/Female					
Nondemented control	10/16	64.24 $\pm$ 9.66	28.17 $\pm$ 1.63	378.36 $\pm$ 198.76	54 $\pm$ 17.49	582.8 $\pm$ 177.27
Mild AD	7/24	74.35 $\pm$ 8.0	6.27 $\pm$ 1.5***	723.43 $\pm$ 277.11****	101.13 $\pm$ 37.47****	418.30 $\pm$ 140.76***
Moderate-severe AD	13/28	76.72 $\pm$ 7.14	17.8 $\pm$ 4.54****	847.43 $\pm$ 378.49****	105.36 $\pm$ 46.10****	333.92 $\pm$ 100.55****

AD, Alzheimer's disease; A $\beta$ <sub>42</sub>, amyloid  $\beta$ 42 protein; pThr<sub>181</sub>-tau, tau phosphorylated at threonine 181; NA, not applicable.

Data are presented as mean $\pm$ SD (standard deviation). Statistical significance of mild AD and moderate-severe AD were calculated compared with nondemented controls using Mann-Whitney test and presented as.

\*\*\* $P$  < 0.001.

\*\*\*\* $P$  < 0.0001.

psychiatric, and cognitive examinations. Patients diagnosed with AD fulfilled the DSM-III-R criteria of dementia<sup>30</sup> and the criteria of probable AD defined by NINCDS-ADRDA.<sup>31</sup> The nondemented controls experienced subjective cognitive symptoms at baseline, but thorough clinical investigation as well as clinical follow-up revealed that they were not affected by a dementia disorder or a neurological disease. Lumbar puncture was performed in all cases as part of routine clinical practice and procedure and analysis of the CSF followed the Alzheimer's Association Flow Chart for CSF biomarkers.<sup>32</sup> All individuals have given informed consent for research before their samples were stored in a biobank. A passive consent procedure was then used where consent for retrospective use of banked samples and basic data were assumed if individuals did not actively retract permission, as instructed in local press advertisements. This study procedure was approved by the local ethics committee at Lund University Sweden.

### Preparation of A $\beta$ oligomers

One mg of lyophilized A $\beta$ <sub>42</sub> peptide was dissolved in 1.5 mL of 50% acetonitrile (acetonitrile:water) and divided into three portions, each containing 500  $\mu$ L (approximately 0.3 mg) and lyophilized. Each tube of 0.3 mg peptide was then dissolved in 200  $\mu$ L hexafluoroisopropanol (HFIP) and incubated for 10–20 min at room temperature. To this solution, 700  $\mu$ L dd H<sub>2</sub>O was added. Tubes were closed with perforated caps allowing HFIP to evaporate. Tubes were then stirred at 500 RPM, using a Teflon-coated micro stir bar for 36 h in the fume hood at room temperature.

### Preparation of recombinant tau oligomers

Recombinant full-length human tau protein (tau 441, 2N4R, 45.9 kDa) was expressed in an *E. coli* system and

subsequently purified from this system as previously described.<sup>33</sup> This recombinant tau was then oligomerized following our published protocol.<sup>34</sup> In brief, tau protein was treated with 8M urea to obtain monomers. Aliquots were stored at  $-20^{\circ}\text{C}$ . Monomeric tau was then dialyzed against 1X PBS and the final volume was adjusted by adding 1X PBS to a final tau concentration of 0.3 mg/mL. To this, A $\beta$ <sub>42</sub> oligomers were added as a seed at a dilution of 1:140 (w/w) and the sample was incubated at room temperature for 1 h on an orbital shaker. This oligomer preparation was purified by fast protein liquid chromatography (FPLC) and used to seed another fresh batch of monomeric tau. This process was continued for another batch (third time) of oligomer preparations to exclude any remaining A $\beta$ <sub>42</sub> oligomer seed. After three rounds of tau oligomer preparation, the final sample did not have any A $\beta$ <sub>42</sub> oligomer (seed) detected by ELISA since A $\beta$ <sub>42</sub>/tau ratio was estimated to be <1:2470000. This final tau oligomer preparation was used to generate standard curves as well as a positive control for all the ELISAs performed with CSF samples.

