Comprehensive Quantitative Profiling of Tau and Phosphorylated Tau Peptides in CSF by Mass Spectrometry Provides New Biomarker Candidates.

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RUNNING TITLE: Detection of Tau and Phosphorylated Tau Peptides in CSF.

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ABSTRACT

Aberrant tau phosphorylation is a hallmark in Alzheimer's disease (AD), believed to promote formation of paired helical filaments, the main constituent of neurofibrillary tangles in the brain. Whilst cerebrospinal fluid (CSF) levels of total tau and tau phosphorylated at threonine residue 181 (pThr181) are established core biomarkers for AD, the value of alternative phosphorylation sites, that may have more direct relevance to pathology, for early diagnosis is not yet known, largely due to their low levels in CSF and lack of standardized detection methods.

To overcome sensitivity limitations for analysis of phosphorylated tau in CSF, we have applied an innovative mass spectrometry (MS) workflow, TMTcalibrator™, to enrich and enhance the detection of phosphoproteome components of AD brain tissue in CSF, and enable the quantitation of these analytes. We aimed to identify which tau species present in the AD brain are also detectable in CSF and which, if any, are differentially regulated with disease.

Over 75% coverage of full length (2N4R) tau was detected in the CSF with 47 phosphopeptides covering 31 different phosphorylation sites. Of these, 11 phosphopeptides were up-regulated by at least 40%, along with an overall increase in tau levels in the CSF of AD patients relative to controls.

Use of the TMTcalibrator™ workflow dramatically improved our ability to detect tau-derived peptides that are directly related to human AD pathology. Further validation of regulated tau peptides as early biomarkers of AD is warranted and is currently being undertaken.

KEYWORDS

Tau, cerebrospinal fluid; biomarkers; phosphorylation; mass spectrometry; Alzheimer's disease, neurodegenerative disease, post translational modifications.

INTRODUCTION

Protein structure and function is modulated by post-translational modifications, the most common modification being protein phosphorylation at serine, threonine and tyrosine residues. It is estimated that one third of all cellular proteins are phosphorylated to some degree, and these phosphorylation events are involved in many regulatory functions including, but not exclusive to, structural integrity, signal transduction, differentiation and proliferation, metabolism and degradation [1]. The reversible phosphorylation of a protein substrate is continuously modulated by protein kinases and phosphatases, and dysregulation of this tight control is a feature of many diseases (ref to a suitable review?). In cancer, where signaling pathways involved in cell cycle control, such as apoptosis and proliferation, are disrupted, there is a long history of understanding the role played by abnormal phosphorylation which has led to many new drug targets (Reference and example here?). The modulation of phosphorylation events in disease is therefore a key area of research.

Aberrant phosphorylation of a number of proteins is also characteristic of Alzheimer's disease (AD) molecular pathogenesis. AD is a progressive neurodegenerative disorder accounting for more than 60% of all dementia cases, and affecting an estimated 36 million

individuals worldwide (Alzheimer's association). The pathological hallmarks of AD include plaques, comprised of the amyloid-beta (amyloid-β) protein, and neurofibrillary tangles (NFTs) containing hyperphosphorylated forms of the microtubule associated protein tau in specific brain regions [2, 3]. Tau exemplifies the aberrant phosphorylation in AD as the protein molecules become hyperphosphorylated and in this hyperphosphorylated state, tau aggregates into paired helical filaments (PHF), the main constituent of the pathological NFTs (REF = Grunke-Iqbal 1986?). This altered phosphorylation is likely to be the result of dysregulation of the kinases and phosphatases that modulate the phosphoproteome. The analysis of the global phosphoproteome alterations in AD (and other diseases) is therefore an important area of research that needs to be explored.

Mass spectrometry (MS) based approaches are ideal for detecting phosphorylation events. Liquid chromatography–tandem MS (LC-MS/MS) based workflows have been employed to identify proteins and their phosphopeptides from a range of sample types. Phosphopeptides only represent a small proportion of peptides generated by tryptic digestion of a complex sample, therefore pre-fractionation and enrichment techniques are required to reduce the complexity and enhance the detection of low abundant proteins and phosphorylation events alongside the more easily detectable high abundant proteins.

The guidelines published by the National Institute of Health [4], indicate that ideally biomarkers should be present in readily accessible samples such as plasma or CSF, and also should be directly derived from the diseased tissue. A complicating issue is that such potential biomarkers are present in very low abundance in CSF and even lower amounts in plasma. Furthermore, the wide concentration span of plasma and CSF proteins and the predominance of plasma albumin and other high-abundant proteins limits the detection of novel biomarker candidates that may aid diagnosis of disease, help monitor effects of treatment in trials and track disease progression. Some studies using mass spectrometry

(Gobom) or HPLC separation of CSF proteins followed by SDS-PAGE and Western blotting using different tau antibodies (Meredith) indicate that tau in CSF is processed to shorter fragments before being released to the CSF, with the N-terminus and mid-domain of the protein being most abundant. However, further studies using approaches with high analytical sensitivity are needed to fully characterize CSF tau, especially its phosphorylated forms, to fully explore the biomarker potential of phosphorylated tau species in the CSF. Currently, the CSF proteins widely accepted as the core biomarkers for AD include total tau levels and tau phosphorylated at threonine (Thr) residue 181 (pThr181) [5]. Despite the wide use of pThr181 as a measure correlating with disease severity, the phosphorylation of tau in the AD brain is known to extend far beyond this one site of interest, with hyperphosphorylation of tau in the brain characteristic of disease. There are also neurodegenerative diseases other than AD (e.g., progressive supranuclear palsy and some forms of frontotemporal dementia) that are characterized by NFT pathology but intriguingly, these are most often normal in regards to pThr181 tau concentrations in CSF (Blennow K et al., Nature Rev Neurol 2010). Potentially, other tau phosphoforms are released into the CSF in these diseases. Previous attempts to use MS workflows to detect tau species in the CSF have proven problematic, with limited observations of phosphorylated tau peptides detected [6, 7]. The challenge is how to find these low abundant peptides in a biofluid with a high protein dynamic range, and hyper-abundant proteins such as albumin. To answer this question, we have applied a novel Tandem Mass Tag® (TMT®) MS approach. TMTcalibrator™ is a tissue-enhanced workflow, combining tissue and peripheral fluid proteomics to allow for the broadest peptide coverage of analytical samples. It was developed to detect and effectively quantify the expression of low abundant peptides and proteins in complex biological samples, including CSF. The approach combines high pH reverse phase chromatography to fractionate the sample, TMT® isobaric labelling to allow six samples to be analyzed simultaneously alongside a 4 point

