Thesis: Validation of salivary tau as a biomarker for Alzheimer disease

Heather Pekeles, Integrated Program in Neuroscience, McGill University, Montreal

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

Table of Contents

Abstract (English)

Biomarkers for Alzheimer's disease (AD) have been studied extensively, however most require expensive or invasive techniques, such as cerebrospinal fluid (CSF) tau. Total tau (t-tau) and phosphorylated tau (p-tau) are abnormally elevated in the brain and CSF of AD patients. Biomarkers for abnormal brain tau could be helpful in delineating which mildly impaired patients will most likely respond to future anti-tau therapy. Tau is also present in salivary gland tissue and saliva. In this study, we wanted to determine if salivary tau could be used as an easily attainable and inexpensive biomarker for AD. Using unstimulated saliva and western blot analysis, we quantified the p-tau/t-tau ratio at different phosphorylation sites. We determined phosphorylation level at serine 404 (S404), serine 396 (S396) and threonine 181 (T181), as well as combined phosphorylation of serine 400, threonine 403, and serine 404 (S400,403,S404). We hypothesized that there would be an elevated salivary p-tau to t-tau ratio in patients with AD compared to cognitively normal elderly controls (NEC). As well, we collected saliva from subjects with mild cognitive impairment (MCI) to determine its usefulness as an early biomarker. We also collected from subjects with frontotemporal dementia (FTD), a group of diseases where patients sometimes have tau pathology, and from subjects with non-tau related neurological conditions, in order to better determine specificity of the test. Additionally, we correlated salivary tau with CSF tau levels in patients who had a lumbar puncture (LP). As well, we hypothesized that abnormal tau levels would correlate with decreased hippocampal volume and impairment in episodic memory scores. We found that for two phosphorylation sites, S396 and S404, p-tau/t-tau is elevated in AD compared to NEC subjects. However, we found that there was great variation in levels in

the AD and other diagnostic groups. This variation limits the utility of the test as a diagnostic biomarker. However, our work adds to the literature on peripheral markers of AD. Future work should explore other phosphorylation sites, and should further investigate the mechanism for increased tau and p-tau in saliva.

Abstract (French)

Les biomarqueurs pour la maladie d'Alzheimer (AD) sont bien étudiés, mais la plupart utilisent des techniques chers ou invasives. Un exemple est la protéine tau dans le liquide céphalo-rachidien (LCR). Le tau "totale" et le tau phosphorylé sont élevés anormalement dans le cerveau et dans le LCR des personnes qui ont la maladie d'Alzheimer. Les biomarqueurs pour le tau dans le cerveau anormalement élevé pourraient être utilisés pour décider quelles personnes avec la déficience cognitive légère (DCL) vont répondre à une immunothérapie "anti-tau". La protéine tau est aussi présente dans la salive et dans le gland salivaire. Dans cette recherche, nous avons voulu voir si le tau salivaire peut être utilisé comme biomarqueur sensitive et spécifique pour la maladie d'Alzheimer. En utilisant la salive non stimulée et des « western blots », nous avons mesuré le niveau de p-tau/t-tau à différents sites de phosphorylation de tau. Nous avons trouvé le niveau de phosphorylation à serine 404 (S404), serine 396 (S396) et thréonine 181 (T181), et aussi le niveau de phosphorylation combiné de serine 400, thréonine 403, et serine 404 ensemble (S400,403,404). Nous avons supposé que les sujets avec AD auraient un niveau de p-tau/t-tau salivaire plus élevé que les personnes âgées en santé. De plus, nous avons pris de la salive des sujets avec une DCL pour voir si notre teste pourrait être utile comme biomarqueur tôt dans la progression de la maladie. Finalement, nous avons pris de la salive des sujets avec de la démence fronto-temporale, un group de

maladie où les personnes ont des fois une pathologie de tau, et des sujets qui ont des maladie qui ne sont pas associé avec la protéine tau, pour mieux comprendre la spécificité du test. Aussi, nous avons corrélé le tau salivaire avec le tau du LCR des patients qui avaient eu un PL. De plus, nous avons pensé que les niveaux de tau anormales corrèleraient avec un réduction en grandeur de l'hippocampe et des résultats plus bas sur des testes de mémoire. Nous avons trouvé qu'à deux sites de phosphorylation, S396 et S404, le niveau de p-tau/t-tau était élevé dans les personnes avec AD. Par contre, nous avons trouvé qu'il y a beaucoup de variation de niveau dans chaque group diagnostique. Cette variation limite l'utilité du test comme biomarqueur diagnostique. Cette recherche est une addition importante à la littérature sur les marqueurs de l'AD qui ne se trouvent pas dans le cerveau. Encore plus de recherche est nécessaire, particulièrement l'étude d'autres sites de phosphorylation et aussi, la façon dont laquelle le tau et p-tau dans la salive deviennent élevés.

Acknowledgements

Thank you to the co-authors of this project, Dr. Hamid Qureshi, Dr. Hemant Paudel, Dr. Hyman Schipper, Dr. Mervyn Gornitsky and Dr. Howard Chertkow. In particular, thank you to Hamid for his hard work and dedication to this project. Thank you to Shelley Solomon (recruitment and testing of normal elderly controls and patients), Chris Hosein (recruitment of patients in the Memory clinic), Jim Nikelski (MRI analysis), Shrisha Mohit (recruitment and processing), Dr. Joe Rochford (statistical advice), Dr. Ana Velly (statistical advice), Nora Kelner (neuropsychology advice), and Kath De Sousa (consent form translation). Thank you to all staff of the Memory and Neurology clinics at the Montreal Jewish General Hospital for allowing us to collect samples for the

project and for providing us with space to do so. Thank you to student volunteers Jessica Wang and Rebecca Kucharsky Hiess, and Dr. Gornitsky's dentistry summer students for their help in collection and processing of samples. A special thank you to Jessica Hier for her help in collection and processing of samples, as well as her dedication to the lab. Thank you to my IPN mentor, Dr. Erik Cook, and my committee members, Dr. Hyman Schipper and Dr. Hemant Paudel for their advice and support. Finally, a huge thank you to my supervisor Dr. Howard Chertkow for his guidance, mentorship, and ingenuity without which this project would have not been possible. Thank you to the whole Chertkow lab for their support throughout my MSc. My work was supported by the Canadian Institutes for Health Research, the Garfield-Weston Fund and Dr. Chertkow's laboratory. This study was approved by the Research Ethics Board of the Jewish General Hospital (January 21st, 2015).

Preface & Contribution of Authors

This project is a collaboration between four labs: Dr. Howard Chertkow's, Dr. Hemant Paudel's, Dr. Hyman Schipper's and Dr. Mervyn Gornitsky's. Dr. Paudel and Dr. Qureshi developed the saliva collection and tau purification protocol used in this study, and Dr. Qureshi ran all the western blots for the project. Dr. Schipper helped develop the methodology of the project. Dr. Gornitsky's lab helped in the recruitment of subjects and saliva processing, and provided storage for the saliva samples. Dr. Chertkow's lab recruited of patients and elderly control, obtained MRI scans, neuropsychology scores, and cerebrospinal fluid. This project was based on a grant funded by the Weston Agency that involved multiple laboratories at the LDI. My role was to supervise the undertaking and carrying out of this complex trial and get it to completion. That included screening of

potential patients for different subject groups and verifying clinical diagnoses, organizing collection of saliva samples by multiple individuals (myself, research assistants, volunteers) across multiple clinics in the hospital, negotiating the logistics of sample collection, maintaining a database of clinical and demographic information to be used in analysis, verifying timely transport of properly labeled and blinded samples to Dr. Paudel's lab for western blot analysis, collection and collation of all results, organizing, choosing, and undertaking the parametric and non-parametric statistical analysis of multiple groups after consultation regarding appropriate statistic tests, analysis of results, organizing presentation and review of the results with the multiple investigators, reporting to the Weston Agency, and writing up results for public presentation.

Introduction and statement of the problem

The World Health Organization estimates that 47.5 million people worldwide are living with dementia[42]. Alzheimer's disease (AD) pathology, the leading cause of dementia[25], is believed to start 10-20 years before the occurrence of the first dementia symptoms[30]. Diagnosis of AD is usually preceded by a period of mild cognitive impairment (MCI). An MCI diagnosis is made if a patient has memory complaint, normal activities of daily living and general cognitive function, abnormal memory for age, and is not demented[28]. The conversion rate of MCI to AD is approximately 10-12% per year[28], but not all MCI individuals go on to dementia. Due to early pathology, potential therapies for AD should be administered as early in progression as possible before severe neurodegeneration and irreversible damage has occurred. Correct diagnosis is important for clinical studies, and can influence course of treatment. Therefore, finding early and easily attainable biomarkers is essential. Many of the current biomarkers for probable AD

require expensive or invasive techniques and therefore are not ideal for wide screening or everyday diagnosis. In our current study, we investigated whether salivary tau, found in unstimulated saliva and analyzed with western blot, can be used as an easily collected and inexpensive biomarker.

Background

According to Kennard (1998), an ideal test for a biochemical marker of AD would: 1) have high sensitivity, 2) have high specificity, 3) be useful for early or pre-symptomatic disease, 4) be able to distinguish familial and sporadic, 5) be inexpensive, 6) be convenient and noninvasive, 7) be based on easily available bodily fluid, 8) be useful for monitoring progression of disease and effects of treatment, and 9) may be routinely used for widespread screening[23].

Various biomarkers for AD have been studied extensively. One source of biomarkers is cerebrospinal fluid (CSF). CSF is collected by lumbar puncture (LP) and analysis is most often done with enzyme-linked immunosorbent assay (ELISA). One such CSF biomarker is $A\beta_{1-42}$. The level of $A\beta_{1-42}$ is reduced in AD, and has an 80% sensitivity and 90% specificity for distinguishing AD from normal controls[4], although has lower specificity in distinguishing AD from non-AD dementia. Another CSF biomarker is total tau (t-tau), which is increased in AD, and has a high sensitivity but low specificity in discriminating AD from other dementias[2]. Finally, phosphorylated tau (p-tau) is another CSF biomarker for AD. There are many tau phosphorylation sites, but the three most commonly used sites are p-tau_{181}, p-tau_{231}, and p-tau_{199}[5,6]. P-tau_{181} is the most studied, and often used in clinical studies. P-tau_{181} is increased in AD and more representative of AD brain pathology than t-tau. CSF p-tau level has a specificity of 80-

100%[20], and may differentiate AD from other neurological disease such as frontotemporal dementia (FTD), Lewy body disease (LBD) and vascular dementia[2,6]. Abnormally increased p-tau and t-tau correlate with an individual's degree of cognitive decline[26,34,41]. Together, CSF $A\beta_{1-42}$, t-tau, p-tau_{181} can be used to identify AD with good accuracy; combined, these biomarkers have better diagnostic ability than when considered in isolation. Unfortunately, CSF biomarkers have a few major drawbacks. In particular, an LP is relatively invasive and requires clinical expertise.

Hippocampal atrophy, seen with MRI, is another well-studied biomarker. In AD, there is atrophy of the medial temporal lobe (MTL), particularly the hippocampus and amygdala[32]. Patients with AD exhibit greater hippocampal atrophy compared to healthy aging[1]. However, this reduction in size is not specific to AD, as it is seen in other neurodegenerative diseases. Furthermore, MRIs are expensive and require technical expertise.

Another biomarker is amyloid imaging with positron emission tomography (PET). A set of radioactive compounds have been developed that can be used to image beta-amyloid plaques in neuronal tissue. These include carbon-11 moieties like Pittsburgh B compound (PiB), and more long-acting F-18 moieties. In AD, PiB is retained in the cingulate, temporal, parietal and frontal cortices[12]. This biomarker can be useful if diagnosis is unclear, for example to distinguish FTD vs. AD if the case is ambiguous[31]. As well, amyloid imaging with PET is most helpful in relatively young patients, when plaques are more specific to AD[27]. However, amyloid imaging with PET is not recommended as a routine test for diagnosis (Molson Lecture, 2015). Another studied biomarker is Fluorine 18 fluorodeoxyglucose positron emission tomography (FDG-PET),

a marker of glucose uptake, a correlate of tissue metabolism. FDG-PET reveals hypometabolism in the temporoparietal regions, posterior cingulate cortex, and frontal lobe even prior to atrophy in AD[36]. Similarly to MRI-derived biomarkers, some disadvantages of PET-derived biomarkers are that they are expensive and require technical expertise.

Although these biomarkers each have limitations, they are not only studied in isolation. Combining clinical information with MRI, FDG-PET, and CSF biomarkers gives the highest accuracy for predicting future MCI conversion to AD[36]. The literature on biomarkers for AD is vast and extends beyond these well-studied biomarkers. For example, one of the most explored plasma biomarker of AD is plasma Aß. However, the results across studies are conflicting in terms of how plasma Aß changes in AD patients[2]. Other potential biomarkers explored in the literature include cytokines, oxidative stress markers, glial and synaptic proteins, cholesterol metabolites and lipoproteins, and transition metals.

Tau in the brain

Tau is a soluble, microtubule-associated protein that is 45-65 kDa in size. Tau is involved in promoting the assembly of tubulin into microtubules, and is predominantly found in axons, binding to and stabilizing microtubules. One of its functions is to regulate axonal transport of organelles such as mitochondria. There are six tau isoforms (3 or 4 microtubule binding repeats, 0, 1, or 2 amino terminal inserts)[3]. Tau's phosphorylation, at many different possible phosphorylation sites, is associated with its ability to form oligomers and aggregates. In AD, brain tau is three to four times more hyperphosphorylated compared to tau of cognitively normal individuals[21]. Importantly,

neurofibrillary tangles (NFTs), aggregates of hyperphosphorylated tau, are linked to degree of dementia[8]. In AD, tangles are predominantly found in the MTL (hippocampus, amygdala, parahippocampal gyrus)[38]. Tau is also affected in other neurodegenerative diseases, together termed tauopathies. In tauopathies, aggregates of abnormally phosphorylated tau accumulate[3,24]. Some tauopathies are specifically associated with the 3R tau isoform, and some with the 4R isoform. For example, 3R tau makes up the Pick's bodies of Pick's disease, and progressive supranuclear palsy (PSP) is associated with the 4R tau[24].

Saliva

Saliva is secreted from the salivary glands, of which there are three major ones: the submandibular, sublingual and parotid glands. Saliva is made up of water (99%), proteins and ions[9]. The compounds in saliva are mostly produced in the salivary glands and secreted, but some pass from blood into saliva through diffusion, active transport or ultrafiltration. Some strengths of saliva as a source of biomarkers are that collection is noninvasive, inexpensive and little training is required for collection. Some challenges of salivary biomarkers are that there is a low concentration of molecules (1000-fold less than blood[29]), and that some people have trouble producing saliva. Difficulty in production can be due to a number of factors, such as medication, disease and hydration level.

The composition of saliva is influenced by collection method[40]. In terms of clinical research, whole saliva is the most frequently used. Whole, unstimulated saliva is collected through the drooling method, the spitting method, the swabbing method, or the suction method. Stimulated saliva, in contrast, is collected by having the subject chew on

paraffin, or by applying citric acid to the tongue[40].

Saliva composition has been studied in a variety of other conditions. For example, one study looked at salivary alpha-synuclein and DJ-1 as potential biomarkers for Parkinson's disease[13]. However, literature on salivary biomarkers for AD is limited. One study, conducted by Shi and colleagues, used mass spectrometry & highly sensitive Luminex assays to assess level of salivary total tau, phosphorylated tau, and $A\beta_{42}$ in AD[37]. The authors found that the p-tau/t-tau ratio was significantly higher in AD patients compared to controls. One limitation of their study is that mass spectrometry is expensive. An ideal biomarker should be inexpensive to collect and measure so that it can be helpful across clinics and laboratories. Furthermore, although not significantly different, the authors found a decrease in total tau in AD subjects compared to controls. This unexpected and counter-intuitive result may be due, in part, to their choice of collection method. The researchers collected saliva by placing a cotton roll in the mouth of the subject between the cheek and gum for one minute, and then the roll was spun inside a salivette. However, a recent review suggested that the use of cotton might not be the best way to collect saliva, as it hinders detection of certain proteins compared to passive drooling[40].