### Antibodies

Antibodies against oligomeric tau as well as the total used for Western blot and ELISA are summarized in Table 3.

### Measuring tau oligomers from CSF samples by ELISA

To measure tau oligomers in CSF samples, 12  $\mu$ L of CSF sample per well was added with 0.05 mol/L sodium bicarbonate (pH 9.6) as a coating buffer in 96-well ELISA plates (NUNC, Polysorp). Each sample was plated in duplicates. Plates were incubated overnight at  $4^{\circ}\text{C}$ . Plates were then washed 1 time with TBST buffer (containing 0.01% Tween 20), followed by blocking for 2 h at room

**Table 3.** Tau antibodies used in this study.

Antibody	Epitope	Application	Species	Source
T22	Tau oligomer	Western blot, ELISA	Rabbit	Generated in-house <sup>28</sup> ; also we made it available from Millipore (#ABN454)
TOMA	Tau oligomer	Western blot	Mouse	Generated in-house <sup>43</sup> ; also we made it available from Millipore (#MABN819)
Tau 5	210-230 aa	Western blot, ELISA	Mouse	Biologend
Tau 13	20-35 aa	Western blot	Mouse	Covance
Tau 12	9-18 aa	Western blot	Mouse	Biologend
Tau-46	404-441 aa	Western blot	Mouse	Covance
TTC-35	Aggregated tau	Western blot	Rabbit	In-house

temperature with 10% nonfat dry milk prepared in TBST buffer. After washing one time with TBST, 100  $\mu$ L of T22 (1:250) diluted in 5% nonfat milk prepared in TBST was added and allowed to react for 1 h at room temperature. Plates were then washed three times with TBST buffer. T22 immunoreactivity was detected, using 100  $\mu$ L of HRP-conjugated anti-rabbit IgG (GE Healthcare, Pittsburgh, Pennsylvania, United States). The secondary antibody (diluted 1:3000 in 5% nonfat milk in TBST) was added, followed by incubation for 1 h at room temperature. Finally, plates were washed three times with TBST and incubated with 100  $\mu$ L of 3,3',5,5'-tetramethylbenzidine (TMB-1 component substrate, from Dako) for 1 h in the dark. The reaction was stopped by adding 100  $\mu$ L 2mol/L HCl and the plates were read at 450 nm in a Polar Star Omega plate reader (BMG Labtech, Cary, North Carolina, United States). We used calibration curves generated with recombinant tau oligomer. The concentration of recombinant tau was measured by Bicinchoninic Acid Assay (BCA method, Pierce) and then used to generate the calibration curves against T22 and Tau 5 antibodies. The concentrations of total tau and tau oligomers for the samples were obtained from their corresponding calibration curves by performing linear regression analyses, using GraphPad prism 6. Graphs were plotted as median and interquartile ranges.

### Western blot analysis of CSF samples

CSF samples from 3 AD patients and 2 controls included in the pilot study were quantified by the BCA method. These CSF samples were loaded and run in 4–12% bis-tris

