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Tau phosphorylation sites serine202 and serine396 are differently altered in chronic traumatic encephalopathy and Alzheimer disease

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Abstract

INTRODUCTION: Chronic traumatic encephalopathy (CTE) is a neurodegenerative tauopathy associated with repetitive head impacts (RHI) typically sustained by contact sport athletes. Post-translation modifications to tau in CTE have not been well delineated or compared to Alzheimer disease (AD).

METHODS: We measured phosphorylated tau epitopes within dorsolateral frontal cortex from post-mortem brains with neither CTE nor AD (n=108), CTE (n=109), AD (n=223), and both CTE and AD (n=33).

RESULTS: Levels of p-tau$_{202}$, p-tau$_{231}$, and p-tau$_{396}$ were significantly increased in CTE. Total years of RHI exposure was significantly associated with increased p-tau$_{202}$ levels (p=0.001), but not p-tau$_{396}$. Instead, p-tau$_{396}$ was most closely related to Aβ_{1-42} levels (p<0.001). The p-tau$_{202}$:p-tau$_{396}$ ratio was significantly increased in early and late CTE compared to AD.

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DISCUSSION: In frontal cortex p-tau$_{202}$ is the most upregulated p-tau species in CTE, while p-tau$_{396}$ is most increased in AD. p-tau$_{202}$ and p-tau$_{396}$ measurements may aid in developing biomarkers for disease.

Keywords

Alzheimer Disease / pathology*; Amyloid beta-Peptides; Brain / pathology; Chronic Traumatic Encephalopathy / pathology*; Football / statistics & numerical data*; Humans; Male; Microtubule-Associated Proteins; tau Proteins; Tauopathies

1. BACKGROUND

CTE is a neurodegenerative disease which has been associated with repetitive head impacts (RHI) sustained in a wide variety of contact sports, such as American football, ice hockey, rugby, wrestling, and association football (soccer) [1–5]. Some of the more common clinical features observed in participants with CTE include impulsivity, aggression, depression, impaired memory, and executive dysfunction [5–7]. Currently, CTE can only be diagnosed after death. The neuropathologic criteria that defines CTE is the perivascular accumulation of abnormal hyperphosphorylated tau (p-tau) in neurons primarily and astroglia found at the depths of cortical sulci [8]. Multiple other pathologies, such as amyloid-beta (Aβ) [9], Lewy bodies, and TDP-43 may also frequently occur in CTE, but the presence of p-tau is the defining feature. The tau epitopes involved in the progression of CTE have not yet been delineated.

Alzheimer's disease (AD), in contrast to CTE, is characterized by the combined presence of Aβ deposits in the form of neuritic plaques (NP) and tau-immunoreactive neurofibrillary tangles (NFT), progressing in a stereotypical pattern first involving the medial temporal lobe [10] [11]. The tau phosphorylation sites involved in the progression of AD have been extensively studied, especially in the temporal cortex, with approximately 30 serine/threonine residues associated with physiological functions [12] [13] [14] [15] [16]. Some of the more commonly recognized phosphorylation sites include Thr181 (p-tau$_{181}$), Ser202 (p-tau$_{202}$), Thr231 (p-tau$_{231}$), and Ser396 (p-tau$_{396}$). Phosphorylation at p-tau$_{231}$ has been shown to be an early event in AD [17], although the temporal phosphorylation patterns of p-tau$_{202}$ and p-tau$_{396}$ remain controversial. Some studies have found that phosphorylation of p-tau$_{396}$ is a later event which succeeds phosphorylation of p-tau$_{202}$ [18] [19], while others suggest that phosphorylation of p-tau$_{396}$ precedes p-tau$_{202}$ [20], or occurs in parallel [21]. Distinctions between the type and pattern of tau pathology in CTE versus AD are still being established [10] [22–24].

Here we set out to determine which p-tau epitopes are most involved at different stages of CTE and compare to different stages of AD. Specifically, we tested the four aforementioned epitopes which are commonly recognized in AD: p-tau$_{181}$, p-tau$_{202}$, p-tau$_{231}$, and p-tau$_{396}$, as well as levels of total Aβ (Aβ$_{1–38}$, Aβ$_{1–40}$, and Aβ$_{1–42}$) in participants with neither CTE nor AD, CTE, AD, and both CTE and AD. Using total years of playing contact sports as a proxy for cumulative RHI exposure [3,9,25–28],we further tested the hypothesis that total years of play would predict increased phosphorylation of CTE relevant tau epitopes. In doing so, we not only have systematically delineated four p-tau epitopes at varying stages

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of both CTE and AD, but have also evaluated any potential relationships between specific phosphorylation sites and type of neurodegenerative disease.

2. METHODS

2.1. Participants

The study goal was to examine pathologic groups including CTE, AD, both, and neither. Participants were selected from three different study groups that included Understanding Neurologic Injury and Traumatic Encephalopathy (UNITE) as part of the Veteran's Affairs-Boston University-Concussion Legacy Foundation (VA-BU-CLF), the Boston University's Alzheimer Disease Research Center (BU ADRC), and Framingham Heart Study (FHS) brain banks. The original study sample included 607 autopsy participants with fresh frozen brain tissue received prior to 10/8/19. Participants were excluded if they had one or more of the following comorbidities: frontotemporal lobar degeneration (both FTLD-TDP and FTLD-tau, including PSP and CBD) (FTLD, n=74), motor neuron disease (MND, n=74), or neocortical Lewy body disease (n=43) resulting in a total of 473 participants. In order to increase the generalizability of our findings, we did not exclude age-related pathologies from our groups, such as primary age-related tauopathy (PART) or limbic-predominant age-related TDP-43 encephalopathy (LATE). Of the 473 brains, 210 were part of the UNITE study, which requires a history of exposure to RHI, including football, ice hockey, rugby, wrestling, and boxing [29]. An additional 179 participants were available from the FHS, a longitudinal and community-based study [30]. The remaining 84 brains were part of the BU ADRC, which recruits control and Alzheimer disease participants during life, as previously described [31] [32]. Total years of all contact sports participation as well as duration of American football was determined from next of kin after death in the UNITE and FHS groups [27]. History of contact sports was unknown in the BU ADRC brain donors. All consents for research participation and brain donation were provided by next of kin. Institutional Review Boards of the Bedford VA Healthcare System and Boston University Medical Center approved all study protocols.

2.2. Pathological Assessment

All brains included in this study were assessed for neuropathological changes of CTE, AD, and other neurodegenerative disorders by using previously published procedures and selection criteria [29,33]. Specifically, brains were hemisected and pathological assessment was performed on the fixed hemibrain; the opposing hemibrain was frozen for biochemical protein quantification. CTE was diagnosed using previously established consensus criteria from the National Institute of Neurological Disorders and Stroke consensus [8]. CTE staging was performed as previously described [3,34]. AD was diagnosed using National Institute of Aging Reagan criteria, including low, intermediate, or high probability of dementia caused by AD [35]. NIA-Reagan criteria was used instead of the more recent NIA-AA AD criteria since the majority of cases were previously evaluated with NIA-Reagan and recent studies suggest the Consortium to Establish a Registry for Alzheimer Disease (CERAD) neuritic plaque score and Braak neurofibrillary tangle stage utilized by NIA-Reagan are the best predictors of cognitive impairment in AD [36].

