

RESEARCH

Open Access



Cerebrospinal fluid phospho-tau T217 outperforms T181 as a biomarker for the differential diagnosis of Alzheimer's disease and PET amyloid-positive patient identification

Nicolas R. Barthélemy^{1,2*}, Randall J. Bateman², Christophe Hirtz¹, Philippe Marin³, François Becher⁴, Chihiro Sato², Audrey Gabelle^{5*} and Sylvain Lehmann^{1*}

Abstract

Background: Cerebrospinal fluid biomarker profiles characterized by decreased amyloid-beta peptide levels and increased total and phosphorylated tau levels at threonine 181 (pT181) are currently used to discriminate between Alzheimer's disease and other neurodegenerative diseases. However, these changes are not entirely specific to Alzheimer's disease, and it is noteworthy that other phosphorylated isoforms of tau, possibly more specific for the disease process, have been described in the brain parenchyma of patients. The precise detection of these isoforms in biological fluids remains however a challenge.

Methods: In the present study, we used the latest quantitative mass spectrometry approach, which achieves a sensitive detection in cerebrospinal fluid biomarker of two phosphorylated tau isoforms, pT181 and pT217, and first analyzed a cohort of probable Alzheimer's disease patients and patients with other neurological disorders, including tauopathies, and a set of cognitively normal controls. We then checked the validity of our results on a second cohort comprising cognitively normal individuals and patients with mild cognitive impairments and AD stratified in terms of their amyloid status based on PiB-PET imaging methods.

(Continued on next page)

* Correspondence: barthelemy.nicolas@wustl.edu; audreygabelle@hotmail.com; s-lehmann@chu-montpellier.fr

¹Laboratoire de Biochimie Protéomique Clinique, Plateforme de Protéomique Clinique, CHU de Montpellier, INSERM, Université de Montpellier, Montpellier, France

⁵Memory Resources and Research Center of Montpellier, Department of Neurology, Gui de Chauliac Hospital, Montpellier, France

Full list of author information is available at the end of the article



© The Author(s). 2020 **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

(Continued from previous page)

Results: In the first cohort, pT217 but not pT181 differentiated between Alzheimer's disease patients and those with other neurodegenerative diseases and control subjects much more specificity and sensitivity than pT181. T217 phosphorylation was increased by 6.0-fold in patients with Alzheimer's disease whereas T181 phosphorylation was only increased by 1.3-fold, when compared with control subjects. These results were confirmed in the case of a second cohort, in which the pT217 cerebrospinal fluid levels marked out amyloid-positive patients with a sensitivity and a specificity of more than 90% (AUC 0.961; CI 0.874 to 0.995). The pT217 concentrations were also highly correlated with the PiB-PET values (correlation coefficient 0.72; $P < 0.001$).

Conclusions: Increased cerebrospinal fluid pT217 levels, more than those of pT181, are highly specific biomarkers for detecting both the preclinical and advanced forms of Alzheimer's disease. This finding should greatly improve the diagnosis of Alzheimer's disease, along with the correlations found to exist between pT217 levels and PiB-PET data. It also suggests that pT217 is a promising potential target for therapeutic applications and that a link exists between amyloid and tau pathology.

Keywords: Alzheimer's disease, Tau proteins, Cerebrospinal fluid

Background

There still exists no widely recognized cure for Alzheimer's disease (AD), the leading cause of dementia worldwide [1, 2]. The diagnosis of AD and the development of suitable drug treatment are highly challenging issues, and there is still a crucial need for biomarkers which can be used to detect its early forms and distinguish it from other neurodegenerative disorders. Recent developments in cerebrospinal fluid (CSF) biochemistry [3] and brain positron emission tomography (PET) imaging [1] biomarker have yielded valuable tools in relation with amyloid- β plaques and neurofibrillary tangles (NFT), respectively. The main CSF biomarker profiles currently used for identifying AD are decreased amyloid-beta 42 (A β 42) levels, evaluated either alone or with respect to A β 40 [4], and increased total and phosphorylated tau levels at threonine 181 (pT181) [3–6]. This approach, based mainly on immunoassays, can be used to discriminate between AD and non-AD pathologies [7–9] [10] and to detect the AD process many years prior to the onset of cognitive symptoms and complaints [7, 11]. However, changes in the total CSF and pT181 tau levels are not entirely specific to AD. They are sometimes observed in other tauopathies, such as progressive supranuclear palsy (PSP) [8] and frontotemporal lobar degeneration (FTLD) [9], as well as dementia of other kinds, where they also reflect neurodegenerative processes. Brain studies on AD have shown the presence of many hyperphosphorylated tau sites [12–15] which might promote tau aggregation and the formation of NFT [16, 17]. These phosphorylated sites might therefore constitute alternative biomarkers of AD in addition to pT181. Finding specific means of detecting the corresponding p-tau isoforms in biological fluids for diagnostic purposes is still a challenging problem as the protein is subject to multiple post-translational modifications (acetylation, ubiquitination, methylation, truncation, etc.) at many sites along its sequence in addition to phosphorylation [18, 19].

To our knowledge, only phosphorylations of threonine 231 (pT231) and serine S199 [10, 20] have been tested for AD diagnosis in CSF using commercially available IVD immunoassay kits [21]. Whether or not the performances of methods using this biomarker are more efficient than those based on pT181 has not yet been clearly established.

Mass spectrometry-based methods are more relevant than immunoassays for assessing changes in the phosphorylation levels of specific sites independently of the total protein levels, as these methods can be used to directly quantify the phosphorylated peptides and their corresponding unmodified counterparts [13–15, 22]. In the present study, we used our latest mass spectrometry (MS) approach, which gives sensitive detection of pT181 and pT217 tau not only in brain extracts, but also in the CSF [23]. We first analyzed a cohort of probable AD patients and patients with other neurological disorders, including tauopathies, and a set of cognitively normal controls. We then checked the validity of our results on a second cohort comprising cognitively normal individuals and patients with mild cognitive impairments and AD stratified in terms of their amyloid status based on Pittsburgh compound B (PiB)-PET imaging methods. The data obtained show that CSF pT217 is a much more highly specific marker than pT181 for detecting both preclinical and advanced AD. We also established the existence of a correlation between patients' pT217 levels and the presence of amyloidosis at an early stage in the disease.

