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CCL11 as a Biomarker for the In Vivo Diagnosis of Chronic Traumatic Encephalopathy

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**CCL11 as a Biomarker for Assessing the Development of Chronic Traumatic
Encephalopathy in Athletes Exposed to Years of Repetitive Traumatic Brain Injury**

A Thesis Presented

By

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Of Claremont Mckenna, Pitzer, and Scripps Colleges

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Senior Thesis in Neuroscience

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ABSTRACT

Chronic traumatic encephalopathy is the neurodegenerative disease that is ascribed to the long term development of cognitive, behavioral, emotional, and motor deficits as a result of the exposure to high amounts of sub concussive traumatic brain injuries. The disease has gained recent popularity in the media for its prevalence in American football as a response to recent research that has suggested the prominence of the disease in nearly every NFL player that is examined post mortem. This has produced a growing concern for the consequences of head impact and participation in contact sports. Despite media attention, little is currently known about the specific causes of the disease and an in life diagnosis is still nonexistent. The present study proposes that the chemokine, CCL11, could prove to be a viable biomarker for recognizing the onset and progression of chronic traumatic encephalopathy. The results of our study suggest that football players who are clinically suspicious of CTE show significantly higher levels of CCL11 in their cerebrospinal fluid than do sedentary controls and noncontact athletes. Our results demonstrate that this increase in CCL11 is correlated with the number of years that a football player had participated in. We also suggest that this increase in CCL11 is associated with a unique immune response through results showing that the CCL11 expression increase is correlated with an increase in the expression of the cytokine IL-4 and substantial decrease in IFN-gamma. The analysis of CCL11 expression levels in the cerebrospinal fluid may prove to be a viable method of diagnosing and providing treatment for patients who may be at risk of chronic traumatic encephalopathy.

INTRODUCTION

Chronic traumatic encephalopathy (CTE) is the neurodegenerative disease characterized by the behavioral and neuropathological symptoms resulting from exposure to recurring head impacts. CTE is most notably associated with tau pathology and the accumulation of neurofibrillary tangles (NFTs) and astroglia, particularly around smaller cerebral arteries and veins at the depths of sulci (Mckee et al. 2013). While a multitude of neurological disorders associate with tau inclusions, there are particular patterns and regional formations of NFTs specific to characterizing Chronic traumatic encephalopathy (Mckee et al. 2016). In fact, the National Institute of Neurological Disorders and Stroke and the National Institute of Biomedical Imaging and Bioengineering have had an ongoing project these past recent years, known as the UNITE project, which has been funded to validate the neuropathological criteria used in the diagnosis of CTE. Because other neurodegenerative diseases like Alzheimer's have very similar clinical presentations with CTE, they often share copathologies, it has become an important area of study to present ways to discriminate CTE from other diseases that express similar neuropathological symptoms. Therefore, biomarkers to discriminate CTE from similar neuropathological diseases will show necessary to aid in diagnoses and treatment of the disease.

Research on chronic traumatic encephalopathy has often focused on the study of athletes who have been exposed to repetitive head impacts (RHIs) as a result of participating in sport. Experimental case reports have ascribed the term CTE to athletes who have been exposed to traumatic brain injuries (TBIs) and exhibited cognitive problems, headaches, a multitude of mood disorders, suicidal ideation, and aggressive behavior (Meehan et al. 2015). Post mortem analysis of athletes who exhibit a variation of these

cognitive deficits have shown a trend of pathological findings. The juxtaposition of these cognitive deficits with post mortem analysis of brain tissue exhibiting specific neural pathological trends has shaped sciences characterization of what has been come to be known as CTE (Meehan et al. 2015). The disease was first described by a pathologist named Harrison Martland who termed the phrase “punch drunk” after noticing specific cognitive and motor deficits occurring in prized boxers around 1928. A more professional medical diagnoses to describe the deficits observed in boxers arose the condition known as dementia pugilistica, in 1937 (Gavett et al. 2011). As the neuropathological research grew and the symptoms coincided with RHIs, the term chronic traumatic encephalopathy was termed by Henry Miller in 1966 (Miller. 1966). The prevailing studies of CTE in recent research have continued to focus the study of CTE in sports athletes, very often in American football players. Unlike other diseases known to attribute contact sports to acute symptoms in the brain, like in post-concussion syndrome, CTE occurs as a later developing disease, oftentimes decades after exposure to RHI (Gavett et al. 2012). Studies done on athletes like that done by Stern and colleagues in 2013 have shown three major of symptoms associated with athletes exposed to RHI: cognitive, behavioral, and mood impairments (Stern et al. 2013). The wide range of expressed defecits associated with the disease have caused some concern in the ability to specifically attribute specific symptoms to the disease (Perrine et al. 2017). Instead, the distinction of the disease has rested on the post mortem evaluation of brain tissues that show extensive tau pathology in athletes who have suffered from one of a variety of these neurological symptoms. With such varying symptoms being expressed in the diagnoses of the disorder across studies, and the overlapping neuropathological indicators of

the disease with other disorders, the study of CTE has many challenges but is still seeing progression.

The clinical features of chronic traumatic encephalopathy are unique to the evaluation of the disease. While the correlation between cognitive dysfunction and exposure to multiple cumulative subconcussive blows to the head has been observed since Martlands “punch drunk syndrome,” the specific clinical signs of CTE have been closely studied in experiments done by Mckee et al. and other researches of recent. The syndrome is often characteristic in early stages as presenting problems with immediate memory, confusion, headaches, and attention deficits. However, CTE has been known to express rather insidious symptoms in its later stages, as the disease often takes multiple years to progress (Mckee et al., 2013). While the rate of the progression varies among patients, there is often a developing worsening of mental abilities, this including: poor judgment, further impaired confusion and disorientation, and an onset of antisocial behaviors (Baylock & Maroon, 2011). Extreme cases of CTE often show clinical symptoms in which patients express highly depressive, often suicidal, trends, as well as high levels of aggressive behavior (Mahar et al. 2017). A number of these symptoms are characteristic of poor prefrontal cortex executive function and limbic system dysfunction (Baylock and Maroon, 2011). This is characteristic of the diseases patchy cortical distribution mostly affecting superficial cortical layers. In its most severe cases, CTE has been known to spread wide throughout the CNS, often affecting areas like the brainstem and spinal cord which can result in the development of dysarthria, dysphagia, impaired speech and motor movements, tremors, and even deafness (Baylock and Maroon 2011). The lack of extensive and longitudinal studies involving patients with CTE leaves a gap in the ability to directly correlate these clinical symptoms with the disease.