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Using CTE staging and the NIA-Reagan criteria, participants were separated into six different pathologic groups: No CTE/No AD (controls), Low CTE (stages I and II), High CTE (stages III and IV), Low AD, AD (intermediate or high), and both CTE & AD. Participants with no evidence of CTE, no evidence of intermediate or high AD, and CERAD scores of zero were labeled as the "No CTE/No AD" group and used as our control group. Other pathologies such as vascular disease, non-neocortical LBD, and limbic TDP-43 pathology were not selected for. Participants having a CTE Stage of I or II and no evidence of intermediate or high AD were determined to represent early stage disease, and thus were termed "Low CTE." Conversely, those with a CTE Stage of III or IV and no evidence of AD were determined to have late stage disease, and were labeled as "High CTE." Low and high stage CTE also correlated with recently suggested consensus CTE staging criteria [34]. Participants who had no evidence of CTE and no evidence of AD, but had a CERAD score of one or greater and a Braak Score greater than zero were placed in the "Low AD" group. Those with a CTE Stage of zero and evidence of AD were classified as "AD." Lastly, participants having both CTE and threshold criteria for intermediate or high AD were termed "CTE & AD."

2.3. Measurements of pathological proteins

Frozen tissue from the gyral crest of the dorsolateral prefrontal cortex was weighed and placed on dry ice. Brain tissue was homogenized in 5:1 volume of freshly prepared, ice cold 5M Guanidine Hydrochloride in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.4), which contained 1:100 Halt protease inhibitor cocktail (Thermo Fischer Scientific, Waltham, MA) and 1:100 Phosphatase inhibitor cocktail 2 & 3 (Sigma-Aldrich, St. Louis, MO) as previously reported [37]. The homogenate was then shaked (regular rocker) overnight at room temperature. The lysate was diluted with 1% Blocker A (Meso Scale Discovery (MSD), Rockville, Maryland, #R93BA-4) in wash buffer according to specific immunoassays. 1:4000 for $A\beta_{1-38}$, $A\beta_{1-40}$, and $A\beta_{1-42}$ (MSD #K15200E-2), and 1:300 for p-tau_{181}, p-tau_{202} (MSD custom kit), total tau and p-tau_{231} (MSD #K15121D-2). Samples were centrifuged at 17,000g and 4°C for 15 minutes. The supernatant was subsequently applied to the immunoassays, and the original homogenate was aliquoted and stored at −80 °C.

To capture tau phosphorylated at Thr residue 181, antibody AT270 was used. The detecting antibody was the biotinylated HT7 that recognizes residue 159–163 of tau (Thermo Scientific, Rockford, IL). To measure p-tau_{396}, a rabbit monoclonal antibody against p-tau_{396} (Abcam, ab156623) was used as the capturing antibody, and HT7 was used as a detecting antibody. Sulfo-tag conjugated streptavidin secondary antibody was used for signal detection by the MSD platform. MSD SECTOR Imager 2400 was used to measure p-tau_{396} levels. Internal calibrators of p-tau and tau were used (MSD). p-tau levels were measured in arbitrary units, which may or may not be related among the different epitopes. Standards with known concentrations were used for Aβ, and all standards and samples were run in duplicate. Measurements were made using the multi-detection SPECTOR 2400 Imager (MSD).

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2.4. Statistical analysis

Statistical analysis was performed using SPSS 26.0 (IBM Corp, Armonk, NY) and Prism v7 and v8 (Graph-Pad Software, La Jolla, CA). The ROUT method [38] was used to detect outliers by directly controlling for false discoveries, with a maximum False Discovery Rate set to Q=1%. Outliers were removed from each amyloid-beta or p-tau group studied, across pathologic disease groups, but were retained for linear regressions which utilized rank-based normalization.

Relative amounts of $A\beta_{1–38}$, $A\beta_{1–40}$, $A\beta_{1–42}$, p-tau_{181}, p-tau_{202}, p-tau_{231}, p-tau_{396} were computed by dividing each data point by the mean of the control group. An analysis of covariance (ANCOVA) was used to adjust for age at death and compare differences of each analyte between CTE pathology groups and, separately, AD pathology groups. The p-tau_{202}:p-tau_{396} ratio was compared between CTE and AD pathology groups. Statistical significance was determined at $p<0.05$. Tau epitope and Aβ levels, as well as total years of contact sports play underwent rank-based normalization for regression analyses [39]. Multiple linear regression analyses were used to evaluate associations between p-tau and predictors total years of playing contact sports and $A\beta_{1–42}$ levels, adjusting for age at death and sex. Two separate regression models were run, in order to separately examine CTE (excluding AD) and AD (excluding CTE).

3. RESULTS

3.1. Participant groups

The participants were separated into six different pathologic groups based on both the presence or absence and level of severity of CTE and AD pathology. The groups differed significantly in age at death, sex, and RHI, as seen in Table 1. In order from youngest to oldest, the Low CTE, control, High CTE, and CTE & AD groups all were younger in comparison to the AD and Low AD groups. Low CTE, High CTE, and CTE & AD groups had a much higher composition of men than the other pathologic groups, as well as a greater likelihood of playing contact sports for a longer period of time.

3.2. Beta-amyloid levels are differentially altered in CTE and AD

3.2.1. Changes in CTE (Figure 1A, 1B, 1C, 1D)—Both Low CTE and High CTE groups had significantly lower levels of $A\beta_{1–38}$ compared to the control group (p=0.019 and p=0.001, respectively). $A\beta_{1–40}$ fold change to control was significantly increased in High CTE (1.5-fold change from control, SEM=0.14, p=0.009), while no differences were observed between the control and Low CTE groups. Similarly, although there was no significant fold change of $A\beta_{1–42}$ between control and Low CTE groups, $A\beta_{1–42}$ was greatly increased in High CTE, statistically significant from Low CTE and control groups (28-fold change from control, SEM=2.9, $p<0.001$). It is evident that while $A\beta_{1–38}$ was a low abundant species in CTE, $A\beta_{1–40}$, and $A\beta_{1–42}$ in particular, were the main Aβ species in High CTE. To further examine the relationship between the two, the $A\beta_{1–42}$:$A\beta_{1–40}$ ratio was computed, and a significant increase was observed in High CTE compared to the Low CTE and control groups (8.5-fold change from control, SEM=0.86, $p<0.0001$). Lastly, a secondary analysis was performed to compare beta-amyloid levels between our High

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CTE and CTE & AD participants (data not shown). As expected, CTE & AD participants exhibited significantly higher $A\beta_{1-38}$, $A\beta_{1-40}$, and $A\beta_{1-42}$ levels ($p<0.001$).

3.2.2. Changes in AD (Figure 2A, 2B, 2C, 2D)—It is evident that Low AD, AD, and CTE & AD groups all had much higher levels of all forms of amyloid-beta ($A\beta_{1-38}$, $A\beta_{1-40}$, and $A\beta_{1-42}$) compared to the control and CTE groups. As expected, levels of each Aβ species increased from Low AD to AD ($A\beta_{1-38}$ $p<0.001$, $A\beta_{1-40}$ $p<0.001$, $A\beta_{1-42}$ $p=0.006$). The Low AD group also displayed significantly increased levels of $A\beta_{1-38}$ ($p<0.001$) and $A\beta_{1-42}$ ($p<0.0001$) compared to controls. However, in AD, the largest fold increase from control was observed in $A\beta_{1-42}$ (93-fold change from control, SEM=4.3, $p<0.0001$). In Low AD, the ratio of $A\beta_{1-42}$:$A\beta_{1-40}$ displayed a 25-fold increase in $A\beta_{1-42}$ levels with respect to $A\beta_{1-40}$ levels, highly significant from both the AD ($p<0.001$) and CTE & AD groups ($p<0.001$). Additionally, although significantly less than the increases observed in Low AD and CTE & AD groups, $A\beta_{1-42}$ was increased 5.8-fold relative to $A\beta_{1-40}$ in AD (SEM=1.3).