Methods

Subjects and samples

The first cohort used in this study focusing on AD and its differential diagnosis originated from the Montpellier Memory Research and Resources Center. All the patients consulting the center underwent a thorough clinical examination, magnetic resonance brain imaging (MRI), and standard neuropsychometric tests including

the Mini-Mental State Examination (MMSE) [24]. Laboratory tests were also performed in order to rule out the presence of dementia linked to thyroid dysregulation, metabolic syndrome, and viral diseases. The cohort included 10 patients with probable AD with high levels of evidence based on the NIA diagnostic criteria [25]. To avoid circular reasoning in this study focusing on tau, we used MRI and CSF amyloid findings rather than ELISA tau and pT181 levels to determine the pattern of the neural lesions present in AD patients. These AD patients were compared with 40 patients with non-AD diseases as follows: 8 patients with frontotemporal lobar degeneration (FTLD) with consensus criteria [26], 9 with Lewy body disease (LBD) based on the McKeith criteria [27], 6 with progressive supranuclear palsy (PSP), 1 with corticobasal degeneration (CBD), 6 with adult chronic idiopathic hydrocephalus (ACIH), 2 with mixed dementia, 2 with vascular dementia with possible AD, 2 with vascular dementia, 1 with brain metastasis, and 5 control subjects without any cognitive complaints and with normal neuropsychological profiles. In this group, lumbar punctures were performed in order to detect any acute cephalalgia or focal neurological signs so as to rule out the presence of central nervous system alterations. Participants' demographics, clinical presentation, MRI data, and neuropsychological and biomarker profiles are presented in Table 1 and SupTable1. All the participants

gave their written informed consent to participate in this study, which was approved by the Montpellier University Hospital's regional Ethics Committee (number 2011-003926-028). CSF samples were collected in polypropylene tubes under standard conditions [28]. CSF A β 42, total tau, and pT181 levels were measured using the standardized commercially available Innostest[®] sandwich ELISA (Fujirebio). The quality/accuracy of the results was ensured by using internal and external quality control (QC) procedures [29]. The QC coefficient of variation obtained on CSF total tau and pT181 in each batch and between batches ranged consistently below 15%. CSF samples were stored at the Montpellier CSF-Neurobank (#DC-2008-417 of the certified NFS 96-900 CHU resource center BB-0033-00031, www.biobanques.eu). Authorization to handle personal data was granted by the French Data Protection Authority (CNIL) under the number 1709743 v0.

The second cohort included amyloid-positive and prodromal AD participants, cognitively normal individuals, and patients with mild cognitive impairments recruited from the Alzheimer's Disease Research Center (ADRC) at the Washington University in Saint Louis (WUSTL) as previously published by Patterson et al. [30]. In this ethically approved cohort, deposition of amyloid plaques was quantified in terms of the mean cortical binding potential (MCP) the [11C]PiB-PET score (amyloid

Table 1 Demographical and cerebrospinal fluid (CSF) biomarker values in the Montpellier (AD and NAD) and the WUSTL (amyloid (-) and (+) cohorts. Results are expressed as means \pm standard deviations (SDs). *Abbreviations:* MMSE Mini-Mental State Examination, AD Alzheimer's disease, NAD non-Alzheimer's disease; *P* significance level of the Student's *t* test

Montpellier cohort		NAD	<i>n</i> = 40	AD	<i>n</i> = 10	
Variable	Mean	SD		Mean	SD	<i>P</i>
Age (years)	69.3	12.1		75.7	10.1	0.1330
Sex (% male)	67.5%	–		20.0%	–	0.0070*
MMSE	21.2	6.2		18.5	4.4	0.2112
E_Tau (pg/mL)	260	182		715	304	< 0.0001
E_pT181 (pg/mL)	42.9	24.6		95.8	25.0	< 0.0001
MS_pT181 (fmol/mL)	23.8	13.2		60.2	27.7	< 0.0001
MS_pT217 (fmol/mL)	0.948	1.066		11.740	5.006	< 0.0001
WUSTL cohort		Amyloid (-)	<i>n</i> = 51	Amyloid (+)	<i>n</i> = 33	
Variable	Mean	SD		Mean	SD	<i>P</i>
Age (years)	62.7	14.3		67.0	16.3	0.2063
Sex (% male)	39.2%	–		57.6%	–	0.1007*
CDR-SB	0.34	1.09		1.77	1.82	0.0004
%E4	17.7%	–		66.7%	–	< 0.0001*
PiB-PET	0.046	0.047		0.670	0.293	< 0.0001
E_pT181 (pg/mL)	47.5	21.5		76.0	25.4	< 0.0001
MS_pT181 (ng/mL)	0.350	0.256		0.596	0.312	< 0.0001
MS_pT217 (ng/mL)	0.057	0.1121		0.202	0.126	< 0.0001

*Chi-squared test for the comparison of two proportions

positive if the PiB-PET MCBP score > 0.1814). CSF was collected and stored as described in the latter study. Total tau and p-tau (181) CSF levels were measured using the standardized commercially available Innostest® sandwich ELISA (Fujirebio). This cohort included 33 amyloid-positive and 51 amyloid-negative participants, based on the results of the PiB-PET: the patients' demographics of participants are described in Table 1.

CSF tau purification and digestion

In the case of the Montpellier cohort, CSF samples (450 μ L) spiked with recombinant 15N-tau-441 (final concentration 100 fmol/mL) were extracted as previously described [31]. After brief perchloric acid precipitation, acidic supernatant was extracted by performing solid phase extraction, dried, and then digested with trypsin. For the phosphorylated peptide quantification, the following synthetic heavy isotope-labeled phosphorylated peptides (AQUA, labeled at the C-terminal residue) TPPAPKpTPPSSGEPPK (pT181) and TPSPpTPPTREPK (pT217) (Thermo Fisher Scientific, Ulm, Germany) were spiked in each sample to obtain a concentration of 100 fmol/mL. In the case of the WUSTL cohort, 800 μ L of CSF supernatant obtained after A β immunoprecipitation [30] and stored at -80°C was used for tau analysis. Thawed supernatants were spiked with 15N tau internal standard (5 ng per sample, notably to control preanalytical variation) and extracted as previously described [22] by performing Tau1 immunoprecipitation. This immunoprecipitation ensured a more specific tau enrichment than chemical purification, reducing LC-MS/MS interference and ensuring a phosphopeptide signal higher by a factor 2 to 3. This could however induce a bias in p-tau detection as Tau1 antibody is sensitive to phosphorylation in its epitope. Though Tau1 failed to recover pS199, pT217 and pT181 phosphorylated isoforms are recovered by this antibody in a similar way to what is observed with some other tau antibodies [23]. Briefly, 5 mM guanidine, 1% NP-40, and protease inhibitor cocktail were added to the sample. Samples were then mixed for 3 h at room temperature with 20 μ L of sepharose beads cross-linked to Tau1 antibody. Beads were precipitated before being rinsed with 0.5 M guanidine and triethylammonium bicarbonate (TEABC) 25 mM. Samples were digested with 400 ng of trypsin. In each sample, the TPPAPKpTPPSSGEPPK (pT181) and TPSPpTPPTR (pT217) AQUA peptides were spiked along with their unphosphorylated counterparts TPPSSGEPPK (T181) and TPSPpTPPTR (T217) at 10 and 100 fmol per sample, respectively. The resulting peptide samples were loaded onto TopTip C18 tips, washed with 0.1% formic acid (FA) solution, and eluted with 60%

acetonitrile (ACN) 0.1% FA solution. Samples were dried in a Speedvac and stored at -80°C . Prior to LC-MS analysis, samples were resuspended in 25 μ L 2% ACN 0.1% FA.

Mass spectrometry assays

In the case of the Montpellier cohort, MS analysis of endogenous and spiked peptides was performed as described elsewhere, using a LC-ESI-Quadrupole-Orbitrap analytical system (Q-Exactive, Thermo Scientific, San Jose, CA). Three microliters of each CSF digested sample was injected. Peptide separation was achieved within 30 min on a C18 column with the mobile phases (A) 0.1% formic acid in water and (B) 0.1% FA in ACN. Gradients used were 0 min-2%, 3 min-4%, 15 min-15%, 15.6 min-52%, 16 min-90%, 17.3 min-90%, 17.5 min-2%, and 30 min-2%. In the case of the WUSTL cohort, experiments were performed as previously described [22] using a Fusion Tribrid mass spectrometer (Thermo Scientific, San Jose, CA). Five microliters of each sample was injected. Peptide separation was achieved within 30 min on a Waters HSS T3 column. Mobile phases were (A) 0.1% FA in water and (B) 0.1% formic acid in ACN. Gradients used were 0 min-0.5%, 3 min-6%, 17 min-15%, 19 min-52%, 20 min-90%, 22 min-90%, 23 min-2%, and 30 min-2%. Data were acquired with both methods in the positive ion mode.