tissue calibrator, two separate phosphopeptide enrichment techniques in parallel, and increased liquid chromatography separation using a 50cm column and long MS gradient (Russell CL, unpublished). In addition to analyzing phosphopeptides enriched by two separate techniques, we also analyze the un-enriched (predominately non-phosphorylated) peptides for comprehensive profiling of a tissue or peripheral fluid sample. In order to enhance the MS data acquisition of the low abundant peptides in the analytical samples of interest, the 4-point calibrator is incorporated into each analysis, made possible by the use of TMT® labels. TMTcalibrator™ has been designed to ensure the calibrator proteins dominate the overall protein content of the TMT®10plex sample. As we are limited by the amount of protein that can be injected onto the analytical LC column, a balance between dominant calibrator and analytical samples must be achieved. We designed the 4-point TMTcalibrator™ to be spiked into the total combined analytical samples at a ratio of 1:4:6:10. These are arbitrary values allowing us to determine a suitable protein load from the six samples for analysis. The six individual CSF samples are then combined, after separate TMT® labelling, at a $2x$:$2x$:$2x$:$2x$:$2x$:$2x$ ratio, falling within the 4-point calibrator range. The overall dominance of the calibrator signals in the TMT® 10plex ensures the vast majority of peptides detected are from tissue-derived proteins. During MS acquisition, where a peptide is present both in the calibrator tissue and the body fluids we obtain both the peptide sequence and quantitative TMT® reporter ion signals from all 10 samples within the same MS/MS spectrum. This allows us to detect peptides that would otherwise be lower than the limit of detection for standard data-dependent acquisition, and results in a list of peptides that are common to the calibrator and the analytical samples.

To investigate the tau species present in the CSF we included a TMTcalibrator™ approach with phosphopeptide enrichment. For this study we used post mortem brain tissue from AD patients with known Braak V/VI classification, and analyzed 3 CSF samples from non-AD

controls and 3 CSF samples from biochemically confirmed AD patients. The aim was to use the proteins present in the brain tissue (the calibrator) to trigger the MS/MS acquisition allowing detection of any tau peptides present in the CSF samples that are common to the AD brain material. In addition to detection, the use of TMT® labelling also allowed us to determine any regulation of the tau peptides detected in the CSF between AD patients and control individuals, albeit in a small cohort.

MATERIALS AND METHODS

Cerebrospinal fluid samples – CSF samples were from patients who sought medical advice because of cognitive impairment. Patients were designated as normal or AD according to CSF biomarker concentrations using cutoffs that are 90% sensitive and specific for AD [8]: total tau >350 ng/L, phospho-tau >80 ng/L and Aβ42 <530 ng/L. None of the biochemically normal subjects fulfilled these criteria. CSF total tau, phospho-tau and Aβ42 levels were determined using INNOTEST enzyme-linked immunosorbent assays (Fujirebio, Ghent, Belgium) by board-certified laboratory technicians according to protocols approved by the Swedish Board for Accreditation and Conformity Assessment (SWEDAC). The study was approved by the regional ethics committee at the University of Gothenburg. The protein concentration of the brain lysate was estimated by Bradford protein assay. Include analysis of serum as a negative control?

Post Mortem Brain Tissue – Brain tissue samples were received from the University of East Finland. Three frozen tissue samples from inferior temporal cortex samples were dissected from larger frozen tissue sections. Samples were selected according to neurofibrillary tangle (NFT) pathology (Braak staging) and represent Stages V and VI of AD-related NFT pathology. Frozen brain tissue was lysed in 8M urea, 75mM NaCl, 50mM Tris (pH 8.2) with

phosphatase and protease inhibitors added, before sonication (20 x 1sec 20% amplitude) on ice and centrifuged at 12,500g for 10mins at 4°C. The protein concentration of the brain lysate was estimated by Bradford protein assay.

In-Solution Tryptic Digest and TMT® labelling – Following solubilization and denaturation in 100mM TEAB buffer and 0.1% SDS, the CSF samples and the AD brain tissue (calibrator) were reduced with 1mM TCEP at 55 °C for 60 min and alkylated with 7.5mM iodoacetamide at room temperature for 60 min. Trypsin was used for digestion at an approximate 1:25 weight ratio of trypsin-to-total protein and incubated at 37 °C overnight (~18 h). Following digestion, the peptides were labeled with one of the TMT®10plex reagents at 15mM and incubated for 60 min at room temperature. To quench the TMT® reaction 0.25% hydroxylamine was added to each sample and incubated for 15 min. The samples were then combined as detailed in the text and incubated for a further 15 min and desalted on RP18 columns.

Fractionation and Phosphopeptide Enrichment – The TMT®10plex was split into two equal aliquots and both fractionated by high pH reverse phase fractionation (Thermo Fisher Scientific kit, product number 84868) into 8 fractions. These were pooled into 4 fractions. A small portion of each fraction was removed for analysis of un-enriched (predominantly non-phosphorylated) peptides, and the remainder enriched for phosphopeptides using either Immobilized Metal Affinity Columns (IMAC) or Titanium dioxide (TiO2). Following enrichment the corresponding enriched fractions from IMAC enrichment and TiO2 enrichment were pooled, to generate 4 fractions, and cleaned up prior to MS using Graphite Spin Columns (Thermo Fisher Scientific). For MS analysis the sample was resuspended in 2% ACN + 0.1% formic acid.

LC-MS/MS - Quantitative analysis was performed using an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) in the positive mode with EASYnLC1000 system and a 50cm EASY-Spray column (Thermo Fisher Scientific). The column temperature was maintained at 40°C, and the peptides were separated at a flow rate of 200 nL/min. Peptides were eluted from the column over a 180 min gradient, from 10% to 30% solvent B (acetonitrile with 0.1% formic acid) in 160 min, followed by an increase to 100% solvent B in 10mins, which was held for a further 10mins. Solvent A was water with 0.1% formic acid. Wash and blank LC–MS/MS runs preceded the analysis and wash runs in between fractions.