3.3. Tau epitopes are differentially altered in CTE and AD

3.3.1. Changes in CTE (Figure 3A, 3B, 3C, 3D)—The High CTE group demonstrated a significant decrease in p-tau_{181} in comparison to the control (0.70-fold change from control, SEM=0.07, $p<0.001$). Participants in the High CTE group displayed a large, highly significant increase in p-tau_{202} levels compared to the Low CTE and control groups (3.2-fold change from control, SEM=0.29, $p<0.001$). This increase of p-tau_{202} was the largest fold increase from control observed among all epitopes studied in CTE. For p-tau_{231}, the High CTE group demonstrated a significant increase compared to the control (1.6-fold change from control, SEM=0.09, $p<0.001$). Lastly, p-tau_{396} displayed a fold increase in High CTE, which was statistically significant from both the Low CTE and control groups (2.0-fold change from control, SEM=0.15, $p<0.001$).

3.3.2. Changes in AD (Figure 4A, 4B, 4C, 4D)—p-tau_{181}, p-tau_{202}, p-tau_{231}, and p-tau_{396} all were significantly higher in AD when compared to either the control or Low AD group. The greatest increase among the epitopes was shown in p-tau_{396}, where an 11-fold increase to control was observed in AD (SEM=2.0, $p<0.001$). Of the four epitopes, p-tau_{202} levels were increased the most in the CTE & AD group (33-fold change to control, SEM=3.2, $p<0.0001$). p-tau_{231} and p-tau_{396} levels were also significantly increased in the CTE & AD group compared with the control, Low AD, and AD groups. Total tau levels did not significantly change within either CTE or AD (data not shown).

3.4. Multiple regression analysis shows total years of play is a significant predictor of p-tau_{202} levels in CTE (Table 2a)

In order to test the hypothesis that total years of playing contact sports is associated with any of the four p-tau epitopes in CTE, we performed multiple linear regression analyses (Table 2). Controls and both Low and High CTE participants were grouped together and associations were tested between each p-tau epitope and total years of contact sports play and $A\beta_{1-42}$ levels, adjusting for sex and age at death. Total years of playing contact sports was a highly significant predictor of p-tau_{202} ($\beta=0.293$, $p=0.001$). There was no significant association of total years of contact sports play with p-tau_{181}, p-tau_{231}, or

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p-tau$_{396}$. Additionally, Aβ$_{1–42}$ was a significant predictor of p-tau$_{231}$ (β=0.213, *p*=0.012) and p-tau$_{396}$ (β=0.195, *p*=0.034). Secondary linear regression models using Aβ$_{1–40}$ as a covariate instead of Aβ$_{1–42}$ showed similar associations between p-tau$_{202}$ and total years of play (β=0.300, *p*=0.001). Aβ$_{1–40}$ was also a significant predictor of both p-tau$_{231}$ (β=0.292, *p*<0.001) and p-tau$_{396}$ levels (β=0.345, *p*<0.001). A sensitivity analysis using total years of playing football rather than total years of playing contact sports showed that years of football participation was even more significantly associated with p-tau$_{202}$ (β=0.383, *p*<0.001) as well as p-tau$_{231}$ (β=0.273, *p*=0.001).

3.5. Multiple regression analysis shows Aβ$_{1–42}$ is most closely related to p-tau$_{396}$ levels in AD (Table 2B)

Multiple linear regressions were performed in order to evaluate the contributions of Aβ$_{1–42}$ on the four p-tau epitopes in AD (Table 2). Participants in control, Low AD, and AD were grouped together. Data for total years of playing contact sports was not available for most participants of the AD cohort, and thus only Aβ$_{1–42}$ was used as a variable, again adjusting for age at death and sex. Increasing Aβ$_{1–42}$ levels in AD were significantly associated with p-tau$_{202}$, p-tau$_{231}$, and p-tau$_{396}$, however the strongest relationship of the four epitopes was observed at p-tau$_{396}$ (β=0.312, p<0.001). Subsequent linear regression models using Aβ$_{1–40}$ instead of Aβ$_{1–42}$ showed similar significant associations with p-tau$_{202}$, p-tau$_{231}$, and p-tau$_{396}$, and a significant association with p-tau$_{181}$ also (β=0.189, p=0.007). Again, the strongest relationship of the four epitopes with Aβ$_{1–40}$ was observed at the p-tau$_{396}$ epitope (β=0.530, p<0.001).

3.6. Ratio of p-tau$_{202}$ to p-tau$_{396}$ is significantly increased in CTE compared to AD at both early and late stages of disease (Figure 5)

Because p-tau$_{202}$ demonstrated the greatest fold-increase in CTE (Fig 3) and was the only epitope significantly associated with RHI (Table 2a) and p-tau$_{396}$ showed the greatest fold-increase in AD (Fig 4) and the strongest association with Aβ (Table 2b), we computed the ratio of p-tau$_{202}$ to p-tau$_{396}$ at both low and high stages of disease. ANCOVA adjusted for age demonstrated that the ratio of p-tau$_{202}$ to p-tau$_{396}$ was significantly increased in Low CTE compared to Low AD (p=0.018). High CTE p-tau$_{202}$:p-tau$_{396}$ ratio was also significantly higher than both early and late stages of AD (compared to Low AD: p<0.001; compared to AD: p=0.030). There were no significant differences between p-tau$_{202}$:p-tau$_{396}$ ratios of Low CTE and High CTE, or Low AD and AD. Lastly, the CTE & AD group had the highest levels of p-tau$_{202}$ relative to p-tau$_{396,}$ a statistically significant increase in comparison to every other group.

In this study we exclusively used guanidine hydrochloride for protein extraction. A comparison study between guanidine hydrochloride and 2% SDS extraction solutions showed equivalent amounts of p-tau$_{202}$, but less p-tau$_{396}$ with 2% SDS. The two extraction techniques showed similar trends between controls, CTE, and AD participants. In addition, we compared the collapsed McKee CTE stages used here with the newly recommended consensus CTE staging system that determines low and high CTE stage based on a summation of regions involved [40] and found excellent agreement (89%) between the two staging systems in 71 cases that had complete data for the new staging determination.

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In addition, analysis using the consensus stages in these participants found the same p-tau levels regardless of the classification system.

4. DISCUSSION

This study examined various tau phosphorylation sites in the dorsolateral frontal cortex of participants with early and late stages of CTE and AD, in order to determine disease specific differences and similarities. Of the four epitopes studied, p-tau$_{202}$ showed the greatest increase compared to control in high-stage CTE, suggesting this site is most involved in CTE. In comparison, in AD p-tau$_{396}$ was increased the most, while participants with both CTE and AD had the greatest increase in p-tau$_{202}$. Multiple linear regression analyses demonstrated that total years of contact sports play, a proxy for RHI, was a significant predictor of p-tau$_{202}$ levels in the frontal cortex. In contrast, A$\beta_{1–42}$ was most strongly associated with the p-tau$_{396}$ epitope in both AD and CTE. The ratio of p-tau$_{202}$ to p-tau$_{396}$ was significantly higher at different stages of CTE and in CTE & AD compared to AD. Overall, in the dorsolateral frontal cortex, serine202 is the most involved tau phosphorylation site in CTE while serine396 is associated with Aβ and AD.