Phosphorylated and non-phosphorylated Tau peptide quantification

Since no labeled p-tau proteins were available, AQUA phosphorylated peptides were used: this approach is generally considered to be the best source of reference material for monitoring endogenous phospho-peptide levels. In the Montpellier cohort, the absolute concentrations of unphosphorylated peptide were computed with respect to the corresponding 15N-labeled peptides, as previously described [31]. The level of each endogenous phosphorylated peptide was calculated using single point calibration methods to compare the area obtained on AQUA phosphorylated peptide counterparts. In the WUSTL cohort, pT217 and pT181 levels were calculated as follows: ptau/tau ratio for each site is calculated by comparison with AQUA internal phosphorylated and unphosphorylated peptide signals. The ptau level is then calculated by multiplying ptau/tau ratio to the t-tau level measured for the corresponding unmodified peptide using the comparison to the 15N peptide signal from recombinant tau internal standard. Tau phosphorylation on other sites, like pS214 or pS184/S185, could bias the estimation of the phosphorylation ratio at T217 or T181 respectively by contributing to the level decrease of corresponding unphosphorylated peptide. However, the phosphorylation occupancy at these sites was respectively measured as 10

to 100 times less than at positions T217 and T181 in the brain and CSF [23] which therefore reduces this potential contribution.

Measurement reproducibility was assessed using quality controls consisting of CSF pools extracted four times independently and analyzed simultaneously within the cohort (see Sup Table 4). The response linearity of the PRM transitions used for phosphorylated peptide quantification was confirmed by the reverse curves obtained on phosphorylated AQUA peptides in pools of CSF extracts.

Statistical analysis

Statistical analyses were performed using the MedCalc software program (19.0.5). With a non-normal distribution, the chi-squared test or Fisher's exact test was performed on the qualitative variables and the Kruskal-Wallis test on the quantitative variables. To test the normal distribution, Student's *t* test was used. Spearman correlations were performed. Receiver operating characteristic (ROC) curves were used to represent the sensitivity and specificity of the amyloid-positive patient detection levels. Significance level was set at $P < 0.05$.

Results

CSF tau and p-tau quantification on the Montpellier AD cohort

The Montpellier cohort used in this study was composed of a relatively small number of samples collected from

thoroughly characterized patients (Sup Table 1). This cohort consisted of AD patients diagnosed with a high level of confidence [25] and non-AD (NAD) patients with various other neurological disorders (PSP, LBD, FTLN) associated with cognitive deficits. These pathologies are often used for the identification of specific AD biomarkers as they represent classical differential diagnosis of AD. As expected, we observed the existence of a significant difference ($P < 0.0001$) between AD and NAD patients in terms of the ELISA values of total tau (E_Tau) and tau pT181 (E_pT181) (Table 1). However, as shown in Fig. 1a and b, several NAD patients obtained values regarded as pathological in the case of both biomarkers (above 400 ng/mL and 60 ng/mL of tau and p-tau (181), respectively [6]). Quantification of pT181 and pT217 by MS also showed the existence of significant differences between AD and NAD populations (Table 1). The distribution of the pT181 values obtained by MS, which was clearly correlated with the ELISA values (Spearman's coefficient 0.773; $P < 0.0001$), also overlapped between the AD and the NAD populations (Fig. 1c), whereas the p217 values discriminated strongly between the two populations (Fig. 1d). In order to investigate the differences observed between pT181 and pT217 more closely, we distributed these values between the various NAD etiologies (Fig. 1e, f). In some well-defined cases of FTLN, LBD, or PSP in which the pT181 values were in the AD range, the pT217 values were

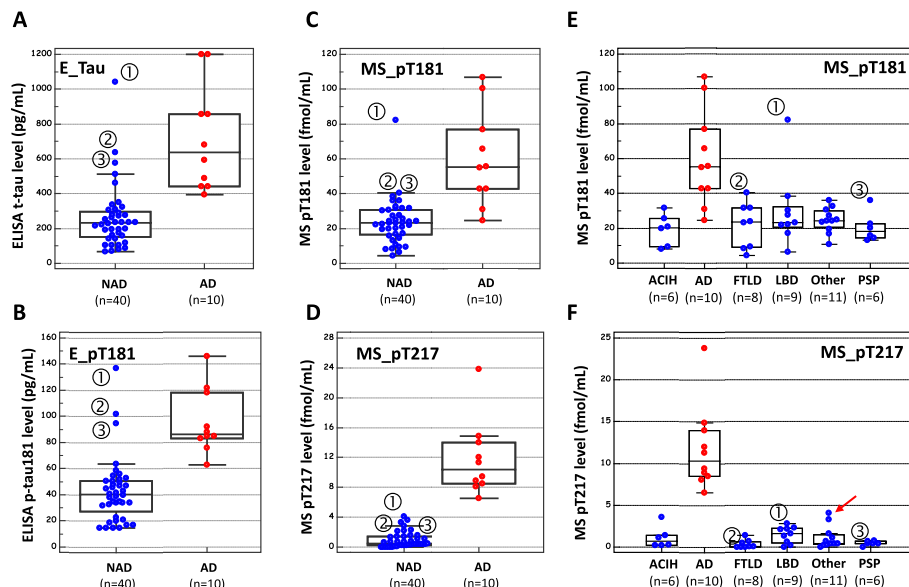


Fig. 1 CSF tau and p-tau levels in the Montpellier AD cohort. CSF concentration of total tau (E_tau) and pT181 (E_pT181) measured by ELISA (in pg/mL) in the NAD and AD population (**a, b**). CSF concentration of pT181 and pT217 measured by quantitative mass spectrometry (MS) (in fmol/mL) in the NAD and AD population (**c, d**) and in the diseases included in the cohort (**e, f**). ①, ②, and ③ indicate participants with high pT181 level but normal pT217 level. The red arrow indicates participants with mixed dementia having higher MS_p217 level than controls. Differences between NAD and AD populations are statically significant (see Table 1). Abbreviations: AD Alzheimer disease, NAD non-Alzheimer disease, FTLN frontotemporal lobar degeneration, LBD Lewy body dementia, PSP progressive supranuclear palsy, ACIH adult chronic idiopathic hydrocephalus

comparable to those measured in NAD subjects (Fig. 1f, black arrows). Apart from being associated with high total tau levels, no obvious clinical signs distinguishing these patients with high p181 levels were detected (SupTable1). We also noted that the two mixed dementia patients (Sup Table 1) had the highest pT217 levels in the NAD population (red arrow).

