

Quantification of Tau in Cerebrospinal Fluid by Immunoaffinity Enrichment and Tandem Mass Spectrometry

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BACKGROUND: Cerebrospinal fluid (CSF) tau is a common biomarker for Alzheimer disease (AD). Measurements of tau have historically been performed using immunoassays. Given the molecular diversity of tau in CSF, the selectivity of these immunoassays has often been questioned. Therefore, we aimed to develop an analytically sensitive and selective immunoaffinity liquid chromatography–tandem mass spectrometry (LC-MS/MS) (IA-MS) assay.

METHODS: IA-MS sample analysis involved the addition of an internal standard, immunoaffinity purification of tau using a tau monoclonal antibody coupled to magnetic beads, trypsin digestion, and quantification of a surrogate tau peptide by LC-MS/MS using a Waters Trizaic nanoTile ultraperformance LC microfluidic device. Further characterization of tau peptides was performed by full-scan MS using a Thermo Orbitrap LC-MS. CSF samples from a cohort of age-matched controls and patients with AD were analyzed by the IA-MS method as well as a commercially available immunoassay.

RESULTS: The IA-MS assay had intra- and interassay imprecision values of 3.2% to 8.1% CV and 7.8% to 18.9% C, respectively, a mean recovery of 106%, and a limit of quantification of 0.25 pmol/L and was able to quantify tau concentrations in all human specimens tested. The IA-MS assay showed a correlation of $R^2 = 0.950$ against a total-tau immunoassay. In patients with AD, tau was increased approximately 2-fold.

CONCLUSIONS: Combining immunoaffinity enrichment with microflow LC-MS/MS analysis is an effective approach for the development of a highly selective assay

to measure total tau and, potentially, other posttranslationally modified forms of tau in CSF.

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Recently, there has been substantial effort to characterize biomarkers of Alzheimer Disease (AD)⁴ that may potentially aid in clinical diagnosis and the development of new therapeutics (1). Much of this effort has been focused on the plaque-forming amyloid β peptides (2) and the microtubule binding protein tau (3) in cerebrospinal fluid (CSF). Tau is preferentially expressed in the cortical axons of the limbic cortex and is thought to be involved in the regulation of microtubule stability. When tau is hyperphosphorylated, primarily by the action of serine/threonine kinases, it dissociates from microtubules, leading to the formation of paired helical fragments that, in turn, aggregate into neurofibrillary tangles and neuropil threads (4). It is hypothesized that the formation of neurofibrillary tangles leads to the loss of synapses and neurons, thus ultimately contributing to the development of dementia (4). The concentrations of CSF tau are thought to be related to the extent of axonal injury, cell death, and neurofibrillary tangle morphology (5). Tau is also extensively posttranslationally modified and some of these modifications (glycosylations, acetylations, and others) may be related to disease progression (6). The combination of CSF amyloid β , total tau, and phospho(Thr181)-tau is currently the most-used biomarker test for AD (7). Tau is also a very difficult protein to quantify. There are 6 isoforms of tau of varying lengths [352, 381, 383, 410, 412, and 441 amino acids (AAs)] as well as an unknown number of potential degradation products circulating in CSF, many containing a variety of modifications. Thus, analytical specificity is

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⁴ Nonstandard abbreviations: AD, Alzheimer disease; CSF, cerebrospinal fluid; AA, amino acid; LC-MS/MS, liquid chromatography–tandem mass spectrometry; IA-MS, selective immuno-affinity LC-MS/MS; mAb, monoclonal antibody; IS, internal standard protein; MSD, Meso Scale Discovery; LOQ, limit of quantification; PTM, posttranslational modification; MRM, multiple reaction monitoring.

of particular importance for the quantification of tau in CSF. In the case of CSF total tau, multiple immunoassays exist that rely on different combinations of capture and detection antibodies with uncertain analytical specificities. There is currently no standardized reference material or reference method identified that can be used to reconcile the differences between assay results (7). Immunoassays have inherent liabilities in analytical specificity, driven mainly by the specificity of the antibody pair used for capture and detection. In contrast to immunoassays, quantitative liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods directly measure the target peptide with analytical specificity based on the peptide’s retention time, *m/z*, and fragmentation profile (8–10). As a result, MS-based assays have been proposed as alternatives to immunoassays when the immunoassay performance is insufficient (2, 11–14) or to confirm the analytical specificity of immunoassays (15).

