**Cerebrospinal fluid tau fragment correlates with tau PET: a candidate biomarker for tangle pathology**

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## Abstract

To date, there is no validated fluid biomarker for tau pathology in Alzheimer's disease (AD), with contradictory results from studies evaluating the correlation between phosphorylated tau in cerebrospinal fluid (CSF) with tau PET imaging. Tau protein is subjected to proteolytic processing into fragments before being secreted to the CSF. A recent study suggested that tau cleavage after amino acid 368 by asparagine endo peptides (AEP) is upregulated in AD. We used immunoprecipitation followed by mass spectrometric analyses to evaluate the presence of tau368 species in CSF. A novel Simoa assay for quantification of tau368 in cerebrospinal fluid (CSF) was developed, while total tau (t-tau) was measured by ELISA and the presence of tau368 in tangles was evaluated using immunohistochemistry. The diagnostic utility of tau368 was first evaluated in a pilot study (AD=20, control=20), then in a second cohort where the IWG-2 biomarker criteria was applied (AD=37, control= 45) and finally in a third cohort where the correlation with [$^{18}$F]GTP1 tau PET was evaluated (AD=38, control=11). The tau368/t-tau ratio was significantly decreased in AD ($p < 0.001$) in all cohorts. Immunohistochemical staining demonstrated that tau fragments ending at 368 are present in tangles. There was a strong negative correlation between the CSF tau368/t-tau ratio and [$^{18}$F]GTP1 retention. Our data suggests that tau368 is a tangle-enriched fragment and that the CSF ratio tau368/t-tau reflects tangle pathology. This novel tau biomarker may be used to improve diagnosis of AD and to facilitate the development of drug candidates targeting tau pathology. Furthermore, future longitudinal studies will increase our understanding of tau pathophysiology in AD and other tauopathies.

**Key words**

CSF biomarkers, tau, fragments, Alzheimer's disease, tangles, pathology

## Introduction

Alzheimer's disease (AD) is the most common form of dementia affecting more than 24 million people worldwide (Ferri et al., 2005). The key pathological findings in brain are neurofibrillary tangles composed of hyperphosphorylated tau protein (p-tau) and amyloid plaques composed of aggregated β-amyloid (Aβ), leading to synaptic and neuronal and brain atrophy (Blennow et al., 2006). Although most evidence indicates that the disease is triggered by Aβ deposition and amyloid plaque pathology, tangle pathology is better linked to cognitive decline and neuronal loss (Delacourte et al., 1999), and there is an urge to better understand how tangle pathology is initiated, mechanisms for spreading and progression of tau pathology, and differences in tau pathophysiology between the neurodegenerative tauopathies. Furthermore, a biochemical marker of tangle pathology would be an important tool to improve the diagnoses of AD and other tauopathies, for future enrichment in treatment trials targeting tau pathology as well as to monitor treatment effects in those trials.

There are three established core cerebrospinal fluid (CSF) biomarkers to support the clinical diagnosis of AD; Aβ42, total tau (t-tau) and p-tau (Blennow et al., 2006). Reduced CSF levels of Aβ42 and increased levels of p-tau are believed to reflect amyloid plaques and the hyperphosphorylation of tau, preceding tangle pathology, respectively, while the increase of total tau reflects increased release of tau to the CSF due to neurodegeneration. In support for t-tau as a general marker for neurodegeneration is the increase of CSF t-tau observed in various acute conditions such as ischemic stroke(Hesse et al., 2000) and acute traumatic brain injury (TBI) (Kay et al., 2003) with the highest levels found in neurodegenerative disorders with rapid neuronal injury and loss, specifically Creutzfeld Jacobs Disease (CJD) (Skillback et al., 2014). However, more recent studies suggest that tau can be actively secreted from neurons and to be present in the extracellular fluid (Kim et al., 2010; Yamada, 2017), suggesting that extracellular tau may play a role in the propagation of tau pathology.