The neurophysiology of brain injury is important to understanding the disease chronic traumatic encephalopathy. Research suggests that it is the acceleration to deceleration force (A/D) that initiates a pathophysiologic process of progressive trauma that defines TBI injury (Gaetz 2004). It is a noticeable characteristic of CTE to not be necessarily correlated with the number of concussions or severity of head blows, but rather, the repeated exposure to subconcussive head blows is what leads to the altered neurophysiology (Breedlove et al. 2012). It may thus be suggested that CTE could correlate to the number of blows sustained (Breedlove et al. 2012). The effect of these A/D forces to the head result in sheer strain within the cranial vault which causes stretching and sheering of neurons and blood vessels(Gaetz 2004). However, it is an area of focus in CTE to examine secondary axotomy specifically to understand how neurodegeneration from TBI can occur over a prolonged period of time. Diffuse axonal injury (DAI) is often considered the affecting factor that influences the altering of the neurophysiology of neurons in patients with TBI exposure (Su and Bell 2016). That is, what occurs in secondary brain injury is often the cause of long term dysfunction of the brain on the molecular level. The lasting excitotoxicity, apoptosis, ischemia, inflammation, mitochondrial dysfunction, and basic neurodegeneration that exists after injury is the defining influence in CTE. According to Su and Bell, DAI is the process of progressing, widespread axonal damage as a result of TBI that is attributed to the multitude of injuries, like inflammation, that result from secondary brain injury (Su and Bell 2016).

The neuropathology of CTE has been an important topic of examination in the detection and diagnoses of the disease. Tauopathy has been the strongest and most relevant indicator of the disease. The hyperphosphorylation of tau and the build up of neurofibrillary tangles (NFTs), as well as glial tangles and astrocytic inflammation, have been defining

factors in the diagnosis of the disease in studies examining post mortem neural tissue. It has been shown that the accumulation of NFTs in neurodegenerative disease correlates with the onset of cognitive decline, however, the exact mechanisms of this process are still to be understood (Spires-Jones et al. 2008). It has been suggested, however, that these tangles may contribute to the secondary brain injury symptoms of apoptosis, in which neural cells die due to impeded cell function as a result of these aggregates of protein clusters (Calcul et al. 2013). Although the list of diseases with tau inclusions stretches wide, the unique distribution of tangles and the perivascular specific accumulation of NFTs appears to distinguish the disease from those that express indistinguishable tau isoforms, like in Alzheimer's disease (AD) (Perrine et al. 2017). Besides the unique accumulation of ptau in CTE diagnosis, other indicators like lower levels of neuritic plaques and NFT formation absent of Abeta deposition have been shown to be unique qualities found to distinguish the disease from other disorders (Geddes et al. 1999). CTE has also shown neurofibrillary degeneration localizing primarily in sulcal depths in the frontal and temporal cortices, superficial cortices, and subpial areas (Mckee et al. 2009). Distinguishing the specificity of neuropathology in CTE compared to other diseases is important in the identification and the diagnosis of the disease. Especially because behavioral and cognitive symptoms can vary greatly among those diagnosed with CTE, attention to the unique neuropathology of the disease will greatly aid in the understanding, prevention, and diagnosis among individuals who are exposed to RHI.

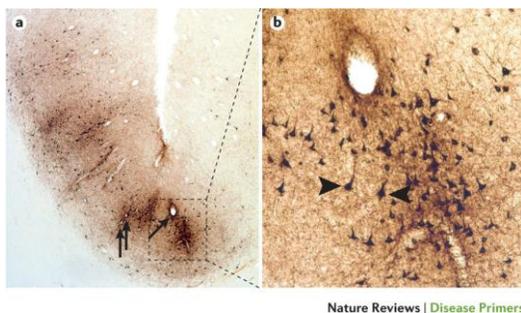


Figure 1. Accumulation of hyperphosphorylated tau in an irregular pattern, clustering specifically at the depths of the cortical sulci. Image taken from (Blennow et al. 2016)

The specific neuropathological features of CTE have evolved into the proposal of individual progressive stages of the disease, intended to specify and allocate diagnosis across individual cases. Mckee et al. proposed in 2013 that the neuropathological features that appear in the progression of degeneration can be broken down into four stages. Levels of atrophy and both NFT accumulation and location are used to diagnose the disorder into one of the four categories: Stage 1 of CTE, as defined by Mckee et al., expresses normal weight brains, NFT accumulation in the superior and dorsolateral frontal cortices, immunoreactive glia, microglia clusters, and axonal swelling. The progression to stage 2 shows NFTs scattered throughout the superficial cortical layers, enlarged third ventricles, and cavum septi pellucidi and pallor of the locus coeruleus and substantia nigra. Stage 3 is diagnosed as a reduction in weight due to substantial increase in atrophy throughout the brain and further ventricular dilatation. NFT accumulation spreads throughout the cortical areas as well as into the olfactory bulbs, amygdala, hippocampus, hypothalamus, and the substantia nigra. Stage four is diagnosed as the most severe case of CTE and is seen as a severe reduction in brain weight, due to atrophy, septal abnormalities, complete depigmentation of the locus coeruleus and substantia nigra, and severe p-tau pathology spreading to the basal ganglia, brainstem,

and spinal cord (Mckee et al. 2013). While the work by Mckee and colleagues can be useful to our understanding of CTE, many limitations to the stage identification still exist, especially in the lack of correspondence between clinical symptoms and the neuropathological diagnoses.