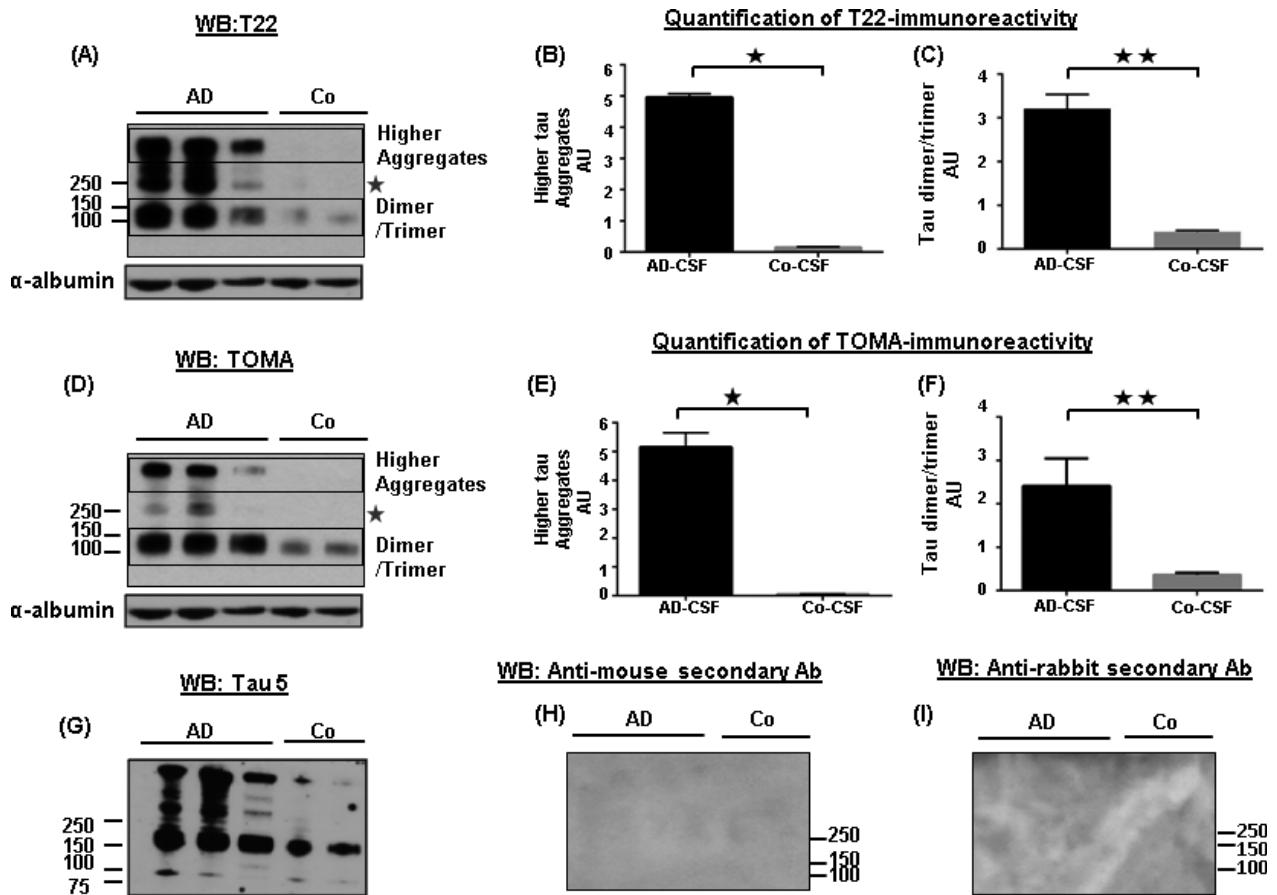
SDS-PAGE gels in nonreducing condition so that each sample contained 35  $\mu$ g of CSF protein. Proteins were transferred onto nitrocellulose membranes. Membranes were first blocked overnight at 4°C in 10% nonfat dry milk prepared in 1X TBS-T (containing 0.01% Tween 20) buffer and were then incubated with primary antibodies T22 (1:250), TOMA (1:100), TTC-35 (1:250), Tau 5 (1:500), Tau 13 (1:1000), Tau 12 (1:1000) and Tau 46 (1:500) diluted in 5% nonfat dry milk solution for 1 h at room temperature. After washing three times (5 min each), membranes were probed with their corresponding secondary antibodies diluted in 5% nonfat dry milk for 1 h at room temperature. Membranes were washed three times (first washing for 15 min and next two washes for 5 min). Immunoreactivity with T22 and TTC-35 was detected with HRP-conjugated anti-rabbit IgG (GE Healthcare) and TOMA, Tau5, Tau 13, Tau 12, and Tau 46 immunoreactivities were detected, using HRP-conjugated anti-mouse IgG (GE Healthcare). Human  $\alpha$ -albumin antibody (1:1000) was used as loading control for the CSF samples and its signal was detected, using an HRP-conjugated anti-human IgG (GE Healthcare). Finally, ECL plus solution (GE Healthcare) was used for signal detection. Bands from immunoblots were normalized by their corresponding  $\alpha$ -albumin signals, and densitometric quantifications were performed, using ImageJ software, National Institute of Health.

