Exploration of serum Lipid-based biomarkers and cognitive behaviours linked to repetitive concussions: The UK Rugby Health study

Toni Robinson and Toni Robinson

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Abstract

Background

Rugby players in both Union and League face a risk of concussion. Repetitive brain trauma, particularly without adequate recovery, can lead to post-concussion syndrome and is associated with an increased risk of neurodegenerative diseases including Alzheimer's, ALS, and Parkinson's.

Method

This study analysed serum biomarkers and cognitive performance in retired male rugby players with a concussion history (n=34) compared with a non-contact sports group (n=22). 24-HC, 25-HC, 27-HC, APOE4, triglycerides, and ceramides were measured using ELISA assays and correlated with each other and previously identified biomarkers from the UK Rugby Health Study. Cognitive parameters assessed included verbal and visual memory, processing speed, and executive function. Standardised cognitive scores were analysed alongside biomarker levels to identify associations between indicators of brain stress, cognitive performance, and concussion history.

Results

Compared to controls, concussed participants showed reduced 24-HC and increased 25-HC, 27-HC, APOE4, triglycerides, and ceramides. Aβ42 positively correlated with 25-

HC (r=0.694, R^2=0.482), 27-HC (r=0.651, R^2=0.424), and ceramide (r=0.876, R^2=0.768). Tau proteins positively correlated with 25-HC (P-Tau181: r=0.906, R^2=0.820; P-Tau231: r=0.441, R^2=0.195) and triglycerides (T-Tau: r=0.826, R^2=0.683), and negatively correlated with 24-HC (P-Tau181: r=0.969, R^2=0.8939; P-Tau231: r=0.639, R^2=0.409). Participants with >5 concussions showed deficits in executive function. Raw and verbal memory positively correlated with 25-HC (Raw: r=0.680, R^2=0.462; Verbal: r=0.578, R^2=0.3343), while psychomotor and processing speed negatively correlated with 27-HC (Psychomotor: r=0.514, R^2=0.264; Processing: r=0.529, R^2=0.280).

Conclusion

Multiple TBIs without adequate recovery may cause neuronal damage, reflected by decreased 24-HC and increased 25-HC, 27-HC, APOE4, ceramides, and triglycerides. These changes correlate with elevated Aβ42 and T-Tau, promoting amyloid plaque and NFT formation, hallmarks of neurodegenerative diseases such as Alzheimer's, Parkinson's, and ALS. A long-term follow-up is needed to track biomarker progression and disease development.

Keywords

Neurodegenerative disease; Concussion; Biomarkers; 24-Hydroxycholesterol; 25-Hydroxycholesterol; 27-Hydroxycholesterol; Apolipoprotein E4; Triglyceride; Ceramide; Alzheimer's; Amyotrophic Lateral Sclerosis

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List of Abbreviations

24-HC	24-Hydroxycholesterol
25-HC	25-Hydroxycholesterol
27-HC	27-Hydroxycholesterol
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
APOE	Apolipoprotein E
APP	Amyloid Precursor Protein
Aβ	Amyloid Beta
$A\beta_{42}$	Amyloid Beta 42
CCL2	Chemokine Ligand 2
CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
CT	Computerised Tomography Scan
CTE	Chronic Traumatic Encephalopathy
DaT	Dopamine Transporter Scan
ELISA	Enzyme-linked immunosorbent assay
HRP	Horseradish Peroxidase
IL-1β	Interlukin-1 beta
IL-6	Interlukin-6
MRI	Magnetic Resonance Imaging Scan
MS	Multiple Sclerosis
mTBI	Mild Traumatic Brain Injury

NFL	Neurofilament Light
NFT	Neurofibrillary Tangles
NHS	National Health Service
P-Tau181	Phosphorylated Tau 181
P-tau217	Phosphorylated Tau 217
P-Tau231	Phosphorylated Tau 231
PBS	Phosphate Buffered Saline
PD	Parkinson’s Disease
PNS	Peripheral Nervous System
RA	Retinoic Acid
RBP4	Retinol Binding Protein 4
RBPs	Retinol Binding Proteins
ROS	Reactive Oxygen Series
SAA	Serum Amyloid Alpha
T-Tau	Total Tau
TBI	Traumatic Brain Injury
TLR	Toll-like Receptors
TMB	Tetramethylbenzidine
TNF-α	Tumor Necrosis Factor alpha

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To everyone that has been a part of this journey—thank you.

1. Introduction

1.1 Traumatic Brain Injury and Concussion

Concussion is a type of traumatic brain injury (TBI) which is typically caused when any type of external force is applied to the head and the brain experiences movement. During the event, the brain will typically accelerate and decelerate with rotational, translational and angular forces (Gennarelli and Thibault, 1982; Collins and Collins, 2006). The rapid change in the speed of rotational force will damage axonal fibres, which disrupts normal brain function. As a result of this, a person can experience pathophysiological changes that include neuronal damage, metabolic dysfunction and a trigger of an inflammatory response (Smith et al., 2013).

A person with concussion may experience, but is not limited to experiencing physical, cognitive and behavioural symptoms such as headaches, nausea, sensitivity to light, mood swings, loss of consciousness, confusion and sleep disturbance. TBI's can be given a degree of severity: mild, moderate and severe, based on a mix of physical, cognitive and behavioural symptoms. As you move up the scale, the symptoms experienced become more severe (McCrory et al., 2017).

Despite many people's beliefs, a loss of consciousness is not always consequential of a concussion. A more accurate way to measure and diagnose a concussion is to understand the severity of the injury and the symptoms (Collins and Collins, 2006). Cognitive tests and neurological exams can be used to diagnose concussion as opposed to imaging that may not pick up on neuronal changes (Chesnutt et al., 2014). With complete physical and cognitive rest, recovering from a concussion should take

7-10 days in most adults, with the length of recovery taking even longer in youth (Chesnutt et al., 2014).

Most people will typically recover well from a concussion, but some will experience long-term effects, known as post-concussion syndrome. Chronic traumatic encephalopathy (CTE) is a little different, in that it develops in cases where concussion is repeated.

1.2 Concussion in Rugby

Rugby is a dual code sport: Union and League, that is of a physical and high-impact nature. Previous research has shown that in the 2018-2019 season in England, 20% of professional rugby players sustained at least one concussion, which was a 16% increase from the previous season (BBC Sport, 2020). A concussion in rugby is most likely to occur when a player is in a situation where a tackle, scrum, ruck or maul has occurred. The tackle is responsible for 64% of head injuries, and 74% of concussions, making it the most likely contact event to cause head injuries and concussions in community-level rugby (Tonder, et al., 2024). This data represents a cause for concern across the board, at all levels in any code of Rugby.

Whilst players experience the same typical symptoms of a non-sports related concussion, the way in which it presents can differ. Some players will experience immediate symptoms following the concussive event, which will result in immediate removal from the field of play. Others may experience a delayed onset of symptoms, where the symptoms can occur hours or even days after the concussive event (McCrory et al., 2017). In both cases, a player will undergo testing at the earliest onset or

detection of any symptoms. Both the Sports Concussion Assessment Tool (SCAT) and Head Injury Assessment (HIA) are used to aid in the diagnosis of a potential concussion (Echemendia et al., 2017).

Upon diagnosis, individuals will enter a period of physical and cognitive rest and recovery. Once this is completed and symptoms subside, the person will follow a strict protocol that involves a steady increase in physical activity, in which the person will be monitored for any reoccurrence of symptoms. If a player's symptoms re-occur, they will go back to the rest and recovery stage as opposed to no re-occurrence and progress to the next stage of the return to play protocol (McCrory et al., 2017). Once a player has progressed through all the stages, and they have no concussion symptoms, they will be declared fit to play.

In a situation where a person has experienced concussion multiple times in their career, the risk of developing long-term neurological issues increases. Chronic Traumatic Encephalopathy (CTE) is a brain disease that has been linked to suffering repeated concussions (McKee et al., 2013). Multiple concussions can also be linked to several other neurological diseases (Alzheimer's, ALS and Dementia), and issues (physical, cognitive and behavioural) (Gavett et al., 2011). To lessen the risk of these, organisations such as World Rugby have established multiple changes to reduce the likelihood of suffering concussion. Rules have been adapted so that tackle height has been lowered to reduce the number of head injuries. They also rolled out head injury protocols and funded further research into concussions (Patricios et al., 2018).

It is understood on a bigger level that concussion is a significant worry in rugby. With this comes an understanding that the only way to tackle the stigma and minimize the impact concussion has on players and their lives (both short and long-term), is to further research the issue. Whilst there are effective management protocols in place for a concussion diagnosis and treatment, research into changes in blood biomarkers could provide a quicker, more accurate diagnosis of concussion. World Rugby is currently funding research into concussion management and prevention through a collaboration with Marker Diagnostics and the University of Birmingham, which has developed a salivary-biomarker test for mild traumatic brain injury (University of Birmingham, 2023). It is research like this, alongside a stronger education on the topic for players, and stricter enforcement of safety measures, will help protect players better from the worsening long-term consequences of concussion developed in a rugby setting.

1.3 Cholesterol Metabolism

Cholesterol and repetitive trauma have been shown to affect lipid metabolism within the brain, making cholesterol and its derivatives a measurable indicator of neurological injury, or recovery (Killen, et al., 2019). Cholesterol is a lipid in the body that is vital for hormone synthesis, functions in cells, bile acid production and metabolism. It is mainly synthesised in the liver, but it can also come from dietary sources such as meat and dairy (Cerqueira et al,. 2016; International et al., 2025). Cholesterol is transported throughout the body by low- and high-density lipoproteins. Low-density lipoproteins (LDL) transport cholesterol to tissue, whilst high-density lipoproteins (HDL) carry cholesterol back to the liver. LDL is known as the ‘bad’ cholesterol as high levels of it can lead to a buildup of cholesterol in

the arteries. HDL is known as the ‘good’ cholesterol because it removes excess cholesterol back to the liver for removal from the body (Molecular Biomedicine 2025).

Cholesterol homeostasis is important to maintain health, with both excess and deficits of cholesterol leading to several different health risks. The body regulates cholesterol levels by increasing or decreasing cholesterol synthesis in response to nutritional intake of cholesterol. Cholesterol metabolism is an important aspect in the maintenance of physical, psychiatric and cognitive health.

In physical health, increased cholesterol levels are of particular concern. High cholesterol levels are a risk factor for developing cardiovascular disease (Rader & Hovingh, 2014). Particularly high levels of LDL cholesterol can contribute to the formation of atherosclerotic plaques, which can lead to strokes and heart attacks (Rader & Hovingh, 2014). Increased cholesterol levels have also been associated with an increased risk of different types of cancers, such as colon and testicular (Ding, et al., 2019). This possibly occurs due to the role of cholesterol in cell proliferation and lipid rafts that affect signalling in cells (Ding, et al., 2019).

In psychiatric health, whilst less common, decreased levels of cholesterol are seen as contributing factors. Cholesterol is needed for the function of both serotonin and dopamine neurotransmitters that play roles in regulating emotions (Cheon, 2023). Cholesterol also affects the excitatory and inhibitory neurotransmitters that affect mood. An imbalance in cholesterol in these neurotransmitters can contribute to disorders such as depression, anxiety and cognitive dysfunction (Cheon, 2023). More specifically, low levels of cholesterol have been linked to an increased risk of depression, with a low supply

of cholesterol-reducing serotonin and dopamine receptors and signalling activity (Cheon, 2023). Anxiety disorder is similar in that a reduction of cholesterol will see a reduction of activity in the excitatory and inhibitory receptors. A study has reported a correlation between bipolar disorder and mood swings, with some cholesterol-based treatments improving symptoms (Fusar-Poli, et al., 2020). Schizophrenia has been linked with cholesterol imbalances and particularly shows a profile of low HDL and high LDL levels (Śmierciak, et al., 2023). These abnormal cholesterol levels affect neurotransmitter receptor function, which pathologically aligns with the disorder.

In neurocognitive health, there is an indifference with both high and low levels being associated with neurocognitive impairment. Whilst an increased cholesterol level led to a cognitive decline through vascular problems, a decrease in cholesterol levels led to impairment of neuronal function and synaptic plasticity (Björkhem & Meaney, 2004). Abnormal cholesterol metabolism has been related to neurodegenerative diseases such as Alzheimer's, Amyotrophic Lateral Sclerosis, and Parkinson's (Petrov, et al., 2016). The link between neurodegenerative diseases and cholesterol levels is based on the understanding that abnormal levels of cholesterol can trigger disruptions in the cholesterol transport proteins, and the formation of amyloid plaques (He, et al., 2024).

Cholesterol metabolism plays a crucial role in the body and brain and has benefits and detriments depending on the balance of cholesterol homeostasis. With dysregulated levels having negative effects on physical, mental and cognitive health, it is important to understand these relationships and treat any ill effects, ensure healthy levels, and maintain this across the life course for good health.

1.4 Neurodegenerative Disease

1.4.1 Neuroinflammation and Oxidative Stress

Neuroinflammation occurs as an inflammatory response to a trauma or disease in the brain. When this happens, the brain experiences a release of pro-inflammatory chemokines such as CCL2 cytokines (e.g., TNF-α, IL-1β, IL-6), and reactive oxygen species (ROS). ROS can include excitotoxicity, mitochondrial dysfunction and synaptic loss, ultimately having negative neuronal effects (Heneka, et al., 2014). Normally, these cells will usually aid in brain protection, but in the case of neuroinflammation they are chronically activated in such a way that they can lead to excessive inflammation and neuronal injury (Amor et al., 2014).

Oxidative stress is caused by an imbalance between the oxidative and antioxidant systems, which favours the oxidative system. The brain is highly susceptible to oxidative stress due to its high oxygen consumption and lipid-rich content (Uttara et al., 2009). When the brain is in a state of oxidative stress, it can cause damage to proteins and lipids, meaning that it has a shared responsibility for the development of neurodegenerative disease (Chen, et al., 2012). Whilst oxidative stress has previously been linked to neurodegenerative disorders, research into its detriments towards neuropsychiatric disorders is now being recognised too (Salim, 2017).

Neuroinflammation and oxidative stress are two processes that can be linked to the progression of neurodegenerative diseases such as CTE, Alzheimer's, ALS and Parkinson's. In neurodegenerative diseases, there is an activation of astrocytes and microglia, that trigger neuroinflammation (Block & Hong, 2005). Upon triggering the

neuroinflammatory response, inflammatory mediators and reactive oxygen species are released, which aids oxidative stress of the brain (Block & Hong, 2005). This onsets a cycle, where being in a state of oxidative stress will trigger more production of microglia and astrocytes. In time this cycle will aid neuronal death and damage to neuronal structures and cellular function (Glass et al.,2010). The cycle will allow for quicker progression of neurocognitive disease and an earlier onset of symptoms than is typically seen in Alzheimer's and ALS (Heneka, et al., 2015; Dexter & Jenner, 2013).

1.4.2 Chronic Traumatic Encephalopathy

Chronic Traumatic Encephalopathy (CTE) is a degenerative brain disease that is associated with repeated head trauma/injuries. Multiple studies are suggestive of CTE developing in people who experience head trauma or injury, namely post-concussion syndrome or repetitive concussions (Vasilevskaya & Tartaglia, 2018). It is pathologically defined by an accumulation of abnormal p-tau (Stern, et al., 2013). The disease presents with worsening symptoms through stages 1 to 4. Stage 1 CTE shows headaches alongside a loss of concentration whilst stage 2 CTE presents with short-term memory loss and depression (McKee, et al., 2013). Additional symptoms in stage 3 include cognitive impairment, and lastly, stage 4 consists of dementia and aggression (McKee, et al., 2013).

CTE can only be diagnosed post-mortem and is most often found in military and contact sports such as rugby and boxing personnel (Butler, et al., 2022). The post-mortem exam will usually show a degeneration of brain tissues alongside deposits of tau where these deposits of tau usually form tangles and are typically found in the cortical sulci, interfering

with neuronal communication, cognitive function, mood and motor stability (Butler, et al., 2022). A post-mortem brain scan study has proposed a staging system for CTE (McKee, et al., 2015). In stage 1 p-tau is found in foci, most typically in superior or lateral frontal cortices, around small vessels at the depth of sulci. Stage 2 CTE has multiple foci of p-tau at the depth of cerebral sulci, with localisation of the spread from the epicentres to the superficial layers of the adjacent cortex. Stage 3 shows a larger number of p-tau patches in the depths of the sulci, with distributed NFTs also found in the hippocampus, entorhinal and peripheral cortices and the amygdala. The final stage 4 shows a drop in brain weight and widespread p-tau pathology that affects most regions of the cerebral cortex, the medial temporal lobe, sparing calcarine cortex in most cases (McKee, et al., 2015).

