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Non-Phosphorylated Tau as a potential biomarker of Alzheimer's Disease:

Analytical and diagnostic characterization

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Abstract

BACKGROUND: Virtually nothing is known about a potential diagnostic role of non-phospho-epitopes of Tau (Non-P-Tau) in cerebrospinal fluid (CSF).

OBJECTIVE: To establish and analytically and clinically characterize the first assay capable to measure concentrations of Non-P-Tau in human CSF.

METHODS: An antibody (1G2) was developed that selectively binds to the Tau molecule non-phosphorylated at the positions T175, T181, and T231, and was used in establishing a sandwich ELISA capable to measure Non-P-Tau in human CSF, following analytical and clinical validation of the method.

RESULTS: The 1G2 antibody shows decreasing reactivity to tau peptides containing phosphorylation at positions T175, T181 and T231. Detection limit of the assay is 25 pg/ml; the coefficients of variation (CV's) of the optical densities of the repeated standard curves were between 3.6 - 15.9%. Median intra-assay imprecision of double measurements was 4.8%; inter-assay imprecision was in the range of 11.2% - 15.3%. Non-P-Tau concentrations are stable in the CSF samples sent to distinct laboratories under ambient temperature; inter-laboratory variation was approximately 30%. The Non-P-Tau CSF concentrations were highly significantly increased in patients with Alzheimer's disease in stage of Mild Cognitive Impairment or dementia (AD/MCI, n=58, 109.2 ± 32.0 pg/mL) compared to the non-demented Controls (n=42, 62.1 ± 9.3 pg/mL, $p < 0.001$). At the cut-off of 78.3 pg/mL, the sensitivity and the specificity were 94.8% and 97.6%, respectively.

CONCLUSION: For the first time, an assay is reported to reliably measure concentrations of non-phosphorylated Tau in human CSF.

Keywords: Biomarkers; Cerebrospinal fluid; Tau; Phosphorylation;

Introduction

A crucial role of the CSF biomarkers in an early diagnosis of Alzheimer's disease (AD) has been extensively discussed, leading to inclusion of the Neurochemical Dementia Diagnostics (NDD) biomarkers into different diagnostic and/or research criteria [1-4]. Such development is not surprising when the need is considered for a reliable early AD diagnosis in drug trials and clinical practice. Research on medical interventions in AD is focusing on the early stages of the disorder, for example mild cognitive impairment (MCI), and hence CSF biomarkers are very helpful to identify individuals in the pre-dementia phase of AD that would benefit from the treatment [5]. This is also reflected in the European Medicines Agency statement that AD CSF biomarkers are useful for the enrichment of the prodromal AD populations in clinical trials [6].

The alterations in CSF occur many years or even decades before the onset of the clinical symptoms of AD [7, 8]. Currently two groups of molecules in the CSF are accepted as the NDD biomarkers: amyloid β ($A\beta$) peptides, which reflect deposition of $A\beta$ (senile) plaques in the brain, and Tau protein along with its hyperphosphorylated forms (pTau), which is linked to the accumulation of neurofibrillary tangles and neurodegeneration (reviewed in [9] and [10]). Although alterations in $A\beta$ metabolism are currently considered the earliest detectable events in AD [7], interventional strategies based on the $A\beta$ hypothesis have been so far disappointing [11, 12]. This calls for more extensive investigation of other hypotheses, of which those related to Tau seem particularly attractive [13]. This is further supported by the observation that cognitive symptoms in AD are directly related to biomarkers of neurodegeneration rather than biomarkers of $A\beta$ deposition (reviewed in [7]); also in neuropathological studies a clear correlation was shown between the degree of post mortem neurofibrillary tangle pathology and a patient's cognitive functions *intra vitam* [14, 15].

