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A mass spectrometry-based method to quantify in parallel Tau and amyloid β 1–42 in CSF for the diagnostic of Alzheimer's Disease

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Abstract

Alzheimer's disease (AD), the most common form of dementia, afflicts about 50 million people worldwide. Currently, AD diagnosis is primarily based on psychological evaluation and can only be confirmed post-mortem. Reliable and objective biomarkers for prognosis and diagnosis have been sought for several years. Together, tau and amyloid β 1–42 ($A\beta_{42}$) in cerebrospinal fluid (CSF) have been shown to provide good diagnostic sensitivity and specificity. Additionally, phosphorylated forms of tau, such as tau pS181, have also shown promising results. However, the measurement of such markers currently relies on antibody-based immunoassays that have shown variability, leading to discrepant results across laboratories. To date, mass spectrometry methods developed to evaluate CSF tau and $A\beta_{42}$ are not compatible. We present in this article the development of a mass spectrometry-based method of quantification for CSF tau and $A\beta_{42}$ in parallel. The absolute concentration of tau and $A\beta_{42}$ we measured are on average 50 ng/mL (7–130 ng/mL) and 7.1 ng/mL (3–13 ng/mL), respectively. Analyses of CSF tau and $A\beta_{42}$, in a cohort of patients with AD, mild cognitive impairment and healthy controls (30 subjects), provide significant group differences evaluated with ROC curves ($AUC_{(control-AD)}$ and $AUC_{(Control-MCI)}=1$, $AUC_{(MCI-AD)}=0.76$), with at least equivalent diagnostic utility to immunoassay measurements in

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Conflict of interest

The authors declare no competing financial interest.

the same sample set. Finally, a significant and negative correlation was found between the tau and Aβ peptide ratio and the disease severity.

For Table of Contents Only
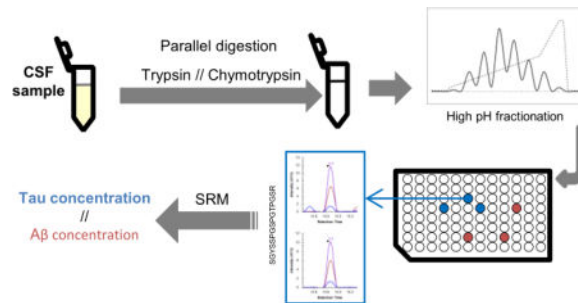
Keywords

Alzheimer's disease; mass spectrometry; SRM; diagnosis; tau; amyloid β; $A\beta_{42}$

Introduction

Diagnosis of Alzheimer's disease (AD), the most common form of geriatric dementia, relies largely on clinical and neuropsychological evaluation, and confirmation of the diagnosis can only be obtained post-mortem (1). Upon autopsy, pathological examination of the AD brain reveals two characteristic hallmarks: neuritic plaques, primarily containing amyloid β (Aβ), of which the 42-amino acid form ($A\beta_{1-42}$) is more prone to aggregation than the 40-amino acid form (2); and neurofibrillary tangles containing tau, including its phosphorylated forms. However, objective biomarkers capable of detecting AD and tracing its course in living patients are an area of substantial interest, leading to studies that have investigated new markers to diagnose or evaluate the disease progression. The candidate markers are many, including proteins and lipid markers in body fluids as well as brain imaging using magnetic resonance imaging or positron emission tomography (3), (4). The most promising biomarkers to date come from cerebrospinal fluid (CSF) of living patients, where the levels of $A\beta_{1-42}$ and tau can be monitored. These studies (5), (6), (7) revealed a decrease of Aβ in the CSF of AD patients, which inversely correlated with the formation of plaques (8), (9). On the contrary, tau and phosphorylated tau increase in CSF of AD patients, with at least some tau forms correlating with the formation of tangles (10), (8), (11). These changes in levels of the two key proteins make them valuable markers of the disease, especially when their concentrations are studied together, and CSF tau and $A\beta_{1-42}$ remain two of the most targeted proteins thanks to their high power of discrimination between healthy control and AD patients as well as their mechanistic association with the disease.

A complication in the use of $A\beta_{1-42}$ and tau as clinical biomarkers is their quantification using different immunoassay platforms, such as ELISA, Luminex, meso scale discovery (MSD) (12), (13), (6), (14), and so on. These platforms depend on antibodies, leading to variability in their inherent efficiency, as well as substantial differences between assays using different antibody pairs, complications due to matrix effects in samples, and difficulty

in measuring the absolute concentration of the markers. The standardization of CSF tau and $A\beta_{1-42}$ measurements using immunoassays between laboratories has proven difficult (15), (16), and this phenomenon is not unique to the AD field (17). For example, a mass spectrometry-based assay has been developed to quantify synuclein isoforms in CSF, showing the value of measuring more than one marker to increase the potential of the assay (18). Similarly, to eliminate the limitations imposed by the use of antibodies, research groups have attempted the quantification of tau (19) or $A\beta_{1-42}$ (20), (21), (22) in CSF using mass spectrometry techniques, such as selected reaction monitoring (SRM), which relies on quantifying the intensity of signal produced by detection of peptides generated from the targeted protein (for more details see (23)). The SRM method not only provides highly selective and sensitive measurements of distinct proteins, but also demonstrates good precision and excellent reproducibility across different laboratories (24), (25).

The major challenge in using SRM to quantify tau and $A\beta$ is their biochemical dissimilarity, leading investigators to use separate assays under specific conditions providing the best detection and quantification for each target, that is, tau (19) or $A\beta$ (20), (21), (22). In particular, the detection of tau, being a protein of low abundance in CSF and, more importantly, being represented by multiple fragments in CSF, remains challenging without initial enrichment. Although using antibodies to enrich both proteins at the same time before analysis would, in theory, increase the sensitivity, this has not been reported as a successful approach; so far each of the previous studies presents the optimal conditions for the quantification of the marker of interest, often using conditions that are not compatible, making it impossible to quantify both biomarkers simultaneously (i.e., in a single mass spectrometry run), or in parallel (where the sample processing for both analytes is similar enough to prepare simultaneously, and no modifications to the MS machinery or protocols are required between analyte runs). Our goal here was to establish a method that would allow the quantification of both tau and $A\beta_{42}$ in a single experiment. This approach would provide not only time savings by analyzing two biomarkers in parallel in one experimental run, but also reduced variation, given that processing the samples in a parallel manner reduces the difference in handling between experiments. A simultaneous or in-parallel strategy also provides additional chances to improve the sensitivity and specificity of the assay by quantifying tau in conjunction with $A\beta_{1-42}$, as demonstrated by immunoassays (e.g., INNO-BIA AlzBio3 Luminex and MSD assays) (26), (27), (14). Here we present a method of fractionation allowing the detection and quantification of peptides from tau and $A\beta$ in parallel, with at least equivalent diagnostic utility to immunoassays.

Materials and Methods

Materials

Trypsin gold (mass spectrometry grade) and chymotrypsin were obtained from Promega (Madison, WI). Glass screw neck vials for liquid chromatography autosampler were from Waters (Milford, MA). Protease Inhibitor Cocktail (PIC), dithiothreitol (DTT), and iodoacetamide (IAA) were obtained from Sigma (St. Louis, MO). Acids and organic solvents were HPLC grade. Recombinant tau 441 and Beta-amyloid (1–42) standards

unlabeled (quantified using amino acid analysis) and ^{15}N tau 441 uniformly labeled and ^{15}N Beta-amyloid (1–42) uniformly labeled were purchased from rPeptide (Bogart, GA).