Lately, not only phosphorylation but also fragmentation of tau has been suggested to play a role in tau pathogenesis where fragmentation has been shown to promote secretion of tau (Kim et al., 2010) and recent studies suggest that disease specific strains of tau are formed (Taniguchi-Watanabe et al., 2016), proposing that the underlying molecular mechanism differ between various tauopathies. There are several publications, which indicate that fragments of tau are present in CSF (Amadoro et al., 2014; Borroni et al., 2009; Cicognola et al., 2019; Ishiguro et al., 1999; Meredith et al., 2013), brain (Amadoro et al., 2014; Arai et al., 2004; Chen et al., 2018; Zhao et al., 2016) and vesicles (Sokolow et al., 2015). The "gold standard" ELISA method used for measurement of t-tau in CSF is based on three monoclonal antibodies binding to the mid region of the protein (Blennow et al., 1995), meaning that the sum of full length tau and all fragments containing the mid region is monitored. Therefore, this ELISA is not able to distinguish between full-length tau or mid-domain fragments, and either N-terminal or C-terminal tau fragments. In light of this, we hypothesize that identifying endogenous fragments of tau and developing assays that specifically measure these fragments may shed light not only on underlying disease mechanisms, but may also have a potential to improve the diagnosis of AD and other tauopathies.

A recent publication described a novel proteolytic pathway of tau (Zhang et al., 2014), and identified that asparagine endopeptidase (AEP) can cleave tau, thereby generating tau fragments or peptides ending at amino acid 368 (based on tau 441 numbering). In addition, an increase in tau368 in brain tissue was found in patients with AD (Zhang et al., 2014).

To this end, the aim of this study was to explore whether tau368 is present in CSF and evaluate if CSF levels of tau368 differ between patients with AD and controls and how CSF levels may relate to tau pathology as measured by tau PET ligand, [$^{18}$F]GTP1 (Sanabria-Bohórquez S, 2019). We also wanted to explore the protein levels and enzymatic activity of AEP in CSF as well as evaluate the relation to CSF t-tau. We therefore developed an assay on

the Simoa platform with analytical sensitivity enough to quantify tau368 in individual CSF samples. By applying the method on three separate clinical cohorts, we demonstrate that the ratio of tau368 and t-tau is significantly decreased in patients with AD and with a strong negative correlation with [$^{18}$F]GTP1 SUVR.

## Material and methods

### Tau368 Simoa assay development and validation

Magnetic beads (Quanterix, Lexington, MA, USA) were conjugated with capture antibody anti-Tau368 (Zhang et al., 2014) at 0.3 mg/mL. Prior to each run, Tau 1-368 recombinant protein calibrator (Zhang et al., 2014) was serially diluted and biotin-labeled detection antibody KJ9A (rabbit polyclonal antibody against tau 243-441, Sigma) was diluted to 3 µg/mL in 50mM Tris (pH 9.0), 0.1 % Tween20, 2 % BSA, 5mM EDTA. For each determination, 150.000 assay beads and 350.000 helper beads were washed and resuspended in 100 µL CSF sample, quality control sample or calibrator. After a washing step 20 µL of detection antibody was added, followed by a 30 min incubation. After a final wash beads were resuspended in 100 µL streptavidin-conjugated β-galactosidase (Quanterix, Lexington, MA, USA) at 150 pM diluted in SBG Diluent (Quanterix, Lexington, MA, USA). The signal, expressed as average enzyme per bead (AEB), was used to extract concentrations and each sample AEB was fitted to a four-parameter logistic curve plotted from the known concentrations of the tau368 calibrator run in parallel with the samples. Calibrator points were run in triplicates while samples were run in duplicates. The Lower Limit of Quantification (LLOQ) and Upper LOQ (ULOQ) were determined by calculating the deviation from the true value of each calibration point. The CV for the back-calculated concentrations of the data from the calibrator curve has to be <25% at LLOQ and ULOQ, while <20% in between. The

Limit of Detection (LOD) was determined by analyzing 16 duplicates of the blank and by adding 3 standard deviations to the mean blank signal (16 replicates/one plate) the concentration is calculated using the calibration curve. Precision is defined by the calculated standard deviation ($SD_r$) and the variation coefficient ($CV_r$) using One-way ANOVA in accordance with ISO 5257-2.

