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Exposure to repeated head impacts is associated with an increase in white matter perivascular macrophages in young individuals

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Thesis

**EXPOSURE TO REPEATED HEAD IMPACTS IS ASSOCIATED
WITH AN INCREASE IN WHITE MATTER PERIVASCULAR
MACROPHAGES IN YOUNG INDIVIDUALS**

by

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ABSTRACT

Neuroinflammation has been linked to the pathogenesis of many diseases, including chronic traumatic encephalopathy (CTE). CTE is a progressive neurodegenerative disease caused by exposure to repeated head impacts (RHI) from a variety of sources, including contact sports and military injury. CTE is characterized neuropathologically by the deposition of hyperphosphorylated tau (p-tau) in neurons as neurofibrillary tangles (NFT) and neurites at the depths of the cortical sulci in an irregular pattern. In addition to p-tau accumulation, there is also an accumulation of pigment-containing macrophages around small blood vessels in the white matter and widespread microglial inflammation in CTE. Macrophage and microglial inflammation can be beneficial to tissue repair, but if persistent, can precipitate neurodegeneration. This study quantified the density of perivascular CD68+ macrophages in the dorsolateral prefrontal (DLF) white matter, a brain region known to be affected early in CTE in post-mortem brain tissue from 46 individuals, 7 controls (mean age: 46.14, SD: 11.39, range: 22–55), 20 individuals exposed to RHI without CTE (mean age: 22.75, SD: 3.65, range: 17–29), and 19 individuals, all of them American football players, with pathologically verified CTE (mean age: 25.11, SD: 2.92, range: 18–29). Brain tissue was provided by the Injury and Traumatic Encephalopathy (UNITE) brain bank and the post-traumatic stress disorder

(PTSD) brain bank. Comparisons were made between controls, individuals exposed to RHI without CTE, and individuals with CTE. Fixed tissue samples of the DLF cortex and white matter were cut at 10 μ m and stained with CD68 to mark perivascular macrophages. Slides were imaged with a brightfield microscope at 40x magnification and analyzed using the HALO image software analysis platform. In the total population, a one-way test of variance (ANOVA) revealed a statistically significant increase in perivascular macrophages, indicated by CD68 positive pixels, in Stage III CTE compared to controls ($p<0.05$), a significant increase in Stage II compared to Stage I CTE ($p<0.05$), and a statistically significant increase in Stage III compared to Stage I CTE ($p<0.01$). The analysis also revealed a trend toward more CD68 pixels in Stage II CTE compared to controls ($p=0.0883$) and a trend toward more pixels in Stage III CTE compared to RHI no CTE ($p=0.0705$). Among the American football players, analyses revealed that Stage II CTE had significantly more perivascular macrophages than Stage I CTE ($p<0.01$), Stage II CTE had significantly more than controls ($p<0.01$), Stage III CTE had significantly more than Stage I CTE ($p<0.05$), and Stage III CTE had significantly more than controls ($p<0.05$). In summary, this study demonstrates that there is an increase in perivascular CD68 positive macrophages in individuals exposed to RHI with and without CTE. Perivascular macrophages and other neuroinflammatory molecules may play a critical role in the pathogenesis of CTE.

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LIST OF ABBREVIATIONS

AD.....	Alzheimer’s Disease
ANOVA	Analysis of variance
BU.....	Boston University
COD	Cause of death
CTE.....	Chronic Traumatic Encephalopathy
DAB	3’-3-diaminobenzidine
DB.....	Defensive back
DKS	Donkey serum
DLF	Dorsolateral prefrontal
FTLD.....	Frontotemporal lobar degeneration
LB	Linebacker
MRI.....	Magnetic resonance imaging
NFL.....	National Football League
NFT	Neurofibrillary tangles
NINDS/NBIB.....	National Institute on Neurological Disorders and Stroke/ National Institute on Biomedical Imaging and Bioengineering
NT	Neuropil threads
OL	Offensive lineman
P-tau	Hyperphosphorylated tau
PBS	Phosphate-buffered saline
PMI	Post-mortem interval

PTSD..... Post-traumatic stress disorder
RHI..... Repeated head impacts
TE..... Tight end
TES Traumatic Encephalopathy Syndrome
TX Triton
UNITE..... Understanding Neurologic Injury and Traumatic Encephalopathy
VA..... Veterans Affairs
WM..... White matter
WR Wide receiver

INTRODUCTION

Millions of people worldwide participate in contact sports, military duty, and other activities resulting in exposure to repetitive head impacts (RHI). Many studies have suggested that sustained RHI exposure causes the development of chronic traumatic encephalopathy (CTE). CTE is a progressive neurodegenerative disease that has been diagnosed in individuals as young as 17 years old. Currently, CTE can only be diagnosed postmortem upon neuropathological examination, and no interventions or therapies exist. The goals of ongoing research are to develop an *in vivo* marker of CTE and to understand better the disease mechanism to promote precautions and safety regulations to prevent RHI exposure.

History of CTE

In 1928, a pathologist, Dr. Harrison Martland, first noticed clinical alterations in boxers exposed to repeated head trauma. He described the neurological deterioration in boxers to be manifesting as cognitive, mood, and behavior issues which he called “punch drunk”(Martland, 1928). Dr. Martland observed that boxers who participated in more fights and took more hits often showed the most severe clinical symptoms (Martland, 1928). Therefore, he noticed the association between the severity and amount of repeated head trauma to the development of punch drunk. In 1937, Dr. Millspaugh coined the term “dementia pugilistica” as a more socially acceptable term for punch drunk (Millspaugh, 1937). After noticing that activities other than boxing, including but not limited to American football, ice hockey, soccer, interpersonal violence, and military blasts, were associated with the development of dementia pugilistica, Dr. Critchley deemed the term

progressive traumatic encephalopathy (Critchley, 1957). Later, chronic traumatic encephalopathy (CTE) became more widely used after the condition of a patient with traumatic encephalopathy did not improve. CTE refers to the diagnosis exclusively made post-mortem based on tissue analysis.

The first female diagnosed with CTE was a woman who had been physically abused for years by her husband. Her behavior and personality were described as “demented” as the abuse worsened over the years (Roberts et al., 1990). She presented with cauliflower ears and upon post-mortem neuropathological examination, she exhibited plaques and distribution of neurofibrillary tangles (NFTs) characteristic of CTE (Roberts et al., 1990). This case is critical because it is an example of exposure to RHI from a source other than contact sports leading to CTE development. The only other woman to have been neuropathologically diagnosed with CTE is a 24-year-old female with autism who participated in head-banging behavior (Hof et al., 1991). These case studies show that both sexes can develop CTE. There are more published cases of men with CTE because men tend to be involved in more risk-taking, physically dangerous activities that are prone to RHI; however, both sexes can develop CTE.

Clinical presentation of CTE

CTE refers to pathological alterations that may or may not be associated with clinical alterations. Since CTE cannot be diagnosed *in vivo* yet, traumatic encephalopathy syndrome (TES) is used to describe the clinical manifestations of exposure to RHI. A panel of 20 clinician scientists met in 2021 to agree upon diagnostic criteria for TES. They agreed upon four criteria of TES, the first three are required to make a definite

diagnosis and then the individual's level of functioning is graded. The first primary diagnosis criteria is substantial exposure to RHI that may or may not have been associated with clinical symptoms, concussion, or signs of traumatic brain injury (TBI) (Katz et al., 2021). The second primary diagnosis criteria is a progressive course of cognitive impairment, neurobehavioral dysregulation, or both. The third primary diagnosis criterion is that the pattern of progressive cognitive deficits is not fully accounted for by other established, preexisting, or acquired psychiatric, medical, or nondegenerative nervous system conditions or disorders (Katz et al., 2021). Lastly, the fourth diagnosis criterion is a level of functional dementia/ dependence from the impact of the cognitive deficits (Katz et al., 2021). It is important to note that a diagnosis of TES does not indicate a CTE diagnosis. Diagnosis of TES will improve as neuroimaging and other biomarkers of CTE pathology improve.