More research is required to fine-tune the specifics such as how many concussions, or how severe these concussions need to be to lead to developing CTE. With other risk factors, mechanisms, epidemiology and treatment of CTE yet to be pinpointed, due to being unable to diagnose this in a person's lifetime, it proves to be an area for further research and understanding (Alosco, et al., 2021).

1.4.3 Alzheimer's Disease

Alzheimer's is a disease of a progressive neurodegenerative nature that is usually categorised into three stages. In the early stages, a person with Alzheimer's will experience short-term memory loss, confusion and a struggle to complete day-to-day tasks (McKhann, et al., 2011). Middle-stage Alzheimer's is usually the longest stage, lasting sometimes for many years. This stage will see a worsening of early-stage issues, with the addition of more symptoms that include changes in sleep patterns and trouble

with controlling the bladder and bowels (Alzheimer's Association, 2024). In the late stages, a person will also develop severe memory loss, alongside the loss of physical and communicative abilities (Alzheimer's Association, 2024).

In most cases, a diagnosis of Alzheimer's can be reached clinically by running neurological and cognitive tests (McKhann, et al., 2011). Other than this, Alzheimer's can be confirmed by biomarker testing. Blood and Cerebrospinal fluid can be tested to determine levels of tau proteins and beta-amyloid levels (Blennow & Zetterberg, 2018). This way of testing is effective due to the pathology behind Alzheimer's disease.

The pathology of Alzheimer's is distinguishable by two main abnormalities: amyloid plaques and neurofibrillary tangles. Amyloid plaques form when there is a build-up of amyloid beta in the tissues that are in between nerve cells (Selkoe and Hardy, 2016). It is thought by many that amyloid beta aids neuronal death. This is believed to happen either directly, or by tau causing an influx of neurofibrillary tangles (Hardy and Higgins, 1992). The tangles are toxic in that they stop cellular proteins from carrying out their normal functions and can cause structures to collapse in the neurons (Iqbal et al., 2016). Both amyloid beta and tau proteins have been recognised in previous research to give insight into potential Alzheimer's diagnosis. Previous research has stated findings of a positively correlated ratio between the two biomarkers resulting in an increased probability of the development of Alzheimer's disease (Mantzavinos & Alexiou, 2017).

1.4.4 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that is pathologically characterised by a loss of motor neurons and nerve cells in the brain and

spinal cord (Matteo, et al., 2007). Whilst most ALS cases are of an unknown cause, it is known that approximately 10% of sufferers have inherited the disease (Alsultan, Waller, Heath and Kirby, 2016). Whilst both inherited and unknown cause ALS to present the same clinically, familial ALS is distinguishable due to a mutation of the free radical scavenging enzyme. Both ways of developing ALS are equally as agonizing for a person to experience, with it ultimately leading to death (Matteo, et al., 2007).

As the disease progresses into its late stages, a person can experience greater difficulty controlling emotions, maintaining weight and breathing (National Institute of Neurological Disorders and Stroke, 2025). ALS is fatal, and eventually, a person with end-stage ALS will become immobile, with most people who have ALS dying due to respiratory failure within 3-5 years from the onset of initial symptoms (National Institute of Neurological Disorders and Stroke, 2025).

ALS is quite difficult to diagnose, with the primary diagnostic route being clinical examination alongside other tests that ultimately rule out other similar diseases (ALS Association, 2024). Recent studies have looked at the potential of biomarkers in both blood and spinal fluids as a diagnostic measure for ALS. A recent study has looked at P-tau and T-tau levels and how they can be used as a diagnostic measure. The study finds that T-tau levels are elevated while the ratio between P-tau/T-tau is lowered (Thapa, et al., 2023).

1.4.5 Parkinson's Disease

Parkinson's disease is the second most common neurodegenerative disease with only Alzheimer's being more common (Lebouvier, et al., 2009). The disease is caused by a

loss of nerve cells in the substantia nigra. These cells are responsible for the production of dopamine. When these nerve cells are damaged, consequently there is a reduction in the amount of dopamine (Zhou et al., 2023). This loss of dopamine is detrimental to neurocognitive health. It is unknown why this loss of neuronal nerve cells occurs, but the current belief is that genetics and environmental factors play a role (Schapira et al., 2017).

Parkinson's is characterised by both motor and non-motor issues that will typically start mild and worsen over time. The main symptoms include a tremor that usually starts in the hand, muscle stiffness that can make moving around more difficult and a slower-than-normal way of moving around (Lees et al., 2009). Whilst some of the main symptoms are physical, this is not to say that a person with Parkinson's won't experience negative psychiatric symptoms such as anxiety and depression or negative neurocognitive symptoms such as impaired memory, visual hallucinations and delusions (Chaudhuri et al., 2006).

The pathological mark of Parkinson's comes in the form of protein aggregates. These deposits are called Lewy bodies and are often found with Lewy neurites, which are mostly axonal (Kouli, et al., 2018). The main component of these aggregates is alpha-synuclein (Kouli, et al., 2018). Lewy bodies and neurites in Parkinson's can be found in the prefrontal cortex and coronal sections of the brain (Kouli, et al., 2018). The only way to find out whether a person has the presence of suspected Lewy bodies is in a post-mortem autopsy. This will confirm the presence of Lewy bodies, and a suspected Parkinson's diagnosis (Alzheimer's Association, 2024).

The standard diagnosis of Parkinson's disease is done based on clinical presentations. This means that an individual will have the disease diagnosed based on their medical history and symptoms, both mentally and physically (Parkinson's Foundation, 2024). To clinically diagnose Parkinson's, a person must have bradykinesia in addition to one of the three following symptoms: trouble with balance, shaking or tremor in any limb whilst at rest, or stiffness of the arms, legs or trunk. Whilst there is no lab or imaging technique available to give a direct or clear diagnosis of Parkinson's, imaging such as MRI, DaT scans and blood work can all be used to aid a diagnosis (Parkinson's Foundation, 2024).

Blood work for diseases like Parkinson's is at the forefront of neurocognitive research. Research is currently looking at whether blood and serum biomarkers can be used to pinpoint an early diagnosis. Some research has shown alpha-synuclein and NFL as promising diagnostic biomarkers, with inflammatory markers, amyloid-beta and tau looking to be further studied (Tonges, et al., 2022).

1.5 Biomarkers

Research is developing to establish links between biochemical biomarkers and neurodegenerative diseases, with limited studies working in concussion. The biomarkers of focus for this project include 24-Hydroxycholesterol, 25-Hydroxycholesterol, 27-Hydroxycholesterol and apolipoprotein e4. Analysis of biomarkers and exosomes was carried out and utilized to make connections with the previously analysed biomarkers and exosomes that were part of the UK Rugby Health study.

1.5.1 24-Hydroxycholeserol

Cholesterol is metabolised in the brain into 24-OHC by 24-hydroxylase. It goes across the blood-brain barrier and into systemic circulation (Gamba, et al., 2015).

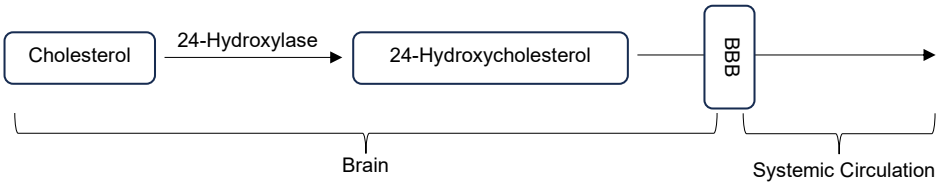


Figure 1: This figure shows how cholesterol is metabolised into 24-hydroxycholesterol by 24-hydroxylase and transported across the blood-brain barrier from the brain into systemic circulation.

24-HC in the brain is important as it aids in the removal of excess cholesterol in the brain. Cholesterol is unable to cross the blood-brain barrier, but when synthesized into 24-HC it becomes more soluble and able to cross the barrier into the bloodstream. This pathway is important as it turns over and regulates the levels of cholesterol in the central nervous system (Russel, et al., 2010).

The levels of 24S-hydroxycholesterol (24-HC) can reflect neuronal health, as 24-HC is the major cholesterol metabolite in the brain, produced by the neuron-specific enzyme CYP46A1 and released into the circulation to maintain cholesterol homeostasis (Leoni & Caccia, 2013; Hughes et al., 2013; Pikuleva & Russell, 2013). Lower 24-HC levels are associated with impaired neuronal survival, synaptic dysfunction, and cognitive deficits, likely due to the accumulation of unprocessed cholesterol disrupting cell membranes, synapses, and myelin sheaths (Leoni & Caccia, 2013; Hughes et al., 2013). Alterations in 24-HC have been observed in neurodegenerative conditions such as Alzheimer's disease

and amyotrophic lateral sclerosis, supporting its potential as a biomarker for disease progression (Leoni & Caccia, 2011; Pikuleva & Russell, 2013). Given its brain origin and measurable presence in plasma, serum, and cerebrospinal fluid, 24-HC provides a clinically accessible indicator of neuronal cholesterol metabolism and neurodegenerative processes (Leoni & Caccia, 2011).

1.5.2 25-Hydroxycholesterol

Cholesterol is metabolised to 25-HC by the 25-hydroxylase enzyme. 25-HC moves both ways across the blood-brain barrier.

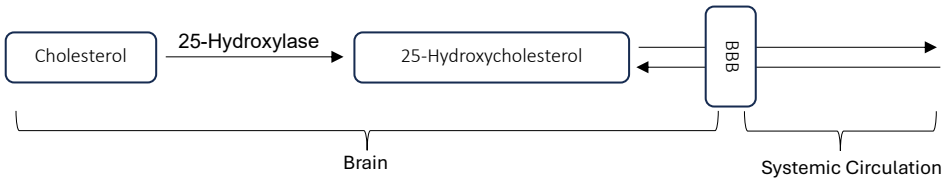


Figure 2: This figure shows how cholesterol is metabolised into 25-hydroxycholesterol by 25-hydroxylase and transported across the brain and systemic circulation via the blood brain barrier.

25-HC is known to play a part in cholesterol homeostasis, apoptosis and signalling in the brain. 25-HC is produced in macrophages and induced by inflammation or infection. 25-HC acts as an amplifier of inflammatory signalling (Gold, et al., 2014). This means that 25-HC can be used as an inflammatory marker, due to its abundance being positively correlated to the amount of inflammatory signalling.

A higher concentration of 25-HC can negatively affect the functionality of the central nervous system (Odnoshivkina, et al., 2022). Increased levels of 25-HC have been shown to reduce the survival of motor neurons (Odnoshivkina, et al., 2022). These higher levels

of 25-HC have been linked to neurodegenerative disease, particularly ALS. A study supports this by finding that the levels of 25-HC are higher in both serum and CSF in a patient with ALS, compared to an age-matched healthy control group (Kim, et al., 2017). Further solidification of the link between levels of 25-HC and ALS is apparent in this research with other findings stating that the severity of a patient's case of ALS shows an association with the concentration of 25-HC (Kim, et al., 2017).

25-HC can be looked at as a potential biomarker for neurodegenerative disease due to its representative role in inflammation in the brain, and its measurability in serum, blood and CSF.

1.5.3 27-Hydroxycholesterol

To a lesser degree, 27-hydroxycholesterol is produced from cholesterol by the enzyme 27-hydroxylase. Unlike 24-OHC, 27-OHC flows from systemic circulation into the brain via the blood-brain barrier.

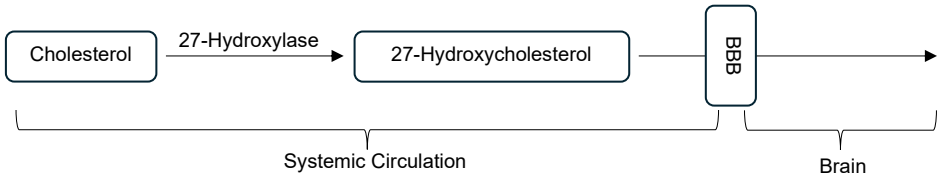


Figure 3: This figure shows how cholesterol is metabolised into 25-hydroxycholesterol by 25-hydroxylase and transported across the blood-brain barrier from systemic circulation into the brain.

27-HC is the major peripheral oxysterol that flows into the brain, and it affects β-amyloid production and elimination (Wu, et al., 2022). 27-HC is one of a few oxysterols that can cross the blood-brain barrier, meaning it is necessary to help maintain cholesterol

homeostasis between the brain and circulation. The dysregulation of this oxysterol can lead to many problems in the brain, with the main ones being oxidative stress, neuroinflammation and neuronal cell degeneration.

Oxidative stress in the brain knocks the balance of cholesterol homeostasis, in a way that it will increase the amount of cholesterol metabolized into 27-HC (Petrov, et al., 2016). It becomes a vicious circle, with higher levels of 27-HC being a trigger of further oxidative stress and neuroinflammation (Gamba, et al., 2015; Björkhem, Cedazo-Minguz, Leoni, & Meaney, 2009). Increased levels of 27-HC in the brain will lead to a higher production rate of toxic β-amyloid plaques, leading to a higher rate of neuronal death. Higher concentrations of 27-HC create a cholesterol imbalance which contributes to improper function of the neurons, synapses and myelin sheaths (Gamba, et al., 2015).

27-HC can be looked at as a diagnostic biomarker due to its role in the maintenance of neuronal health, with altered levels of oxysterol providing signalling for the ongoing progression of neurodegenerative disease.

1.5.4 Apolipoprotein E4

Apolipoprotein E (APOE) is a protein that is vital for redistributing cholesterol and lipids to neurons by binding to receptors on cell surfaces (Liu, et al., 2013). The protein is typically produced in the liver and brain but can also be found in other tissues. There are three isoforms APOE2, APOE3 and APOE4, which are all minimally different in their amino acid make-up but have distinguishing implications on health (Mahley & Huang, 2012). APOE alleles are all involved in lipid metabolism in the brain. Specifically, APOE4 plays a significant role in transporting cholesterol and other lipids in the brain. Any

disruptions to levels of APOE4 levels can dysregulate lipid metabolism and impact neuronal health.

In the central nervous system, APOE is typically expressed in astrocytes, but it can also be produced when neurons are affected by stress, injury or conditions caused by ageing (Zhang, et al., 2023). The presence of APOE4 in neurons can cause a build-up of amyloid beta and tau proteins that are recognised as aggravators for neuroinflammation and neuronal damage (Mattos, et all., 2004).

The E4 allele can have negative neuronal effects that arise from interactions between the amino-terminal domain and the carboxyl-terminal domain (Zhang, et al., 2023). The APOE4 that is produced in damaged neurons has a relation to the formation of NFTs, whilst any APOE4 produced in the astrocytes I s related to the formation of $A\beta_{42}$ (Zhang, et al., 2023). When the levels of APOE4 increase, there is an influx of both the levels of NFTs and $A\beta_{42}$, thus increasing the level of neuroinflammation and neuronal damage (Mattos, et al., 2004).

Pathologically, Alzheimer's is recognisable due to a build-up of $A\beta_{42}$ plaques and NFT's. There have been many links made in previous literature between the levels of APOE4 and Alzheimer's Disease in particular (Mattos, et al., 2004). APOE4 can be looked at and considered as a biomarker for neurodegenerative disease due to its role in lipid metabolism, the formation of NFTs and $A\beta_{42}$, and its measurability in serum and blood.

1.5.5 Triglycerides

Triglycerides are a type of lipid that is made of three fatty acids that are attached to a molecule of glycerol (Dimache, et al., 2021). When food is consumed, excess calories are converted and stored as triglycerides. This acts as an energy source that can be utilised by the body between meals. Triglycerides don't directly function in or supply energy to the brain, but their levels can have indirect impacts on neuronal health and cognition (Dimache, et al., 2021).

High levels of triglycerides are often associated with negative brain health for a few reasons. The increased levels of triglycerides have been linked to a condition called atherosclerosis, a condition where plaque builds up in the arteries and restricts blood flow (Talayero & Sacks, 2011). This is problematic as it reduces circulation and can damage brain tissue.

The increased levels have also been linked to increased neuroinflammation and an increased risk of damage to the blood-brain barrier (Dimache, et al., 2021). Triglyceride accumulation has been linked to inflammation that may accelerate brain cell damage, neurodegeneration, and cognitive decline. This neuroinflammation has been linked to neurodegenerative diseases such as Alzheimer's, ALS and Parkinson's. When the blood-brain barrier becomes compromised harmful toxins can enter the brain that can potentially damage brain cells and increase the risk of developing neurodegenerative disease.

Elevated levels of triglyceride can contribute to insulin resistance (Howard, 1999). It does this by releasing fatty acids into the bloodstream that interfere with insulin signalling. Insulin typically plays a role in both neuronal signalling and memory formation. This

impairment in insulin signalling can affect cognitive function and contribute to the potential development of neurodegenerative disease.