## Pilot study

The control group (n = 20) consisted of patients with minor neurological or psychiatric symptoms, and with basic (cell count, albumin ratio, IgG index) and core (t-tau, p-tau and Aβ42) CSF biomarkers levels within normal ranges. The AD group consisted patients admitted for clinical evaluation of AD (n = 20), and with normal basic CSF biomarkers, but with the core AD CSF biomarkers showing a typical AD profile. The cut-offs used to for a biomarker positive profile was CSF t-tau > 400 ng/L, Aβ42 < 550 ng/L and p-tau > 60 ng/L (Table 1). The CSF sample aliquots used were de-identified left-over aliquots from clinical routine analyses, following a procedure approved by the Ethics Committee at University of Gothenburg (EPN 140811).

## Clinical study

The AD group (n = 37) consisted of patients fulfilling the criteria for probable AD outlined by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (McKhann et al., 1984) as well as the IWG-2 biomarker criteria (Dubois et al., 2014). The control group consisted of cognitively healthy volunteers (n = 45) without history, symptoms of signs of significant neurological or psychiatric disorders. The applied IWG-2 criteria for allocation to the AD group was low CSF concentration of Aβ1-42 (< 550ng/L) together with high CSF level of t-tau (> 400 ng/L) or p-

tau181 (> 80 ng/L). The study was approved by the Ethical Committee at Lund University (Dnr 2013-494 and 2014-467). Demographic data from both cohorts are presented in Table 2.

## [$^{18}$F]GTP1 PET study

*Participants.* Baseline CSF indices and [$^{18}$F]GTP1 PET scans were obtained from a subset of research participants enrolled in two separate studies, as previously described in Sanabria et al. 2018 (submitted manuscript) (Table 3). Briefly, GN30009 (NCT02640092) is a longitudinal study designed to evaluate the natural history of tau pathology in individuals with prodromal AD, in individuals with mild or moderate AD dementia, and in healthy volunteers (HV); and e0048 was a study designed to evaluate the test/retest reliability of [$^{18}$F]GTP1 binding parameters in individuals with AD dementia and HV. For GN30009, all participants in the AD subgroups were required to have a screening [$^{18}$F]florbetapir PET scan that was deemed Aβ positive by visual read by two central raters and a screening brain magnetic resonance imaging (MRI) scan that was consistent with a diagnosis of AD and did not show significant evidence of non-AD neurological disease that might contribute to cognitive impairment.

Imaging: The imaging assessments for both studies were performed at the same imaging center. PET images were acquired on a Siemens HR+ or Siemens Biograph 6 PET-CT scanners. Images were corrected for attenuation, random coincidences, scatter, and decay. Images were reconstructed using harmonization parameters derived from a Hoffman 3-D brain phantom studies using F-18 in each scanner to insure the same image resolution in all images.

*[$^{18}$F]GTP1 PET imaging*. [$^{18}$F]GTP1 is a novel tau tracer that selectively binds to pathological tau in AD and demonstrates high selectivity for tau pathologies over Aβ pathologies (Sanabria- Bohórquez S, 2019) [$^{18}$F]GTP1 scans were performed as previously

described. In brief, [$^{18}$F]GTP1 images were acquired over a 30-minute window starting 60 minutes post-injection after a mean (SD) bolus injection of 343 (31) MBq. [$^{18}$F]GTP1 standardized uptake value ratios (SUVRs) were calculated using the cerebellar gray as reference. Data is reported for an AD temporal meta-ROI (Jack et al., 2017).

*MR imaging.* MRI was performed to determine participant eligibility, for co-registration with [$^{18}$F]GTP1 and [$^{18}$F]florbetapir PET images, and for volumetric analyses. The 3D sagittal T1-weighted MPRAGE sequences were used for volumetric analyses. Images were collected with 1 mm$^2$ in plane resolution, 1.0-1.2 mm slice thickness, 256 mm x 256 mm matrix, and a 240 mm FOV.

## CSF collection and biomarker analyses

All CSF samples were collected by lumbar puncture in the L3/L4 or the L4/L5 interspace in the morning. Up to 20 mL of CSF was collected in a polypropylene tube and immediately transported to the local laboratory and centrifuged (10 min at 1800 g at 20° C). The supernatant was gently mixed to avoid possible gradient effects, aliquoted in polypropylene tubes and stored at –70° C.