The source of tau in saliva has yet to be firmly established. As described by Shi and colleagues, the tau in saliva is unlikely to come from blood via passive diffusion, as tau is not detected in blood and has a relatively large size[37]. The authors suggest the possibility that acinar epithelial cells of salivary glands secrete tau. Therefore salivary tau levels may reflect pathological changes in AD salivary glands as well as in the brain[37]. This suggestion is supported by a study by Conrad and colleagues demonstrating tau

mRNA expression in salivary glands[10]. Another suggestion for the protein's presence in saliva is that because the salivary glands are close to the CNS, tau might be secreted from the nerves that innervate salivary glands[37].

Rationale and hypotheses

Rationale

Based on the study by Shi and colleagues[37], as well as preliminary data of our own, we believed salivary tau could be developed as a reliable and easily attainable biomarker for AD. Our overarching aim was to determine if salivary tau could be used as a sensitive and specific clinical biomarker. Our method of tau analysis, the western blot, is less expensive than mass spectrometry used by Shi and colleagues[37], and has the potential to be carried out in almost any lab. In addition, we used unstimulated saliva, a more favorable method of collection for the detection of proteins[40].

Specific Aims and Hypotheses

AIM 1: We wanted to determine if AD subjects have abnormal salivary p-tau to t-tau ratio compared to NEC subjects. We hypothesized that there would be an elevated p-tau to t-tau ratio in patients with AD compared to NEC subjects. We also collected from subjects with non-tau related neurological conditions, in order to better determine specificity of the test. We hypothesized that patients with other brain diseases not associated with tau, such as multiple sclerosis, epilepsy, and chronic stroke would have normal salivary tau levels.

AIM 2: We wanted to determine the salivary tau levels of MCI patients compared to controls and AD patients. We hypothesized that abnormally elevated salivary tau would be detected in a significant number of MCI patients. Many MCI patients go on to

get dementia, so we expected that some patients would exhibit similar pathology to early AD, and therefore show similar salivary tau level differences to AD patients.

AIM 3: We wanted to determine if abnormally elevated salivary tau correlates with impaired episodic memory scores and other neuropsychology scores such as executive function. Initially, in AD and MCI, tau is mainly deposited in the medial temporal region[7], which includes areas important for episodic memory function. Therefore, we hypothesized that abnormal salivary tau would correlate with decreased episodic memory scores more than other neuropsychology scores, such as measures of executive function.

AIM 4: We wanted to determine if there was a correlation between salivary p-tau and t-tau levels and subjects' CSF p-tau and t-tau levels. We hypothesized that salivary p-tau/t-tau levels would correlate with subjects' CSF p-tau/t-tau levels.

AIM 5: We wanted to determine the relationship between abnormally elevated salivary tau and hippocampal volume. We hypothesized that abnormally elevated salivary tau would correlate with decreased hippocampal volume.

AIM 6: We wanted to see if a salivary tau test could be used for FTD subjects. We hypothesized that some FTD subjects would exhibit elevated p-tau/t-tau ratios compared to NECs subjects.

Methods

Saliva collection, processing and analysis (for all aims):

All subjects were asked not to eat or drink 30-40 minutes before saliva collection, in order to have clean samples, and because eating and drinking can alter saliva composition. All subjects were asked to give informed consent, and given a short medical

questionnaire. Collection was done in the morning, between 9-12 pm when possible, except for a few subjects who gave samples in the early afternoon. This set time frame is to control for diurnal variation, which has not yet been investigated with respect to salivary tau. However, the possibility that time of day may effect tau level and phosphorylation is important to consider because other salivary proteins have shown diurnal variation. For example, the levels of protein carbonyls, a marker of oxidative stress, were found to differ depending on the time of day[39].

The protocol for processing saliva was established by Dr. Hamid Qureshi in Dr. Hemant Paudel's lab at the Lady Davis Institute. The protocol was established to optimize tau detection and to limit tau degradation. We collected unstimulated saliva by having the subject spit four to five milliliters into a sterile 50 ml polypropylene tube. The saliva was then transferred immediately to an identical tube with inhibitor cocktail already in it, and kept on ice. The inhibitor cocktail contained protease inhibitor to help limit the degradation of tau. The saliva was transferred into the tube containing the inhibitor cocktail so that the subject was never at risk of ingesting the cocktail. The saliva was transferred into multiple 1.5 mL eppendorf tubes, and then put in a hot water bath (100°C) for 20 minutes. The hot water bath was to help with the purification of tau, as the tau protein is heat resistant. The tubes were centrifuged at 5000 rpm or 10 000 rpm for 10 minutes at 4°C[*]. The speeds and time were determined by Dr. Qureshi so that tau

[*] As of April 16th 2015, we reduced the speed to 5000 rpm in case some tau was being lost in the pellet. Although not ideal in terms of maintaining consistency, we are confident we can pool them for a few reasons. We analyzed a sample with one aliquot spun at 5000 rpm and one at 10 000 rpm, and found less than a 2% difference between them. As well, the 5000 and 10 000 rpm samples are distributed across diagnostic groups. Finally, all samples were spun again at 10 000 rpm once thawed, prior to western blot analysis, minimizing possible discrepancy.

remained in the supernatant, but other unwanted residue did not. The supernatant of each eppendorf tube was extracted and all supernatants from one subject combined and vortexed in a 15 mL tube to create a homogenous sample. The sample was then re-distributed in 0.5 mL aliquots into 1.5 mL eppendorf tubes. The 0.5 mL aliquots were labelled with a code, and stored in -80°C freezer for later analysis in order to limit any tau degradation over time.

Salivary tau levels were analyzed, blind to diagnosis, in Dr. Paudel's lab. Antibodies for total tau and specific phosphorylated tau sites were used. Antibodies for specific sites T181, S396, and S404 were used, as well as an antibody for S400, T403, and S404 together. The tau4 antibody was used for the measure of "total tau". Collected and frozen samples were thawed, and the supernatant was passed through an anion exchange column and then chromatographed through a cation exchange column. The fractions were then analyzed for tau using western blot. Due to our large number of samples, western blots were run in two rounds. In the first round, 150 samples were run and in the second round, 200 samples were run, for a total of 350 samples analyzed.

AIM #1: We collected saliva from AD and NEC subjects. The AD patients were recruited from the Memory and Neurology clinics of the Montreal Jewish General Hospital, where diagnoses were established by neurologists or geriatricians highly trained in diagnosis of neurodegenerative diseases. I recruited the subjects with the help of research coordinators. We excluded subjects with "mixed" pathology, or a mixed AD/Parkinson's diagnosis. The NEC, aged 60 and above, were recruited to come in to the Memory clinic in the morning. I recruited the subjects with the help of research

coordinators. They were given the Montreal Cognitive Assessment (MoCA), and had to score a 25 or higher to be included as a control. The MoCA was chosen due to its short length (~15 min) and higher sensitivity than the MMSE to distinguish normal controls from MCI[33]. The NEC were also asked about subjective memory impairment (SMI) with the Jessen question[22]. The Jessen question for SMI is: "Do you feel like your memory is becoming worse" With the possible answers: "No", "Yes but it does not worry me", or "Yes and it worries me"[22]. Subjects who answered "Yes and it worries me" were placed in the SMI sub-category. Subjects with SMI with self-reported concern were shown to have similar risk of AD dementia as those with early MCI[22]. Therefore, in addition to considering the NEC group as a whole, we wanted to determine if this sub-category of SMI subjects showed increased salivary tau levels compared to the other non-SMI NEC subjects. As well, we analyzed saliva from 76 healthy young normal controls (YN), aged 18-60 to see if there is an age-related difference in salivary tau levels. These volunteer subjects were recruited from clinics of the JGH. Many were family members of patients being seen for unrelated reasons. To better determine specificity, we also collected saliva from patients with other brain diseases that are not associated with abnormal tau, such as chronic stroke, epilepsy, and multiple sclerosis. We collected saliva samples from 12 of these neurology patients (NEUR) recruited in the Neurology clinics of the Jewish General Hospital. Although in stroke there is an acute increase in CSF tau, there is no change in phosphorylated tau[19], and we only included subjects with chronic stroke (at least 3 months since the stroke). Thus, we would expect normal salivary p-tau in these subjects, further supporting that differences in salivary tau is AD-related and not just a result of a neurological disease. The Neurology patients were also given a MoCA when possible or

their score was obtained from their treating neurologist. They were only recruited if their MoCA was greater than 25/30.

AIM #2: Saliva was analyzed from 55 patients with MCI, diagnosed by clinicians in the Jewish General Hospital's Memory clinic. I recruited the subjects with the help of research coordinators. We determined if their salivary p-tau and t-tau ratios were significantly different from AD patients and NEC. Not all MCI subjects will go onto to develop AD, but a significant portion of them will. A conversion of a group of MCI subject to AD was found to be 10-12% per year for 4 years[28]. If salivary tau were a good marker, we would expect that a significant portion of MCI subjects to have similar salivary tau profiles to AD subjects. Since MCI is not always due to AD pathology, in the future, the test could have predictive value in terms of which MCI patients will likely go on to develop AD.

AIM #3: Episodic memory tests as well as other neuropsychology tests are carried out on many of the subjects who come in to the Memory clinic at the Jewish General Hospital. We determined if episodic memory scores correlated with abnormal salivary tau more than other neuropsychology scores such as tests of executive function. The rationale for this test score investigation is that in AD, brain regions affected by NFTs are mainly areas associated with episodic memory. Furthermore, NFTs are associated with degree of dementia. Thus, we expected a correlation between abnormal salivary tau levels and episodic memory scores, which would support the role of salivary tau as a biomarker for AD. As our measure of episodic memory, we used the logical memory 2 (ie., delayed memory on a paragraph recall test, maximum score = 25) score from the Wechsler Memory Scale (WMS), and for general cognitive function we used the clock drawing test

(CDT) (maximum score = 10).. Finally, we also did a correlation with the subjects' MoCA scores (maximum score = 30).

AIM #4: CSF p-tau and t-tau levels were obtained from 12 subjects getting an LP (for diagnostic or research purposes). The CSF samples were sent to Athena diagnostics, an American company that carries out diagnostic testing for neurological diseases, for CSF analysis. Athena diagnostics uses the p-tau 181 site as a measure CSF p-tau. We collected saliva from 12 subjects who had undergone a LP for CSF tau levels. A correlation was used to determine if CSF tau levels correlated with salivary tau levels.

AIM #5: Hippocampal volumes were obtained from 12 subjects getting an MRI for another research project and who consented to have their scans used for this study. All 12 subjects were run through Freesurfer software (using the CBRAIN processing cluster) by Jim Nikelski (Dr. Chertkow's lab) to determine the size of the left and right hippocampus of these subjects, while accounting for intracranial capacity. We determined if abnormal salivary tau levels correlated with decreased hippocampal volume. Since brain tau pathology is correlated with decreased hippocampal volume, we expected salivary tau level to as well.

AIM #6: We collected saliva from 16 FTD subjects from the Memory and Neurology clinics. These had clinical diagnosis of frontotemporal dementia, supported by imaging results on MRI and FDG-PET. All subtypes (behavioural variant, aphasic variant, and mixed) were accepted.

Statistical analysis

Kruskal Wallis tests and Mann Whitney U tests were used to determine differences in our round 1 data of NEC, MCI and AD subjects. In our initial analysis of

round 2 data, we did statistical analysis following with our hypotheses. Mann Whitney U tests were used to determine differences in p-tau/t-tau in AD compared to NEC, in FTD compared to NEC, and in NEUR compared to NEC. However, doing multiple Mann Whitney Us increases the likelihood of obtaining a false significance. We therefore also ran Kruskal Wallis Tests for AD, NEC, NEUR and FTD, and Mann Whitney Us for pairwise contrasts with a Bonferroni Holmes correction to correct for multiple comparisons. Mann Whitney U tests were used to determine differences in p-tau/t-tau in YN compared to NEC and in SMI NEC compared to non-SMI NEC subjects. Spearman correlations were used for all salivary tau correlations with CSF tau, hippocampal volume, and neuropsychology scores.

Results

In our first round of western blots we looked at 150 subjects including NECs, MCI and AD subjects. In our second round of western blots we looked at 200 subjects, including NEC, AD, FTD, NEUR and young normal (YN) subjects.

First Round results:

150 subjects were run in the first round, but only 148 were analyzed. One subject was excluded due to an eventual diagnosis of "Mixed pathology". One NEC was excluded due to not having a MoCA score obtained. Demographic information for round one data is listed in Table 1. For S404, part of the western blot did not turn out, and therefore there was a reduced sample size for this site for this round of data.

The Shapiro-Wilks tests for normality suggested the data from each site were not from a normally distributed population, $ps<0.05$. Data for each phosphorylation site (S396, T181, S404 and S400,403,404) were therefore analyzed using non-parametric

tests.

The medians followed the same pattern at each site with NEC having the lowest and AD having the highest median p-tau/t-tau levels. A two-tailed Kruskal-Wallis test performed on the data revealed that diagnostic group did not differentially influence p-tau/t-tau levels at any of the phosphorylation sites, $Hs(3) \leq 3.263$, $ps > 0.05$ (Figure 1).

When we initially analyzed round one results, we excluded a few extreme outliers, whose p-tau/t-tau value exceeded 10. When outliers were excluded, a Mann-Whitney U test performed on the data revealed that AD subjects had significantly elevated p-tau/t-tau levels at S396, S404 and S400,403,404, $U(45,45)=768.00$, $U(19,18)=95.00$ $U(46,45)=780$ respectively, $ps<0.05$ (Figure 2).

Second Round results:

200 subjects were included in the second round of analysis. Eight subjects were excluded because there t-tau levels were undetectable by western blot. Two subjects were excluded upon receiving a final diagnosis not within criteria of any of the groups. One subject was excluded due to having given a saliva sample twice. A total of 189 saliva samples were analyzed in round two. Demographic data for subjects included in the analysis are shown in Table 2.

Similarly to round one data, the Shapiro-Wilks tests for normality suggested the data from each site were not from a normally distributed population, $ps<0.05$. Data for phosphorylation site(s) (S400,403,404, S404, S396 and T181) were therefore analyzed using non-parametric tests.

Our first step was to compare p-tau/t-tau levels of AD and NEC subjects, at each site (Figure 3). Two tailed Mann-Whitney U tests performed on the data revealed that the

p-tau/t-tau ratio of AD subjects was greater than NEC subjects at S396 (Figure 4 and 5) and S404 (Figure 6 and 7), $U(41,44)=596.00$ and $U(41,44)=655.00$, respectively, $ps<0.05$. Two tailed Mann-Whitney U tests performed on the data revealed that the p-tau/t-tau ratio of AD subjects did not differ significantly from NEC at S400,403,404 or T181, $U(41,44)=738.00$, and $U(41,44)=791.00$ respectively, $ps>0.05$.

Age group analysis

Given the significant difference in our AD and NEC subjects at S396 and S404, our second step was to compare p-tau/t-tau levels of NEC and YN subjects, at these two sites. Two-tailed Mann-Whitney U tests performed on the data at each site revealed that age group did not differentially influence pS404/tau4 levels, $U(44,76)=1542.000$, or pS396/tau4 levels, $U(44,76)=1413.000$, $ps>0.05$ (Figure 8).

Gender analysis

Because of an unequal female to male ratio in our groups (Table 2), we looked to see if there was a gender difference at any of these two sites in our NEC group. A two-tailed Mann-Whitney U test performed on the data revealed that gender did not differentially influence pS396/tau4 levels, $U(30,14)=158.000$, or pS404/tau4 levels, $U(30,14)=192.000$, $ps>0.05$.