In the case of Alzheimer's disease, amyloid beta is a pathological hallmark. Studies have looked at how triglycerides affect the production of amyloid beta (Dimache, et al., 2021). When the levels of triglyceride increase, so does the build-up of amyloid plaques. The link between triglycerides to Alzheimer's is clearer and more understandable when considering the pathological hallmark of Alzheimer's, warranting the investigation of triglycerides in this study.

The overall increase in triglycerides has been linked to negative neuronal health and the risk of neurodegenerative disease. Whilst triglycerides don't directly supply the brain with energy, increased levels can contribute to neuroinflammation, insulin resistance, restricted circulation and damage to the blood-brain barrier. It is important to maintain a healthy level of triglycerides to support a good level of overall brain health (Howard, 1999).

1.5.6 Ceramides

Ceramides are a type of lipid that plays a role in the maintenance of the structure and function of cell membranes. Ceramides belong to the sphingolipid family and are typically found in the outer layer of skin cells. They also have important functions in other parts of the body, including the brain. In the brain, ceramides help maintain the integrity and fluidity of the membranes that surround neurons and other brain cells, ensuring effective communication with each other (Hannun & Obeid, 2008). They also play a part in the formation of myelin sheaths that cover nerve fibres and allow for faster transmission of electrical signals in the brain. The signalling pathways that ceramides are involved in

include cell growth regulation and apoptosis (Pettus, et al., 2002). Excess ceramide has been linked to neuronal death because of the increased rate of apoptosis.

Whilst apoptosis is one negative effect of increased levels of ceramide, there are many more. Increased levels of ceramides have also been linked to neuroinflammation and insulin resistance (Lee, et al., 2020). Neuroinflammation has been linked to many neurodegenerative diseases such as Alzheimer's, ALS and Parkinson's. Research suggests that ceramide accumulation contributes to inflammation that may accelerate brain cell damage, neurodegeneration, and cognitive decline. Insulin resistance is problematic as insulin typically plays a role in neuronal signalling and memory formation. An increase in ceramide levels can disrupt the brain's insulin signalling, which will in turn impair cognitive function and contribute to the potential development of neurodegenerative disease (He, et al., 2014).

Much like triglycerides, ceramides can affect the blood-brain barrier. Similarly to triglyceride, an increase in ceramides can disrupt the blood-brain barrier, potentially allowing harmful toxins and inflammatory molecules to cross into the brain (Ouro, et al., 2022).

Ceramides have been linked in various ways to the potential development of Alzheimer's disease (Fagan, 2012). The pathological hallmark of Alzheimer's is a build-up of amyloid plaques. Research suggests that Ceramides may promote the formation of these plaques or increase their toxicity to neurons. Higher levels of ceramide have been linked to people with early-stage Alzheimer's (Katsel, et al., 2007).

An increase in ceramides overall has been linked to many potential neurodegenerative diseases, due to the contribution to neuronal death and neuroinflammation. Maintaining healthy ceramide levels is crucial for protecting brain health, supporting cognitive function, and preventing potential neurodegenerative diseases.

1.5.7 Tau proteins

Total tau is representative of the total concentration of tau proteins found in the body. These proteins are normally found in axons where they are involved in stabilizing microtubules that provide structural support and help transport nutrients between cells (Wolfe, 2012). By stabilizing microtubules, tau ensures efficient axonal transport, which is necessary for communication between the neurons, and neuronal survival. Efficient axonal transport is necessary for the delivery of nutrients, signalling molecules, and other components to help aid with the maintenance of synaptic function and plasticity (Wolfe, 2012). Tau proteins exist in many different isoforms that affect microtubules in different ways and have distinct roles in regulation dynamics (Wolfe, 2012). The activity of tau is regulated by phosphorylation, which will either increase or decrease the amount that is bound to microtubules (Wolfe, 2012).

Tau proteins can experience pathological change in which they become hyperphosphorylated. This can lead to the formation of neurofibrillary tangles that can disrupt neuronal function and lead to cell death (Lee, et al., 1991). Hyperphosphorylated tau will bind less with the microtubules, causing a build-up of hyperphosphorylated tau in the neurons, whilst making the microtubule structures less stable. A less stable microtubule structure can lessen axonal transportation (Lee, et al., 1991). The

accumulation of the hyperphosphorylated tau also in the neurons can develop into neurofibrillary tangles that interfere with synaptic pathways and neuronal connections, causing cognitive and memory impairment (Lee, et al., 1991). These tangles that accumulate and eventually lead to neuronal death are a known marker of neurodegenerative disorder (Blennow, Hampel, Weiner, & Zetterberg, 2010). Neurodegenerative diseases with the pathological hallmark of tau presence are also known as tauopathies (Lee, et al., 1991).

Levels of tau can be looked at as a biomarker for neurodegenerative disease (Blennow, et al., 2010). High or elevated total tau levels can be correlated with neuronal damage and are used well when looked at in combination with other biomarkers to diagnose and monitor the progression of neurodegenerative disease (Blennow, et al., 2010). Looking at these levels of tau can help with the early diagnosis of disease, particularly Alzheimer's (Blennow, et al., 2010).

1.5.8 P-Tau

Tau proteins are found in the axonal region of the neuron and typically provide stabilisation to microtubules that are commonly found in nerve cells and most tissues. When tau becomes hyperphosphorylated, the ability to stabilise and bind the microtubules is reduced, which ultimately can lead to synaptic impairment and neuronal death (Rawat, et al., 2022). This phosphorylated tau is a component of neurofibrillary tangles.

In abundance, p-tau damages axonal transport, which in turn will disrupt the distribution of mitochondria. This disturbance to the equilibrium results in an imbalance of mitochondria in the neurons (Rawat, et al., 2022). One of the responsibilities of

mitochondria in the brain is to form reactive oxygen species. When the balance of mitochondria is affected, a person can exhibit oxidative stress. Oxidative stress is a typical pathological state that occurs when the levels of reactive oxygen species become elevated and cause damage to other cells (Rawat, et al., 2022). Research is suggestive that because the brain is such a high energy-demanding body part, the smallest of changes to energy metabolism can cause problems in the central nervous system (Rawat, et al., 2022). This is why energy metabolism imbalance is a consistent feature of neurodegenerative diseases such as Alzheimer's, ALS and Parkinson's.

Upon hyperphosphorylation, tau proteins form neurofibrillary tangle aggregates. The accumulation of these abnormal aggregates is recognised by microglia as a signal that cells are damaged or distressed (Rawat, et al., 2022). When recognised, the microglia are triggered to release pro-inflammatory cytokines and chemokines (Rawat, et al., 2022). The release of both enables further phosphorylation and accumulation of tau. In other words, it creates a loop (Rawat, et al., 2022). This loop is causing further inflammation and build-up of tau pathologies, which will worsen the overall condition of the brain (Rawat, et al., 2022). Whilst the loop continues to repeat, the levels of cytokines and chemokines will continue to increase. The raised levels of cytokines contribute to the neurodegenerative process by worsening the level of neuronal death and synaptic dysfunction.

P-tau refers to the various phosphorylated tau protein variants as a group of tau proteins. When identifying specific p-tau, they are numbered according to where tau is specifically phosphorylated at threonine. So, p-tau 181 is specifically phosphorylated at threonine 181. This specificity can affect the function of p-tau differently and correlate to different

tau pathologies. This research looks specifically at three specific p-tau: 181, 217 and 231. P-tau 181 has been specifically linked to the presence of neurofibrillary tangles. Elevated p-tau 181 levels have been correlated to cognitive decline in Alzheimer's patients whilst being able to differentiate between Alzheimer's and non-Alzheimer patients (Rawat, et al., 2022). P-tau 217 has established the same links but has been suggested as more sensitive for the early detection of neurodegenerative diseases than p-tau 181 and 231 (Rawat, et al., 2022). P-tau 231 differs only from the other two in that it can be reflective of specific cognitive impairments, whilst being able to add extra information about the tau-related pathologies of neurodegenerative disease (Rawat, et al., 2022).

P-tau is valuable in research where neurodegenerative disease is concerned. In particular, the increase in p-tau concentration in CSF, blood and serum and its association with a higher accumulation of NFTs in the brain is what helps us recognise p-tau as a potential biomarker for diseases like Alzheimer's. When looked at in conjunction with other biomarkers such as amyloid beta, a strong pathological profile can be built, from which conclusions can be drawn when wanting to provide a clinical assessment of disease development. Whilst p-tau is a valuable pathological tool, it is understood that more research is still required to understand the extent to which this biomarker can provide evidence for neurodegenerative diseases.

1.5.9 Amyloid Beta

Amyloid beta is formed when the amyloid precursor protein (APP) is broken down by the beta-secretase and gamma-secretase enzymes to form amyloid-beta monomers (Chen, et al., 2017). The amyloid precursor protein can vary in length from 695 to 770 amino

acids (Chen, et al., 2017). Amyloid beta produced from this process can have peptides that vary in size from 37 to 49 residues, with amyloid-beta 42 being one of the more common forms (Chen, et al., 2017). Amyloid-beta aggregates into varying molecules that include oligomers, protofibrils and amyloid fibrils (Chen, et al., 2017). Oligomers are soluble and can spread through the brain. Amyloid fibrils are bigger in size and insoluble, with them being able to further aggregate into toxic amyloid plaques (Chen, et al., 2017). These plaques, being toxic, are thought to lead to neurodegeneration (Chen, et al., 2017).

Amyloid-beta 42 can be dangerous when overproduced in the brain, with an association of increased levels being connected to both the damage and plaque deposits in the synapses in the brain (Dementias Platform UK, 2021). Amyloid-beta 42 accumulates in the hippocampus, and when the concentration rises, a person can experience neuronal damage. This damage can translate into many symptoms such as confusion and memory loss that are also typical of neurodegenerative diseases (Dementias Platform UK, 2021).

Amyloid beta 42 has already been researched in relation to neurodegenerative disease, but the credibility of it being a biomarker improves when looked at in conjunction with other factors (Chen, et al., 2012; Lee, et al., 1991). Tauopathies and amyloid beta are referenced in research to show a correlation between each other, and the development of neurodegenerative disease (Lee, et al., 1991).

1.5.10 Neurofilament Light

Neurofilaments are cytoskeletal proteins that are specific to neurons made up of 3 smaller units called neurofilament light-chain (NFL), neurofilament medium-chain (NfM) and neurofilament heavy-chain (NfH) (Freedman, et al., 2024). This research has a particular

focus on NFL due to it being more widely studied as a biomarker for potential neurodegenerative disorders (Freedman, et al., 2024). NFL congregates into neurofilaments that are vital for dendritic branching and the growth and stability of the axons in the nerves. It is also important for the regeneration of post-traumatic axons (Pekny, et al., 2021). The damage of axons leads to NFL being released into the extracellular space rather than the CNS. This is why an elevated level of NFL in this extracellular space can be indicative of axonal damage and neurodegenerative diseases that include Alzheimer's, ALS and Parkinson's (Pekny, et al., 2021).

Other recent studies have made connections between NFL and concussions. The research has explored the idea that raised levels of NFL are correlative to several head traumas suffered. This is the case in a study that looks at the Ice Hockey players compared to a control group (Kawata, et al., 2018). A pre-season baseline is taken and compared to a post-season screening. When levels are compared to the control group, there is a notable increase in NFL among some Ice Hockey participants. The study reports that this increase does return to normal after approximately 3 months (Kawata, et al., 2018). This study enforces the idea that inflicted NFL levels could be indicative of a person experiencing or having experienced a recent concussion. It also suggested that NFL levels can be used as a predictor for recovery times, with a higher level of NFL correlating to a longer recovery period (Kawata, et al., 2018).

The links made in other research provide a reason to look at NFL in this research as a biomarker for the development of potential neurodegenerative disease. More research is necessary to understand how and at what lengths this biomarker can be used.

1.5.11 Retinol Binding Protein 4

Retinol Binding Protein 4 (RBP4) is a lipid metabolic protein. It is a specific transport protein for retinol in blood. RBP4 is produced in the liver and is secreted by hepatocytes after being loaded with retinol and has bound to transthyretin (TTR) (Steinhoff, et al., 2022). RBP4 binds to specific membrane receptors after retinol is delivered to cells (Steinhoff, et al., 2022). Maintaining an appropriate level of retinoids through this process in the body is important for many physiological processes including vision, growth, and development. The balance and regulation of retinoids is known as retinoid homeostasis.

In the brain, retinoid homeostasis is responsible for brain development, neural function, and the overall maintenance of cognitive performance. Low levels of RBP4 can affect the brain by disrupting the retinoid signalling pathways. These pathways are crucial for neural development, synaptic plasticity, and neurotransmission. The disruption of these pathways can lead to impaired cognitive function and neurological disorders (Steinhoff, et al., 2022).

Amyotrophic Lateral Sclerosis (ALS) is among other neurological disorders that have been linked to low levels of RBP4 in previous literature. A relationship has been reported for lower levels of RBP4 being associated with ALS. “It was found that in the multivariable model, increasing RBP4 concentration was associated with reduced odds for ALS … RBP4 was inversely related to risk for and prognosis of ALS” (Rosenbohm, et al., 2018).

Many links between levels of RBP4 and neurological disorders have been established in previous literature. More research into these relationships is needed to solidify the understanding of them and make them more credible in future.

1.5.12 Serum Amyloid A

Serum amyloid A is an apolipoprotein that is known for being a high-density lipoprotein. It is responsible for an inflammatory response to an injury or trauma to the body (Ye & Sun, 2015). It does this by acting as a chemotactic agent in immune cells and helping the transport of cholesterol from injured cells to the liver to be excreted (Sorić Hosman, Kos and Lamot, 2021). It also aids the inflammatory response by being a modulator of macrophage and neutrophil cells (Sorić Hosman, Kos and Lamot, 2021). The response from serum amyloid alpha is positively correlated to the level of neuroinflammatory response, suggesting that the levels of the apolipoprotein can be used as a biomarker for traumatic brain injury (Wicker, et al., 2019).

We can hypothesise that multiple brain injuries can alter levels of serum amyloid A, but this has not yet been researched or proven. Research has shown that elevated levels are typically found in a disorder that has an association with inflammation (Ye & Sun, 2015). We know that concussion triggers a neuroinflammatory response, which can trigger a surplus of serum amyloid A in the liver as it is an acute-phase protein (Wicker, et al., 2019). Because there is typically a link between traumatic brain injuries and the likelihood of developing a neurodegenerative disease, we can consider the use of serum amyloid A as a potential biomarker for neurodegenerative disease.

1.5.13 Exosome Size

Exosomes are extracellular vesicles that are generated by all cells, with relation to immune response, cardiovascular disease, and central nervous system-related diseases. They can carry proteins, lipids, nucleic acids and other metabolites and aid intercellular

communication in both health and disease (Kalluri & LeBleu, 2020). The vesicles can intrinsically regulate intracellular pathways and being able to do this means they can be utilised in both diagnostic and therapeutic control of neurodegenerative diseases (Kalluri & LeBleu, 2020).

In other research, exosomes have been looked at as a biomarker for both TBI and concussion due to their ability to be able to cross the blood-brain barrier (Karnati, et al., 2019). They are reflective of the environment they come from, which means they can provide specific pathological changes when someone experiences a concussion or TBI. This information is accessible because the internal parts of the exosome are protected from enzymatic degradation by the membranes (Karnati, et al., 2019). Exosomes can also give good insight as they have a presence of both amyloid beta and phosphorylated tau proteins (Karnati, et al., 2019). Both components are regarded as pathological hallmarks for multiple neurodegenerative diseases such as Alzheimer's, ALS and Parkinson's (Guedes, et al., 2020). Exosomes are explained as a 'vehicle' for the spreading of tau proteins in the central nervous system (Karnati, et al., 2019). The accumulation and spread of tau proteins is believed to be one of the pathological explanations for the development of neurodegenerative disease that has TBI as its onset (Karnati, et al., 2019).

Previous work in the UK Rugby Health study has measured exosomes and compared them to the levels of tau and amyloid beta proteins (Alanazi, et al., 2024). Where possible, the exosome measures have provided insight into biomarkers as a tissue and the environment they come from. Looking at exosomes in this way will give scope to understand specific cells and how they respond to concussive events and traumatic brain

injuries. It could also provide better measurability of tau and amyloid beta, as they are found at low levels in circulation.

1.6 Cognitive Parameters

Cognitive parameters are important for diagnosing potential neurodegenerative disease. Most initial stages for diagnosing neurodegenerative disease include clinical testing, which considers a person's functionality, based on cognitive parameters. Most clinical tests will look to hit the main groups when diagnosing neurodegenerative disease. They will look at cognitive and psychological symptoms such as memory, function, attention, speech and emotional balance impairments whilst taking note of any other diagnostic symptoms a patient may be experiencing (Albert et al., 2011). Whilst it is time-consuming and has a requirement of special training to be carried out, clinical testing is the least invasive and most effective way to test (Lezak, Howieson, Bigler and Tranel, 2012). It can be done multiple times, making assessment of the severity and disease progression easily accessible.