Currently available AD biomarkers ($A\beta$ 1-42, $A\beta$ 42/40 Ratio, Tau, and pTau) offer accuracy in the range of 80-85% (depending on the clinical setting), which certainly requires improvement. Therefore, rationale of the current study was to develop an assay that might

improve the diagnosis of neurodegeneration, and to further enrich set of available CSF AD biomarkers. Therefore, we have raised a high-affinity monoclonal tau antibody clone 1G2, strongly recognizing two KTPP motifs on the tau molecule comprising T175 and T181. Whereas the diagnostic role of Tau and its phosphorylated forms, in particular those phosphorylated at threonine 181 (pTau181) has been extensively studied in the last decade, we are not aware of any study addressing the diagnostic role of CSF non-phospho-epitopes of Tau (Non-P-Tau); furthermore, we have not found any reports of an assay capable to measure CSF concentrations of Non-P-Tau. Hence it seemed relevant to establish such an assay and to investigate whether Non-P-Tau could be interesting as a potential novel biomarker of AD.

Materials and Methods

1. Selection and preparation of immunogen

Mice were immunized to generate mouse IgG antibodies directed to Tau protein and characterized by high binding affinity. IgG immune response of mice was tested by estimation of titres of mice sera to Tau2N4R (full length Tau or Tau2N4R) coated on ELISA plates. For selection of most superior antigen mice were immunized 4 times using different Tau preparations as antigens. Antigens were recombinant human Tau2N4R, Tau2N4R aggregates and phosphorylated Tau2N4R aggregates as described below. Comparison of humoral IgG responses after 63 d immunization period showed highest IgG titres of sera from mice immunized with phosphorylated Tau2N4R aggregates followed by mice immunized with Tau2N4R aggregates whereas sera of mice immunized with recombinant human Tau2N4R showed low IgG titres but high IgM titres (data not shown). Therefore, as best immunogenic preparation phosphorylated Tau2N4R aggregates were selected and used for further immunization and booster of mice before B cell selection.

For preparation of the antigen, 2mg/500µl recombinant human tau (2N4R isoform 441 amino acids) was incubated for 20h with 3µg activated MAPK13 (Life Technologies, USA) in 10 mM Tris (pH 7.4), 11 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 1mM AEBSF, 0.5 mM Na₃VO₄, 0.01% TritonX-100, 1 mM ATP at 30°C. Fresh ATP was supplemented after 10h incubation. Tau

aggregation was started by adding 20fold-aggregation mix (1M MOPS pH 6.5, 1M NaCl, 8mg/ml heparin, 2mM AEBSF) after heat inactivation of MAPK13 and phosphorylated tau was incubated 48h at 37°C. Monomeric (fraction 45-47) and aggregated phosphorylated Tau protein (fractions 21-23) were separated using a Sephacryl S500 gel filtration column as described earlier [16].

2. Immunisation and antibody development

50 µg of each antigen in complete Freud adjuvant (Sigma-Aldrich, USA) were used for first immunization of Balb/c mice by subcutaneous injection. Thereafter animals were immunized 4 x using incomplete Freud adjuvant during 56 days followed by blood sampling and sera generation after centrifugation at 4.000 x g for 1 min. Mice sera were tested on coated ELISA plates (Thermo Scientific, USA) using Tau2N4R with 100 ng/ml. Sera were diluted between 1:1000 and 1:1.000.000 in phosphate buffered saline containing 0.05 % Tween 20 (Serva, Germany) followed by incubation on coated plates blocked with 3% bovine serum albumin fraction V (Serva). Bound mice IgG antibodies were detected using goat anti-mice IgG antibody HRP conjugated (Dianova, Germany) and TMB/H₂O₂ staining. Mice with highest IgG titre to full length Tau were used for further immunisation followed by stimulation of B-cells by 3 x intravenous booster injections of antigen in 0.02 M phosphate buffered saline pH 7.4 (PBS).

Spleen cells were collected and fused to mouse myeloma cells X63Ag8.653 using PEG 1500 (Roche Diagnostic GmbH, Mannheim, Germany). Fused cells were selected using Hypoxanthine-Aminopterin-Thymidine (HAT) medium (Sigma-Aldrich) with Hybridoma Fusion and Cloning Supplement (HFCS) (Roche) during 14 days. Clones were selected regarding their reactivity to Tau2N4R in combination with selection for highest affinity to Tau2N4R in ELISA related to their IgG production. Different clones with IgG antibodies to Tau2N4R could be found of which clone 1G2 could be selected because of its IgG antibody showed highest affinity to Tau2N4R coated ELISA plates. Cells were re-cloned twice using the limiting dilution method resulting in monoclonal hybridoma cell line 1G2.