A Top Speed HCD method with a 3 second cycle time was utilized and the parameters were as follows: MS; Spray voltage: 2000V; ion transfer tube temperature: 275 °C; detector: Orbitrap; scan range (m/z): 400-1400; resolution: 120000; AGC target: $5x10^5$. MS/MS: HCD; Isolation mode: quadropole; collision energy: 35%; detector: Orbitrap; Resolution: 30000; AGC target: $1x10^{4.}$

Peptide Identification and Quantification – LC-MS/MS data was initially processed within Proteome Discoverer (PD) v1.4 (Thermo Scientific) using the Spectrum Files node.

Spectrum selector was set to default and the SEQUEST HT node was set up to search data against the Uniprot Homo sapiens FASTA database (taxon ID 9606 - Version September 2014). The reporter ions quantifier node was set up to measure the raw intensity values for TMT® 10plex monoisotopic ions (126, 127N, 127C, 128N, 128C, 129N, 129C, 130N, 130C, 131). Oxidation (M), phosphorylation (S, T, Y), and deamidation (N, Q) were set as variable modifications. Static modifications included carbamidomethylations (C) and TMT10 (N-terminus and K). 1% FDR and 1 x Rank 1 peptide per protein filters were applied and all raw intensity values exported to an Excel text file for downstream processing and filtering using in-house software.

Data Normalization and analysis – TMT® reporter ion intensities for every PSM across the 6 analytical samples within a TMT® 10plex set were normalized using the sum scaling technique [9-11] to minimize the effect of experimental bias and systematic errors on quantitative peptide quantitative values. The TMT® reporter ion intensities of the 4 calibrator channels were scaled using correction factors computed based on expected ratios relative to 129C channel in each TMT® 10plex set. To measure a correspondence between expected calibrator signal intensities to observed calibrator signal intensities a linear model was fitted and applied to every peptide spectral match (PSM). Corresponding R^2 (coefficient of variance) values were computed for each PSM. Post sum-scaling, peptide level intensities were determined by summing all normalized PSM intensities specific to a peptide sequence. Protein level quantification was determined by computing the median value of log2 peptide ratios. These peptide level ratios were determined as the median of sum-scaled and normalized peptide intensities across all 6 analytical samples within a TMT® 10plex. Protein level quantitation was specific to a protein accession ID, distinguishing between different isoforms. Statistical analysis was carried out using a two-sample T test. All data pre-processing steps were performed using in house R scripts (R version 3.1.2 - "Pumpkin Helmet").

RESULTS

TMTcalibrator™ with phosphopeptide enrichment provides broad coverage of the analytical samples of choice with the unique ability to match tissue and body fluid expression of identical peptides and proteins. In this study, over 34,000 unique un-phosphorylated peptides were observed. In addition, more than 11,000 unique phosphopeptides were detected and quantified in our dataset, mapping to more than 11,500 phosphosites. These peptides correspond to over 19,000 proteins, which match in excess of 4,000 protein groups.

We explored this extensive dataset for the presence of peptides aligned to the sequence of human tau 441 (accession number P10636-8). Considering all tau peptides we achieved 86% coverage of the protein. These peptides matched across the entire sequence of the protein, including peptides at both the C and N-termini (Figure 1). In total, we detected 168 unique tau peptides including 115 phosphopeptides, present in at least one of the CSF samples or in the brain tissue analyzed. To determine which of these are common to both the brain material and the CSF samples, the dataset was filtered based on the presence of a signal in the individual TMT® reporter ion channels. When missing values were removed, we found 87 tau peptides covering 76.8% of tau 441, that were present in all 10 TMT® channels, meaning these peptides were detectable in all six CSF samples analyzed, and in the 4-point brain lysate calibrator. Peptides were detected spanning the majority of the sequence, including the C- and N-termini, and the middle region. Residues 127 – 155 and 291 – 321 were the main regions under-represented in the dataset, but this is likely due to a high number of tryptic cleavage sites resulting in very small peptides that are not detectable. We also identify a large peptide that is specific to isoform 1 of tau (758 residues, accession number P10636-1) indicating the presence of this tau isoform in the CSF. 47 of the 87 peptides detected in all 10 channels were phosphorylated (Table 1), representing the most comprehensive detection of phosphorylated tau in CSF to date. Phosphorylation was seen at multiple sites (Table 1), with singly and multiply phosphorylated tau peptides present. In total we observed 31 different tau phosphosites that were annotated with a high confidence score (>75%) by the PhosphoRS tool embedded within the Proteome Discoverer software, 20 of which were present in all of the samples analyzed (as shown in Table 1 alongside peptide sequence). Phosphosites annotated with a question mark (?) could not be confidently assigned to a single amino acid residue within that peptide sequence. Site localization within the phosphopeptide is therefore ambiguous in these instances. Add negative control data (serum)?

A benefit of using TMT® to analyze multiple samples simultaneously is that the relative levels of peptides and proteins detected between sample groups can be accurately determined. This allows us to compare the levels of the tau peptides and phosphopeptides detected in the CSF of non-AD individuals with the levels seen in the AD CSF patients. Following bioinformatics processing, a log2 fold change is attributed to each peptide and protein detected (see methods) and using this parameter to filter the data, we found, as expected, an overall increase in total tau levels in the CSF of AD patients compared to the non-AD control samples analyzed. Overall, a 40% increase was seen, based on the log2 fold change of all un-phosphorylated tau peptides (n=40) (Figure 2). Additionally, 11 phosphopeptides were up-regulated by at least 40% in the CSF of AD patients compared to controls. The significance value of this regulation is also incorporated into the data sheet produced, and this can also be used to further filter the data. The most significant of the tau phosphopeptides regulated in AD compared to controls was a doubly phosphorylated peptide, phosphorylated at Ser231 and Thr235 (Figure 3 A and B) and a singly phosphorylated peptide, phosphorylated at Ser64 (Figure 3 C and D) (numbering according to tau441).

The AD biomarker literature largely focuses on tau phosphorylation at Thr181, and measurement of this phosphorylation site by ELISA is one of the three well established CSF biomarkers. We detect five different peptides phosphorylated at Thr181 in the whole dataset, including singly (Figure 4A) and multiply phosphorylated peptides (Figure 4B). Summing the overall change of all unique pThr181 peptides observed results in the expected increase in AD CSF levels compared to non-AD controls (Figure 4C). Taking the same approach of summing the signal intensities of all of the peptides at each of the phosphosites, we can generate an overall tau phosphorylation site map, and see phosphosites that are regulated in AD CSF (Figure 5).