### Spike recovery of recombinant tau oligomers

A mixture of pooled CSF samples from 9 control subjects was spiked with two different concentrations of recombinant tau oligomers, 600 pg/mL and 1200 pg/mL (prepared as described earlier). The spiked CSF sample and another aliquot of the same CSF sample mixture without any spike were then analyzed by ELISA, using T22 antibody. Percent recovery of the spike sample was measured as compared with nonspiked sample.

### Statistical analysis

All raw data obtained from ELISA assays and Western blot quantifications were first analyzed by Excel. Statistical analyses of these data were performed, using GraphPad prism 6 software. Bar graphs from densitometric quantification for Western blot bands were plotted as median and interquartile ranges and the percentage of oligomeric tau out of total tau were expressed as mean  $\pm$  standard deviation. The nonparametric Mann–Whitney test was used as a test of significance for the ELISA assays with CSF samples and results were represented as median with interquartile range. To compare the ratio of oligomeric



**Figure 1.** Detection and biochemical characterization of tau oligomers in AD-CSF. (A, D) Representative Western blots of CSF samples from 3 AD patients and 2 controls with anti-tau oligomer antibodies, T22 and TOMA. Immunoblotting with T22 and TOMA antibodies clearly showed tau aggregates in AD compared to controls. Tau aggregates of differing molecular weights were noted, ranging from 100 kDa and above. (B, E) Densitometric quantifications of higher molecular weight of tau from the Western blots probed with T22 and TOMA antibodies (indicated in boxes) showed elevated tau oligomers in the AD CSF samples ( $P = 0.01$ ). (C, F) Densitometric quantifications of dimer/trimer revealed significantly increased tau oligomers in AD ( $P = 0.005$  and  $P = 0.008$ , respectively) (indicated in boxes). Bands were normalized with  $\alpha$ -albumin, used as loading control. Bars represent median with interquartile ranges. A comparatively weaker band of tau oligomers was also noticed in the AD-CSF samples at 250 kDa molecular weight with both T22 and TOMA antibodies (indicated with star). (G) Probing with Tau5 antibody showed co-localization of signal from dimer/trimer as well as from highest band observed in the blots. (H, I) Anti-rabbit IgG and anti-mouse IgG secondary antibodies were used to show the specificity of the antibodies used. AU, arbitrary unit; CSF, cerebrospinal fluid.

tau to total tau in the CSF between moderate to severe AD, mild AD, and nondemented control groups, ANOVA with Dunnet's multiple comparisons test was used. A cut-off value of 0.05 was considered significant for statistical analyses.