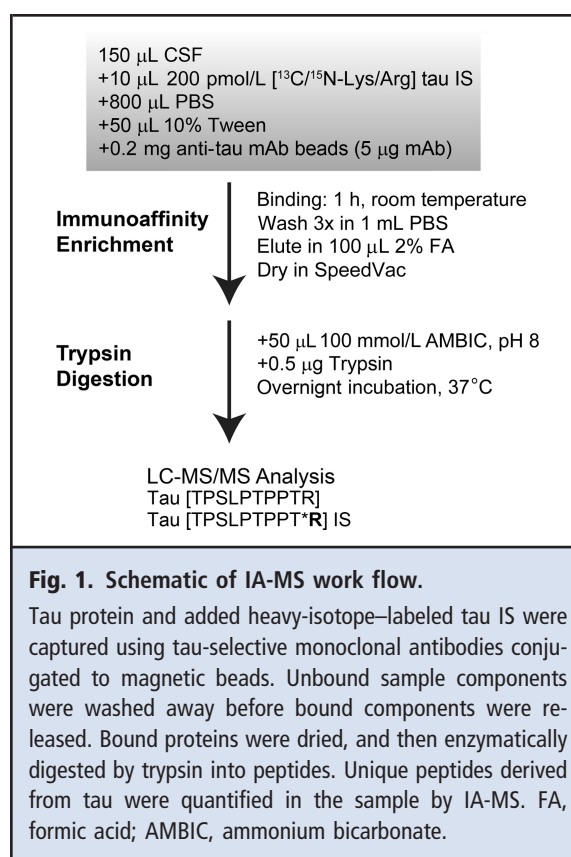
Here we describe the development of a quantitative selective immunoaffinity LC-MS/MS (IA-MS) assay to measure tau in CSF. The assay employs a monoclonal antibody (mAb) to selectively enrich tau from routinely available volumes of CSF, followed by tryptic digestion to produce proteotypic peptide fragments that can be quantified by LC-MS/MS. The use of microflow chromatography, combined with a microfluidic device, provides enhanced analytical sensitivity with the robustness required for clinical assays (16).

Methods and Materials

REAGENTS

Trypsin Gold, mass spectrometry grade, was purchased from Promega. Synthetic peptides corresponding to the tryptic surrogate tau analytes were produced at New England Peptide. A purified recombinant tau calibration standard (Tau 381 isoform) was produced by Proteos using a pET3a vector in *Escherichia coli*, Ni-NTA (nickel-nitrilotriacetic acid), and size-exclusion chromatography, purified to >95% as estimated by Coomassie-stained SDS-PAGE analysis, and quantitated by AA analysis (AAA Service Laboratory). A ¹³C-¹⁵N Arg/Lys-labeled tau (412-AA isoform) internal standard protein (IS) was produced by Promise Proteomics. In addition, the 6 tau isoforms were obtained from rPeptide.

Mouse monoclonal and rabbit affinity purified polyclonal anti-tau antibodies were both raised against a 166-AA tau fragment containing the central region of the protein that is shared among tau isoforms. One resulting clone (13A6) was chosen for use in the immunoaffinity enrichment of tau. Epitope mapping for 13A6 was performed by covalently conjugating synthetic tau peptides to a Luminex bead set. Purified



mAb was incubated with the mixture of peptides on beads, washed, and subsequently incubated with phycoerythrin-labeled anti-mouse IgG reporter antibodies. The beads were then analyzed on a Luminex FLEXMAP 3D® instrument to detect binding. For immunoaffinity enrichment, anti-tau antibodies were covalently coupled to MyOne Tosylactivated Dynabeads (Life Technologies) following the manufacturer’s recommended protocol.

CALIBRATION AND QC SAMPLES

For IA-MS analysis, an 8-point calibration curve was prepared daily from 50 pmol/L to 0.39 pmol/L using a 2-fold serial dilution of recombinant Tau 381 in PBS. Low (2.0 pmol/L), medium (17.1 pmol/L), and high (40.7 pmol/L) QC samples were prepared using CSF (remnant healthy and postmortem) obtained from Bioreclamation. A recombinant Tau 441 calibrator included with the Meso Scale Discovery (MSD) kit was used for immunoassay analysis, as recommended.