Neurology and FTD Subjects

Two-tailed Mann-Whitney U tests performed on the data at each site revealed that NEUR subjects did not differ significantly from NEC in pS396/tau4 levels, $U(44,12)=237.00$ (Figure 9), and pS404/tau4 levels, $U(44,12)=188.00$ (Figure 10), and $ps>0.05$.

Two-tailed Mann-Whitney U tests performed on the data at each site revealed that FTD subjects had significantly greater pS396/tau4 levels, U(44,16)=148.00 (Figure 11), and pS404/tau4 levels, U(44,16)=117.00 (Figure 12), compared to NEC, ps<0.05.

SMI information

A two tailed Mann-Whitney U test performed on the data revealed that the p-tau/t-tau ratio of SMI subjects did not differ significantly from non-SMI subjects, at any of the phosphorylation sites, ps>0.05 (Figure 13).

CSF Correlation

A Spearman correlation (n=12) showed CSF p-tau/t-tau and salivary pT181/tau4 were not significantly correlated, r_s=0.168 p>0.05 (Figure 14). Furthermore, CSF p-tau/t-tau and salivary p-tau/t-tau at sites S396 and S404 were not correlated, r_s=0.357 and r_s=0.070 respectively, ps>0.05.

Hippocampal Volume Correlation

A Spearman correlation (n=12) showed that salivary pS396/tau4 was not significantly correlated with either the left hippocampus or right hippocampus volume, r_s=-0.413 and r_s= -0.483 respectively, ps>0.05 (Figure 15). Likewise, a Spearman correlation (n=12) showed that salivary pS404/tau4 was not significantly correlated with either the left hippocampus or right hippocampus volume, r_s=0.049 and r_s= -0.133 respectively, ps>0.05 (Figure 16).

Episodic memory scores

Using first round data, we correlated scores from the MoCA, logical memory 2 of the WMS and the CDT with p-tau/t-tau in MCI and AD subjects who had undergone neuropsychology testing.

Spearman correlations showed that salivary pS396/tau4 (n=21) and pS404/tau4 (n=8) were not significantly correlated with MoCA scores, r_s=-0.125 and r_s=-0.109 respectively, ps>0.05 (Figure 17). As well, Spearman correlations showed that salivary pS396/tau4 (n=21) and pS404/tau4 (n=6), were not significantly correlated with logical memory 2 scores of the WMS, r_s=-0.165 and r_s=-0.058 respectively, ps>0.05 (Figure 18). Finally, Spearman correlations showed that salivary pS396/tau4 (n=23) and pS404/tau4 (n=8) were not significantly correlated with the CDT scores, r_s=-0.099 and r_s=-0.600 respectively, ps>0.05 (Figure 19).

Kruskal Wallis for the groups together

A two-tailed Kruskal Wallis test performed on the pS396/tau4 data revealed a significant diagnostic group effect, $Chi^2(3)=12.973$, $p<0.05$ (Figure 20). Subsequent pairwise comparison tests, conducted using Mann-Whitney tests with a Bonferroni-Holmes correction, indicated that the median pS396/tau4 level of both the AD and FTD groups were significantly greater than the NEC group, ps<0.05. No other pairwise contrast was significant (ps>0.05).

A two-tailed Kruskal Wallis test performed on the pS404/tau4 data revealed a significant diagnostic group effect, $Chi^2(3)=15.900$, $p<0.05$ (Figure 21). Subsequent pairwise comparison tests, conducted using Mann-Whitney tests with a Bonferroni-Holmes correction, indicated that the median pS404/tau4 level of the FTD group was significantly greater than the NEC group, $p<0.05$. No other pairwise contrast was significant (ps>0.05).

Discussion

AD patients had significantly elevated p-tau/t-tau ratios compared to NEC at S396 and S404. Even when using a strict correction for multiple comparisons, pS396/tau4 was still significantly elevated in AD compared to NEC subjects (Figure 20). We can also take a slightly different approach at looking at our results from S396 and S404 sites. At S396, 75% of AD subjects are above 0.96 p-tau/t-tau level, whereas this is the median for NEC subjects (1.00) (Figure 5). In S404, 75% of AD subjects are above 1.23, compared to NEC, where the median is 1.39 (Figure 7). However, at all the sites and in all diagnostics groups we explored, there was large variation in p-tau/t-tau levels. This variation limits the utility of the test as a diagnostic biomarker. Various factors may have contributed to this heterogeneity.

AD group

We showed that AD subjects had significantly higher levels of salivary tau than age-matched normal, when measured at sites S396 and S404. The most problematic finding for the use of this test as a biomarker is the heterogeneity of phosphorylation level in our AD group. A portion of the AD subjects did not have elevated p-tau/t-tau in their saliva. Several explanations for this important variability are worth mentioning. One possible explanation for this variation is that AD subjects may have variation in tau pathology amongst one another. One study found that only 90% of patients clinically diagnosed with probable or possible AD had AD neuropathology[16]. Alternately, it may be that they all had tau levels elevated in the brain, but for unknown reasons, some AD individuals fail to express the tau peripherally in salivary gland tissue. Finally it is possible that it is in all the salivary tissue, but secreted only in a subgroup of AD individuals. We believe that we controlled for other technical issues related to saliva

collection, but there may have been unknown technical limitations which impaired our measurements nevertheless.

T181 is the most extensively studied tau phosphorylation site, in terms of use as a CSF biomarker. However, in our results, we demonstrated no significant difference between AD and NEC at pT181 site (Figure 3). As well, salivary p-tau/t-tau did not correlate with CSF p-tau/t-tau (Figure 14). These two findings weaken the notion that salivary tau mirrors CSF and brain tau changes. Because we do not know the precise mechanism by which tau is found in saliva, it is difficult to know the specific reasons for the heterogeneity in our results. Clearly more work is required to elucidate tau biology outside the brain.

NEC group

Despite an overall p-tau/t-tau median lower than AD subjects, a high p-tau/t-tau level was found in some of our NEC subjects at various sites. Although neocortical NFTs are mostly absent in non-demented subjects, NFTs can be present in elderly subjects with no dementia[18]. Some elderly seem to be resilient to the neurotoxicity of plaques and tangles, and are sometimes referred to as "high-pathology non-demented controls" or having "asymptomatic AD"[35]. One study, which conducted autopsies on elderly volunteers, found senile plaques and NFTs in many subjects who had not shown cognitive impairment[11]. The authors make note that their group was from well-educated volunteers[11]. In our study, the elderly subjects recruited were mostly volunteers who had been involved in previous research studies, and had on average, greater than 15 years of formal education, and therefore may have some capacity to compensate for the accumulation of neurofibrillary pathology.

Although Jessen and colleagues found SMI and early MCI with self-reported concerns were associated with the same risk of AD[22], we found no difference in salivary p-tau/t-tau between our SMI NEC and non-SMI NEC groups.

MCI group

Individuals with MCI who later convert to AD have a similar CSF biomarker pattern to people with AD[5]. In terms of our MCI cohort, the large variation in p-tau/t-tau levels makes sense. Although there is increased probability of going on to get AD in MCI subjects[28], there is a high degree of heterogeneity underlying MCI[35]. It is very likely that not all of our MCI subjects have underlying AD pathology, and not all will go on to develop AD.

FTD group

FTD, a group of diseases that includes some tauopathies, were found to have higher p-tau/t-tau levels than NEC subjects, although there was still considerable variation within the group. Interestingly, the variation in the FTD group was less than in the AD group. The variation may be due to the fact that not all FTD underlying pathologies are taupathies. Although this test would not be ideal for differentiating AD and FTD, the use of different isoform specific antibodies could create for a more specific test to distinguish between FTD and AD subjects. Hampel and Teipei suggest that for FTD, CSF tau may have limited value for discriminating FTD from AD or healthy aging due to its pathological heterogeneity (it has many subtypes)[17]. Therefore, further work could look at specific subtypes of FTD and abnormal salivary tau.

Neurology patients

As we hypothesized, the median p-tau/t-tau level in our NEUR subjects was not different from NECs. However, our NEUR patients still had great variability in p-tau/t-tau (Figure 9 and 10). Although the majority of subjects had low p-tau/t-tau levels, there were a few with high levels. Specifically, high p-tau/t-tau levels were found in some of the subjects with chronic stroke. In acute stroke, there is an increase CSF tau, but not CSF p-tau[19]. Although we were looking at chronic stroke, it is possible that it still influenced the salivary phosphorylation levels, as we do not know much about the timeframe related to salivary tau abnormalities. Perhaps salivary tau changes at a later time point than CSF tau. Further knowledge and research into the mechanism by which tau and p-tau ends up in saliva may help elucidate why some of our neurology patients, thought not to have brain tau pathology, also exhibited an elevated salivary p-tau/t-tau ratio.

YN vs NEC group

The fact that the YN and NEC did not differ at pS396 or S404 levels of p-tau/t-tau supports that age was most likely not contributing substantially to the difference between the NEC and AD group at these sites. However, it is surprising that there were some relatively high levels amongst the YN controls and the reason for this remains to be elucidated.

CSF

We found no significant correlation between CSF p-tau/t-tau and salivary p-tau/t-tau. Athena diagnostics, where CSF samples were analyzed, uses pT181 as the exclusive site for measuring p-tau/t-tau, and so we used this same site to look for a correlation. CSF biomarkers, although used for research, are not yet recommended for routine clinical

use[14]. One issue to keep in mind is consistency of CSF measurements across clinical centres[14]. We only used Athena Diagnostics to look for CSF p-tau/t-tau measures, but sending samples to other centers may have shown some variability in levels. Another limitation is the time difference between when the LPs were administered and when saliva was collected. The LPs were carried out from 2012 to 2015. Therefore, for some subjects, there was a substantial delay, whereas for other subjects, the collection of CSF and saliva was done on the same day. This discrepancy amongst subjects and long delay for some subjects are limitations of our study, but due to the relatively invasive nature of LPs, were unavoidable.

Hippocampal Volume

Although we found a decrease in hippocampal volume with increased salivary p-tau/t-tau, we found this correlation to be not significant (Figure 15 and 16). One limitation if this analysis is the time difference between when the subject received an MRI and when saliva was collected.

Episodic memory scores

Tau pathology in AD is primarily in the medial temporal lobe. Thus, if salivary tau was a good reflection of brain tau, we would have expected a correlation between salivary tau and episodic memory scores, as measured with logical memory 2 score of the WMS, but not on a test of general cognitive function, such as the CDT. Salivary p-tau/t-tau did not correlate with logical memory 2, CDT or MoCA scores at either S396 or S404. One limitation is the time difference between when the subject received neuropsychology testing and the date of saliva collection, although we only considered subjects who had neuropsychology testing in the same year that they gave a saliva sample. Another

limitation is that the majority of subjects used for these correlations were MCI subjects (although a few were AD subjects), and therefore not all of them will necessarily go on to develop AD, and may have varied underlying neuropathology.

Some sites but not others?

One important question to consider is why only two out of the four sites we considered showed significant differences in p-tau/t-tau levels between AD and NEC. Surprisingly, one of the most studied CSF phosphorylation sites, T181, did not show a difference between the two groups. Furthermore, no correlation between the CSF p-tau/t-tau ratio (which uses T181) and salivary p-tau/t-tau was found. One possibility is that some sites are simply better peripheral markers than others. Since we do not know how p-tau ends up in saliva, we need more information with regards to the upstream mechanisms that result in salivary phosphorylated tau to help us understand why some sites are more indicative than others.

Tau outside of the brain and peripheral manifestations of AD

Another question to consider is what is how tau gets into the saliva. Shi and colleagues (2011), after detecting tau in saliva using mass spectrometry, identified possible reasons for this finding. Firstly, since the salivary glands are near the CNS, it is possible that salivary tau is released from nerves that innervate the salivary glands[37]. Their second suggestion is that tau is expressed and secreted by the acinar epithelial cells of the salivary glands[37]. Interestingly, tau mRNA is found in salivary glands[10]. Further work is needed on establishing the mechanism for which tau and phosphorylated tau end up in saliva.

Although salivary tau is likely not an ideal biomarker for AD, our findings do suggest it represents a peripheral manifestation of AD. A review by Francois et al. summarized findings of biomarkers for AD in peripheral tissues, providing literature support for significant biological changes appearing in non-neural tissues like fibroblasts, blood and buccal cells[15]. For example, total tau protein was elevated in buccal cells of AD compared to age-matched controls[15]. Thus, while our study is not the first to find markers of AD outside of the brain cavity, it adds to the current literature of peripheral manifestations of AD. Our findings and those of others call into question the notion of AD as strictly a disease of the brain.

Additional limitations and future directions

We only considered four amongst the many phosphorylation sites that are hyperphosphorylated in AD pathology. Future work should consider other possible phosphorylation sites with different site-specific antibodies. Other sites may have better specificity.

Another limitation of our study is that tau4 was used as an antibody to measure total tau. However, tau4 only is specific for the 4R tau isoform. The tau4 antibody was chosen because it is reliable and more easily quantified than tau5, which has affinity for both the 3R and 4R tau isoform. In adult humans, there is both the 3R tau isoform and the 4R tau isoform present. Because the tau4 antibody only has affinity for the 4R tau isoform, the 3R isoform was not included in our measure of total tau.

Further research into tau and its presence and phosphorylation in salivary glands is needed. Further exploration of the mechanism by which tau ends up in saliva may add to our understanding of the large variation within each diagnostic group. It would be

interesting to know if abnormal salivary tau correlated with tau pathology at autopsy, which is outside the timeframe of this study.

Further research into the stability of salivary tau is essential – that is, to take the saliva of the same person multiple times in a year to see if there is variation day to day, which would also be necessary to explore in terms salivary tau's usefulness as a biomarker. The stability of salivary p-tau/t-tau levels over the short term is important to consider. Collection today might yield a different p-tau/t-tau level compared to a collection on another day. Furthermore, the diurnal variation of salivary tau may be important to consider, as this variation has been found in other salivary compounds[39].

Conclusions

Our analysis of salivary tau/p-tau revealed a significant difference between AD patients and cognitively healthy elderly subjects at two phosphorylation sites, S396 and S404. There was no such elevation in NEC compared to YN subjects, nor in other neurological diseases compared to NEC subjects. The elevation in saliva did not correlate with (indirect) brain measures – hippocampal volume and CSF tau - nor did it correlate with other neuropsychology tests. There was also a significant increase in p-tau/t-tau level in FTD subjects at these same sites. However, the large variation in levels of AD subjects and in the other diagnostic groups suggests this test may not be useful as a clinical biomarker. Finally, the meaning of this peripheral abnormality in AD requires further elucidation.

References

[1]Apostolova L, Green A, Babakchanian S, Hwang K, Chou Y, Toga A, Thompson P (2012) Hippocampal atrophy and ventricular enlargement in normal aging, mild

cognitive impairment (MCI) and Alzheimer disease. Alzheimer Dis Assoc Disor 26(1): 17-27.

[2]Babic M, Svob Strac D, Muck-Seler D, Pivac N, Stanic G, Hof P, Simic G (2014) Update on the core and developing cerebrospinal fluid biomarkers for Alzheimer disease. Croat Med J 55:347-65.

[3]Ballatore C, Lee VM, Trojanowski JQ (2007) Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. Nat Rev Neurosci. 8:663-72.

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

[5]Blennow K, Vanmechelen E, Hampel H (2001) CSF total tau, AB42 and phosphorylated tau protein as biomarkers for Alzheimer's disease. Molecular Neurobiology 24: 87-97.

[6]Blennow K, Zetterberg H (2013) The application of cerebrospinal fluid biomarkers in early diagnosis of Alzheimer Disease. Med Clin N Am: 369-376

[7]Braak H, Braak E (1991) Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol (Berl) 82:239-59.