DISCUSSION

Tau in the CSF is believed to be of diagnostic importance, correlating with the severity of disease with regard to AD and neurodegeneration. Extensive efforts have been made to further characterize the nature of CSF tau, with previous studies largely utilizing immuno-detection methods, relying on anti-tau antibodies [5, 12]. Immuno-detection approaches have had limited success in characterizing the tau in CSF, as the methods are limited by the availability, specificity and sensitivity of the antibodies. Furthermore, tau is known to be heavily modified by post translational modifications (PTMs), with acetylation, phosphorylation, and glycosylation events on numerous residues reported within the protein sequence [13]. These PTMs can interfere with the detection of tau by anti-tau antibodies and therefore impact on the success of immuno-detection based approaches. Mass spectrometry (MS) provides an ideal analytical tool to overcome the specificity limitations of antibody based studies but lacks sufficient sensitivity for comprehensive profiling in a complex sample such as CSF. Here we have designed and implemented a new MS based method to extensively characterize tau in both AD brain tissue and CSF simultaneously to determine which tau species are present in the CSF. Using this innovative approach, we have detected tau and phospho-tau peptides in the CSF derived from both central and N- and C- terminal regions and, in addition, by utilizing TMT® labeling, observations of differential regulation of tau peptides in AD compared to non-AD controls have been made.

Here we used TMT® labeled peptides derived from post mortem brain material to enhance the intensity of the equivalent CSF peptides and drive the MS/MS acquisition, allowing the detection of low abundant peptides common to the brain and CSF. Although this TMTcalibrator™ study was not specifically targeting tau in the AD brain or the CSF, nor enriching for tau peptides upstream of MS analysis, peptides covering 86% of the full tau 441 protein were detectable. 87 different tau peptides common to the brain and the CSF were

observed, almost half of which were found to be phosphorylated (n=47) representing the most extensive characterization of CSF tau reported to date. These peptides covered multiple phosphosites, including but not limited to, the widely studied Thr181 residue. Phosphorylation of tau at any of the sites reported here could be useful diagnostic biomarkers, and could also be used to monitor the efficacy of novel drugs targeting aberrant tau phosphorylation, for example kinase inhibitors. Additional tau peptides were also measured but were either exclusive to the brain material, or not present in all six CSF samples.

Previous work by groups investigating tau in the CSF has had mixed results, largely due to the differences in sample preparation and up-front processing. Studies using MS to detect CSF tau have demonstrated some success [6, 7], and the most recently reported work identified 18 non-phosphorylated peptides in the CSF using a two-step pre-fractionation workflow and monitoring peptides using a spiked heavy labelled recombinant tau protein [14]. Until now, this is the most successful attempt at characterizing tau in the CSF. TMTcalibrator™ has been designed to avoid immuno-enrichment steps, and does not require depletion or filtering of the samples. The combination of fractionation, enrichment for phosphopeptides, analysis of both enriched and un-enriched material and a long LC gradient has resulted in an extensive coverage of the peptides and proteins present in the AD brain and CSF.

A further benefit of this approach is the use of isobaric TMT® labelling. This has allowed for the simultaneous analysis of six CSF samples in one experiment, and allows relative quantitation of tau peptides and phosphopeptides in the CSF of AD compared to non-AD controls to be made. As expected, the overall tau levels were elevated in the CSF from AD patients relative to control, non-AD individuals. We observed a 40% increase in total tau levels, and this is in line with previous reports using MS to analyze tau levels [7] but lower

than the increase found using immunoassays [15]. The levels of 11 tau phosphopeptides were also seen to significantly increase in AD by at least 40%, including phosphorylation at Thr181. The detection of multiply phosphorylated peptides around the Thr181 residue leads us to question the accuracy of the reported immuno-based approaches commonly used to measure phospho-tau levels, as phosphorylation at multiple sites is likely to interfere with antibody-antigen binding.

The regulation of the phosphorylation events on tau also showed a relative decrease at some phosphosites, most pronounced on Ser285 and Ser289, and down regulation at phosphosites has recently been reported in another study [14]. We hypothesize that this could be the result of extensive aggregation of the tau proteins in AD resulting in reduced levels in the CSF. Alternatively, it is known that O-GlcNAcylation and glycosylation of tau results in a reciprocal down regulation of phosphorylation [16], so it may be that modifications to the tau peptide sequence that are not captured in this study, such as glycosylation, are either directly or indirectly affecting the levels of phosphorylation at certain sites.

Monitoring an extensive array of tau phosphorylation sites in CSF will likely gain importance, particularly as our understanding of their individual and combined roles in disease etiology increases and drug development activities shift away from anti-amyloid strategies to focus on tau and ways to prevent tangle formation where there are a number of experimental molecules currently in development [17].

The ability to characterize tau in the CSF is not only important in AD biomarker research, but could also have significant impact in the discovery of biomarkers for other tauopathies. The TMTcalibrator™ approach could be used to detect the tau species present in the CSF of patients with other neurodegenerative tauopathies, as designed here, or also designed to directly compare CSF from different diseases, for example, AD CSF compared to

frontotemporal dementia (FTD) CSF. There is a need for distinct CSF biomarkers of different tauopathies as the ELISA CSF total tau measurements cannot distinguish AD from some other neurodegenerative diseases; the specificity is only approximately 50% when comparing AD to FTD or vascular dementia [18], and it is considered a general marker of axonal damage, as seen for example in stroke [19], Creutzfeldt-Jakob disease [20] and traumatic brain injury [21]. The ability to detect and relatively quantify phosphorylated tau in the CSF is of great benefit as it is likely to be differences in the PTMs of tau that help to better distinguish AD and other dementias. In contrast to the poor specificity of total CSF tau, phosphorylation at the widely reported Thr181 residue is able to differentiate between AD and FTD [5, 18], and it is reasonable to think that alternative phosphosites will be of equal importance in tauopathy and neurodegenerative disease biomarker discovery.

One limitation of this study is the small sample set used. Confirmation of these findings will require analysis in a larger cohort of CSF samples, and experimental work is already underway to validate the preliminary findings presented here.