In the clinical study, CSF t-tau and tau phosphorylated at threonine 181 (p-tau) were determined using a sandwich enzyme linked immunosorbent assay (ELISA) (INNOTEST® htau Ag and PHOSPHO_TAU (181P); Innogenetics, as previously described (Blennow et al., 1995; Vanmechelen et al., 2000). CSF Aβ42 was measured using a sandwich ELISA (INNOTEST® β-amyloid$_{1-42}$), specifically constructed to measure Aβ starting at amino acid 1 and ending at amino acid 42 (Andreasen et al., 1999). In the GTP 1 study, t-tau was measured using INNOTEST assay while tau phosphorylated at 181 and Aβ1-42 were analysed using Elecsys (Hansson et al., 2018). The protein levels of AEP in CSF were analyzed using a

sandwich ELISA (Human Total Legumain Duoset ELISA Kit, R&D®) according to the manufacturer's instruction.

## Immunohistochemistry

Immunohistochemical analysis of the tau antibodies was carried out on brains donated to the Queen Square Brain Bank for Neurological Disorders, UCL Institute of Neurology, University College London. Seven micron thick formalin fixed paraffin embedded tissue sections were cut from the frontal cortex of pathologically diagnosed Alzheimer's disease cases and neurologically normal controls. Tau immunohistochemistry required pressure cooker pre-treatment in citrate buffer pH 6.0. Endogenous peroxidase activity was blocked with 0.3% H202 in methanol and non-specific binding with 10% dried milk solution. Tissue sections were incubated with the primary anti-tau 368 and AT8 antibodies for 1 hour at room temperature, followed by biotinylated anti-mouse IgG (1:200, 30 minutes; DAKO) and ABC complex (30 minutes; DAKO). Colour was developed with di-aminobenzidine/$H_20_2$(Lashley et al., 2011). Sections were viewed and imaged on a Nikon Eclipse.

## Statistics

GraphPad Prism 7.02 and R were used for the statistical analyses and a p-value lower than 0.05 was considered significant. P-values were not adjusted for multiple comparisons. The Kruskal-Wallis test was used to test significant differences between groups followed by Mann-Whitney test for the comparison of two groups. Spearman correlation was used to test for correlation. Multiple regression was used to test for the association between CSF tau measures and disease stage adjusting for age and gender. A log transformation was applied to the various CSF measures for all multiple regression analyses.

## Results

### Immunoprecipitation-mass spectrometry (IP-MS)

IP of tau from CSF using the polyclonal antibody KJ9A followed by MS analyses confirmed the presence of endogenous species ending at amino acid 368 (Fig. 1). In the Fig. the fragment ion spectrum of the endogenous tau peptide 324-368 is also shown. The IP-MS method is described in Supplementary Material and methods

### Simoa tau368 assay performance

The calibration range for the assay was set to 4.88-625 pg/mL. The LOD was set to 3.6 pg/mL. Calibration curve data from five assay runs were used to determine the ULOQ and LLOQ. LLOQ was set to 4.88 pg/mL and ULOQ to 625 pg/mL. The precision ($CV_r$) was < 7.9% and the between-run $CV_{Rw}$ was below 12.3%.

### Tau368 in CSF in the clinical cohorts

In the pilot study, there was a significant increase of tau368 CSF concentrations in patients with AD compared to controls ($p< 0.001$) (Fig. 2a). There was a significant correlation between CSF levels of t-tau and tau368 within both the AD group ($p= 0.013$, rho= 0.53) and the control group ($p=0.014$, rho=0.55) (Fig. 2c and 2d). The ratio of tau368 to t-tau was significantly decreased in patients with AD, compared to controls ($p < 0.001$) (Fig. 2b).