CTE can present with progressive clinical and behavioral symptoms. In 2009, Dr. Ann McKee reviewed the clinical and neuropathological alterations of the 45 previously published CTE cases and included additional CTE cases (McKee et al., 2009). Since then, there have been two National Institute on Neurological Disorders and Stroke/ National Institute on Biomedical Imaging and Bioengineering (NINDS/NIBIB) consensus meetings to define neuropathological criteria for the diagnosis of CTE. CTE is characterized clinically by behavioral and personality changes, dementia, gait disturbances, and parkinsonism (McKee et al., 2009). The first behavioral symptoms of CTE include deteriorations in memory and attention, disorientation, headaches, and dizziness (McKee et al., 2009). As the neurodegeneration progresses, additional

symptoms include dementia and poor judgment (McKee et al., 2009). Severe cases of CTE include tremors, impeded speech, vertigo, deafness, and slowing of muscular movements (McKee et al., 2009).

Neuropathology of CTE

The neuropathology of CTE is distinct from other neurodegenerative tauopathies, such as Alzheimer's Disease (AD) and frontotemporal lobar degeneration (FTLD). The first published article on the neuropathology of CTE was in 1959 by Dr. John Arthur Corsellis. He noted the defining features of CTE to be NFTs in the temporal cortex, noticeable separation of the septum pellucidum between the lateral ventricles, a decrease in pigment of the substantia nigra, and scarring in the cerebellum (Corsellis & Brierley, 1959).

In 2013, Dr. Ann McKee and colleagues analyzed the brains of 85 individuals. They developed criteria for a hierarchical staging scheme for the spectrum of disease seen in CTE based on the severity of hyperphosphorylated tau (p-tau) pathology. The scheme ranges from Stage I and Stage II (mild) to Stage III and Stage IV (severe). Stage I CTE is characterized neuropathologically by p-tau NFTs by blood vessels in the sulcal depths, mostly in the dorsolateral frontal and superior cortices (McKee et al., 2013). The most common behavioral manifestations of Stage I were headaches and loss of attention and concentration (McKee et al., 2013). In Stage II CTE, p-tau pathology spreads to the superficial layers of the cortex, amygdala, CA1 of the hippocampus, entorhinal cortex, hypothalamus, and the substantia nigra (McKee et al., 2013). There were also axonal

varicosities in the white matter (McKee et al., 2013). Additional Stage II behavioral symptoms include depression, mood swings, and short-term memory loss (McKee et al., 2013). Stage III pathology includes mild cerebral atrophy and extensive NFTs throughout the hippocampus, amygdala, nucleus basalis of Meynert, hypothalamus, mammillary bodies, substantia nigra, locus coeruleus, and the dorsolateral frontal, superior frontal, insular, septal, temporal pole, entorhinal, inferior orbital, and parietal cortices (McKee et al., 2013). In the white matter of the frontal and temporal cortices, distorted axonal profiles and severe axonal loss were reported (McKee et al., 2013). Stage III manifests clinically as memory loss, executive dysfunction, aggression, and explosivity. mood swings, depression, and aggression (McKee et al., 2013). In the most severe stage of CTE, Stage IV, there is significantly decreased brain weight, neuronal loss in the cortex, and widespread, severe p-tau pathology throughout the cerebrum, basal ganglia, spinal cord, and diencephalon (McKee et al., 2013). The distorted axonal profiles and axonal loss in the white matter are also more severe (McKee et al., 2013). Clinically, Stage IV is marked by severe cognition and executive function impairment, dementia, paranoia, and gait, visuospatial, and language difficulties (McKee et al., 2013). Unfortunately, the progressive pathology of CTE worsens the clinical features that may eventually lead to death.

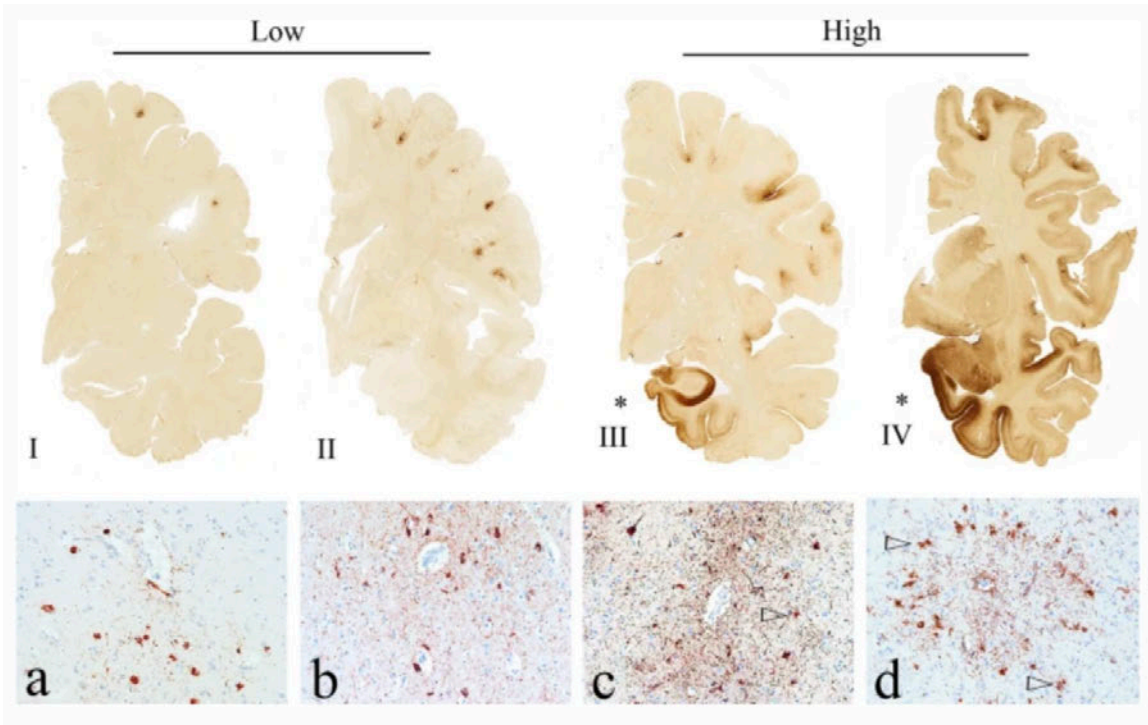


Figure 1 The pathognomic lesion in CTE across the hierarchical staging scheme. Representative pathology images in Stages I, II, III, and IV CTE. Asterisk in III represents neurofibrillary degeneration in the entorhinal cortex and hippocampus. Asterisk in IV represents neurofibrillary degeneration in the entorhinal cortex and amygdala. A–D all x200 magnification. (McKee et al., 2023)

As technology and science progressed, and more cases were studied, a more detailed criteria for CTE diagnosis emerged. The first NINDS/NIBIB consensus meeting in 2016 consisted of seven neuropathologists who blindly reviewed 25 cases. The panel agreed that there were similar features across all CTE cases. Those features include p-tau NFTs in neurons and astrocytes around blood vessels in the neocortex in an abnormal pattern, p-tau NFTs in the superficial layers of the cerebral cortex, and p-tau NFTs at the depths of cerebral sulci (McKee, Cairns, et al., 2016). Notably, the neuropathologists determined that the pathological lesions in CTE are distinct from other age-related tauopathies. Age-related tauopathies do not have p-tau astrocytes accompanied by p-tau

neurons in perivascular regions at the depths of cerebral sulci, which is diagnostic of CTE (McKee, Cairns, et al., 2016). Also, they used the earlier proposed McKee staging scheme to characterize individuals further. The consensus group met again to review and refine the pathological criteria and staging scheme proposed by the first consensus.