IMMUNOAFFINITY ENRICHMENT AND PROTEIN DIGESTION

The work flow for the optimized IA-MS analysis of tau in CSF is shown in Fig. 1. CSF samples (150 μL), calibrators, and QC samples were added to wells of a

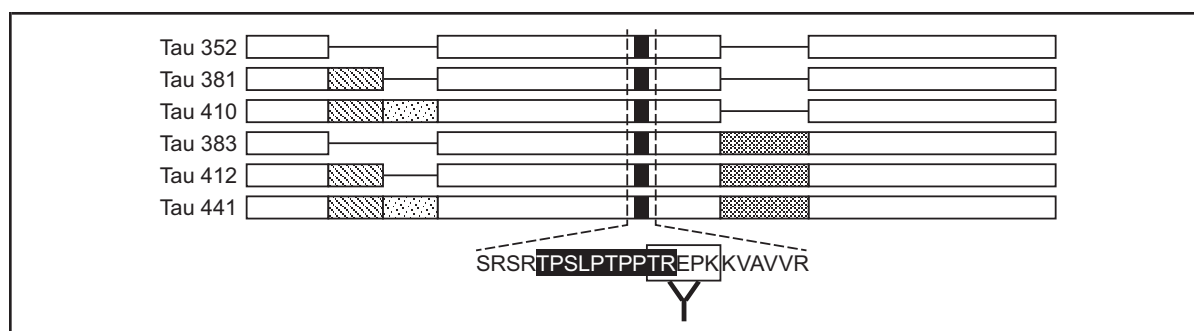


Fig. 2. Features of tau AA sequence.

Regions of consensus between human tau isoforms are aligned with regions of isoform-specific sequence shown with shaded boxes. The tryptic peptide used as the analyte in this assay is shown in the black box. The peptide epitope to which mAb 13A6 binds is shown in the open box.

1.8-mL deep-well plate, and the volume was adjusted to 1 mL using PBS. The following reagents were added to each sample: 10 μ L 200 pmol/L Tau 412 IS, 50 μ L 10% Tween-20, and 10 μ L suspension (5 μ g mAb on 0.2 mg beads) of anti-tau mAb-coated magnetic beads. The plate was sealed and mixed on a plate shaker (Eppendorf MixMate) at ambient temperature for 1 h at 1200 rpm. The magnetic beads were washed 3 times with 1 mL PBS with the aid of a plate stand with magnetic posts that pulled the magnetic beads to the side of each well to allow for liquid aspiration without noticeable bead loss. Proteins were eluted from the washed beads in 100 μ L 2% formic acid with constant shaking at 1200 rpm at ambient temperature for 20 min, transferred to a new 96-well plate, and evaporated to dryness at 45 $^{\circ}$ C in a SpeedVac (Thermo Scientific) using a high-vacuum pump (Thermo VLP120). The dried proteins were resuspended in a mixture of 0.5 μ g trypsin in 50 μ L 100 mmol/L pH 8.0 ammonium bicarbonate and digested overnight with mixing at 1200 rpm at 37 $^{\circ}$ C. The digested samples were moved to a 4 $^{\circ}$ C autosampler for analysis.

MASS SPECTROMETRY

Instrument-specific methods and settings used for the LC-MS/MS quantification of tau in CSF and LC-MS profiling for the characterization of tau posttranslational modifications are described in the Data Supplement that accompanies the online version of this report at <http://www.clinchem.org/content/vol60/issue4>.

ANALYTICAL VALIDATION

Intraassay imprecision was determined by measuring the concentrations of 6 replicates of the QC samples on a single run. Interassay imprecision was determined by measuring the QC samples on 6 different runs. Dilution linearity was determined by diluting CSF up to

1:32 in PBS before analysis. Recovery was performed by spiking CSF with 1.3, 3.9, and 10.9 pmol/L Tau 381 calibration standard before immunoaffinity precipitation. The limit of quantification (LOQ) was determined by measuring the interassay %CV ($n = 5$) of samples with decreasing concentrations of tau and estimation of the lowest concentration that could be measured with an interassay CV <20%.

HEALTHY AND AD SAMPLES

A panel of age-matched CSF from donors with normal cognition ($n = 50$) and donors with a diagnosis of AD ($n = 50$) were obtained from PrecisionMed. Tau concentrations were determined by both IA-MS and immunoassay [Human Total Tau kit (K151LAE-2) from MSD, following the manufacturer's instructions and using single measurements]. Statistical analysis (t -test and correlation analysis) and plotting of the data were performed using GraphPad Prism 5.

