

Beta-amyloid deposition in chronic traumatic encephalopathy

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Abstract Chronic traumatic encephalopathy (CTE) is a neurodegenerative disease associated with repetitive mild traumatic brain injury. It is defined pathologically by the abnormal accumulation of tau in a unique pattern that is distinct from other tauopathies, including Alzheimer's disease (AD). Although trauma has been suggested to increase amyloid β peptide (A β) levels, the extent of A β deposition in CTE has not been thoroughly characterized. We studied a heterogeneous cohort of deceased athletes and military veterans with neuropathologically diagnosed CTE ($n = 114$, mean age at death = 60) to test the hypothesis that A β

deposition is altered in CTE and associated with more severe pathology and worse clinical outcomes. We found that A β deposition, either as diffuse or neuritic plaques, was present in 52 % of CTE subjects. Moreover, A β deposition in CTE occurred at an accelerated rate and with altered dynamics in CTE compared to a normal aging population (OR = 3.8, $p < 0.001$). We also found a clear pathological and clinical dichotomy between those CTE cases with A β plaques and those without. A β deposition was significantly associated with the presence of the *APOE* $\epsilon 4$ allele ($p = 0.035$), older age at symptom onset ($p < 0.001$), and

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older age at death ($p < 0.001$). In addition, when controlling for age, neuritic plaques were significantly associated with increased CTE tauopathy stage ($\beta = 2.43$, $p = 0.018$), co-morbid Lewy body disease (OR = 5.01, $p = 0.009$), and dementia (OR = 4.45, $p = 0.012$). A subset of subjects met the diagnostic criteria for both CTE and AD, and in these subjects both A β plaques and total levels of A β 1-40 were increased at the depths of the cortical sulcus compared to the gyral crests. Overall, these findings suggest that A β deposition is altered and accelerated in a cohort of CTE subjects compared to normal aging and that A β is associated with both pathological and clinical progression of CTE independent of age.

Keywords Chronic traumatic encephalopathy · Alzheimer's disease · Beta-amyloid · Neurodegenerative disorders · Dementia · Tau

Abbreviations

AD	Alzheimer's disease
CTE	Chronic traumatic encephalopathy
CTE-AD	Chronic traumatic encephalopathy with AD
CERAD	Consortium to establish a registry of AD
A β	Amyloid β peptide
APP	Amyloid precursor protein
NP	Neuritic plaques
DP	Diffuse plaques
RTBI	Repetitive traumatic brain injury
ELISA	Enzyme-linked immunosorbent assay
OR	Odds ratio
ptau231	Tau phosphorylated at threonine 231
PET	Positron emission tomography

Introduction

Chronic traumatic encephalopathy (CTE) is a neurodegenerative disease likely caused by repetitive traumatic brain injury (RTBI). Many contact sports have now been linked to CTE, including American football, boxing, hockey, soccer, and rugby. In addition, we have found CTE in military personnel exposed to an explosive blast [10, 29]. The clinical features of CTE typically manifest years or decades after the initial RTBI and consist of impairments in mood (depression, suicidality, irritability), behavior (explosivity, violence, impulsivity), cognition (impaired memory, executive dysfunction, diminished concentration), and motor functioning (parkinsonism, dysarthria, gait changes, weakness) [32, 33, 46]. The reported clinical symptoms following RTBI can vary widely, and different subtypes have been identified in neuropathologically confirmed cases of CTE [46]. Research clinical diagnostic criteria have recently been proposed [32], but investigations to establish

reliability and diagnostic accuracy in predicting underlying CTE pathological changes are ongoing [32]. In some cases the clinical features can overlap with and be clinically indistinguishable from those of Alzheimer's disease (AD). An analysis of neuropathologically confirmed CTE without co-morbid disease demonstrated that in 11 % of subjects overall and in 40 % of demented subjects the clinical presentation is indistinguishable from AD. Moreover, as in AD, there is a spectrum of pathology in CTE, and some, but not all, subjects with pathological CTE will have dementia or CTE-related clinical symptoms [46]. A more complete understanding of the pathology may help explain and predict the clinical outcomes.

CTE is a tauopathy characterized by neurofibrillary tangles, tau-positive astrocytes, and tau-positive cell processes that preferentially involve the cortical sulci, medial temporal lobe, diencephalon, and brainstem. In CTE the tau pathology is characteristically perivascular, most pronounced at the sulcal depths, and preferentially involves the superficial cortical layers. This pattern of tau pathology is distinct from other tauopathies, including Alzheimer's disease. Recently, the US National Institute of Neurological Disorders and Stroke convened a panel of expert neuropathologists to establish consensus criteria for CTE [42]. It was determined that the pathognomonic lesion in CTE was an abnormal perivascular accumulation of tau in neurons, astrocytes, and cell processes in an irregular pattern at the depths of the cortical sulci, and that CTE could be reliably distinguished from other tauopathies including AD, progressive supranuclear palsy, argyrophilic grain disease, corticobasal degeneration, Guamanian Parkinsonism Dementia Complex, and primary age-related tauopathy [4].

The role of amyloid β peptide (A β) in CTE has been controversial. At one point, A β deposition was thought to be a universal feature [39], but subsequent analyses found A β pathology within only a subset of individuals. In our reported cohort of 68 athletes and military veterans with CTE, 44 % had some deposition of primarily diffuse plaques, and 10 % met the criteria for AD [30]. Some epidemiologic evidence exists that suggests moderate to severe TBI is a risk factor for AD [13, 24, 27, 38] although most reports linking TBI and AD have relied on a clinical diagnosis of probable AD without neuropathological verification and a definitive link has yet to be established [17]. It has been shown that acutely following TBI, amyloid precursor protein (APP) and A β increase in tissue and CSF, and there can be rapid formation of diffuse A β plaques in the cortex [9, 16, 36, 40, 41]. Therefore, the release of A β into surrounding tissue following injury may be a basis for plaque formation [41, 43]. The relation of A β deposition in CTE to genetic factors, other pathological lesions, and to the clinical course has yet to be determined.

Here we set out to test the hypotheses that A β deposition is altered in CTE compared to a control population, A β accumulation in CTE differs from AD, and the presence of diffuse or neuritic plaques increases pathological severity and worsens clinical outcomes in CTE. To test these hypotheses we examined the brains of 114 participants with a history of RTBI and compared the pattern of neuropathological lesions in subjects with and without A β to each other, to the brains of individuals with neuropathologically confirmed AD without a known history of RTBI, and to the reported pathology of a large non-selected autopsy cohort of 2332 normal aging cases [2].

Materials and methods

Subjects

A total of 114 subjects were evaluated from Boston University's Chronic Traumatic Encephalopathy Center including former athletes, military veterans, and civilians with a history of repetitive mild traumatic brain injury. Next of kin provided written consent for participation and brain donation. Institutional review board approval for brain donation was obtained through the Boston University Medical Center and the Edith Nourse Rogers Memorial Veterans Hospital, Bedford, MA. Institutional review board approval for post-mortem clinical record review, interviews with family members, and neuropathological evaluation was obtained through Boston University Medical Center. The brains from an additional 319 subjects from the Boston University AD Center were included for comparative analysis.