Previous investigations in the UK Rugby Health Study have combined cognitive performance measures with biomarker data to examines neurological outcomes in retired rugby players (Hind et al.,2021, Alanazi et al, 2024). Cognitive parameters were assessed to understand a broader picture of neurocognitive function. The measures explored associations between biomarkers and neurodegenerative disease and provide insight into the potential link between repeated head injury and cognitive decline.

1.7 Project Aims

This project aims to investigate the relationship between blood biomarkers in retired rugby players who have experienced multiple concussive episodes, comparing them to a control group of non-contact athletes. Previous studies suggest that the concentration of biomarkers can change in response to neuroinflammation or brain injury, which could provide insight into the biological processes underlying repeated concussive events.

The hypothesis is that repeated head trauma in rugby players may influence blood biomarkers, potentially serving as early indicators of neurodegenerative disease or its onset. The study aims to 1) analyse and compare concentrations of 24, 25, and 27 Hydroxycholesterol and APOE4 between retired rugby players and non-contact athletes; 2) determine the association between biomarkers and cognitive function. Raised levels of a biomarker may be representative of neuroinflammatory processes or oxidative stress. Alternatively, a decrease in levels could lead to tissue damage and a reduced ability to remove neurodegenerative proteins. This allows for the analysis of multiple correlations and relationships between biomarkers that can be linked to pathological hallmarks for neurodegenerative disease.

By comparing these biomarkers in athletes with a history of concussion to a control group, the study aims to better understand how repeated concussive events can accelerate neurodegenerative processes and contribute to cognitive decline. The study aims to further understand the pathophysiological mechanisms linked to concussion, and how, when these mechanisms are disrupted, biomarker levels are disrupted, ultimately linking to cognitive decline and neurodegenerative disease. These findings could pave the way

for more precise biomarkers to assess risk and inform early detection and interventions for rugby players, and others involved in high-contact sports.

2. Materials and Methods

2.1. Materials

Material Used	Manufacturer	ID
24(S)-Hydroxycholesterol ELISA Kit	Abcam	AB204530
Human 25-Hydroxycholesterol ELISA Kit	BT LABS	EA0143Hu
27-Hydroxycholesterol (27-HC) ELISA Kit	Abbexa	ABX257403
APOE4 ELISA Kit	Thermofisher	EHAPOE4
Human Ceramide (CER) Elisa Kit	AFG Scientific	EK710698
Triglyceride (TG) Elisa Kit	Abbexa	ABX257659
Phosphate-buffered saline (PBS)	Abcam	AB270748

Table 1: Table to show materials used, where from and identification codes.

2.2. Methods

2.2.1. Participants

The average age of a UK-based male group of retired rugby players (n=34) that have shown a previous record of concussion, and a male non-contact sports group (n=22) is 43.21 ± 9.82. The mean values for weight and age at retirement of the entire cohort are 103.63 ± 22.68 kg and 33.79 ± 7.34 years, respectively.

More specifically, the mean values for age, weight and BMI in the retired rugby player group are 40 ± 6.6 years, 101.3 ± 12.61 kg and 29 ± 3.9, correspondingly, with the retired rugby group retiring 7 years earlier on average. The retired rugby group consists of 24 Rugby Union and 12 Rugby League players, where 29 players played at a professional level, and 5 players played at an amateur level. Alternatively, the averages for age, weight

and BMI in the control group are 47.7 ± 12.6 years, 84.2 ± 12.7 kg and 26 ± 3.3, subsequently.

The retired rugby player group have at least 5 recorded concussions. In this research, they are considered as a concussed group when compared to the non-contact sport control group. This will allow this research to make distinctions between biomarker concentrations in the control versus the concussed group and establish significance by calculating the P-value. Multiple biomarkers were considered when looking to establish links between how biomarkers respond differently to traumatic brain injury, and the relationships or interactions between these. Further assessment uses analysis of variance (ANOVA) between two biomarkers or a biomarker and cognitive parameter to try and further establish any relationships that are statistically significant $F<0.05$.

2.2.2. 24-Hydroxycholesterol ELISA

Abcam's 24(S)-Hydroxycholesterol kit is a competitive in-vitro ELISA that is used for the quantitative measurement of 24-HC levels in a sample. Serum samples were diluted 10-fold in PBS (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4) before starting the assay. 100 µL of both standard and diluted serum samples were added to the plate wells that were pre-coated with the capture antibody (goat anti-rabbit IgG). All standards and samples were run in duplicate. 50 µL of diluted conjugate is added to all the wells before the plate is sealed and incubated at room temperature for an hour. When incubated at room temperature, the antibody will bind 24-HC or the biotin conjugate. This competitive binding occurs because free 24-HC in the sample and the biotinylated 24-HC have similar affinity for the specific antibody, so higher

endogenous 24-HC reduces binding of the biotin conjugate. Each well on the plate is washed with 400 µL, 3 times over, using wash buffer (tPBS containing 0.05% Tween-20**,** where Tween-20 is present at ~3.4 mM) leaving only the bound 24-HC and biotin conjugate. 200 µL of Streptavidin HRP conjugate is added to each well, which binds the biotinylated 24-HC. Again, the plate is sealed and incubated for 30 minutes at room temperature. The wash process is again repeated to remove the excess Streptavidin-HRP conjugate. 200 µL of TMB substrate is added, and then an HRP-catalysed reaction results in the solution turning blue. At the molecular level, HRP (horseradish peroxidase) oxidises TMB (3,3',5,5'-tetramethylbenzidine) using hydrogen peroxide, forming a blue chromophore whose concentration reflects how much biotinylated 24-HC was bound. The plate is sealed and incubated at room temperature for 30 minutes, a final time. Finally, 50 µL of stop solution (2 M sulfuric acid) is added to prevent any further reactions, turning the solution yellow by protonating the oxidised TMB.

The resulting colour is read at 450 nm in a plate reader, with the amount of absorbance shown being inversely proportional to the levels of 24-HC in the sample/standard. All values must be multiplied by 10 to get a true concentration value, due to the dilution of samples being done at 10-fold.

2.2.3. 25-Hydroxycholesterol ELISA

BT Labs 25-Hydroxycholesterol kit is a competitive sandwich ELISA that is used for the quantitative measurement of 25-HC levels in a sample. 50 µL of standard and serum samples were aliquoted to plate wells that are pre-coated with the 25-HC capture antibody. All standards and samples were run in duplicates. 50 µL of biotinylated antigen

is added to each well and the plate is covered with a plate sealer and incubated for one hour at 37°C. Whilst being incubated at 37°C, the antibody will bind 25-HC or the biotin conjugate through competitive binding, where endogenous 25-HC competes with the biotin-labelled analogue for the same antibody binding sites. When the incubation period is complete, the plate is washed with 300 µL of wash buffer, typically PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4) containing 0.05% Tween-20 (approximately 3.4 mM), 5 times over, leaving only the bound 25-HC and biotin conjugate. After washing, 50 µL of avidin-HRP is added to the wells. Avidin binds tightly to biotin via high-affinity non-covalent interactions, allowing HRP to be linked proportionally to the amount of biotinylated antigen bound. The plate is covered with a new seal and incubated again for one hour at 37°C. After incubation, the seal is removed, and the wash process is repeated to remove the excess avidin-HRP. 50 µL of substrate solution A is added to each well followed by adding 50 µL of substrate solution B to each well. These typically contain TMB and hydrogen peroxide, which react in the presence of HRP to form a blue oxidised product. The plate is covered with a fresh seal and incubated for 10 minutes at 37°C, a final time. Finally, 50 µL stop solution, usually 2 M sulfuric acid, is added, preventing further reaction and turning the solution yellow by converting the oxidised TMB into its stable, protonated form. The intensity of colour change is measured at 450 nm on a plate reader, with the amount of absorbance shown being inversely proportional to the levels of 25-HC in the sample/standard.

2.2.4. 27-Hydroxycholesterol ELISA

Abbexa's 27-Hydroxycholesterol kit is a competitive ELISA that is used for the quantitative measurement of 27-HC levels in a sample. Serum samples were diluted 10-fold in PBS

before the assay; PBS typically contains 10 mM phosphate buffer (about 8 mM Na2HPO4 and 2 mM KH2PO4), 137 mM NaCl and 2.7 mM KCl at pH 7.2–7.4. In the competitive format, free 27-HC in the sample competes with the 27-HC that is pre-coated on the well surface for a limited amount of biotin-labelled 27-HC (Detection Reagent A). When 50 µL of standard, diluent buffer, and sample are added to the wells followed immediately by 50 µL of Detection Reagent A, the biotin-labelled conjugate binds either to the free 27-HC in solution or to the immobilised antigen. A higher concentration of 27-HC in the sample results in less biotin-labelled conjugate binding to the plate. The diluent buffer supplied in such kits is usually PBS-based and typically contains about 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl and additives such as 1 percent BSA and 0.05 percent Tween-20. After the 45-minute incubation at 37°C, the plate is washed three times with wash buffer, which is normally PBS containing approximately 0.05 percent Tween-20 (the molar components remain 10 mM phosphate and 137 mM NaCl). This removes unbound conjugates. Then 100 µL of Detection Reagent B is added; this reagent is generally streptavidin–HRP in a stabilised PBS buffer of similar ionic composition. After a 30-minute incubation at 37°C, the plate is washed five times to remove unbound conjugate. Next, 90 µL of TMB substrate is added; TMB substrate solutions typically contain TMB and hydrogen peroxide (around 3 mM) in a 10–50 mM citrate or acetate buffer at pH around 5. Wells containing more HRP-bound conjugate develop a stronger blue colour. The plate is incubated for 20 minutes at 37°C and then 50 µL of stop solution, normally 1–2 M sulfuric acid, is added to convert the colour to yellow and stabilise the signal. The absorbance at 450 nm is then measured, and the intensity is inversely proportional to the amount of 27-HC present in

the sample because higher sample concentrations prevent binding of the biotin-labelled conjugate to the plate.

2.2.5. APOE4 ELISA

Thermofisher's Human Apolipoprotein E4 ELISA is a solid-phase sandwich ELISA that is used for the detection and quantitative measurement of human apolipoprotein E4 in serum, plasma or cell culture media. In a sandwich ELISA, antigen in the sample is captured between two antibodies: a capture antibody that is immobilised on the plate and a detection antibody that binds a different epitope on the same antigen, allowing highly specific and sensitive quantification.

Serum samples were diluted 200-fold with PBS before starting the assay; PBS typically contains 10 mM phosphate buffer (8 mM Na2HPO4 and 2 mM KH2PO4), 137 mM NaCl and 2.7 mM KCl at pH 7.2–7.4. 100 µL of standard and diluted serum samples were aliquoted into the plate wells that are coated with Human Apolipoprotein E4. The wells were covered and incubated for 2.5 hours at 4°C to allow the antigen in the samples to bind to the immobilised capture antibody. The solution in the wells was discarded and washed by filling each well with 300 µL of wash buffer and emptying it four times; wash buffers for ELISA are typically PBS containing 0.05 percent Tween-20, so the molar components remain approximately 10 mM phosphate and 137 mM NaCl. After this, 100 µL of biotin conjugate is added to each well and the plate is sealed for an incubation period of 1 hour at room temperature, during which the biotin-labelled detection antibody binds to any captured Apolipoprotein E4. The solution is again discarded, and the wash process is followed to remove any unbound conjugate. 100 µL of Streptavidin-

HRP is added to each well, followed by sealing the plate and another incubation period of 45 minutes at room temperature with gentle shaking; streptavidin-HRP solutions are typically supplied in PBS-based stabilising buffers containing 0.1–1 percent BSA and 0.05 percent Tween-20. For a final time, the solution is removed, and the wash process is followed. 100 µL of TMB substrate is added to each well, with the substrates beginning to turn blue as the HRP enzyme catalyses the oxidation of TMB. TMB substrate solutions generally contain TMB and hydrogen peroxide (around 3 mM) in a mildly acidic citrate or acetate buffer of approximately 10–50 mM. The plate is covered and incubated in the dark at room temperature with gentle shaking for 30 minutes. After the final incubation period, 50 µL of stop solution, usually 1–2 M sulfuric acid, is added into each well promptly and uniformly to halt any further reactions and convert the blue product to a yellow one that is measurable at 450 nm. The intensity of the yellow colour is read on a microplate reader at 450 nm, with the absorbance values returned being directly proportional to the levels of Apolipoprotein E4 in the standards or serum samples.

2.2.6. Human Ceramide (CER) Elisa Kit

AFG Scientific's Ceramide Kit is a quantitative ELISA that is used for the measurement of ceramides in a sample. This kit uses a typical sandwich ELISA format in which the antigen in the sample is captured by an immobilised antibody on the plate and then detected by an enzyme-linked secondary reagent, allowing direct proportionality between signal intensity and ceramide concentration. The 96-well plate is pre-coated with a capture antibody specific to ceramide. Samples are diluted five-fold by adding 40 µL of sample diluent followed by 10 µL of each sample into the designated wells, while 50 µL of each

standard is aliquoted into standard wells; sample diluent in ELISA kits is usually PBS-based and typically contains 10 mM phosphate buffer (8 mM Na2HPO4 and 2 mM KH2PO4), 137 mM NaCl, 2.7 mM KCl, and stabilisers such as 1 percent BSA and 0.05 percent Tween-20. All standards and samples are run in duplicate and the plate is covered and incubated at 37°C for 40 minutes, allowing ceramide in the sample to bind to the immobilised capture antibody. Each well is then washed with 300 µL of wash buffer five times; such wash buffers are generally PBS containing 0.05 percent Tween-20, maintaining the ionic strength of 10 mM phosphate and 137 mM NaCl while enabling efficient removal of unbound material. After washing, 50 µL of HRP conjugate is added to each well (except the blank) and the plate is again covered and incubated at 37°C for 40 minutes. The conjugate binds to the captured ceramide, forming the complete antibody–antigen–enzyme complex. The wash process is repeated to remove excess HRP. Next, 50 µL of Chromogen A is added to all wells followed by 50 µL of Chromogen B, producing a blue colour as the HRP enzyme catalyses the chromogenic reaction; these chromogens function similarly to TMB substrates, typically containing peroxide and aromatic electron-donor compounds dissolved in mildly acidic buffers of around 10–50 mM. Light is avoided during this step. The plate is covered and incubated at 37°C for 20 minutes. Following incubation, 50 µL of stop solution, normally 1–2 M sulfuric acid, is added to each well, converting the blue substrate into a stable yellow product measurable at 450 nm. The absorbance at 450 nm is directly proportional to ceramide concentration, and all recorded values are multiplied by five to account for the initial dilution.

2.2.7. Triglyceride (TG) Elisa Kit

Abbexa's Triglyceride Kit is a competitive ELISA that is used for the measurement of triglyceride in a sample. In a competitive ELISA, analyte present in the sample competes with antigen immobilised on the plate for a limited amount of detection reagent, causing an inverse relationship between colour intensity and analyte concentration. The 96-well plate is pre-coated with a capture antibody specific for triglyceride. 50 µL of Standard Diluent buffer is added to the zero-control well; such diluent buffers are commonly PBS-based and contain approximately 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, 1 percent BSA and 0.05 percent Tween-20. 50 µL of standard and 50 µL of samples are aliquoted into the relevant wells in duplicate. Immediately, 50 µL of Detection Reagent A is added to each well and the plate is covered and incubated for 1 hour at 37°C. During this time, endogenous triglyceride in the sample competes with the immobilised antigen for binding to the biotin-labelled detection component. Each well is then washed with 350 µL of wash buffer three times; wash buffer is typically PBS containing 0.05 percent Tween-20, giving approximately 10 mM phosphate and 137 mM NaCl. Following washing, 100 µL of Detection Reagent B is added and the plate is incubated for 30 minutes at 37°C; Detection Reagent A and B together form the HRP-conjugate, often via biotin–streptavidin interaction. The wash process is repeated five times to remove excess conjugate. 90 µL of TMB substrate is added to each well, producing a blue colour as the HRP enzyme catalyses oxidation of TMB; TMB substrates typically contain hydrogen peroxide at around 3 mM in a 10–50 mM citrate or acetate buffer at pH about 5. The plate is then incubated for 10–20 minutes at 37°C. Immediately after this incubation, 50 µL of stop solution, usually 1–2 M

sulfuric acid, is added to convert the blue oxidised TMB to a yellow product measurable at 450 nm. The resulting absorbance at 450 nm is inversely proportional to the concentration of triglyceride in the standards or samples, because higher amounts of sample triglyceride prevent binding of the HRP-linked detection complex to the plate.