3. Characterization of antibody 1G2

The antibody 1G2 was tested for reactivity to Tau-Isoforms (rPeptide, USA) in Western Blot. Tau isoforms were separated on 4-16% SDS polyacrylamide gels (Serva, Germany) and proteins were transferred onto nitrocellulose membranes (0.45 μ m, Serva) using semi dry blotting chamber (Analytik Jena, Germany). After 1 h blocking using 5% skim milk powder in Tris buffer pH 10 immunostaining was performed using 1G2 antibody 1 μ g/ml concentrated in blocking buffer overnight at room temperature followed by washing using Tris buffer and anti-mouse IgG antibody HRP conjugated (Dianova, Germany) at room temperature for 2 h. After additional washing bound antibodies were stained using TMB staining kit for Western blots (Analytik Jena). Antibody isotype was determined using mouse immunoglobulin isotyping strip test (Roche, Suisse).

Epitope mapping of 1G2 antibody was performed on 12er peptides of Tau2N4R spotted on nitrocellulose. Arrangement of peptides was starting from amino acid 1 to amino acid 441 with 10 amino acids overlapping between peptides. Membranes were incubated with antibodies, and staining was performed as described above for Western blot.

After finding the binding motifs KTHP and RTHP including amino acids 175, 181 and 231 of Tau2N4R it was evaluated if binding of antibody 1G2 to its binding sites is influenced by phosphorylation of T175, T181 or T231, respectively. Therefore reactivity of 1G2 was tested in direct ELISA on ELISA plates coated with Tau2N4R and peptides of Tau amino acid sequences 172-184 and 228-240, respectively as well as both peptides with phosphorylations at T175, T181 and T231, respectively. Tau2N4R and peptides were coated as described above with concentration of 100 ng/ml in coating buffer followed by blocking of coated plates. Antibody 1G2 was incubated at dilutions of 100 ng/ml – 0.4 ng/ml at room temperature for 1 h followed by antibody detection using anti-mouse IgG HRP conjugated and TMB staining.

Additionally, inhibition experiments were performed using sandwich ELISA as described below where capturing of Tau2N4R was inhibited by competition with peptides of Tau amino acid sequences 172-184 and 228-240 and both peptides with phosphorylations at T175, T181 and T231, respectively. 1G2 coated plates were pre-incubated 10 min with peptides

at concentration of 1 µg/ml in dilution buffer followed by titration of Tau2N4R inside this inhibition solution according to the protocol of sandwich ELISA how described below.

4. Development of Sandwich-ELISA for detection of non-phosphorylated Tau

Maxisorb® plates (Thermo Scientific, USA) were coated with 5µg/ml 1G2 antibody in 0.05 mol/l Na₂CO₃/NaHCO₃, pH 9.6 with 120 µl per well at 4-8°C overnight. Coated plates were aspirated and blocked using 50 mM Tris buffer pH 7.5 containing 0.15 M NaCl and 0.05% Tween 20 (washing buffer) containing 3 % BSA fraction V (Serva, Heidelberg, Germany). Lyophilized standards were designed using different concentrations of Tau2N4R (rPeptide) as well as controls and negative controls by means of phosphate buffered saline buffer containing BSA and stabilizers. The assay was optimised studying different incubation protocols, and 3 h room temperature on orbital shaker appeared appropriate for antigen binding in the first step. Washing was performed 5 times using 50 mM Tris buffer pH 7.5 containing 0.15 M NaCl and 0.05% Tween 20 and followed by 90 min incubation with HRP conjugated detection antibody 7E5 (Supplementary Material) at room temperature. This antibody is described as anti-human Tau antibody that binds Tau2N4R between amino acids 156-165. After 5 additional washing steps, the plate was developed with TMB substrate solution for 30 min in the dark followed by termination with 1.5 M H₂SO₄. Optical density (OD) was measured at 450nm as well as 450/620 nm.