In the clinical validation study CSF concentrations of tau368 were significantly increased in patients with AD compared to controls ($p=0.005$) (Fig. 3a). When comparing the ratio of

tau368 to t-tau the ratio was decreased in patients with AD, compared to controls ($p < 0.001$, Fig. 3b). There was a correlation between total tau and tau368 within the AD group ($p = 0.002$, rho= 0.57) and control group ($p < 0.001$, rho= 0.90) (Fig. 3c and 3d). There was a correlation between p-tau181 and tau368 in the control group ($p < 0.001$, rho= 0.95) and within the AD group ($p=0.001$ rho= 0.57) (data not shown). There was no correlation between tau368 and Aβ42 within the AD group ($p=0.23$) but a significant correlation within the control group ($p < 0.001$, rho= 0.58) (data not shown).

There was a significant association between age and CSF levels of tau368, but adjusting for age did not affect the significant group differences. There was no significant association between age or gender and CSF levels of t-tau, p-tau, the ratio between tau368/t-tau or Aβ42.

## AEP in CSF

The protein levels of AEP were significantly lower in patients with AD (Fig. 4a, $p = 0.036$) while there was no difference in enzymatic activity of AEP ($p = 0.63$, data not shown). There was no association between age or gender with levels or enzymatic activity of AEP. The enzymatic activity method is described in Supplementary Material and methods.

## Immunohistochemistry

Staining with anti tau368 in normal control (Figure 5a) present nuclear and cytoplasmic neuronal staining. In AD brain tissue (Figure 5b and 5c) staining with anti tau368 antibody present nuclear and cytoplasmic neuronal staining (b) along with staining of the filamentous structures observed in neurofibrillary tangles (c).

## [$^{18}$F]GTP1 imaging

In the [$^{18}$F]GTP1 cohort, 6 amyloid PET-negative cognitively-normal controls, 5 amyloid PET-positive cognitively-normal controls, and 38 amyloid PET-positive prodromal, mild or moderate AD patients with paired [$^{18}$F]GTP1 scans and CSF were analyzed. [$^{18}$F]GTP1 SUVR increased step-wise with disease severity ($p<0.001$), and was elevated in amyloid positive cognitively-normal controls. CSF levels of t-tau and p-tau were highly correlated (spearman r=0.96, AD only spearman r=0.99) and both correlated with [$^{18}$F]GTP1 SUVR within 2 decimal points of precision (both t-tau and p-tau spearman rho=0.61, $p<0.001$, adjusting for age: partial spearman r=0.56, $p<0.001$). The correlation of p-tau with [$^{18}$F]GTP1 SUVR was reduced after removal of the amyloid PET-negative and amyloid PET-positive cognitively-normal controls (spearman r=0.50, $p<0.001$). CSF levels of the tau368 fragment alone was not correlated with [$^{18}$F]GTP1 SUVR (spearman rho=0.06, p=0.689) (Wildsmith, , 2017). However, the ratio of tau368/t-tau in CSF decreased with increasing disease severity ($p<0.001$) and was numerically lower in amyloid positive controls vs. amyloid negative controls (p=0.131, Fig 6A). A numerically larger correlation was seen with the ratio of tau368/t-tau, which was negatively associated with [$^{18}$F]GTP1 SUVR in the temporal meta-ROI (spearman rho= -0.73 $p<0.001$, Fig 6B) including after adjustment for age (partial spearman r = -0.71, $p<0.001$)or after removing the amyloid PET-negative and amyloid PET-positive cognitively-normal controls from the analysis (spearman rho = -0.57, $p<0.001$).

# Discussion

In the present study we demonstrate that a fragment of tau ending at amino acid 368 (tau368) is present in CSF and can be quantified using a novel Simoa method. The assay was successfully validated and showed a robust performance with between assay $CV_{RW}$ of less than 12.3%. In three independent cohorts, we found that absolute levels of Tau368 in CSF are

significantly increased in AD compared to controls (Fig. 1a and 2a). We observed an association between age and CSF levels of tau368, but adjusting for age did not affect the significant group differences.