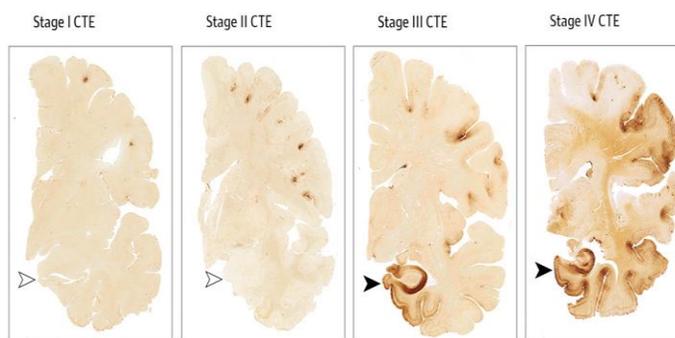


Figure 2. Pathological distinction of the four stages of CTE, as proposed by Mckee et al.. Table used from Mckee et al. *The spectrum of disease in chronic traumatic encephalopathy.* (Mckee et al., 2013)

While research on CTE has focused on comparing known cognitive deficits with specific neuropathological characteristics found in port mortem brain analysis, there still does not exist a clinical diagnosis for this condition (Barrio et al. 2015). For example, the disease gained a lot of attention in the media recently as a result of studies done on American football players by Mez and colleagues. Mez conducted a study on 202 deceased football players of varying levels of play and found that up to 87 percent expressed neuropathologically diagnosed CTE (Mez et. al, 2017). This study suggested the high relation between CTE and prior participation in football. Studies done on correlations between football players and TBI have suggested that there are multiple characteristics of the sport that could influence the development of CTE. For example, a study done by Stamm and colleagues showed that the age at which exposure to football starts in an individual correlates

with altered corpus callosum white matter microstructure as well as later developing cognitive impairment, in retired football players (Stamm et al., 2015). The cumulative repetitive mild traumatic brain injuries characteristic of the sport have been shown to make CTE have a well known association with the sport (Mez et al., 2017).

Research into in vivo diagnosis of the disease to help prevent and control long term neural deficits in active athletes who may be exposed to further mild TBIs is a topic still of high interest. For example, efforts like that taken by Barrio and colleagues in 2015 have suggested a way to obtain in vivo characterizations of the disease, specifically in American football players. Barrio suggests the use of PET imaging and imaging agents, like [F-18]FDDNP, to establish topographic brain locations of insoluble protein aggregates in athletes known to have suffered from years of RHIs and mTBIs. The protein marker used, [F-18]FDDNP, however, is not CTE specific and binds to β -amyloid aggregates, which are more prominent in Alzheimer's disease and may occur prominently in aging brains, as well (Teng et al. 2011). The benefit of protein binding agents like FDDNP is that it can cross the blood brain barrier and allows for in vivo analysis without relying on potentially harmful invasive techniques, like probing. Other techniques, like fmri analysis, suggests other ways to use imaging to conduct non invasive analysis of the brain for subjects exposed to a history of mTBIs. A study done by Little and colleagues, for example, used fmri imaging to look for correlations in brain tissue volume of subjects exposed to a history of TBI versus a control (Little et al. 2014). The problem with imaging techniques to utilize neurobiological assessment to ultimately diagnose patients with chronic traumatic brain injuries, however, rests in the lack of advancement in our current imaging tools. Little and colleagues, for example, suggest that we need an imaging modality that meets all of four criteria: accessible

and safe in patients with acute injury, imaging equally sensitive to the range of injury severity that we see in the disease, equally sensitive to the transition from acute to chronic development, and appropriate for the identifying the earliest of pathological symptoms as they transition to being a neurodegenerative disease (Little et al. 2014). Without these criteria met, the use of imaging techniques as a practical and efficient way to help diagnose and prevent further injuries in TBI cases is hindered in its ability to identify who will potentially be at risk for neurodegeneration (Little et al. 2014). For this reason, other methods of identification of the disease, like identifying a biomarker in the blood or cerebrospinal fluid of patients with the neurodegenerative disease, are still being considered.

Chemokines are small proteins that stimulate the recruitment of leukocytes, they act as secondary pro-inflammatory mediators (Graves and Jiang, 1995). Essentially, chemokines and their receptors are able to mediate the action and response, as well as the residence, of all immune cells (Palomino and Marti, 2015). Currently, there has been approximately 50 endogenous chemokines ligands and 20 G-protein-coupled receptors of these proteins. The interaction between these chemokines, and the immune cells that they coordinate with, allows for the triggering of a series of biochemical and cellular coordinated events during immune response (Griffith et al., 2014). The physiological significance of chemokines as mediators is derived from their specificity. Unlike other leukocyte chemo-attractants, members of the chemokine family, like CCL11, are known to induce recruitment of well-defined leukocyte subsets (Graves and Jiang, 1995). Many chemokines are considered pro-inflammatory and can be released as a result of an immune response at a site of infection (Palomino and Marti, 2015). The chemokine CCL11 is a part of one of two families of chemokines, known as CC or beta chemokines (Palomino and Marti, 2015). CCL11, as a

chemoattractant responsible for eosinophil recruitment, has been shown to play a large role in the response to tissue injury and lung dysfunction (Monchy et al. 1985).

CCL11 is a chemokine, also known as eotaxin-1, that was first identified as an eosinophil chemoattractant found in the peripheral immune system in allergic inflammation, asthma, atopic dermatitis, and inflammatory bowel disease (Cherry et al. 2017). Although it has been identified as a main factor in the periphery, the cytokine has been shown to be penetrable to the blood brain barrier (Baruch et al. 2013). Also important to CCL11 is that it has been shown in mice brain that microvascular pericytes secrete these chemokines, like CCL11 as an immune response to inflammatory insults in cultures of astrocytes, pericytes, and microglia (Kovac, Erickson, and Banks 2011). CCL11 has been shown to have age related increases in both humans and mice. The age related component of CCL11 is important because CCL11 levels are also known to associate with the decrease of neurogenesis in mice brains, specifically decreasing the number of Dcx-positive cells in the dentate gyrus (Villeda et al., 2011). Some highlighted features of the chemokine CCL11 by Villeda and colleagues shows that the plasma blood levels of the protein are associated with a decrease in neurogenesis and an impairment of learning and memory. This suggests that the decline in neurogenesis and cognitive impairments can be linked with changes in blood-borne factors, like the expression of CCL11 (Villeda et al., 2011). The noticeable connection between the increase in systemic chemokine levels having a direct effect on the central nervous system is important for the present study.

The role of chemokines like CCL11 has been extended to research in humans. The increased regulation of 6 chemokines, including CCL11 were found to be expressed in significantly higher numbers in the cerebrospinal fluid of patients who suffer from

neuropathic pain, compared to healthy controls (Backyrd et al., 2017). These findings suggest a causal relationship between the presence of inflammation in neural disorders due to the activity of these microglial related chemokines in the reactivity to neuroinflammation during neuronal dysfunction. CCL11 has also been shown to play a role in bone resorption, Kindstedt and colleagues found that endogenous CCL11 was uptaken by osteoclast and played a key role in osteoclast stimulation and concomitant increase during bone resorption (Kindstedt et al., 2017).