5. Determination of the intra- and inter-assay imprecision; repeatability of the standard curves

Intra-assay imprecision was calculated from the duplicate analyses of one hundred CSF samples, and expressed as median of the range-to-average of the duplicates.

Inter-assay imprecision was determined with three quality control (QC) samples prepared from the pooled human CSF samples. Briefly, CSF pools were stored at -80°C, and thawed once for the preparation of the three QC samples (coded QC1, QC2, and QC3) for this study. After the preparation, aliquots were re-frozen and kept at -80°C. Immediately before the

analysis, one set of the three QC samples was freshly thawed. The samples were assayed on eight ELISA plates on eight different days, by the same operator. The readings of the OD's were performed in two modes: (A) without the reference wavelength (i. e. measurement at 450 nm only) and (B) with the subtraction of the OD's obtained at the reference wavelength (620 nm). The imprecision for each QC sample is expressed as the percentage coefficients of variation (CV's) defined as the standard deviation divided by the average of eight measurements. To test repeatability of the standard curves, six independent runs were performed on six different days.

6. Inter-center quality control

For the inter-center study, four pairs of pooled CSF samples were prepared; from each pair, each of the nine participating centers received one aliquot frozen on dry ice and one aliquot shipped at ambient temperature. The latter had to be deep frozen upon arrival and kept at -80°C until the analyses. The participating centers were informed that they would receive eight samples (four frozen and four in liquid status) but not that the frozen and liquid samples were matched.

7. Clinical characterization: Patients and samples handling

The study on the human samples was approved by the ethical committee of the University of Erlangen-Nürnberg. All patients, or their close relatives, gave their written informed consents. For the clinical validation, the analyses were performed in the CSF samples from very carefully selected and characterized patients with dementia due to AD (n=32) or MCI with AD pathology (MCI-AD, n=26) (Positive Group, n=58) and Non Demented Controls (Control Group, n=42). AD/MCI patients were diagnosed and sub-classified according to the current recommendations from the NIA-AA working groups [1, 2], including analyses of the "classic" four AD biomarkers (cut offs in brackets): A β 1-42 (600 pg/mL), A β 42/40 ratio (0.05), Tau (300 pg/mL), and pTau181 (60 pg/mL). The characteristics of the groups are presented in table 1. The samples were collected by lumbar puncture (LP) into polypropylene test tubes according to a protocol described elsewhere [17], centrifuged, aliquoted, and stored at -80°C until the analyses.

8. Statistical analysis

If not stated otherwise, the results are presented as averages $\pm$ standard deviations (SD's). Imprecision is reported as coefficients of variation (CV's) or, in case of duplicate determinations, as ranges-to-averages and their medians. The area under the receiver operating characteristic (ROC) curve is reported with the corresponding 95% confidence interval (95% CI). Statistical comparison of the patient groups was done with the Mann-Whitney test. The cut off for the separation of the patient groups, and the corresponding sensitivity and the specificity, were calculated at the maximized Youden Index. Regression model was fitted to test the effect of age adjusted for diagnostic categories on the concentrations of Non-P-Tau. The analyses were performed with Statistica 12, Stata 14.1, and MedCalc. A $p < 0.05$ was considered significant.

Results

1. Analytical selectivity; intra- and inter-assay imprecision

Using partially phosphorylated tau protein aggregates as antigen we raised a high-affinity monoclonal tau antibody clone 1G2 strongly recognizing two KTPP motifs and weakly recognizing one RTPP motif on the tau molecule (Fig.1, Fig.S1). Antibody 1G2 binds to all 6 isoforms of Tau protein in Western blot (data not shown). Specificity for tau binding of 1G2 has been verified by immunohistochemistry on tau knock-out mice (Fig.S2). Isotype of antibody 1G2 was determined as IgG2a. Epitope mapping with staggered 12aa Tau peptides comprising the amino acid 172-184 and 228-240 in a phosphorylated or unphosphorylated version of T175, T181 or T231, respectively reveals binding sites of 1G2 to the non-phosphorylated peptide sequences KTPP (174-177), KTPP (180-183) and RTPP (230-233) (Fig. 1, Fig. 3). The antibody had a decreasing reactivity to Tau peptides containing multiple phosphorylation at positions T175, T181 and T231 (Fig. 2a). Sandwich ELISA using 1G2 as capture antibody and Tau2N4R as antigen showed complete inhibition of Tau2N4R capturing by Tau 172-184 peptide phosphorylated at T175 or T181, respectively whereas Tau 228-240 peptide showed slightly inhibition only. Both peptides together phosphorylated at positions T175, T181 and T231 have