Clinical assessment

Concussion and RTBI history, history of cognitive and behavioral changes and clinical status leading up to death were determined through post-mortem interviews with next of kin performed by physicians, neuropsychologists, and doctoral candidates trained to assess for RTBI, dementia, and neurodegenerative diseases (JM, TS, DHD, PHM, and RAS). Interviewers were blind to the results of the neuropathological examination at the time of interview, and the neuropathologists were blind to the results of the clinical interviews at the time of neuropathological examination and diagnosis. Informants were interviewed before receiving the results of the neuropathological examination. The interview was semi-structured and conducted by telephone. Medical record review was also performed (JM, TS, PK, DHD, PHM, TDS, ACM, and RAS). Recorded clinical measures included the presence or absence of CTE symptoms

including mood, behavioral, or cognitive changes (reviewed in [32]), age of symptom onset, presence of symptoms associated with parkinsonism (e.g., tremor, bradykinesia, rigidity, gait disturbance, ataxia, dysarthria, dysphagia), and behavioral or cognitive variant of CTE [46]. We recorded dementia status using a conservative definition of dementia based on a diagnosis made during life. In the subset of AD subjects selected for ELISA and A β plaque burden, subjects and their informants were asked during life about a history of contact sports play or head injury including RTBI and those without such history were selected for comparison.

Pathological diagnoses

Chronic traumatic encephalopathy

The diagnosis and staging of CTE followed the definitions and criteria described previously [30] and adapted to include the following: (1) perivascular foci of hyperphosphorylated tau immunoreactive neurons, astrocytes, and cell processes; (2) irregular cortical distribution of hyperphosphorylated tau immunoreactive neurons and astrocytes with a predilection for the depths of cerebral sulci; and (3) hyperphosphorylated tau-positive neurons in the cerebral cortex located preferentially in the superficial layers. Supportive features included clusters of subpial and periventricular astrocytic tangles in the cerebral cortex, diencephalon, basal ganglia and brainstem. CTE stages varied from I to IV based on the extent and severity of tau pathology as previously described [30, 45]. Briefly, Stage I CTE is characterized by isolated perivascular foci of hyperphosphorylated tau within neurons, astrocytes, and cell processes present at the sulcal depths. In stage II the tau pathology extends to involve the superficial cortical layers into the gyral crest. In stage III there is additional involvement of the parietal and temporal lobes as well as medial temporal lobe tau pathology (i.e. hippocampus, amygdala, entorhinal cortex). By stage IV all cortical lobes are involved with severe tau pathology. In addition, there is abnormal tau accumulation within the diencephalon, brainstem, and cerebellum.

Alzheimer's disease

Neuropathological diagnosis of AD required an intermediate or high degree of neuropathological changes as defined by the ABC score. Subjects with AD changes had a low degree of neuropathological change as defined by the ABC score [15, 34]. Neuritic plaques were assessed with Bielschowsky silver staining and using the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) scoring system for plaque pathology [31].

Lewy body disease

The presence or absence of Lewy bodies was determined by immunohistochemical staining for alpha-synuclein. The regions examined in each case included the olfactory bulb, medulla, substantia nigra, amygdala, hippocampus, and cingulate gyrus.

Immunohistochemistry

Human tissue was fixed in periodate-lysine-paraformaldehyde, and tissue blocks were paraffin-embedded and cut at 10 μm for immunohistochemistry. Antigen retrievals were performed by formic acid treatment for 2 min for A β antibodies. Sections were incubated at 4 °C overnight with antibodies to phosphorylated PHF-tau (AT8; Pierce Endogen, Rockford IL; 1:2000), A β 1-40 (AB5074P; EMD Millipore, Billerica, MA; 1:2000), A β 1-42 (AB5078P; EMD Millipore; 1:2000), alpha-synuclein (rabbit polyclonal; Chemicon, Temecula, CA; 1:15,000), and pTDP-43 (pS409/410 mouse monoclonal; Cosmo Bio Co, Tokyo, Japan; 1:2000). For determination of A β deposition in the medial temporal lobe (section of the hippocampal formation, parahippocampal gyrus, transentorhinal region, and portions of occipitotemporal gyrus), the monoclonal anti-A β antibody (Clone 4G8; Covance, Dedham, MA, 1:100,000) was used. After three washes with PBS (pH 7.4), sections were treated with biotinylated secondary antibody and labeled with a 3-amino-9-ethylcarbazol HRP substrate kit (Vector Laboratories). Sections were counterstained with Gill's Hematoxylin (Vector Laboratories H-3401) and then coverslipped with Permount mounting medium.

Quantitative ELISA measurement of A β and tau

The buffer conditions, protease inhibitors, and centrifugation protocols have been reported previously [51]. Briefly, a 4-mm tissue punch was used to isolate and remove gray matter from the gyral crests and sulcal depths of the middle frontal gyrus and neighboring sulci and superior temporal gyrus and sulcus. Brain tissue was homogenized in fivefold volume of 5 M Guanidine Hydrochloride/50 mM Tris-HCL, pH 8.0, with protease inhibitors (Thermo Scientific, 78439) and phosphatase inhibitors (Sigma, P5726 and P0044). Tissue was homogenized using a mechanical homogenizer for 25 strokes followed by ultrasonic disruption on ice. The homogenates were shaken at room temperature overnight. Samples were diluted tenfold with 0.4 % Block Ace (Dainippon Pharmaceuticals Co, Japan) and centrifuged at 14,000 rpm for 15 min at 4 °C. Enzyme-linked immunosorbent assay (ELISA) was performed for A β 1-38, 1-40, and 1-42 using a multiplex plate from Meso Scale Discovery (MSD, Rockville, MD, USA) as well as

for levels of phosphorylated tau (Thr231) following the manufacturer's instruction.

A β plaque deposition was determined using A β 1-40 and A β 1-42 immunostaining and quantified as reported previously [37]. Briefly, digital images were captured at 200 \times magnification and a threshold optical density was obtained, which discriminated staining from background. The amyloid burden was defined as the total percentage of cortical surface area covered by either A β 1-40 or A β 1-42 deposition over three sections and was calculated for the bottom third of the sulcus (depth of the sulcus) and the top third of the gyrus (gyral crest).

APOE genotyping

DNA was extracted from brain tissue samples using a Qia-gen QIAamp DNA extraction kit (Qiagen, Valencia, CA, USA). Two single nucleotide polymorphisms (National Center for Biotechnology Information SNPs rs429358 and rs7412) were examined using TaqMan assays (Applied Biosystems, Foster City, CA, USA). Allelic discrimination was automated using the manufacturer's software. Positive controls, consisting of DNA of each of the 6 possible APOE genotypes (ϵ 2/ ϵ 2, ϵ 2/ ϵ 3, ϵ 2/ ϵ 4, ϵ 3/ ϵ 3, ϵ 3/ ϵ 4, ϵ 4/ ϵ 4), were included on each plate and genotyped with restriction isotyping.