Characterization of subjects and sample collection

The Institutional Review Boards at the University of California at San Diego, Oregon Health Science University and the University of Washington (UW) approved this study. "Reference CSF" samples were used to develop and assess the assay, and consisted of pooled samples discarded following clinical testing at Harborview Medical Center, UW. After collection and clinical assessment, the "reference CSF" samples were stored at 4°C until transfer to our laboratory. Upon reception, the samples were treated with PIC at the manufacturer's suggested concentration. Subsequently, the samples were pooled, aliquoted and stored at −80°C until further use. A total of 30 subjects, including 10 patients with AD, 10 patients with mild cognitive impairment (MCI) and 10 age- and sex- matched healthy controls were used. All CSF samples were obtained after informed consent from patients who underwent thorough medical history evaluation, physical and neurological examinations, laboratory tests, and neuropsychological assessments. The inclusion and exclusion criteria have been previously described (28). Briefly, AD dementia patients were diagnosed with probable AD according to NINDS-ADRDA criteria (29). MCI subjects had a Mini Mental Status Examination (MMSE) score between 24–30, a memory complaint, objective memory loss measured by education adjusted scores on Wechsler Memory Scale Logical Memory II, a Clinical Dementia Rating (CDR) of 0.5, absence of significant levels of impairment in other cognitive domains, essentially preserved activities of daily living, and an absence of dementia. The control subjects consisted of healthy community volunteers and had no signs or symptoms of cognitive impairment or neurological disease; all control subjects had a MMSE score between 28 and 30, a CDR score of 0, and New York University paragraph recall scores (immediate and delayed) of > 6. The summary of demographics of subjects is provided in Table 1; there was no significant group difference on age (p=0.13).

All CSF samples were collected by lumbar puncture in the morning, processed and stored at −80°C following standard clinical protocols and quality control procedures as described previously (28). At the UW, the samples were thawed, treated with 10% protease inhibitor cocktail (PIC) and aliquoted before once again being frozen and stored at −80 °C until further analysis.

Sample preparation and protein digestion

Uniformly labeled ^{15}N Ttau 441 and ^{15}N Beta-amyloid (1–42) were spiked into 100 μL CSF samples to a final concentration of 5 fmol/μL and 20 fmol/μL respectively; and the proteins were precipitated with 20% trichloroacetic acid (final concentration). After 2h of incubation at −20°C, the samples were centrifuged at 15000 × g for 15min at 4°C and washed twice with 180 μL of cold acetone. Dried pellets were solubilized in 50 μL of urea solution (8M for trypsin digest or 4M for chymotrypsin digest) and the total amount of protein was determined using the Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The protein mixture was reduced, for 1h at 37°C, with 5mM DTT (final concentration) and alkylated for 30 min, in the dark at room temperature, with 15 mM iodoacetamide (final concentration). After dilution (1:10, v:v) of the protein sample with

25mM ammonium bicarbonate, enzymes, trypsin or chymotrypsin, was added at the approximate ratio 1:25 (enzyme:protein, w:w). Samples were then incubated overnight at 37°C with agitation. After the incubation, the digestion was quenched by adding trifluoroacetic acid (TFA, 0.4%, final concentration) and acetonitrile (ACN, 2% final concentration). The peptides were finally desalted using microspin columns, C18 silica (Nest Group, Southborogh, MA), following the manufacturer's protocol. After desalting, peptides were dried under vacuum and resuspended in 25 μL of 2% ACN, 0.1% formic acid. The peptide concentration was determined using BCA protein assay kit. For the high pH fractionation, the volume equivalent to 35 μg of peptides was transferred to a glass vial and the volume was adjusted to 70μL.

High pH peptide fractionation

The peptide separation was performed on a U3000 nanoHPLC system (Dionex, Thermo Scientific) following the method published by Shi *et al.* (30). In brief, 50 μL of peptide samples diluted to 0.5 μg/μL were loaded and separated on C18 column (home-packed 50 cm long, 200 μm ID, Jupiter® 300Å C18 3μm diameter, from phenomenex, Torrance, CA, USA). The separation was performed using a ratio between two buffers, 10 mM ammonium formate in water at pH 9 (buffer A) and 10 mM ammonium formiate in 90% ACN (buffer B) at a flow rate of 2.2μL/min. The gradient applied was first loading and equilibrating the column for 30 min at 1% B, followed by gradient increase to 5% B within 2 min, 60 min gradient up to 45% B, increase to 90% B in 13 min, 3 min at 90% B, decrease to 1% B within 3 min, and 14 min at 1% B. Fractions were collected every 3 min from the beginning of the gradient in collection tubes containing 18.4 μL of 2% ACN, 0.1% formic acid.

Selected reaction monitoring (SRM) assay

Quantification of target peptides from tau and $A\beta_{1-42}$ by SRM was performed on a TSQ Vantage, triple quadrupole mass spectrometer (Thermo Scientific) coupled to a nanoAcquity UPLC (Waters). Four (4 μL) microliters of protein digest or high pH fractions was loaded on C18 trap column (20 mm long, 75μm ID) and separated on a reversed-phase home-packed fused silica column, 75 μm i.d. × 30 cm (100 Å Magic C18AQ; Michrom/Bruker, Auburn, CA, USA) at a flow rate of 0.3 μL/min using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in ACN as mobile phase B. Elution was carried out using a binary gradient increasing from 2 to 30% B in 15 min, 30% to 80% B in 1 min, 80% B for 5 min, 80% to 2% B in 1 min, finally the column was re-equilibrated with 2% B for 8 min. All MS experiments were performed in positive ion mode with source temperature set to 350°C and spray voltage to 2.1 kV. Scheduled SRM was performed with 8 min retention time windows for most of the peptides and an instrument cycle time of 2000 ± 500ms. Dwell times were optimized based on the number of concurrent transitions but in all cases they were at least 50 ms. Collision energies for each transition were calculated using Skyline software, which have previously been tested for accuracy in our hands. Both of the Q1 and Q3 peak widths (FWHMs) were set to be 0.70.

To construct the calibration curves for the target peptides, recombinant unlabeled and ^{15}N-labeled proteins were used. Standard proteins were digested in 50 μg of BSA following the conditions of the CSF digest. Unlabeled protein standard was resuspended in Buffer A and

serial dilution was performed to yield concentrations ranging from 100 pmol/mL to 0.013 pmol/mL (4.6 µg/mL to 0.6ng/mL) for tau and 1000 pmol/mL to 0.13 pmol/mL (4.5µg/mL to 0.6ng/mL) for $A\beta_{1-42}$. Digested ^{15}N-labeled peptides were spiked at 50 pmol/mL final concentration for tau and 200 pmol/mL final concentration for $A\beta_{1-42}$ on the serially diluted standards. Four microliters of each standard was injected on column for SRM analysis (see table S-1 for transitions list).

SRM Data Analysis

All SRM raw data files were uploaded and processed in Skyline software ver. 3.5.0.9319 (McCoss Lab, University of Washington). Typical settings applied include 0.055 Th match tolerance m/z, default peak integration and Savitzky–Golay smoothing algorithm. Manual inspection and evaluation of data sets were done to ensure correct peak detection and integration. Quantification was performed using peak areas of at least the three best performing transitions for both unlabeled and labeled peptides (see table S-1 for the best transitions).