[8]Brion JP (1998) Neurofibrillary tangles and Alzheimer's disease. Eur Neurol 40(3):130-40.

[9]Carpenter GH (2013) The secretion, components, and properties of saliva. Annu. Rev. Food Sci. Technol 4:267-76.

[10]Conrad C, Vianna C, Freeman M, Davies P (2002) A polymorphic gene nested within an intron of the tau gene: Implications for Alzheimer's disease. PNAS 99(11) 7751-7756.

[11]Davis DG, Schmitt FA, Wekstein DR, Markesbery MD (1999) Alzheimer neuropathologic alterations in aged cognitively normal subjects. J Neuropathol Exp Neurol, 58:376-388

[12]De-Paula V, Radanovic M, Diniz B, Forlenza O (2012) Alzheimer's disease. Subcellular Biochemistry 65, 329-352.

[13]Devic I, Hwang H, Edgar JS, Izutsu K, Presland R, Pan C, Goodlett DR, Wang Y, Armaly J, Tumas V, Zabetian CP, Leverenz J, Shi M, Zhang J (2011) Salivary a-synuclein and DJ-1: potential biomarkers for Parkinson's disease. Brain 134:1-5.

[14]Ewers M, Mattson N, Minthon L, Molinuevo JL, Antonell A, Popp J, Jessen F, Herukka SK, Soininen H, Maetzler W, Leyhe T, Burger K, Taniguchi M, Urakami K, Lista S, Dubois B, Blennow K, Hampel H (2015) CSF biomarkers for the differential diagnosis of Alzheimer's disease: a large-scale international multicenter study. Alzheimer's & Dementia 11: 1306-1315.

[15]Francois M, Leifert W, Martins R, Thomas P, Fenech M (2014) Biomarkers of Alzheimer's disease risk in peripheral tissues; focus on buccal cells. Current Alzheimer Research 11:519-531.

[16]Galasko D, Hansen L, Katzman R, Wiederholt W, Masliah E, Terry R, Hill R, Lessin P, Thal L (1994) Clinical-neuropathological correlations in Alzheimer's disease and related dementias. Arch Neurol 51:888-895.

[17]Hampel H, Teipel SJ (2004) Total and phosphorylated tau proteins: evaluation as core biomarker candidates in frontotemporal dementia. Dement and Geriatr Cogn Disord 17:350-354.

[18]Haroutunian V, Purohit D, Perl D, Marin D, Khan K, Lantz M, Davis K, Mohs R (1999) Neurofibrillary tangles in nondemented elderly subjects and mild Alzheimer disease. Arch neurol 56(6): 713-718.

[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. Neuroscience Letters 297: 187-190.

[20]Hoglund K, Fourier A, Perret-Liaudet A, Zetterberg H, Blennow K, Portelius E. (2015) Alzheimer's disease – Recent biomarker developments in relation to updated diagnostic criteria. Clin Chim Acta 449: 3-8.

[21]Iqbal K, Liu F, Gong C, Grundke-Iqbal I (2010) Tau in Alzheimer Disease and Related Tauopathies. Curr Alzheimer Res, 7(8): 656-664.

[22]Jessen F, Wolfgruber S, Wiese B, Bickel, H, Mosch E, Kadusziewicz H, Pentzek M, Riedel-Heller S, Luck T, Fuchs A, Weyerer S, Werle J, van den Bussch H, Scherer M, Maier W, Wager M (2014) AD dementia risk in late MCI, in early MCI, and in subjective memory impairment. Alzheimer's & Dementia 10: 76-83.

[23]Kennard M (1998) Diagnostic markers for Alzheimer's disease. Neurobiology of aging 19(2): 131-132.

[24]Lee G, Leugers C (2012) Tau and tauopathies. Progress in Molecular Biology and Translational Science 107: 263-292.

[25]Mayeux R, Stern Y (2012) Epidemiology of Alzheimer disease. Cold Spring Harb Perspect Med 2(8):a006239.

[26]McKhann GM, Knopman DS, Chertkow H, et al (2011) The diagnosis of dementia due to Alzheimer's disease: Recommendations from the National Institute on Aging and the Alzheimer's Association workgroup. Alzheimers Dement 7:263-9.

[27]Ossenkoppele R, Jansen WJ, Rabinovici GD, Knol DL, van der Flier WM, van Berckel BNM, Scheltens P, Jelle Visser P, Amyloid PET Study Group (2015) Prevalence of Amyloid PET Positivity in Dementia Syndromes A Meta-analysis. JAMA 313(19): 1939-1949.

[28]Petersen RC, Smith GE, Waring SC, Ivnik RJ, Tangalos EG, Kokmen E (1999). "Mild cognitive impairment: clinical characterization and outcome". *Arch. Neurol.* **56** (3): 303–8.

[29]Pfaffe T, Cooper-White J, Beyerlein P, Kostner K, Punyadeera C (2011) Diagnostic potential of saliva: current state and future applications. Clinical Chemistry 57(5): 675-687.

[30]Price J, Morris J (1999) Tangles and plaques in nondemented aging and “preclinical” Alzheimer’s disease. Ann Neurol 45:358-68.

[31]Rabinovici GD, Rosen HJ, Alkalay A, Kornak J, Furst AJ, Agarwal N, Mormino EC, O’Neil JP, Janabi M, Karydas A, Growdon ME’ Jang JY, Huang EJ, DeArmond SJ, Trojanowski JQ, Grinberg LT, Gorno-Tempini ML, Seeley WW, Miller BL, Jagust WJ (2011) Amyloid vs FDG-PET in the differential diagnosis of AD and FTLD. Neurology 77:2034-2042.

[32]Reitz C, Mayeux R (2014) Alzheimer disease: Epidemiology, diagnostic criteria, risk factors and biomarkers. Biochemical pharmacology 88:640-651.

[33]Roalf D, Moberg P, Xie S, Wolk D, Moelter S, Arnold S (2013) Comparative accuracies of two common screening instruments for classification of Alzheimer's disease, mild cognitive impairment, and healthy aging. Alzheimer's & Dementia 9:529-537.

[34]Rowe CC, Villemagne VL. Amyloid imaging with PET in early Alzheimer disease diagnosis. The Medical clinics of North America 2013;97:377-98.

[35]Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT (2011) Neuropathological alterations in Alzheimer disease. Cold Spring Harbor Perspectives in Medicine 1:a006189

[36]Shaffer JL, Petrella JR, Sheldon FC, Choudhury KR, Calhoun VD, Coleman RE, Doraiswamy PM, Alzheimer's Disease Neuroimaging Initiative (2013) Predicting cognitive decline in subjects at risk for Alzheimer disease by using combined cerebrospinal fluid, MR imaging, and PET biomarkers. Radiology, 266(2): 583-91.

[37]Shi M, Sui YT (2011) Salivary Tau Species are Potential Biomarkers of Alzheimer's Disease. J Alzheimers Dis 27: 299-305.

[38]Shin J, Lee S, Kim S, Kim Y, Cho S (2008) Multitracer PET imaging of amyloid plaques and neurofibrillary tangles in Alzheimer's disease. NeuroImage 43: 236-244.

[39]Su H, Gornitsky M, Geng G, Velly A, Chertkow H, Schipper H (2008) Diurnal variations in salivary protein carbonyl levels in normal and cognitively impaired human subjects. Age (Dordr.) 30:1–9;

[40]Wang J, Schipper HM, Velley AM, Mohit S, Gornitsky M (2015) Salivary biomarkers of oxidative stress: A critical review. Free Radic Biol Med 85:95-104.

[41]Wiltfang J, Lewczuk P, Riederer P, et al. (2005) Consensus paper of the WFSBP Task Force on Biological Markers of Dementia: the role of CSF and blood analysis in the early and differential diagnosis of dementia. World J Biol Psychiatry 6:69-84.

[42]Dementia Fact Sheet (March 2015). World Health Organization. Available from: http://www.who.int/mediacentre/factsheets/fs362/en/

Tables and Figures

Table 1. Demographic information for round one data

	N*	F:M	Median age (IQR)
AD	46	22:24	80 (9)
NEC	47	32:15	73 (6)
MCI	55	32:23	78 (14)

* For S404, part of the western blot did not turn out, and therefore there was a reduced sample size for this site for this round of data (n= 19, 20, 16, for AD, NEC and MCI respectively).

Table 2. Demographic information for round two data

	N	F:M	Median age (IQR)
AD	41	24:17	80 (8)
NEC	44	30:14	72 (7)
FTD	16	5:11	71.5 (10)
NEUR	12	7:5	55 (11)
YN	76	45:31	32 (22)

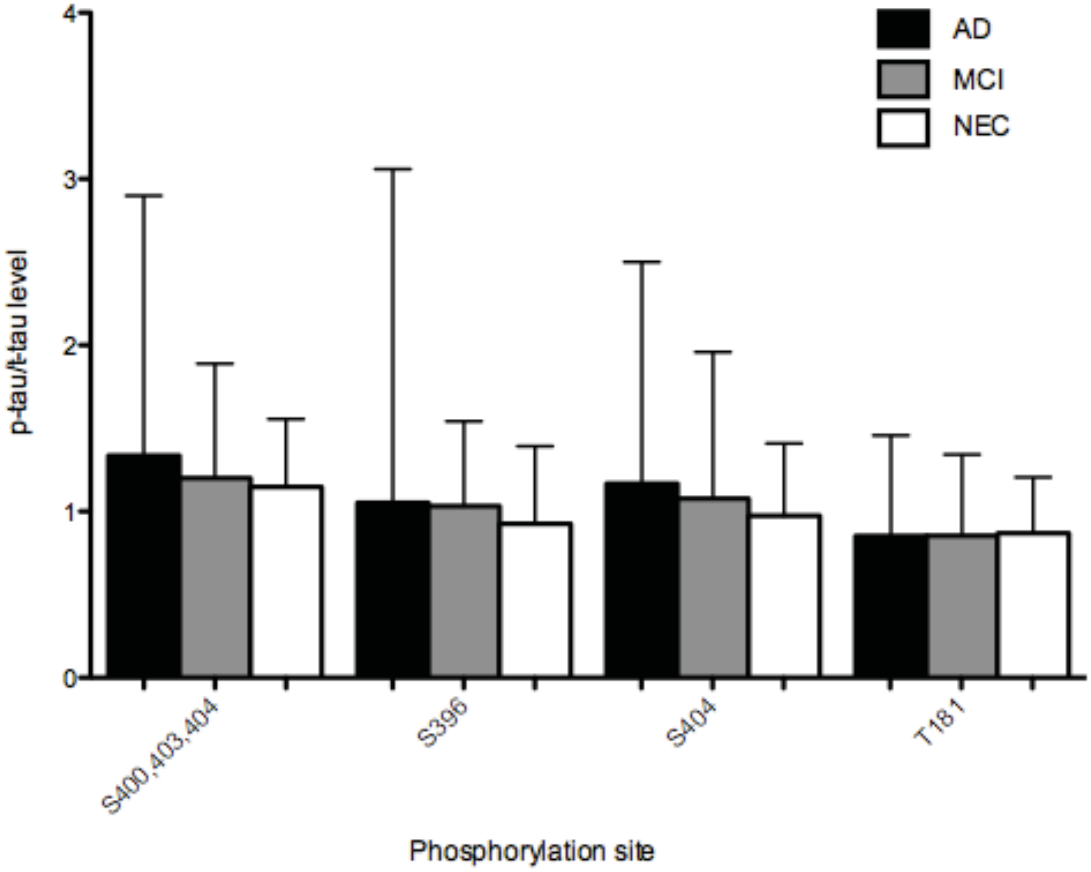

Figure 1: Median p-tau/t-tau levels in AD (n=46 for all sites, except S404 where n=19), MCI (n=55 for all sites, except S404 where n=16) and NEC (n=47 for all sites, except S404 where n=20) at each phosphorylation site. Error bars=IQR. All sites no significant (n. s.) difference ps>0.05

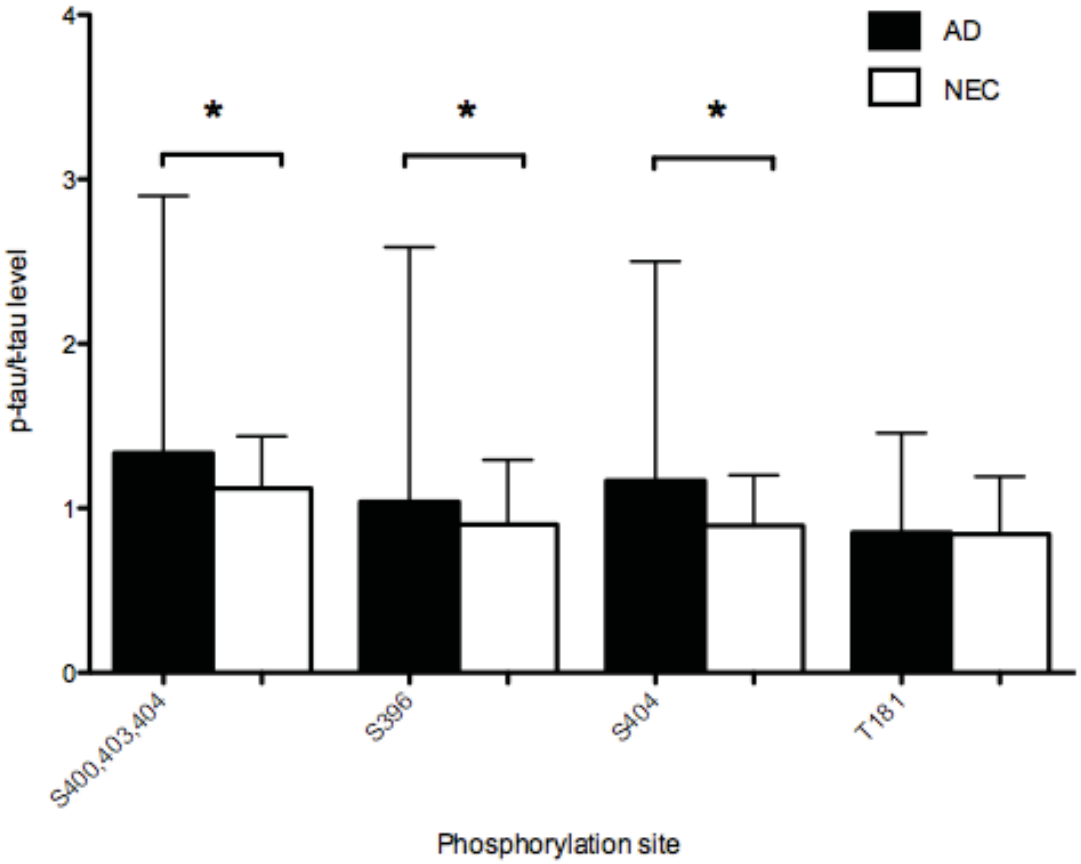

Figure 2: Median p-tau/t-tau levels in AD (n=46 for S400, 403, 404 and T181, n=45 for S396, n=19 for S404) and NEC (n=45 for S400,403,404 and S396, n=46 for T181, n=18 for S404) at each phosphorylation site, with outliers (>10 p-tau/t-tau level) excluded. Error bars=IQR. * ps<0.05

Figure:

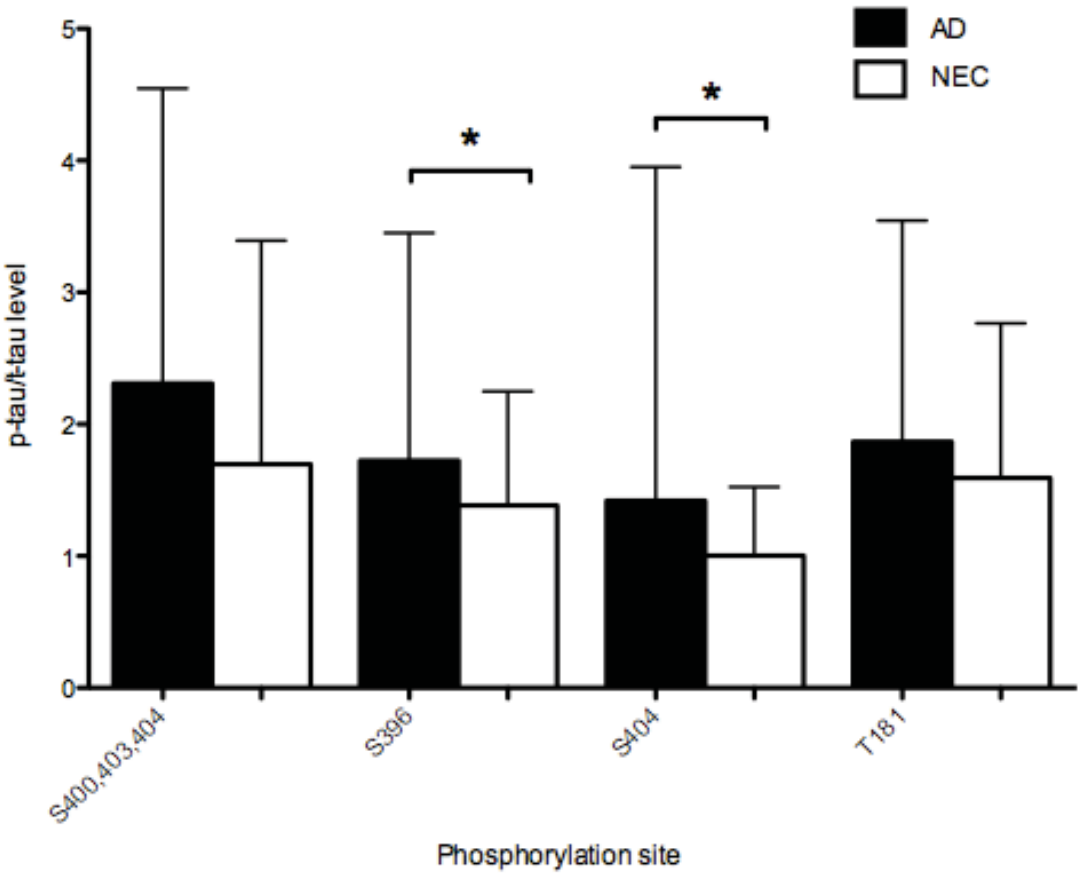

Figure 3: Median p-tau/t-tau levels in AD (n=41) and NEC (n=44) at each phosphorylation site. Error bars=IQR *ps<0.05

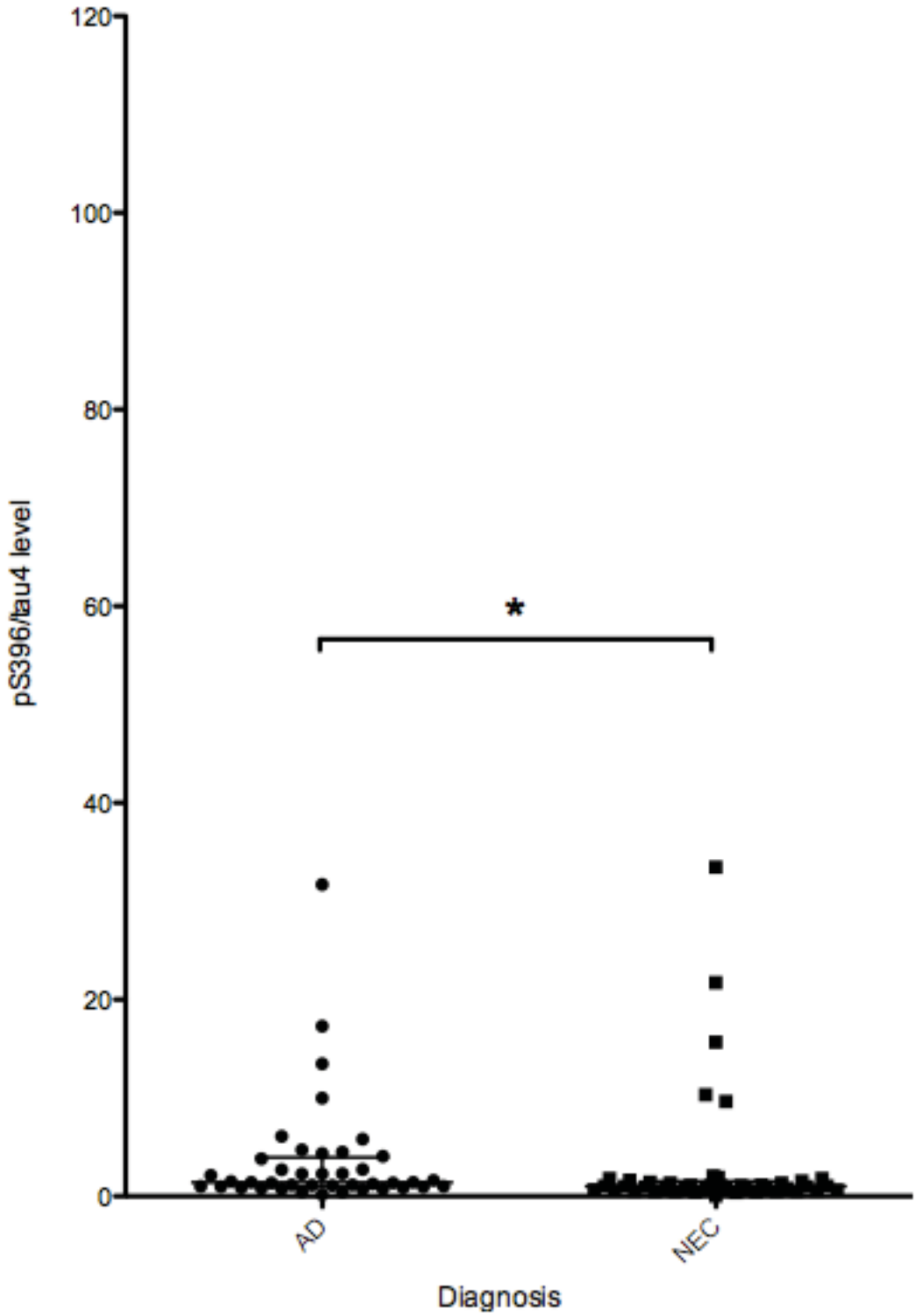

Figure 4. Scatterplot of pS396/tau4 levels in AD (n=41) and NEC (n=44) subjects, *p<0.05. The middle line indicates the median. The lowest and highest line indicate the IQR.

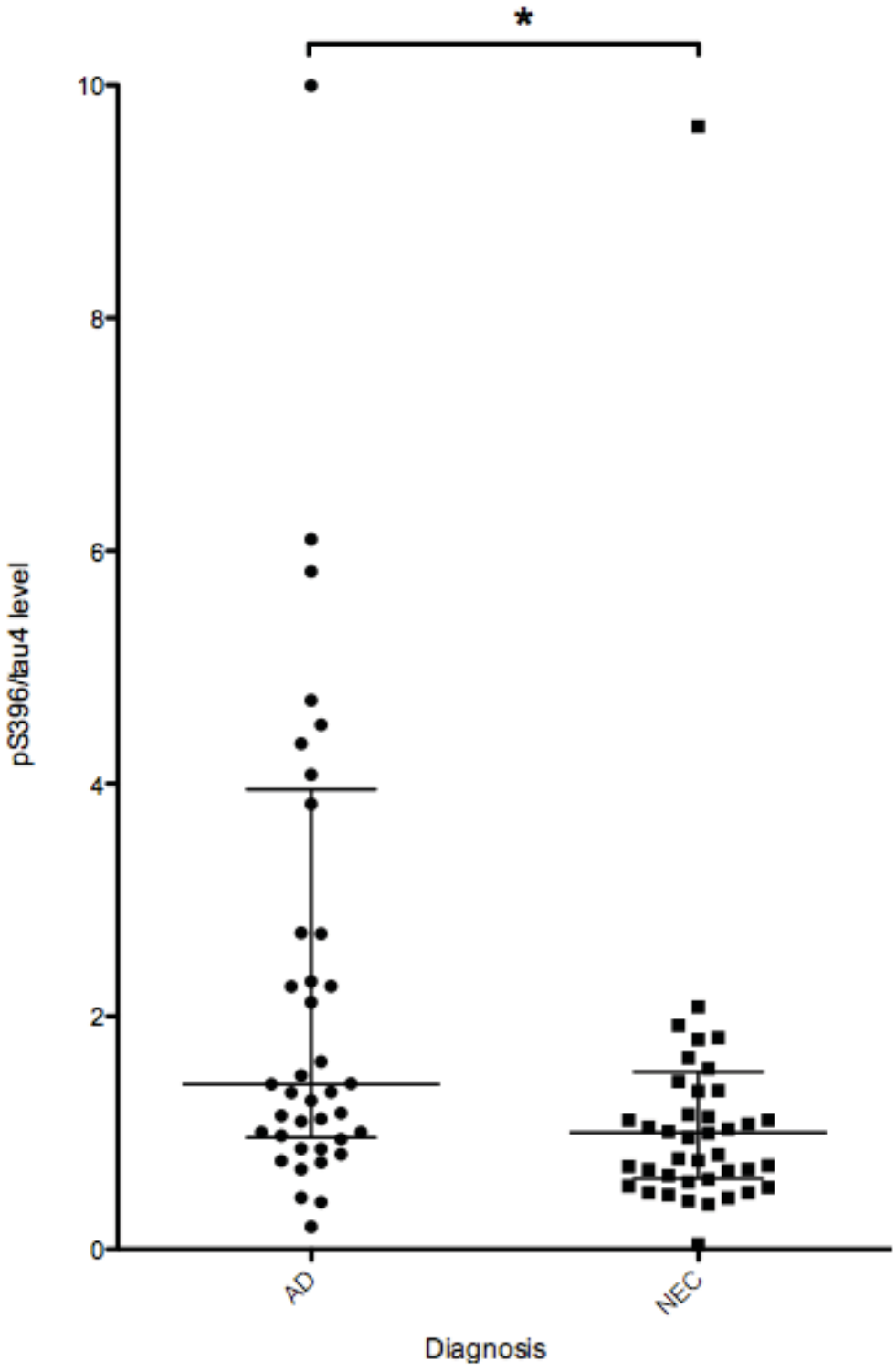

Figure 5. Scatterplot of pS396/tau4 levels in AD and NEC subjects cutoff at 10, *p<0.05. The middle line indicates the median. The lowest and highest line indicate the IQR.

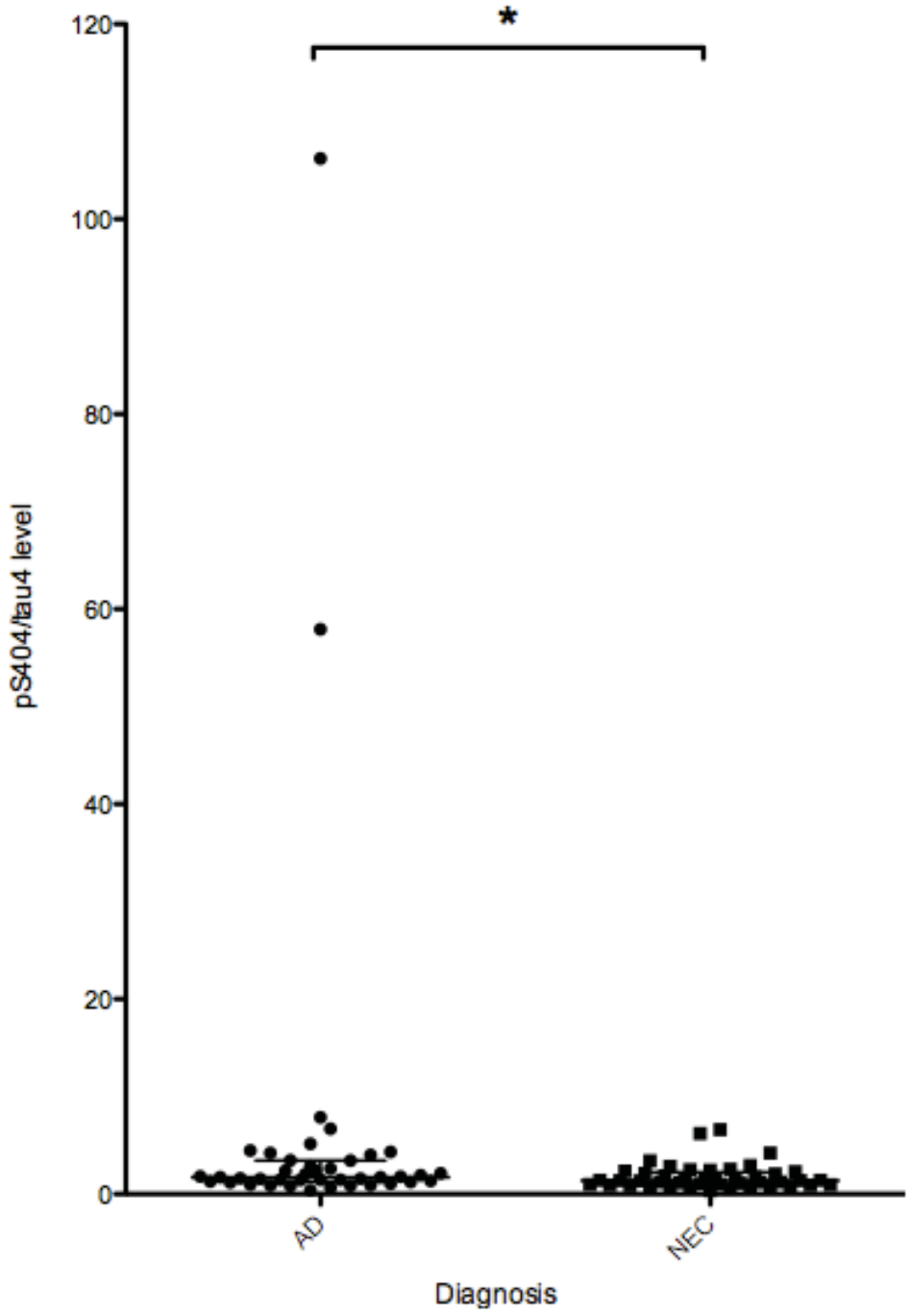

Figure 6. Scatterplot of pS404/tau4 levels in AD (n=41) and NEC (n=44) subjects, *p<0.05. The middle line indicates the median. The lowest and highest line indicate the IQR.