CSF total-tau, p-tau correlation, and ratio in the Montpellier AD cohort

In order to present the changes in tau phosphorylation state independently of the tau phosphorylation levels, pT181 and pT217 were plotted against their corresponding unmodified counterparts T181 and T217 (Fig. 2). One sample corresponding to the patient with brain metastasis (black arrow) was taken to be an outlier, since the unphosphorylated and phosphorylated levels did not follow the same variation. In addition, the correlation between the isoforms was highly significant in both NAD and AD populations (see rho Spearman's coefficient and P value in the figure). Interestingly, the slopes of the correlations were higher in the AD population than in the NAD population, especially in the case of pT217 (regression formula for AD $y = 0.426 + 0.0797x$, for NAD $y = 0.734 + 0.00364x$; slope of the ANCOVA comparison $P < 0.001$). This suggests that a process of hyperphosphorylation occurs independently of the increase in tau levels in AD. To further confirm this hypothesis, we determined the p-tau to tau ratio for each phosphorylated site in the AD and NAD populations (Fig. 3a). This confirmed the occurrence of both T181 and T217 hyperphosphorylation in AD patients, corresponding to 1.3- and 6.0-fold increases in the

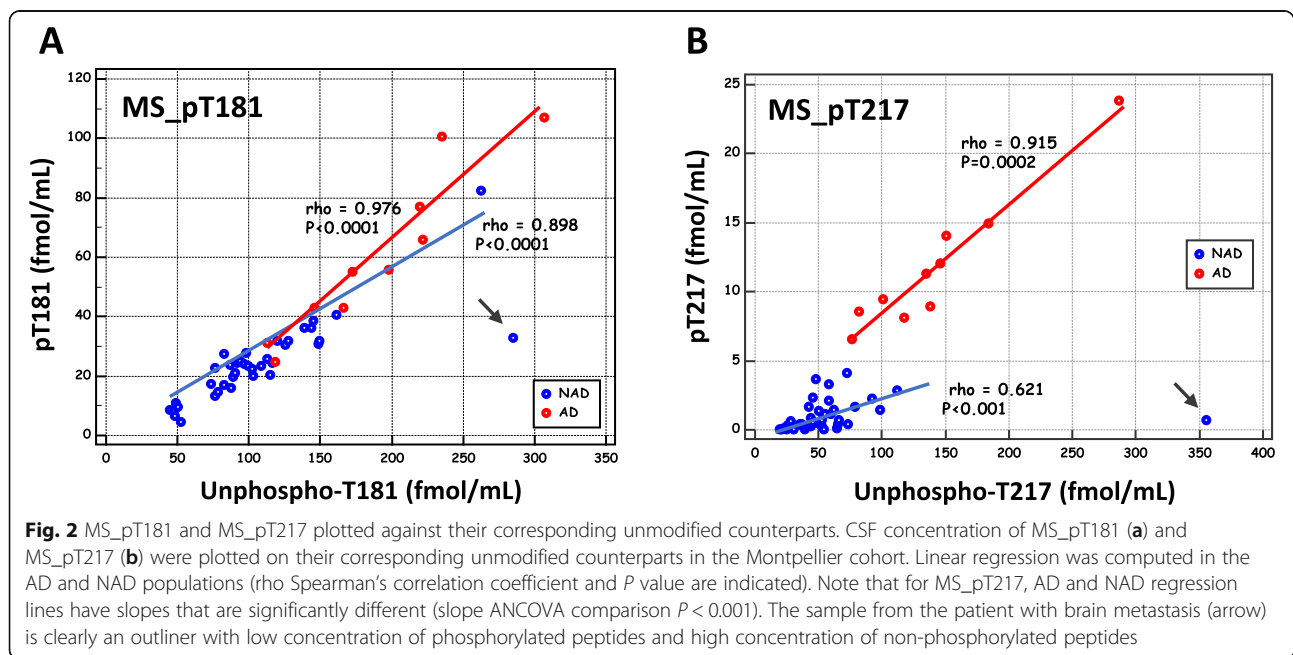
T181 and T217 phosphorylation states, respectively, in AD vs. NAD patients (see also Sup Table 2).

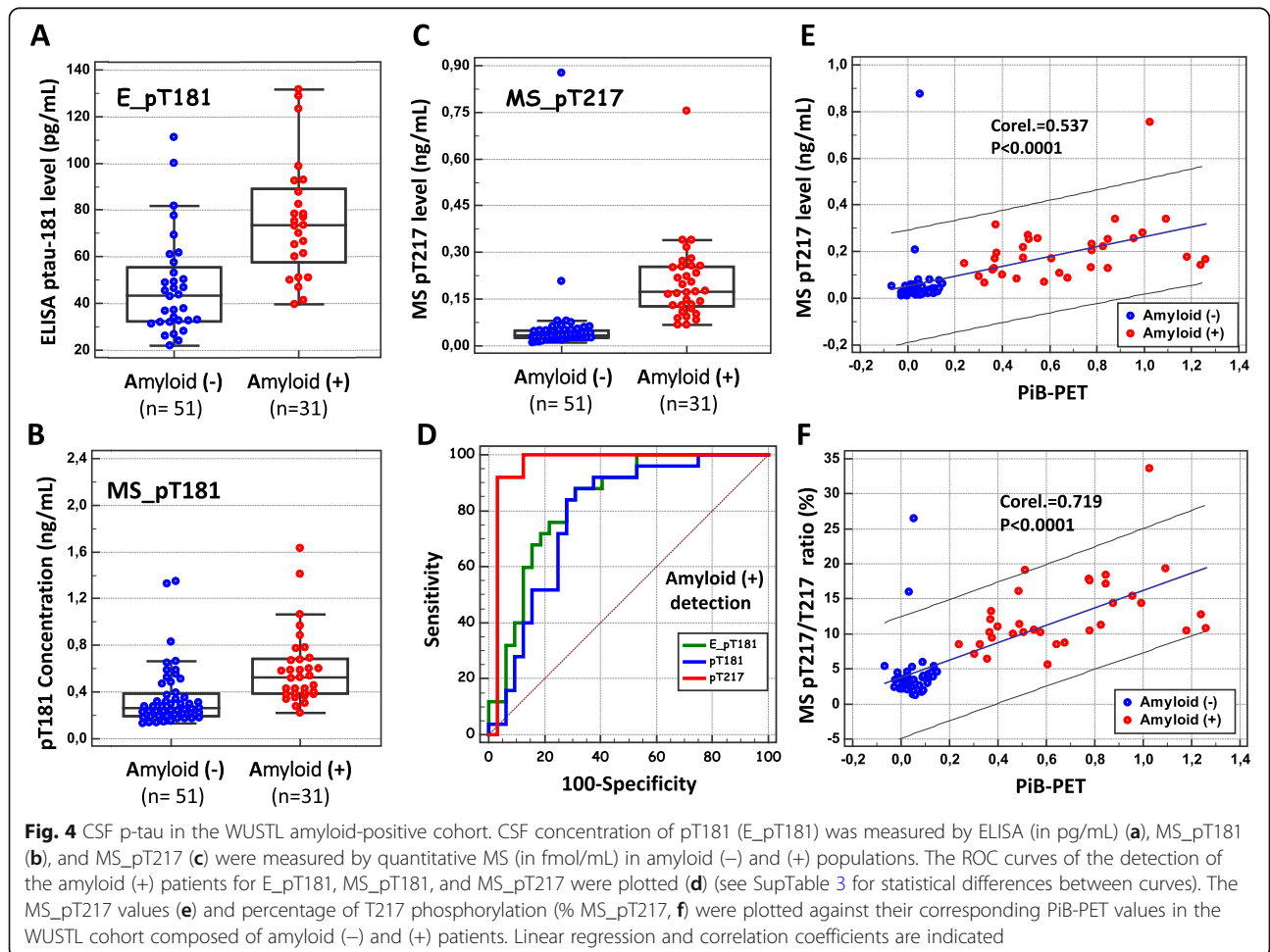
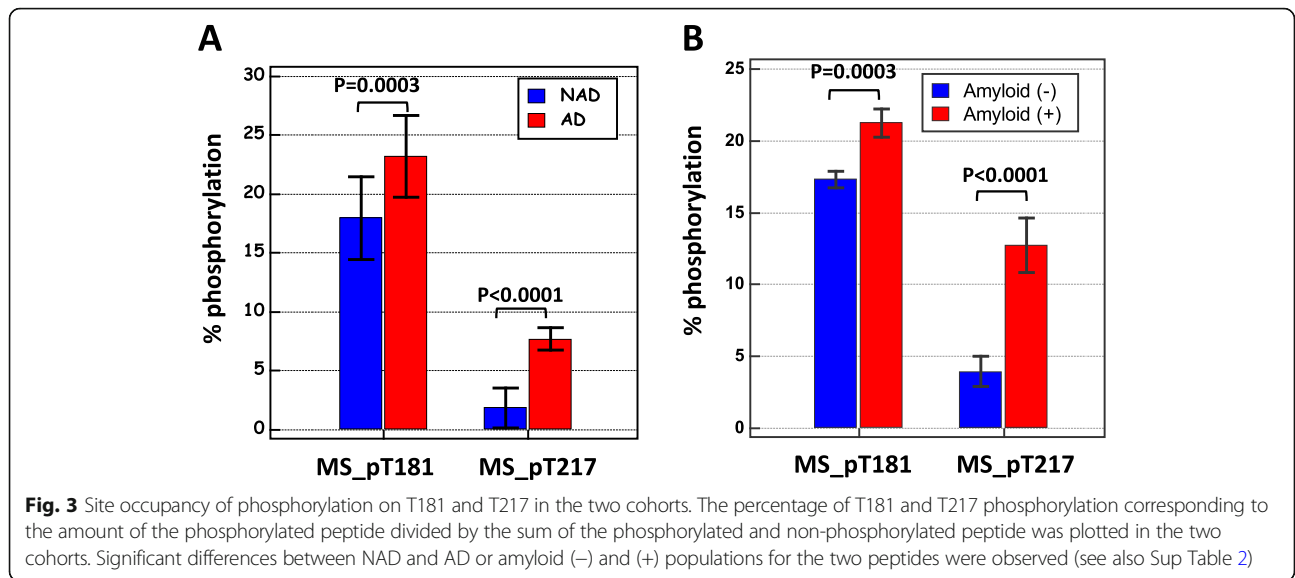
CSF tau pT181 and pT217 levels in the WUSTL cohort