Statistical methods

Both SPSS version 20.0 (IBM Inc., Chicago, IL, USA) and SAS version 9.3 (SAS Institute, Cary, NC, USA) were utilized for statistical analyses. Significance was set a priori at $\alpha = 0.05$. Neuropathologically confirmed CTE tauopathy cases were divided into two subgroups based on the presence or absence of A β plaques and further distinguishing between diffuse and neuritic types. Group comparisons were based on demographic (e.g., age at death), clinical (e.g., symptom onset, presentation subtype), genotype (APOE), exposure (e.g., sport, RTBI history), and lesion (e.g., A β , LBD, TPD-43) characteristics. Spearman's rank order correlation was used to determine the statistical association between CTE stage and all linear variables of interest. For non-linear independent variables, the Wilcoxon-Mann-Whitney *U* test was used for independent variables with only two groups (e.g., presence of neuritic plaques). A two-sample Chi square test weighted by sample size and a logistic regression model were used to compare the frequency of A β deposition in the medial temporal lobe by age in our CTE cohort to a published non-selected community-based autopsy series of 2332 subjects [2]. The Chi square statistical method is nonparametric and, therefore, makes no assumptions about the population distribution. Linear regression analysis was performed to determine the

Table 1 Frequency of A β deposition in CTE

	<i>n</i>	Mean age at death (years)	APOE ϵ 4 % (+/–) ^a	DP freq (%)	NP freq (%)
CTE (all)	114	60	33 % (27/55)	52	36
Stage I	13	35	33 % (3/6)	15	0
Stage II	30	52	32 % (7/15)	33	20
Stage III	34	62	23 % (6/20)	44	27
Stage IV	37	75	44 % (11/14)	87	70
AD	319	80	59 % (13/9)	84	100

CTE Chronic traumatic encephalopathy, DP diffuse plaques, NP neuritic plaques, AD Alzheimer's disease

^a % of both ϵ 4 heterozygotes and homozygotes out of those cases with genotypes (*n* = 82)

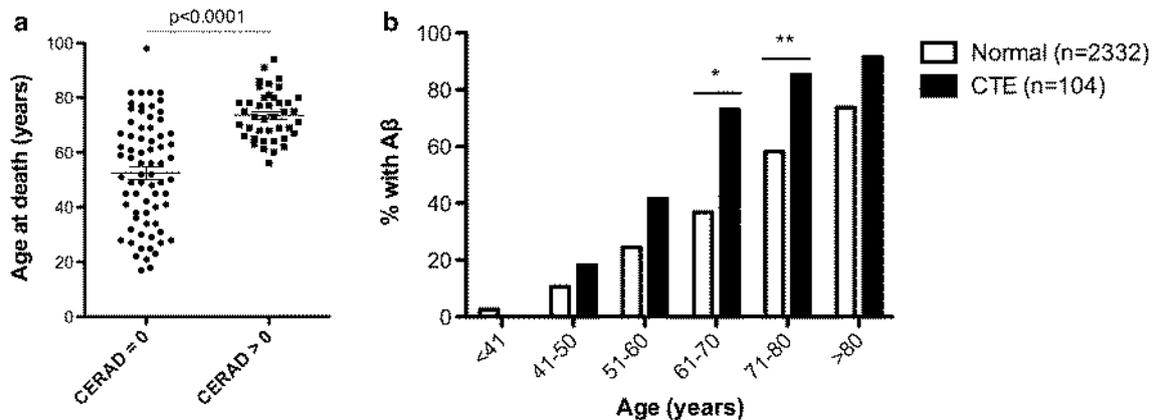


Fig. 1 The presence of neuritic A β plaques (NPs, CERAD > 0) was associated with age at death and was accelerated in CTE. **a** Subjects with neuritic plaques were significantly older at death than those without neuritic plaques (CERAD = 0). **b** When compared to the

gradual age-dependent increase in the frequency of A β plaques in the medial temporal lobe of a non-selected autopsy series [2], the frequency of A β plaques in CTE is significantly higher in subjects in their 60 s (* $\chi^2 = 13.7$, $p < 0.001$) and 70 s (** $\chi^2 = 7.76$, $p = 0.005$)

relationship of A β deposition and age at death and CTE stage. The interaction effect between age and A β pathology in predicting CTE stage was calculated when appropriate. Logistic regression models were used to generate odds ratios (OR) while adjusting for age and CTE stage when appropriate.

Results

Beta-amyloid pathology in CTE, AD, and aging

We first set out to determine whether the frequency of plaques differed in our cohort of CTE versus AD and normal aging. Beta-amyloid plaques can be either diffuse or neuritic as defined by the presence of abnormal tau-positive neurites. In our cohort of 114 subjects with CTE (mean age at death = 60 years, all men), we found A β deposition in the form of diffuse plaques in 52 % with A β immunostaining and neuritic plaques (CERAD > 0) in 36 % using Bielschowsky silver staining. The percent of cases with both diffuse and neuritic A β deposition increased with CTE stage

(Table 1). When compared to AD subjects from the Boston University's AD Center, stage IV CTE had a similar frequency of diffuse A β plaques. However, there were significantly fewer neuritic plaques in stage IV CTE compared to AD (Table 1, $Z = -9.94$, $p < 0.001$).

The mean age at death was significantly higher in CTE subjects with A β plaques compared to those without (Fig. 1a; Table 3). To evaluate the age-dependent increase of A β deposition in our CTE cohort and to determine whether it differs from normal aging, we grouped the age at death of our subjects into decades and plotted the frequency of cases positive for A β deposition in the medial temporal lobe (available in $n = 104$ subjects) as assessed by the anti-A β antibody 4G8 (Fig. 1b). A β pathology first appeared in CTE cases aged 41–50, with a frequency that was significantly higher in later decades (Fig. 1b). The odds of developing A β pathology in CTE increased 2.7-fold for every decade ($p < 0.0001$). A clear age-dependent increase in A β plaque deposition was also reported in the medial temporal lobe using identical methods in a non-selected community-based autopsy series of 2332 subjects [2]. When compared to this normal aging population, the

Table 2 *APOE* allele frequencies in CTE

Group	$\epsilon 2$ allele count <i>n</i> (%)	<i>p</i> value (Z score)*	$\epsilon 3$ allele count <i>n</i> (%)	<i>p</i> value (Z score)*	$\epsilon 4$ allele count <i>n</i> (%)	<i>p</i> value (Z score)**
CTE overall	12 (7.3 %)	0.726 (−0.348)	120 (73 %)	0.298 (−1.04)	32 (20 %)	0.069 (1.48)
CTE without A β	5 (6.4 %)	0.596 (−0.534)	64 (82 %)	0.258 (1.13)	11 (14 %)	0.386 (−0.294)
CTE with DPs	6 (7.0 %)	0.711 (−0.368)	56 (65 %)	0.012 (−2.51)	23 (27 %)	0.002 (2.92)
CTE with NPs	3 (5.4 %)	0.459 (−0.742)	39 (70 %)	0.219 (−1.23)	14 (25 %)	0.022 (2.01)
Population norms, ages 42–70 years [28]	758 (8.1 %)		7205 (77 %)		1439 (15 %)	