Results

ASSAY DEVELOPMENT

A blast homology comparison (17) between the 6 tau isoforms was performed to identify conserved regions among the isoforms (Fig. 2). An *in silico* tryptic digest revealed multiple peptides amenable to MS analysis that were conserved between the 6 isoforms of human tau. Of these, 3 peptides were selected as potential total-tau surrogate peptides based on sequence uniqueness, reduced likelihood of posttranslational modifications (PTMs), and amenability for LC-MS analysis (listed with sequence information based on Tau 441): TPSLTPPTR (AA212–221), LQTAPVPMPDLK (AA243–254), and IGLSDNITHVPGGGNK (AA354–369). Multiple reaction monitoring (MRM) transitions and instrument properties for peptides studied are included in online

Table 1. Selected tau derived peptides and associated PTMs identified by data-dependent analysis of immunoaffinity-enriched postmortem CSF.

Peptide sequence	Mascot score	Peak intensity	% PTM intensity (PTM intensity/combined intensity)
TPSLTPPTR	44.55	8.7E+06	
TPS(Phos)LTPPTR	20.83	1.5E+05	1.7%
STPTAEDVTAPLVDEGAPGK	71.57	2.3E+04	
ST(Phos)PTAEDVTAPLVDEGAPGK	50.61	2.6E+04	53%
SPVVS GDTSPR	32.35	7.5E+04	
SPVVS GDTSPR(Phos)PR	60.35	4.7E+04	39%
LQTAPVMPDLK	64.24	4.1E+06	
LQTAPVPM(Ox)PDLK	46.58	1.4E+06	25%

Supplemental Table 1. For each of these peptides, 2 product ion transitions were monitored to aid in peak identification. Analysis of a tryptic digest of recombinant Tau 381 as well as an equimolar mix of synthetic versions of the 3 monitored tau peptides revealed that the peptide TPSLTPPTR provided the most intense MRM signal intensity, and the primary transition for this peptide was selected as the primary readout for tau throughout method development and validation.

Heavy-isotope-labeled (^{13}C - ^{15}N -Lys/Arg) recombinant tau was spiked into CSF samples before immunoaffinity purification and subsequent digestion and analysis. The value of using a protein IS over a peptide IS was that it could be added at the beginning of the sample preparation procedure and, therefore, accounts for variations of both immunoaffinity enrichment and digestion. The suitability of using a single isoform recombinant protein as an IS for all endogenous isoforms was tested to ensure there was no bias in immunoaffinity enrichment. For this test, tau IS was mixed with postmortem CSF. The ratio of light to heavy tau was compared with or without immunoaffinity enrichment ($n = 4$). The omission of the immunoaffinity step was possible because of the much higher concentration of tau in postmortem CSF. A *t*-test could not detect a change in the light/heavy tau ratio, indicating that the mAb (clone 13A6) did not preferentially enrich one tau over the other (data not shown).

Several antibodies (affinity-purified rabbit polyclonal antibodies to human tau and multiple mouse monoclonal antibodies) were initially tested for the immuno-pull down step. A single monoclonal clone developed in house (clone 13A6) was selected for use in this assay because of availability and good assay performance. To ensure that clone 13A6 equally enriches all tau isoforms without bias, recoveries of each recombinant isoform of tau were tested and compared. Recoveries for all 6 isoforms ranged from 60% to 75% and

did not reveal any bias among isoforms (data not shown).

Epitope mapping performed on the 13A6 mAb clone revealed binding of a peptide with sequence TREPK (tau AAs 220–224). The AAs “TR” of this epitope were also contained within the peptide selected for MRM analysis, TPSLTPPTR (Fig. 2).

Following antibody selection, multiple parameters of the tau IA binding, wash, elution, and digestion steps were optimized to allow for optimal tau recovery. The minimum quantity of beads required for optimal tau recovery was determined to be at least 0.05 mg beads; the trypsin digestion was found to be optimal using 0.5 μg trypsin per sample overnight.

LC-MS/MS PROFILING OF TAU IN CSF

Immunoaffinity purified and digested tau from a human postmortem CSF sample was analyzed by full-scan MS with data-dependent MS/MS for identification of peptides. From 1 sample, 34 unique tau peptides were observed. Both unmodified and modified peptides were observed (Table 1), including the 3 peptides initially identified using in silico techniques as potential total-tau surrogate peptides. One of these, IGSLDN ITHVPGGGNK was observed as predicted but was also observed in some samples with a missed cleavage IGSLDNITHVPGGGNKK (data not shown), suggesting that trypsin digestion might be problematic for this peptide. A second candidate peptide, LQTAPVMPDLK, was identified but observed to be oxidized, with a resulting peak intensity roughly one-fourth that of the unoxidized peptide. The third peptide, TPSLTPPTR, was detected using this methodology with high peak intensity and found to be only minimally phosphorylated. In comparison, several other tau peptides were identified that were phosphorylated to a much greater extent, including SPVVS GDTSPR and the isoform specific peptide STPTAEDVTAPLVDEGAPGK.

