Use of the tau protein-to-peptide ratio in CSF to improve diagnostic classification of Alzheimer’s disease

Karl Hansson, Rahil Dahlén, Oskar Hansson, Elin Pernevik, Ross Paterson, Jonathan M. Schott, Nadia Magdalinou, Henrik Zetterberg, Kaj Blennow, Johan Gobom

Abstract

Cerebrospinal fluid (CSF) tau and phospho-tau are well established biomarkers of Alzheimer’s disease. While these measures are conventionally referred to as ‘total tau’ (T-tau) and ‘phospho-tau’ (P-tau), several truncated and modified tau forms exist that may relay additional diagnostic information. We evaluated the diagnostic performance of an endogenous tau peptide in CSF, tau 175–190, in the phosphorylated and non-phosphorylated state. A liquid chromatography-mass spectrometry (LC-MS) method was established to measure these peptides in CSF and was used to analyze two independent clinical cohorts; the first cohort included patients with Alzheimer’s disease (AD, n = 15), Parkinson’s disease (PD, n = 15), progressive supranuclear palsy (PSP, n = 15), and healthy controls (n = 15), the second cohort included AD patients (n = 16), and healthy controls (n = 24). In both cohorts T-tau and P-tau concentrations were determined by immunoassay. While tau 175–190 and P-tau 175–190 did not differentiate the study groups, the separation of AD and controls by T-tau (area under the ROC Curve (AUC) = 95%) and P-tau (AUC = 92%) was improved when normalizing the ELISA measurements to the concentrations of the endogenous peptides: T-tau/tau 175–190 (AUC = 100%), P-tau/P-tau 175–190 (AUC = 95%). The separation between patients and controls by T-tau (AUC = 88%) and P-tau (AUC = 82%) was similarly improved in the second cohort by taking the ratios of T-tau/tau 175–190 (AUC = 97%) and P-tau/P-tau 175–190
(AUC = 98%). In conclusion, our results suggest that the performance of the AD biomarkers T-tau and P-tau could be improved by normalizing their measurements to the endogenous peptides tau 175–190 and P-tau 175–190, possibly because these endogenous tau peptides serve to normalize for physiological, and disease-independent, secretion of tau from neurons to the extracellular space and the CSF. Finally, the observations made here add to the general applicability of mass spectrometry as a tool for rapid identification and accurate quantification of biomarker candidates.

Keywords

Alzheimer’s disease, microtubule-associated protein tau, endogenous peptides, biomarker, peptidomics
Abbreviations

- Amyloid beta (Aβ)
- Area under the ROC curve (AUC)
- ELISA (enzyme-linked immunosorbent assay)
- Guanidinium hydrochloride (GdnHCl)
- LC-MS (liquid-chromatography coupled to mass spectrometry)
- Phospho-tau protein (P-tau)
- PRM (Parallel reaction monitoring)
- Receiver operating characteristic (ROC)
- SIL peptide (Stable isotope labelled peptide)
- Total tau protein (T-tau)

Introduction

The core pathological hallmarks of Alzheimer’s disease are cortical plaques, primarily made up of aggregated Aβ1-42, and neurofibrillary tangles constituted of a heterogeneous mixture of hyperphosphorylated fragments of microtubule-associated protein tau (Blennow et al., 2006). Current CSF biomarkers of Alzheimer’s disease reflect neurodegeneration (total tau; T-tau) and plaque and tangle pathologies (Aβ1-42, and phosphorylated tau; P-tau). Increased abundance of amyloid plaques directly correlates with a decrease in CSF Aβ1-42 concentration. While increased CSF T-tau is observed in other neurological conditions such as stroke and
Creutzfeldt-Jakob disease, as a consequence of neuronal loss, P-tau is specifically increased in Alzheimer’s disease (Blennow et al., 2010).