APOE apolipoprotein E gene, CTE chronic traumatic encephalopathy, *DPs* diffuse plaques, *NPs* neuritic plaques; significant *p* values in bold (Z test versus population norms)

* Two-tailed, ** one-tailed

frequency of A β deposition in our CTE cohort was higher in CTE subjects in the decades between the 50 and 90s and significantly higher for CTE subjects in their 60s ($\chi^2 = 13.7$, $p < 0.001$) and 70s ($\chi^2 = 7.76$, $p = 0.005$), and the overall risk of developing plaque pathology was higher for the CTE group (Fig. 1b). In fact, the odds of developing plaque pathology were 3.8 times higher in CTE than in normal aging ($p < 0.0001$). To test the hypothesis that A β plaque frequency in CTE and normal aging are not derived from a common distribution we performed a weighted two-sample Chi square test. Importantly, this demonstrated a distinct distribution of A β by age in the CTE cohort compared to normal aging ($\chi^2 = 721$, $p < 0.0001$). This suggests that A β deposition in CTE is not simply accelerated aging, but rather a distinct process with altered dynamics.

APOE $\epsilon 4$ allele is enriched in CTE subjects with A β

We next set out to determine whether the proportion of *APOE* $\epsilon 4$ was enriched in CTE cases overall and in those with A β versus those without. First, we tested the hypothesis that the proportion of $\epsilon 4$ alleles is elevated in our CTE cohort overall. Although there were more $\epsilon 4$ alleles in the overall CTE cohort (20 %) when compared to an age-matched normative sample (15 % from [28]), the difference was not quite significant (Table 2, $Z = 1.48$, $p = 0.069$, *z* test). Similar to what was reported previously in a subset of these cases [46], there was a significantly greater proportion of $\epsilon 4$ homozygotes ($Z = 1.94$, $p = 0.026$) and a non-significant increase in heterozygotes in the overall CTE cohort compared to the normative sample. To test the hypothesis that the $\epsilon 4$ proportion is elevated in CTE subjects with A β , but not in those without, we compared the proportions of these different groups to a control population [28]. In CTE subjects without either diffuse or neuritic plaques, the proportion of $\epsilon 4$ alleles (14 %) was not significantly different from population norms (15 %). However, the proportion of

$\epsilon 4$ alleles was significantly greater than a control population in CTE subjects with diffuse plaques (27 %, $Z = 2.92$, $p = 0.002$) or neuritic plaques (25 %, $Z = 2.01$, $p = 0.022$). We also examined the other *APOE* allele frequencies. In CTE subjects with diffuse plaques there was a decrease in the number of $\epsilon 3$ alleles (65 %) compared to the control population (77 %, $Z = -2.51$, $p = 0.012$). Differences in the $\epsilon 2$ allele frequency were not detected (Table 2). Finally, to directly test the hypothesis that the $\epsilon 4$ allele is associated with A β in our CTE cohort, we performed a Fisher's exact test and found that $\epsilon 4$ was significantly associated with the presence of diffuse plaques ($p = 0.035$), but did not quite reach significance for neuritic plaques ($p = 0.086$).

Sulcal versus gyral A β levels

If traumatic injury influences the deposition of A β , we would hypothesize that more A β would be present at the sulcal depths due to the putative stress concentration there [45]. Therefore, we next examined a subset of cases that met the neuropathological criteria for both AD and CTE (CTE-AD; Supplementary Table 1) for differences in A β levels at the sulcal depths of the middle frontal sulcus versus the gyral crests. We also examined subjects with CTE and neuritic plaques that did not reach the full criteria for an AD diagnosis (CTE + NPs). These groups were compared to subjects without any history of RTBI or professional contact sports play, including those with pathologically diagnosed AD, and to those with neuritic plaques that did not have AD (AD changes; Supplementary Table 1). In subjects with only AD, there was no difference in A β 1-40 or A β 1-42 plaque deposition between the sulcus and the gyrus (Fig. 2). However, in CTE-AD subjects, the A β 1-40 plaque burden was significantly greater in the sulcus compared to the gyral crest ($t = 2.21$, $p = 0.029$, Student's *t* test), but this difference was not present for A β 1-42. The A β 1-42 plaque burden was similar between AD and