Normalizing the levels of tau368 in CSF to total tau suggest that only a small proportion of tau ends at 368, approximately between 3-8%. When comparing the ratio of tau368 to total tau in CSF, we found a decrease in patients with AD, suggesting that a lower proportion of tau368 compared to total tau are secreted into CSF in AD than in controls (Figure 1b, 2b and 6b). One hypothesis is that fragmentation patterns change in disease, and that tau cleaved by AEP contains the microtubule binding domain, giving it an increased propensity for aggregation and tangle formation, as compared with the mid- to N-terminal tau fragments measured by the t-tau CSF assay, with less tau368 being excreted into CSF in patients with AD. It was difficult to generate evidence to support the hypothesis that AEP activity changes in disease using in vitro assays.In the present study, levels of the CSF AEP protein were found to be significantly decreased in patients with AD (Fig. 4). However, we did not find any difference in terms of activity and there was no correlation between the levels and activity of AEP nor any effect from age on the levels or activity of AEP (data not shown). Biochemically, the AEP enzyme is highly regulated by its specificity for asparagine residues and pH (Li et al., 2003; Zhang et al., 2014). The pH in CSF is close to physiological, but as soon as CSF is tapped into tubes the pH increases close to pH 10 (Cunniffe et al., 1996). This may affect the activity of AEP in CSF *in vitro*. In the paper by Zhan et al AEP activity was shown to increase with age (Zhang et al., 2014).

That being said, other data supports a shift in fragmentation in disease, including a recent study noticing a shift in fragmentation patterns in late stage AD brains (Chen et al. 2018). It

seems that C-terminal fragments containing the MTBD may not be secreted as readily into CSF. In support for such a hypothesis are the C-terminal half of tau in CSF are low (Barthelemy et al., 2016) while levels of N-terminal tau are comparable or even higher than mid-region tau (Meredith et al., 2013). In agreement with the hypothesis that C-terminal tau fragments such as tau368 are retained in the brain and lower amounts secreted to the CSF, a study performing biochemical characterization of tau in the sarkosyl insoluble fraction of brain tissue homogenates, showed that C-terminal tau fragments were abundant (Arai et al., 2004).

Further data to support that fragmentation patterns change in disease and that c-terminal fragments like tau368 may remain intracellular enabling aggregation, is the difference in the strength of the correlations between total tau and tau368, comparing the controls with the AD group. In the clinical study (Figure 3) there is a strong correlation in the control group (r= 0.90) but only a moderate correlation in the AD group (r=0.57). If the tau368 fragment is related to pathology, as in a patient with AD having tangles, the amount of tau fragment released may be affected, which is not the case in controls. In controls, lacking tangle pathology, tau368 may be present in the CSF due to the normal secretion/leakage. However, in the pilot study (Figure 2) there was a moderate correlation between total tau and tau368 in both AD and control groups (0.53 and 0.55 respectively). Potential explanations for this discrepancy may be the lower number of patients included in the pilot study, as well as the less stringent biomarker cut offs.

We show by immunohistochemistry on brain tissue from AD cases that the anti-tau368 antibody stains tau fragments ending at amino acid 368 that are enriched in tangles. This lends further support for CSF tau368 being a biomarker of tangle pathology. In addition, strong support that AEP dependent truncation of tau facilitates the formation of tangles come from a

recent study demonstrating that an AEP inhibitor prevents the formation of tau368 which leads to reduced phosphorylation and aggregation of tau (Zhang et al., 2017).

Of potential clinical relevance, we also show a strong negative association of the CSF tau368/t-tau ratio and tau PET ([$^{18}$F]GTP1 SUVR), which is not impacted by age, a factor which is known to influence tau levels, further supporting that this ratio may reflect the underlying tau pathology. While significance was not reached due to the small sample size, the correlation of the tau368/t-tau with [$^{18}$F]GTP1 SUVR was higher than for t-tau or p-tau. Similarly, previous studies using the standard assays for CSF t-tau and p-tau have only found weak or intermediate correlation between CSF tau measures and other tau PET ligands (Chhatwal et al., 2016; Gordon et al., 2016). The present results are also in agreement with our previous study using a different tau PET tracer, [$^{18}$F]THK5317 (Leuzy et al., 2019), where we demonstrated a stronger correlation between [$^{18}$F]THK5317 SUVR and CSF tau368, compared to that with CSF t-tau. Further exploration of the performance of tau368/t-tau ratio will be needed in larger cohorts to understand whether or not it will provide additional utility to the standard CSF tau biomarkers. For example, the fragment ratio may enable more sensitive detection of changes in AD pathology and cognition, similar to [$^{18}$F]GTP1 SUVR (Sanabria- Bohórquez S, 2019; Teng et al., 2019), and even potentially be able to detect changes in pathology prior to symptom onset in cognitively normal individuals. In comparison with t-tau and p-tau whose levels increase in early AD and plateau, [$^{18}$F]GTP1 SUVR and the tau368/t-tau ratio increased or decreased, respectively, in cognitively-normal amyloid PET positive individuals, and levels continued to change with disease severity. Assessment of CSF from longitudinal studies will be needed to assess whether or not the tau368/ t-tau fragment ratio can detect changes over time like tau PET (Pontecorvo et al., 2019) and be potentially used to monitor disease progression. The reduction in the CSF