Tau was first identified by Weingarten et al. in 1975 and described as a heat-stable protein essential for microtubule assembly (Weingarten et al., 1975). Subsequent studies showed in more detail that the functions of tau include stabilising and promoting microtubule polymerisation, as well as regulating axonal transport and affecting neurite growth (Drechsel et al., 1992; Johnson and Stoothoff, 2004; Mietelska-Porowska et al., 2014; Saftig and Bovolenta, 2015; Seiberlich et al., 2015; Feinstein et al., 2016). In 1986, tau was associated with neurodegeneration and Alzheimer’s disease as it was found to be the principal component of paired helical filaments (Grundke-Iqbal et al., 1986), a subunit or precursor of neurofibrillary tangles. Further, it was found that this impairment of the function of tau is dependent on the extent of protein phosphorylation, and that hyperphosphorylation; extensive phosphorylation of proline-rich motifs in the protein c-terminus, is a characteristic of pathological tau (Grundke-Iqbal et al., 1986; Bretteville and Planer, 2008).

Several forms of tau are expressed in humans as a result of alternative splicing and post-translational modification. A number of these alternate forms have been detected in CSF and there are indications that some of these may be altered in Alzheimer’s disease (Meredith et al., 2013).

In a previous, explorative study a large number of endogenous peptides in human CSF were identified (Hansson et al., 2017). Among the identified peptides were a number of small endogenous peptides from tau, five of which spanned the diagnostically interesting site threonine-181, used to indicate degree of tau phosphorylation (Vanmechelen et al., 2000; Itoh et al., 2001; Vanmechelen et al., 2001; Schönknecht et al., 2003; Hampel et al., 2004; Lewczuk et al., 2004).
In the present study we evaluated the potential of an endogenous peptide corresponding to tau 175-190, in the non-phosphorylated and phosphorylated state, as biomarkers of Alzheimer’s disease and other neurodegenerative disorders. A liquid-chromatography-mass spectrometry (LC-MS) method based on parallel reaction monitoring (PRM) was established, using synthetic stable isotope-labelled peptide standards. The method was employed for analysis of a clinical cohort consisting of healthy controls and patients diagnosed with Alzheimer’s disease, Parkinson’s disease and progressive supranuclear palsy, and findings were verified in a second, larger cohort consisting of Alzheimer’s disease patients and controls from a different medical centre.

Materials and Method

Ethical considerations

Discovery cohort…

CSF samples collected at the National Hospital for Neurology and Neurosurgery, London were used for research with written consent from all participating patients and approval from regional ethics committees.

CSF samples

Samples from two clinical cohorts were included in this study. The preliminary discovery trial was performed on CSF sampled from a cohort consisting of a total of 60 individuals divided into four groups of healthy controls (n=15), and patients clinically diagnosed with Alzheimer’s disease (n=15), Parkinson’s disease (n=15) and progressive supranuclear palsy (n=15). A secondary validation cohort consisted of a total of 40 individuals of healthy controls (n=24) and clinically diagnosed Alzheimer’s disease patients (n=16).
Stable isotope labelled peptide standards

Synthetic isotope-labelled (SIL) peptide standards (AQUA™ peptides, Thermo Scientific) of endogenous tau 175-190, 175-TPPAPKTPSSGEPPK-190, and P-tau 175-190, 175-TPPAPK-[T(PO$_3$H$_2$)]-PPSSGEPPK-190 (the amino acid numeration corresponds to Isoform Tau-F, UniProt accession number: P10636-8), were acquired from Thermo Fisher Scientific. The heavy SIL peptides incorporated a C-terminal lysine residue labelled with six $^{13}$C atoms: 175-TPPAPKTPSSGEPP-[K($^{13}$C$_6$)]-190 and 175-TPPAPK-[T(PO$_3$H$_2$)]-PPSSGEPP-[K($^{13}$C$_6$)]-190. The peptide content of each standard was determined by quantitative amino acid analysis performed by the manufacturer.