The role of CCL11 in the diagnosis of CTE is critical because of the lack of biomarkers that are available for in vivo characterizations of the disease. Chronic traumatic encephalopathy has recently been gaining fame for its effect on individuals exposed to rTBI, especially athletes. However, this correlating diagnosis between brain defects and rTBI has been solely based on post-mortem evaluations of the brains of diseased athletes. Therefore, for our understanding of the disease to help promote in life diagnosis of CTE, identifying a biomarker that can accurately identify the existence of the disease will provide the ability to save lives and prevent further neurodegeneration.

CCL11 presents itself as an ideal biomarker for CTE as it has already been shown to present significantly increased levels of expression in in vitro examinations of brain tissue of deceased athletes with the disease, compared to that expressed in both controls and deceased patients with Alzheimer's disease(AD) (Cherry et al., 2017). Cherry and colleagues took brain slices from the dorsolateral frontal cortex of deceased athletes who were exposed to years of rTBI and clinically diagnosed with CTE. Comparatively, they took these measurement using enzyme linked immunosorbent assay in 23 of these athletes to compare against measurements from 18 non athlete controls as well as 50 individuals diagnosed with

AD. The study found significant increases in CCL11 fold changes in the DLFC of diagnosed CTE patients, but also, importantly, found significant increases of the chemokine expression in the cerebrospinal fluid of CTE patients, compared to that of both controls and AD patients (Cherry et al., 2017). The study also found that CCL11 levels were closely correlated to the RHIs received by the athletes in the study, and also correlated to the phosphorylated tau present in the brain examination (Cherry et al., 2017). The results of this study are especially significant because they suggest a biomarker that is expressed differently in CTE as it is with AD, two neurodegenerative diseases known to be very similar in their pathological symptoms and clinical presentation. It is therefore still unclear and still to be studied why CTE and AD express levels of CCL11 differently.

A study done by Baruch and colleagues has suggested an important aspect among brain function that upon immune response, the choroid plexus (CP) releases CCL11 into the CSF (Baruch et al. 2013). This increase of CCL11 was shown to be correlated with the expression of the ratio between two other cytokines in the CSF (Baruch et al., 2013). Baruch and colleagues suggested that the expression of the cytokine IL-4 (interleukin-4) was greatly increased in the CP of older mice, compared to that of the cytokine, IFN- γ (interferon gamma) (Baruch et al., 2013). The relationship between these two cytokine expression may play a role in the upregulation of CCL11 found in CTE patients as the expression of IFN- γ has been shown to be elevated in the brains of patients with AD (Belkhefha et al., 2014). The results of these studies suggest hypotheses for the observed upregulation of CCL11 in CTE patients compared to similar pathological diseases, as well as controls. It could be suggested that high levels of IL-4, when not properly balanced by sufficient levels of IFN- γ , could lead to the accumulation of CCL11 expression in chronic traumatic encephalopathy (Villeda et al.,

2011). The specific factors that these two cytokines play when expressed in the body have been previously described as a mutual antagonistic relationship (Paludan, 1998). This antagonistic relationship between the two cytokines is defined by the role of IL-4 in promoting T cell type 2 differentiation, and inhibiting T cell type 1 differentiation, while IFN- γ has been shown to have the defining role of inhibiting type 2 cell differentiation, and type 1 cell promotion (Paludan, 1998). Therefore, the expressed ratio of these two cytokines within the body can have major implications as to the type of immune activity being expressed in an individual. It can be suggested that the measurable expression of a cytokines like IL-4, IFN- γ , and CCL11 can give insight to the existence of a specific immune response occurring within a patient expressing neurodegenerative symptoms.

CCL11 expression may therefore lead to cognitive impairment when it is upregulated in response to the shifting immune response in CTE. Baruch and colleagues suggest that the upregulation of CCL11 coexpressed with high levels of IL-4 and low levels of IFN- γ , likely have a role in the cognitive impairments seen in aging (Baruch et al, 2013). It could be suggested that the TBI induced CCL11 expression in the CSF, along with immune response changes due to inflammation specific to TBI, specifically in the ratio of IL-4 to IFN- γ , could result in the cognitive impairment expressed by CTE patients with high exposure to TBIs. The upregulation of CCL11 in both aging and TBI exposure could suggest a similar neurodegenerative process occurring in both CTE as well as healthy aging. Research suggests a similar change in immune cell regulation that is uniquely analogous in both aging and chronic neurodegeneration specific to repetitive head impacts. Therefore, the expression of these cytokines could provide insight into the potential risk of a patient exposed to rTBIs for developing CTE.

The activation of chemokines into the CSF in response to TBI has already been suggested by studies done by Szmydynger-Chodobska and colleagues (Szmydynger-Chodobska et al., 2011). The assumed correlation suggests that the release of chemokines like CCL11 may be the result of a neuroimmune response expressed in response to TBI. The resulting up regulation of CCL11 in CTE may thus be the result of progressive CCL11 production in the CP accumulating from multiple TBIs, providing the chronic accumulation of CCL11 levels expressed in the CSF (Cherry, Olschowka, and O'Banion, 2014). The correlation between reactive astrocytes and microglia being prominent in CTE pathology and the ability of these cells to release chemokines, may also suggest insight to the observed difference in the CCL11 expression in CTE compared to AD (Cherry et al., 2017). Research on the clinical and pathological representation of CTE and studies showing the unique functions of the chemokine CCL11, provide the foundations for the present study. Considering the recent suggested results of Cherry and colleagues, that the chemokine is significantly expressed in higher amounts in post mortem analysis, identifies CCL11 as an ideal biomarker to detect for diagnosing CTE using in vivo examination of patients with exposure to rTBIs.