2.2.8. The CNSVS Battery

Cognitive parameters were measured using the Vital Signs Computerized Neurocognitive Assessment, which was developed as a routine clinical screening tool (Gualtieri & Johnson, 2006). The clinical evaluation battery is made up of seven tests: verbal memory (VBM), visual memory (VIM), finger tapping (FTT), symbol digit coding (SDC), the Stroop Test (ST), a test of shifting attention (SAT) and the continuous performance test (CPT). These tests are used widely by neuropsychologists with acknowledgement of their reliability and validity. The tests are also acknowledged to be sensitive to most of the causes of cognitive dysfunction or impairment. The data provided was collected when participants attended testing appointments as part of the UK Rugby Health Study.

2.2.8.1. Verbal and Visual Memory Tests (VBM and VIM)

The verbal memory test is a variation of the Rey Auditory Verbal Learning Test (Rey, 1964; Taylor, 1959). In the Vital Signs CNS version of the test, 15 words are shown one by one, every two seconds, on a screen (Gualtieri & Johnson, 2006). The participant is asked to remember the 15 words before being shown a list of 30 words. Among the 30 words, the original 15 are mixed randomly. When the participant recognises one of the 15 target words, they press the space bar. After the other 6 tests, the participant is shown 30 words, again with the 15 target words mixed in, and asked to recognise them.

The visual memory test is also a variation of a Rey test where it uses geometric figures rather than words (Rey, 1964; Taylor, 1959). 15 figures are shown, and the participant is asked to remember them before being shown 30 figures (Gualtieri & Johnson, 2006). Among the 30 figures, the original 15 are mixed randomly. When the participant recognises one of the 15 target words, they press the space bar. After 5 other tests, the participant is shown 30 figures, again with the 15 target figures mixed in, and asked to recognise them.

The scores of verbal memory and visual memory are recorded separately, as well as being summed to generate a raw memory score.

2.2.8.2. The Finger Tapping Test (FTT)

The finger-tapping test is a simple test that asks the participant to tap the space bar as many times as they can in 10 seconds (Gualtieri & Johnson, 2006). The test consists of one practice, then three runs. It is repeated with testing on the left hand. The finger-tapping test score is generated by an average of the scores on the left and the right hand.

2.2.8.3. Symbol Digit Coding (SDC)

The symbol digit coding test will first give the participant a training session on how the numbers are linked to digits (Gualtieri & Johnson, 2006). The test then will begin, showing one screen at a time with 8 symbols and 8 empty boxes below. The aim is for the participant to type the corresponding digit (from 2 to 9) in the empty box. The test will continue showing 8 symbols and 8 empty boxes per screen for 120 seconds. The score for the symbol digit coding test is generated by using the correct number of responses in 2 minutes.

The number of right and left taps from the finger-tapping test and the number of correct responses from the symbol digit coding test are combined to form a score for psychomotor speed.

2.2.8.4. The Stroop Test (ST)

The Stroop Test that Vital Signs uses consists of four colours/colour words and the space bar (Gualtieri & Johnson, 2006). The test is made up of three sections. In the first section, the words red, yellow, blue and green appear in black text at random, and the participant should press the space bar as soon as they appear. This will generate the score for simple reaction time. In the next section, the same words appear but this time in different colours. The participant is asked to tap the space bar when the colour of the word matches the word. This part of the test generates a complex reaction time score. The final part of the test is the same as the second, but instead, the participant is asked to tap the space bar when the colour of the word does not match the word. This also generates a complex reaction time score, called the colour-word reaction time. An error score is also created. Taking an average of the scores from sections 2 and 3 gives a score for processing speed.

2.2.8.5. The Shifting Attention Test (SAT)

The shifting attention test is designed to measure a participant's ability to shift from one set of instructions to another accurately (Gualtieri & Johnson, 2006). In the test, the participants are asked to match geometric objects by either shape or colour when three figures appear on a screen. The images are placed with one on the top (either a square or circle),and two on the bottom (a square and circle each). All figures are either red or

blue. The top figure is the one that will need to be matched to either figure on the bottom. The rules for matching this are random and do change. This test goes on for 90 seconds with the goal of choosing the correct match as many times as the participant can. The score for cognitive flexibility can be generated by subtracting the number of response errors on the SAT and Stroop test, from the number of correct responses.

2.2.8.6. The Continuous Performance Test (CPT)

The Continuous Performance Test in Vital Signs asks the participant to respond to the letter “B” which is the target stimulus, but not any other letter (Gualtieri & Johnson, 2006). In a timeframe of 5 minutes, the participant will see 200 letters, of which 40 are the target letter. The letters are shown randomly, with the target stimulus being shown 8 times per minute. The test is scored into three categories: correct responses, commission errors and omission errors. The test will also report the participant’s reaction time to choose for each variable. The score for complex attention is generated by adding the number of errors committed in the CPT, the SAT and the Stroop.

2.2.9. Data Analysis

All ELISA absorbance readings at 450 nm were recorded using a microplate reader. For each sample, the mean of the technical replicates was calculated and used for further analysis and plotting to reduce the effect of pipetting or assay variability. Standard curves were generated for each ELISA by plotting the mean absorbance of the standards against their known concentrations. For sandwich ELISAs (e.g., ceramide, ApoE4), a regression model was fitted to the standard curve to interpolate sample concentrations. For competitive ELISAs (e.g., triglycerides, 27-hydroxycholesterol),

standard curves were also fitted using a regression model, and sample concentrations were determined inversely from the standard curve.

Any sample dilution factors were applied to the calculated concentrations to obtain final values (for example, ceramide values were multiplied by five to account for the five-fold dilution). Data are presented as mean ± standard deviation (SD) for technical replicates. Where multiple groups were compared, statistical analyses was performed. All data processing, curve fitting, and statistical analyses were performed using either GraphPad Prism or Excel with significance accepted at $p<0.05$ or $p<0.01$.

Data were analysed using descriptive and inferential statistics. Outliers were retained because, in human blood biomarker research, extreme values may reflect genuine biological variability rather than error. This ensures the analysis captures the full range of physiological differences.

3. Results

The study was designed to quantify multiple lipid-related biomarkers using ELISA assays, enabling direct comparison of ceramide, triglyceride, apolipoprotein E4 and 27-hydroxycholesterol levels between concussed and non-concussed groups. This was not investigated between different codes of rugby, to allow the focus to stay on differences between contact and non-contact athletes.

All ELISA's were performed using technical replicates, and the mean of these replicates were used for plotting and analysis.

3.1. Biomarkers

3.1.1. 24-Hydroxycholesterol

In this research, a sandwich ELISA was carried out to calculate the levels of 24-HC in serum samples. The first step of this was to establish levels of absorption in standard samples of a known concentration of 24-HC.

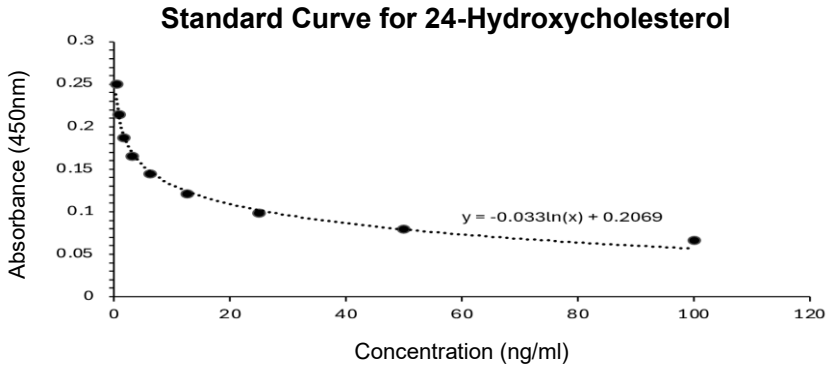


Figure 4: Standard curve for 24(S)-Hydroxycholesterol ELISA Assay.

Figure 4 shows the original plot of a known concentration and the correlating absorbance values. Inverse proportionality results lead us to take the logarithmic values of concentration to be able to fit a linear standard curve.

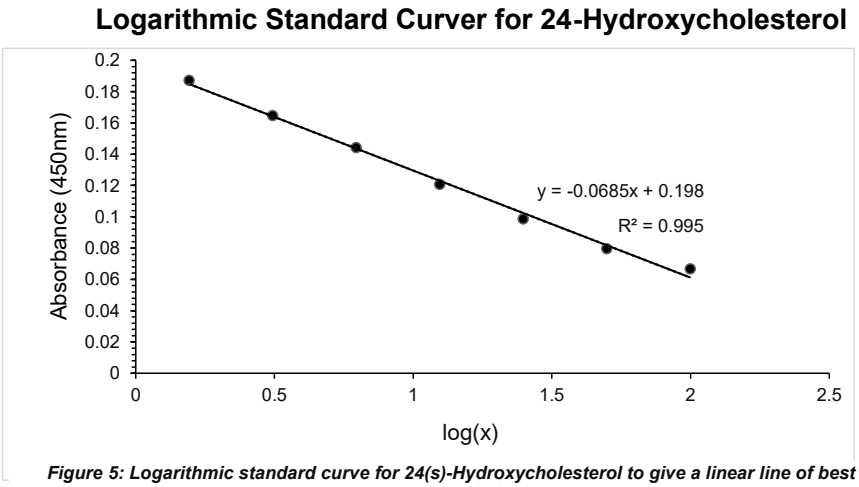


Figure 5: Logarithmic standard curve for 24(s)-Hydroxycholesterol to give a linear line of best fit for correlation.

Figure 5 shows the logarithms of the same values of known concentrations against absorbance to produce the linear standard curve required. Figure 6 shows this and shows the reliability of the standard testing by producing a line of best fit with $R^2 > 0.95$. The equation of the standard curve can be used to work out unknown concentrations from samples where we have an absorbance value, with inverse log and dilution factors being considered.

The linear standard curve gives an equation that can be used to work out unknown concentrations. The absorption values from unknown concentration samples can be taken and input into the equation to work out the unknowns. From this, a comparison can, be drawn between the control group (n=10) and the retired rugby player group (n=10).

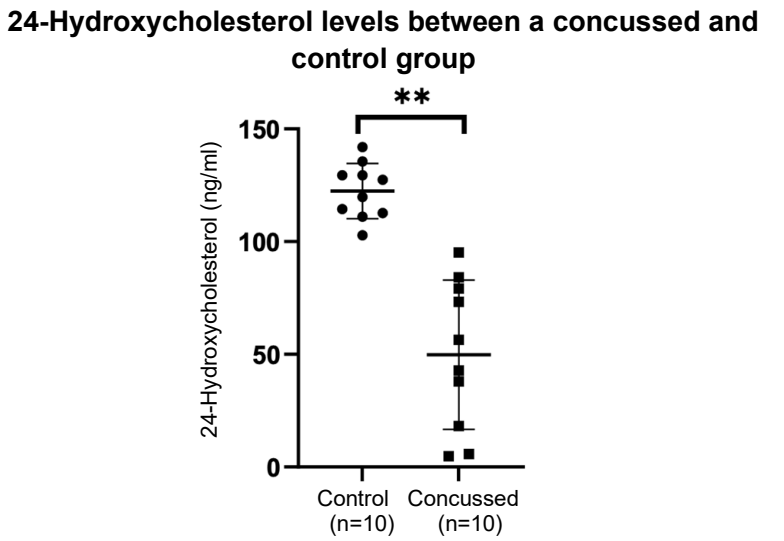


Figure 6: 24-HC serum comparison between a concussion and control group.

*A decrease in the 24-HC serum concentration is seen in the concussed group. Dots show the individua data points in the control group whilst squares show the individual data points in the concussed group. Bars show the median and upper and lower quartiles. Test for significance shows ** as P<0.01. Statistical analysis and graphing were carried out by using the Mann Whitney U Test on GraphPad prism.*

Figure 6 shows a significant ($p<0.01$) reduction in the concentration of 24-HC in the retired rugby player group (concussed). These findings are expected and in line with other research where there is a reduced concentration of 24-HC in study participants with TBI history or neurodegenerative disease. This result gives a foundation for further investigation into interactions and correlations between 24-HC and other biomarkers that were previously investigated in the UK Rugby Health Study. There was no statistical significance when correlating 24-HC with 25-HC, T-Tau, RBP4, $A\beta_{42}$, P-Tau 217, NFL or Exosome size. A Positive correlation was found with statistical significance when correlating between 24-HC and SAA ($p = 0.0147$, $r = 0.773$ $R^2 = 0.0597$). Negative correlations were found with statistical significance when correlating between 24-HC and

27-HC (p = 0.0464, r = 0.486 R^2 = 0.235), P-Tau231 (p = 0.0479, r = 0.639 R^2 = 0.409) or P-Tau181 (p = 0.0358, r = 0.969 R^2 = 0.939).

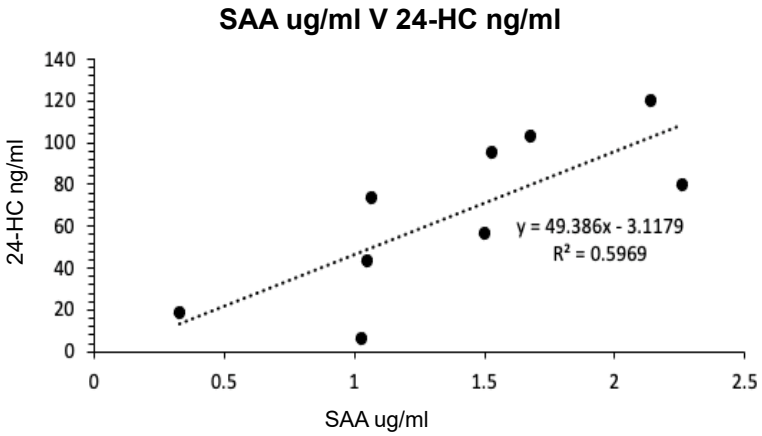


Figure 7: Positive correlation between SAA and 24-HC. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel. SAA data was taken from the UK Rugby Health Project.*

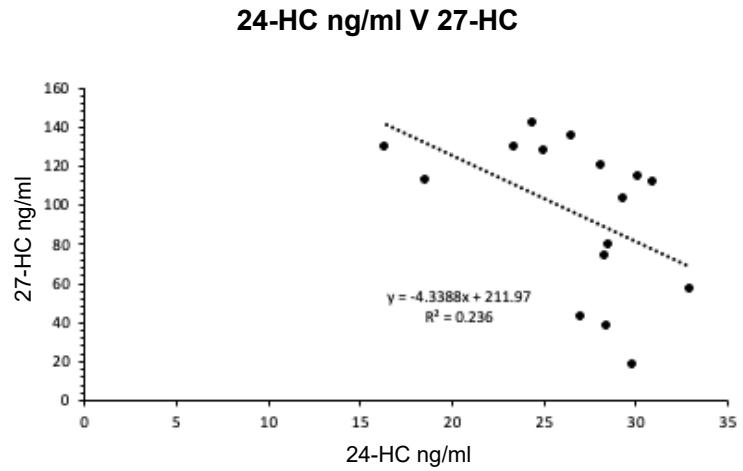


Figure 8: Negative correlation between 27-HC and 24-HC. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel.*

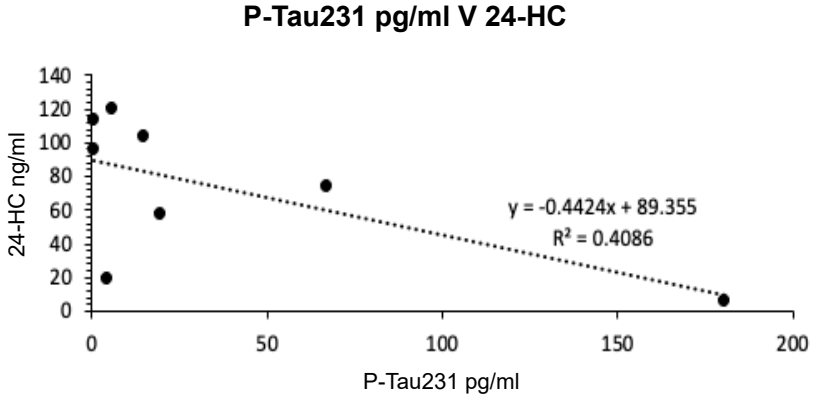


Figure 9: Negative correlation between P-Tau231 and 24-HC. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel. P-Tau231 data was taken from the UK Rugby Health Project.*

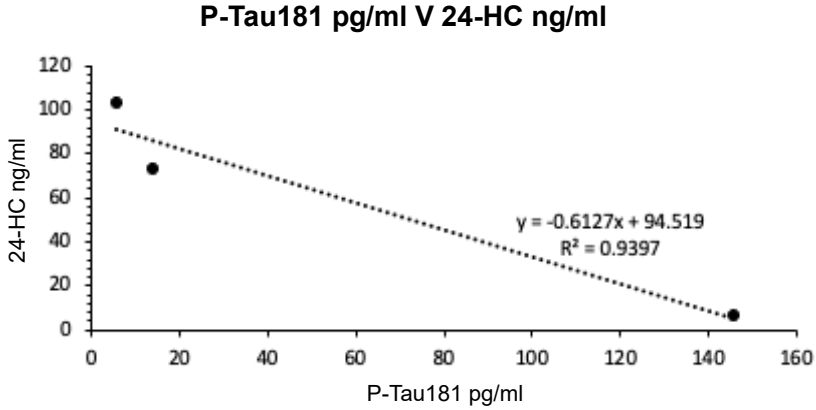


Figure 10: Negative correlation between P-Tau181 and 24-HC. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel. P-Tau181 data was taken from the UK Rugby Health Project.*

3.1.2. 25-Hydroxycholesterol

In this research, a sandwich ELISA Assay was carried out to calculate the concentration of 25-HC in serum samples. To achieve this, samples with a known concentration of 25-HC were used against their absorbance values to develop a standard curve. The data and standard curve were gathered and developed as part of the UK Rugby Health Project.