Preparation of standard samples

Peptide calibration curves were prepared using a diluted plasma pool as matrix, acquired from patients undergoing routine diagnosis. The samples were prepared by combining a fixed concentration of 20 fmol/mL of each of the two SIL peptides with a varying concentration of their non-labelled counterparts. Non-labelled tau 175-190 was prepared in concentrations ranging from 4-40 fmol/mL (1-10 fmol on column), and P-tau 175-190 between 2-20 fmol/mL (0.5-5 fmol on column). Subsequently the standards were spiked into the 1:200 diluted plasma (protein concentration 0.6 mg/mL), and prepared in aliquots of 250 µL according to the protocol described below. Samples for method precision evaluation were prepared by spiking five 250 µL CSF aliquots with 40 and 10 fmol/mL of SIL peptide tau 175-190 and P-tau 175-190, respectively.

Sample preparation for recovery of endogenous CSF peptides

Aliquots of 250 µL of CSF were spiked with synthetic peptide standards corresponding to 5 fmol/mL of tau 175-190 and 2.5 fmol/mL of P-tau 175-190. Samples were buffered by addition
of 50 µL 0.5 M triethylammonium bicarbonate, pH 8.5±0.1. Guanidinium hydrochloride (GdnHCl, 150 µL, 8 M) was added, followed by shaking (600 rpm) at room temperature for 10 min to dissociate protein/peptide structures aggregates. The samples were finally diluted by an addition of 1 mL de-ionised water to reduce the GdnHCl concentration to 0.8 M.

A molecular weight cut-off ultracentrifugation filter (Vivacon 2, 30 kDa, Sartorius) was used to selectively separate the endogenous CSF peptides from the bulk CSF proteins. All centrifugation steps were performed at 20°C and at a centrifugal rate of 2.500 x g for a specified length of time. Conditioning and equilibration of the filter was performed by sequential addition and centrifugation (2 x 20 minutes) of 2 mL 1 M GdnHCl followed by 1.5 mL 25 mM triethylammonium bicarbonate. Subsequently the flow through was discarded and the collection tube was rinsed with de-ionised water. The whole sample volume was added to the filter and centrifuged (40 minutes), the filtrate was left in the collection tube and a final addition to the filter and centrifugation (30 minutes) of 1 mL 25 mM triethylammonium bicarbonate was performed to rinse out remaining peptides. The filtrate was acidified by an addition of 25 µL 10 % formic acid, pH was subsequently adjusted to 3 through titration of 20 % phosphoric acid.

Solid phase extraction (SPE) was employed to remove salts and other contaminants from the peptide filtrate. SPE cartridges (1 mL Sep-Pak C18, Waters) were attached to a vacuum manifold and conditioned by sequential through flow of: 1 mL 84 % acetonitrile, 0.1 % formic acid; 1 mL 1 M GdnHCl and 1 mL 84 % acetonitrile, 0.1 % formic acid; the cartridge was then equilibrated by two additions of 0.1 % formic acid. The sample was added to the cartridge in portions, was subsequently washed by two additions of 0.1 % formic acid and finally eluted into a 1.5 mL polypropylene tube (LoBind, Eppendorf) by 1 mL 84 % acetonitrile, 0.1 % formic acid. The solvents were evaporated through vacuum centrifugation and the samples were subsequently stored at -80°C until analysis.
Targeted LC-MS analysis

The endogenous peptide samples were re-dissolved in 6.5 µL 2% acetonitrile, 0.1 % formic acid and gentle agitation for 20 min. 5 µL of the sample was loaded on an Ultimate 3000 rapid separation HPLC (high performance liquid chromatography) nano-flow system (Dionex), configured in trap column mode (trap column: Acclaim® PepMap 100 (Thermo Fisher Scientific), 75 µm x 2 cm, C₁₈, 100 Å pore size, 3 µm particle size; separation column: Acclaim® PepMap C₁₈ (Thermo), 75 µm x 500 mm, 100 Å pore size, 2 µm particle size). Two aqueous mobile phases: A) 0.1 % formic acid and B) 84% acetonitrile, 0.1 % formic acid, were applied at a constant flow of 150 nL/min in the following 70 minute gradient: t (min) =0, B=3%; t=10, B=2%; t=45, B=30%; t=46, B=80%; t=50, B=80%; t=50.5, B=3%; t=70, B=3%.