Several complications arise from the potential use of CCL11 as a biomarker for in vivo diagnosis of CTE. The chemokine has often been found to be associated with age dependence in mice studies conducted to identify the distinct pathological role that CCL11 plays in immune responses and expression in the CP, brain tissue, and CSF. In the study conducted by Cherry and colleagues, the samples of brain tissue used to measure the expression of CCL11 in CTE patients were all taken from deceased athletes who were 50 years of age or older. As mentioned previously, plasma levels of CCL11 have been shown by Villeda et al. to be expressed in much higher levels in patients aged between 65-90 than

patients aged 25-50 (Villeda et al., 2014). The effect of age may therefore play a variable role in the expression of CCL11 in patients who are seeking diagnosis of CTE. Further research would benefit from the examination of the differences in CCL11 expression in patients of varying age who have been exposed to comparatively equal levels of rTBI. In the comparative study between CTE and AD done by Cherry et al., CCL11 levels in patients with CTE were only demonstrated to differ from that of deceased controls as well as deceased AD patients. CTE is well known to share copathologies with multiple other diseases. Therefore, the use of the biomarker CCL11, with the further examination of the coexpression of other symptoms using imaging techniques, like PET or fmri, as well as correlating with years played, clinical symptoms, and amount of TBIs exposed to, will help in the in vivo diagnosis of CTE.

HYPOTHESIS

The development of the neurodegenerative disease, CTE, has been a research based on post mortem examination of brain tissue. The dangers of the disease for individuals with a history of repetitive head impacts has gained recently progression recognition, however, a reasonable and noninvasive way to diagnose the disease during life has yet to be suggested. The chemokine CCL11 serves as a potentially ideal biomarker for the in vivo diagnosis of CTE. The in vivo measurement of CCL11 in the cerebrospinal fluid of athletes with a history of exposure to rTBI could serve as a reliable indicator for the diagnosis and prevention of CTE. The present study hypothesizes that athletes who are at risk of CTE due to their exposure to high amounts of TBI in contact sports for an extended period of time will express higher levels of the chemokine CCL11, as measured using ELISA, in their CSF

compared to athletes without exposure to rTBI as well as sedentary controls. The study further hypothesizes that this increase CCL11 expression is the result of a unique immune response occurring, which will be suggested by measuring higher expression levels of the ratio between the two Cytokines IL-4 and IFN- γ . We hypothesize that the increased expression of this ratio will correlate with the participants expression of CCL11, and that CCL11 expression will correlate with the number of years participating in american football.

METHODS

Patients

A total of 81 participants (24 American football players, 27 competitive swimmers, and 30 controls) were recruited for a clinical assessment and a spinal tap procedure as part of the study assessing biomarker expression in athletes exposed to repetitive mTBI. All participants were males in the age range between 25-33. Participants were recruited based on a minimal involvement in sport, at least 10 years, versus controls who were designated based on a sedentary lifestyle (no involvement in athletic sports), however, involvement in sport ranged between subjects (10-22 years participation). Patients with 10+ years of exposure to american football underwent a lumbar puncture to provide a CSF sample, due to their clinical suspicion of CTE. Participants with 10+ years exposure to competitive swimming, as well as the sedentary controls, were given a complete disclaimer to the potential side effects of the invasive procedure. Therefore, participants gave full consent to undergo lumbar puncture for the experimental comparison of CCL11 levels in their CSF. All participants were given incentive to participate in the experiment by receiving a 100 dollar cash compensation after undergoing the procedure.

Participants in the football group were recruited based on retirement from high school, college, and NFL football teams and were selected based after undergoing a clinical assessment. Age matched control participants were recruited from on an online ad and completed the clinical assessment before taking part in the experiment to ensure they matched the age and sedentary/swim requirements. Swimmers were selected as having been engaged in competitive swim for a minimum of 10 years.

Clinical assessment

A clinical assessment was performed by each subject prior to participation in the study. General information including athletic history, education, demographic information, drug use, sleep, and health were assessed during an interview with the researchers.

Participants proceeded to fill out two forms used to determine TBI history, similar to the evaluation used by (Cherry et al., 2017): the Ohio State University TBI Identification Method Short Form (Corrigan and Bogner, 2007) and the executive functioning self report assessment developed by Seichepine and colleagues (Seichepine et al. 2013). Scores on the TBI assessments were used to denote a clinical suspicion of CTE. Participants in the football group who signed up for the study but did not show evident problems with executive functioning, as measured in clinical assessment, were disqualified from participating in the study.

Spinal Tap

A standardized lumbar puncture was performed on the 81 participants in the study in order to extract cerebrospinal fluid for analysis. 10 mL of CSF were extracted using an atraumatic needle and aseptic techniques with all subjects in the sitting position as done similarly by Rembach and colleagues (Rembach et al., 2015). Patients were locally

anesthetized before inserting the atraumatic hollow needle into the subarachnoid space in the lumbar area (between L4/L5). Upon insertion into the subarachnoid space 10 mL of CSF were collected by gravity flow into a polypropylene tube. The CSF was then quickly moved to ice (4° C) and it was centrifuged for 10 minutes at 2,000 x g as done by Rembach and colleagues (Rembach et al., 2015). The resulting supernatant produced about 300 µL of product, which was transferred into a separate 1.5 mL polypropylene tube and held at -80°C to be used for further analysis.

CSF testing and CCL11 measurement (ELISA)

Obtained CSF supernatant was thawed to room temperature to be used in the analysis of targeted biomarker expression. The supernatant was run through an enzyme-linked immunosorbent assay (ELISA) kit for analytical analysis. CSF was run undiluted using an R&D Quantikine Human CCL11 ELISA kit as explained by the R&D systems assay procedure protocol. This CSF biomarker assessment is similar to that used by Cherry and colleagues in their post mortem analysis of CCL11 expression in football players (Cherry et al. 2017). Two other kits were used to measure the expression of two other cytokines, IL-4 and IFN- γ . Kits from R&D systems were also used for this assessment, Human IL-4 protein, 6507-IL kit and Human IFN-gamma protein, 285-IF kits were used to assess expression of each of these chemokines separately, in individual participants. The wells of the ELISA kits come pre coated with the target-specific antibody for each selected chemokine in the supplied microplates. This precoating of the ELISA kits allows for controlling variability that could result from performing other kit methods that would require applying consistent levels of the specific antibody. Therefore, when a specific amount of each sample of CSF are added to the wells, a proportional measure of the chemokine expression can be measured through

the created enzyme-antibody-target complex signal produced once a substrate solution is added.