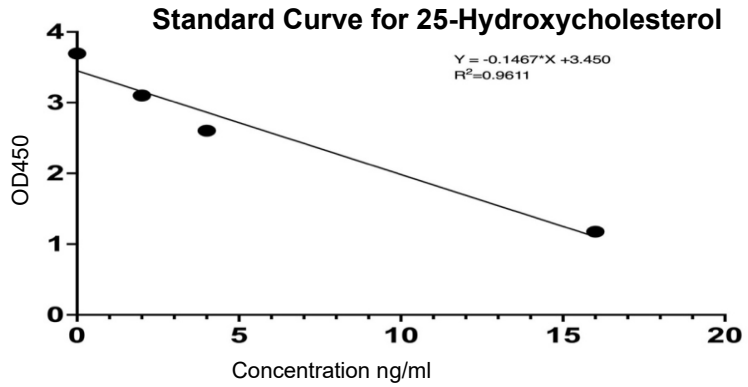


Figure 11: Standard curve for 25-Hydroxycholesterol - *Data was taken from the UK Rugby Health Project.*

Figure 11 shows concentrations against absorbance to produce a linear standard curve required. Figure 6 shows this and shows the reliability of the standard testing by producing a line of best fit with $R^2 > 0.95$.

The standard curve developed can provide an equation that can be used to work out unknown concentrations of the participant samples. Absorption values from the ELISA can be taken and input into the equation to find the relative concentration values. The resulting concentration values for samples and their respective ID codes were provided to be used in this research. This allowed for a 25-HC serum concentration comparison between the control group (n=13) and the retired rugby player group (n=6).

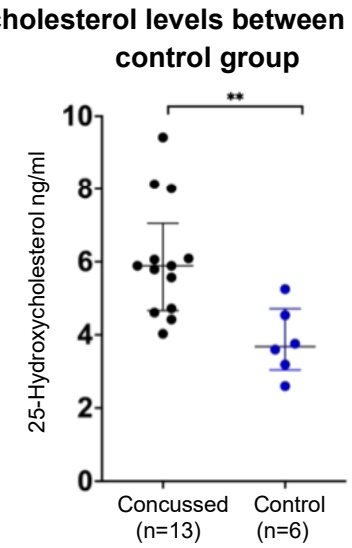


Figure 12: 25-HC serum comparison between a concussion and control group.

*An increase in the 25-HC serum concentration is seen in the concussed group. Black dots show the individual data points in the concussed group whilst blue dots show the individual data points in the control group. Bars show the median and upper and lower quartiles. Test for significance shows ** as P<0.01. Statistical analysis and graphing were carried out by using the Mann Whitney U Test on GraphPad prism.*

Figure 12 shows the comparison shows a significant ($p<0.01$) increase in the concentration of 25-HC in the retired rugby player group (concussed). These findings are consistent with other research where there is an increased concentration of 25-HC in study participants with increased neuroinflammatory markers. This result gives reason for further investigation into interactions and correlations between 25-HC and other biomarkers that were previously investigated in the UK Rugby Health Study. There was no statistical significance when correlating 25-HC with 24-HC, 27-HC, T-Tau, SAA, RBP4, P-Tau217, NFL or Exosome Size, respectively. Positive correlations with statistical significance were found when correlating between 25-HC and $A\beta_{42}$ ($p = 0.00844$, $r = 0.694$

R^2 = 0.482), P-Tau231 (p = 0.0231, r = 0.441 R^2 = 0.195) or P-Tau181 (p = 0.0494, r = 0.906 R^2 = 0.820).

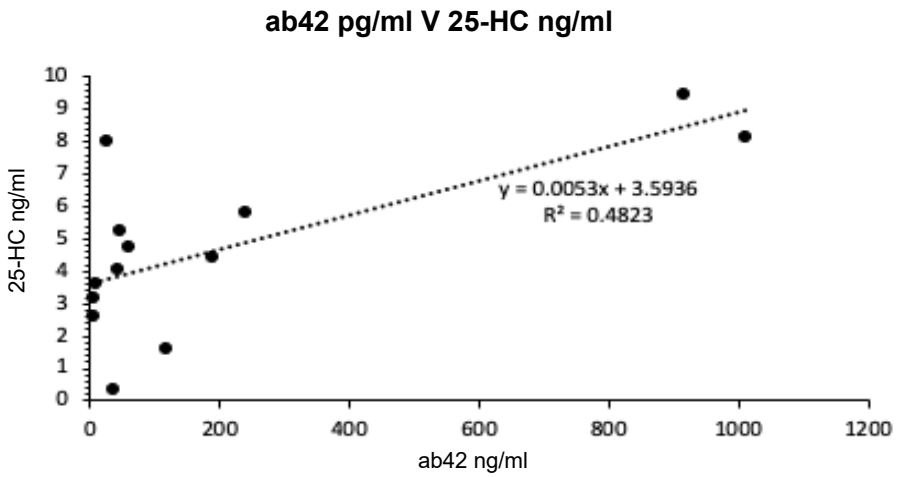


Figure 13: Positive correlation between $A\beta_{42}$ and 25-HC. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel. The $A\beta_{42}$ data was taken from the UK Rugby Health Project.*

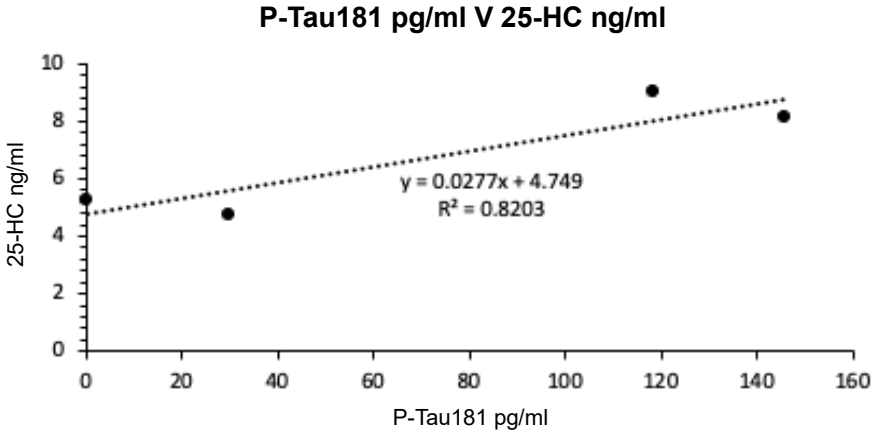


Figure 14: Positive correlation between P-Tau181 and 25-HC. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel. P-Tau181 data was taken from the UK Rugby Health Project.*

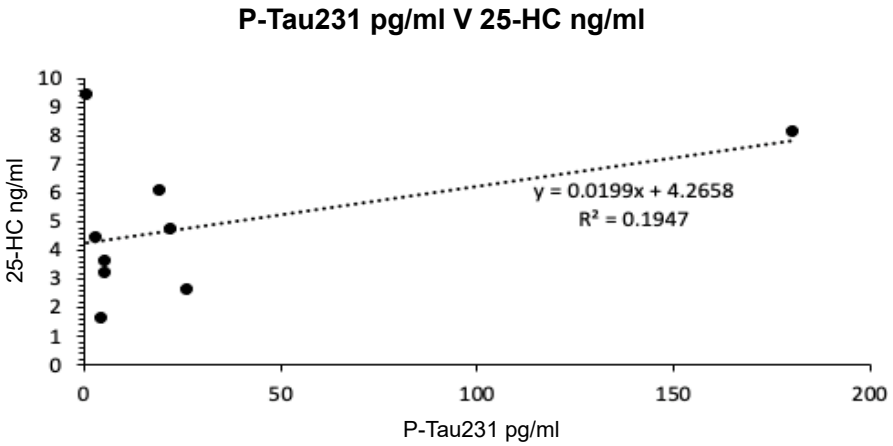


Figure 15: Positive correlation between P-Tau231 and 25-HC. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel. P-Tau231 data was taken from the UK Rugby Health Project.*

3.1.3.

3.1.4. 27-Hydroxycholesterol

In this research, a sandwich ELISA was carried out to calculate the levels of 27-HC in serum samples. The first step of this was to establish levels of absorption in standard samples of a known concentration of 27-HC.

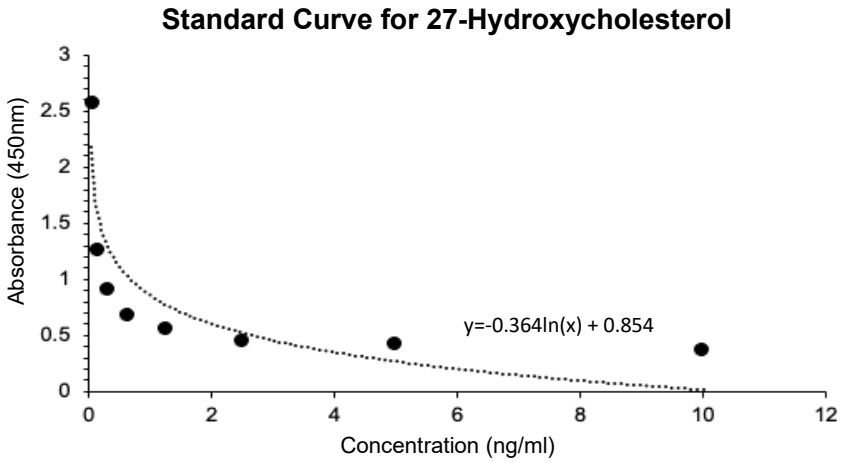


Figure 17: Standard curve for 27-Hydroxycholesterol ELISA Assay

Figure 17 shows the original plot of a known concentration and the correlating absorbance values. Inverse proportionality results lead us to take the logarithmic values of concentration to be able to fit a linear standard curve.

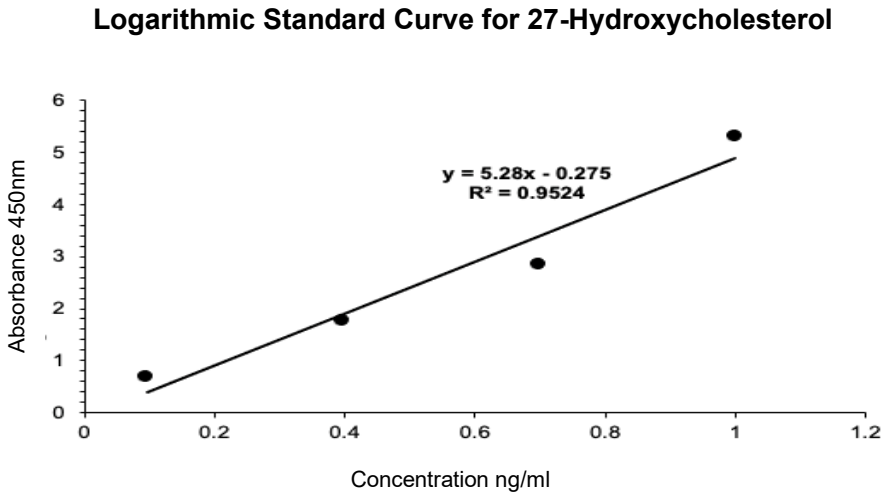


Figure 18: Logarithmic standard curve for 27-Hydroxycholesterol to give a linear line of best fit for correlation.

Figure 18 shows the logarithms of the same values of known concentrations against absorbance to produce the linear standard curve required. Figure 18 shows this and shows the reliability of the standard testing by producing a line of best fit with $R^2 > 0.95$. The equation of the standard curve can be used to work out unknown concentrations from samples where we have an absorbance value, with inverse log and dilution factors being considered,

The linear standard curve gives an equation that can be used to work out unknown concentrations. The absorption values can be used in the equation to work out the 27-HC serum concentrations. This allows for comparison between a retired rugby player group (n=13) and a non-contact sports control group (n=13).

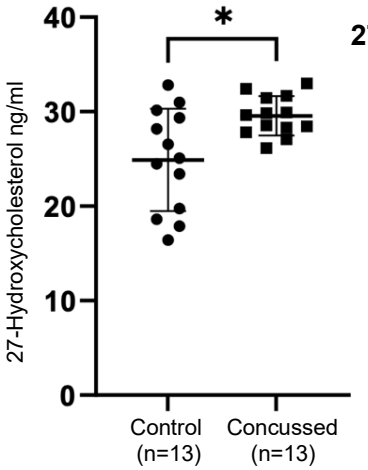


Figure 19: 27-HC serum comparison between a concussion and control group.

*An increase is shown in the 27-HC serum concentration in the concussed group. Dots show the individual data points in the control group whilst squares show the individual data points in the concussed group. Bars show the median and upper and lower quartiles. Test for significance shows * as P<0.05. Statistical analysis and graphing were carried out by using the Mann Whitney U Test on GraphPad Prism.*

Figure 19 shows a significant (P<0.05) increase in the concentration of 27-HC in the retired rugby player group (concussed). This result is in keeping with other previous studies and research. This result gives the foundation for further investigation into interactions and correlations between 27-HC and other biomarkers that were previously investigated in the UK Rugby Health Study. There was no statistical significance when correlating 27-HC with 25-HC, T-Tau, RBP4, P-Tau217, P-Tau181, NFL or Exosome Size, respectively. A positive correlation with statistical significance was found when correlating between 27-HC and $A\beta_{42}$ ($p = 0.0218$, $r = 0.651$ $R^2 = 0.424$). Negative correlations with statistical significance were found when correlating between 27-HC and 24-HC ($p = 0.0464$, $r = 0.486$ $R^2 = 0.235$), SAA ($p = 0.0236$, $r = 0.354$ $R^2 = 0.122$) or P-Tau231 ($p = 0.0132$, $r = 0.716$ $R^2 = 0.513$).