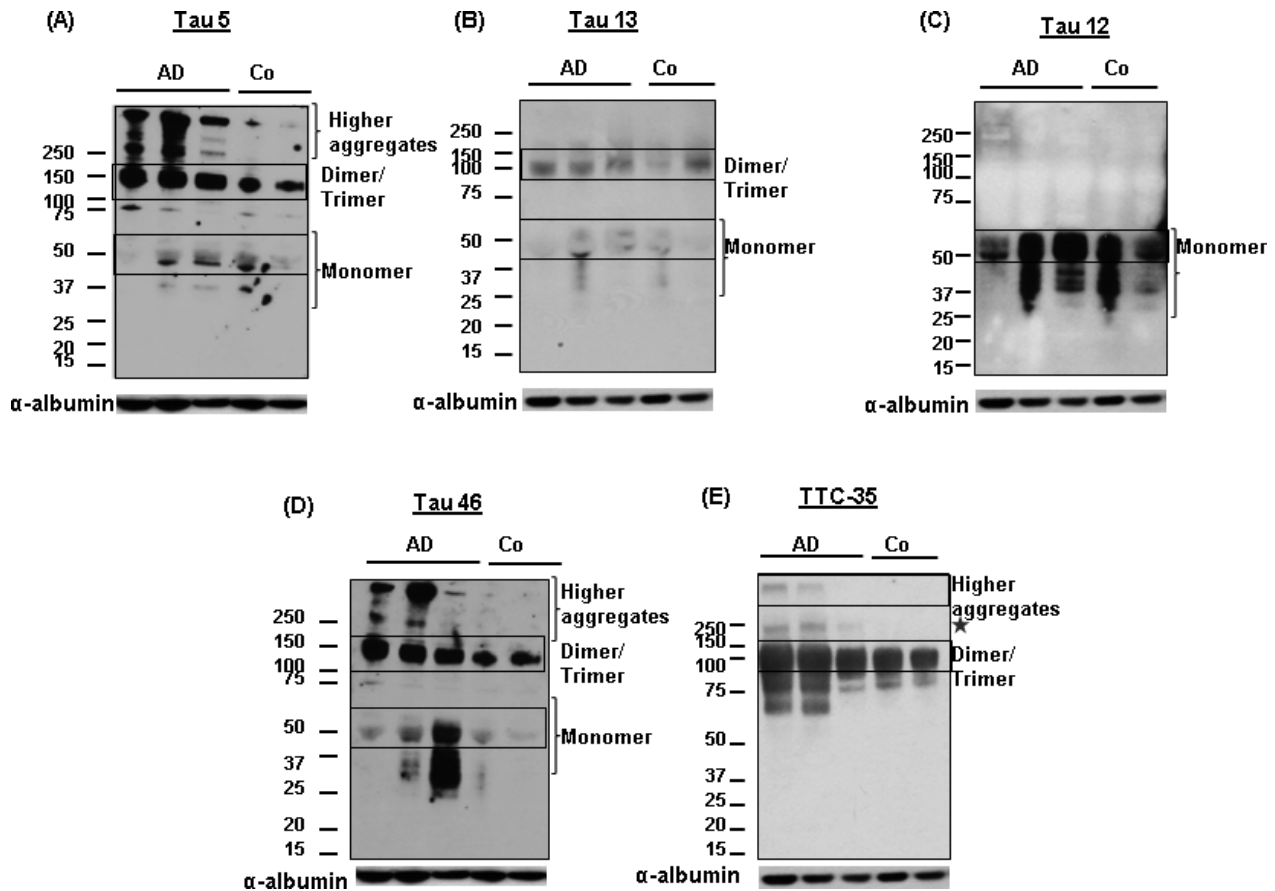
## Results

### Oligomeric tau aggregates in AD-CSF

Western blot analysis of 3 AD and 2 control CSF samples from the pilot study, using the anti-tau oligomer antibodies T22 and TOMA clearly showed tau oligomers in the AD samples, while the signals in the controls were not as

intense (Fig. 1A–F). Tau oligomers detected by these antibodies exhibited different orders of aggregation ranging from 100 kDa in molecular weight and above. The bands observed at 100–150 kDa molecular weight, immunoblotted with T22 and TOMA, appeared to correspond to dimers and/or trimers of tau and were elevated with both the antibodies ( $P < 0.001$ ). There was no tau detected below the dimer/trimer bands (Fig.S1A and B). Guzman-Martinez et al. had demonstrated tau oligomers in the platelets of AD patients showing bands between 100 and 190 kDa molecular weights, which is in accordance with our observation.<sup>35</sup> Tau aggregates at the dimer or trimer size range have also been observed in synaptosome-enriched fraction from AD brains.<sup>36</sup> Using T22 and