According to R&D Systems product details, the Eotaxin Quanktikine ELISA kit used is a Solid Phase Sandwich ELISA. It has a 96-well strip plate with an incubation time of 3.5 hours. The process can be used to measure relative mass values for natural human Eotaxin. Therefore, ELISA allows for a measureable comparison of each of our target Cytokine expression levels across participants in each group.

Stats

Cytokine expression levels were baselined using mean expression levels obtained from sedentary lifestyle, age matched participants who served as controls in the experiment. Mean cytokine expression for this group was averaged and used to measure fold changes of biomarker expression in the TBI and non-TBI control groups. All stats were run using SPSS. CCL11 Fold changes were compared using one way analysis of covariance (ANOVA) between sedentary control group, football group, and competitive swim group. IL-4 and IFN- γ fold changes were also compared between the three groups using ANOVA. We compared IL-4 and IFN- γ levels using the ratio of the two cytokines, baselined with the ratio observed in the sedentary control group. A bivariate regression was run to analyze the correlation between expressed CCL11 levels and expressed ratio of IL-4 to IFN- γ . A bivariate regression analysis was conducted to measure the correlation between number of years exposed to repetitive TBI, using the number of years participating in football, with the measured CCL11 expression in the CSF of football players.

RESULTS

Results in figure 3 display the significant increases in CCL11 expression in the cerebrospinal fluid of football players who are suspected to have developed CTE, compared to both sedentary controls and athletes with no prior exposure to rTBI. Expression levels were baselined using the mean values of the sedentary control group. CCL11 expression in the CSF, as measured by ELISA, significantly discriminates football players from controls when controlling for age and gender.

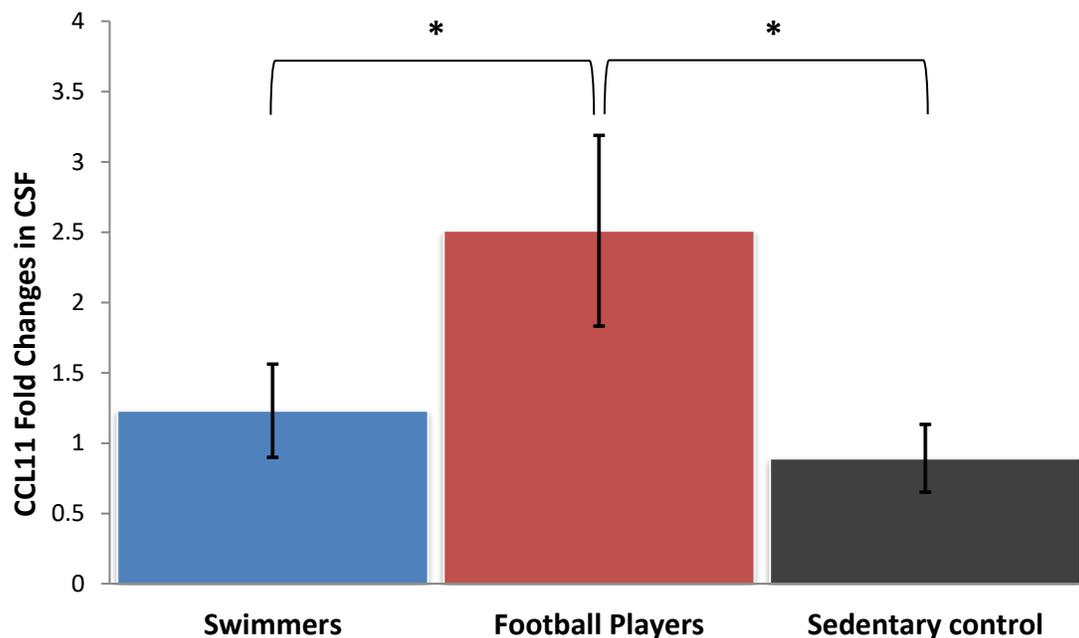


Figure 3. CCL11 protein levels expressed as fold changes, measured using ELISA. Bar graph shows mean expression and standard error, compared using ANOVA. Red shows football players group (N=25), Black shows sedentary control group (N=30), and blue shows swimmer controls group (N=27).

The histogram in figure 4. shows the level of expression of the two Cytokines as a ratio of IL-4 to IFN- γ , as measured by ELISA, between the three groups, football,

swimming, and sedentary. Expression levels were baselined using the mean values recorded for the sedentary control group. Results show significant increase in expressed ratio of interleukin-4 to interferon-gamma in football group compared to both swimmers and the sedentary control. No significant difference in expressed ratio was found between swimmers and sedentary control.

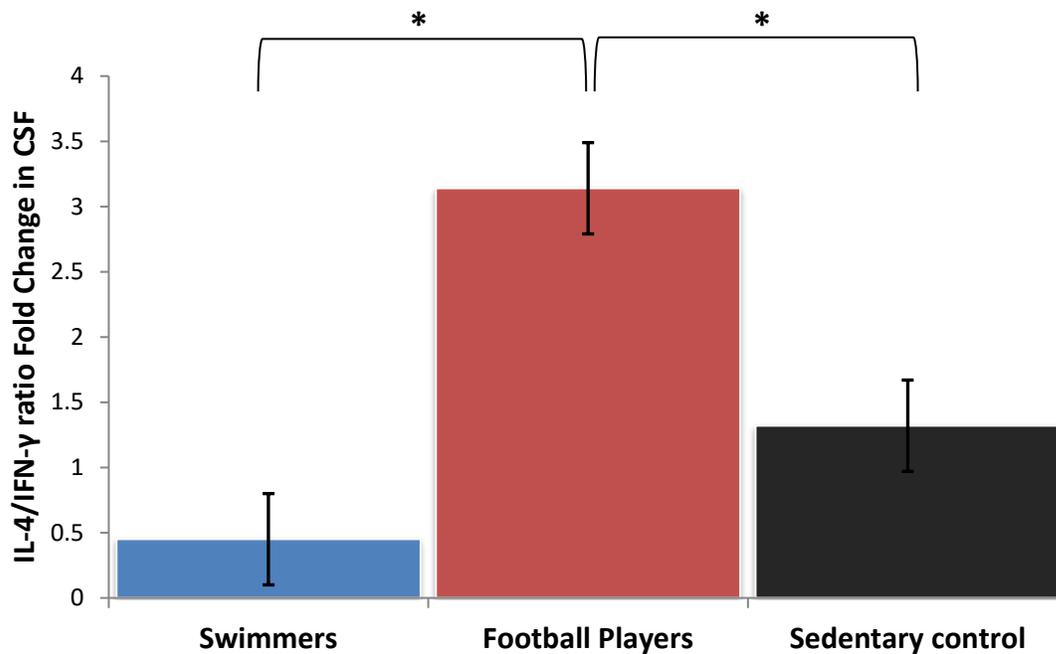


Figure 4. Expression of two cytokines IL-4 and IFN- γ represented as fold changes of the ratio between the two proteins between football (red, N=25), swim (blue, N=27), and sedentary groups (black, N=30). Bar graph shows the mean with standard error bars, compared using ANOVA.