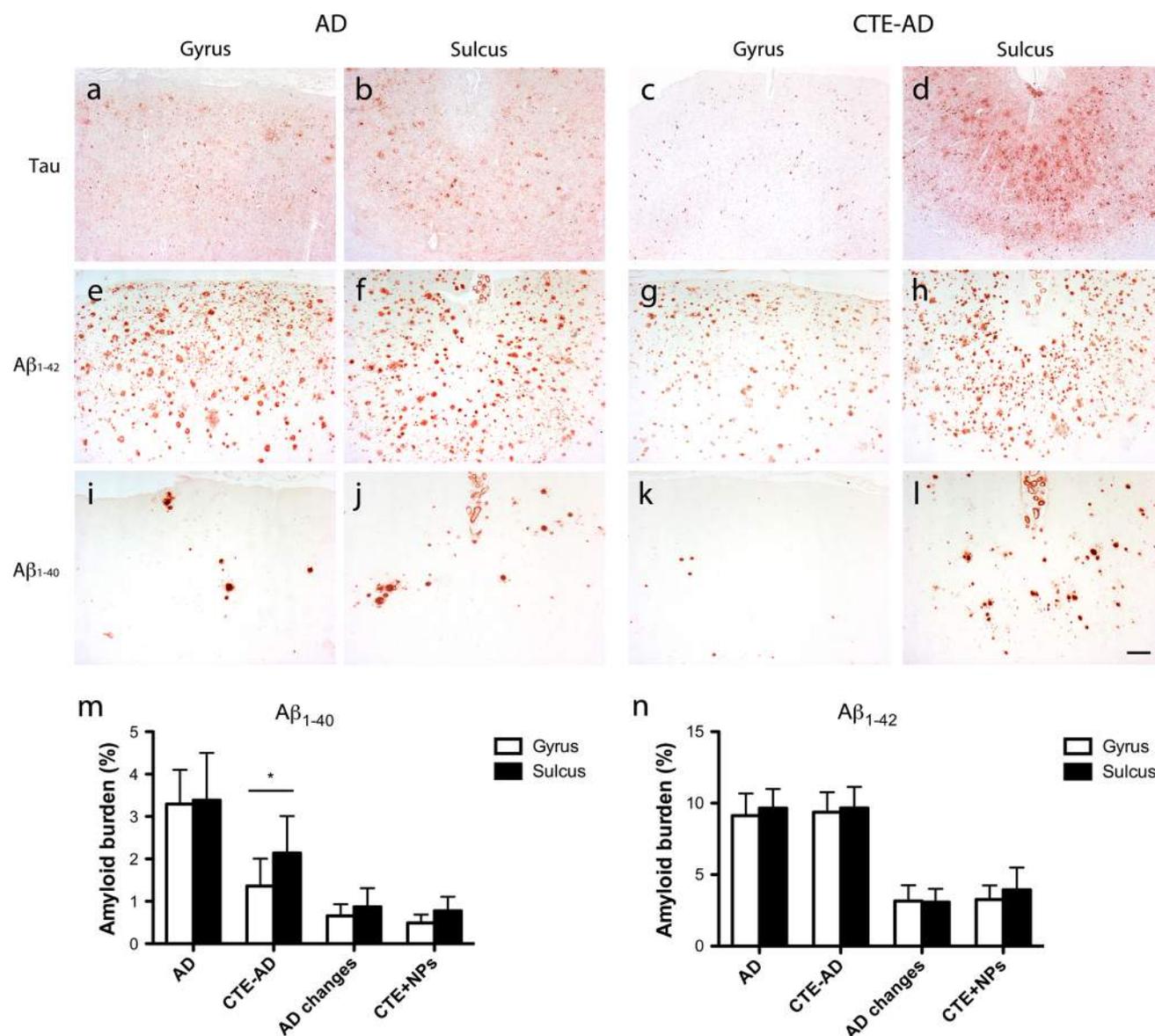


Fig. 2 A β and tau pathologies were concentrated within the sulcal depths as compared to the gyral crests of the dorsolateral frontal cortex in CTE-AD. **a–d** Immunostaining for tau showed a greater accumulation of tau-positive neurons, astrocytes, and cell processes in the sulcus of CTE-AD subjects (**d**) as compared to the gyrus (**c**), but no difference in AD subjects (**a, b**). **e–l** Immunostaining for A β ₁₋₄₀ and A β ₁₋₄₂ showed no difference between the gyrus (**e, i**) and sulcus (**f, j**) of AD subjects. However, there were a greater number of plaques in the sulcus of subjects with CTE-AD (**h, l**) as compared to the gyrus

(**g, k**) although the difference in A β ₁₋₄₀ was most pronounced (**k, l**). Scale bar 200 μ m. (**m, n**) Quantitation of the percent amyloid burden showed significantly increased A β ₁₋₄₀ (**m**), but not A β ₁₋₄₂ (**n**) within the sulcus compared to the gyrus in the dorsolateral frontal cortex of CTE-AD subjects (* $p = 0.029$, Student's t test). There was no difference between sulcal or gyral plaque burden for A β ₁₋₄₀ or A β ₁₋₄₂ in subjects with AD, AD changes, or CTE with neuritic plaques (CTE+NPs)

CTE-AD subjects. In contrast, there was a significantly lower A β ₁₋₄₀ plaque burden in the gyral crest in CTE-AD compared to AD subjects (Fig. 2, $t = -2.80$, $p = 0.013$). Subjects with AD changes and subjects with CTE + NPs had similar plaque burden levels for both A β ₁₋₄₀ and A β ₁₋₄₂ in both the gyrus and the sulcus, and all were significantly less compared to the AD subject groups ($p < 0.05$, Student's t test).

To determine whether total levels of various species of A β were elevated in the sulcus compared to the gyrus in subjects with CTE compared to those without, we next used ELISA to measure the total levels of A β ₁₋₃₈, A β ₁₋₄₀, and A β ₁₋₄₂ isolated from the sulcal depths of the middle frontal cortex and compared them to levels in the gyral crests. Similar to the data on A β plaque burden, we found significantly more A β ₁₋₄₀ in the sulcus compared to the

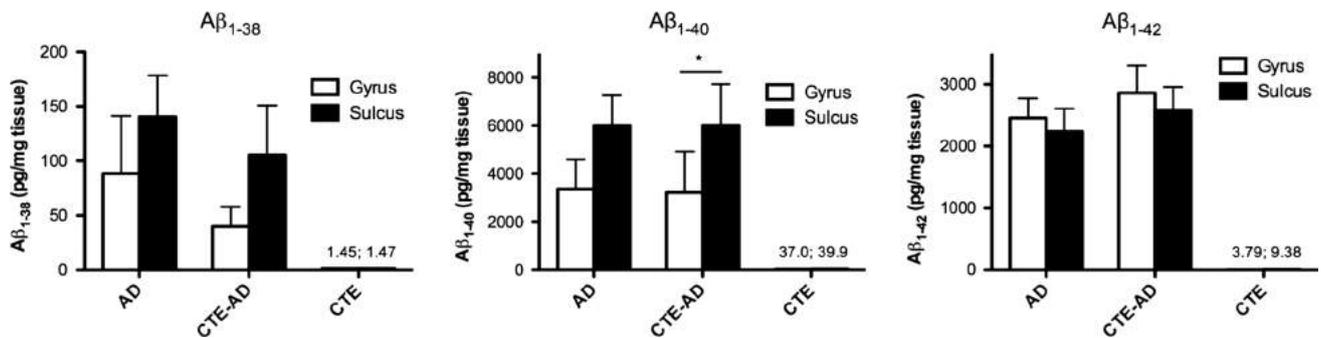


Fig. 3 Total levels of Aβ in subjects with AD, CTE-AD, or CTE alone as measured by ELISA. Subjects with both CTE and AD (CTE-AD) had a significantly higher Aβ₁₋₄₀ burden within the sulcus compared to the gyrus (* $p = 0.045$, paired t test). There was no difference

between sulcal or gyral Aβ₁₋₄₂ levels in subjects with AD or CTE-AD. Subjects with CTE alone had low levels of Aβ₁₋₃₈, Aβ₁₋₄₀, and Aβ₁₋₄₂ that were not significantly different between sulci and gyri

gyrus in CTE-AD subjects (paired $t = 1.965$, $p = 0.045$) and no difference between sulcal and gyral levels of Aβ₁₋₄₂ (Fig. 3). Surprisingly, we also found a trend of elevated Aβ₁₋₄₀ levels in the middle frontal sulcus compared to the gyrus of AD subjects without a history of RTBI; however, this did not reach significance ($t = 2.16$, $p = 0.059$, paired t test). As expected, subjects with CTE without neuritic plaques had very low levels of Aβ₁₋₃₈, Aβ₁₋₄₀, and Aβ₁₋₄₂ (Fig. 3).