The second NINDS/NIBIB consensus in 2021 consisted of eight neuropathologists blindly reviewing 27 cases from a new broad range of sources to propose a model for the diagnosis of CTE. At this consensus, the McKee staging scheme was inconsistent, so they denoted CTE severity as “Low CTE” or “High CTE”. Low CTE consists of Stage I and Stage II CTE, while High CTE consists of Stage III and Stage IV CTE. The diagnosis was completed on a point scale performed on the most severe region of pathology; cases with less than five points were considered to be Low CTE, and cases with five or more points were High CTE (Bieniek et al., 2021). Each NFT present in the gyrus, superficial cortical layers, CA2 of the hippocampus, CA4 of the hippocampus, amygdala, mammillary body, thalamus, entorhinal cortex, and cerebellar dentate nucleus was counted as one point each (Bieniek et al., 2021). Therefore, Low CTE is characterized by NFTs in cortical regions, and High CTE has more widespread NFT pathology. The neuropathologists agreed with the previous consensus that the minimum threshold for CTE diagnosis was a single lesion of p-tau in neurons at the depth of a cortical layer around a blood vessel (Bieniek et al., 2021). These two consensuses have developed consistent diagnostic criteria based on post-mortem neuropathological examination for the diagnosis and severity of CTE.

CTE in sports

CTE gained national media attention when Dr. Bennet Omalu was the first to report CTE in a retired professional American football player in 2005. Given American football's significant impact on culture, Dr. Omalu's research greatly influenced the American public. Upon neuropathological evaluation of the former National Football League (NFL) player, he noted amyloid plaques, neuropil threads (NTs), and NFTs in the neocortex consistent with CTE (B. I. Omalu et al., 2005). An individual is considered an NFL player if they have played in at least one regular season NFL game.

In 2020, the largest group of American football players with post-mortem diagnosed CTE were analyzed. In this group of 366 male individuals, Spearman's rho correlations showed that a higher CTE stage (determined using the McKee CTE staging scheme) was associated with higher scores on p-tau severity and density and that the severity and distribution of p-tau was age-dependent (Alosco et al., 2020). Also, p-tau pathology was most prevalent across the individuals in the DLF cortex, superior temporal cortex, entorhinal cortex, locus coeruleus, and amygdala, with the lowest CTE stage and CTE in the youngest brain donors exhibiting pathology in the DLF cortex and locus coeruleus (Alosco et al., 2020).

CTE has also been characterized in individuals exposed to RHI from all levels of various contact sports, military service, and head-banging behaviors. In 2011, Dr. Omalu reported CTE in 7 out of 8 professional American football players, 2 out of 4 professional wrestlers, and one boxer (B. Omalu et al., 2011). Dr. McKee's 2013 examination of 85 individuals exposed to RHI from American football, ice hockey, military service, boxing,

and head-banging behaviors showed p-tau pathology consistent with CTE (McKee et al., 2013). Brain banks worldwide have reported CTE in the brains of bull riders, boxers, soccer, rugby, ice hockey, and American football players. CTE is important in the context of sports because it affects not only professional athletes but also athletes who played only in college, high school, or at a community level.

Differences between RHI and CTE

There has been a debate on whether the relationship between CTE and RHI is causal or associative. However, all neuropathologically diagnosed CTE cases have been reported to have some history of RHI exposure. Establishing causation is important to emphasize CTE mitigation efforts on a large scale. Recently, researchers evaluated studies on CTE in relation to RHI exposure, and by using the Bradford Hill criteria for causation, they concluded that RHI causes CTE (Nowinski et al., 2022). RHI is defined as head impacts from exposure to concussive and subconcussive events. Since RHI causes CTE, ideally, it can be prevented with proper safety efforts. The amount of time an individual is exposed to RHI, not the number of concussions and sub-concussions, is associated with the p-tau pathology characteristic of CTE (McKee et al., 2013). This isn't to say that CTE doesn't have more than one cause, but it is important to recognize the strength of the causal relationship between RHI and CTE.

With the number of young children participating in contact sports increasing, CTE has become a growing public health concern. Research suggests that the age at first exposure to RHI might play a role in the pathological and clinical response to RHI (McKee, Alosco, et al., 2016). If an individual begins being exposed to RHI in contact

sports early in life, they will accumulate more RHI if they remain in the sport. It also has been shown that RHI precedes CTE. Clinical symptoms of CTE tend to arise at least two years after the first exposure to RHI (McKee, Alosco, et al., 2016). Now that the causal relationship between RHI and CTE has been established, future efforts can focus on minimizing and eliminating RHI as a means to prevent CTE.

Dorsolateral prefrontal cortex implicated in CTE

Prior to this study, all large-scale neuropathological and clinical studies on individuals diagnosed with CTE had been conducted on older individuals (over the age of 45). Since this study is focused on younger individuals aged 17–29, we investigated a brain region where the earliest stages of CTE have been reported in the youngest individuals. The dorsolateral prefrontal (DLF) white matter is the region of interest in this study. Although the initial p-tau pathology is seen in the DLF gray matter, perivascular pigment-containing macrophages are often seen in the DLF white matter. In young individuals with CTE and individuals with Stage I CTE, p-tau pathology first arises in the dorsolateral, superior, or lateral frontal cortex (McKee et al., 2013). The early pathology in these regions leads to clinical symptoms such as lack of insight, poor executive function, and disinhibition (McKee et al., 2013). Since it has been shown that age and duration of exposure to RHI have roles in CTE development, looking at young individuals sheds light on some of the earliest changes that occur in CTE in the absence of common age-related comorbidities.

White vs. gray matter alterations in CTE

To date, most research on CTE has been performed on the gray matter; however, some recent studies have shown there to be white matter (WM) alterations such as axonal injury, myelin loss, and WM degeneration associated with CTE. An *in vivo* imaging study on men playing in the NFL showed that an increased volume of WM signal abnormalities detected by magnetic resonance imaging (MRI) was associated with higher cumulative head impact scores (Alosco et al., 2017). In the former NFL players, an increased volume of WM signal abnormalities was correlated with worse performance on tests of executive function and psychomotor speed, both functions implicated in CTE (Alosco et al., 2017). Since the primary pathology of CTE is the accumulation of p-tau at the depths of gray matter in the sulci, a study investigated possible WM alterations at those sulcal depths. Researchers found that the white matter adjacent to sulci with high levels of p-tau pathology had substantial axonal disruption (Holleran et al., 2017). WM refraction has also been detected in individuals with CTE. Among American football players diagnosed with CTE, more years of football play was associated with more severe WM refraction (Alosco et al., 2019). Worse WM refraction severity also correlated with an increased likelihood of dementia (Alosco et al., 2019). This proves that white matter is a novel target for future CTE therapeutics.

Perivascular macrophages in CTE

A proposed mechanism of the immune response to CTE is that upon RHI, the recruitment of inflammatory macrophages perpetuates disease propagation and inflammatory response. Initially, the recruitment of macrophages to a damaged area

removes dead tissue, promotes recovery, and prevents further infection. Macrophages also release reactive oxygen species and inflammatory cytokines. However, too much of this initially beneficial signal might be damaging. In CTE, p-tau NFTs accumulate near small blood vessels, which causes subsequent macrophage recruitment to the vessels because macrophages are recruited to areas of damage to reduce pathologic protein accumulation and repair the damage (Ransohoff & Brown, 2012). Once the macrophages are near the blood vessels, they can perpetuate neuroinflammation and further progress the pathology of CTE.