We combined years of play in multiple sports to look at the effect of total years of contact sport participation. Multiple linear regression analysis showed that years of play was a significant predictor of p-tau$_{202.}$ However, duration of play is likely not equivalent across sports types. The majority of the study participants played American football, and after limiting the sample to include only total years of football play, our results were strengthened. In addition, the association between years of play and ptau$_{231}$ went from being almost significant to highly significant when only football years were included in the model, suggesting that ptau$_{231}$ is also affected by RHI. Overall, in our groups football years is a main driver of the findings relating total years of contact sport participation and p-tau.

Much of what is known about tau phosphorylation has been derived from the study of AD [41] [42] [43] [19]. In line with our own findings, p-tau$_{202}$ has previously been shown to be increased in AD compared to Low AD [44]. Additionally, hyperphosphorylation at p-tau$_{202}$ and p-tau$_{231}$ has been identified as a critical step in prompting microtubule instability [45] [46], a key component in the biological mechanism of degeneration that is likely common to AD and CTE. Here we show that in the frontal cortex in AD, p-tau$_{396}$ is increased the most. Prior studies support the involvement of p-tau$_{396}$ in AD and suggest that phosphorylation at this site is a vital step in disrupting normal microtubule assembly and developing neurofibrillary pathology [47] [48]. On the other hand, Neddens and colleagues found that p-tau$_{231}$ showed the most evidence of progression with AD pathology using immunofluorescent techniques [44]. We also found p-tau$_{231}$ to have significantly increased from Low AD to AD and to be significantly associated with Aβ in AD.

The variability of tau phosphorylation across different brain regions may in part explain why levels of p-tau$_{181}$ in the frontal cortex showed no significant increase in CTE compared to controls, and only a slight increase from Low AD to AD. CSF p-tau$_{181}$ is an established biomarker for AD, and blood p-tau$_{181}$ is emerging as biomarker and predictor of Aβ and tau pathology [49] [50] [51]. This likely reflects tau phosphorylation changes in the temporal

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lobe, which is preferentially affected early in AD and later in CTE. Future studies should also focus on quantifying levels of p-tau$_{202}$ and p-tau$_{396}$ in CSF and blood. In a preliminary study, plasma exosomal tau levels were observed to be increased in a group of professional football athletes in comparison to controls [52]. If p-tau levels in these compartments reflect the changes observed here in the dorsolateral prefrontal cortex, then p-tau$_{202}$ could potentially serve as a clinical biomarker for high stage CTE and p-tau$_{396}$ as a biomarker for AD. The p-tau$_{202}$:p-tau$_{396}$ ratio may also be relevant for distinguishing between early stages of both diseases.

Few studies have investigated the role of p-tau$_{202}$ or other phosphorylation sites in the low and high stages of CTE. A previous study using antibody CP13 to detect p-tau$_{202}$ and PHF1 for p-tau$_{396}$ described similar staining between CTE and AD [23]. However, this finding is constrained by both the small sample size and the design of the study. Immunohistochemistry techniques can only detect the presence, and not the relative levels of an epitope. Additionally, measurements were taken from a variety of brain regions depending on each individual case. Drawing from a much larger sample size and using biochemical measurements, our study was able to discriminate quantitative levels of tau epitopes that differed in CTE and AD. Furthermore, we demonstrated an association between years of contact sports play and levels of p-tau$_{202}$.

Examining what is known about specific kinases and phosphatases that act upon these specific epitopes may provide additional insight into mechanisms of tau phosphorylation in CTE and AD. One study examining tau phosphorylation by glycogen synthase kinase 3β (GSK3β) demonstrated that phosphorylation at p-tau$_{202}$ was a direct process, while p-tau$_{396}$ was a two-step mechanism and required prephosphorylation at two other sites by a priming kinase and subsequently GSK3β [53]. This difference in the mechanism of phosphorylation by GSK3β at p-tau$_{202}$ versus p-tau$_{396}$ may in part be due to the differing pathological mechanisms of CTE and AD. In cases of CTE and traumatic brain injury (TBI) in particular, the process of tau phosphorylation and protein accumulation may be much more rapid than in AD [54]. The two-step phosphorylation at p-tau$_{396}$ might correspond with a slower progression of AD pathology. Indeed, phosphorylation at p-tau$_{396}$ by GSK3β has been identified as an important step in the progression of AD [55] [56] [57]. On the other hand, GSK3β may play a role in tau phosphorylation at Thr175 in CTE [58] and inhibition of GSK3 may have therapeutic effects in reducing TBI- related symptoms [59].

This study has several limitations. Although tau is phosphorylated at over 30 different sites [12] [13] we limited our evaluation to the four epitopes commonly implicated in AD and tauopathies and with available and reliable quantitative immunoassays. It is likely that there are other p-tau epitopes similarly implicated at different stages of CTE and AD that were simply not included in our study, such as p-tau217 [60]. Future studies should consider evaluating a wider variety of p-tau epitopes to more thoroughly delineate the different phosphorylation sites implicated in CTE and AD. In addition, tau phosphorylation may be altered by tau isoforms, which have been shown to change in high stage CTE [24].

CTE can be a patchy disease that is sometimes limited to the sulcal depths. However, due to the difficulty of getting cortex from the sulcus in frozen coronal slabs, we performed

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our measurements within the gyral crest. Given that the majority of CTE tau pathology is found at the sulcal depths [61], measuring tau from the gyri likely under-estimated the levels of p-tau in CTE in comparison to the p-tau levels in AD, especially in low stage CTE. Additionally, because pathological assessment and biochemical measurements were performed on opposite hemispheres, it is possible that the frozen section biochemistry did not always capture a tau CTE lesion. These factors may explain why we observed no differences in p-tau between controls and Low CTE. Looking at verified lesions and changes at the sulcal depths may be more helpful in identifying Low CTE changes in future studies. Although the dorsolateral prefrontal cortex is affected in early CTE, it is also affected in early stages of amyloid deposition. Future studies should aim to examine additional brain regions to clarify regionality of disease processes. It is also worth noting that a significant number of participants in the High CTE group displayed neuritic plaques, which may have obscured CTE-related changes. Lastly, although this is the largest study to date, variables such as age, sex, and presence of comorbid pathologies necessarily differed between groups. Although we statistically adjusted for some of these factors in our regression analysis, future studies with larger numbers will be necessary to confirm these findings.

In this study we investigated four of the more commonly recognized phosphorylation sites and their roles in the progressive tauopathies of CTE and AD. We found that in the dorsolateral frontal cortex, p-tau$_{202}$ was consistently the most upregulated site in CTE, and was significantly predicted by total years of playing contact sports, a proxy for RHI. In AD, p-tau$_{396}$ was the most increased and strongly associated with A$\beta_{1–42}$. The ratio of p-tau$_{202}$ to p-tau$_{396}$ was significantly increased in CTE compared to AD across all stages of disease. These findings may aid in the neuropathological diagnosis and in the development of biomarkers to discriminate between CTE and AD.