Immuno-based Assay

Levels of tau and $A\beta_{1-42}$ were assessed by MSD immunoassays and published previously (14), which generate comparable data to the commonly used AlzBio3 Luminex assay.

Statistical analysis

All statistical analyses were performed in Mathematica (Mathematica software, Champaign, IL, USA, www.wolfram.com/mathematica V11.0.0.0) and GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com Prism 6.0). Analysis of Variance (ANOVA) followed by posthoc Tukey HSD test were used to compare group differences between AD, MCI and controls for both MSD and SRM assay. Receiver Operating Characteristic (ROC) curves for analytes were generated to evaluate their sensitivities and specificities in distinguishing AD from healthy control subjects. The "optimum" cutoff value for a ROC curve was defined as the value associated with the maximal sum of sensitivity and specificity. Spearman correlation was used to determine correlation between CSF Aβ or tau MSD assay measurements and the SRM results. Values with p<0.05 were considered significant in the analyses.

Results

Method development

We developed a mass spectrometry-based method for the quantification of tau and Aβ in parallel. In our initial protocol we processed the samples with trypsin, followed by the direct injection of the sample in the LC-MS system for SRM measurement. With this approach, trypsin cleavage of Aβ generates one peptide corresponding to amino acids in positions 17 to 28 of $A\beta_{1-42}$ (see figure 1A), easily detectable in CSF, while tau peptides remain barely detectable and/or unreliable. To improve the detection of tau peptides, we added a high pH fractionation prior to the LC-MS measurement (see synopsis in figure 1B). By simplifying the sample with the fractionation, we were able to reliably detect and quantify two peptides from tau in reference CSF, covering the sequences 194 to 208 and 211 to 220 of tau 441.

The fractionation allowed us to increase the signal intensity for both heavy and light peptides. For example, from the same reference CSF sample, the signal intensity for the heavy tau peptide SGYSSPGSPGTPGSR (peptide 194–208) was increased sixty fold and the light signal was brought above the detection and quantification limits. For the tau peptide TPSLPTPPTR (peptide 211–220), the heavy signal intensity was increased five fold and the light signal was brought above the noise signal to a more acceptable level for its quantification. Figure 1B shows the signal after fractionation for those two peptides (see also a comparison in figure S-1). A third peptide from tau showed a reproducible detection in reference CSF, however, the expression of this peptide remains quite low and close to the limit of quantification. Figure 2A shows the difference of concentration of the most intense peptides (TPSLPTPPTR and SGYSSPGSPGTPGSR) and the third peptide of low intensity (STPTAEDVTAPLVDEGAPGK). We estimate the difference of concentration to approximately 7.2 fold in the same reference CSF sample. The peptide STPTAEDVTAPLVDEGAPGK is part of the N2 region of tau, which is not expressed in every tau isoform. This would explain the difference in expression between this peptide and the others, as well as the lower intensity of this peptide.

Trypsin digestion followed by high pH fractionation resulted in reliable detection of one quantifiable peptide representative of Aβ. During our initial evaluation of the assay, when tested in pooled samples, the pattern of expression of Aβ did not show any differences between control, MCI and AD patients (data not shown). This could be explained by the fact that the peptide measured (LVFFAEDVGSNK) is shared between several proteoforms of Aβ and thus is not representative of $A\beta_{1-42}$. The only peptide able to discriminate $A\beta_{1-42}$ from the other proteoforms would be the C-terminal peptide (GAIIGLMVGGVVIA, figure 1A), which was not etectable under these conditions.

To detect the C-terminal portion of $A\beta_{42}$ we used chymotrypsin, which generated two detectable peptides—AEDVGSNKGAIIGL and MVGGVVIA (figure 1A). The latter peptide represents the last eight amino acids of $A\beta_{1-42}$ and is thus specific to this proteoform. Figure 2B shows the difference in expression of the peptides generated by chymotrypsin. We estimate approximately 6.8 fold differences between both peptides within the same reference CSF sample, which likely illustrates the difference in quantity between the peptide shared among proteoforms of Aβ and the C-terminal peptide of $A\beta_{1-42}$. This C-terminal peptide of $A\beta_{1-42}$ includes the amino acids that differentiate $A\beta_{1-42}$ from $A\beta_{1-40}$ and $A\beta_{1-38}$. However, as we do not measure the entire proteoform, we differentiate in the text the full form "$A\beta_{1-42+}$" from the form we measure "$A\beta_{42}$". Because of the low abundance of the C-terminal peptide of $A\beta_{42}$, samples digested by chymotrypsin are also fractionated with our high pH method prior to SRM measurement to reduce the noise and increase the sensitivity of the detection. Finally, the expression of the Aβ peptides LVFFAEDVGSNK, from trypsin digest and AEDVGSNKGAIIGL, from chymotrypsin digest show a good correlation (p<0.001), indicating comparable digests (figure S-2).

To validate the reproducibility, we performed three repetitions of the assay using reference CSF, evaluating the variation between each sample preparation. Table 2 summarizes the characteristic of each peptide monitored in this assay (standard curves are shown on figure S-3).

Comparison of the SRM assay to the corresponding immunoassay on CSF samples

We tested our SRM assay in CSF from a small cohort of clinical samples from control subjects or patients with MCI or AD, which we have previously measured for tau and $A\beta_{1-42}$ concentrations using immunoassays. For tau, among the three peptides detectable in our SRM assay only two were quantifiable in all samples, while the third (STPTAEDVTAPLVDEGAPGK), positioned at the N-terminal region of the protein, could be detected in eleven samples and quantified in eight. However, while this peptide provides the lowest concentration for tau, the comparison of the SRM concentrations with the immunoassay results reveals values on average twelve times higher than the concentrations obtained by immunoassay.

To compare our assay with traditional immunoassays, we determined the correlation between the quantification obtained in the current SRM assay with the data previously obtained with MSD assay for the same set of samples. The concentrations, obtained with the peptides TPSLPTPPTR and SGYSSPGSPGTPGSR, that could be quantified by SRM in every sample analyzed, significantly correlated (ρ=0.74 and 0.82 respectively and p<0.001 for both peptides, figure S-4A and B) with the concentrations measured using the immunoassay. Both peptides were estimated to have similar concentrations in each sample. When the expression level of both peptides were averaged, we estimated the average concentration to be close to ninety times higher than the concentration obtained by immunoassay (correlation ρ=0.83, p<0.0001), likely due to the antibodies capturing and detecting a sub-population of tau species in CSF (figure 3A). The average would compensate for any variation between samples, due to changes in digest, post-translational modifications, and so on. The correlation for the peptide STPTAEDVTAPLVDEGAPGK was only borderline significant (ρ=0.52, p=0.07, figure S-4C), probably resulting from the small number of samples. Considering that this peptide is not representative of tau in the majority of the sample, we disregarded this peptide in the rest of the analysis.

The discrepancies between immunoassay and SRM raise the question of the accuracy of either of the assays. When comparing the values obtained by the immunoassay on the standards used for mass spectrometry (quantified by amino acid analysis), we observed a more than 3 fold decrease between the expected concentration and the measured value (Table S-2), suggesting that the immunoassay might underestimate the "true" concentration. Inversely, the quantification of the MSD standards by SRM showed that the concentration was 30 times higher than the labeled concentration for the standard (Table S-2). Taken together, those differences led to more than a hundred fold difference between the two assays, which may at least partially explain the discrepancies in CSF tau concentrations measured by the immunoassay and SRM.