no inhibition effect on Tau2N4R capturing. (Fig. 2b). Figure 3 presents a schema of the Tau molecule, highlighting the binding sites addressed in this study as well as the corresponding kinases known to phosphorylate them [18-20]. The configuration of the sandwich ELISA where the epitopes of capturing antibody 1G2 and detection antibody 7E5 are located close to each other has the additional advantage of detecting short tau fragments.

The median intra-assay range-to-average imprecision of one hundred double measurements was 4.8% (interquartile range 2.4 - 8.9%). Among these duplicate determinations, none resulted in the imprecision exceeding 20%, which normally would lead to a repetition of the measurement in the everyday diagnostic routine. The results of the inter-assay imprecision are presented in table 2. The comparison of the two reading modes (with and without wavelength correction) shows clearly better precision when the OD is read out without correction; correspondingly, we used the non-corrected reading mode for all measurements in this study.

2. Standard curves

The detection limit found was 25 pg/mL using recombinant Tau 2N4R (data not shown). The plots of the OD's versus the defined concentrations of the standards are presented in figure 4. The goodness of fit of the average standard curve was > 0.99 . The CV's of the OD's obtained in six repetitions of the standard curves were between 3.6 - 15.9%.

3. Inter-center comparison

The results of the inter-center comparison (averages of the reported concentrations and the corresponding CV's) are presented in table 3 and, as representative examples of two pairs of samples, in figure 5. We observed an inter-center variation of approximately 30% for all eight samples, whereas the differences between the concentrations in the pairs sent frozen and under ambient conditions were practically neglectable.

4. Clinical characterization

First, linear regression model was fitted modelling Non-P-Tau as a function of age and diagnoses (categorical variable with three categories: Controls, MCI, and AD-dementia). In this model, we did not observe significant effect of age ($\beta = -0.039$, $p = 0.81$), and similarly difference between effects of AD-dementia and MCI was insignificant ($\beta = 4.42$, $p = 0.61$); highly significant differences were observed between effects of Controls and AD-dementia ($\beta = -45.9$, $p < 0.001$) and between Controls and MCI ($\beta = -50.3$, $p < 0.001$). Therefore, for further analyses we combined MCI and AD-dementia categories into one group (MCI/AD). The results are presented in fig. 6. The concentrations were highly significantly increased in the AD/MCI group (109.2 ± 32.0 pg/mL) compared to the Controls (62.1 ± 9.3 pg/mL, $p < 0.001$, fig. 6a). At a cut-off of 78.3 pg/mL, the sensitivity and the specificity were 94.8% and 97.6%, respectively. The area under the ROC curve (fig. 6b) was 0.976 (95% CI: 0.923 to 0.996).

Discussion

In this paper, we present the development, and the analytical and clinical validation of an assay capable to specifically measure the concentrations of Tau molecules using non-phosphorylated epitopes in human CSF. NDD relies currently on the CSF biomarkers of two groups: amyloid β peptides and Tau proteins [10]. The latter includes also phosphorylated epitopes, which emerged from the observation that the Tau molecule is hyperphosphorylated in Tau deposits in the AD brain [13, 21, 22]. Whereas increased CSF concentration of Tau is a sensitive marker for neurodegeneration but entirely unspecific for AD, the increased concentration of pTau molecules seems much more AD specific [23, 24]. Threonin 181 and Threonin 231 are two main phospho-Tau positions addressed in the assays utilized for AD diagnostics [23, 25, 26]; pTau181 and pTau231 have been reported to characterize with similar diagnostic performance [25]. In contrast, to our best knowledge, pTau175 has been recently reported in the context of FTD and ALS [27]. Diagnostic utility of different Tau and phospho-Tau isoforms in the CSF was reported by Meredith et al [28], who concluded that discrimination between AD and controls relies on the subset of Tau molecules measured. On the other hand, virtually nothing is known about a

possible diagnostic utility of non-phosphorylated Tau epitopes. A recent meta-analysis of CSF biomarkers pointed to a greater predictive value of total tau CSF measurements [29].