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Declarations of interest:

Outside of the submitted work, WX received research support from the National Institutes of Health, Veterans Health Administration, Biomedical Laboratory Research and Development, and Veterans Health Administration, Clinical Sciences Research and Development Merit Awards. He reports being principal investigator and co-investigator on clinical trials, and has a patent pending regarding the diagnosis of AD using machine learning. JDC received grant support from the Department of Veterans Affairs Career Development Award and National Institute of Aging Boston University AD Center. YT reports receiving grant support to the Boston University School of Public Health. MLA has received a grant from the National Institute of Neurological Disorders and Stroke (NINDS), royalties from Oxford University, consulting fees from Corino Therapeutics, Inc., and honoraria from the International Neuropsychological Society, American Academy of Neurology, and Neuroscience Grand Rounds at Allegheny General Hospital. JM received support for the submitted work from the NIH and DOD

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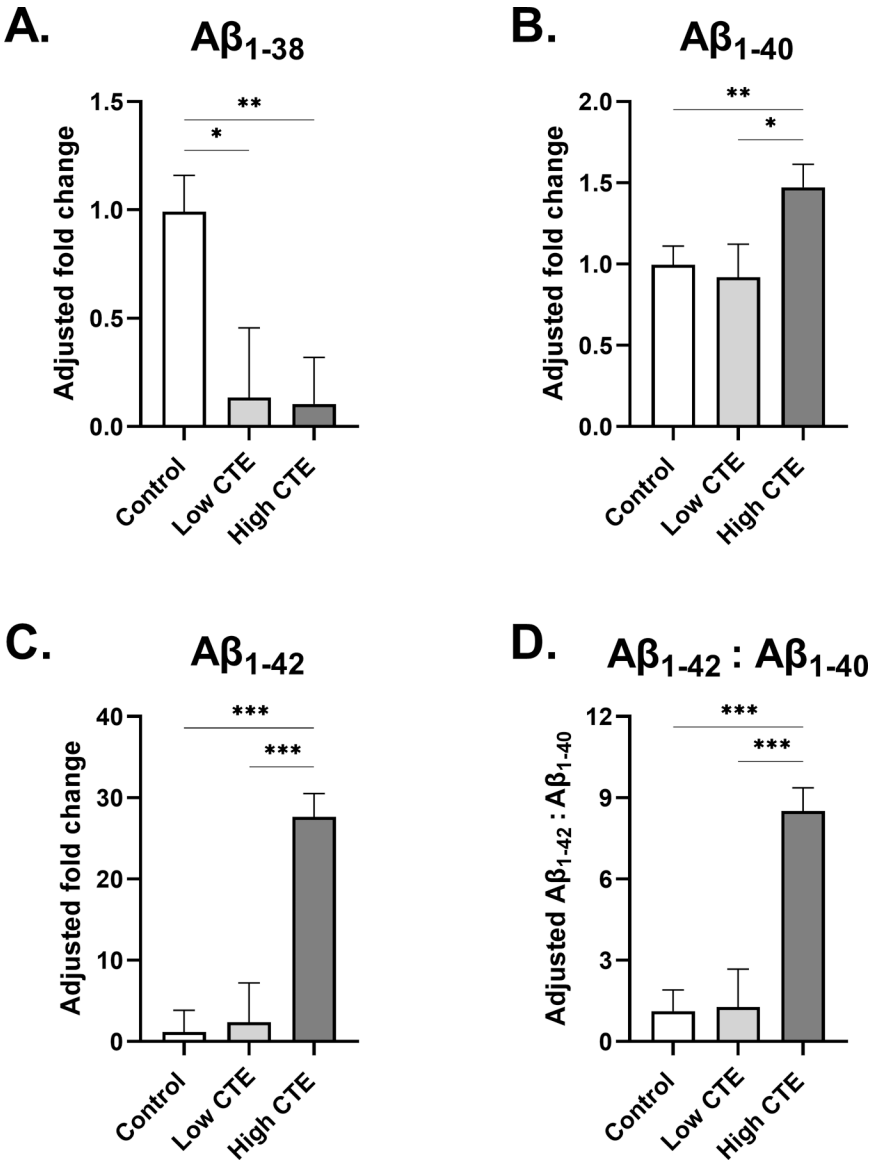

Fig. 1. Beta-amyloid levels in frontal cortex of low and high stage CTE

(A-C) Estimated marginal mean and SEM of fold change from control is displayed. **(A)** Participants with low and high stage CTE displayed significantly decreased levels of $A\beta_{1–38}$ in comparison to control. **(B)** $A\beta_{1–40}$ levels in participants with High CTE were slightly increased in comparison to both control and Low CTE groups. **(C)** Like $A\beta_{1–40}$, no significant difference in $A\beta_{1–42}$ levels was observed between participants of control and Low CTE groups. Participants with High CTE displayed a highly significant, 28-fold increase in $A\beta_{1–42}$ levels in comparison to the control. **(D)** Estimated marginal mean and SEM of $A\beta_{1–42}$:$A\beta_{1–40}$ ratio is displayed. The High CTE group had significantly increased $A\beta_{1–42}$:$A\beta_{1–40}$ levels compared to both control and Low CTE groups. For **(A-D)** *p<0.05, **p<0.01, ***p<0.001, ANCOVA adjusting for age at death.

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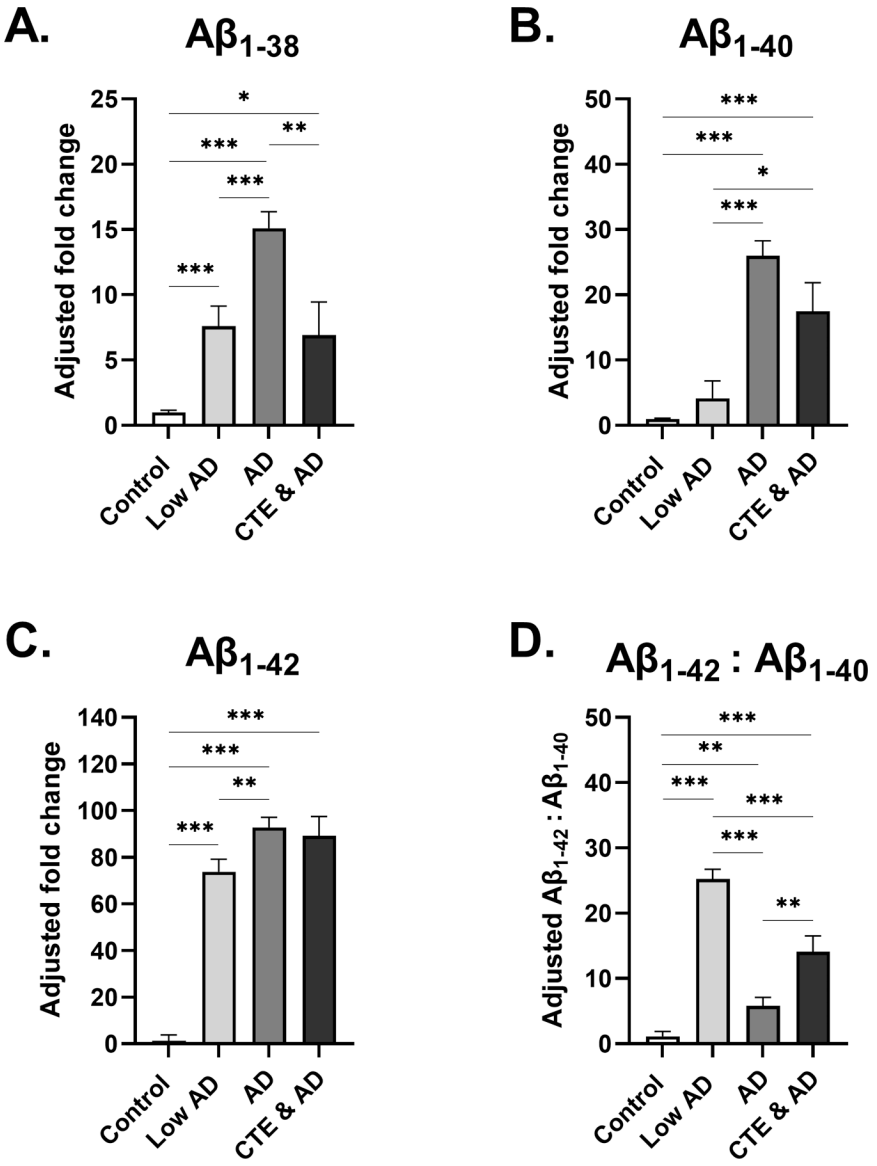