Regarding the $A\beta$ peptides targeted in this assay, the chymotryptic peptide corresponding to the C-terminal portion of $A\beta_{42}$ significantly correlates (ρ=0.56, p<0.05) with the previous data (figure 3B). The concentration for $A\beta_{42}$ obtained by SRM are however thirty two times higher than the concentration measured with immunoassay for $A\beta_{1-42}$ specifically. On the contrary, the tryptic peptide LVFFAEDVGSNK and the chymotryptic peptide AEDVGSNKGAIIGL did not correlate with the immunoassay values (figure S-5A and B). Additionally, the shared peptides characterize the global expression of amyloid β, including

its truncated forms, such as $A\beta_{1-40}$, $A\beta_{1-38}$, P3 and so on., but exclude the soluble form of APP such as sAPP-α and sAPP-β. We thus evaluated the ratio of $A\beta_{42}$ we measured in our assay over the global expression of amyloid β ($A\beta_{42}$/Amyloid β). To do so we divided the expression of the peptides produced by chymotrypsin –MVGGVVIA (C-terminus of $A\beta_{1-42}$) divided by AEDVGSNKGAIIGL (shared between several amyloid β forms, such as $A\beta_{1-40}$, $A\beta_{1-38}$, P3 etc.). Interestingly, the ratio ($A\beta_{42}$/Amyloid β) significantly correlated ($\rho=0.52$, $p<0.05$) with the immunoassay values (figure 3C).

Evaluation of the discrimination of the assay

To further validate the performance of our assay, we evaluated the benefits provided by the SRM assay comparing healthy control, patients with MCI patients and AD patients (n=10 in each group). The SRM results for tau showed significant differences between healthy control, MCI and AD subjects ($p<0.001$, figure S-6A). Aβ on the contrary, shows a nonsignificant decrease in MCI and AD (p=0.46, figure 4A). This can be explained by the proteoform targeted in our assay. By measuring the C-terminal peptide of $A\beta_{1-42}$ the quantities are exposed to interferences from other proteoforms that contain this region of the peptide, for example, forms truncated at the N-terminal portion. The difference between healthy control and demented patients was, however, improved with the ratio $A\beta_{42}$/Amyloid β, which displays a significant difference between healthy control and MCI and AD ($p_{(Ctl-AD)}<0.0001$, $p_{(Ctl-MCI)}<0.0001$, figure 4B)

Taken together as a ratio (Tau/Aβ) the comparison of healthy control, MCI and AD subjects (figure 4C) shows significant differences ($p<0.001$), similar to the immunoassay data ($p<0.0005$, figure 4D). Furthermore, this is illustrated by the ROC curve, presented in figure 5A, indicating that the tau/Aβ ratio could discriminate MCI patients and healthy control or AD patients and healthy control quite well (AUC= 0.96 for both conditions). In contrast the ROC curve comparing AD and MCI displays a moderate level of discrimination (AUC=0.71), possibly reflecting the heterogeneity of MCI disease, which features a possible evolution to AD. Those values remain fairly comparable to the ROC curves obtained with the same set of samples measured with MSD ($AUC_{(control-AD)}= 1$, $AUC_{(Control-MCI)}=0.85$, $AUC_{(MCI-AD)}=0.69$, figure S-7). Finally, to isolate the performance of the relative level of $A\beta_{42}$, we normalized it to the total level of Amyloid β (i.e. $A\beta_{42}$/Amyloid β) and compared the use of the ratio tau/($A\beta_{42}$/Amyloid β) to the ratio tau/$A\beta_{42}$. We showed first that the $A\beta_{42}$/Amyloid β ratio significantly improves the difference between MCI and AD patients ($p<0.05$, figure 5B). The additional ratio also improved the discrimination between healthy control, MCI and AD demonstrated with the ROC curve ($AUC_{(Control-AD)}= 1$, $AUC_{(Control-MCI)}=1$, $AUC_{(MCI-AD)}=0.76$, figure 5C). It should be noted that, due to the small sample size, the ROC curve analysis here remains quite preliminary, and thus further evaluation is needed to validate this result in future studies using a larger number of cases.

Finally, to further evaluate the performance of the assay, we compared the new ratio tau/($A\beta_{42}$/Amyloid β) to the neurological evaluation of the patients (MMSE). The ratio significantly and negatively correlated ($\rho=-0.65$, $p<0.001$) with mental state of the patients (figure 5D). The correlation between tau/Aβ and MMSE was not demonstrated before; however, using the data obtained by MSD, this correlation was also true (data not shown).

The improvement provided by the new ratio as well as the statistical evalutation of the analytes measured in our assays are sumarized in table 3.

Discussion

This study presents important advancements regarding measuring tau and $A\beta_{42}$ in CSF using antibody-free, mass spectrometry-based methods. First, we made it possible to measure CSF tau and $A\beta$ peptides in parallel. Second, we developed methods to quantify peptides specifically derived from tau and $A\beta_{42}$, and when tested on a small cohort of samples, significant diagnostic group differences were observed. Third, we show that in our assay the ratio of $A\beta_{42}$ to the global expression of amyloid β significantly improves the diagnostic utility of our SRM assay. And fourth, we also showed a significant negative correlation between the ratio generated with our assay and the MMSE score.

The rationale for developing a new assay for both analytes together is mainly the adaptation to the downstream method of measurement. Indeed, the most universal method of liquid chromatography coupled to mass spectrometry is a reverse phase method under acidic conditions, using acetonitrile as the organic solvent for elution. The previous successful methodologies used to quantify either tau or $A\beta_{1-42}$ employed liquid chromatography separation more specific to their purposes. For the tau peptides, the mobile phase contained water and formic acid and the elution was performed with a mix of methanol and formic acid. On the contrary, the nondigested form of $A\beta_{1-42}$ was transported in a liquid phase containing water, acetonitrile and ammonia and eluted in a buffer containing acetonitrile, methanol and trifluoroethanol. These liquid chromatography methods are quite different, and would thus either require replacing the liquids in the chromatography system between runs, or using two liquid chromatography systems in parallel. In these cases it is not practical to measure both analytes in parallel in a single system. Therefore, we sought to identify a fractionation strategy suitable for solving multiple challenges: first, allowing separation and detection of both $A\beta_{42}$ and tau peptides under the same conditions, and second, sufficient simplification of the CSF sample to allow observation of low-abundance tau peptides without predigestion enrichment, such as was necessary in previous studies (19), (31), (32). The strategy we developed allows the simultaneous detection of peptides from tau and other protein species like $A\beta$. For the postdigestion fractionation, several options were available (ion exchange, SPE, IEF, etc.). We chose high pH reverse phase fractionation, as it is highly robust and produces fractions compatible with mass spectrometry with limited processing between the fractionation and the mass spectrometry measurement. In addition this method was previously employed for $A\beta$ peptides and proven valuable (33).