Effect of Aβ on CTE tauopathy

To test the hypothesis that the development of Aβ pathology relates to an increased severity and progression of CTE tauopathy, several regression analyses were employed. The CTE stage is a measure of the degree and distribution of tau pathology [30] and was significantly increased in subjects with co-morbid neuritic plaque pathology (Fig. 4a). In the overall CTE cohort, age was also significantly associated with CTE stage. Therefore, we also tested for an interaction between neuritic plaques and age on CTE stage. The overall linear regression model showed that CTE stage was significantly predicted by neuritic plaques ($\beta = 2.43$, $p = 0.018$) when adjusted for age ($\beta = 0.029$, $p = 0.001$) and the two-way interaction (age \times neuritic plaque, $\beta = -0.028$, $p = 0.047$). Taken together, Aβ pathology and age accounted for almost half of the total variance in the progressive development of CTE stage ($r^2 = 0.47$) although the contribution of age to the development of CTE tau pathology had a small effect (β coefficient <0.25). Figure 4b illustrates the nature of this interaction by separately plotting the regression of age and CTE stage in subjects with neuritic plaques versus those without. Notably, there was a significant association between age at death and CTE stage in subjects lacking neuritic plaques ($\beta = 0.032$, $p < 0.001$), but not in CTE subjects with neuritic plaques ($\beta = 0.004$, $p = 0.740$). The slope of the linear regression

line between age and CTE stage was significantly greater in CTE subjects without neuritic plaques compared to those with neuritic plaques ($F = 4.147$, $p = 0.044$). This demonstrates that the presence of neuritic plaques modifies the relationship between age and CTE stage. When broken down by decade, CTE stage was significantly increased in subjects in their 60s with neuritic plaques, but not younger subjects (Fig. 4c). After age 70, the average CTE stage plateaued to near its maximum level for subjects both with and without NPs. To examine this relationship even further, we employed binary logistic regression and found the odds of a case progressing to the most severe stage of CTE, stage IV tauopathy, was 3.90 times higher in subjects presenting with co-morbid neuritic plaques compared to those without ($p = 0.008$) while adjusting for age. Overall, these data demonstrate that the presence of neuritic plaques was associated with more severe CTE-related tauopathy independent of age.

Quantitative levels of tau phosphorylated at threonine 231 (ptau231) were also measured by ELISA in the middle frontal cortex in a subset of subjects (Supplementary Table 1). ptau231 was significantly increased in CTE subjects with neuritic plaques compared to those without (Supplementary Figure 1, $t = 2.10$, $p = 0.045$) and was further increased in CTE-AD subjects. CTE-AD subjects had similar levels of ptau231 as AD subjects despite an average age at death almost a decade earlier (70 years in CTE-AD versus 79 years in AD). This further supports the hypothesis that Aβ deposition is associated with more severe tau pathology in CTE.

Clinical and mTBI exposure associations

We next set out to determine whether the presence of Aβ was associated with worse clinical outcomes. First, we found no significant difference between the number of years of athletic exposure. Subjects with diffuse or neuritic

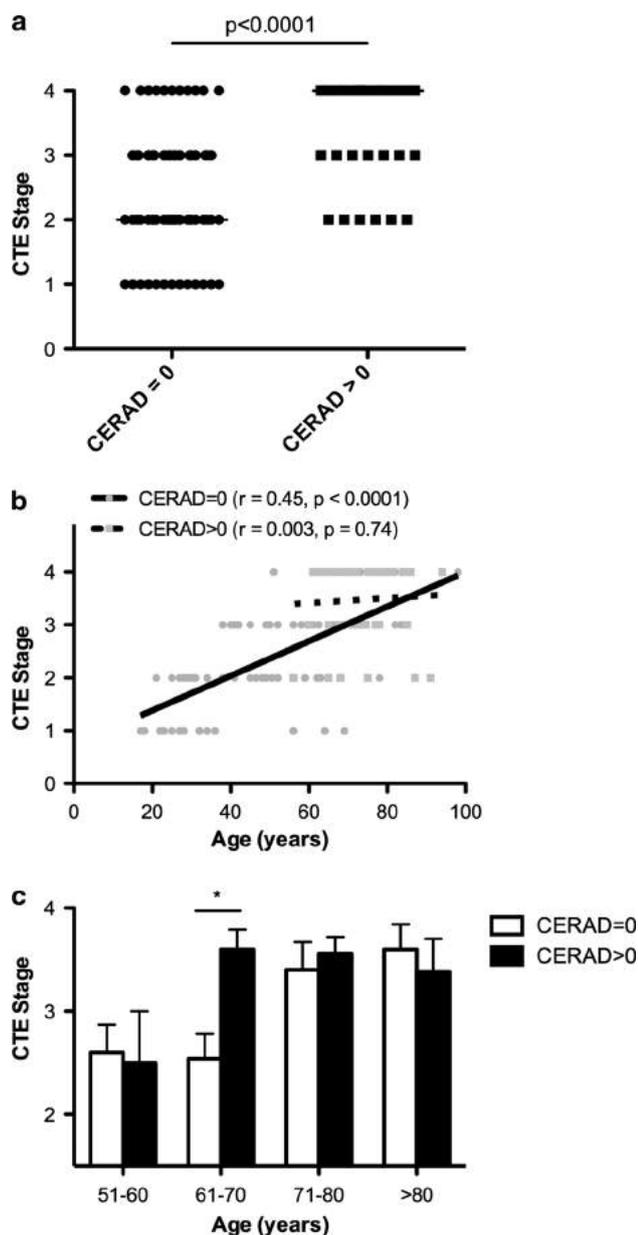


Fig. 4 CTE subjects with neuritic A β plaques (CERAD > 0) had an accelerated tauopathy. **a** The median CTE stage was significantly greater in CTE subjects with neuritic plaques ($*p < 0.001$, Mann–Whitney U test). **b** Scatter plots of CTE tau pathology (stage) versus age are shown with separate regression lines for subjects without neuritic plaques (CERAD = 0) and those with neuritic plaques (CERAD > 0). There was a significant correlation between age and CTE stage in subjects without neuritic plaques, but not in CTE subjects with neuritic plaques. The slope of the regression line between age and CTE stage was significantly greater ($p = 0.044$) in CTE subjects without compared to those with neuritic plaques. **c** The increase in CTE stage in subjects with neuritic plaques was significant in the 7th decade of life ($*p < 0.002$, Mann–Whitney U test), while older subjects had similarly elevated CTE stages

plaques did have an increased number of reported concussions compared to CTE subjects without A β , but this difference was not significant (Table 3). Both the age at death

and the age at symptom onset were significantly greater in CTE subjects with A β (either diffuse or neuritic plaques) compared to those without A β (Table 3, $p < 0.001$).

There was a significantly greater frequency of dementia in subjects with A β compared to those without. A logistic regression analysis controlling for age shows that dementia remained significantly enriched in the CTE with A β subgroups (Table 3). In fact, the odds of developing dementia were 3.9 times higher in persons with diffuse plaque pathology (OR = 3.93, $p = 0.013$) while controlling for age ($p < 0.001$) and 4.5 times higher in persons with neuritic pathology (OR = 4.45, $p = 0.012$) while controlling for age ($p < 0.001$). In addition, the proportion of subjects with CTE clinical symptoms was greater in those subjects with either diffuse or neuritic plaques (neuritic: $p = 0.028$, Pearson χ^2 test), although this difference was not significant when controlling for age (Table 3).

Two distinct clinical presentations of CTE have been reported: some subjects present with cognitive symptoms (cognitive variant) and others present with behavioral or mood symptoms (behavioral-mood variant) [46]. The frequency of the cognitive variant was increased in CTE subjects with A β , but this difference did not reach significance (Table 3).