Mass analysis was performed on an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fisher Scientific) connected to the HPLC via a Nanospray Flex™ electrospray ionisation interface (Thermo Scientific) equipped with a stainless steel emitter. The mass spectrometer was running in positive ion mode and ionisation was performed over a static spray voltage of 1700 V to a heated transfer tube set to 275°C. Mass spectrometric acquisition combined two separate methods running in tandem; a full ms-only method, followed by a parallel reaction monitoring (PRM) method. The full ms method spectra were recorded at an orbitrap resolution of 50,000, scanning the mass-to-charge range 350-1400 m/z, with an automatic gain control target set to 1.0e6 with a maximum injection time of 100 milliseconds, no ms/ms scans were acquired following the full scan. The PRM method scanned for one of the four targeted peptides consecutively, applying the quadrupole with a mass window of 1 m/z as a mass filter coupled to an inclusion list of precursor ions (see Table 1). Selected ions were fragmented using higher-energy collision induced dissociation individually optimised in a separate trial (data not shown). Following fragmentation full ms/ms scans were acquired at an orbitrap resolution of 60,000, with an AGC target set to 1.0e5 and a maximum injection time of 118 milliseconds.
Peptide quantification and statistical analysis

The acquired raw ms/ms-data was analysed using the software Skyline v. 3.7 (MacLean et al., 2010). Quantification of the endogenous peptides was based on the calculated peak area-ratio between light and heavy isotopologues. The software was set to filter for y-type and precursor ions in the mass-range 400-1000 m/z, but was otherwise unrestricted. Previous trials had shown that transition y_{14}^{++} was consistently the most prominent product ion and was therefore used for quantitation, remaining transitions and precursor ions were used to confirm correctness of identification.

The calculated peak area-ratios obtained in Skyline were converted to molar concentrations and subsequently exported to GraphPad Prism (v. 7.03) for statistical analysis. Mann-Whitney (unpaired) test and Spearman r were applied to investigate group differences and correlations. All statistical analysis were performed at a confidence level of 99 % (P=0.01).

Immunassay analysis

CSF T-tau and P-tau concentrations were measured in both cohorts and Aβ_{1-42} concentrations were measured only in the validation cohort, using sandwich enzyme-linked immunosorbent assay (ELISA; INNOTEST®: β-AMYLOID 1-42, hTAU Ag and PHOSPHO-TAU 181P; Fujirebio, Ghent, Belgium).

Results

Development of a mass spectrometric assay for tau 175-190 and P-tau 175-190

A mass spectrometric method based on parallel reaction monitoring (PRM) for measurement of tau 175-190 and P-tau 175-190 in CSF was established on a quadrupole Orbitrap mass spectrometer. Synthetic peptide analogues labelled with $^{13}$C$_6$–Lys at position 16 in the amino
acid sequence were used as internal standards. Chromatographic separation was performed using a nano-flow HPLC system equipped with a C_{18} reversed phase column in a trap-column configuration. The LC was interfaced to the mass spectrometer via a nano-electrospray ion source operated in the positive ion mode.

For both peptides, the +3 ions were the most prominent charge state, and thus chosen as precursor ions. Higher collisional dissociation resulted in six prominent y-ions from the respective peptides (Figure 1a, b, Table 1). The fragmentation patterns of the SIL peptide standard (blue) were identical to those of the respective endogenous peptides in CSF (red), confirming the identities of the respective peptides. For both peptides, y_{14}^{++} was used for quantification, while the other fragments were used to verify the identity of the peptide.

Calibration curves were constructed by spiking human plasma, diluted 1:200, with different amounts of unlabelled peptides and a fixed amount of SIL peptides (Figure 2). For tau 175-190 the curve showed good linearity (R^2=0.997) over the tested concentration range 8-40 fmol/ml (2-10 fmol on column). For P-tau 175-190 the curve was linear (R^2=0.986) over the concentration range 2-20 fmol/ml (0.5-5 fmol on column).