Another difficulty was the discrimination of $A\beta_{1-42}$ from the other proteoforms of amyloid β. To circumvent this problem, as trypsin did not generate peptides specific to $A\beta_{42}$ that could be detected with mass spectrometry under our conditions, we utilized a different enzyme. Indeed, chymotrypsin digestion provided us with two peptides that covered the shared part of the peptide sequence and the C-terminal part of $A\beta_{1-42}$. With the peptide common to several amyloid β proteoforms, we were able to compare the digests between enzymes, certifying the reliability of the digest. On the contrary, the C-terminal peptide was more representative of the expression of $A\beta_{1-42}$, and it correlated with the expression of

$A\beta_{1-42}$ previously measured by immunoassay (14). Comparing the absolute concentrations measured with SRM with the immunoassay results we observed higher concentrations with SRM. The concentrations measured here, in the lower nanogram per mililiter range, are comparable to previous studies (20) where the concentrations of $A\beta$ were in the higher picogram to lower nanogram per milliliter range. The slight difference between the SRM assays could result from several factors such as sample collection, handling and preparation. Additionally, recent discoveries (34) showed that the ratio of $A\beta_{1-42}$ to either $A\beta_{1-40}$ or $A\beta_{1-38}$ improves the diagnostic value of $A\beta_{1-42}$. While we did not measure peptides from $A\beta_{1-40}$ or $A\beta_{1-38}$ in our assay, we did monitor an additional peptide, which is shared between several proteoforms of amyloid β, including $A\beta_{1-40}$ and $A\beta_{1-38}$. The ratio of these two peptides ($A\beta_{42}$/Amyloid β) allowed us to improve the diagnostic significance of our assay, similar to the findings using $A\beta_{1-40}$ or $A\beta_{1-38}$. Developing assays to monitor these two peptides specifically may be desirable in the future, but it is also worth noting that the C-terminal peptide we monitor in the current assay is eight amino acids long, and is already at the lower limit regarding the peptide length measurable with SRM. The C-terminal peptides for $A\beta_{1-40}$ and $A\beta_{1-38}$, using chymotrypsin, would be six and four amino acid long respectively, making their detection difficult. Thus, alternative strategies and additional assay development will be necessary to measure these proteoforms specifically.

The level of expression of tau measured by SRM was substantially higher than that obtained with immunoassays. This difference resulted from a combination of multiple factors. First, the immunoassay appears to underestimate even the amount of standard proteins (Table S-2). Second, tau is a protein displaying a large variety of fragments, either produced by alternative splicing (35), (36) or by post-translational modification as already observed in the brain tissues (37). The isoform or isoforms included in an immunoassay depend on the antibodies used to capture and detect the analyte. As a result, the quantitative values provided by the immunoassay represent a portion of the isoform of tau forms. On the contrary, the SRM approach likely combines more isoforms (unmodified ones in this study) and measures the total concentration for all forms containing the target peptide(s). Thus a direct comparison between immunoassay and SRM remains difficult. Thrid, the immunoassays, to which we compared our results, targeted total tau protein, including its isoforms, according to the manufacturer. However, considering that capture and detection antibodies must bind at two different positions along the sequence of the protein, any cleavage or truncation of tau between those two epitopes would lead to an absence of detection and thus reduce the final concentration, which is at least a theoretical possibility given that the specificity of the antibodies for each tau isoforms remains to be completely defined. Additionally, tau is known to be highly modified and post-translational modifications (PTMs) can certainly result in changes in antibody affinity in immunoassays and reduction of SRM signal. Of note, analyzing PTMs of tau and $A\beta$ species is currently being carried out in our laboratory. Fourth, Barthelemy *et al.* (32) showed that the apparent expression of tau peptides varies depending on their position within the sequence, which suggested that depending on the position of the capture and detection antibodies' epitopes, the measured tau concentrations may vary significantly. With the limited information regarding the commercial kit that was used for the immuno-detection of tau, we cannot compare any further the absolute concentration measured by SRM and the concentration

obtained by immunoassay. However, this may also explain why we see differences between our three tau peptides, knowing that STPTAEDVTAPLVDEGAPGK is located at the N-terminal portion of the protein, in the N2 region of tau, and TPSLPTPPTR and SGYSSPGSPGTPGSR are part of the core of tau. In addition, Barthelemy *et al.* (32) also observed a twenty fold difference when comparing their ELISA and SRM assays, illustrating the difference we could expect when comparing ELISA and SRM on the same region of the protein. When comparing the absolute concentration for tau, measured with SRM, our current values are consistent with previous studies (19), and the concentrations in both cases are in the nanogram per milliliter range. Despite the inherent differences between mass spectrometry and immuno-based methods, it is possible to measure similar trend of expression with SRM and immunoassays.

More importantly, we confirmed the diagnostic utility of our assay through validating the power of the assay for the discrimination of AD, MCI and healthy control subjects. As described in previous studies (38), a ratio gathering the expression from $A\beta$ and tau proteins performs better than each protein taken individually. Also, based on the results of the current assay, we are able to differentiate MCI and AD patients from healthy controls. Nevertheless, as we showed here that the ratio $A\beta_{42}$/Amyloid β improves the diagnostic efficiency of $A\beta_{42}$ we gathered this ratio with the expression of tau, creating a slightly more complex ratio, that is tau/($A\beta_{42}$/Amyloid β). This combination of values was also an improvement as it allowed a significant discrimination of healthy control and MCI patients. Moreover, with our current set of data and our improved ratio, we identified a significant negative correlation between the tau/$A\beta$ ratio and the MMSE, indicating a relationship between this ratio and the disease severity. This correlation suggests the potential of using tau/$A\beta$ ratio as a disease progression marker, although it requires additional investigation. For example, a longitudinal sample cohort could be used to track time-related changes during the progression of AD. As a methodology study and proof of concept, we only utilized a small number of clinical samples (30 in total), and thus our results will require further confirmation with larger sample cohorts in future studies. However, the significant group differences and correlations we observed with such a small sample size in this study suggest the robustness of our SRM assay.