Table 2. CSF tau assay imprecision. Inter- and intraassay %CV for CSF tau QC samples.

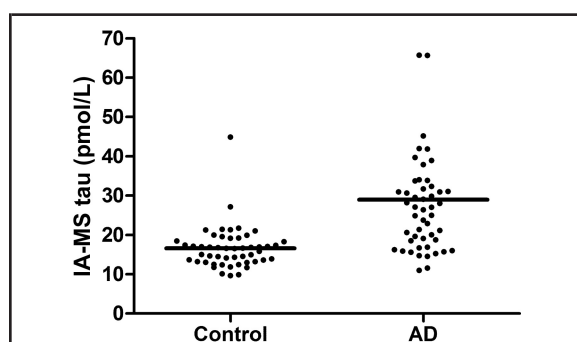
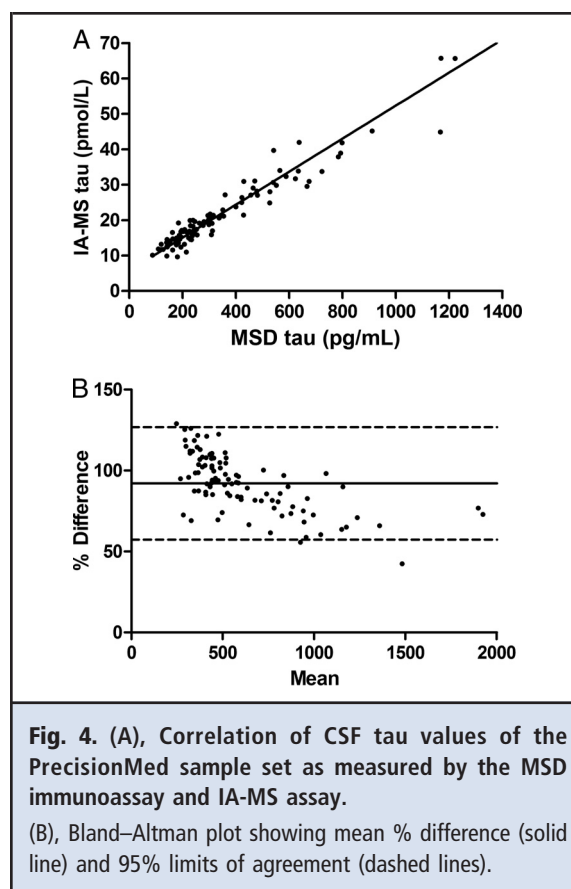
	Interday (n = 6)			Intraday (n = 6)		
	Mean, pmol/L	SD	% CV	Mean, pmol/L	SD	% CV
Low	2.0	0.4	18.9	2.0	0.2	8.1
Medium	17.1	1.5	9.0	15.3	0.5	3.2
High	40.7	3.2	7.8	38.9	1.5	3.8

ANALYTICAL VALIDATION

Intra- and interassay imprecision were found to be <10% and 20%, respectively (Table 2). Dilution linearity experiments revealed that dilutions of CSF up to 1:32 were acceptable (<20% change from undiluted) (see online Supplemental Table 2). Spike recoveries at 3 concentrations were tested with 1 CSF sample, from a donor without AD, containing 10.3 pmol/L tau. Recoveries of 102% (low spike), 114% (3.9 pmol/L spike), and 102% (10.9 pmol/L spike) were observed (see online Supplemental Table 3). The LOQ of the assay (CV <20%) was calculated to be 0.25 pmol/L.

ANALYSIS OF CSF SAMPLES

Tau concentrations were measurable in all CSF samples by both IA-MS and immunoassay. The IA-MS identified a significant difference ($P < 0.0001$) in tau concentrations between healthy controls and patients with AD (mean of control=17 pmol/L, AD=29 pmol/L) (Fig. 3). Similar results were seen with the MSD total-tau assay (mean control, 229 pg/mL; AD, 503 pg/mL). The fold difference between the AD and control group was slightly higher when using the immunoassay to measure tau (2.2- vs 1.7-fold). The 2 methods had a correlation of $R^2 = 0.950$, $S_{y/x} = 3.3$ (Fig. 4A). Conversion of the IA-MS data from pmol/L

**Fig. 3.** Distribution of tau concentrations for control and AD groups as measured by the IA-MS assay.**Fig. 4.** (A), Correlation of CSF tau values of the PrecisionMed sample set as measured by the MSD immunoassay and IA-MS assay.