Due to its unfolded, highly hydrophilic nature, Tau is a highly phosphorylation-prone protein with 85 (in case of the longest isoform, i. e. 2N4R) potential phosphorylation sites. In experimental conditions, about half of them have been observed to be phosphorylated to some extent (currently reviewed in [13]). Main role of Tau proteins is stabilization of axonal structures, whereas phosphorylation-dephosphorylation directly regulates its association and dissociation from the neuron's microtubules. Under physiologic conditions, Tau contains on average two phosphorylated sites; this number can increase to 7-8 sites under pathological conditions such as AD [29]; but with many phosphorylation sites being modified only in a low percentage range.

Immunization strategy using three different immunization variants for testing of best antigen to stimulate an anti-Tau response resulted in highest IgG antibody titer of mice immunized with phosphorylated and aggregated Tau protein (data not shown). Because of the high amino acid sequence homology of 90 % between human and mouse tau we assumed that using phosphorylated and aggregated tau protein could reduce self-tolerance and increases humoral immune response. Phosphorylation of Tau with kinases (MAPK13 in this case) does not result in a complete phosphorylation yield of any given phosphorylation site. This, paradoxically, has resulted in the development of a non-phospho-epitope Tau antibody, in spite that a phosphorylated protein was used for immunization. The monoclonal antibody 1G2 has shown to bind to a TPP sequence in its unphosphorylated form flanked by a basic amino acid at the N-terminus as a core signature of the epitope. This resulted in three binding sites on 2N4R Tau containing 441 amino acids but binding of 1G2 antibody to Tau2N4R depends on KTHP motives that are present at T175 and T181 whereas binding to each of these both sites is not influenced by phosphorylation of the other site. Therefore availability of these unphosphorylated KTHP motives could provide a very strong avidity of the antibody for maximum of both free binding sites. Further, based on the results of our analysis showing a concentration that is about one-third of total Tau, we may assume that the Tau population

detected by the 1G2 antibody is correspondingly approximately one-third of the total Tau in the CSF; hence we can assume that strongest avidity obtained by using two binding sites on a single tau molecule could be a requirement for detection of such low concentrations.

Analytical characterization of the assay revealed its very good performance, with reasonable intra- and inter-assay precision, and standard curve repetition, comparable to the parameters observed in other NDD assays [30]. Noteworthy is that among duplicate determinations of one hundred patients' samples, none resulted in the imprecision exceeding 20%, which would force repetition of the measurement in the everyday diagnostic routine. The inter-center study with the paired frozen/non-frozen QC samples indicated no differences between the two preanalytical sample handling and shipping procedures: in virtually all cases the results obtained from a frozen sample were practically identical, in terms of the measured concentrations as well as the imprecision, as the results obtained from a matched sample shipped under ambient temperature. We believe this has important practical implications, since patients samples can be shipped to a distant laboratory at ambient temperature and without freezing, which significantly decreases costs of shipment, at least as long as a sample is delivered within 4-5 days. Furthermore, QC samples based on native CSF can be similarly shipped at ambient condition (for example, in the kit). On the other hand, similarly to other CSF biomarkers routinely used in AD diagnostics, Non-P-Tau measurements are currently characterized by a relatively high inter-center variability (approximately 30%) [31, 32]. This situation definitely should be improved; however, before successful measures are undertaken to reduce inter-center variability, establishing center-specific reference ranges for diagnostic purposes could help solve the problem of diagnostic-relevant interpretation.