We next investigated the levels of tau pT181 and T217 in a second cohort of patients with no cognitive complaints or only mild cognitive impairments attending the Knight ADRC at WUSTL [30, 32]. This cohort was stratified using PiB-PET in 33 amyloid-positive (+) and 51 amyloid-negative (-) participants (Table 1). To improve the sensitivity of the assay, Tau was immunoprecipitated with the Tau1 antibody (see the "Methods" and [22]). The values of pT181 measured using either ELISA or quantitative MS were found to differ significantly between amyloid (+) and (-) populations (Table 1) but overlapped significantly (Fig. 4a, b). By contrast, the pT217 also differed but overlapped less conspicuously (Fig. 4c). In order to compare the performances of these biomarkers, we computed the area under the curve (AUC) of the ROC plotting the sensitivity and specificity for amyloid (+) patient detection at different cutoffs (Fig. 4d). pT217 (AUC, 0.961) was found to be more sensitive and specific than pT181, as measured by performing either ELISA (AUC, 0.833) or MS (AUC, 0.785) (Sup Table 3). We also computed the p-tau/tau ratios of pT181 and pT217 phosphorylation (Fig. 3b) and confirmed the hyperphosphorylation of both sites in amyloid (+) patients (showing a 1.2- and a 3.5-fold increase, respectively, Sup Table 2).

Relationship between CSF biomarkers and PiB-PET

We then examined whether there existed a correlation between amyloid PiB-PET and the total and phosphorylated





tau levels in CSF from both amyloid (+) and amyloid (−) patients. Both pT181 and pT217 levels were found to be significantly correlated with the PiB-PET data, as shown in Sup Table 4 (correlation coefficients 0.418 and 0.537, respectively) and illustrated in Fig. 4e, f and in SupFigure 2. We also examined whether the p-tau/tau ratio for T217 was also correlated with the PiB-PET findings. As shown in Fig. 4f, pT217/T217 ratio was significantly correlated with the PiB-PET data, giving an even better coefficient than with the pT217 levels (correlation coefficients 0.689 and 0.719, respectively).

Discussion

Using targeted quantitative MS to quantify low-abundance phosphorylated tau peptides (pT181 and pT217) and their unmodified counterparts, we found compelling evidence that patients' CSF pT181 and pT217 levels had undergone AD-specific changes. These changes were characterized by an increase both in the total concentrations and in the occupancy of phosphorylation on each site measured as p-tau/tau ratios. They were particularly pronounced in the case of CSF pT217, which was 3 to 6 times more phosphorylated in preclinical to advanced AD patients from two independent, well-characterized cohorts in comparison with the corresponding control values. This finding is consistent with the increased pT217 levels detected in the brains of AD patients measured using MS methods [13, 15, 33] or with anti-p-tau antibody AT100, which recognizes pT212, pS214, and pT217 [33]. It is also in keeping with the fact that pT217 has been detected in aggregated tau extracted from AD patients' brains [33, 34]. As far as the possible pathological consequences of T217 phosphorylation are concerned, it was previously established that preventing this process by mutating threonine 217 to alanine reduces the ability of p-tau to promote microtubule assembly in vitro [33] and to bind to SH3 domains such as the BIN1 SH3 domain [35]. All in all, these findings show that pT217 is linked to the pathophysiology of AD. The mechanism underlying T217 phosphorylation is still a matter of debate, which is also the case with the majority of tau phosphorylation sites. GSK-3, PKA, and the stress-activated protein kinases SAPK4/p38 or JNK2 generating the AT100 epitope might be involved [33, 34, 36]. Further experiments on pT217 would help to explain its high, early, specific contribution to the positive amyloid imaging results. These findings also suggest that T217 is an interesting new candidate for developing a targeted therapeutic approach in the field of AD [36]. Our results clearly show that CSF pT217 outperforms pT181 as a means of AD diagnosis. The detection of pT181 is generally held, however, to be the "gold standard" for detecting AD in the CSF [37, 38], and its recent detection in blood also suggests