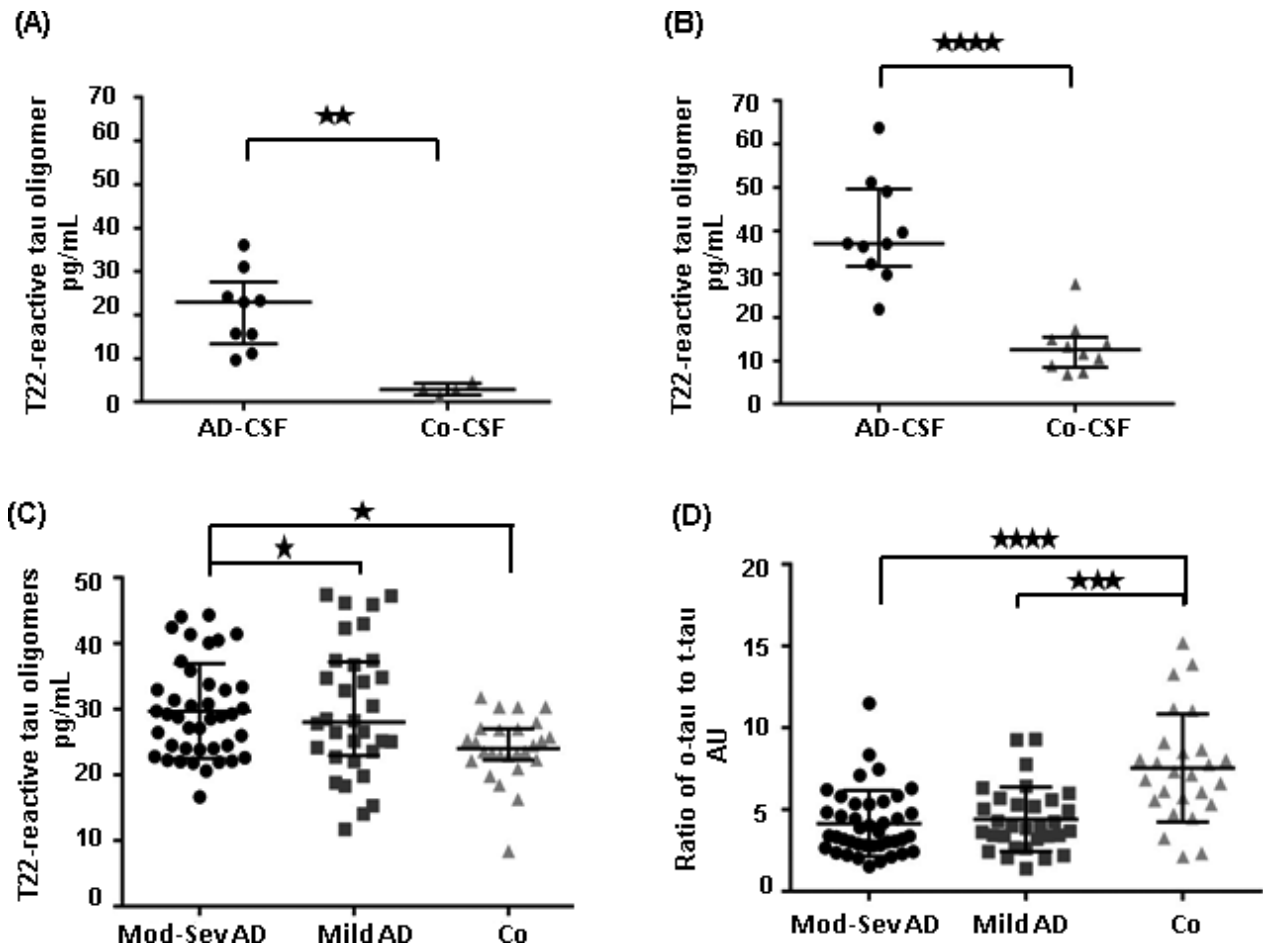




**Figure 2.** Confirming tau oligomers in CSF from multiple independent studies by biochemical analyses. (A–E) Representative Western blot showing differing orders of tau oligomers versus monomer in CSF, using Tau 5, tau 13, Tau 12, tau, 46 and a rabbit polyclonal antibody TTC-35. Immunolabeling with rabbit polyclonal TTC-35 demonstrated the presence of both higher tau aggregates as well as its dimer/trimer (indicated in boxes). Tau 13 antibody detected both tau dimer/trimer and monomer (indicated in boxes). Tau 46 demonstrated high-molecular weight tau as well as its monomer. Whereas, Tau 12 antibody explicitly demonstrated monomeric tau. A band was noted in AD-CSF samples at 250 kDa molecular weight (indicated with star) that was also noticed with T22 and TOMA antibodies. CSF, cerebrospinal fluid.