Animal models have been used to investigate many human diseases, including CTE. Rodents are biologically similar to humans and have been used to model CTE. These studies have shown that mice exposed to RHI develop p-tau deposition but not the pathognomic CTE lesion (Tagge et al., 2018). This could be because rodent brains are lissencephalic, so they are smooth and lack the sulcal depths susceptible to the pathognomic lesion humans develop in their gyrencephalic brains. A mouse model showed that experimental head impact injury induces traumatic microvascular injury, blood-brain barrier disruption, and secondary inflammation shown by perivascular macrophages, reactive astrogliosis, and perivascular microgliosis (Tagge et al., 2018). One of the four mice brains in that study showed p-tau indicative of early-stage CTE (Tagge et al., 2018).

The CD68 antibody was used in this research to mark perivascular macrophages. CD68 is associated with a more phagocytotic and neuroinflammatory type of macrophage (Rabinowitz & Gordon, 1991). A study on 224 neuropathologically diagnosed cases with

CTE showed a significant increase in inflammatory macrophage recruitment around blood vessels showing p-tau pathology in the DLF cortex compared to controls (Cherry et al., 2020). There were more macrophages around all blood vessels in high-stage CTE (Stage III and Stage IV) when compared to low-stage CTE (Stage I and Stage II) (Cherry et al., 2020). Central nervous system-derived macrophages also were recruited to the CTE pathognomonic lesion (Cherry et al., 2020). The controls had no history of exposure to RHI.

Notably, a case of a 17-year-old high school American football player with CTE showed perivascular clusters of activated macrophages around a small blood vessel in the subcortical white matter (Tagge et al., 2018). Researchers determined the perivascular macrophages to indicate resolved micro-hemorrhage (Tagge et al., 2018). Given this previous research, the mechanism of early immune cell recruitment, specifically perivascular macrophages, to areas of damage from RHI is of growing interest in hopes of reducing neuroinflammation and disease propagation.

Gaps in research

Most research on CTE has been performed on older individuals (over 45), but CTE has been reported in individuals as young as 17. This study explores novel targets investigating the role of age and length of exposure to RHI in CTE development, specifically in the white matter. There have been many studies on how CTE affects professional American football players. Still, the risk for CTE in young amateur contact sports athletes at the collegiate, high school, and local community levels have yet to be

determined. With more young people participating in contact sports and military combat, CTE is a prevalent public health issue.

This research aims to explore the earliest pathological changes that occur in the white matter of young individuals with CTE. I hypothesize that the duration of exposure to RHI will be associated with more CD68+ macrophages and that this will be further increased by greater severity of CTE. Since all individuals exposed to RHI in this study are under the age of 30, it is hypothesized that the inflammatory response of perivascular macrophages is among the earliest signs of disease. The amount of CD68+ perivascular macrophages (indicated by positive CD68 positive pixels) in the DLF white matter will be quantified across RHI exposure duration, CTE pathology, CTE stage, different sources of RHI exposure, and primary position in a cohort of young individuals.

Current research is guided toward finding biomarkers to diagnose CTE while individuals are alive. Understanding the progression of CTE in young individuals will allow biomarkers for low-stage CTE to be investigated. It is imperative to develop such biomarkers because it will allow precautions to be implemented while individuals are still young and will cease CTE progression.

METHODS

Human subjects

The RHI exposure groups (RHI no CTE and CTE) include 39 brains from individuals from ages 17–29 who donated their brains to the Understanding Neurologic Injury and Traumatic Encephalopathy (UNITE) brain bank from 2008 to the present day (Table 1). Each individual was evaluated by four board-certified neuropathologists (Dr. Ann McKee, Dr. Bertrand Huber, Dr. Thor Stein, and Dr. Victor Alvarez) for a complete neuropathological diagnosis of CTE, Alzheimer's Disease, frontotemporal degeneration, accumulation of Lewy bodies, and motor neuron disease based on previously established criteria for each disease (Bieniek et al., 2021). Phone and online interviews were conducted with informants to gather information on the donor's social/occupational histories, demographics, cause of death, athletic history (type of sport played, level of sport played, age of exposure, primary position played, and length of time playing each sport), neuropsychiatric history, medical history, and military history (branch, location of service, and length of time exposed to combat) (Stern et al., 2013). Researchers conducting these interviews were blinded to the neuropathological analysis results, and the donor's informants were interviewed before receiving the results of the neuropathological evaluation. Institutional review board approval for brain donation was obtained through the Boston University (BU) Alzheimer's Disease and CTE Center, the Human Subjects Institutional Review Board of the BU School of Medicine, and the Veteran's Association (VA) Bedford Healthcare System. Next of kin or the legally authorized representative provided written consent for individual brain donation and

participation in the study.

The presence and stage of CTE were diagnosed by the previously mentioned four board-certified neuropathologists. They utilized the established 2013 McKee CTE staging scheme ranging from Stage I and Stage II (mild) to Stage III and Stage IV (severe) based on the density and regional deposition of p-tau NFTs and neuropil neurites (McKee et al., 2013). In summary, Stage I CTE is characterized by one or two isolated perivascular areas of p-tau NFTs and neuropil neurites at the depths of the cerebral sulci in the frontal, parietal, or temporal cortices (McKee et al., 2013). In Stage II CTE, there are three or more neuropil neurites in more than one cortical region, and p-tau NFTs are present in the superficial cortical layers (McKee et al., 2013). Reactive microglia and axonal swelling are also present in the white matter in Stage II CTE (McKee et al., 2013). Multiple neuropil neurites, widespread degeneration of the entorhinal and perirhinal cortices, hippocampus, and amygdala, and diffuse p-tau in the frontal, insular, temporal, and parietal cortices are seen in Stage III CTE (McKee et al., 2013). In Stage IV CTE, p-tau NFTs and neuropil neurites are prolifically spread throughout the cerebral cortex, epithalamus, hypothalamus, thalamus, subthalamus, and brain stem (McKee et al., 2013). Neuronal loss, decrease in brain weight, white matter atrophy, myelin loss, and astrocytic p-tau pathology are also present in Stage IV CTE (McKee et al., 2013). Of the individuals in this study diagnosed with CTE, 17 (89%) of the individuals were diagnosed with mild CTE (Stage I or Stage II), and 2 (11%) of the individuals were diagnosed with severe CTE (Stage III or Stage IV).

Donors were excluded if their post-mortem interval (PMI) was greater than 106

hours or the quality of the tissue was poor. PMI was rounded up to the nearest hour if it ended in 30 or more minutes, and it was rounded down to the nearest hour if it ended in less than 30 minutes. Cases were excluded from analysis if they met the neuropathological criteria for comorbid conditions such as AD, Lewy body disease, Parkinson's disease, or FTLD. Also, cases were eliminated if there were incomplete brain fragments or if the individual's cause of death was hypoxia because hypoxia initiates a neuroinflammatory response that provides a potential confound to this study.

Cohort design

The three sex and age-matched cohorts are as follows — one group diagnosed with CTE based on neuropathological consensus (“CTE”), one group exposed to RHI with no CTE diagnosis (“RHI no CTE”), and a control group with neither CTE diagnosis nor RHI exposure (“Control”). Inclusion criteria for the RHI group included exposure to RHI (e.g., contact sports, domestic violence, military service) regardless of clinical symptoms. RHI exposure and severity were determined using the cumulative head index, which combines individual self-reported measures of athletic exposure and objective measures based on position(s) played, which were obtained from previously published studies.

Table 1 Demographic and exposure characteristics of the total cohort. Data are expressed as mean \pm standard deviation in the age at death and contact sports exposure rows and expressed as n in the remaining rows.

*no COD or PMI information was available for the control cohort.