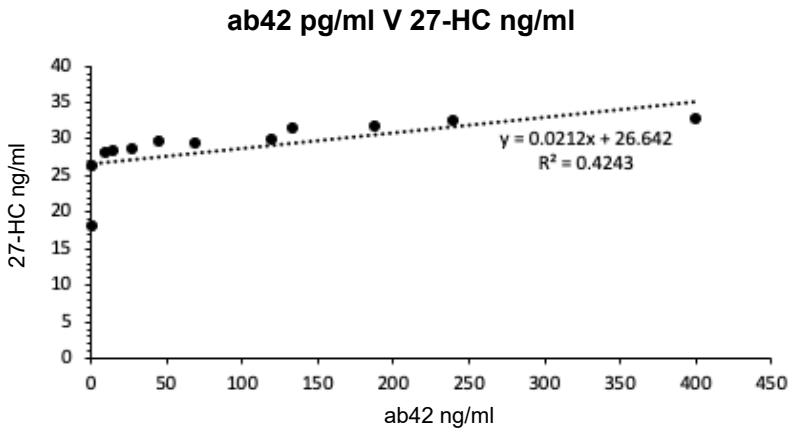


Figure 20: Positive correlation between $A\beta_{42}$ and 27-HC. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel. The $A\beta_{42}$ data was taken from the UK Rugby Health Project.*

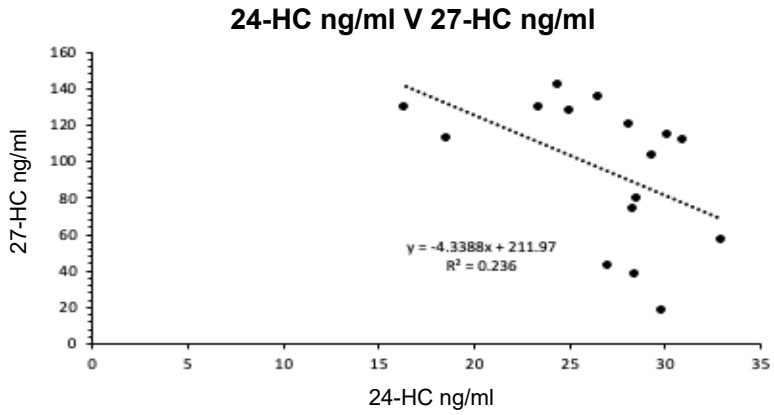


Figure 21: Negative correlation between 24-HC and 27-HC. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel. 24-HC data was taken from the UK Rugby Health Project.*

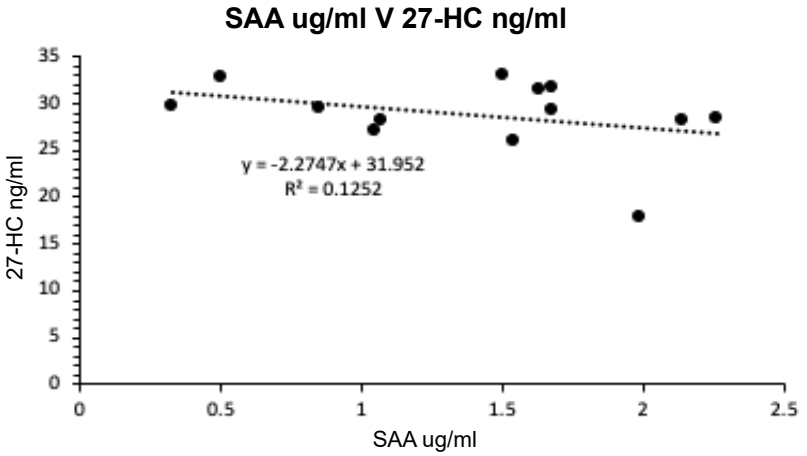


Figure 22: Negative correlation between SAA and 27-HC. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel. 24-HC data was collected by me. data was taken from the UK Rugby Health Project.*

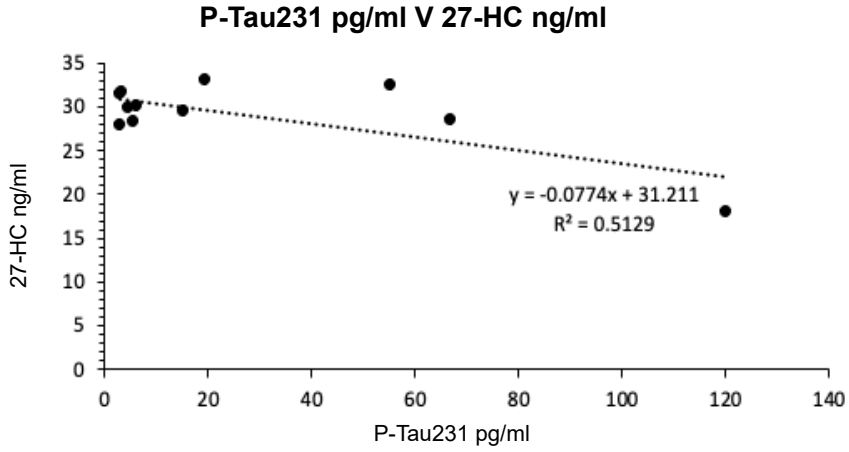


Figure 23: Negative correlation between P-Tau231 and 25-HC. *Points are individual data points. Statistical testing and graphing carried out on Microsoft Excel. P-Tau231 data was taken from the UK Rugby Health Project.*

3.1.5. APOE4

In this research, a sandwich ELISA was carried out to calculate the levels of APOE4 in serum samples. The first step of this was to establish levels of absorption in standard samples of a known concentration of APOE4.

Commented [PC1]: Mistake, ApoE4

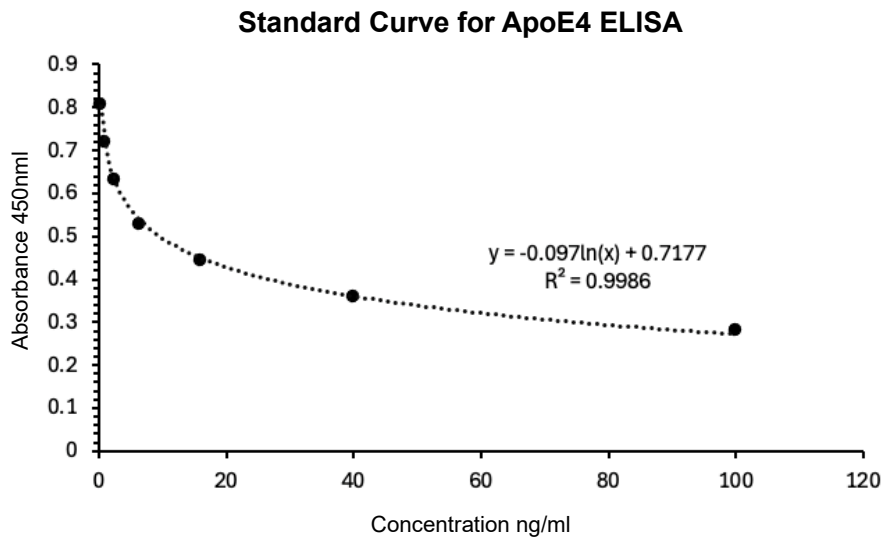


Figure 24: Standard curve for ApoE4 ELISA assay.

Figure 24 shows the original plot of a known concentration and the correlating absorbance values. Inverse proportionality results lead us to take the logarithmic values of concentration to be able to fit a linear standard curve.

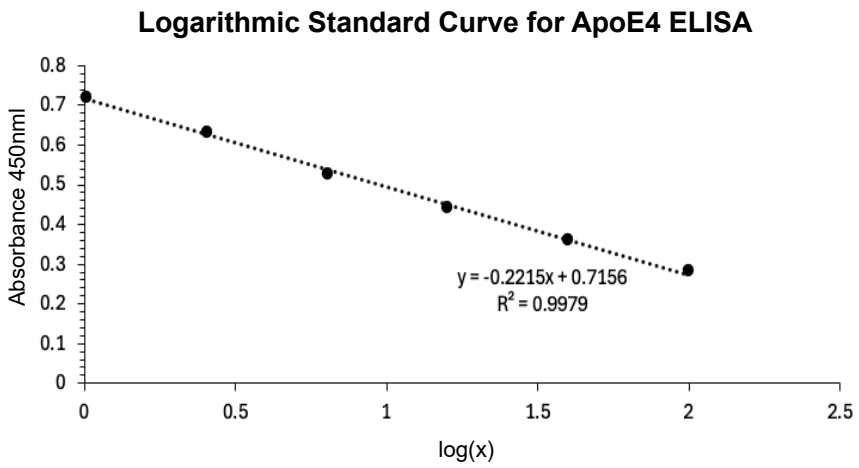
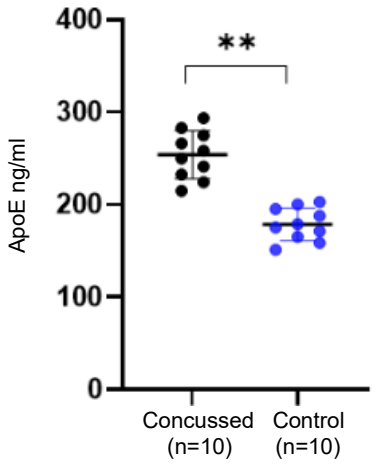


Figure 25: Logarithmic standard curve for APOE$ to give a linear line of best fit for correlation.

Figure 25 shows the logarithms of the same values of known concentrations against absorbance to produce the linear standard curve required. Figure 24 shows this and shows the reliability of the standard testing by producing a line of best fit with $R^2 > 0.95$. The equation of the standard curve can be used to work out unknown concentrations from samples where we have an absorbance value, with inverse log and dilution factors being considered.

The linear standard curve can be used to work out the unknown concentrations. The absorption values can be used in the equation to work out the APOE4 serum concentration. A comparison can be made between a retired rugby player group (n=10) and a non-contact sports control group (n=10).



ApoE4 levels between a concussed and control group

Figure 26: ApoE4 serum comparison between a concussion and control group.

*An increase in the apoE4 serum concentration is seen in the concussed group. Black dots show the individual data points in the concussed group whilst blue dots show the individual data points in the concussed group. Bars show the median and upper and lower quartiles. Test for significance shows ** as P<0.01. Statistical analysis and graphing were carried out by using the Mann Whitney U Test on GraphPad prism.*

Figure 26 shows a significant ($p<0.01$) increase of APOE4 in the retired rugby player group (concussed). These findings are expected and in line with other research where there is an increased concentration of APOE4 in study participants with TBI history or neurodegenerative disease. This result gives a foundation for further investigation into interactions and correlations between APOE4 and other biomarkers that were previously investigated in the UK Rugby Health Study. There was no statistical significance when correlating APOE4 with SAA, RBP4, T-Tau, P-Tau217, P-Tau213, ab42, NFL, 25-HC or 27-HC. There were not enough data points to correlate between APOE4 and P-Tau181. Positive correlations with statistical significance were found when correlating between APOE4 and Exosome Size ($p = 0.00638$, $r = 0.949$, $R^2 = 0.901$). A negative correlation with statistical significance was found between APOE4 and 24-HC ($p = 0.00301$, $r = 0.658$, $R^2 = 0.433$).

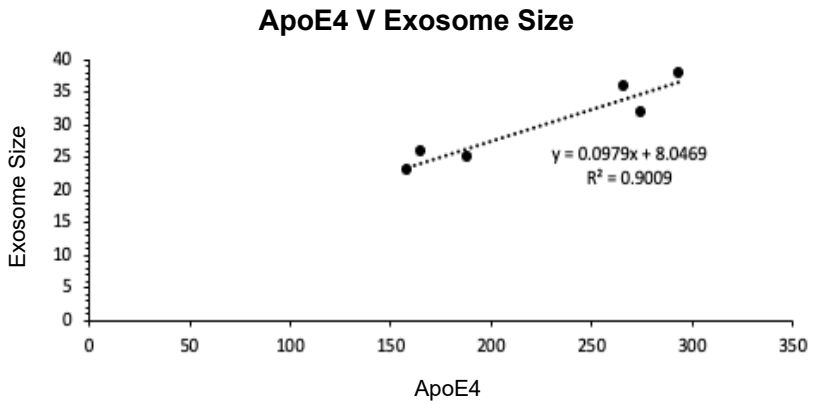


Figure 27: Positive correlation between apoE4 and Exosome Size. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel. Exosome size data was taken from the UK Rugby Health Project.*

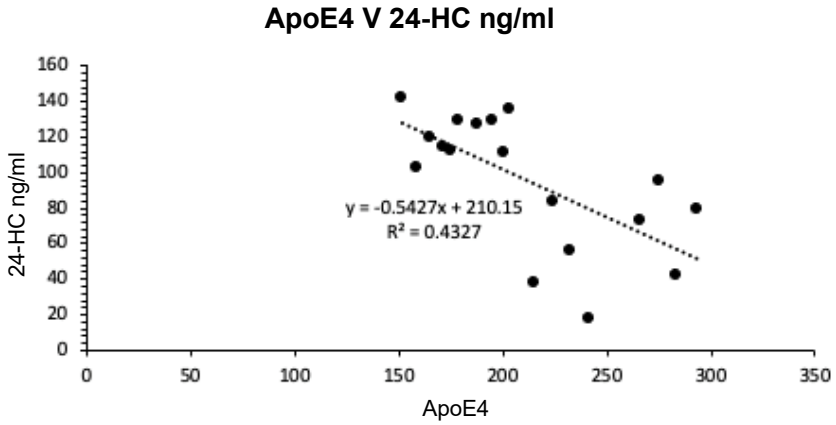


Figure 28: Negative correlation between SAA and 24-HC. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel.*

3.1.6. Triglycerides

In this research, a sandwich ELISA was carried out to calculate the levels of Triglyceride in serum samples. The first step of this was to establish levels of absorption in standard samples of a known concentration of Triglyceride.

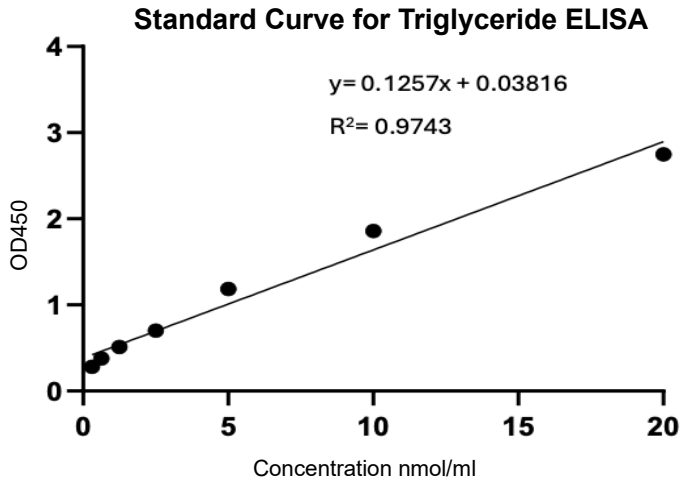
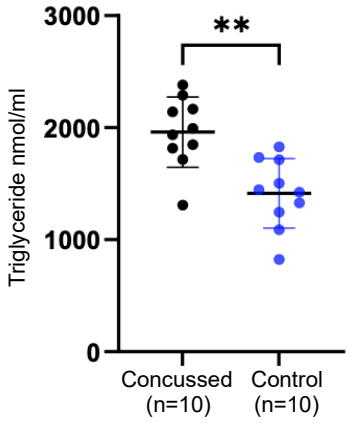


***Figure 29: Standard curve for Triglyceride ELISA Assay**. Data was taken from the UK Rugby Health Project.*

Figure 29 shows the linear standard curve required that gives an equation that can be used to work out unknown concentrations. Figure 29 shows its reliability by producing a line of best fit with $R^2 > 0.95$.

The absorption values can be taken from unknown concentration samples and input into the equation to work out the unknowns. From this, a comparison can be drawn between the control group (n=10) and the retired rugby player group (n=10).



Triglyceride levels between a concussed and control group

Figure 30: Triglyceride serum comparison between a concussion and control group.

*An increase in Triglyceride serum concentration is seen in the concussed group. Blue dots show the individual data points in the control group whilst black dots show the individual data points in the concussed group. Bars show the median and upper and lower quartiles. Test for significance shows ** as P<0.01. Statistical analysis and graphing were carried out by using the Mann Whitney U Test on GraphPad Prism.*

The comparison shows a significant ($p<0.01$) increase in the serum concentration of Triglyceride in the retired rugby player group (concussed). These findings are expected and in line with other research where there is a raised concentration of Triglyceride in study participants with neurodegenerative disease. This result gives the foundation for further investigation into interactions and correlations between Triglycerides and other biomarkers that were previously investigated in the UK Rugby Health Study.

There was no statistical significance when correlating Triglyceride with Ceramide, SAA, RBP4, P-Tau 231, P-Tau 217, NFL, Exosome size, 25-HC or 27-HC. Positive correlations were found with statistical significance when correlating between Triglyceride and T-Tau ($p = 0.0219$, $r = 0.826$, $R^2 = 0.683$) and APOE4 ($p = 0.00143$, $r = 0.727$, $R^2 = 0.528$). A negative correlation was found with statistical significance when correlating between Triglyceride and 24-HC ($p = 0.0246$, $r = 0.558$, $R^2 = 0.312$). No correlation could be found between Triglyceride and P-Tau 181 as there were not enough data pairs to test against each other for statistical significance.