In conclusion, we present here the most comprehensive characterization of CSF tau in AD using a new MS technique designed to enhance the detection of low abundant proteins in peripheral bodily fluids using an AD brain tissue trigger and numerous phosphorylation sites on tau could be detected and quantified in CSF, many of which have not previously been observed. Furthermore, the unique experimental design has allowed for differential regulation of these phosphosites between AD and non-AD CSF to be investigated. The results indicate that tau in the CSF is extensively modified by phosphorylation events and further studies are warranted to determine the value of these modifications as potential biomarkers of disease. Analysis of further samples is necessary to confirm disease association, and these measurements are ongoing in a more extensive cohort at the time of submission of this manuscript.

Whilst this report has focused exclusively on tau, this was an unbiased study and there were over 100 other proteins representing multiple facets of AD pathology that were regulated in the disease group CSF samples. Based on these preliminary findings the same TMTcalibrator™ approach can be used to provide important pharmacodynamic information pertaining to new therapeutic compounds during pre-clinical development and to validate translational biomarkers in early human studies. The deployment of TMTcalibrator™ as a systems biology tool could therefore offer significant benefits as we strive to find better therapeutic strategies to combat dementia.

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Add grants?

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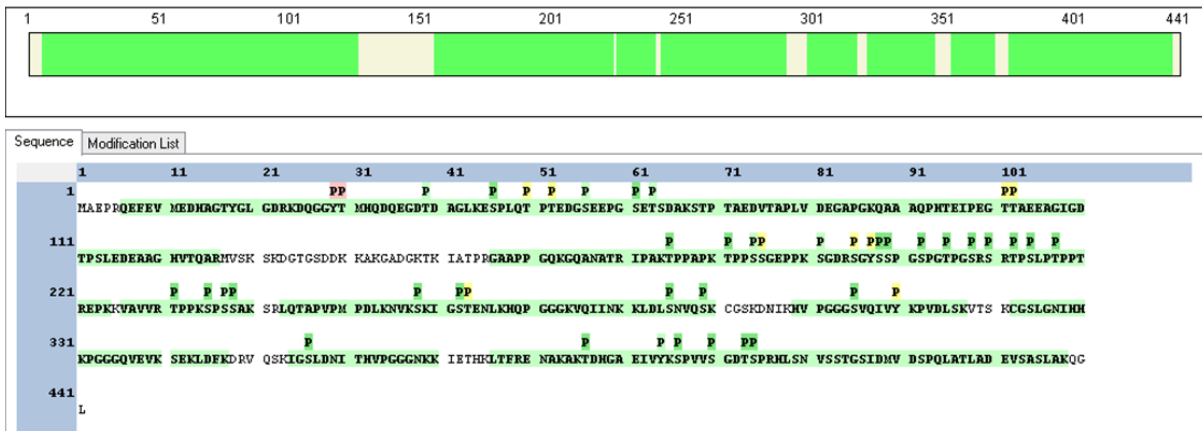

Figure 1. 86% tau coverage was seen in this study with peptides covering all regions of tau 441. In this image the green highlighted regions represent regions of the protein sequence detected.

Peptide Sequence	Phosphorylation Site	Number of Phosphosites
akTDHGAEIVykSPVVSGDTSPR	S396;	1
akTDHGAEIVYksPVVsGDTSPR	Y?394;S?396;S400;	2
akTDHGAEIVykSPVVSGDtSPR	Y?394;S?396;T?403;S?404;	2
akTDHGAEIVYksPVVsGDTsPR	Y?394;S?396;T403;S404;	3
esPLQTPTEDGSEEPGSETSDAk	S46;	1
eSPLQTPTEDGSEEPGsETSDAk	S61;	1
iGsTENLk	S262;	1
iGsTEnLkHQPGGGk	S?262;T?263;	1
iGStEnLkHQPGGGk	S?262;T?263;	1
iGStENLkHQPGGGk	S?262;T?263;	1
iGsTENLkHQPGGGk	S262;	1
kLDLSNVQsk	S289;	1
kLDLSnVQsk	S289;	1
lDLsNVQsk	S285;S289;	2
lDLSNVQsk	S289;	1
qAAAQPHTEIPEGtTAEEAGIGDTPSLEDEAAGHVTQAR	T?101;T?102;	1
sGYSsPGSPGTPGSR	S?195;Y?197;S?198;	1
sGYssPGSPGTPGSR	S?195;Y?197;S?198;S?199;	2
sGySSPGSPGtPGSR	S?198;S?199;T205;	2
sGYSsPGsPGTPGSR	S199;S202;	2
sGYSSPGsPGTPGSR	S202;	1
sGYsSPGSPGTPGSR	S202;	1
skIGsTEnLk	S262;	1
skIGStENLkHQPGGGk	S?258;S?262;	1
skIGsTEnLkHQPGGGk	S?258;S?262;T?263;	2
skIGStEnLkHQPGGGk	S?258;S?262;T?263;	1
skIGsTENLkHQPGGGk	S258;	1
sPVVSGDtSPR	S?400;T?403;S?404;	1

sPVVsGDTSPR	S?400;T?403;S?404;	1
sPVVSGDTsPR	T?403;S?404;	1
sRtPsLPtPPTR	T212;S214;T217;	3
sRTPsLPTPPTREPk	T212;S?214;T?217;	2
sRtPSLPtPPTREPk	T212;S214;	2
sRtPsLPTPPTREPk	T212;S214;	2
sRtPsLPtPPTREPk	T212;S214;T217;	3
tDHGAEIVYksPVVSGDTSPR	S396;	1
tDHGAEIVYkSPVVsGDTsPR	S396;S?400;T?403;S?404;	2
tDHGAEIVYksPVVsGDTSPR	S396;S400;	2
tDHGAEIVykSPVVSGDtSPR	S396;T?403;S?404;	2
tDHGAEIVYksPVVsGDTsPR	Y?394;S?396;S?400;T?403;S?404 ;	3
tPPAPktPPSSGEPPk	T181;	1
tPPAPktPPSSGEPPkSGDR	T181;	1
tPsLPTPPTREPk	S214;	1
tPSLPtPPTREPk	T?212;S?214;T217;	2
vAVVRtPPkSPSSAk	T231;	1
vAVVRtPPkSPssAk	T231;S?235;S?237;S238;	3
vAVVRtPPkSPSsAk	T231;S235;	2

Table 1. List (alphabetical) of phosphorylated tau peptides detected in all samples detailing the peptide sequence, phosphorylation site (numbering according to tau 441), and number of phosphosites on the peptide. A ? next to the phosphosite denotes a site that cannot be determined by the software.