The precision of the assay was evaluated by analysing five CSF samples prepared separately, resulting in a CV of 5.97% for tau 175-190 and 6.13% for P-tau 175-190.

**The tau ratio and P-tau ratio improve the diagnostic accuracy of tau in a discovery cohort**

To evaluate whether CSF tau 175-190 and P-tau 175-190 are altered in neurodegenerative disorders, CSF samples from 60 individuals clinically diagnosed with Alzheimer’s disease (n=15), Parkinson’s disease (n=15), progressive supranuclear palsy (n=15), and healthy control subjects (HC, n=15) were analysed using the developed PRM method. The core Alzheimer’s
disease biomarkers T-tau and P-tau were also measured, using ELISA (for demographics, see Table 2).

No significant difference in the concentrations of either peptide were observed in any of the disease groups (Figure 3a and b, Table 3), however, T-tau (measured by ELISA) correlated more strongly to tau 175-190 (measured by PRM) in the controls as well as in Parkinson’s disease and progressive supranuclear palsy compared to in Alzheimer’s disease (Figure 3c, Table 4). The same observation was made for P-tau (Figure 3d, Table 4).

These observations suggested that the endogenous tau peptides may reflect the proportion of CSF tau that is secreted under physiological conditions, independently of pathological processes, and prompted us to test if Alzheimer’s disease-control group separation by T-tau and P-tau could be improved by normalizing T-tau and P-tau concentration to those of the respective endogenous peptides.

Figure 4a and b show scatter plots of the group separations for T-tau and for the ratio of T-tau/tau 175-190. The effect size for separating Alzheimer’s disease from controls (Table 3) was larger for the T-tau ratio (Cohen’s $d = 1.98$) than for T-tau (1.72). Similarly, a larger effect size was observed for the P-tau ratio ($d = 1.72$) than for P-tau ($d = 1.53$; Figure 4c, d).

The mean relative increase in Alzheimer’s disease for the T-tau ratio (139%; Supplementary Table S1) was similar to that of T-tau (146%), but the CVs for the tau ratios in both the Alzheimer’s disease (31.5%) and controls (11.8%) groups were smaller compared to those of t-tau (Table 3; $CV_{AD}=39.2; CV_{HC}=24.7$). Similar results were observed for the P-tau ratio ($CV_{AD}=28.5; CV_{HC}=10.7$) compared to P-tau ($CV_{AD}=31.1; CV_{HC}=21.1$).

Receiver operating characteristic (ROC) curve analysis showed an improved diagnostic performance in separating Alzheimer’s disease from HC for both the T-tau ratio (area under the
curve (AUC =100%) compared to t-tau (AUC=95.1%), and for the P-tau ratio (AUC=95.3%) compared to P-tau (AUC=92%; Figure 4 e and f).

The degree of phosphorylation was calculated, for the tau protein as the ratio of [P-tau]/([t-tau]), and for the endogenous peptides as [P-tau 175-190]/[t-tau 175-190]. There was no apparent difference in the degree of phosphorylation for any disease group (Supplementary Table S2).

**Verification of the results in a second cohort**

To verify the findings from the discovery cohort, tau 175-190 and P-tau 175-190 were measured in a second cohort, consisting of CSF samples from 40 patients from a different medical centre, with clinical diagnosis of Alzheimer’s disease (n=16) and HC (n=24; for demographics, see Table 5). The core biomarkers, T-tau, P-tau, and Aβ1-42 were determined by ELISA.