tau368/t-tau ratio in AD and the association with tau PET could be analogous to the reduction in CSF Aβ42, which reflects deposition of the peptide into plaques (Blennow et al., 2015), and may reflect an alteration in the fragmentation of tau that begins at the early stages of disease.

This is the first study in which the specific C-terminal tau368 fragment of tau has been quantified in CSF. It is not known how such tau fragments are produced or how they are secreted to the CSF. Partly, the CSF is produced through passive diffusion from the brain parenchyma, therefore it closely reflects brain pathological processes. Pre-clinical studies demonstrate that t-tau is present in the interstitial fluid (ISF) (Yamada et al., 2011). There are also pre-clinical studies suggesting that tau fragments are present in the ISF (Meinhardt, 2017). These observations support the findings of tau fragments in the present study.

To conclude, we have specifically quantified a tangle-enriched fragment of tau in CSF, ending at amino acid 368, and our data support that the ratio between tau368 and t-tau is a marker for the deposition of tau368 into tangles by the strong negative correlation to tau PET. It is possible that the turnover of tau is disease specific and there is evidence that fragmentation of tau leads to conformational changes, aggregation and possibly acting as a seed for propagation to other cells. In this case, an assay to specifically monitoring such a fragment would also increase our knowledge on disease mechanisms and it would complement Tau PET imaging as a biomarker of tangle pathology.

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## Declaration of interest

HZ has served at advisory boards for Roche Diagnostics, Eli Lilly and Wave, has received travel support from Teva and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. KB has served as a consultant or at advisory boards for Alzheon, BioArctic, Biogen, Eli Lilly, Fujirebio Europe, IBL International, Merck, Novartis, Pfizer, 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. OH has acquired research support (for the institution) from Roche, GE Healthcare, Biogen, AVID Radiopharmaceuticals, Fujirebio, and Euroimmun. In the past 2 years, he has received consultancy/speaker fees (paid to the institution) from Lilly, Roche, and Fujirebio. KH has served as a consultant for Eisai and Abbvie. KW, SS, PM, GK, and RW are employees of and receive a salary from Genentech, Inc. The other authors report no conflicts of interest.

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**Figure legends**

**Figure 1.** Figure 1 summarizes the endogenous peptides immunoprecipited by antibody KJ9A from cerebrospinal fluid along with an example of a fragment ion spectrum of the endogenous tau peptide (324)SLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGN(368). These data confirms the presence of endogenous tau species ending at amino acid 368.

**Figure 2.** Tau368 levels (A) and the ratio Tau368 to Total Tau (B) in CSF from patients with Alzheimer's disease (AD) and controls. There was a significant difference in both absolute levels ($p<0.0001$) and ratio ($p<0.0001$). Data are presented as median (interquartile ranges). Figure 2C show the correlation between tau368 and total tau within the AD group ($p=0.0128$, $r=0.53$) and (D) within the control group ($p=0.0143$, $r=0.55$).

**Figure 3**. Figure 3a display the significant difference of CSF Tau368 when comparing AD and controls ($p<0.0001$) and Figure 3b display the ratio Tau368 to Total Tau, which was significantly lower in patients with AD ($p<0.0001$). There was a positive correlation between Tau368 and Total-Tau within controls ($p<0.0001$, $r=0.90$) and AD ($p=0.0002$, $r=0.57$). Data are presented as median (interquartile ranges).