that this finding may lead to some interesting applications [39]. The fact that pT181 detection has been so widely used in clinical practice is no doubt due to the high levels of this peptide detected in AD patients (here it amounted to around 20% of the total tau) in comparison with other phosphorylated sites such as pT217 and to the availability of high-performance immunodetection assays which can be used for pT181 quantification. The present limitations to AD diagnosis encountered using CSF amyloid and tau biomarkers [3–6, 40–42] might be overcome if pT217 is used instead of pT181. It is our belief that the future of accurate AD diagnosis will include pT217 or other phosphorylation sites which are more specific AD markers than pT181. Universal cutoff values of pT217 optimal for AD detection will have to be determined on larger cohorts, and using reference material. To evaluate the added diagnostic value of pT217, we also compared the performance of pT181 and pT217 alone or as a ratio with A β 1-42 (SupFigure 1 and SupTable3). In the Montpellier cohort, the added value of the ratio pTau/A β 1-42 was not apparent which might be related to the poor performance of A β 1-42 in confirmed/advanced AD. In the WUSTL cohort, which focuses on amyloidosis, the added value of the ratio was present especially for pT181. Additional experiments with larger cohorts will be needed to have an optimal clinical use of the detection of pT217 alone, or most likely in combination with amyloid biomarkers. The present results also show that the pT217 levels are strongly indicative of the amyloid plaque load, as measured with PiB-PET. This finding is in line with longitudinal studies showing that amyloid impairments are involved in tau pathology [11, 43–46]. The molecular mechanism whereby amyloid and Tau pathologies contribute to AD might depend on the influence of A β on kinases such as GSK-3 β [47, 48], which might result in the phosphorylation of tau at many sites, including T181 and pT217 [34]. Further studies focusing on T217 phosphorylation, using PET-tau imaging or other approaches, are now required in order to understand the exact role of pT217 in the cascade of molecular events that lead to AD. The finding that the pT217/T217 ratio (the percentage occupancy of phosphorylation on T217) is highly correlated with the amyloid load suggests the occurrence of a series of changes that could be used to follow the progression of the disease and/or improve the possibility of predicting the risk of cognitive decline in individuals who have converted/are converting to clinical AD [44].

Limitations

This study has several limitations. First, the present AD cohort was rather small, which is nevertheless counterbalanced by the detailed clinical and biological data on the patients. In addition, the second cohort was stratified based on the presence of brain amyloidosis, which is not

always equivalent to AD. Lastly, this study does not include any longitudinal data that might have served to document the changes in biomarkers with respect to the progression of the disease.

Conclusions

In conclusion, the results of this study suggest that pT217 is a more accurate biomarker than pT181, which could be used to improve the diagnosis and follow-up of preclinical to advanced cases of Alzheimer's disease. It should therefore constitute a promising new target for therapeutic applications. These findings also suggest/show that amyloid plaques contribute to the processes responsible for tau pathophysiology.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s13195-020-00596-4>.

Additional file 1: SupFigure 1. ROC curves of biomarker combinations. The ROC curves for the detection of the amyloid (+) patients in the WUSTL cohort (Panel A) or the AD patients in the Montpellier cohort (Panel B) for A β 1–42, MS_pT181, MS_pT217, MS_pT181/ A β 1–42 and MS_pT217/ A β 1–42 were plotted. AUC values and statistical differences between curves of the panel A are reported in SupTable 3. **SupFigure 2.** CSF p-tau181 correlation with PiB-PET. The MS_pT181 values (panel A) and % of T181 phosphorylation (% MS_pT181, panel B) were plotted against their corresponding PiB-PET values in the WUSTL cohort composed of amyloid (–) and (+) patients. Correlation coefficients are indicated.

Additional file 2: SupTable 1. Clinical and MRI characteristics of the Montpellier cohort. **SupTable 2.** Percentage of T181 and T217 phosphorylation. **SupTable 3.** AUC of phosphorylation of the different sites. **SupTable 4.** Correlation between CSF biomarkers and PiB-PET. **SupTable 5.** Internal quality control and reproducibility of the two methods.

Abbreviations

ACIH: Adult chronic idiopathic hydrocephalus; ACN: Acetonitrile; AD: Alzheimer's disease; ADRC: Alzheimer's Disease Research Center; AQUA: Absolute quantitation; AUC: Area under the curve; A β : Amyloid β ; CBD: Corticobasal degeneration; CI: Confidence interval; CSF: Cerebrospinal fluid; FA: Formic acid; FTLD: Frontotemporal lobar degeneration; LBD: Lewy body dementia; LC-ESI: Liquid chromatography electrospray ionization; MCBP: Mean cortical binding potential; MCI: Mild cognitive impairment; MMSE: Mini-Mental State Examination; MRI: Magnetic resonance brain imaging; NAD: Non-Alzheimer disease; NFT: Neurofibrillary tangles; PET: Positron emission tomography; PiB: Pittsburgh compound B; PSP: Progressive supranuclear palsy; QC: Quality control; ROC: Receiver operating characteristic; SD: Standard deviation; TEABC: Triethylammonium bicarbonate; WUSTL: Washington University in Saint Louis

Acknowledgements

Part of the nano-LC-MS/MS analyses (first cohort, discovery) was performed using facilities at the Functional Proteomics Platform of Proteomics Pole of Montpellier-Languedoc-Roussillon. We would like to thank Serge Urbach and Martial Severo for their assistance with the MS experiments, Vitaliy Ovod for his work in assembling clinical data from the WUSTL cohort, Paul Moiseyev for his assistance with the sample management at WUSTL, Nicholas Kanaan for providing Tau1 antibodies, Isabelle Huvent for providing 15N Tau recombinant protein, and Joel Ménard for his ongoing support in the ProMarA study.

Authors' contributions

Barthelemy and Lehmann had full access to all the data in the study and take responsibility for their presentation and the accuracy of the analysis. Concept and design: Barthelemy, Bateman, Gabelle, and Lehmann. Acquisition, analysis, and interpretation of the data: Barthélemy and Bateman. Drafting of the manuscript: Barthelemy, Hirtz, Marin, Becher, Sato, Gabelle, and Lehmann. Funding obtained: Bateman, Gabelle, and Lehmann. Administrative, technical, and material support: Hirtz, Marin, Becher, and Sato. The author(s) read and approved the final manuscript.

Funding

This research was supported by the 2010 National PHRC "ProMarA" project on the "Use of targeted quantitative proteomics and metabolic labeling with stable isotopes for the diagnosis and the investigation of neurological disorders and in particular Alzheimer Disease" and through the National French Alzheimer effort ("Plan Alzheimer 2009-2012"). This work (second cohort, validation) was supported by the following grants: NIH R01 NS065667 (RJB PI), NS095773 (RJB PI), and received financial support from the Alzheimer's Association Research Fellowship (NRB) and the Coins for Alzheimer's Research Trust (CS PI). The funder/sponsor had no role in the design or conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and the decision to submit the manuscript for publication.

Availability of data and materials

The datasets used for the analyses are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All the participants gave their written informed consent to participate in this study, which was approved by the Montpellier University Hospital's regional Ethics Committee (number 2011-003926-028).

Consent for publication

Not applicable.

Competing interests

Dr. Nicolas Barthélemy is a recipient of the Alzheimer's Association Research Fellowship which supported this work. He declares that there are no conflicts of interest involved. Pr Sylvain Lehmann received institutional support from Montpellier University Hospital and the French National Research Agency for biomarker research. He received honoraria from Thermo Fisher, Roche, and Fujirebio for serving on scientific advisory boards. He is a shareholder at the Spot-to-Lab start-up company, which was not involved in this particular research. Dr. Audrey Gabelle received funds from the Fondation Philippe Chatrier. She declares that there are no conflicts of interest involved in this paper. Dr. Randall Bateman has received honoraria from Janssen, Pfizer, and Roche as a speaker; from Eisai as a consultant; and from Merck and Pfizer as an Advisory Board member. Washington University has submitted the US nonprovisional patent applications "Methods of Diagnosing and Treating Based on Site-Specific Tau Phosphorylation" (co-inventors RJB, NB) and "Central Nervous System Tau Kinetic Measurements as Diagnostic and Theragnostic Biomarkers" (co-inventors RJB, CS) and the provisional patent application "Quantification of Tau Isoforms, Modification, and Truncation for Assessment of Tauopathies" (co-inventors RJB, CS). Dr. Philippe Marin, François Becher, Christophe Hirtz, and Chihiro Sato (préciser Dr. ou Professor avant chaque nom) also declare that there are no competing interests.