The results for T-tau, P-tau and their respective ratios are shown as scatter plots in Figure 5 (a-d) and are summarized in Table 6. Similarly as in the discovery cohort, neither tau 175-190 nor P-tau 175-190 differed between Alzheimer’s disease patients and controls (Supplementary Figure S1). T-tau and P-tau showed a significant (p<0.01) group separation (Figure 5a and c), but with more overlap between the groups compared to the discovery cohort. The T-tau and P-tau ratios improved group separations (Figure 5b and d), with similar mean relative increase in Alzheimer’s disease (Supplementary Table S3), and with lower intra-group variation (Table 6). The effect size for separating Alzheimer’s disease from controls was larger for the T-tau ratio (Cohen’s d = 1.59) than for T-tau (1.25). Similarly, a larger effect size was observed for the P-tau ratio (1.64) than for P-tau (1.03).

Contrary to the discovery cohort, the correlation between T-tau and tau 175-190 was similar for Alzheimer’s disease patients and controls; the correlation between P-tau and P-tau 175-190 was
even slightly higher in the Alzheimer’s disease group (Supplementary Figure S1, Supplementary Table S4). ROC curve analysis (p=0.01) showed improved group separation for the T-tau ratio (AUC=0.97) and the P-tau ratio (AUC=0.98) compared to t-tau (AUC=0.88) and P-tau (AUC=0.82; Figure 5 e, f). As in the discovery set, the degree of phosphorylation did not differ between the groups (Supplementary Table S5).

We tested if tau 175-190 and P-tau 175-190 could be used interchangeably for normalization by calculating the ratios of t-tau/P-tau 175-190 and P-tau/tau 175-190 (Supplementary Figure S2 and S3, and Supplementary Tables S8 and S9).

To investigate whether the endogenous tau peptides reflect a protein-specific variation or a variation of more general nature, we tested if ratios of Aβ1-42 to tau 175-190 and P-tau 175-190 would improve group separation similarly as normalization to Aβ1-40 does. However, Aβ1-42/tau 175-190 and Aβ1-42/P-tau 175-190 both resulted in an increased intra group CV, decreased effect size and a decreased AUC for separating Alzheimer’s disease patients and controls compared to using the Aβ1-42 concentration alone (Supplementary Figure S4 and Supplementary Table S6).

**Discussion**

In this paper, we explore the potential of an endogenous CSF peptide from microtubule-associated protein tau, in phosphorylated and non-phosphorylated state, as a biomarker of neurodegenerative diseases, particularly Alzheimer’s disease. By ‘endogenous peptides’ we mean in this context peptides naturally present in the neat CSF, *i.e.*, not produced by *in vitro* proteolysis of CSF proteins with trypsin, as is the procedure most commonly applied in proteomics.
The peptide chosen for the current study was one out of seven endogenous peptides from tau that were identified in an explorative peptidomic analysis (Hansson et al., 2017). In that study, endogenous CSF peptides isolated by molecular weight cut-off (30 kDa) ultracentrifugation were subjected to extensive fractionation and analysed by nano-LC-MS on a high-resolution mass spectrometer operated in the data-dependent mode, resulting in the identification of over 18,000 peptides in the molecular mass range 470 – 5,900 Da. The peptides originated from 1,918 proteins, many of which are associated to neurodegeneration, synaptic loss, axonal damage, or Alzheimer’s disease. The large number of endogenous peptides in CSF raises several questions: where and through what processes are they produced and shed into the CSF? Do their concentrations correlate to those of their parent proteins? Can they be used as disease biomarkers and, if so, do they relay information on pathologies different from that of their parent proteins?

The peptide tau 175-190 was selected for the current study because it spans threonine-181, phosphorylation of which is widely regarded as an Alzheimer’s disease specific biomarker (Vanmechelen et al., 2000; Itoh et al., 2001; Vanmechelen et al., 2001; Schönknecht et al., 2003; Hampel et al., 2004; Lewczuk et al., 2004). Further, the peptide could be detected both in the phosphorylated and non-phosphorylated state, enabling measurement of the degree of phosphorylation at Thr-181. We observed, however, no difference in the concentration of neither the phosphorylated, nor the non-phosphorylated form of tau 175-190, or in the degree of phosphorylation, in AD, PD or PSP patients, compared to healthy controls in the discovery cohort, nor between Alzheimer’s disease and healthy controls in the validation cohort.