The graph in figure 5 shows the correlation of the expressed IL-4 and IFN- γ ratio to the expression of the chemokine CCL11 for both the football group and the sedentary control and athletic control groups. A bivariate regression showed significant correlation between the expression of the Chemokines, as measured using ELISA. All groups are represented in the

scatterplot, control groups are represented by red squares, illustrating the low CCL11 expression corresponding to a low IL-4/IFN- γ ratio expressed. The blue diamonds represents the football group, which expressed increasingly high CCL11 levels with correspondingly high increases in IL-4 expression.

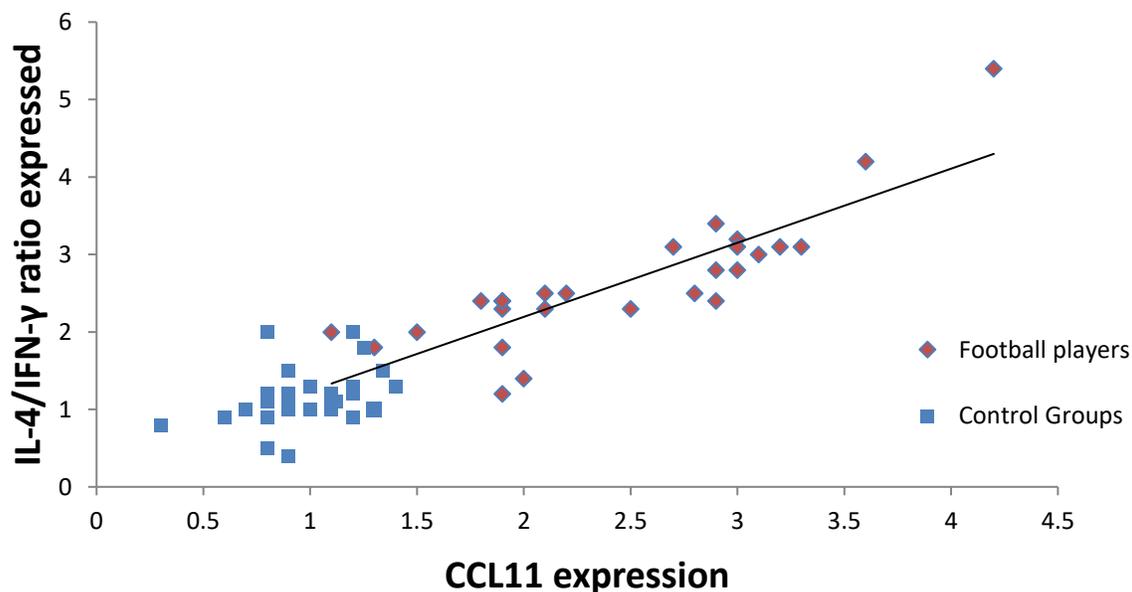


Figure 5. Correlation between CCL11 and IL-4/IFN- γ ratio expression levels of subjects from all groups, football players (Red triangles, N=25) and control groups (blue squares, N=57). Bivariate regression shows positive correlation between cytokine levels as measured by ELISA.

The graph in figure 6. shows the positive correlation of CCL11 expression, as measured by ELISA, to the time that subjects of the football group had participated in competitive football. The number of years participating in football show a strong and significant correlation to the expression of the chemokine CCL11 in the CSF of football

players competing for a minimum of 10 years. High CCL11 levels in football players show to increase continually with the time of exposure to the sport

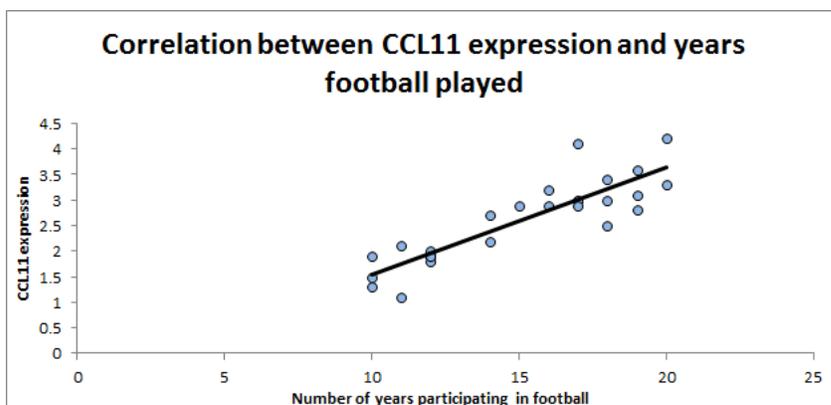


Figure 6. Increased CCL11 expression correlates significantly with the number of years participating in football (N=25). Bivariate regression shows high R value and a significantly low p value.

DISCUSSION

Athletes exposed to high levels of repetitive traumatic brain injury expressed significantly higher levels of the chemokine CCL11 in their CSF, compared to both swimmers and sedentary controls (Fig. 1). The significance in these results suggest that the chemokine can be used as a viable biomarker for athletes at risk of developing CTE. The subjects used in the football group were selected based on a clinical suspicion of CTE, which was judged based on their score on the Ohio State University TBI Identification Form. Therefore, the study presents the ability for the chronic development of cognitive and motor symptoms in athletes who have been exposed to high levels of participation in contact sports to be clinically diagnosed with CTE, using the biomarker CCL11. Research has shown that CCL11 levels in the CSF are increased in diseased athletes who had an official diagnosis of CTE (Cherry et al., 2017), however, the importance of the present study suggests that the

expression of CCL11 in live athletes at risk of CTE is significantly distinguishable from patients not exposed to TBI. Further evidence for the use of CCL11 as a biomarker for CTE is produced in figure 4, which illustrates the correlation in the relationship between years participating in contact football and the expression of CCL11 in the CSF of the athlete. While there may be multiple factors in football that could differentiate the amount of risk of TBI in an individual player (e.a. Position played, level of play, equipment used, etc.), the number of years played may be the best indicator of the number of RHIs that a player is exposed to. Thus, the years of football played provides the best representation to the athletes exposure to TBI. As the amount of exposure to TBI increases in an athlete's career, theoretically so does the progression of the disease, and as hypothesized,so does the expression levels of CCL11 (Fig. 4).