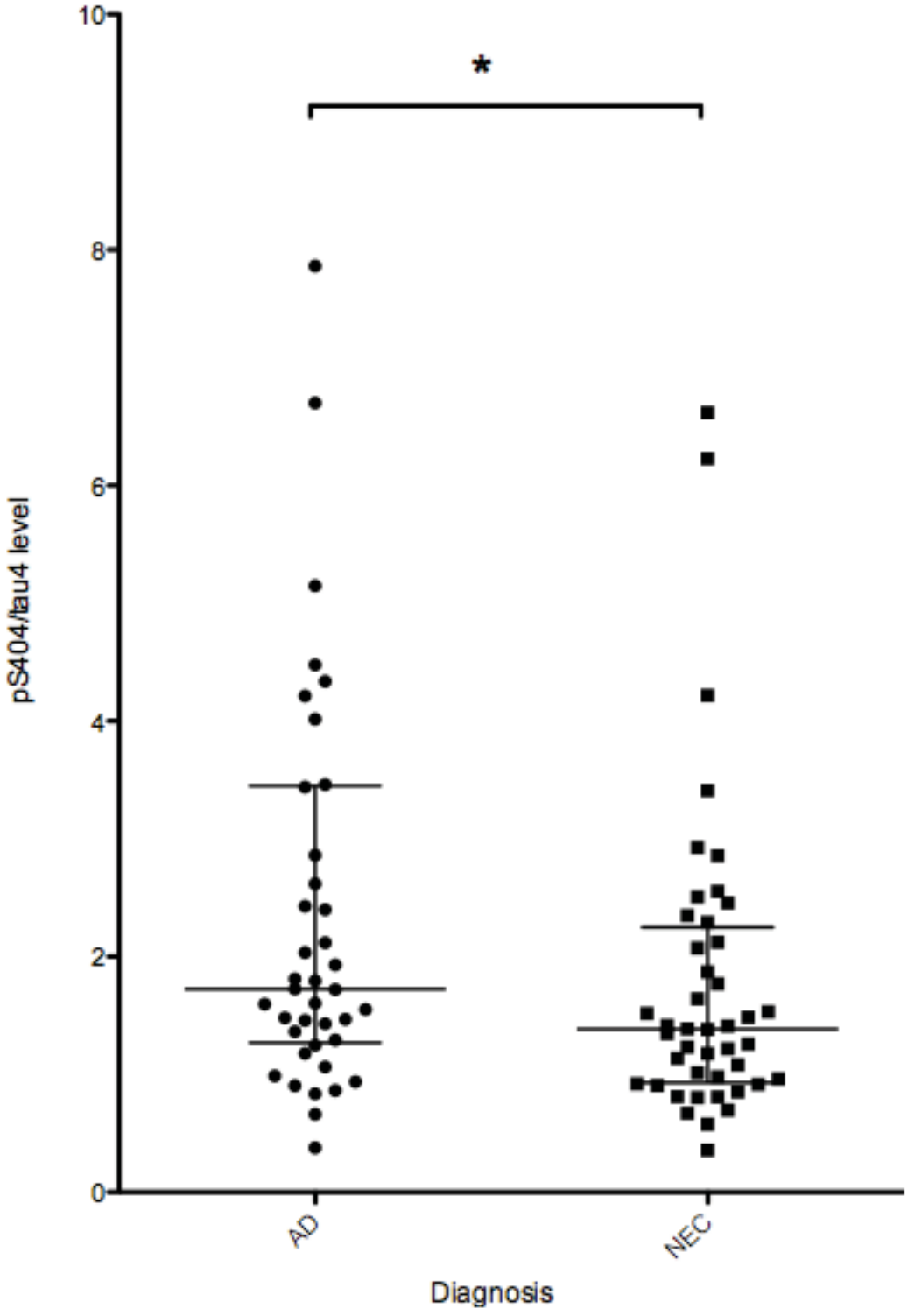

Figure 7. Scatterplot of pS404/tau4 levels in AD and NEC subjects cutoff at 10, *p<0.05. The middle line indicates the median. The lowest and highest line indicate the IQR.

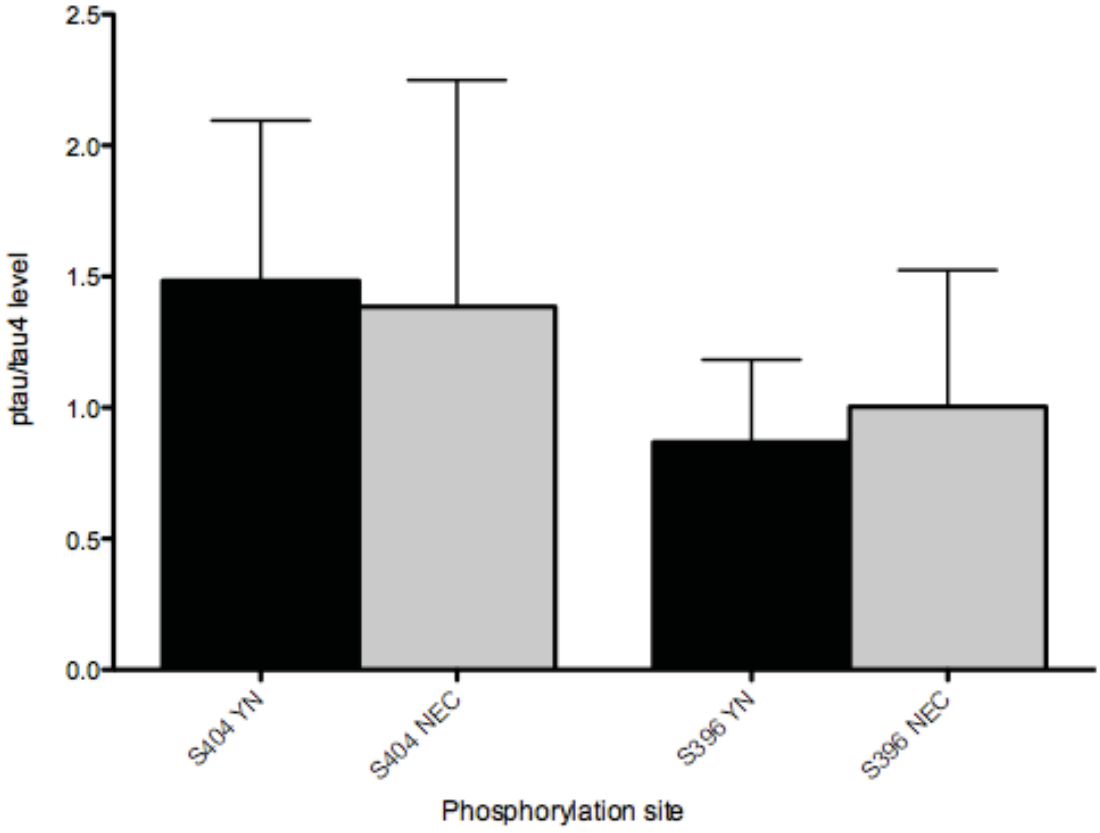

Figure 8. Median p-tau/t-tau levels in YN (n=76) and NEC (n=44) subjects. Error bars=IQR. n.s. ps>0.05.

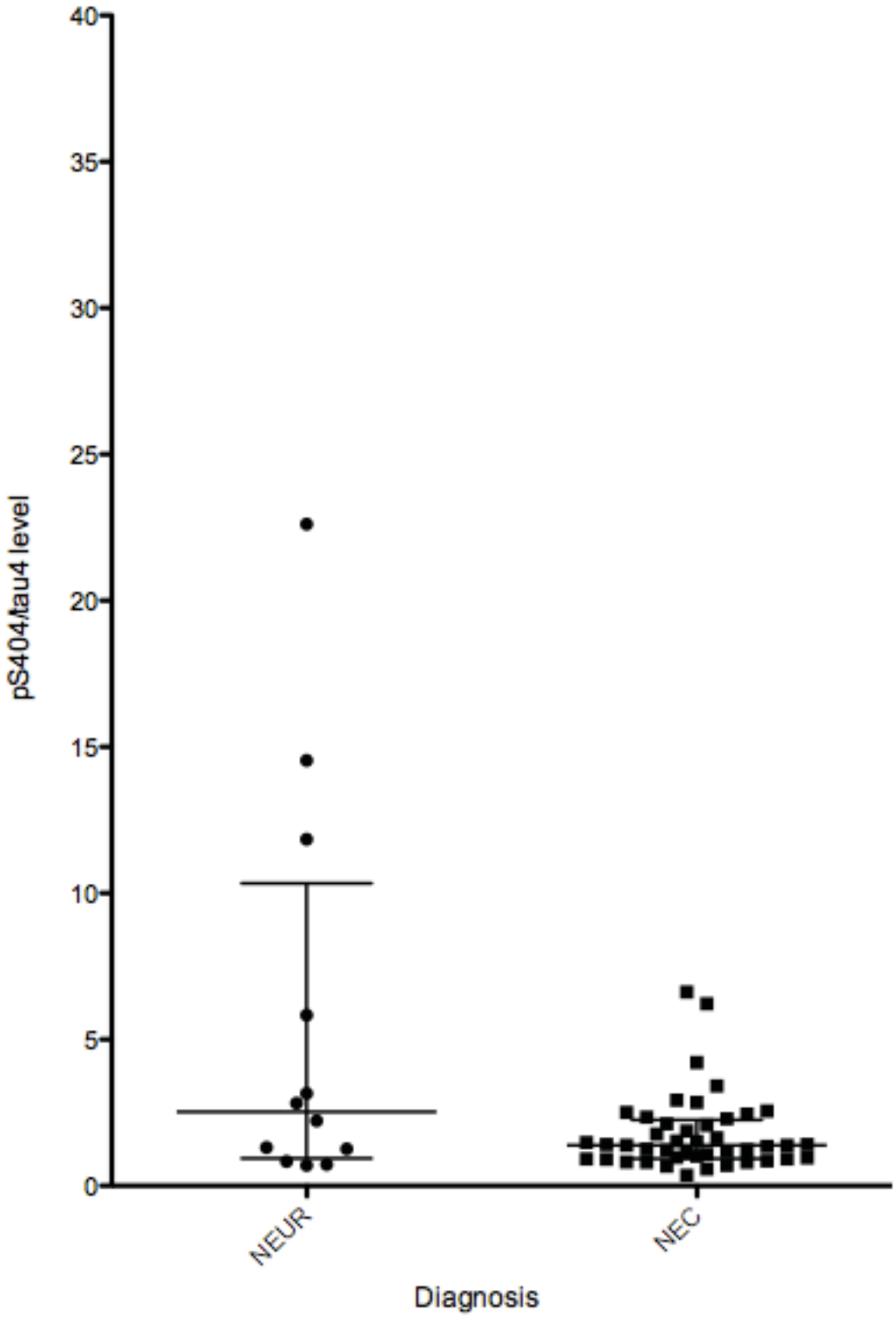

Figure 9. Scatterplot of pS396/tau4 levels NEUR (n=12) and NEC (n=44) subjects, n.s. $p>0.05$. The middle line indicates the median. The lowest and highest line indicate the IQR.

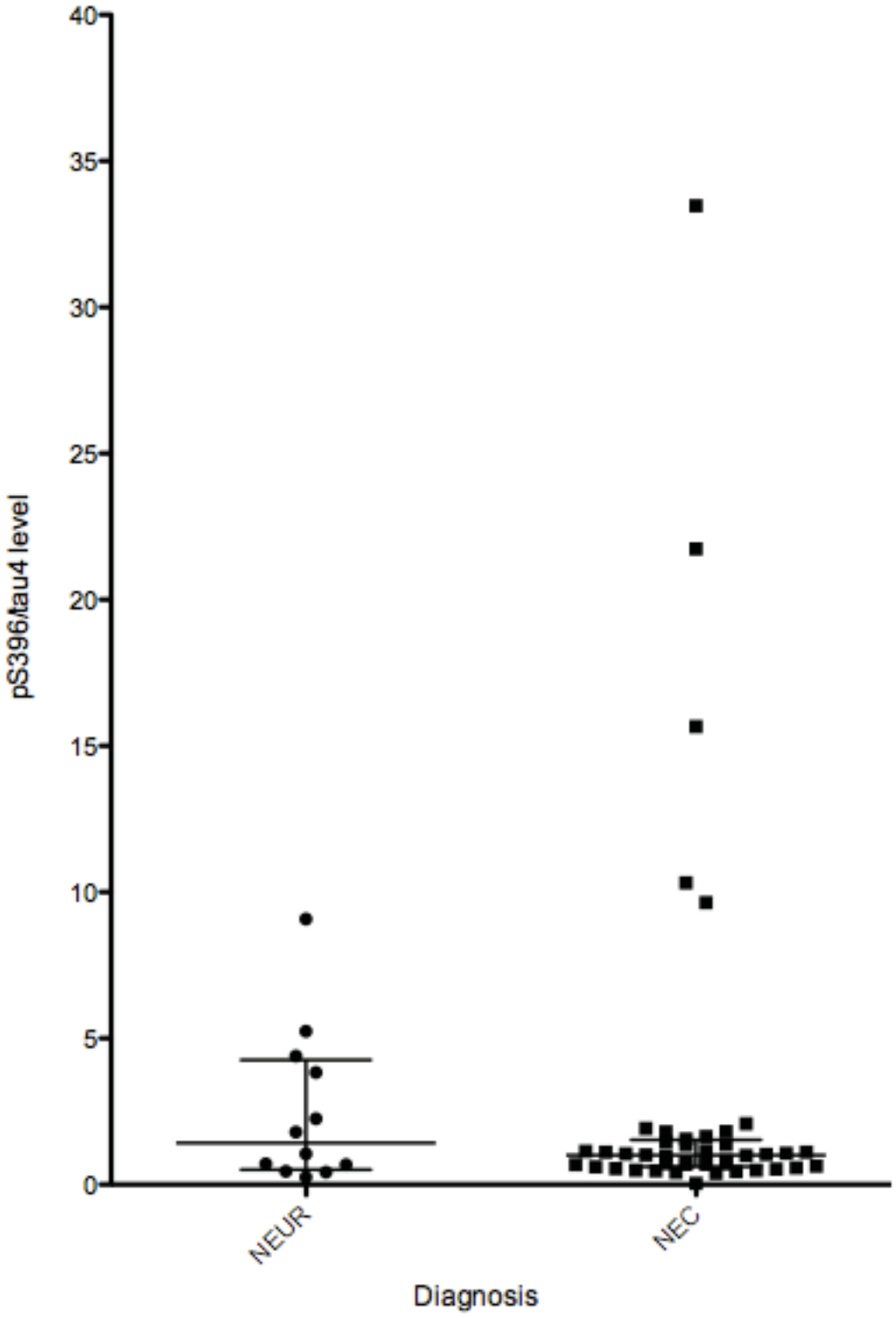

Figure 10. Scatterplot of pS404/tau4 levels NEUR (n=12) and NEC (n=44) subjects, n.s. $p>0.05$. The middle line indicates the median. The lowest and highest line indicate the IQR.

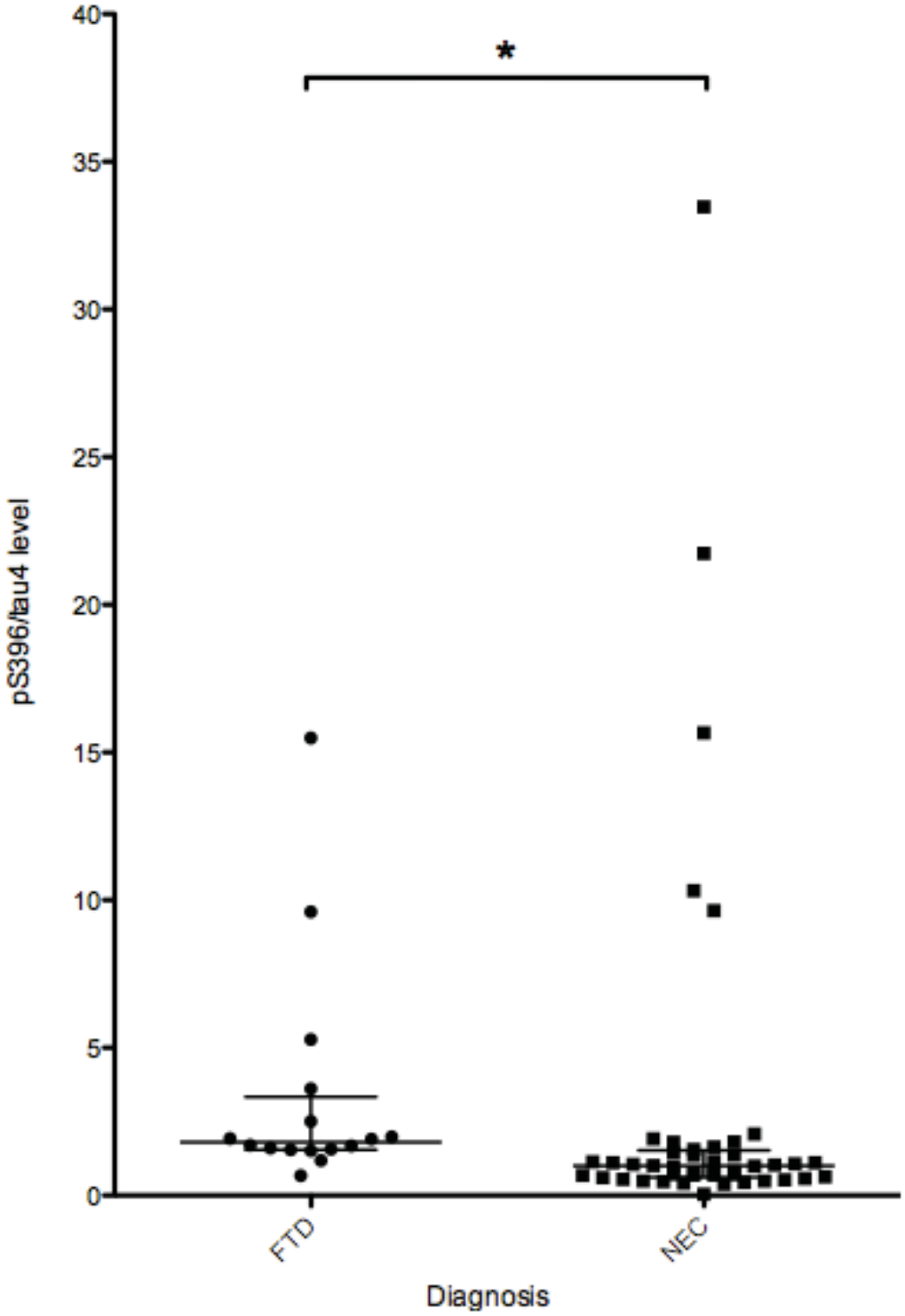

Figure 11. Scatterplot of pS396/tau4 levels in FTD (n=16) and NEC (n=44) subjects, *p<0.05. The middle line indicates the median. The lowest and highest line indicate the IQR.