TOMA, a few other bands were noted above 250 kDa in AD-CSF samples, but were not detected in control samples. Densitometric quantification of higher molecular weight bands labeled with T22 and TOMA antibodies (as indicated in blue box) showed significant increase in the AD-CSF samples ( $P < 0.05$ ) (Fig 1B and E). Tau5 antibody confirmed the dimer/trimer bands as well as higher molecular weight bands (Fig 1G). It also detected monomeric tau (Fig 2A). Probing with only anti-rabbit and anti-mouse secondary antibodies did not show any signal in these CSF samples (Fig 1H and I). Additionally, we tested these CSF samples with two N-terminal tau antibodies, Tau 13, and Tau 12, C-terminal antibody Tau 46, and another in-house rabbit polyclonal antibody, TTC-35 that recognizes aggregated tau (Fig 2B–E). Tau 13 antibody detected monomeric tau between 60 kDa and

37 kDa molecular weight in both AD and control CSF samples that were not recognized by the anti-tau oligomeric antibodies. Tau 12 antibody also detected monomer in these samples. Additionally, Tau 46 antibody detected both high-molecular weight tau and monomeric tau monomer. TTC-35 antibody reacted with the higher aggregates of tau which was observed only in the two AD-CSF samples and also with tau dimer/trimer. A much weaker signal is observed at 250 kDa molecular weight in the two AD samples, further verifying the tau oligomers in these CSF samples. We performed two independent pilot studies with ELISA, using T22 antibody to confirm tau oligomers in the CSF samples observed from Western blot. ELISA results were used to quantify tau oligomers interpolated from the calibration curve which was generated against recombinant tau oligomers (Fig 3A and B).



**Figure 3.** The ratio of tau oligomers to total tau is decreased in AD. (A, B) CSF samples from two independent pilot studies were analyzed by ELISA, using T22 antibody. T22-reactive tau oligomers from these ELISA assays were then quantified using a standard curve generated against recombinant tau oligomers. AD-CSF samples clearly showed significant level of tau oligomers as compared to controls ( $P = 0.002$  and  $P < 0.0001$ , Mann-Whitney test). Data points were represented as median with interquartile ranges. (C) A double blinded analysis of large cohort of CSF samples from moderate-severe AD ( $N = 41$ ), mild AD ( $N = 31$ ) and nondemented controls ( $N = 26$ ) was performed using T22 antibody in ELISA. Tau oligomers were quantified from this analysis using a standard curve generated against recombinant tau oligomers. Tau oligomers were significantly increased in groups of moderate to severe AD and mild AD patients when compared with controls ( $P < 0.05$ , Mann-Whitney test). (D) The ratio of oligomeric tau to total was plotted for all the 3 groups of CSF samples. The oligomeric tau to total tau ratio was significantly increased in control groups in comparison with mild AD and then moderate to severe AD groups ( $P < 0.0001$  between both the groups). Data were represented as median with interquartile ranges. One-way ANOVA with Dunnett's multiple comparisons test was used for statistical analyses. CSF, cerebrospinal fluid. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$ .

Both the studies showed significant presence of tau oligomers in AD-CSF samples in comparison with control CSF ( $P = 0.002$  and  $P < 0.0001$ , respectively).

We quantified the dimer/trimer signal at 100–150 kDa molecular weight detected by T22, TOMA and Tau 5 antibodies in the Western blot. Intensity values from T22 to Tau 5 signals were compared and percentage of oligomeric tau (mostly at dimer/trimer) was measured. Similar percentage quantification was also performed for oligomeric tau (dimer/trimer) detected by TOMA and Tau 5 antibodies (Supplementary Fig 1D and E). Approximately, 35–40% of the total tau detected by Tau 5 antibody was

detected as tau oligomers in AD ( $P < 0.05$  for T22,  $P < 0.005$  for TOMA). To rule out any matrix interference in the CSF sample assay, we spiked the CSF samples with various concentrations of recombinant tau oligomers and the spiked CSF samples were assayed by ELISA for oligomers with around 100% recovery (Fig. S1C).