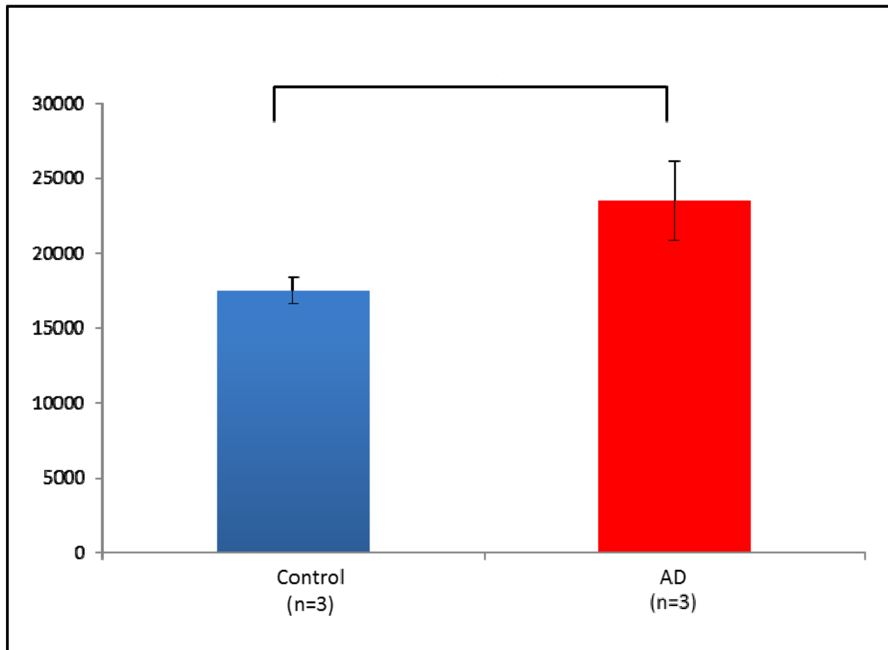

Figure 2. Total tau levels are seen to increase by 40% in the CSF of AD patients compared to non-AD control individuals (p=0.049).

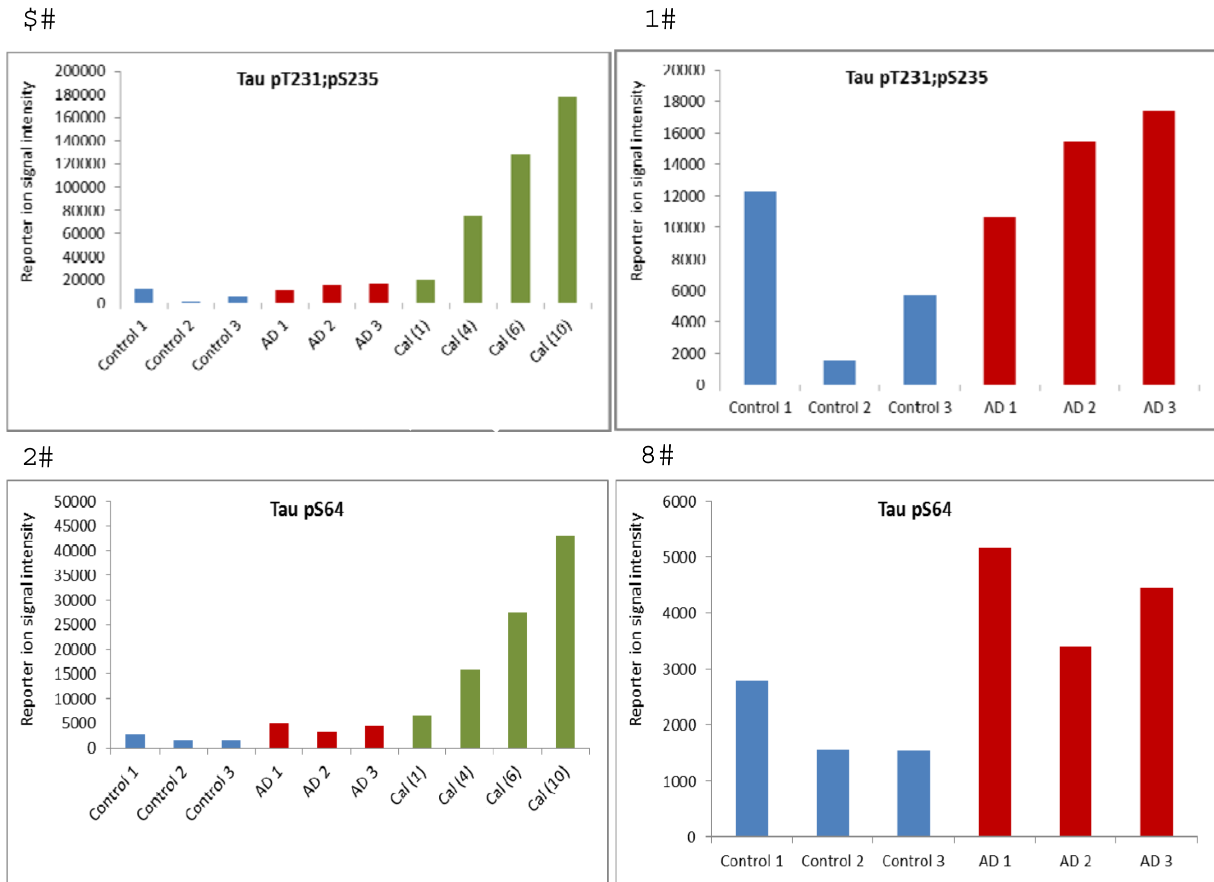

Figure 3. Regulation of phosphotau peptides in seen in the CSF of AD patients compared to non-AD controls. Doubly phosphorylated Thr231;Ser235 peptide is shown with the 4-point calibrator signal (green) (A) and without the calibrator (B) to better see the regulation between control (blue) and AD (red) CSF levels. Similarly, single site phosphorylation at Ser64 is shown with (C) and without (D) the calibrator signal.