	Control	RHI no CTE	Stage I CTE	Stage II CTE	Stage III CTE	Total
n	7	20	10	7	2	46
Age at death (years)	46.14 \pm 11.39	22.75 \pm 3.64	25.40 \pm 2.46	23.86 \pm 3.39	28.00 \pm 1.41	27.28 \pm 9.63
Contact sports exposure (years)	0 \pm 0	9 \pm 5.09	10.80 \pm 3.29	9.83 \pm 3.55	14.00 \pm 3.24	8.12 \pm 5.42
PMI (hours)		39.53 \pm 22.98	60.0 \pm 33.55	34.25 \pm 21.52	23	43.44 \pm 26.63
Race						
White	2	15	6	5	2	30
Black	1	2	3	1	0	7
Hispanic	3	0	0	0	0	3
Other	1	2	1	0	0	4
COD						
Suicide		14	7	3	1	25
Accidental		5	2	1	0	8
Overdose						
Injury		1	0	1	0	2
Drown		0	0	1	0	1
Cardiovascular		0	1	0	0	1
Other		0	0	0	1	1
Highest Level of Play						
Youth	n/a	0	0	0	0	0
High School	n/a	13	3	2	0	18
College	n/a	5	6	4	1	16
Professional	n/a	0	1	1	1	3
Primary Exposure						
None	7	0	0	0	0	7
Football	0	9	10	7	2	28
Ice Hockey	0	3	0	0	0	3
Wrestling	0	1	0	0	0	1
Soccer	0	3	0	0	0	3
Veteran	0	1	0	0	0	1
Other	0	3	0	0	0	3
Sex						
Male	7	17	10	7	2	43
Female	0	3	0	0	0	3

Primary position at highest level of play

Tight end	n/a	2	0	0	1	3
Linebacker	n/a	0	5	3	1	9
Defensive back	n/a	0	2	1	0	3
Quarterback	n/a	1	0	1	0	2
Offensive lineman	n/a	3	1	0	0	4
Wide receiver	n/a	2	0	0	0	2
Forward	n/a	2	0	0	0	2
Winger	n/a	1	0	0	0	1
Midfielder	n/a	2	0	0	0	2
Multiple	n/a	2	2	1	0	5

Controls

The control group consists of 7 brains from individuals ages 22–55 who donated their brains to the post-traumatic stress disorder (PTSD) brain bank at the Jamaica Plains VA (Table 1). The PTSD brain bank collects human tissue from Veterans and non-Veterans with PTSD diagnoses presently or in the past. The inclusion criteria for controls in this study were no exposure to RHI, no CTE diagnosis, and no other neurodegenerative disease diagnosis. Control cases were excluded if they met the neuropathological criteria for comorbid conditions such as AD, Lewy body disease, Parkinson’s disease, or FTLTD.

Region of interest

The region of interest was the white matter of the DLF cortex. The DLF cortex was selected because it is often one of the first regions affected by CTE pathology. CTE in the youngest donors and lowest stage CTE have shown p-tau deposition in the DLF cortex (Alosco et al., 2020). Gray matter pathology in CTE has been quantified, while white matter pathology has not yet been quantified.

Preparation of DLF cortex sections

All brain tissue that arrives at the UNITE and PTSD brain banks is processed by fixation in periodate-lysine-paraformaldehyde and stored at 4°C for 24–48 hours prior to embedding. To embed the tissue in paraffin, it is first rinsed with running tap water for one hour. Then, it is dehydrated with 70%, 80%, and 95% ethanol, respectively, for 45 minutes in each concentration, followed by three washes in 100% ethanol for one hour for each wash. The slides then go into two washes of xylene for an hour each. Then, the tissue goes into three paraffin washes for an hour each, is embedded in a paraffin block, and stored at room temperature. Paraffin-embedded blocks containing the DLF cortex were cut into 10µm thick sections using an Eprelia HM 325 Rotary Microtome and floated in a bath of 40°C distilled water before being put on glass microscope slides.

Immunohistochemistry

The DLF cortex slides underwent single-labeled immunohistochemistry. Slides were deparaffinized, starting in three washes of xylene for five minutes each. Then, the slides were transferred to 100% ethanol for two washes for five minutes each. The slides were then transferred to one wash of 95%, 70%, and 50% ethanol, respectively, for five minutes each. Slides were rinsed with distilled water and put in the citrate antigen retrieval solution (pH = 6.0). The sections were boiled at 100°C in the citrate antigen retrieval solution to expose the antigen for labeling. Once the slides cooled to room temperature, they were washed with distilled water twice for two minutes each time. To reduce nonspecific background staining, slides were incubated with 3% donkey serum (DKS) in 0.4% phosphate-buffered saline (PBS)-Triton (TX) for 30 minutes. After

blocking, slides were washed with PBS twice for two minutes each. All sections were then incubated with 1:15,000 Anti-CD68 (Abcam) in 1% DKS in 0.4% PBS-TX for one hour at 20°C. Sections were washed with PBS three times for two minutes each. Then, sections were incubated with 1:250 Anti-rabbit secondary antibody in 1% DKS in 0.4% PBS-TX for 30 minutes at 20°C. Sections were then washed again with PBS three times for two minutes each. Then, 3'3'- diaminobenzidine (DAB) chromagen was added to the sections at room temperature until suitable staining developed, which usually took around 5–10 minutes. After being rinsed with distilled water, sections were coverslipped using a Permount mounting medium.

Microscopy

Immunostained slides were scanned in brightfield and digitized at 40x magnification using the Vectra Polaris System. They were visualized using a brightfield Olympus Thermo Fisher microscope at 40x magnification with a numerical aperture of 0.95 and a resolution of 261 nm. The average area of the ROI is 175905218.62 μm^2 .

Perivascular macrophage criteria

The inclusion criteria for a vessel in the white matter were a clear, defined lumen and an area of at least 200 μm^2 at 40x magnification. Borders of approximately 5 μm were included around each vessel to accommodate the perivascular macrophages.

Image analysis

The slides were annotated using the Indica Laboratory HALO image analysis platform to differentiate the white and gray matter. The white matter of the slides was analyzed, while the gray matter was disregarded. HALO was also used to generate an algorithm to delineate the vessels in the white matter of the slides. After each vessel was annotated, another algorithm was created to generate an output of the percentage of positive pixels (indicated by positive Anti-CD68 staining) in the vessels in the white matter. The algorithm used color and intensity thresholds to detect the Anti-CD68 immunostaining as positive pixels and background staining as negative pixels. The number of positive pixels was normalized to the number of total pixels to account for variations in the vessel size.

Statistics

All statistical analyses were performed with SPSS version 27.00 (IBM) and Prism v9 (GraphPad Software). Linear regression showed that PMI and age at death had no effect on the density of CD68 positive pixels. In addition, one-way ANOVA and Bonferroni post hoc analyses exhibited that cause of death (COD), race, and sex had no effect on the density of CD68 positive pixels. Therefore, they were not treated as covariates when performing statistical analyses. In the total population, one-way ANOVA and Bonferroni post hoc analyses were performed to test the differences between the density of CD68 positive pixels between the groups (Control, RHI no CTE, and CTE), across CTE stages, and between the various routes of primary exposure to RHI. Similarly, in the football population, ANOVA and Bonferroni post hoc analyses

were executed to compare the density of CD68 positive pixels between the groups (Control, RHI no CTE, and CTE), across diagnosed CTE stages, between the highest level of football played, and between the primary position played at the highest level.

Shapiro-Wilk testing demonstrated that the total years of play had a normal distribution in the total population and the football population and were sufficient to perform linear regression analysis. Linear regressions were performed with total years of play in the total and football population as independent predictor variables and density of CD68 positive pixels as the dependent variable.

RESULTS

Perivascular Macrophage Morphology

Imaging at 40x revealed CD68+ macrophages infiltrating into the perivascular space in all groups (Figure 2).