Our findings support the hypothesis that TBI injury leads to a specific immune change as a response to the inflammation caused by head injury that is distinguishable from other neurodegenerative diseases that may share similar pathologies. The bodies development of an immune response when exposed to repetitive TBI is specific to the the long term neurodegeneration caused by repetitive exposure to traumatic brain impacts. With evidence supporting that CCL11 production increases both in response to traumatic brain injury, as well as in parallel with normal aging, the age control of participants in the study suggest that the significant difference in the expression of the protein, between groups, can indeed be attributed to the exposure to TBI. Therefore, the studies ability to demonstrate differences in the chemokine expression between participants of the same age group is significant to our understanding of CTE. To further support the hypothesis that there is a developing immune response specific to CTE, the increase in IL-4 levels with

correspondingly lower levels of IFN- γ were measured and shown to correlate with the increased expression of the chemokine CCL11 (Fig. 3). These results support the hypothesis that the significance in the increased expression of CCL11 in participants exposed to TBI is due to a particular shift in inflammatory response of immune cells, specific to chronic backlash from traumatic brain injury. With the molecular mechanisms of these two cytokines having been shown to be vital in the regulation of immune reactions, the study suggests a major role of immune cell expression to the development of the cognitive degeneration observed in CTE (Paludan, 1998). Figure 2 shows the increased ratio of IL-4 to IFN- γ , illustrating that there is an observed change in expression of immune cells in the CSF in athletes exposed to rTBI.

While on average CTE suspicious participants expressed higher levels of CCL11, the exact mechanisms of disease development and its relation to the chemokine expression are still in question. Due to the relatively younger age group selected to participate in our study, our results suggest that the expression of CCL11 in CTE subjects may be measurable as early as the beginning of onset of cognitive dysfunction. This hypothesis is supported by research that has shown CCL11 to enhance excitotoxic neuronal death by producing reactive oxygen species in microglia (Parajuli et al., 2015). Therefore, increased CCL11 expression may also be responsible for the accumulation of neurofibrillary tangles and in the pathological indicators of the disease. Results from previous research suggests that astrocytic responses to inflammation release CCL11, which has been shown to trigger oxidative stress, therefore a probable factor in producing the particular clinical pathology that has come to define CTE (Parajuli et al., 2015). It is thus suggested that the long developing symptoms of CTE can be attributed to the neurodegenerative effects of the immune response of inflammatory

astrocytes accumulating long term from repetitive and mild traumatic brain injuries. The results are important for the evaluation, diagnosis, and potential treatment methods for those at risk of CTE.

The present studies results may suggest that the effect of the inflammation from RHI on the CSFs immune cell presentation can develop in correlation to the symptomatic development of the disease (Fig. 4). However, development of the disease can differ greatly between individuals. Therefore, the use of CCL11 as a biomarker could benefit from studies that compare expression levels between multiple age groups as well as groups of varying disease severity. With correlations being shown between the number of years played and the expression levels of the protein, we suggest that expression levels may correlate with the severity of disease. The number of years played is relatively representative of the number of RHIs that a subject is exposed to. However, further research could also benefit from investigating how the expression of clinical symptoms are correlated with varying expression of CCL11 levels. Considering the extended, chronic development of such a disease, symptoms may not be noticeable until much later in age, often times long after exposure to any RHI has occurred. Therefore, using the chemokine as a reliable biomarker may need rely on the ability to catalog expression levels for individuals of varying age, length of participation, and symptom severity. With a more knowledgeable account of how expression levels vary among individuals, measuring expression levels of CCL11 in active athletes may give insight into their risk of developing CTE.

Another factor of the present study in need of consideration is the methodological use of younger participants that express TBI symptoms, in the present study, must rely on the clinical suspicion of CTE. These athletes therefore were not clinically diagnosed with CTE,

because that would require further invasive tests to analyze brain weight and tau accumulation. Therefore research must consider the possibility of other head impact injuries may exist in the participants used for the football group. It is suggested in the study that CCL11 expression is unique to chronic inflammatory response of CTE, however, participants who were recently exposed to RHI may still be at risk for other acute acting head injuries. Therefore, because athletes were chosen based their score on TBI assessment forms, they are athletes with neural defects occurring after their exposure to TBI, and not able to be specifically diagnosed with CTE.

The use of athletes not exposed to rTBI as a control in the present study was to ensure that exercise levels or fitness were not differentiating factors in CCL11 expression. This control of the study helped preserve that the expressed chemokine levels were in fact due to TBI exposure, specifically, and therefore an indicator of CTE development. Therefore, the study suggests that the protein expression increase, compared to the sedentary controls, is due to exposure to TBI, not the exposure to a more active lifestyle. Also, the use of solely male participants were to control for gender related chemokine expression that could be detected within groups. Therefore, further research could benefit from analyzing how gender may affect the specific expression of CCL11, IL-4, and IFN- γ .

Thus, CCL11 is presented in this study as a working biomarker for in life diagnosis of CTE. An in life analysis of the protein expression levels in an athlete's CSF could alert the individual to the onset of chronic developing neurodegeneration due to their prolonged exposure to RHI. With the ability to diagnose the disease, severely developing cognitive problems in athletes who participate in sports like football, rugby, boxing, and hockey can potentially be prevented. The study also gives insight into treatment methods that could be

used to treat athletes at risk of CTE. Due to the suggested impact of immune response changes specific to CTE, those at risk of CTE may benefit from treatments that target immune cell production. Neuropreventive treatments could also be considered as to maintain immune responses in athletes who are exposing themselves to high amounts of repetitive head impacts. Research into immunomodulation in order to prevent age related cognitive decline may also, therefore, become useful in the prevention of chronic developing neurodegeneration that occurs in participants exposed to repetitive mild traumatic brain injuries.

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