In summary, to the best of our knowledge, this is the first time that CSF tau and $A\beta_{42}$ are targeted in parallel with SRM assay. Using the technology established here AD and MCI subjects were differentiated from healthy controls, with at least equivalent diagnostic utility to immunoassays. Furthermore, regarding the diagnostic power of both markers separately, it would be acceptable to use only one of those markers for the diagnostic of MCI and AD, but it is also clear that the combination of both markers makes this assay more robust. Although our SRM assay needs to be further validated in larger cohorts, this could at least serve as a reference method for CSF tau and $A\beta_{1-42}$ measurements. The validation of this assay will also need to include additional pathologies, especially other tauopathies such as progressive supranuclear palsy and certain forms of frontotemporal dementia. Also, with the same technology, we are currently investigating additional biomarkers that could monitor other aspects of AD pathology and combining them with tau and $A\beta_{42}$, with the intension to further differentiate AD from other dementias and closely follow the disease progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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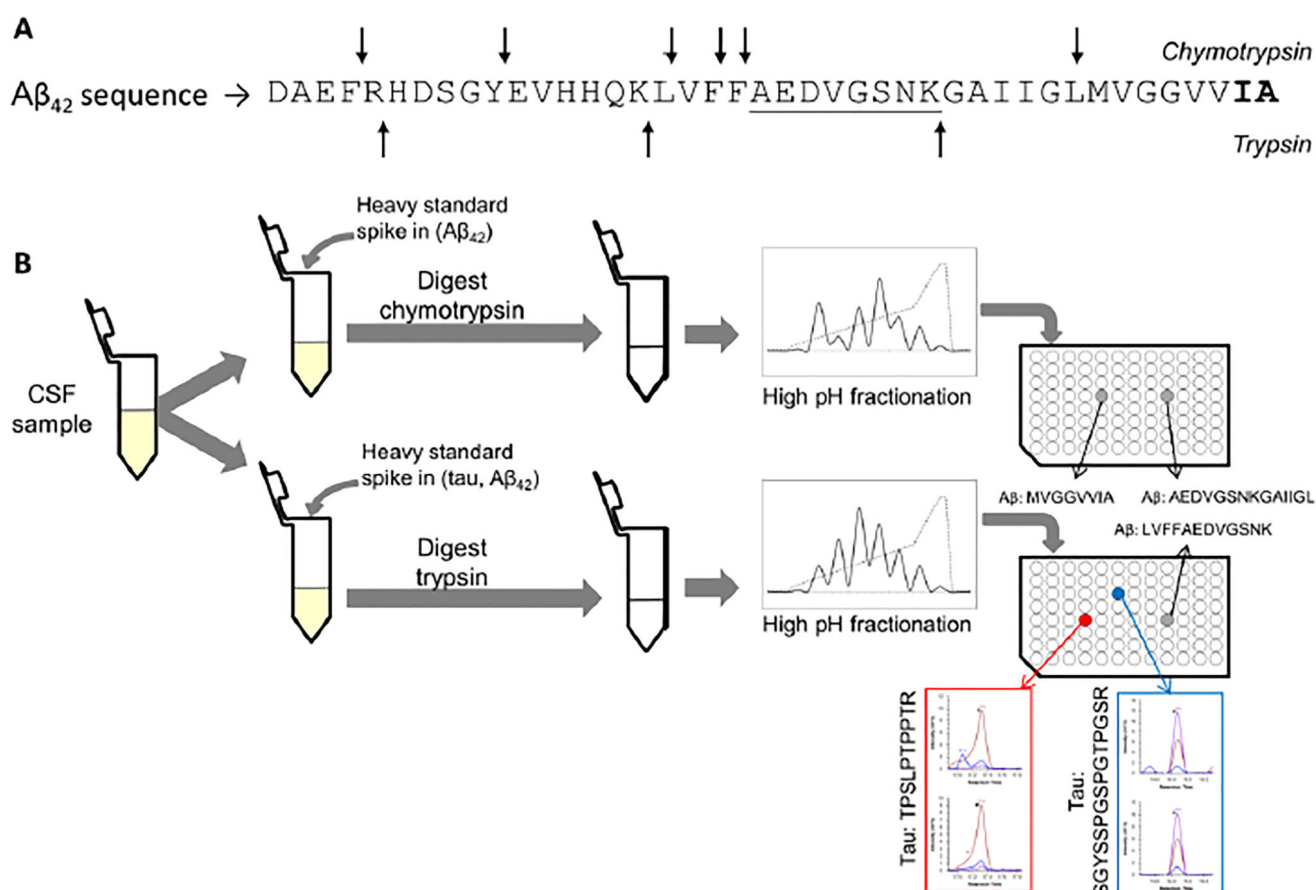
Figure 1.
(A) Sequence of Aβ$_{1-42}$, with the cleavage sites for chymotrypsin (arrows at top of the sequence) and cleavage sites for trypsin (arrows at the bottom of the sequence). The part of the sequence included in both peptides measured after trypsin and chymotrypsin digest is underlined. The amino acid sequence specific to Aβ$_{42}$ is represented in bold at the C-terminal end of the sequence. (B) Schematic illustrating the design of our experiment, showing the digest performed in parallel with trypsin or chymotrypsin, the separate fractionation of the peptides generated by each digest and the analysis of the fraction containing the peptides of interest with LC-MS.
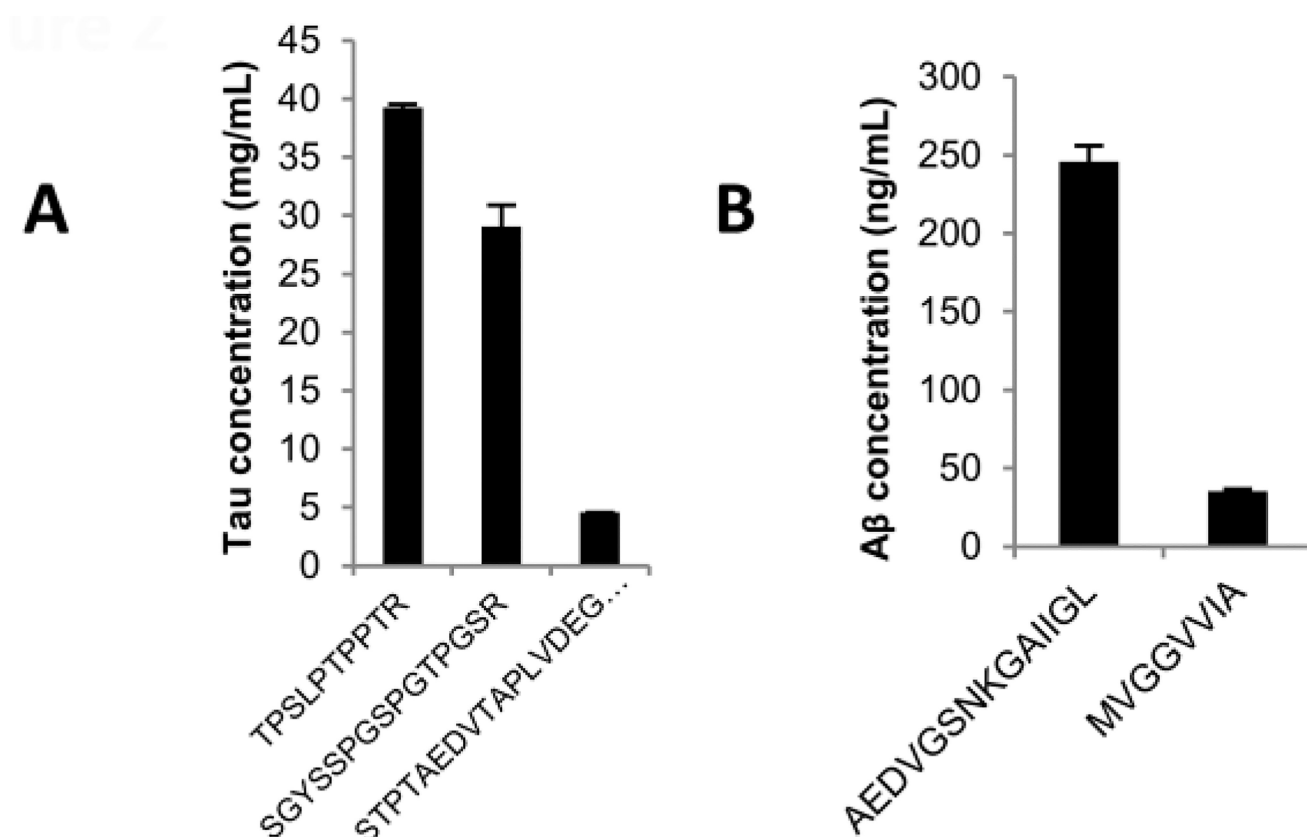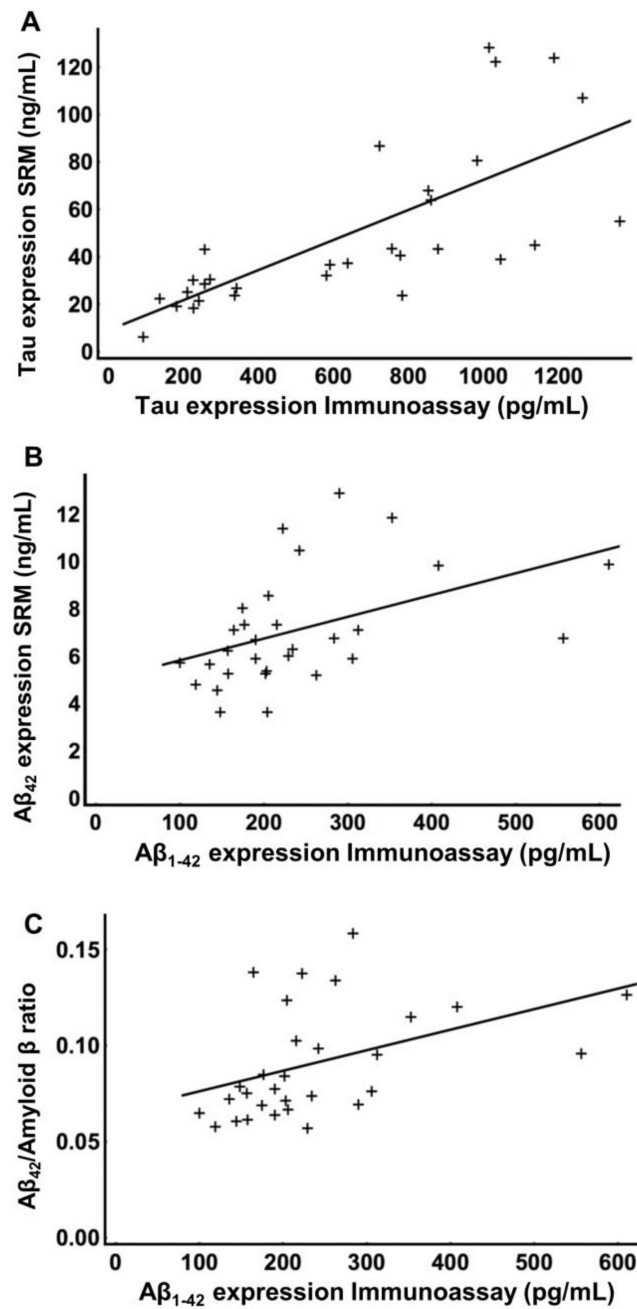
Figure 2.
(A) Histogram illustrating the difference of expression of the tau peptides: TPSLPTPPTR and SGYSSPGSPGTPGSR are part of the core of the protein and have higher level of expression; STPTAEDVTAPLVDEGAPGK is located at the N-terminal portion of the protein and displays a lower level of expression. (B) Histogram illustrating the difference of expression of the Aβ peptide shared between amyloid β proteoforms (AEDVGSNKGAIIGL) and the peptide specific to Aβ$_{42}$ (MVGGVVIA).