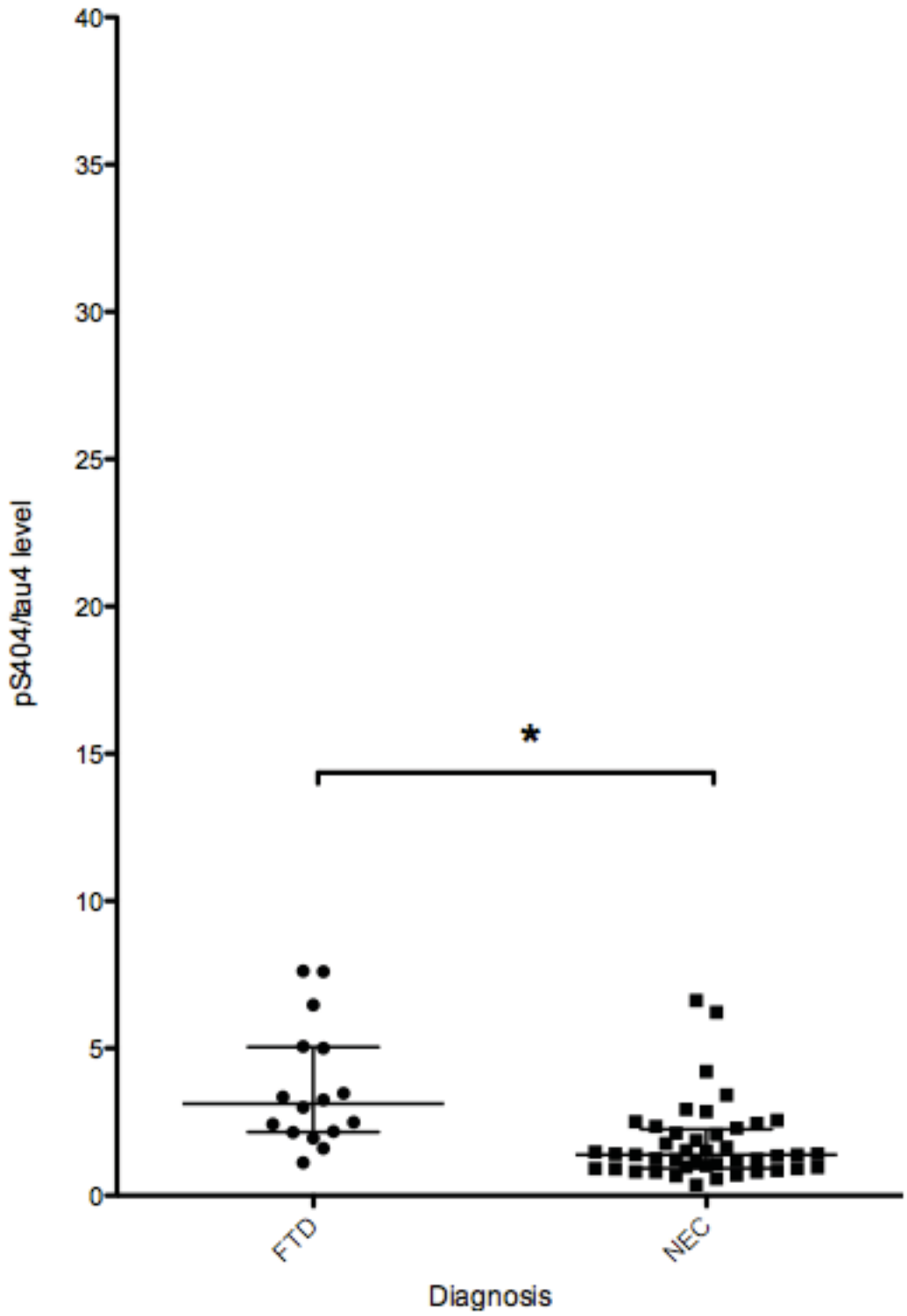

Figure 12. Scatterplot of pS404/tau4 levels in FTD (n=16) and NEC (n=44) subjects, *p<0.05. The middle line indicates the median. The lowest and highest line indicate the IQR.

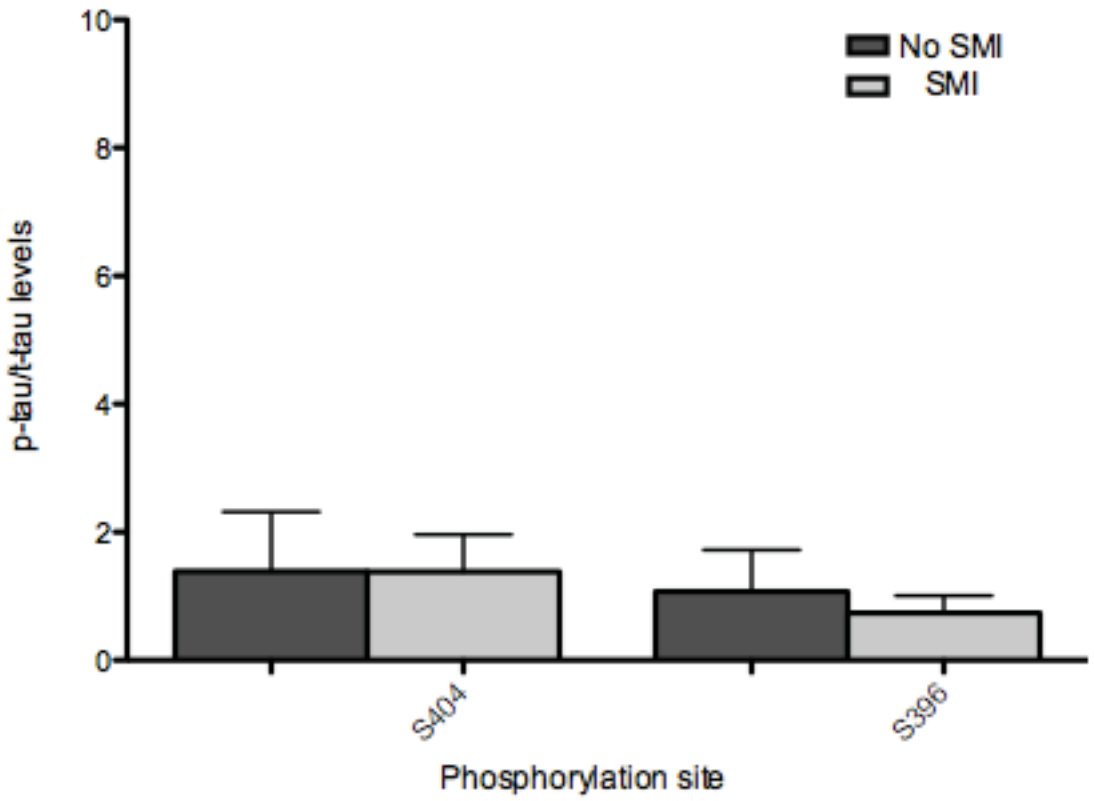

Figure 13. Median p-tau/t-tau levels at S404 and S396 in NEC without (n=33) and with (n=11) SMI. Error bars=IQR. n.s. ps>0.05

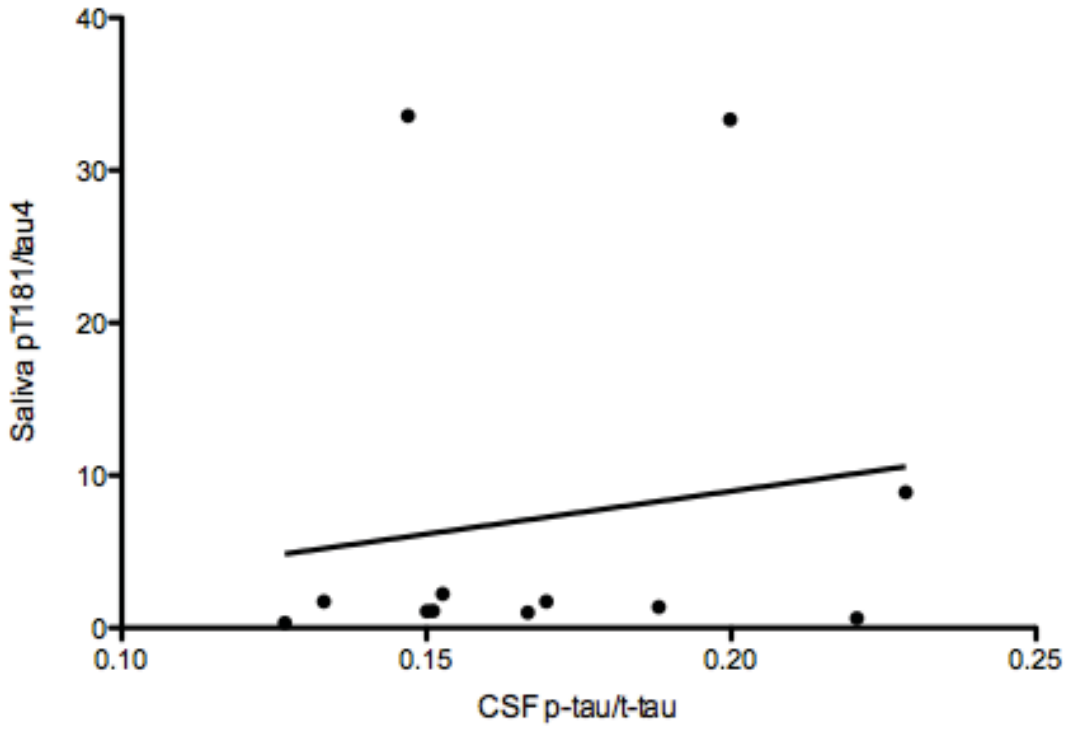

Figure 14: Regression line for CSF p-tau/t-tau and salivary pT181/tau4. A spearman correlation (n=12) showed the two measures were not significantly correlated, r_s=0.168 p>0.05.

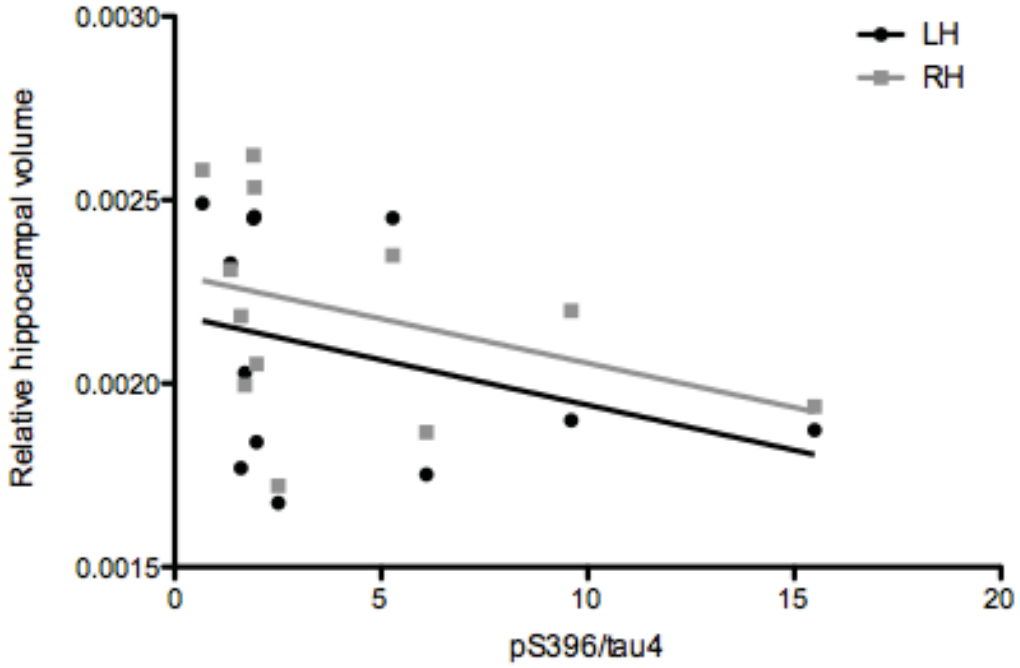

Figure 15: Correlation (n=12) of salivary pS396/tau4 with left and right hippocampus volume, r_s=-0.413 and r_s= -0.483 respectively, n.s. ps>0.05. Relative hippocampal volumes were obtained by dividing hippocampal volume (left and right hippocampus individually) by the subject's estimated total intracranial volume (to standardize across subjects).

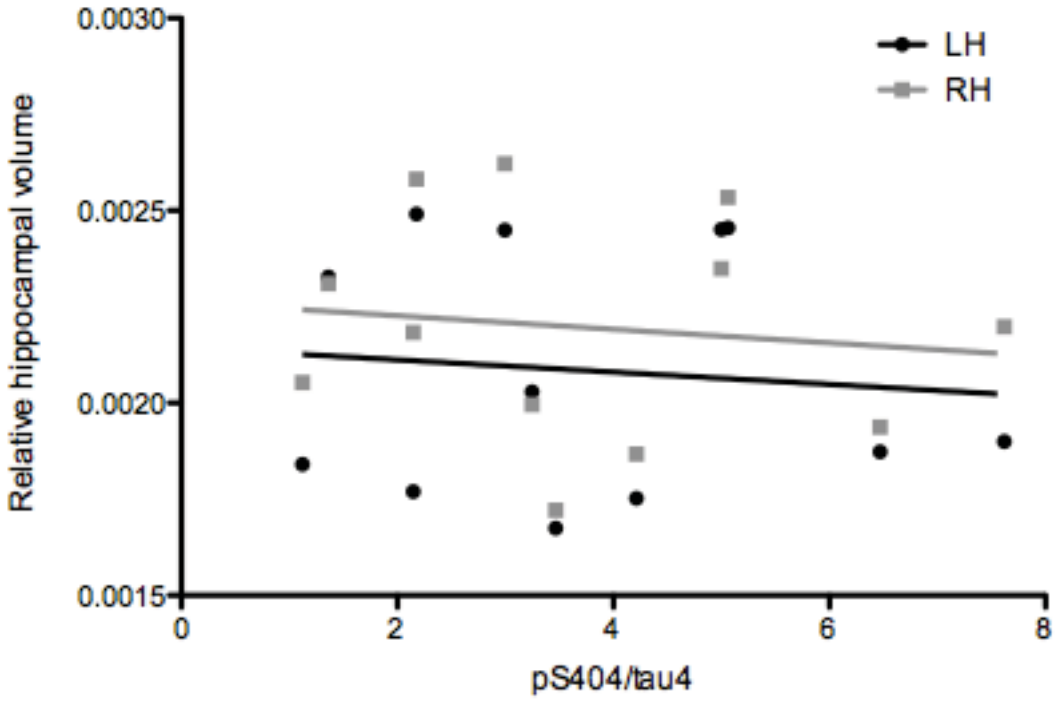

Figure 16: Correlation (n=12) of salivary pS404/tau4 with left and right hippocampus volume, r_s=0.049 and r_s= -0.133 respectively, n.s. ps>0.05. Relative hippocampal volumes were obtained by dividing hippocampal volume (left and right hippocampus individually) by the subject's estimated total intracranial volume (to standardize across subjects).