Fig. 2. Beta-amyloid levels in frontal cortex of low and high stage AD

(A-C) Estimated marginal mean and SEM of fold change from control is displayed. **(A)** Participants with AD displayed significantly increased $A\beta_{1–38}$ in comparison to control, Low AD, and CTE & AD groups. **(B)** In participants with AD, $A\beta_{1–40}$ fold change to control was significantly increased from the control and Low AD group. **(C)** $A\beta_{1–42}$ fold change to control was increased even further in participants with Low AD, AD, and CTE & AD, with $A\beta_{1–42}$ displaying a 93-fold increase with respect to control. **(D)** Estimated marginal mean and SEM of $A\beta_{1–42}$:$A\beta_{1–40}$ ratio is displayed. $A\beta_{1–42}$ was increased relative to $A\beta_{1–40}$ in all three groups. However, the greatest increase in $A\beta_{1–42}$:$A\beta_{1–40}$ levels was observed in the Low AD group, which was highly significant from controls, AD, and CTE & AD groups. For **(A-D)** *p<0.05, **p<0.01, ***p<0.001, ANCOVA adjusting for age at death.

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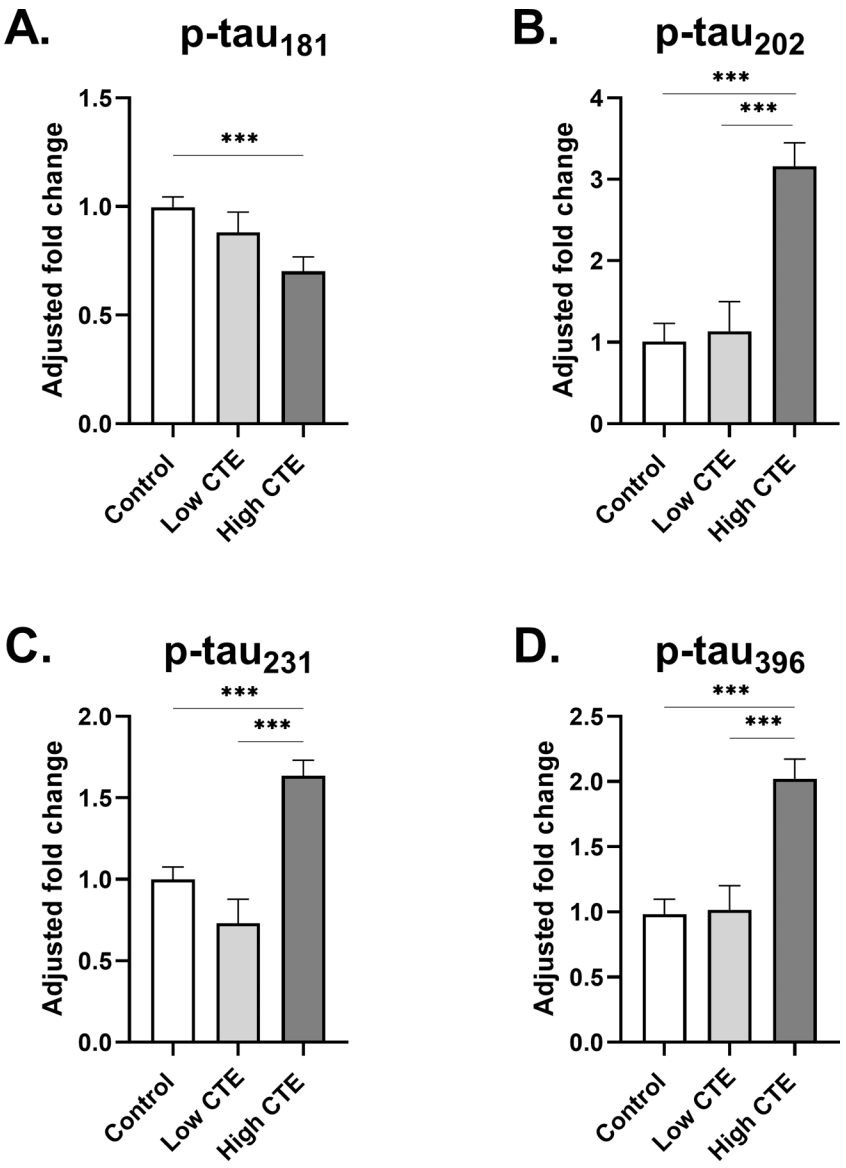

Fig 3: Phosphorylated tau levels in frontal cortex of low and high stage CTE.

(A-D) Estimated marginal mean and SEM of fold change from control is displayed. **(A)** A significant fold decrease of p-tau$_{181}$ with respect to control was observed in participants with High CTE. **(B)** A large 3.2-fold increase of p-tau$_{202}$ levels was observed in High CTE, highly significant in comparison to both Low CTE and control groups. **(C)** In comparison to the control, p-tau$_{231}$ levels were slightly but significantly increased in participants with High CTE. **(D)** There was a significant fold increase in p-tau$_{396}$ compared to both the control group and the Low CTE group. For **(A-D)** *p<0.05, **p<0.01, ***p<0.001, ANCOVA adjusting for age at death.