Figure 3.
(A) Correlation of the expression of the tau peptides (y-axis, ng/mL) with the immunoassay concentration (x-axis, pg/ml). (B) Correlation of the SRM-based expression of $A\beta_{42}$ (y-axis, ng/mL) with the corresponding immunoassay values (x-axis, pg/mL). (C) and correlation of the ratio $A\beta_{42}$/Amyloid β (y-axis) with the corresponding immunoassay values (x-axis, pg/mL).
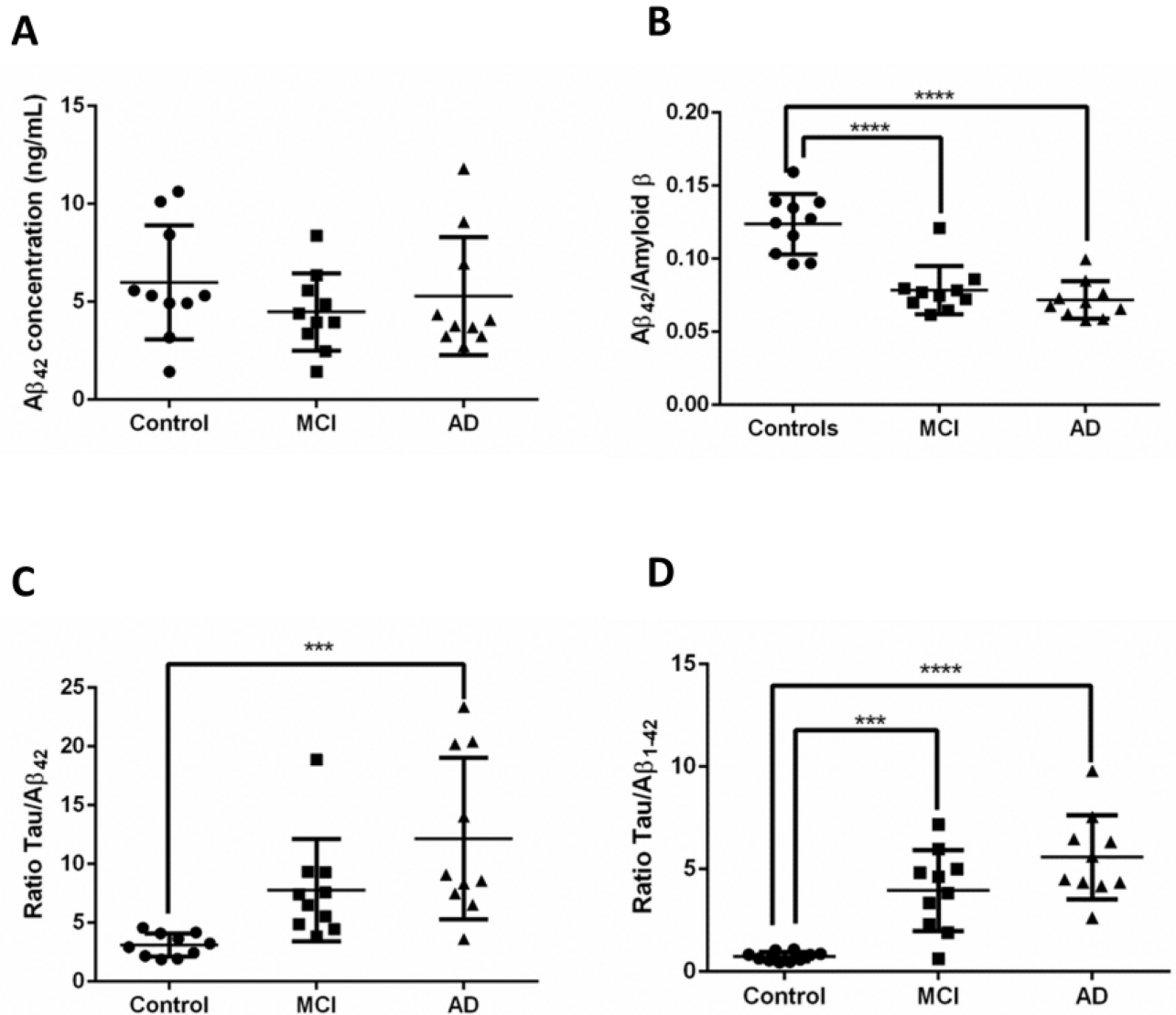
Figure 4.
(A) Aβ$_{42}$, measured with SRM indicates no significant differences between healthy control, MCI patients and AD patients. (B) The ratio Aβ$_{42}$/Amyloid β displays a significant ($p < 0.0001$) difference between healthy control, MCI patients ($p_{(control-MCI)} < 0.0001$) and AD patients($p_{(control-AD)} < 0.0001$). (C) The ratio tau/Aβ, measured with SRM indicates a significant ($p < 0.001$) difference between healthy control, MCI patients and AD patients ($p_{(control-AD)} < 0.0005$). (D) The ratio tau/Aβ measured with MSD displays a significant ($p < 0.001$) difference between healthy control, MCI patients ($p_{(control-MCI)} < 0.005$) and AD patients($p_{(control-AD)} < 0.0001$).
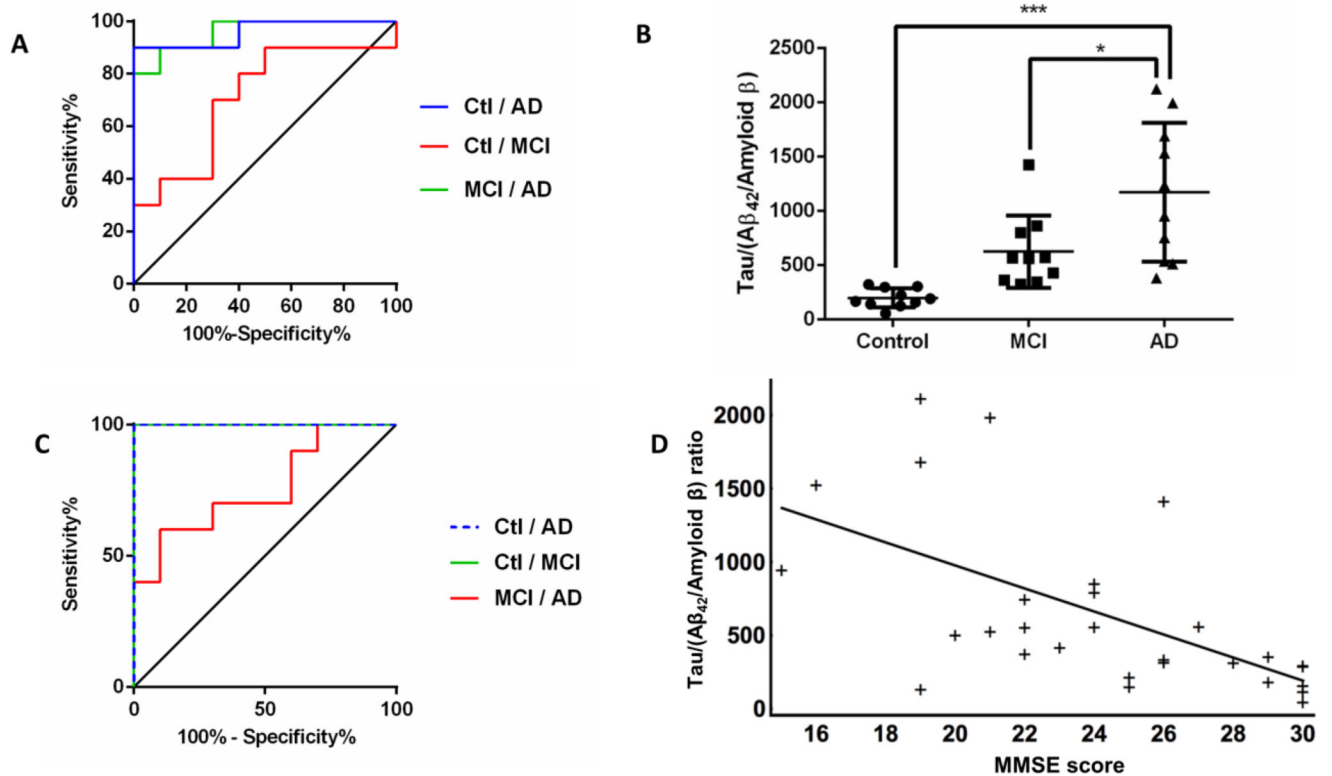
Figure 5.
(A) the ROC curve based on the ratio tau/Aβ obtained with our SRM assay shows a good discrimination comparing healthy control to AD (blue, AUC= 0.96) or to MCI (green, AUC= 0.96). The discrimination between MCI and AD remains fair (red, AUC= 0.71). (B) The ratio Tau/(Aβ$_{42}$/Amyloid β) indicated a significant (p<0.0001) difference between healthy controls and AD patients ($p_{(Ctl-AD)}$<0.0001) as well as a significant difference between MCI and AD patients ($p_{(MCI-AD)}$<0.05). (C) The ROC curve based on the ratio tau/(Aβ$_{42}$/Amyloid β) shows a good discrimination comparing healthy control to AD (dashed blue, AUC= 1) or to MCI (green, AUC= 1). The discrimination between MCI and AD remains fair (red, AUC= 0.76). (D) Based on the results of our SRM assay, the ratio Tau/(Aβ$_{42}$/Amyloid β) shows a significant negative correlation with the MMSE score (ρ=−0.65, p<0.001).