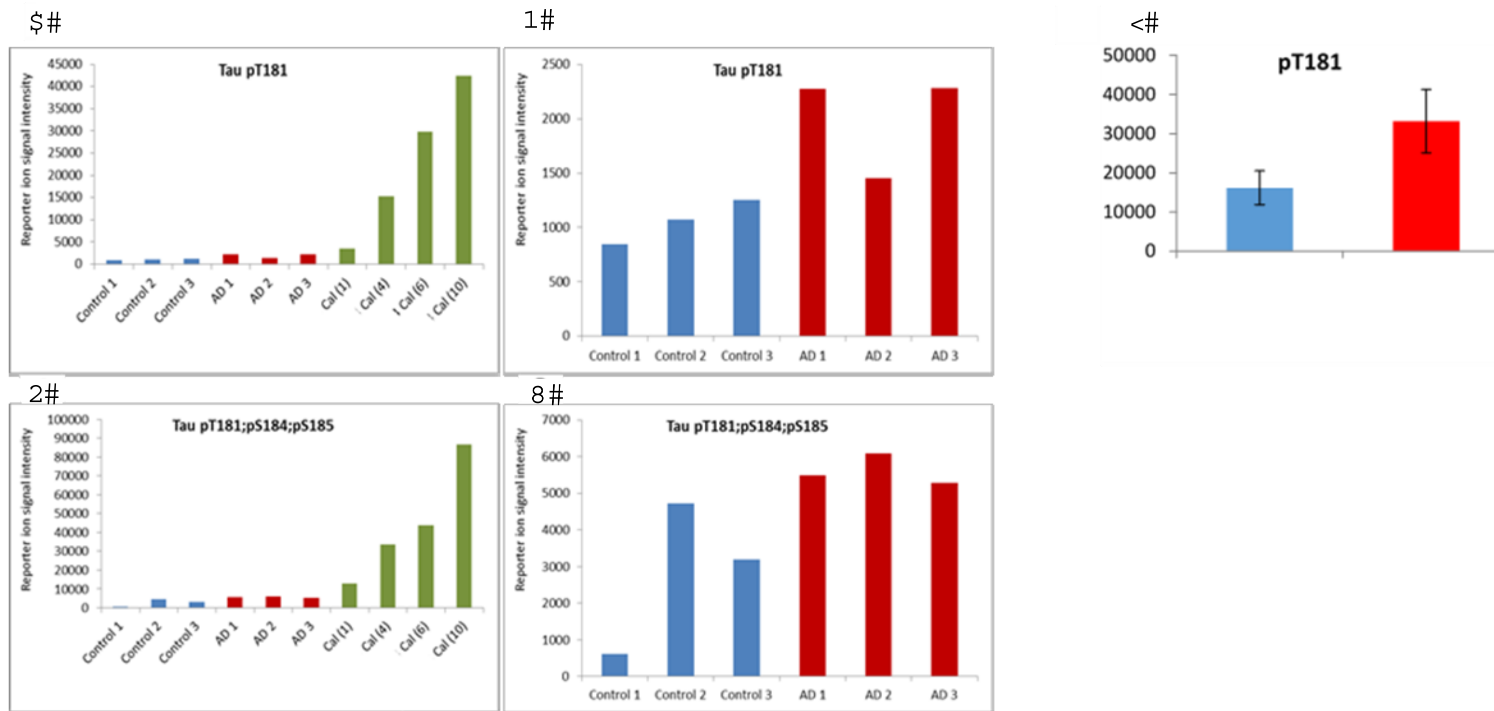

Figure 4. Regulation of tau phosphorylated at Thr181. Singly phosphorylated peptide is shown with (A) and without (B) the calibrator signal to better show the increased levels in AD CSF (red) compared to non AD controls (blue), Similarly, triply phosphorylated Thr181;Ser184;Ser185 is also shown with (C) and without (D) the calibrator signal. Overall levels of tau phosphorylated at Thr181 were seen to increase in the CSF of AD patients compared to non-AD controls (E). Include an F-panel with correlation of pT181 to INNOTEST P-tau181?

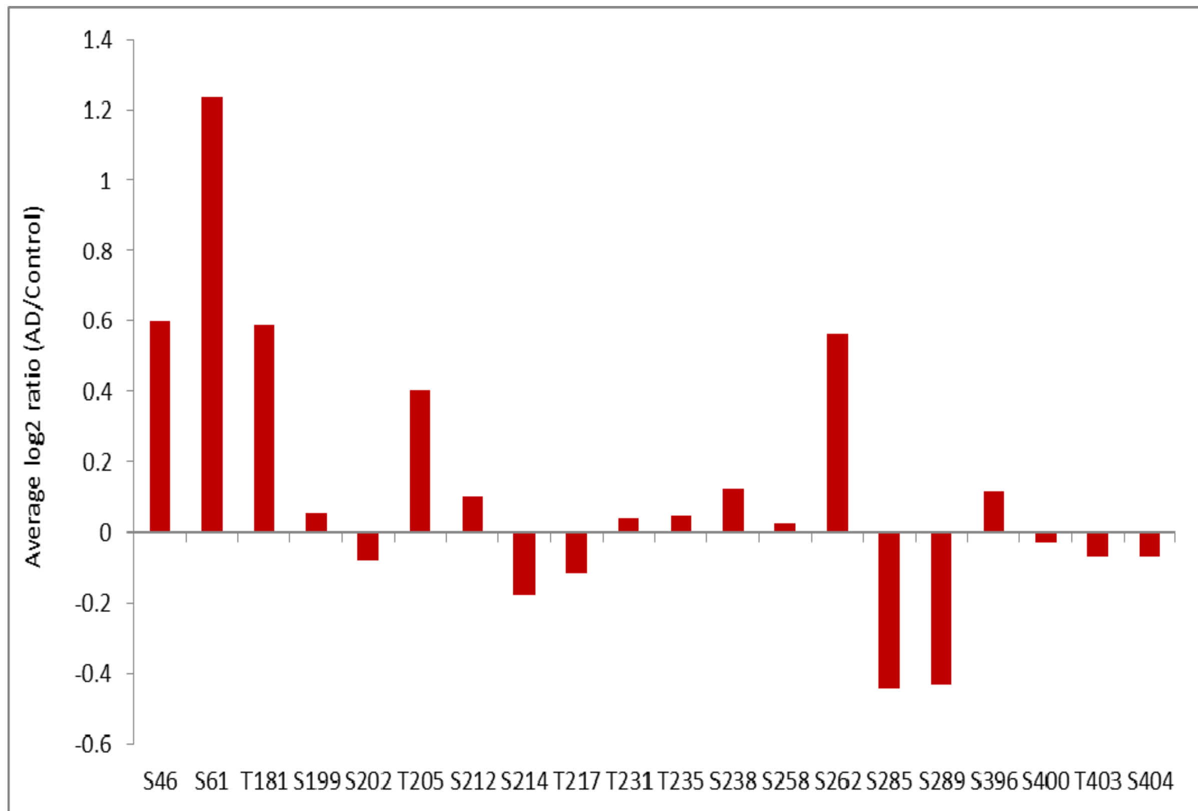

Figure 5. Tau phosphosite map showing the average log2 fold change in AD CSF compared to non-AD controls for each individual phosphosite measured in all 10 TMT® channels.