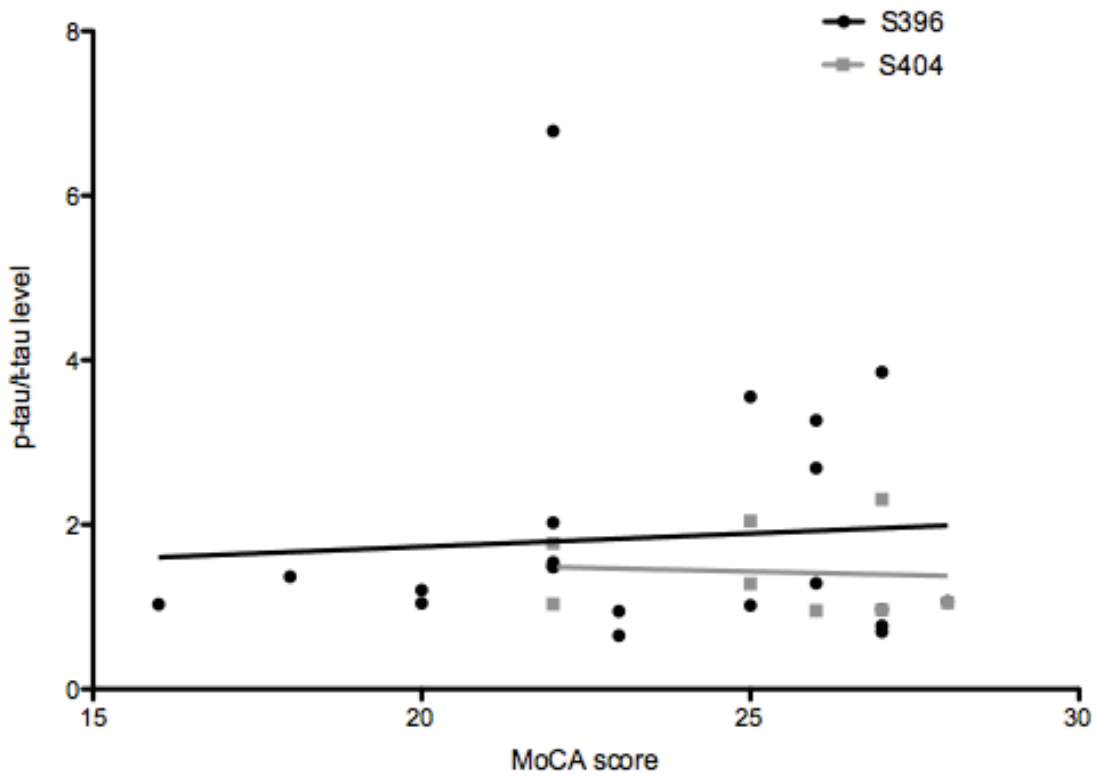

Figure 17: Correlations of salivary pS396/tau4 (n=21) and pS404/tau4 (n=8) with MoCA scores, r_s=-0.125 and r_s=-0.109 respectively. n.s. ps>0.05.

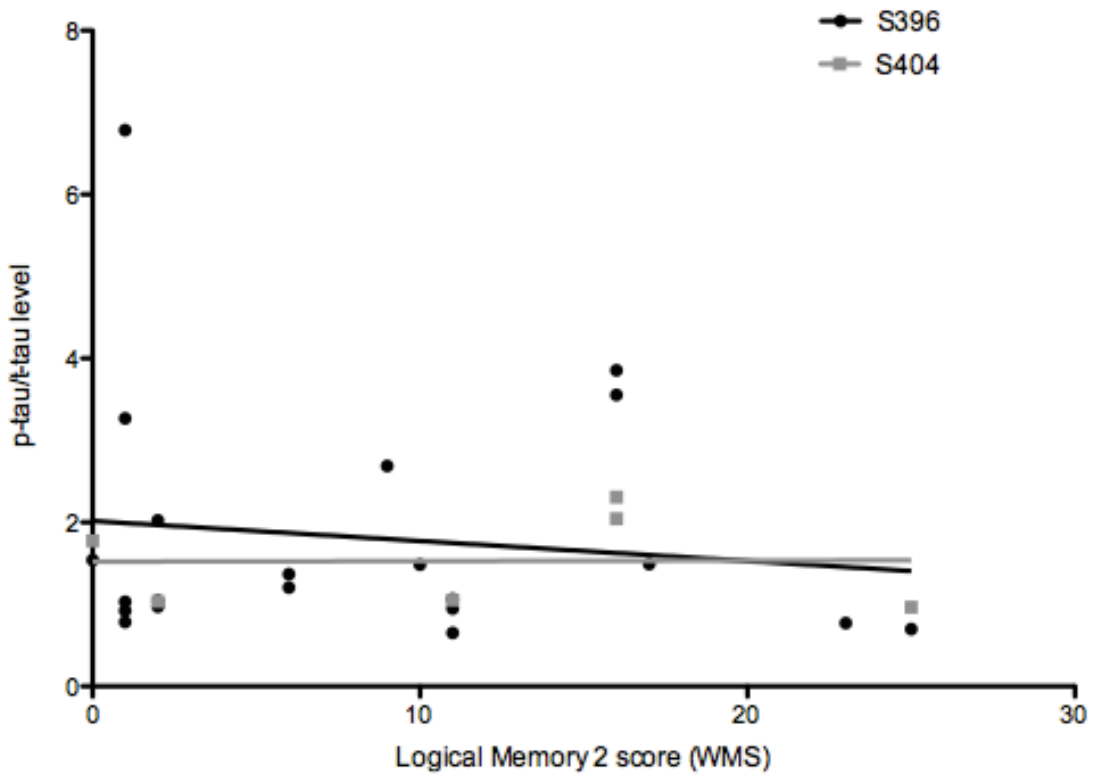

Figure 18: Correlations of pS396/tau4 (n=21) and pS404/tau4 (n=6) with logical memory 2 scores of the WMS, r_s=-0.165 and r_s=-0.058 respectively. n.s. ps>0.05.

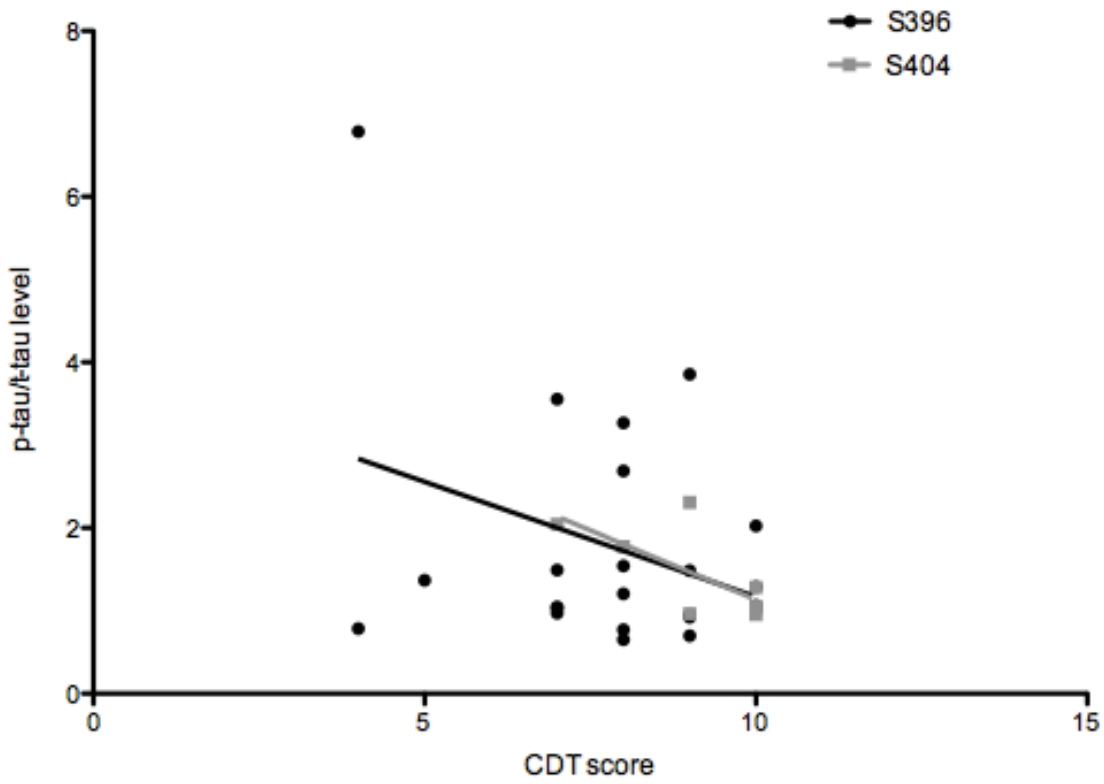

Figure 19: Correlations of salivary pS396/tau4 (n=23) and pS404/tau4 (n=8) with CDT scores, r_s=-0.099 and r_s=-0.600 respectively, n.s. ps>0.05.

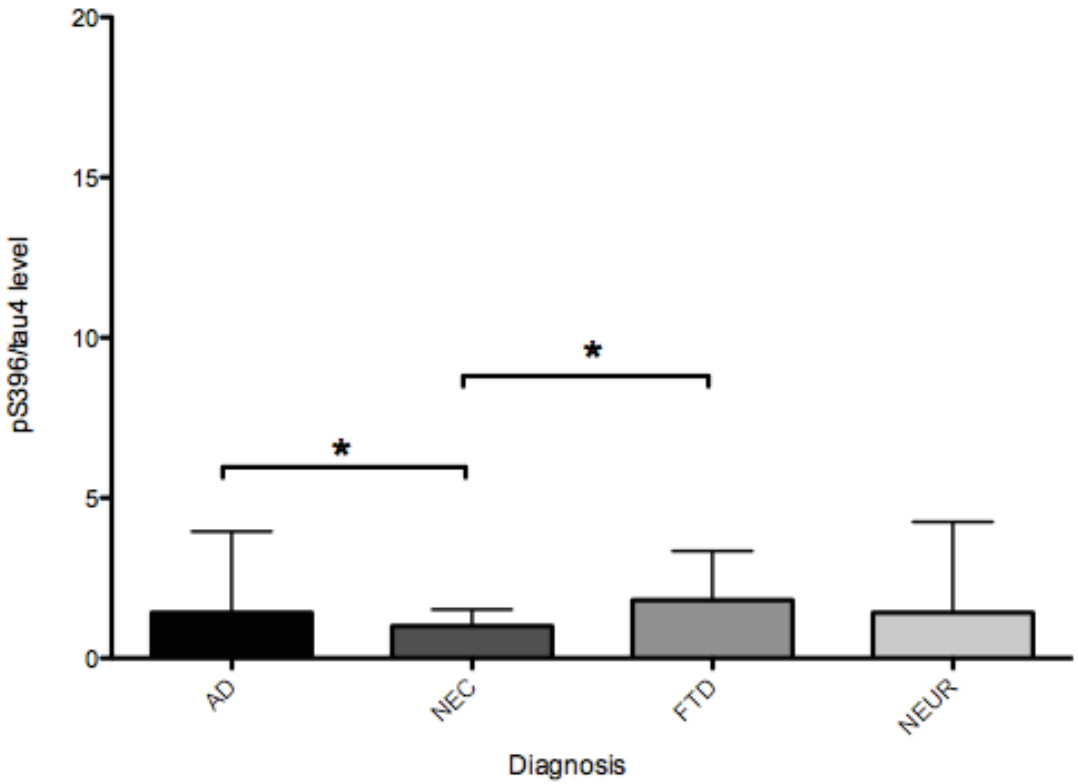

Figure 20: Median pS396/tau4 in AD (n=41), NEC (n=44), FTD (n=16) and NEUR (n=12) subjects. Error bars=IQR. *ps<0.05

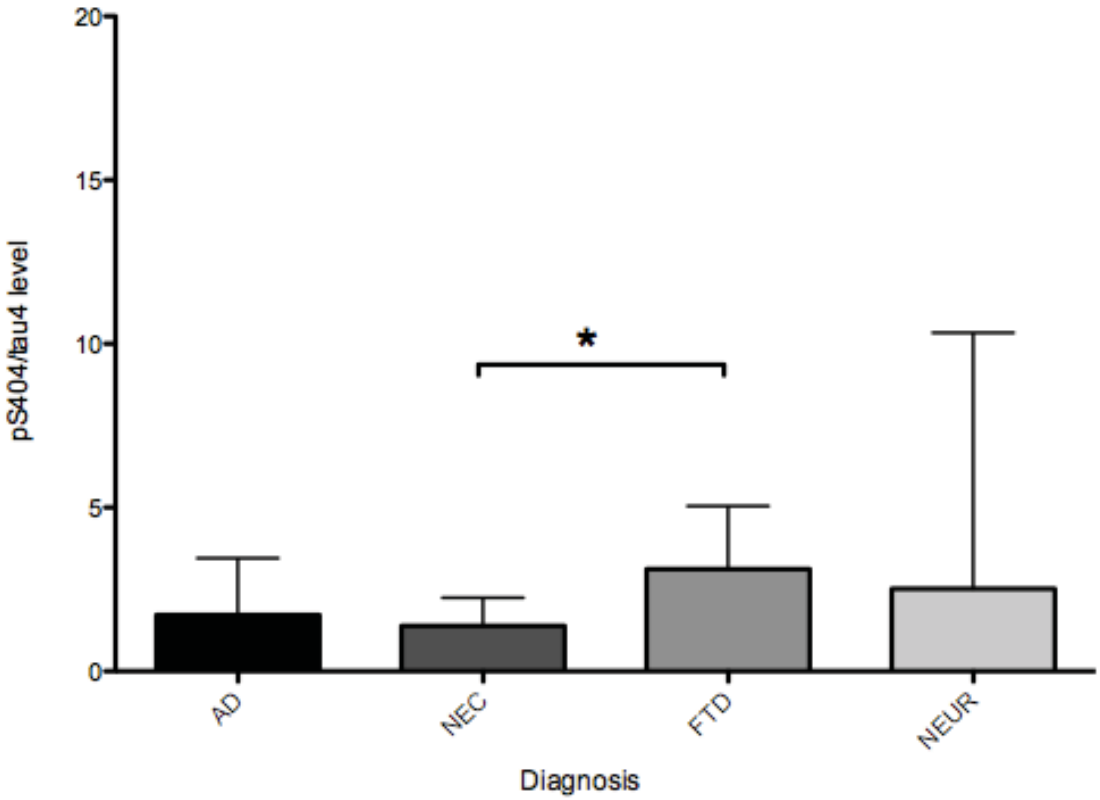

Figure 21: Median pS404/tau4 in AD (n=41), NEC (n=44), FTD (n=16) and NEUR (n=12) subjects. Error bars=IQR. *ps<0.05