Motor symptoms including parkinsonism have been reported in a subset of CTE subjects. Moreover, Lewy body disease has been associated with A β deposition [11]. We, therefore, tested whether A β is associated with Lewy body disease and parkinsonism in our CTE cohort. We found a significantly higher frequency of both Lewy body disease pathology and parkinsonism in CTE subjects with A β compared to those without. A logistic regression analysis controlling for age shows that both Lewy body pathology (neuritic: OR = 5.01, $p = 0.009$; diffuse: OR = 7.09, $p = 0.019$) and parkinsonism (diffuse: OR = 8.86, $p = 0.050$) are significantly enriched in the CTE with A β subgroups (Table 3).

The CTE cohort is a heterogeneous group with multiple comorbidities, including a history of substance abuse (present in 44 % of subjects, $n = 94$) and symptoms of depression (65 %). To test whether these comorbidities may influence the association of A β with dementia, parkinsonism, and Lewy body disease, we performed a logistic regression analysis controlling for them. A β deposition was still significantly associated with dementia, parkinsonism, and Lewy body disease, but neither a history of substance abuse nor symptoms of depression were significantly associated (data not shown). Furthermore, to test whether these comorbidities are associated with A β deposition in the CTE cohort, we performed a logistic regression analysis with the presence of diffuse plaques as the dependent variable and found a positive association with age ($\beta = 0.081$, $p < 0.001$), but a non-significant negative association with a history of substance

Table 3 Clinical, exposure, and pathological findings in CTE with and without A β deposition

	CTE		CTE with DPs			CTE with NPs		
	<i>n</i>	Mean \pm SEM	<i>n</i>	Mean \pm SEM	<i>p</i> *	<i>n</i>	Mean \pm SEM	<i>p</i> *
Age at death (year)	55	48.2 \pm 2.6	59	71.6 \pm 1.4	<0.001	41	73.6 \pm 1.4	<0.001
Athletic exposure (year)	51	15.0 \pm 1.0	46	15.9 \pm 0.9	0.500	31	16.7 \pm 1.2	0.22
Concussion no.	48	11.5 \pm 3.0	41	24.2 \pm 6.6	0.071	28	17.5 \pm 5.4	0.99
Age at Sx onset (year)	43	36.2 \pm 2.3	42	54.2 \pm 2.3	<0.001	30	56.4 \pm 2.7	<0.001

	CTE	CTE with DPs		CTE with NPs			
	Freq (+/–)	Freq (+/–)	<i>p</i> **	<i>p</i> [^]	Freq (+/–)	<i>p</i> **	<i>p</i> [^]
CTE Sx	76 % (37/12)	90 % (43/5)	0.059	0.419	94 % (31/2)	0.028	0.164
Dementia	20 % (10/39)	73 % (35/13)	<0.001	0.013	82 % (27/6)	<0.001	0.012
Cognitive variant	58 % (23/17)	76 % (31/10)	0.067	0.382	76 % (22/7)	0.15	0.597
Parkinsonism	2.1 % (1/48)	25 % (12/36)	0.001	0.050	27 % (9/24)	0.010	0.113
LBD pathology	3.7 % (2/52)	35 % (19/35)	<0.001	0.019	42 % (16/22)	<0.001	0.009
TDP-43 pathology	67 % (37/18)	84 % (48/9)	0.030	0.810	93 % (38/3)	0.003	0.097

For statistical tests, CTE with DPs compared to CTE without DPs (labeled CTE in table) and CTE with NPs compared to CTE without NPs (data not shown)

DP diffuse plaques, NP neuritic plaques

* Student's *t* test, ** Pearson χ^2 test, ^ logistic regression controlling for age, significant *p* values are in bold

Table 4 CTE Stage and frequency of A β deposition by sport or military history

	<i>n</i>	Mean age at death (years)	CTE stage mean \pm SEM	<i>p</i> value*	APOE ϵ 4 % (+/–) ^a	DP freq (%)	NP freq (%)
Boxing	10	67	3.80 \pm 0.13	–	29 % (2/5)	70	50
Football	88	61	2.78 \pm 0.11	0.002	35 % (22/41)	51	34
Hockey	5	57	2.60 \pm 0.40	0.012	40 % (2/3)	40	40
Military	9	55	2.22 \pm 0.12	0.002	20 % (1/4)	44	33

DP diffuse plaques, NP neuritic plaques

* Mann–Whitney *U* test compared to boxing, significant *p* values are in bold

^a % of both ϵ 4 heterozygotes and homozygotes out of those cases with genotypes

abuse ($\beta = -0.762$, $p = 0.148$) and symptoms of depression ($\beta = -0.088$, $p = 0.874$). Results were similar for neuritic plaques suggesting that substance abuse and depression do not influence A β deposition in the CTE cohort.

Because forces associated with RTBI likely differ with the type of exposure, we hypothesized that A β deposition would also differ with the type of exposure. Thus we compared A β plaque deposition across CTE subjects with different exposure histories, specifically boxing, American football, hockey, and military combat (Table 4). Overall, we found that boxers in our series had a significantly greater CTE stage when compared to football players, hockey players, or military veterans (Table 4, Mann–Whitney *U* test). The boxers also had the greatest frequency of neuritic plaques (50 %) compared to football players (34 %), hockey players (40 %), and military veterans (33 %), although the difference was not significant. Similarly, boxers had a greater, but non-significant, frequency of

diffuse plaques compared to football or hockey players or military veterans (Table 4).

Discussion

Here we show that A β plaque deposition was present in 52 % of all subjects with pathologically diagnosed CTE in our series (mean age at death = 60 years). We find that A β deposition occurred at a younger age and at an accelerated rate in our cohort of subjects with a RTBI history and a neuropathological diagnosis of CTE when compared to a community-based autopsy series [2] (Fig. 1b). In addition, there were elevated levels of A β 1–40 within the sulcus compared to the gyrus in subjects with CTE-AD. When A β plaques were present in CTE, they were significantly associated with more severe tau and Lewy body pathology and worse clinical outcome independent of the effect of age.

While it is possible that the presence of A β simply represents the development of co-morbid Alzheimer's disease pathology, A β deposition occurred at an accelerated rate in subjects with a neuropathological diagnosis of CTE when compared to a community-based cohort (Fig. 1b), which suggests that exposure to RTBI or the presence of hyperphosphorylated tau might be a modifying factor in A β accumulation. In fact, a weighted two-sample Chi square test demonstrated that the age-dependent distribution of A β was significantly different in our CTE cohort compared to normal aging ($\chi^2 = 721$, $p < 0.001$), suggesting that RTBI is not simply accelerating an aging process, but also may be altering the normal dynamics of A β deposition. All the subjects in our CTE cohort were men, which is not the case in the normal aging cohort. However, men develop A β pathology at a significantly slower rate than women [2], suggesting that the actual increase in frequency of A β deposition in CTE may be greater than what we report. There was a high frequency of substance abuse history (44 %) and symptoms of depression (65 %) in the CTE cohort. However, neither of these comorbidities was significantly associated with A β deposition, suggesting that other factors drive plaque formation in CTE. Moreover, the pattern of A β deposition was altered in CTE-AD subjects with elevated levels of A β 1-40 at the sulcal depths compared to the gyral crests. This sulcal predilection of A β corresponds to regions of axonal injury in gyrencephalic animals after acceleration–deceleration injury [44], which may be a result of stress concentration at the sulcal depths following trauma [3]. Altogether, these findings suggest that repetitive mild traumatic injury may accelerate and alter A β accumulation and deposition.