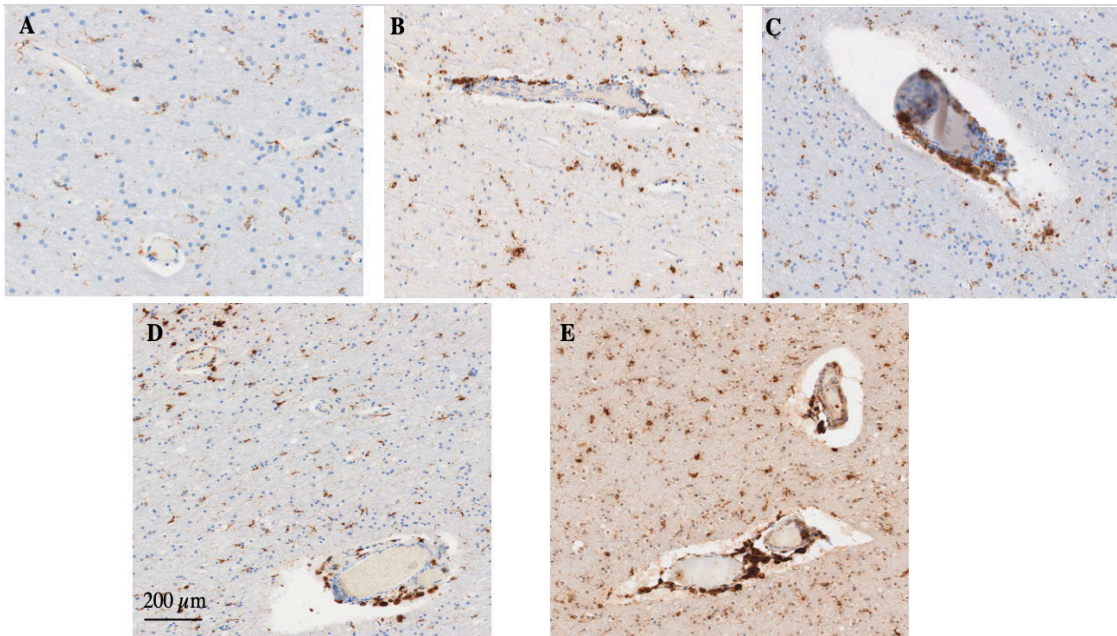


Figure 2 CD68+ perivascular macrophage morphology between groups.

Representative images of CD68+ macrophages infiltrating perivascular space in the white matter of the DLF cortex across groups. All images were taken at 40x magnification. A) 22-year-old male control, scale bar 200 μm . B) 29-year-old male with RHI exposure, but no CTE pathology, scale bar 200 μm . C) 29-year-old male with Stage I CTE, scale bar 200 μm . D) 26-year-old male with Stage II CTE, scale bar 200 μm . E) 27-year-old male with Stage III CTE, scale bar 200 μm .

Trend of increased density of CD68 positive pixels associated with increased years of play in total population

A linear regression was performed on the total years of play in the total population to determine if it was associated with the density of CD68 positive pixels in the vessels. There was a trend towards the increased density of CD68 positive pixels associated with increased total years of play ($r^2= 0.072$, $p=0.094$) (Figure 3).

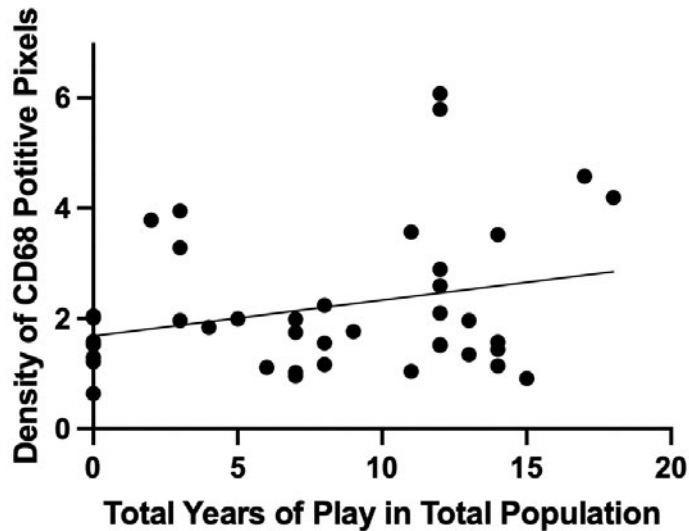
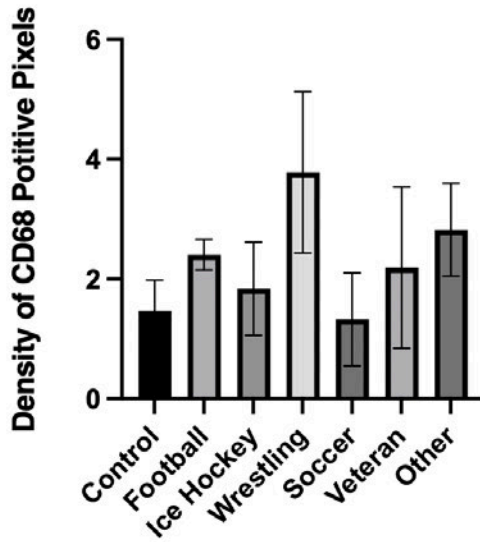


Figure 3 Trend of the density of CD68 positive pixels increasing with more years of play in the total population. Linear regression analysis revealed a trend toward increased CD68 positive pixel density associated with increased total years of play. The line represents the regression line ($r^2=0.072$, $p=0.094$).

Density of CD68 positive pixels not associated with different routes of primary exposure in the total population

To investigate whether different primary routes of exposure to RHI were associated with the density of CD68 positive pixels in the vessels, an ANOVA was performed across the various routes of exposure in the total population. There were no statistically significant differences between any of the routes of exposure ($p=0.416$) (Figure 4).



Primary Exposure in Total Population

Figure 4 Comparing the density of CD68 positive pixels across primary routes of exposure to RHI in the total population. One-way ANCOVA and Bonferroni post hoc analysis were conducted across the routes of exposure, and none of the differences were significant ($p=0.416$).

Perivascular macrophage density increases as CTE pathology becomes more severe in the total population

To test the hypothesis that exposure to RHI would be associated with increased perivascular macrophage density, a one-way ANOVA and Bonferroni post hoc analyses were performed across the groups in the total population. Stage III CTE had significantly more perivascular macrophages than controls ($p<0.05$) (Figure 5). Also, there was a statistically significant increase in CD68 positive pixels in Stage II compared to Stage I CTE ($p<0.05$) and a statistically significant increase in Stage III compared to Stage I CTE ($p<0.01$) (Figure 5). Analysis revealed a trend toward more CD68 pixels in Stage II CTE compared to controls ($p=0.0883$) and a trend toward more pixels in Stage III CTE compared to RHI no CTE ($p=0.0705$) (Figure 5). However, there were no statistically

significant differences between RHI no CTE and control ($p=0.5677$), Stage I CTE and control ($p=0.988$), Stage I CTE and RHI no CTE ($p=0.3737$), Stage II CTE and RHI no CTE ($p=0.4642$), and Stage III CTE and Stage II CTE ($p=0.2484$) (Figure 5).