(B), Bland-Altman plot showing mean % difference (solid line) and 95% limits of agreement (dashed lines).

to pg/mL using an estimated mean protein molecular weight of 40 kDa yielded a correlation slope of 1.8, which may reasonably be accounted for by differences in the calibration standards used in this IA-MS assay and those included in the MSD assay. A Bland-Altman plot comparing the 2 assays (Fig. 4B) indicated that, at higher concentrations (>500 pg/mL), the immunoassay overestimated the concentration of tau compared to the IA-MS method; however, a more definitive assessment should be made with a larger sample set.

Discussion

We present here an MS-based assay to measure tau in CSF. An MS-based method represents an orthogonal technique that can confirm the analytical specificity of more commonly used immunochemistry-based assays. It is also a powerful platform that can be applied to measure specific modifications of proteins, such as tau. The low CSF concentrations of tau in healthy individuals and many diseased patients require advanced techniques in sample preparation and analysis, such as the use of immunoaffinity enrichment for concentrations of tau protein, as well as the use of robust ultraperfor-

mance LC microflow LC-MS/MS to enable measurements of low-abundance peptides. Tau immunoaffinity enrichment was accomplished before trypsin digestion using an anti-tau mAb that binds to a region shared between all tau isoforms (TREPK) and partially overlaps with the surrogate peptide analyte (TPSLPT-PPTR). For detection, the LC-MS/MS Trizaic nanoTile system was found to be sufficiently robust for clinical analysis, with fast cycle times, reproducible peptide retention times, and no need for user intervention during the course of hundreds of sample analyses.

MS has been proposed as a good alternative platform for the development of a quantitative assay for tau because it can best measure tryptic peptides that are conserved across protein isoforms and are infrequently modified (total tau) with high specificity. Furthermore, MS methods can also be developed to measure sequences that are not conserved across isoforms or that contain 1 or more PTMs. Historically, quantitative LC-MS/MS has been most commonly employed to measure relatively abundant protein analytes; however, with the development of IA-MS and more sensitive instruments, the quantification of low-abundance proteins is now possible and is becoming more common (18, 19). This technique increases the analytical sensitivity through sample concentration and removal of the majority of matrix proteins. Additional analytical sensitivity gains can be achieved through the use of low-flow chromatography, which increases ionization efficiency, but usually at the expense of throughput and robustness (20).

Our approach for the development of a CSF tau assay was to quantify a tryptic peptide present in all isoforms and that was not (or was minimally) posttranslationally modified. Because tau present in CSF is likely to have heterogeneous mass due to multiple isoforms and posttranslational modifications, molar units more accurately describe tau in CSF. Therefore, concentration units for our MS-based assay were expressed as pmol/L rather than the standard pg/mL units typically used by immunoassays.

Developing an MS assay suitable for clinical sample analysis requires the meeting of key requirements for assay imprecision. The use of heavy isotope-labeled tau protein as an IS, added at the very beginning of the procedure before immunoaffinity enrichment, was required to account for variability throughout sample processing. Extensive up-front testing of the IS ensured that the tau IS was recovered equally with the endogenous tau.

Tau values correlated between the 2 assays, but some scatter was seen around the correlation line. This may indicate that although both assays seemed to rec-

ognize the same analyte, there may be some subtle differences in analytical specificity, which was also reflected by the slight change in fold-change between patients with AD and controls measured by both assays. Therefore, our IA-MS assay provides a useful tool that can be used in the better understanding and qualification of immunoassays. This is of particular value for current collaborative efforts, such as with the Global Consortium for the Standardization of CSF Biomarkers (21), which is working to establish standardized reference methods for the measurement of clinical AD biomarkers.

The LOQ of our assay rivals or surpasses that of some immunoassays. Additionally, the specificity of MS can be used to understand modifications or changes in the tau protein that are relatively uncommon but may have a profound relationship with disease progression. MRM analysis can easily be developed to monitor multiple tau-derived peptides, including those containing posttranslational modifications. Analysis of protein turnover (synthesis and clearance) can also be accomplished by an established IA-MS method having sufficient ability to detect the incorporation of heavy isotope AAs in newly synthesized protein. Therefore, the establishment of an IA-MS assay to a protein implicated in human disease such as tau provides an opportunity to open up new lines of scientific discovery.

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