The presence of A β was associated with disease progression in CTE. Subjects with neuritic plaques had a significantly increased stage of CTE tauopathy even when controlling for age. Although an association between neuritic plaques and advanced tau pathology might be expected given that neuritic plaques require tau-positive neurites and are associated with neurofibrillary tangles in AD, the pattern of tau pathology associated with the various CTE stages is unique, and an association between A β deposition and tauopathy severity in CTE has not previously been shown. We further found that levels of ptau231 in CTE were increased in the presence of A β . Along with this increase in tau pathology, there was a significantly higher frequency of dementia in the presence of A β with the age-adjusted odds of developing dementia in CTE 4.5 times higher in persons with A β pathology than without. Taken together, this suggests that CTE with A β pathology is a distinct subtype of CTE—one that has a more aggressive pathology and clinical course.

Deposition of A β is strongly associated with Lewy body disease, the formation of alpha-synuclein inclusions,

and higher levels of insoluble alpha-synuclein [11, 20]. In vitro studies have shown that A β promotes the formation of alpha-synuclein oligomers and polymers as well as inclusions in transfected cell lines [25]. In our CTE cohort we found a significant association of A β with LBD even when controlling for age. Moreover, the age-adjusted odds for developing parkinsonism was 8.9 times higher in CTE subjects with diffuse A β deposition compared to those without A β . Thus, A β may lead to Lewy body pathology and parkinsonism in addition to causing a more severe tauopathy in CTE.

The $\epsilon 4$ allele of *APOE* is a common genetic factor that appears to render individuals susceptible to A β deposition. The *APOE* $\epsilon 4$ allele is a major risk factor for Alzheimer's disease with homozygotes possessing a more than tenfold greater risk for developing AD dementia and heterozygotes a threefold greater risk [8, 26]. In clinical control populations, the $\epsilon 4$ allele is associated with biomarkers indicating greater A β deposition [35, 50]. Moreover, the $\epsilon 4$ allele is associated with increased A β levels following TBI [6], and several studies have demonstrated worse clinical outcomes in $\epsilon 4$ carriers following a traumatic brain injury or concussion [19, 21, 23, 48]. Here we show that the *APOE* $\epsilon 4$ allele was significantly associated with A β deposition in CTE. In fact, when subjects with A β were excluded, there was no significant difference in the proportion of $\epsilon 4$ alleles between CTE and population norms although our sample size was small. Although additional effects of the $\epsilon 4$ allele cannot be ruled out, it may be that the $\epsilon 4$ allele increases the likelihood of A β accumulation and deposition following RTBI and this, in turn, worsens the pathological and clinical outcomes.

Although CTE is a distinct disease, there are similarities to AD. Trauma has long been thought to be a risk factor for developing AD, and moderate to severe TBI has been shown to acutely increase A β levels and plaque formation [16, 36]. An increased percentage of Thioflavin-S-positive plaques has been shown in subjects many years after single TBI compared to age-matched controls, suggesting that even a single TBI may accelerate and alter A β deposition [18]. Axonal injury is a proposed source of elevated A β in acute and moderate to severe TBI, and we found increased A β at the depths of sulci in CTE where there is also evidence of significant axonal injury in animal models of mild TBI. This suggests that the axonal injury provoked by mild TBI might also alter the chronic deposition of A β in CTE. We found increased A β 1-40 at the sulcal depths compared to the gyral crests, but the level of A β 1-42 was unchanged in subjects with both CTE and Alzheimer's disease. The reason for this difference is unclear. Neuritic plaques are composed of both A β 1-40 and A β 1-42 while diffuse plaques are composed of A β 1-42 [5, 12, 14]. Therefore, the increase in A β 1-40 may result in more neuritic plaques in CTE.

Abundant genetic and experimental evidence suggests that A β can lead to the abnormal phosphorylation and accumulation of tau [1]. Our finding that the deposition of A β in CTE was associated with more severe tau pathology supports the hypothesis that A β can accelerate a tauopathy. These findings have important implications for diagnosing CTE before death. The interpretation of blood and cerebrospinal fluid biomarkers and positron emission tomography (PET) neuroimaging using both amyloid and tau ligands will need to consider the possibility of A β pathology in persons older than 40 with CTE (see Fig. 1).

There also may be differences in tau and amyloid pathology depending on the type of traumatic exposure. Studies in boxers using anti-A β immunohistochemistry with formic acid pretreatment demonstrated diffuse cortical A β plaques in 27 of 28 (96.4 %) subjects [39, 49]. In contrast to the original reports of CTE in boxers, the majority of cases in our cohort were football players. The nature, frequency, and intensity of traumatic impacts in different sports may lead to differences in CTE pathology. Here we show that boxers had a significantly greater CTE stage compared to football players, hockey players, or military veterans (Table 4). There was also a trend toward greater frequency of A β deposition in boxers although the difference was not significant, which may be due to the small sample size.

There are several limitations to our study. This is an autopsy-based study involving a heterogeneous cohort of individuals with a history of RTBI that came largely by self or family referral. The study subjects may not be representative of the entire RTBI population. Moreover, the clinical histories are retrospectively obtained and are subject to bias. Our subjects lived in the United States, and although the prevalence of AD does not vary much between the USA and European countries [47], it is unknown how differences in ethnicity may affect A β deposition. Ethnic differences have been shown to affect ϵ 4 allele frequency. For instance, a recent study showed ϵ 4 frequency increased with latitude [22]. We compared our cohort to a large meta-analysis that included data from multiple countries [28], and other US-based studies found similar ϵ 4 allele frequencies [7, 50], supporting the use of this control population. Future longitudinal, prospective studies are needed to address the frequency and type of pathologies present in population-based cohorts and to obtain less restrictive clinical histories and evaluations. Better quantitation of subject exposure to RTBI and correlation to in vivo biomarkers as well as neuropathological diagnoses at autopsy will be critical to clearly understand the role of RTBI on A β aggregation and deposition and CTE pathogenesis.

Overall, our work suggests that CTE with A β pathology is a distinct pathological subtype of CTE that shows a greater degree of tauopathy, a greater likelihood of LBD, and has a worse clinical outcome compared with CTE

without A β . RTBI may alter and accelerate the deposition of A β in some individuals, and A β , in turn, may accelerate tau and Lewy body pathologies and worsen the clinical course in CTE. It remains to be determined whether individuals susceptible to A β deposition can be identified in life with biomarkers, what genetic factors, in addition to *APOE* ϵ 4, may influence susceptibility to A β deposition, and what minimum burden of head trauma is necessary for induction of CTE and modulation of A β deposition.

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