The observations that tau 175-190 and P-tau 175-190 did not increase in Alzheimer’s disease and correlate more strongly to ELISA measurements of T-tau and P-tau, respectively, in the healthy, PD and PSP patients, compared to AD, suggests that the endogenous peptides are shed into the CSF through processes different from those responsible for the increased CSF tau in
AD. This observation prompted us to test the diagnostic performance of the ratio of T-tau to tau 175-190 (tau ratio) compared to t-tau alone. We found that the tau ratio improved the group separations. This finding was confirmed in a second cohort. The effect was mainly due to decreased intra-group variation in all study groups, leading to very low variation, particularly in the control group, while the mean relative difference between Alzheimer’s disease patients and controls was unchanged. These results thus indicate that the variation in the concentration of the endogenous peptides is not related to Alzheimer’s disease.

Improving the diagnostic performance of a biomarker by taking the ratio of the biomarker to a non-biomarker endogenous peptide from the same protein has previously been reported (Hansson et al., 2007; Wiltfang et al., 2007) and is by now well-established for the Alzheimer’s disease biomarker Aβ₁-₄₂ in using the Aβ₁-₄₂/₄₀ ratio (Dumurgier et al., 2015; Lewczuk et al., 2015). Similarly to the tau ratio, the improvement observed for the Aβ₁-₄₂/Aβ₁-₄₀ ratio compared to Aβ₁-₄₂ alone, results from decreased intra group variation while the relative difference between Alzheimer’s disease and healthy do not change.

It is not known what the variation in Aβ₁-₄₀ accounts for; it has been suggested that it might reflect variability in the efficiency of processing the amyloid precursor protein or differences in the expression profile of APP on the cell surface (Lewczuk et al., 2015). Alternatively, the ratio of Aβ₁-₄₂ to Aβ₁-₄₀ may normalize for differences in CSF dynamics, e.g. production, flow along the spinal cord, and absorbance. Analogously for tau, one may speculate that there is a pool of secreted tau – or tau-derived peptides – different from that released into the CSF during the process of Alzheimer’s disease pathological neurodegeneration, that displays a normal variation in abundance and that is not affected in Alzheimer’s disease.

What is the source of variation that the endogenous tau peptides reflect? That tau 175-190 and P-tau 175-190 correlate significantly (p<0.01) to Aβ₁-₄₀ suggests that the effect of
normalization, to some extent, is to reduce variation of more general nature, such as CSF dynamics (Supplementary Table S7). However, using tau 175-190 or P-tau 175-190 to normalize Aβ1-42 measurements resulted in poorer group separation than for the Aβ1-42/Aβ1-40 ratio or Aβ1-42 alone (Supplementary Table S6), suggesting that the variation in tau 175-190 and P-tau 175-190 to some extent also is protein specific.

Do tau 175-190 and P-tau 175-190 relay the same information? The peptides correlate strongly in both Alzheimer’s disease patients and healthy controls (Supplementary Table S7) and when using tau 175-190 to normalize P-tau and, vice versa, P-tau 175-190 to normalize t-tau, group separations were significantly better compared to the non-normalized t-tau and P-tau results, in both cohorts (Supplementary Table S8 and S9). Comparing t-tau/tau 175-190 with t-tau/P-tau 175-190 or P-tau/P-tau 175-190 with P-tau/tau 175-190 showed relatively small differences.

At this point it is not clear where the tau peptides are formed. We do not believe that they are generated in vitro. Possibly, they are generated as part of the normal tau synthesis; for example, it is known that short peptides derived from most proteins are produced by the proteasome and presented on the cell surface in a MHC-I context as part of the cell’s self-declaration of protein synthesis.