Table 1

Patients information

	Total	Sex (M/F)	Age (±SD)	MMSE (±SD)
Control	10	5/5	72.4 (±5.0)	27.6 (±3.6)
MCI	10	5/5	74.4 (±8.1)	25.1 (±2.1)
AD	10	5/5	67.4 (±9.5)	19.4 (±2.5)

Table 2

Characteristics observed for each peptide monitored in our tau and $A\beta_{42}$ assay. Mean, Standard deviation (SD) and coefficient of variation (CV) were obtained with repeats of a reference sample

			Mean	SD	CV	Linerity range (fmol/ul)	LLOD*	LLOQ*
		TPSLPTPPTR	39.2	0.321	0.8%	0.10–50	0.78	1.56
	Tau	SGYSSPGSPGTPGSR	29.1	1.84	6.3%	0.10–50	0.1	0.1
Trypsin		STPTAEDVTAPLVDEGAPGK	4.57	0.013	0.3%	0.10–6.25	0.1	0.1
	$A\beta$	LVFFAEDVGSNK	227.879	9.666	4.2%	15.6–500	15.6	15.6
		AEDVGSNKGAIIGL	245.987	9.897	4.0%	15.6–1000	15.6	31.25
Chymotrypsin	$A\beta$	MVGGVVIA	35.150	1.355	3.9%	3.9#–500	0.49	0.98

*
The LLOD is the lowest measurement with a CV<20%. The LLOQ is the lowest value with a CV<10%

#
for the peptide MVGGVVIA, LLOD and LLOQ are below the linear range. However, no experimental values are below the lowest value of the linear range.

Table 3

Summary of the statistical values for the analytes monitored with SRM

Protein or ratio	Significance	P(Ctl-AD)	P(Ctl-MCI)	P(MCI-AD)	AUC(Ctl-AD)	Sensitivity/Specificity	AUC(Ctl-MCI)	Sensitivity/Specificity	AUC(MCI-AD)	Sensitivity/Specificity
Tau	<0.0005	<0.0001	ns	<0.05	1	80%/100%	0.92	100%/100%	0.77	80%/70%
Aβ42	ns	ns	ns	ns	0.62	70%/80%	0.68	70%/80%	0.51	30%/90%
Aβ42/Amyloid β	<0.0001	<0.0001	<0.0001	ns	0.98	90%/100%	0.96	90%/100%	0.66	65%/65%
Tau/Aβ42	<0.001	<0.001	ns	ns	0.96	80%/100%	0.96	100%/100%	0.71	80%/100%
Tau/(Aβ42/Amyloid β)	<0.0001	<0.0001	ns	<0.05	1	100%/100%	1	100%/100%	0.76	60%/90%

Non-significant values are reported as ns