Finally, we performed a double-blinded clinical study, including CSF samples from 41 moderate to severe AD, 31 mild AD, and 26 nondemented controls. We measured tau oligomer levels in these samples, using T22 (Fig 3A). The total tau concentration was determined, using sandwich ELISA (Innotest hTAU-Ag, Innogenetics, Gent,

Belgium). Increased levels of tau oligomers were observed in AD samples as compared to the controls ( $P = 0.01$ ). The ratios of oligomeric tau to total tau were plotted separately for each of the three groups of samples, and we found that the control group had the highest ratio followed by mild AD, whereas the moderate to severe AD group exhibited the lowest value (Fig 3B). The ratio was significantly lower in both the moderate to severe AD and mild AD groups than in the control group ( $P < 0.0001$ ).

## Discussion

AD is a devastating disorder that heavily impacts society, both emotionally and economically. Given the aging population, and the fact that AD is the most common form of dementia in the elderly, it is expected that the prevalence of this grave condition will increase. AD can be definitively diagnosed only after death by combining clinical assessment and the histopathological analysis of the autopsied brain. Earlier, AD was only diagnosed once the clinical symptoms have become sufficiently apparent. This is problematic, because the destructive process of degeneration in the brain begins almost a decade prior to the appearance of the first symptoms.<sup>37</sup> This argues almost unequivocally for the significance of identifying tools, techniques or methods to diagnose AD in the preclinical stage. Although the exact mechanism of neurodegeneration is still not known in detail, the role of tau undergoing posttranslational modification, conformational changes and aggregation has received much attention in recent years. The levels of tau in CSF are being used as a complementary biomarker, and the quantification of both t-tau and p-tau (threonine 181) have been validated by frequent testing of human CSF samples in several clinical studies. Increased concentration of tau was detected almost 15 years before the onset of symptoms was expected in the CSF of AD patients.<sup>38</sup> A growing number of studies point to the roles of oligomeric assemblies of pathogenic proteins in toxicity and propagation of AD. Here, we present our findings from multiple independent studies showing that the levels of CSF tau oligomers are present and are increased in the CSF of AD patients. Tau aggregations at different molecular weights were noticed in these patients. Our observation suggests that tau oligomer levels in human CSF have the potential to serve as a valuable biomarker for AD. We also found increased levels of tau oligomers in AD samples compared to samples from mild AD and controls, and in fact the levels descended in that order. Measurements of both the levels of tau oligomers and the ratio of oligomeric tau to total tau, once reproduced across multiple laboratories using different cohorts, have the potential to serve as an indicator for disease progression.

Although CSF  $A\beta$  and tau, with their limitation to standardization and sensitivity, reveal the disease pathology to some extent, only a handful of fluid biomarkers have been identified for AD after extensive research.<sup>39</sup> We have previously demonstrated that tau oligomers also act as the pathological species in the disease progression of progressive supranuclear palsy (PSP) patients.<sup>40</sup> Moreover, tau oligomers have been identified in cortical and striatal brain regions in patients with other amyloid diseases, such as Huntington's disease.<sup>41</sup> These observations further support testing the levels of tau oligomers in the CSF of other tauopathies as well as other neurodegenerative diseases. Although, our results suggest that CSF tau oligomers could be used as a potential biomarker in combination with the core biomarkers, additional studies, especially longitudinal ones, are required. Such studies are critical not only to diagnose the disease pathology more accurately, but also to prepare for evaluating the upcoming and exciting anti tau therapeutic approaches which have been initiated recently in clinical trials.<sup>42</sup> Identifying novel CSF biomarkers for AD may supplement the traditional neuropsychological and imaging diagnostic techniques used for early diagnosis, and may become a useful surrogate for evaluating the pharmacological action of therapies targeting AD and other tau-associated dementias.

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## Author Contributions

R.K., U.S., E.P., and K.B. contributed to the concept and design of the study. K.B., H.Z., E.P., R.W., and D.G. provided the materials for this study. U.S., E.P., K.B., and H.Z. participated in data acquisition. R.K., U.S., and E.P. analyzed and interpreted the data. U.S. and E.P. prepared the figures. R.K. and U.S. drafted the manuscript. All authors contributed in revising the manuscript.

## Conflict of Interest

Rakez Kayed is the inventor of patent and patent applications on the compositions and methods related to tau oligomers and antibodies.



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## Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1.** Biochemical quantification of tau oligomers in AD-CSF samples.