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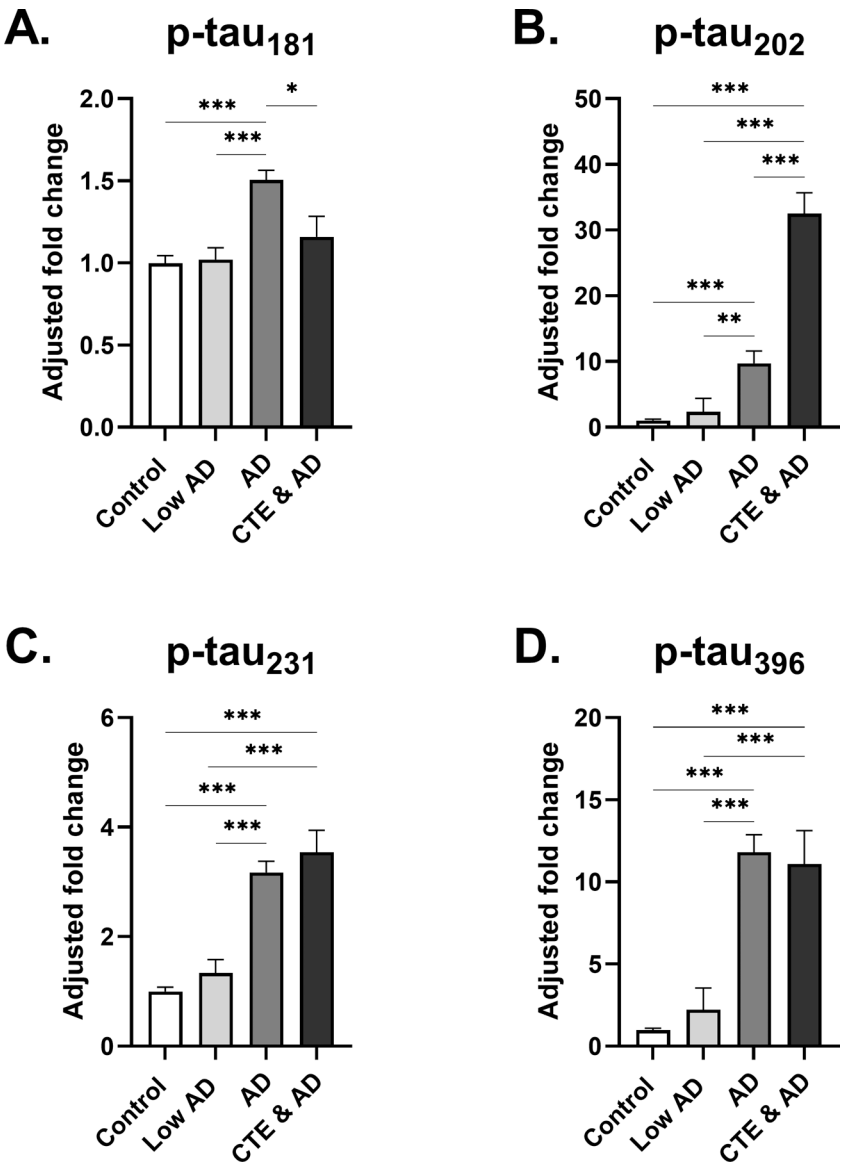

Fig 4: Phosphorylated tau levels in frontal cortex of low and high stage AD.

(A-D) Estimated marginal mean and SEM fold change from control is displayed. **(A)** A small increase in p-tau$_{181}$ fold change to control was observed in the AD group, highly significant compared to both the control and Low AD group. **(B)** Participants with AD showed increased fold change to control of p-tau$_{202}$, with less significance distinguishing from Low AD. Additionally, participants in CTE & AD group showed a highly significant, 33-fold increase in p-tau$_{202}$ fold change to control. **(C)** Levels of p-tau$_{231}$ were significantly increased in AD and CTE & AD groups in comparison to the Low AD and control groups. **(D)** Similar to trends observed in p-tau$_{202}$ and p-tau$_{231}$, participants displayed significant increases in p-tau$_{396}$ levels from Low AD to AD, and from Low AD to CTE & AD. Additionally, a large 11-fold increase of p-tau$_{396}$ was observed in the AD group. For **(A-D)** *p<0.05, **p<0.01, ***p<0.001, ANCOVA adjusting for age at death.

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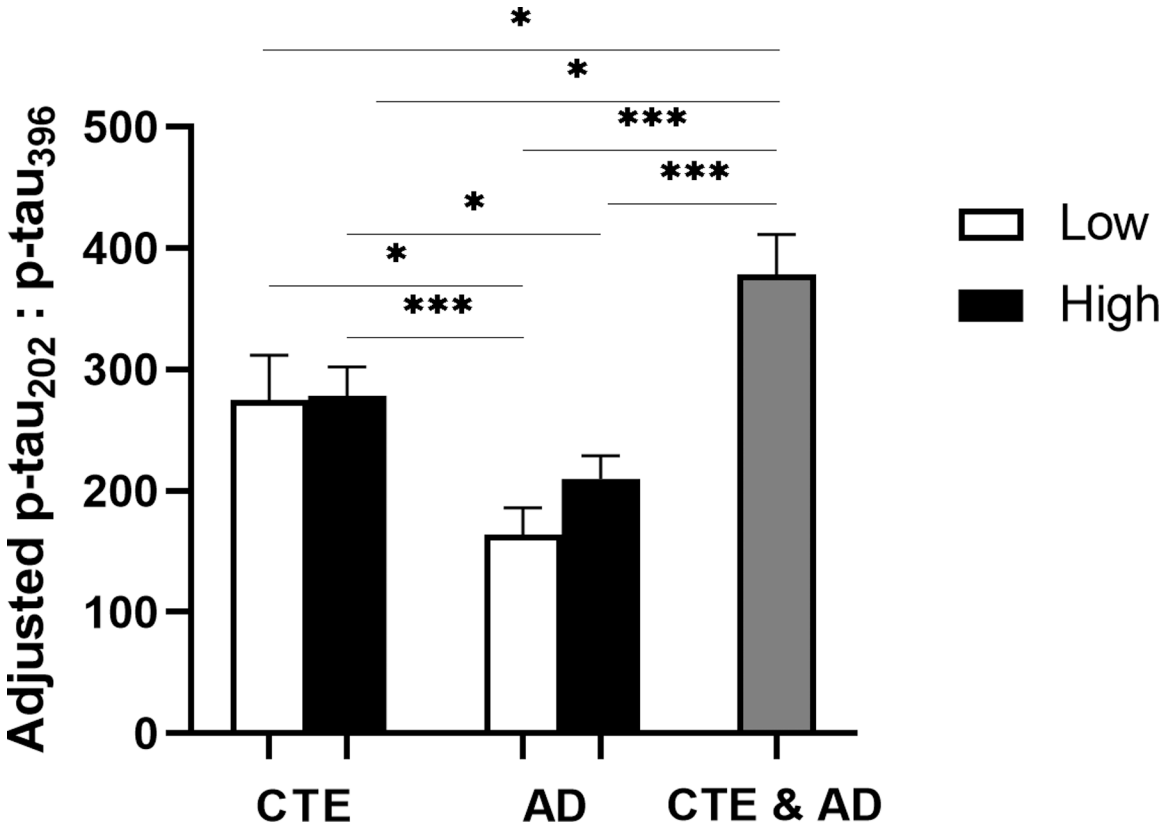

Fig 5: Ratio of p-tau$_{202}$ to p-tau$_{396}$ is increased in low and high stage CTE compared to AD.

Estimated marginal mean and SEM of ratio of p-tau$_{202}$ to p-tau$_{396}$ is displayed. The ratio of p-tau$_{202}$:p-tau$_{396}$ was significantly higher in Low CTE as compared to Low AD. p-tau$_{202}$:p-tau$_{396}$ was also significantly increased in high stage CTE with respect to both the Low AD and AD groups. No significant differences in p-tau$_{202}$:p-tau$_{396}$ were observed between stages of the same disease. CTE & AD group demonstrated the greatest increase of p-tau$_{202}$:p-tau$_{396}$ ratio, significant from low and High CTE and Low AD and AD groups. *p<0.05, **p<0.01, ***p<0.001, ANCOVA adjusting for age at death.