In conclusion, our study shows that the performance of T-tau and P-tau as Alzheimer’s disease biomarkers can be improved by normalizing the measured CSF concentrations to those of the endogenous peptides tau 175-190 and P-tau 175-190. A limitation of the study is that immunoassays (which were used to measure t-tau and P-tau) often do not correlate well with mass spectrometry (which was used to quantify the endogenous peptides), which may add inter-analytical variation to the tau ratio. Recently, mass spectrometric methods have been reported for quantification of CSF tau based on analysis of tau peptides produced by tryptic cleavage (McAvoy et al., 2014). It is possible that measuring both tau protein and endogenous peptide
concentrations with a mass spectrometric method would yield decreased analytical variation and thereby improved performance of the tau ratio as a biomarker of Alzheimer’s disease.

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**Figure Legends**

**Figure 1.** PRM of tau 175-190 and P-tau 175-190. MS/MS spectra of the [M+3H]^3+ ion of (a) tau 175-190 and (b) P-tau 175-190. The six most prominent y ions are labelled in the spectra. PRM chromatograms of (c) tau 175-190 and (d) P-tau 175-190. The spectra and chromatograms of the endogenous form of the respective peptide coloured red and the SIL peptide is coloured blue.

**Figure 2.** Calibration curves for (a) tau 175-190 and (b) P-tau 175-190, with instrument response calculated as the MS/MS peak area ratios of the $^{12}$C$_6$- (light) and $^{13}$C$_6$- (heavy) SIL peptides multiplied by the concentration of spiked-in heavy SIL peptide as a function light SIL peptide concentration.

**Figure 3.** CSF tau 175-190 and P-tau 175-190 concentrations in the discovery cohort. Scatter plots of (a) tau 175-190 and (b) P-tau 175-190 in patients with Alzheimer’s disease (AD), Parkinson’s disease (PD), Progressive supranuclear palsy (PSP), and healthy controls (HC).

**Figure 4.** Scatter plots of group separation and ROC analysis of diagnostic accuracy in the discovery cohort. The ability to separate disease groups/healthy controls based on (a) t-tau concentrations. (b) t-tau/tau 175-190 molar ratio. (c) P-tau concentrations and (d) P-tau/P-tau 175-190 molar ratio. (e, f) ROC curve analysis showing the effect on diagnostic precision when separating Alzheimer’s disease from healthy controls based on protein concentration alone compared to protein/peptide ratio.

**Figure 5.** Scatter plots of group separation and ROC analysis of diagnostic accuracy in the validation cohort. The ability to separate Alzheimer’s disease from healthy controls based on (a) t-tau concentrations. (b) t-tau/tau 175-190 molar ratio. (c) P-tau concentrations and (d) P-tau/P-tau 175-190 molar ratio. (e, f) ROC curve analysis showing the effect on diagnostic
precision when separating Alzheimer’s disease from healthy controls based on protein concentration alone compared to protein/peptide ratio.
Supplementary Figure Legends

**Figure S1:** (a) CSF tau 175-190 and (b) P-tau 175-190 (b) concentrations in the validation cohort. Alzheimer’s disease = AD; healthy controls = HC. Scatter plots of (c) tau 175-190 and t-tau, and of (d) P-tau 175-190 and P-tau.

**Figure S2:** Performance of (a) t-tau/tau 175-190 versus (b) t-tau/P-tau 175-190, and of (c) P-tau/P-tau 175-190 versus (d) P-tau/tau 175-190 in the discovery cohort. Alzheimer’s disease = AD; healthy controls = HC, PD = Parkinson’s disease; PSP = progressive supranuclear palsy.

**Figure S3:** Performance of (a) t-tau/tau 175-190 versus (b) t-tau/P-tau 175-190, and of (c) P-tau/P-tau 175-190 versus (d) P-tau/tau 175-190 in the discovery cohort. Alzheimer’s disease = AD; healthy controls = HC.

**Figure S4:** Effect on group separation by normalizing Aβ1-42 to tau 175-190 and P-tau 175-190. (a) CSF Aβ1-42 concentration; (b) Aβ1-42 normalised to tau 175-190; (c) P-tau 175-190.
References