**Figure 4**. Figure 4 presents data on cerebrospinal fluid (CSF) levels of asparagine endopeptidase (AEP) protein levels. Levels of AEP were significantly reduced in Alzheimer's

disease (AD) compared to controls (p= 0.036). Data are presented as median (interquartile range).

**Figure 5.** Tau368 immunohistochemistry: Normal control (a) nuclear and cytoplasmic neuronal staining present. In Alzheimer's Disease (b and c) nuclear and cytoplasmic neuronal staining present (b) along with staining of the filamentous structures observed in neurofibrillary tangles (c). Scale bar in a represents 50µm in a and b; 20µm in c.

**Figure 6**. (A) The ratio of tau368/t-tau in CSF decreases with disease severity (B) [$^{18}$F]GTP1 SUVR and tau368/t-tau ratio in CSF are negatively correlated (spearman r= -0.73, p<0.001, AD only spearman r= -0.57, p<0.001) (triangles, amyloid PET negative; circles, amyloid-PET positive; black, cognitively-normal; red, prodromal AD; blue, mild AD; green, moderate AD).

**Table 1. Demographic data from brain tissue study**

| | Age at death (years) mean (range) | Gender (M/F) | Disease duration (years) (mean) | Braak stage (Tau) |
|---|---|---|---|---|
| AD (n = 9) | 72 (62-86) | 6/3 | 11 | 5 & 6 |
| PA (n =5) | 88 (85-95) | 2/3 | NA | 3 & 4 |
| FAD (n = 9) | 55 (37-70) | 4/5 | 9 | 5 & 6 |
| Control (n = 4) | 83 (71-95) | 2/2 | NA | 1,2 &3 |

AD= Alzheimer's disease, PA= pathological aging, FAD= familiar AD, NA= not applicable. The following mutations were found in FAD: PSEN1 A434T & T291A, E120K exon 5 PSEN1, PSEN1 mutation, PSEN1 I202F Pre 200 PS1/ R47 H, APP V717I, PS1 after 200, PSEN1 Intron 4 Pre 200 PS1/ R47 H, PSEN1 S132A and APP V717I.

**Table 2. Demographic data in all three clinical cohorts.**

| **Pilot Study** | | | | | |
|---|---|---|---|---|---|
| | Age (years) mean (range) | Gender (M/F) | p-tau (ng/L) mean (range) | t-tau (ng/L) mean (range) | Aβ42 (ng/L) mean (range) |
| AD (n=21) | 75 (59-85) | 9/12 | 92 (71-195) | 735 (520-1370) | 362 (220-460) |
| Control (n=20) | 63 (42-84) | 14/6 | 40 (26-53) | 217 (120-260) | 806 (670-1050) |
| **Clinical study** | | | | | |
| AD (n=37) | 73 (53-84) | 13/24 | 82 (50-136) | 728 (304-1396) | 372 (191-533) |

| | | | | | |
|---|---|---|---|---|---|
| Control (n=45) | 73 (60-83) | 15/30 | 46 (19-74) | 330 (88-564) | 913 (217-1698) |
| **GTP 1 study** | | | | | |
| Control (n=11) | 64 (51-71) | 6/5 | 20 (11-36) | 386 (165-660) | 908 (542-1363) |
| Prodromal AD (n=15) | 70 (56-83) | 8/7 | 32 (11-72) | 619 (204-1332) | 685 (392-1102) |
| Mild AD (n=13) | 70 (59-80) | 5/8 | 37 (16-86) | 699 (327-1558) | 720 (393-1032) |
| Moderate AD (n=10) | 72 (68-76) | 8/2 | 37 (20-72) | 719 (379-1392) | 522 (309-931) |

Biomarker data are presented in ng/L. M=men, F=female, AD=Alzheimer's disease, t-tau= total tau, p-tau tau phosphorylated at 181. INNOTEST ELISA assays were used to analyze Aβ1-42, t-tau and p-tau. With the exception of Aβ1-42 and p-tau in the GTP1 study, which was analyzed using Elecsys assays.