We did not observe significant difference of Non-P-Tau concentrations between patients in the MCI and the dementia stages of AD. This is not surprising, as it is well known that CSF biomarkers, including those related to neurodegeneration (Tau), are altered already at the early MCI stage, and show only slight dynamics in further course of the disease (reviewed in [7]). Similarly, when adjusted for diagnostic categories, we did not observe correlation of Non-P-Tau with age.

To properly interpret the clinical characterization results, particularly the ROC curve, Youden Index, and the corresponding sensitivity and specificity, it has to be taken into consideration that the NDD biomarkers, including Tau and pTau181, were used to classify the patients. Indeed, this significantly improves the categorization of the groups [3, 33], but meanwhile limits the estimation of the overall diagnostic value of a novel candidate biomarker. In other words, subjective component of the clinically-based diagnoses is reduced, but any direct comparison of the diagnostic performance of any novel assay with those existing is impossible.

We are aware of at least two limitations of our study: (a) in our clinical part, we included only patients with AD and non-demented controls. Certainly it would be interesting to see if Non-P-Tau could be helpful in differential diagnostics of other dementing conditions, particularly tauopathies (for example, fronto-temporal lobar degeneration). (b) as already stated, our strategy of patient inclusion relies on the application of "classic" NDD biomarkers to support clinical and neuropsychological diagnoses. Therefore, a further study with non-NDD biased subjects, whose diagnoses are, for example, confirmed by neuropathology, is certainly needed. We also believe that additional studies are necessary to check long term repeatability with different assay production batches.

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Figure legends

Fig. 1. Epitope mapping of 1G2 antibody on 12 amino acid peptides of 2N4R TAU2N4R with 10 amino acid overlapping. Peptides are spotted on NC membranes 10 amino acids overlapped. Membranes blocked with 5% skim milk powder in Tris buffer pH 10 containing 0,1 % Tween 20. After 1 µg/ml 1G2 was incubated in blocking buffer at room temperature overnight followed by 3 x washing using Tris buffer and anti-mouse IgG antibody HRP conjugated. Staining was performed using Western blot staining solution based on TMB. Antibody 1G2 binds to peptide

sequences containing KTTP or RTTP motif. Spots of these 12 amino acid peptides showing reactivity of 1G2 are described for line A and line B of the NC membrane.

Fig. 2. Specificity of 1G2 antibodies.

2a. Direct ELISA on Tau2N4R or Tau peptides coated plates for analysis of 1G2 binding. to antigens. . Peptides comprised amino acid sequence 172-184 of Tau 2N4R containing T175 and T181, respectively and amino acid sequence 228 -240 of Tau 2N4R containing T231. Additional peptide sequences 172-184 phosphorylated at position T175 [T175Pi] or phosphorylated at position T181 [T181Pi] and peptide 228-240 phosphorylated at position T231 [T231Pi] were tested, respectively. Bound 1G2 was detected using anti-mouse-IgG antibody HRP conjugated followed by TMB staining.

2b. Sandwich ELISA was used for inhibition of Tau2N4R capturing by 1G2 antibody by competition with peptides of amino acid sequence 172-184 containing T175 and T181 and amino acid sequence 228-240 containing T231 compared with inhibition effects of these peptides with different phosphorylations at position T175 [T175Pi], T181 [T181Pi] or T231 [T231Pi], respectively. Captured antigen by 1G2 was detected using 7E5 antibody HRP conjugated followed by TMB staining.

Fig. 3. Schematic presentation of the Tau molecule, the three binding sites addressed in this study, and the corresponding kinases.

Fig. 4. Reproducibility of the standard curves of the assay; presented are average OD's and their standard deviations; the insert presents the zoom-in of the four lowest standards.

Fig. 5. Results of the inter-center variability study: presented are the reported concentrations of two pairs of QC samples (A/E and B/F) sent frozen (filled symbols) and under ambient temperature (open symbols).

Fig. 6. Non-P-Tau concentrations in the patients' groups, whose clinical and neuropsychological diagnoses were supported by the "classic" AD biomarkers.

6a. Concentrations in the patients' CSF (circles) with medians and inter-quartile ranges (bars).

The long horizontal bar presents the cut off at the maximal Youden Index.

6b. Receiver Operating Characteristic Curve.

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