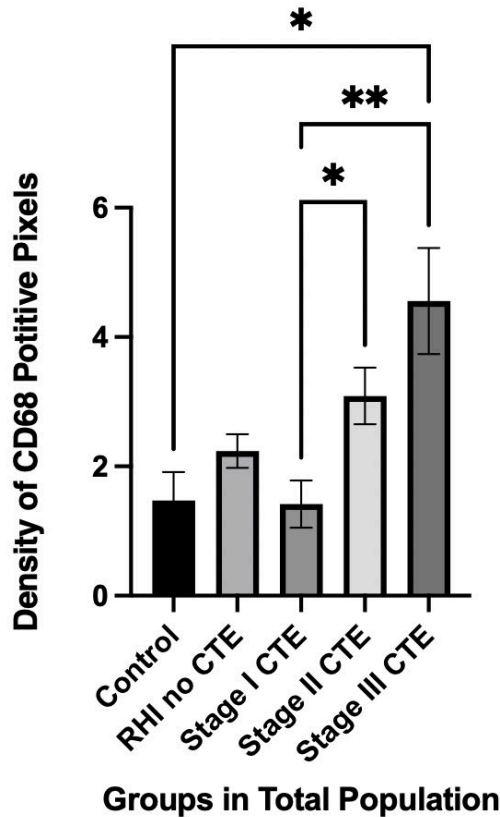
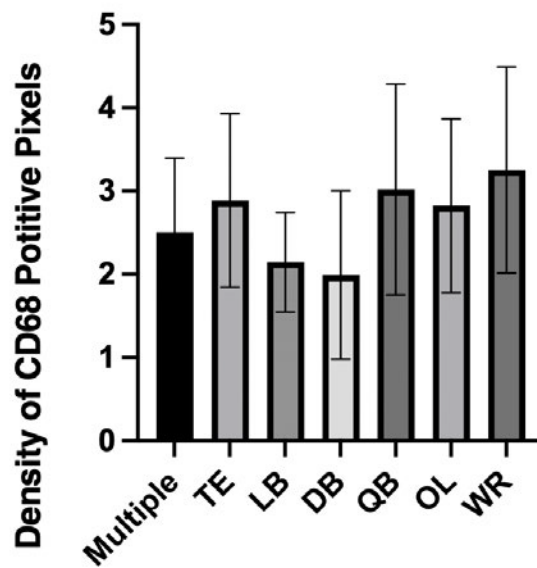


Figure 5 Density of CD68 positive pixels increases as CTE pathology becomes more severe in total population.

One-way ANOVA and Bonferroni post hoc analysis were conducted. Increased density of positive pixels in Stage III CTE compared to controls ($p<0.05$). Increase in CD68 positive pixels in Stage II compared to Stage I CTE ($p<0.05$) and a statistically significant increase in CD68 positive pixels in Stage III compared to Stage I CTE ($p<0.01$). Trend toward more CD68 pixels in Stage II CTE compared to controls ($p=0.0883$) and a trend toward more pixels in Stage III CTE compared to RHI no CTE ($p=0.0705$). No statistically significant differences between RHI no CTE and control ($p=0.5677$), Stage I CTE and control ($p=0.988$), Stage I CTE and RHI no CTE ($p=0.3737$), Stage II CTE and RHI no CTE ($p=0.4642$), and Stage III CTE and Stage II CTE ($p=0.2484$).

Density of CD68 positive pixels is not associated with primary position played in the football population

An ANOVA analysis was performed across the primary positions played in football to investigate if it was associated with the density of CD68 positive pixels. None of the differences in CD68 positive pixels between positions were statistically significant ($p=0.963$) (Figure 6).



Primary Position Played in Football Population

Figure 6 Graph quantifying the CD68 positive pixels in the vessels across different primary football positions played. One-way ANOVA and Bonferroni post hoc analysis were conducted across the positions, and none of the differences were significant ($p=0.963$).

Perivascular macrophage density increases as CTE pathology becomes more severe in the football population

Similarly to the total population, it was hypothesized that exposure to RHI would be associated with an increased amount of perivascular macrophages, indicated by CD68 positive pixels, in the football population. A one-way ANOVA and Bonferroni post hoc

analyses were performed across all groups. The analyses revealed that Stage II CTE had significantly more CD68 positive pixels in the vessels than Stage I CTE ($p < 0.01$), Stage II CTE had significantly more than controls ($p < 0.01$), Stage III CTE had significantly more CD68 positive pixels when compared to Stage I CTE ($p < 0.05$), and Stage III CTE had significantly more than controls ($p < 0.05$) (Figure 7). However, there were no significant differences between Stage III and Stage II CTE ($p = 0.2693$), between Stage III CTE and RHI no CTE ($p = 0.2020$), between Stage II CTE and RHI no CTE ($p = 0.2175$), between Stage I CTE and RHI no CTE ($p = 0.2916$), between Stage I CTE and controls ($p = 0.990$), or between RHI no CTE and controls ($p = 0.4322$) (Figure 7).

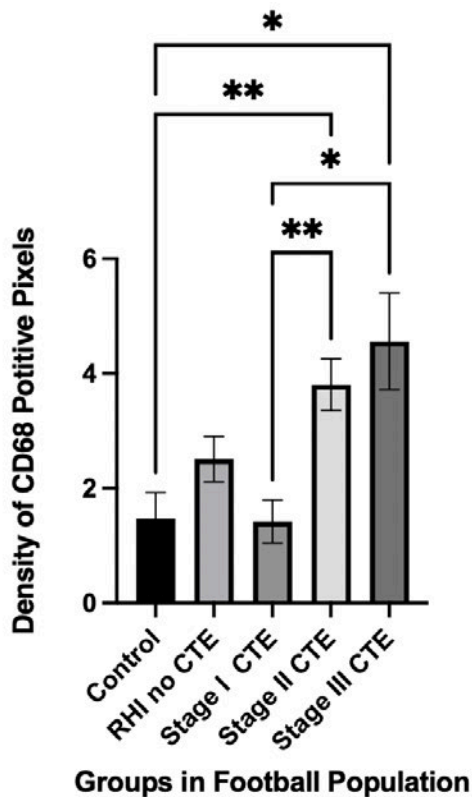


Figure 7 Density of CD68 positive pixels increases as CTE pathology becomes more severe in the football population. One-way ANOVA and Bonferroni post hoc analysis were conducted. Stage II CTE had significantly more CD68 positive pixels in the vessels

than Stage I CTE ($p < 0.01$), Stage II CTE had significantly more pixels than controls ($p < 0.01$), Stage III CTE had significantly more CD68 positive pixels when compared to Stage I CTE ($p < 0.05$), and Stage III CTE had significantly more pixels than controls ($p < 0.05$). No significant differences between Stage III and Stage II CTE ($p = 0.2693$), between Stage III CTE and RHI no CTE ($p = 0.2020$), between Stage II CTE and RHI no CTE ($p = 0.2175$), between Stage I CTE and RHI no CTE ($p = 0.2916$), between Stage I CTE and controls ($p = 0.990$), or between RHI no CTE and controls ($p = 0.4322$).

Increased density of CD68 positive pixels associated with increased years of play in the football population

To test the hypothesis that more years of playing football would be associated with an increased density of CD68 positive pixels in the vessels, a linear regression was performed. The linear regression revealed a significant association between more CD68 positive pixels associated with more years of playing football ($r^2 = 0.125$, $p = 0.047$)

(Figure 8).

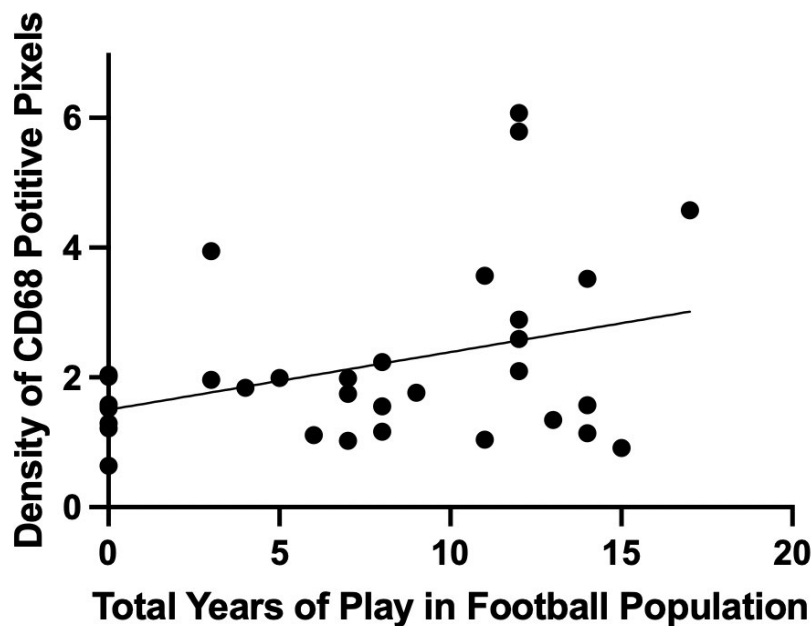


Figure 8 Plot of the density of CD68 positive pixels increasing with more years of play in the football population. Linear regression analysis revealed increased CD68 positive pixel density associated with increased total years of play. The line represents the regression line ($r^2 = 0.125$, $p = 0.047$).