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Table 1

Demographic, clinical, and pathological measures between pathology groups

	no CTE/no AD (control)	low CTE	high CTE	low AD	AD	CTE and AD	*p* value
Sample size (*n*)	108	37	72	93	130	33	
Cohort							
UNITE	42 (38.9%)	35 (94.6%)	71 (98.6%)	9 (9.7%)	21 (16.2%)	32 (97.0%)	<0.001[b]
FHS	52 (48.1%)	1 (2.7%)	0 (0%)	66 (71.0%)	59 (45.4%)	1 (3.0%)	<0.001[b]
BUADC	14 (13.0%)	1 (2.7%)	1 (1.4%)	18 (19.4%)	50 (38.5%)	0 (0%)	<0.001[b]
Age at death (S.E.M)	70.2 (2.5)	53.7 (2.9)	73.6 (1.2)	86.1 (1.0)	83.5 (0.8)	75.6 (1.5)	<0.001[a]
Sex m/f (%male)	67/41 (62.0%)	36/1 (97.3%)	71/1 (98.6%)	52/41 (55.9%)	71/59 (54.6%)	33/0 (100%)	<0.001[b]
RHI y/n (%exposed)	41/31 (56.9%)	34/0 (100%)	70/0 (100%)	10/44 (18.5%)	23/41 (35.9%)	31/0 (100%)	<0.001[b]
Total years[*] (S.E.M)	6.6 (1.2)	15.1 (1.9)	17.0 (0.7)	1.3 (0.5)	3.2 (0.7)	15.9 (1.8)	<0.001[a]
Sport y/n (%played)							
football	33/39 (45.8%)	33/1 (97.1%)	69/1 (98.6%)	9/45 (16.7%)	19/45 (29.7%)	30/2 (93.8%)	<0.001[b]
hockey	5/67 (6.9%)	2/32 (5.9%)	5/65 (7.1%)	1/53 (1.9%)	3/61 (4.7%)	4/28 (12.5%)	0.492[b]
rugby	1/71 (1.4%)	4/30 (11.8%)	4/66 (6.1%)	0/54 (0%)	1/63 (1.6%)	1/31 (3.1%)	0.036[b]
wrestling	2/70 (2.8%)	3/31 (8.8%)	11/59 (15.7%)	0/54 (0%)	1/63 (1.6%)	1/31 (3.1%)	0.001[b]
boxing	0/72 (0%)	2/32 (5.9%)	7/63 (10.0%)	1/53 (1.9%)	1/63 (1.6%)	1/31 (3.1%)	0.029[b]
Other[†]	11/61 (15.3%)	4/30 (11.8%)	2/68 (2.9%)	2/52 (3.7%)	1/63 (1.6%)	2/30 (6.3%)	0.011[b]
Braak Stage (*n*)							
Stage 0	38 (35.2%)	14 (38.9%)	2 (2.9%)	0 (0%)	0 (0.7%)	0 (0%)	
Stage I	20 (18.5%)	6 (16.7%)	0 (0%)	17 (18.5%)	0 (0.7%)	0 (0%)	
Stage II	33 (30.6%)	11 (30.5%)	9 (13.2%)	32 (34.8%)	0 (0.7%)	0 (0%)	
Stage III	15 (13.9%)	2 (5.6%)	40 (58.8%)	33 (35.9%)	7 (5.6%)	0 (0%)	
Stage IV	2 (1.9%)	3 (8.3%)	11 (16.2%)	6 (6.5%)	19 (15.2%)	3 (9.1%)	
Stage V	0 (0%)	0 (0%)	4 (5.9%)	2 (2.2%)	31 (24.8%)	8 (24.2%)	
Stage VI	0 (0%)	0 (0%)	2 (2.9%)	2 (2.2%)	68 (54.4%)	22 (66.7%)	

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	no CTE/no AD (control)	low CTE	high CTE	low AD	AD	CTE and AD	*p* value
CTE Stage *n* (%)							
Stage I		20 (51.4%)	0 (0%)			1 (3.0%)	
Stage II		17 (45.9%)	0 (0%)			2 (6.1%)	
Stage III		0 (0%)	38 (52.8%)			7 (21.2%)	
Stage IV		0 (0%)	34 (47.2%)			23 (69.7%)	
CERAD Score *n* (%)							
0	108 (100%)	35 (94.6%)	40 (56.3%)	0 (0%)	0 (0.7%)	0 (0%)	
1	0 (0%)	2 (5.4%)	29 (40.8%)	77 (82.8%)	40 (32.0%)	7 (21.2%)	
2	0 (0%)	0 (0%)	2 (2.8%)	16 (17.2%)	49 (39.2%)	19 (57.6%)	
3	0 (0%)	0 (0%)	0 (0%)	0 (0%)	36 (28.8%)	7 (21.2%)	
Thal Phase *n* (%)							
0	44 (69.8%)	26 (70.3%)	15 (21.1%)	0 (0%)	0 (0%)	0 (0%)	
1	6 (9.5%)	5 (13.5%)	13 (18.3%)	2 (6.5%)	0 (0%)	0 (0%)	
2	2 (3.2%)	0 (0%)	7 (9.9%)	3 (9.7%)	1 (1.9%)	0 (0%)	
3	10 (15.9%)	4 (10.8%)	11 (15.5%)	13 (41.9%)	4 (7.7%)	2 (6.2%)	
4	1 (1.6%)	2 (5.4%)	15 (21.1%)	5 (16.1%)	14 (26.9%)	6 (18.8%)	
5	0 (0%)	0 (0%)	10 (14.1%)	8 (25.8%)	33 (63.5%)	24 (75.0%)	

Data are presented as mean (S.E.M.) years for age at death and total years and as # yes/# no (%) unless otherwise indicated.

CTE chronic traumatic encephalopathy, *AD* Alzheimer disease, *RHI* repetitive head impacts

[*]Total years of contact sports participation primarily available from UNITE and FHS groups. Includes participants who played multiple sports.

[†]includes soccer, lacrosse, mixed martial arts, karate, equestrian, professional wrestling, and martial arts

[a]ANOVA

[b]χ^2 test for proportions between all pathology groups

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Table 2:

Multiple linear regression analyses of associations with p-tau epitopes in CTE and AD

(a) Associations with tau epitopes in participants with and without CTE (excluding AD)								
	pTau181		**pTau202**		**pTau231**		**pTau396**	
Predictor	**β**	**p-value**	**β**	**p-value**	**β**	**p-value**	**β**	**p-value**
Contact sports play (yrs)	−0.106	0.275	**0.293**	**0.001**	0.157	0.067	0.092	0.318
$A\beta_{1-42}$ (pg/mg)	−0.060	0.532	0.019	0.836	**0.213**	**0.012**	**0.195**	**0.034**
(b) Associations with tau epitopes in participants with and without AD (excluding CTE)*								
	pTau181	**pTau202**	**pTau231**	**pTau396**				
Predictor	**β**	**p-value**	**β**	**p-value**	**β**	**p-value**	**β**	**p-value**
$A\beta_{1-42}$ (pg/mg)	0.129	0.071	**0.205**	**0.006**	**0.249**	**<0.001**	**0.312**	**<0.001**

Two different models were run for each of the p-tau epitopes in CTE (a) and AD (b) using multiple linear regression analysis with total years of playing contact sports and $A\beta_{1-42}$. All variables displayed underwent rank-based normalization, and both models adjusted for age at death and sex. β indicates standardized beta value. Significant associations are shown in bold.

For **(a)** participants with and without CTE: for pTau181 n=168, for pTau202 n=180, for pTau231 n=202, and for pTau396 n=180.

For **(b)** Participants with and without AD: for pTau181 n=253, for pTau202 n=244, for pTau231 n=289, and for pTau396 n=259.

*Total years of playing contact sports not available for most participants in AD cohort