Author details

¹Laboratoire de Biochimie Protéomique Clinique, Plateforme de Protéomique Clinique, CHU de Montpellier, INSERM, Université de Montpellier, Montpellier, France. ²Department of Neurology, Washington University School of Medicine, Saint-Louis, MO, USA. ³Institut de Génomique Fonctionnelle, CNRS UMR5203, INSERM U1191, Université de Montpellier, Montpellier, France. ⁴Service de Pharmacologie et d'Immunoanalyse (SPI), Laboratoire d'Etude du Métabolisme des Médicaments (LEMM), CEA, INRA, Université Paris Saclay, F-91191 Gif-sur-Yvette cedex, France. ⁵Memory Resources and Research Center of Montpellier, Department of Neurology, Gui de Chauliac Hospital, Montpellier, France.

Received: 26 September 2019 Accepted: 9 March 2020

Published online: 17 March 2020

References

- Association As. 2018 Alzheimer's disease facts and figures. *Alzheimers Dement.* 2018;14(3):367–429.
- Piton M, Hirtz C, Desmetz C, Milhau J, Lajoix AD, Bennys K, et al. Alzheimer's disease: advances in drug development. *Journal of Alzheimer's disease : JAD.* 2018;65(1):3–13 Epub 2018/07/25.
- Duits FH, Prins ND, Lemstra AW, Pijnenburg YA, Bouwman FH, Teunissen CE, et al. Diagnostic impact of CSF biomarkers for Alzheimer's disease in a tertiary memory clinic. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2015;11(5):523–32 Epub 2014/08/27.
- Gooblar J, Carpenter BD, Coats MA, Morris JC, Snider BJ. The influence of cerebrospinal fluid (CSF) biomarkers on clinical dementia evaluations. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2015; 11(5):533–40 e2 Epub 2014/07/16.
- Mouton-Liger F, Wallon D, Troussiere AC, Yatimi R, Dumurgier J, Magnin E, et al. Impact of cerebro-spinal fluid biomarkers of Alzheimer's disease in clinical practice: a multicentric study. *J Neurol.* 2014;261(1):144–51 Epub 2013/10/29.
- Lehmann S, Schraen S, Paquet C, Bombois S, Delaby C, Dorey A, et al. A diagnostic scale for Alzheimer's disease based on cerebrospinal fluid biomarker profiles. *Alzheimers Res Ther.* 2014;6:38.
- Buchhave P, Minthon L, Zetterberg H, Wallin AK, Blennow K, Hansson O. Cerebrospinal fluid levels of beta-amyloid 1-42, but not of tau, are fully changed already 5 to 10 years before the onset of Alzheimer dementia. *Arch Gen Psychiatry.* 2012;69(1):98–106 Epub 2012/01/04.
- Borroni B, Gardoni F, Parnetti L, Magno L, Malinverno M, Saggese E, et al. Pattern of Tau forms in CSF is altered in progressive supranuclear palsy. *Neurobiol Aging.* 2009;30(1):34–40 Epub 2007/08/22.
- Marelli C, Gutierrez LA, Menjot de Champfleur N, Charroud C, De Verbizier D, Touchon J, et al. Late-onset behavioral variant of frontotemporal lobar degeneration versus Alzheimer's disease: interest of cerebrospinal fluid biomarker ratios. *Alzheimers Dement (Amst).* 2015;1(3):371–9 Epub 2016/05/31.
- Hampel H, Buerger K, Zinkowski R, Teipel SJ, Goernitz A, Andreasen N, et al. Measurement of phosphorylated tau epitopes in the differential diagnosis of Alzheimer disease: a comparative cerebrospinal fluid study. *Arch Gen Psychiatry.* 2004;61(1):95–102.
- Bateman RJ, Xiong C, Benzinger TL, Fagan AM, Goate A, Fox NC, et al. Clinical and biomarker changes in dominantly inherited Alzheimer's disease. *N Engl J Med.* 2012;367(9):795–804 Epub 2012/07/13.
- Wang JZ, Xia YY, Grundke-Iqbal I, Iqbal K. Abnormal hyperphosphorylation of tau: sites, regulation, and molecular mechanism of neurofibrillary degeneration. *JAD: Journal of Alzheimer's disease.* 2012. Epub 2012/06/20.
- Mair W, Muntel J, Tepper K, Tang S, Biernat J, Seeley WW, et al. FLEXITau: quantifying post-translational modifications of tau protein in vitro and in human disease. *Anal Chem.* 2016;88(7):3704–14 Epub 2016/02/16.
- Dammer EB, Lee AK, Duong DM, Gearing M, Lah JJ, Levey AI, et al. Quantitative phosphoproteomics of Alzheimer's disease reveals cross-talk between kinases and small heat shock proteins. *Proteomics.* 2015;15(2–3): 508–19 Epub 2014/10/22.
- Hanger DP, Byers HL, Wray S, Leung KY, Saxton MJ, Seereeram A, et al. Novel phosphorylation sites in tau from Alzheimer brain support a role for casein kinase 1 in disease pathogenesis. *J Biol Chem.* 2007;282(32):23645–54 Epub 2007/06/15.
- Noble W, Hanger DP, Miller CC, Lovestone S. The importance of tau phosphorylation for neurodegenerative diseases. *Front Neurol.* 2013;4:83 Epub 2013/07/13.
- Delacourte A, Buee L. Normal and pathological Tau proteins as factors for microtubule assembly. *Int Rev Cytol.* 1997;171:167–224 Epub 1997/01/01.
- Sergeant N, Bretteville A, Hamdane M, Caillet-Boudin ML, Grognet P, Bombois S, et al. Biochemistry of Tau in Alzheimer's disease and related neurological disorders. *Expert review of proteomics.* 2008;5(2):207–24 Epub 2008/05/10.
- Iqbal K, Liu F, Gong CX, Grundke-Iqbal I. Tau in Alzheimer disease and related tauopathies. *Curr Alzheimer Res.* 2010;2010 Epub 2010/08/04.
- Buerger K, Teipel SJ, Zinkowski R, Blennow K, Arai H, Engel R, et al. CSF tau protein phosphorylated at threonine 231 correlates with cognitive decline in MCI subjects. *Neurology.* 2002;59(4):627–9 Epub 2002/08/28.
- Santos JRF, Bauer C, Schuchhardt J, Wedekind D, Waniek K, Lachmann I, et al. Validation of a prototype tau Thr231 phosphorylation CSF ELISA as a potential biomarker for Alzheimer's disease. *J Neural Transm (Vienna).* 2019; 126(3):339–48 Epub 2019/02/16.
- Sato C, Barthélemy NR, Mawuenyega KG, Patterson BW, Gordon BA, Jockel-Balsarotti J, et al. Tau kinetics in neurons and the human central nervous system. *Neuron.* 2018;97(6):1284–98 e7 Epub 2018/03/24.
- Barthélemy NR, Mallipeddi N, Moiseyev P, Sato C, Bateman RJ. Tau phosphorylation rates measured by mass spectrometry differ in the intracellular brain vs extracellular cerebrospinal fluid compartments and are differentially affected by Alzheimer's disease. *Frontiers in aging neuroscience.* 2019;11:121 Epub 2019/06/11.
- Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res.* 1975;12(3):189–98 Epub 1975/11/01.
- McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR Jr, Kawas CH, et al. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2011; 7(3):263–9 Epub 2011/04/26.
- McKhann GM, Albert MS, Grossman M, Miller B, Dickson D, Trojanowski JQ, et al. Clinical and pathological diagnosis of frontotemporal dementia: report of the Work Group on Frontotemporal Dementia and Pick's Disease. *Arch Neurol.* 2001;58(11):1803–9 Epub 2001/11/16.
- McKeith IG. Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): report of the Consortium on DLB International Workshop. *Journal of Alzheimer's disease : JAD.* 2006;9(3 Suppl):417–23 Epub 2006/08/18.
- Del Campo M, Mollenhauer B, Bertolotto A, Engelborghs S, Hampel H, Simonsen AH, et al. Recommendations to standardize preanalytical confounding factors in Alzheimer's and Parkinson's disease cerebrospinal fluid biomarkers: an update. *Biomark Med.* 2012;6(4):419–30 Epub 2012/08/25.
- Dumurgier J, Verduyck O, Paquet C, Bombois S, Chaulet C, Laplanche JL, et al. Intersite variability of CSF Alzheimer's disease biomarkers in clinical setting. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2013;9(4):406–13 Epub 2012/11/13.
- Patterson BW, Elbert DL, Mawuenyega KG, Kasten T, Ovod V, Ma S, et al. Age and amyloid effects on human central nervous system amyloid-beta kinetics. *Ann Neurol.* 2015;78(3):439–53 Epub 2015/06/05.
- Barthélemy NR, Fenaille F, Hirtz C, Sergeant N, Schraen-Maschke S, Vialaret J, et al. Tau protein quantification in human cerebrospinal fluid by targeted mass spectrometry at high sequence coverage provides insights into its primary structure heterogeneity. *J Proteome Res.* 2016;15(2):667–76 Epub 2016/01/09.
- Schindler SE, Bollinger JG, Ovod V, Mawuenyega KG, Li Y, Gordon BA, et al. High-precision plasma beta-amyloid 42/40 predicts current and future brain amyloidosis. *Neurology.* 2019; Epub 2019/08/03.
- Yoshida H, Goedert M. Sequential phosphorylation of tau protein by cAMP-dependent protein kinase and SAPK4/p38delta or JNK2 in the presence of heparin generates the AT100 epitope. *J Neurochem.* 2006;99(1):154–64 Epub 2006/09/22.
- Zhou Y, Shi J, Chu D, Hu W, Guan Z, Gong CX, et al. Relevance of phosphorylation and truncation of tau to the etiopathogenesis of Alzheimer's disease. *Front Aging Neurosci.* 2018;10:27 Epub 2018/02/24.
- Lasorsa A, Malki I, Cantrelle FX, Merzougui H, Boll E, Lambert JC, et al. Structural basis of tau interaction with BIN1 and regulation by tau phosphorylation. *Front Mol Neurosci.* 2018;11:421 Epub 2018/11/30.
- Hanger DP, Anderton BH, Noble W. Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. *Trends Mol Med.* 2009;15(3):112–9 Epub 2009/02/28.
- Lewczuk P, Esselmann H, Bibl M, Beck G, Maler JM, Otto M, et al. Tau protein phosphorylated at threonine 181 in CSF as a neurochemical biomarker in Alzheimer's disease: original data and review of the literature. *J Mol Neurosci.* 2004;23(1–2):115–22 Epub 2004/05/06.
- Blennow K, Vanmechelen E, Hampel H. CSF total tau, Abeta42 and phosphorylated tau protein as biomarkers for Alzheimer's disease. *Mol Neurobiol.* 2001;24(1–3):87–97.
- Mielke MM, Hagen CE, Xu J, Chai X, Vemuri P, Lowe VJ, et al. Plasma phospho-tau181 increases with Alzheimer's disease clinical severity and is associated with tau- and amyloid-positron emission tomography.