DISCUSSION

CTE p-tau pathology is initially focal, perivascular, and cortical and becomes more widespread and severe as the disease progresses (McKee et al., 2023). Neuroinflammation has been implicated in CTE pathogenesis. Previously published studies have shown that microglial inflammation is increased after RHI and increases with CTE severity (Cherry et al., 2016). Macrophages detect and phagocytose potentially harmful foreign particles, and have anti-inflammatory or pro-inflammatory properties. They are known to wrap around blood vessels and extend their filopodia through the vessel wall into the blood vessel lumen (Lapenna et al., 2018). In CTE, perivascular macrophages could have beneficial effects, such as clearing axonal damage; however, they could also have harmful effects, such as exacerbating inflammation resulting in disease progression. This study used CD68 to stain perivascular macrophages in the white matter of the DLF cortex in young individuals. More perivascular CD68+ macrophages (measured by the density of CD68 positive pixels) were associated with greater CTE severity and greater total years of play in the total and football population. Additional findings in this study demonstrate no significant difference between types of RHI exposure and the density of CD68 positive pixels. This study also showed a trend of increasing perivascular macrophages in Stage II CTE compared to controls and a trend toward more perivascular macrophages in Stage III CTE compared to RHI no CTE in the football population. However, those differences were not statistically significant in the total population. In the American football players, the density of CD68 positive pixels was not significantly associated with the highest level of play or the primary position

played.

The total population in this study included RHI exposures from various sources, including American football, ice hockey, wrestling, soccer, and military injury. In 2015, Montenigro et al. conducted a study investigating the phenotype of CTE between different primary sports exposures. They found that impacts from boxing cause greater strain to the midbrain and cerebellum, resulting in a greater frequency of motor symptoms in confirmed CTE cases than those from American football players (Montenigro et al., 2015). The types of head impacts and forces athletes endure differ by the contact sport, which is why this study created a subset of just American football players from the total population to study further independent variables and keep the primary exposure the same. American football was chosen because it was the most common form of exposure (n=28).

In both the total and American football populations, this study established an increase in the density of CD68 positive pixels as CTE pathology became more severe. There were significantly more CD68 positive pixels in Stage II compared to Stage I CTE and when comparing Stage III to Stage I CTE in both population groups. In the American football population, Stage III and Stage II CTE had significantly more perivascular macrophages than controls. In the total population, Stage III CTE had significantly more perivascular macrophages than controls, and there was a trend towards more perivascular macrophages when comparing Stage II CTE to controls and when comparing Stage III CTE to RHI no CTE. However, the difference in perivascular macrophages between Stage III and Stage II CTE, RHI no CTE and controls, Stage I CTE and controls, Stage I

CTE and RHI no CTE, and Stage II CTE and RHI no CTE were not statistically significant in either population. This result shows that increased neuroinflammation is associated with a higher CTE Stage, as evidenced by increased density of perivascular CD68. It can be hypothesized that the CD68 perivascular macrophages are one mechanism by which CTE pathology spreads and becomes more severe because as the CTE stage progresses, there are more perivascular macrophages. Perivascular macrophages have been implicated in multiple pathological central nervous system conditions. Beyond their beneficial role of detecting and phagocytosing potentially harmful agents, perivascular macrophages have been shown to promote inflammation (Lapenna et al., 2018). Uncontrolled inflammation often contributes to disease progression. In a mouse model of induced allergic encephalomyelitis, Polfiet et al. demonstrated that infected mice had an increased number of perivascular macrophages in the brain (Polfiet et al., 2002). Upon selectively eliminating the perivascular macrophages in the brain, the disease progression was reduced (Polfiet et al., 2002). Considering this and the results of this study, the downregulation of perivascular macrophages might be a promising therapeutic target to reduce CTE progression.

This study also found a significant positive linear regression trend towards more CD68 positive macrophages associated with more years of play in the total sample and the American football players. Years of play serve as a proxy for cumulative RHI exposure. Although it is not a perfect measurement, as, for example, an athlete playing as a starting offensive lineman for a year may have more RHI exposure than a punter who rarely played for a year. It is important to note that since this study was conducted on

young individuals, the years of exposure are less than that of previous studies on CTE pathology in middle-aged and older individuals. This study shows that even in young individuals with relatively few years of RHI exposure, there is increased neuroinflammation as years of play increase. These findings emphasize the importance of years of exposure, no matter how few, in CTE pathology. In a sample of 266 individuals, Mez et al. reported a direct correlation between the years of American football played and the odds of developing CTE (Mez et al., 2020). Mez et al. found that for every 2.6 years of American football played, the odds of developing CTE double (Mez et al., 2020). Both neuroinflammation, indicated by an increased amount of perivascular macrophages, and odds of developing CTE are directly related to the time playing contact sports.

In addition, this study reports no significant difference in CD68 positive pixels between the various forms of exposure to RHI in the total population. All forms of exposure to RHI might be indifferent in terms of neuroinflammation, but future studies should research this further and include a larger sample size from each form of exposure.

Since sports positions and the highest level of play should not be compared between multiple sports because they differ, those independent variables were just tested in the American football population. The density of CD68 positive pixels was compared across the reported highest levels of play and the primary position played among former American football players. Both analyses revealed no significant differences. In 2015, Bieniek et al. reported no significant difference in CTE pathology in American football players across different highest levels of play (Bieniek et al., 2015). Therefore, the

finding that CD68 perivascular macrophages are not associated with varying highest levels of American football play is consistent with the literature. Likewise, Mez et al.'s 2020 publication surveying 266 former American football players reported no association between primary American football position and CTE pathology (Mez et al., 2020). The finding of this study is consistent with that as well.

In conclusion, these results demonstrate that changes in perivascular macrophages may play a role in the pathology of CTE in young individuals. Perivascular macrophages have proven to be involved with more severe CTE pathology and increased years of play.

Limitations of the current study

A significant limitation of this study is the small sample size which greatly reduces the statistical power and could result in a false negative, type II error. There were only seven controls, and their age range was older than the rest of the sample. As individuals get older, comorbidities such as AD, FTLN, and Lewy Body Disease become more common, which is a potential confounding factor. Another limitation of this study is the small sample size of individuals with RHI exposure from sources other than American football. There are n=3 ice hockey and soccer players and n=1 former wrestlers and veterans. Those sample sizes are too small to make a significant comparison between them.

Future directions

The findings reported in this study that more CD68 perivascular macrophages are associated with more severe CTE pathology and more years of play warrant further investigation. The limitations of the current study could be readily addressed in future studies by increasing the sample size of each group and getting a younger and larger control population. This study showed that even in the youngest individuals with the earliest CTE stages, CD68+ macrophages infiltrate the perivascular space in the white matter. This study shows that perivascular macrophages might be important in the diagnosis or therapeutic targets of CTE.

Future research should continue to research the clinical and neuropathological alterations in young individuals because they may differ from older individuals. Investigating the earliest disease mechanisms of CTE in young individuals will allow us to understand the consequences of RHI exposure better. Many children participate in contact sports beginning at an amateur level, and RHI prevention and safety precautions are necessary.

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