ABBREVIATIONS

Aβ – Amyloid beta; AD - Alzheimer's disease; CSF – Cerebrospinal fluid; FTP – frontotemporal dementia; IMAC – Immobilized Metal Affinity Columns; LC-MS/MS – Liquid chromatography tandem mass spectrometry; MS – Mass spectrometry; NFT – Neurofibrillary tangles; PHF – Paired helical filaments; pThr181 – Phosphorylation of Threonine 181; PTMs – Post translational modifications; Ser – Serine; Thr – Threonine; Tandem Mass Tags® - TMT®; TiO_2 – Titanium dioxide.

REFERENCES

[1] Cohen P (2002) The origins of protein phosphorylation. *Nat Cell Biol* **4**, E127-130.

[2] Selkoe DJ (1991) The molecular pathology of Alzheimer's disease. *Neuron* **6**, 487-498.

[3] Blennow K, de Leon MJ, Zetterberg H (2006) Alzheimer's disease. *Lancet* **368**, 387-403.

[4] McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* **34**, 939-944.

[5] Vanmechelen E, Vanderstichele H, Davidsson P, Van Kerschaver E, Van Der Perre B, Sjogren M, Andreasen N, Blennow K (2000) Quantification of tau phosphorylated at threonine 181 in human cerebrospinal fluid: a sandwich ELISA with a synthetic phosphopeptide for standardization. *Neurosci Lett* **285**, 49-52.

[6] Portelius E, Hansson SF, Tran AJ, Zetterberg H, Grognet P, Vanmechelen E, Hoglund K, Brinkmalm G, Westman-Brinkmalm A, Nordhoff E, Blennow K, Gobom J (2008) Characterization of tau in cerebrospinal fluid using mass spectrometry. *J Proteome Res* **7**, 2114-2120.

[7] McAvoy T, Lassman ME, Spellman DS, Ke Z, Howell BJ, Wong O, Zhu L, Tanen M, Struyk A, Laterza OF (2014) Quantification of tau in cerebrospinal fluid by immunoaffinity enrichment and tandem mass spectrometry. *Clin Chem* **60**, 683-689.

[8] Hansson O, Zetterberg H, Buchhave P, Londos E, Blennow K, Minthon L (2006) Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study. *Lancet Neurol* **5**, 228-234.

[9] Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139-140.

[10] Paulson JN, Stine OC, Bravo HC, Pop M (2013) Differential abundance analysis for microbial marker-gene surveys. *Nat Methods* **10**, 1200-1202.

[11] De Livera AM, Dias DA, De Souza D, Rupasinghe T, Pyke J, Tull D, Roessner U, McConville M, Speed TP (2012) Normalizing and integrating metabolomics data. *Anal Chem* **84**, 10768-10776.

[12] Meredith JE, Jr., Sankaranarayanan S, Guss V, Lanzetti AJ, Berisha F, Neely RJ, Slemmon JR, Portelius E, Zetterberg H, Blennow K, Soares H, Ahlijanian M, Albright CF (2013) Characterization of novel CSF Tau and ptau biomarkers for Alzheimer's disease. *PLoS One* **8**, e76523.

[13] Russell CL, Koncarevic S, Ward MA (2014) Post-translational modifications in Alzheimer's disease and the potential for new biomarkers. *J Alzheimers Dis* **41**, 345-364.

[14] Barthelemy NR, Gabelle A, Hirtz C, Fenaille F, Sergeant N, Schraen-Maschke S, Vialaret J, Buee L, Junot C, Becher F, Lehmann S (2016) Differential Mass Spectrometry Profiles of Tau Protein in the Cerebrospinal Fluid of Patients with Alzheimer's Disease, Progressive Supranuclear Palsy, and Dementia with Lewy Bodies. *J Alzheimers Dis* **51**, 1033-1043.

[15] Blennow K, Hampel H (2003) CSF markers for incipient Alzheimer's disease. *Lancet Neurol* **2**, 605-613.

[16] Smet-Nocca C, Broncel M, Wieruszeski JM, Tokarski C, Hanoulle X, Leroy A, Landrieu I, Rolando C, Lippens G, Hackenberger CP (2011) Identification of O-GlcNAc sites within peptides of the Tau protein and their impact on phosphorylation. *Mol Biosyst* **7**, 1420-1429.

[17] Qian X, Hamad B, Dias-Lalcaca G (2015) The Alzheimer disease market. *Nat Rev Drug Discov* **14**, 675-676.

[18] Hampel H, Buerger K, Zinkowski R, Teipel SJ, Goernitz A, Andreasen N, Sjoegren M, DeBernardis J, Kerkman D, Ishiguro K, Ohno H, Vanmechelen E, Vanderstichele H, McCulloch C, Moller HJ, Davies P, Blennow K (2004) Measurement of phosphorylated tau

epitopes in the differential diagnosis of Alzheimer disease: a comparative cerebrospinal fluid study. *Arch Gen Psychiatry* **61**, 95-102.

[19] Hesse C, Rosengren L, Andreasen N, Davidsson P, Vanderstichele H, Vanmechelen E, Blennow K (2001) Transient increase in total tau but not phospho-tau in human cerebrospinal fluid after acute stroke. *Neurosci Lett* **297**, 187-190.

[20] Blennow K, Johansson A, Zetterberg H (2005) Diagnostic value of 14-3-3beta immunoblot and T-tau/P-tau ratio in clinically suspected Creutzfeldt-Jakob disease. *Int J Mol Med* **16**, 1147-1149.

[21] Zetterberg H, Hietala MA, Jonsson M, Andreasen N, Styrud E, Karlsson I, Edman A, Popa C, Rasulzada A, Wahlund LO, Mehta PD, Rosengren L, Blennow K, Wallin A (2006) Neurochemical aftermath of amateur boxing. *Arch Neurol* **63**, 1277-1280.