- Alzheimer's & dementia : the journal of the Alzheimer's Association. 2018; 14(8):989–97 Epub 2018/04/08.
40. Gabelle A, Dumurgier J, Vercauysse O, Paquet C, Bombois S, Laplanche JL, et al. Impact of the 2008-2012 French Alzheimer Plan on the use of cerebrospinal fluid biomarkers in research memory center: the PLM Study. *Journal of Alzheimer's disease : JAD*. 2013;34(1):297–305 Epub 2012/11/29.
 41. Ewers M, Mattsson N, Minthon L, Molinuevo JL, Antonell A, Popp J, et al. CSF biomarkers for the differential diagnosis of Alzheimer's disease: a large-scale international multicenter study. *Alzheimer's & dementia : the journal of the Alzheimer's Association*. 2015;11(11):1306–15 Epub 2015/03/26.
 42. Schoonenboom NS, Reesink FE, Verwey NA, Kester MI, Teunissen CE, van de Ven PM, et al. Cerebrospinal fluid markers for differential dementia diagnosis in a large memory clinic cohort. *Neurology*. 2012;78(1):47–54 Epub 2011/12/16.
 43. Jack CR Jr, Vemuri P, Wiste HJ, Weigand SD, Lesnick TG, Lowe V, et al. Shapes of the trajectories of 5 major biomarkers of Alzheimer disease. *Arch Neurol*. 2012;69(7):856–67 Epub 2012/03/14.
 44. Vos SJ, Xiong C, Visser PJ, Jasielec MS, Hassenstab J, Grant EA, et al. Preclinical Alzheimer's disease and its outcome: a longitudinal cohort study. *The Lancet Neurology*. 2013;12(10):957–65 Epub 2013/09/10.
 45. Lleo A, Alcolea D, Martinez-Lage P, Scheltens P, Parnetti L, Poirier J, et al. Longitudinal cerebrospinal fluid biomarker trajectories along the Alzheimer's disease continuum in the BIOMARKAPD study. *Alzheimer's & dementia : the journal of the Alzheimer's Association*. 2019; Epub 2019/04/11.
 46. Sutphen CL, Jasielec MS, Shah AR, Macy EM, Xiong C, Vlassenko AG, et al. Longitudinal cerebrospinal fluid biomarker changes in preclinical Alzheimer disease during middle age. *JAMA neurology*. 2015;72(9):1029–42 Epub 2015/07/07.
 47. Hernandez F, Gomez de Barreda E, Fuster-Matanzo A, Lucas JJ, Avila J. GSK3: a possible link between beta amyloid peptide and tau protein. *Exp Neurol* 2010;223(2):322–325. Epub 2009/09/29.
 48. Jin M, Shepardson N, Yang T, Chen G, Walsh D, Selkoe DJ. Soluble amyloid beta-protein dimers isolated from Alzheimer cortex directly induce Tau hyperphosphorylation and neuritic degeneration. *Proc Natl Acad Sci U S A*. 2011;108(14):5819–24 Epub 2011/03/23.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more [biomedcentral.com/submissions](https://www.biomedcentral.com/submissions)