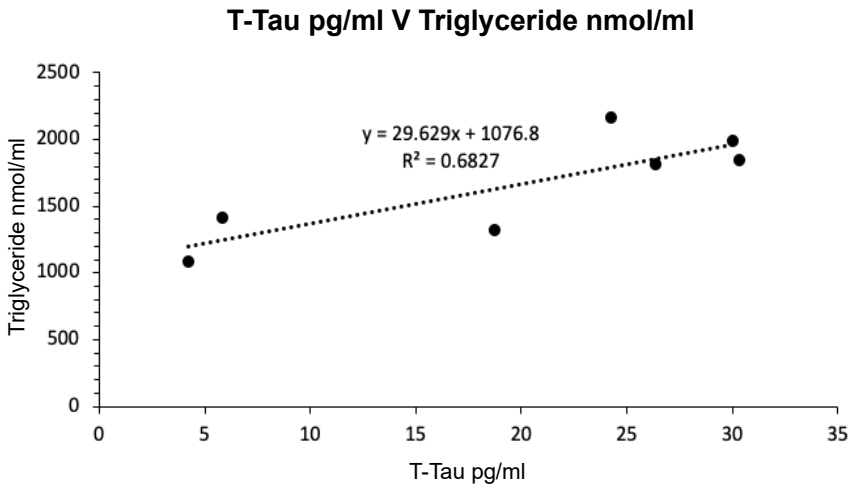


Figure 31: Positive correlation between Triglyceride and T-Tau. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel. T-Tau data was taken from the UK Rugby Health Project.*

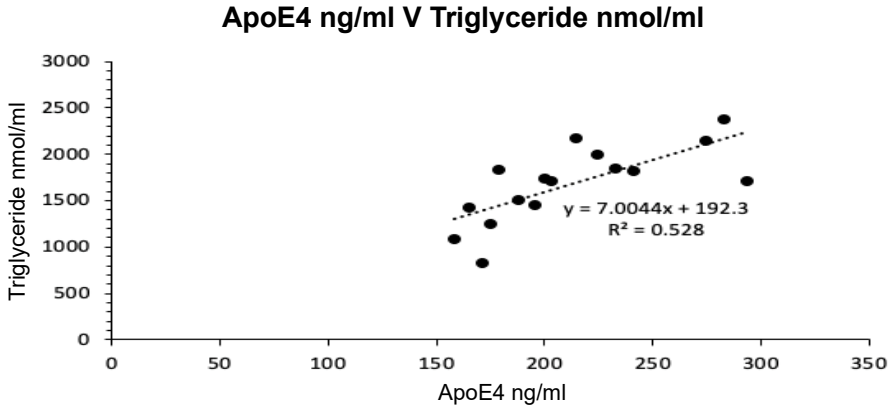


Figure 32: Positive correlation between Triglyceride and ApoE4. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel.*

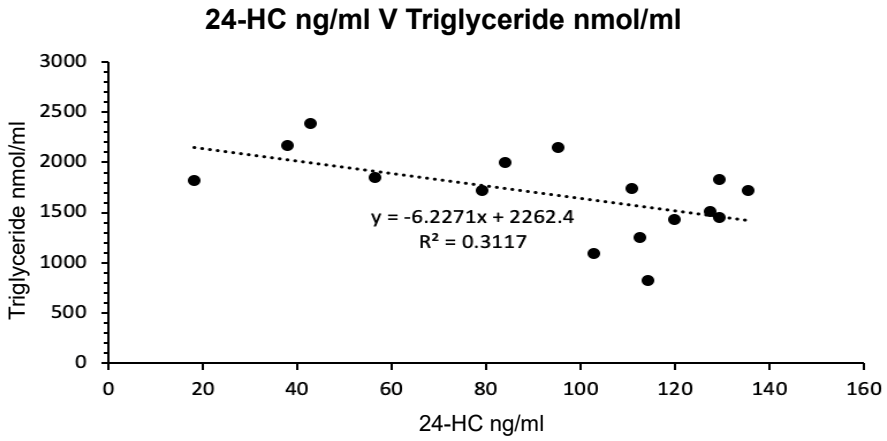


Figure 33: Negative correlation between Triglyceride and 24-HC. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel.*

3.1.7. Ceramides

In this research, a sandwich ELISA was conducted to determine the levels of Ceramide in serum samples. The first step of this was to establish levels of absorption in standard samples of a known concentration of Ceramide.

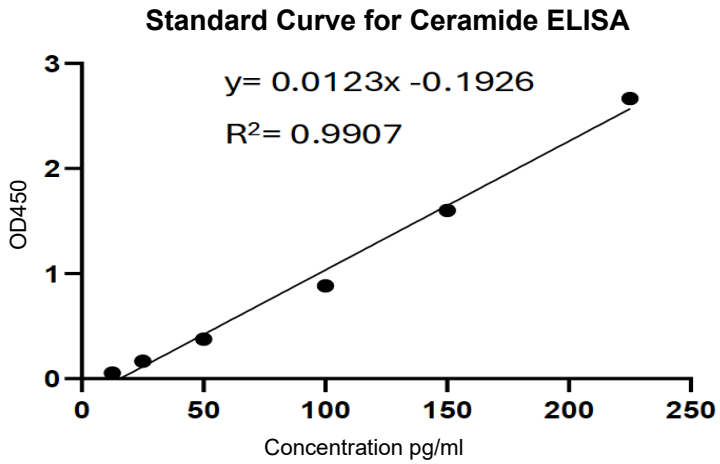
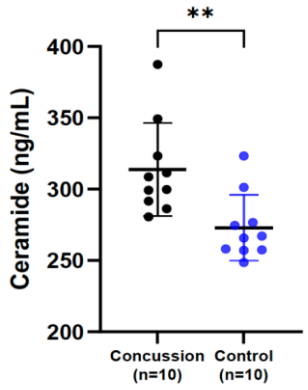


Figure 34: Standard curve for Ceramide ELISA Assay. *Data was taken from the UK Rugby Health Project.*

Figure 34 shows the linear standard curve required that gives an equation that can be used to work out unknown concentrations. Figure 34 shows its reliability by producing a line of best fit with $R^2 > 0.95$.

The absorption values can be taken from unknown concentration samples and input into the equation to work out the unknowns. From this, a comparison can, be drawn between the control group (n=10) and the retired rugby player group (n=10).



Ceramide levels between a concussed and control group

Figure 35: Ceramide serum comparison between a concussion and control group.

*An increase in Ceramide serum concentration is seen in the concussed group. Blue dots show the individual data points in the control group whilst black dots show the individual data points in the concussed group. Bars show the median and upper and lower quartiles. Test for significance shows ** as P<0.01. Statistical analysis and graphing were carried out by using the Mann Whitney U Test on GraphPad Prism*

A significant ($p<0.01$) increase in Ceramide serum concentration is seen in the concussed group. There was no statistical significance when correlating Ceramide with Triglyceride, SAA, RBP4, P-Tau 231, P-Tau 217, NFL, Exosome size, 24-HC, 25-HC or 27-HC. A positive correlation was found with statistical significance when correlating between Ceramide and $A\beta_{42}$ ($p = 0.0220$, $r = 0.768$, $R^2 = 0.876$. No correlation could be found between Ceramide and P-Tau 181 as there were not enough data pairs to test against each other for statistical significance.

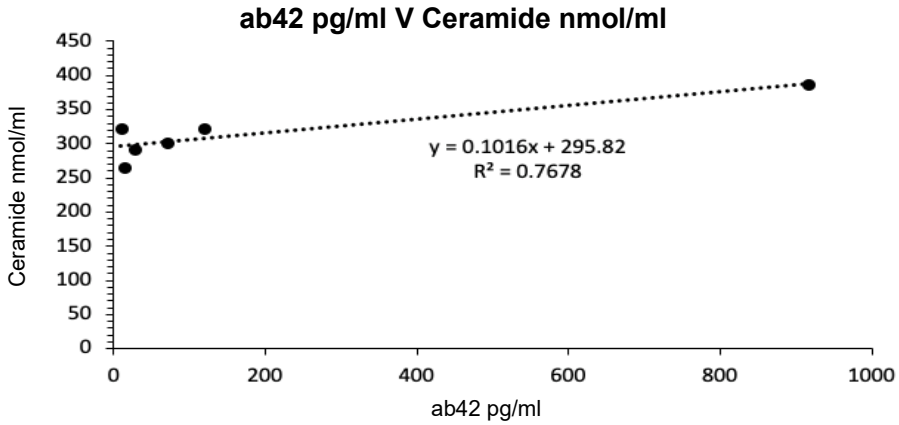


Figure 36: Positive correlation between Ceramide and $A\beta_{42}$. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel. $A\beta_{42}$ data was taken from the UK Rugby Health Project.*

3.2. Cognitive Parameters

The cognitive parameters tested and analysed in this research include the raw scores for memory, verbal memory, visual memory, psychomotor speed, reaction time, complex attention, cognitive flexibility, processing speed, executive function, simple attention and motor speed. These parameters were measured using the Vital Signs Computerized Neurocognitive Assessment that was developed as a routine clinical screening tool. These tests are used by many health professionals who acknowledge the reliability and validity of the results. The tests run as part of the assessment are sensitive to most of the causes of cognitive dysfunction or impairment. The raw test scores were provided by Professor Paul Chazot as part of the UK Rugby Health Study.

An initial investigation of the test score results was carried out to test for significance between a group who had experienced 5 or more concussions, compared to a group who suffered from none. The group with 5 or more concussions are considered as “concussed group” whilst the latter is considered as the control. The scores were used to calculate the neurocognitive index (NCI), which gave an overall score for neurological function.

	Concussed	Control	P-Value
NCI	89.83±13.44	104.±2.16	0.049(*)
Memory	91.7±16.81	110.25±8.73	0.076
Psychomotor speed	97.43±14.46	106.25±7.37	0.249
Reaction time	86.04±27.	91.5±14.55	0.700
Complex attention	86.57±24.1	109.±3.16	0.079
Cognitive flexibility	87.35±15.74	104.±5.03	0.049(*)
Processing speed	97.32±15.46	110.25±9.22	0.061
Executive function	87.86±15.24	104.±5.48	0.025(*)
Simple attention	88.70±24.44	99.±14.67	0.215

Table 2: A table that compares cognitive function between two groups. *Comparison between a concussed and control group. * = P<0.05. Data was obtained from the UK Rugby Health Project.*

The table shows the Initial findings between the concussed and control group. The P-value returned from the comparison reports significance when $P<0.05$. Neurocognitive index, cognitive flexibility and executive function all returned significant scores for the P-value. An initial basis can be formed when looking at 3 cognitive parameters that are strongly significant and can be used as an indicator to further analyse cognitive parameters.

The collected cognitive scores were further investigated in this research against the ELISA analysis from 24-HC, 25-HC, 27-HC and APOE4. Positive correlations were found with statistical significance when correlating between 25-HC and raw memory ($p = 0.0213$, $r = 0.680$, $R^2 = 0.462$) or verbal memory ($p = 0.0424$, $r = 0.578$, $R^2 = 0.334$). Negative correlations were found with statistical significance when correlating between 27-HC and psychomotor speed ($p = 0.0499$, $r = 0.514$, $R^2 = 0.264$) or processing speed ($p = 0.0491$, $r = 0.529$, $R^2 = 0.280$). The cognitive parameters did not show any statistical significance when correlated against 24-HC or APOE4.

This part of the research aims to investigate some of these potential links between the cognitive parameter scores and levels of other tested biomarkers. Where this is not possible, a basis may be formed upon which more research is likely necessary.

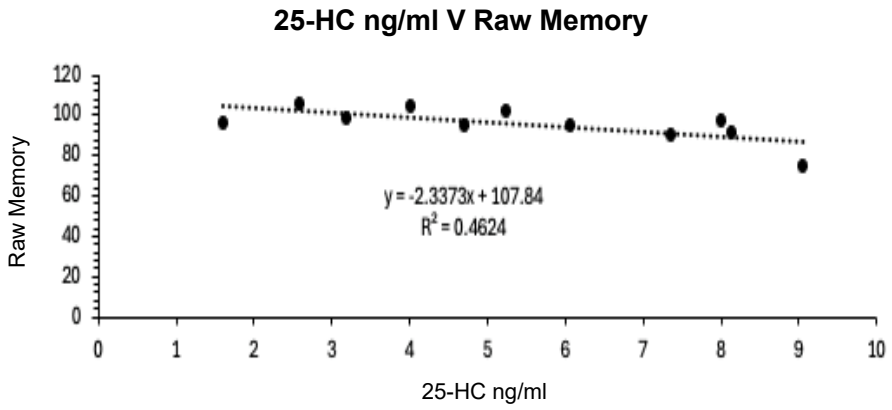


Figure 37: Negative correlation between 25-HC and verbal Memory. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel.*

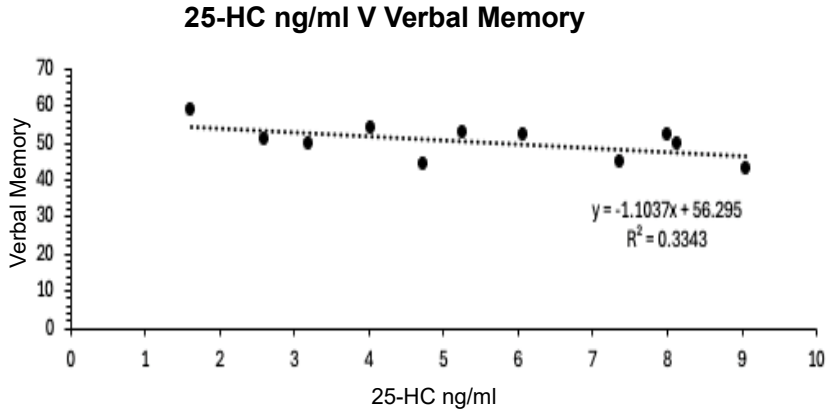


Figure 38: Negative correlation between 25-HC and Verbal Memory. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel. Verbal Memory data was taken from the UK Rugby Health Project.*

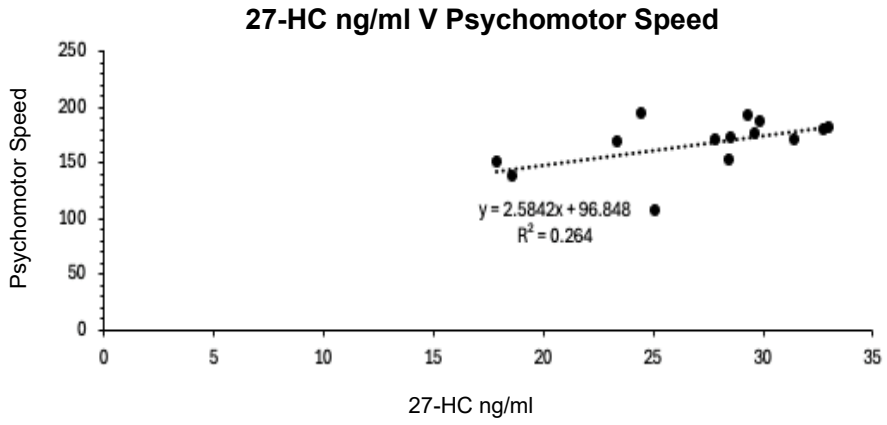


Figure 39: Positive correlation between 27-HC and Psychomotor Speed. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel.*

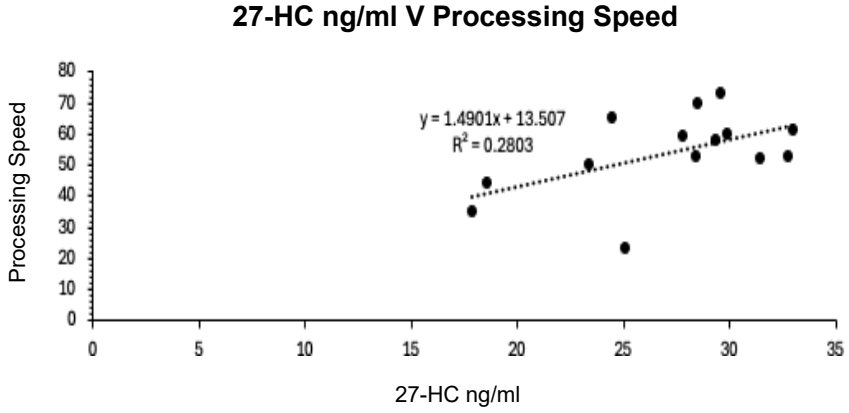


Figure 40: Positive correlation between 27-HC and Processing Speed. *Dots are individual data points. Statistical testing and graphing carried out on Microsoft Excel.*

4. Discussion

The ideas built in this research suggest evidence that there are varying levels of biomarkers between a non-contact sports control group and a retired rugby player concussed group. These findings give a basis of evidence that allows for further development of the idea that serum measurable prodromal biomarkers could be predictive of neurodegenerative diseases such as Alzheimer's, Amyotrophic Lateral Sclerosis, Parkinson's, and perhaps CTE.

4.1. Biomarkers

4.1.1. 24-Hydroxycholesterol

In this research, it is found that the levels of 24-HC were reduced in the serum of the retired rugby concussion group when compared to the levels of the healthy non-contact control group (**Figure 6**). This reduction was statistically significant (** = $P<0.05$). There was some statistical significance ($P<0.05$) found between 24-HC and four other biomarkers. There is evidence of a positive correlation with statistical significance between 24-HC and SAA (**Figure 7**). Statistically significant negative correlations are established between 24-HC and 27-HC (**Figure 8**), PTau231(**Figure 9**) or P-Tau181 (**Figure 10**).

These results are in keeping with other research, thus making them significant as they consolidate previous ideas of 24-HC as a potential biomarker for concussions and neurodegenerative disease. Research around 24-HC as a diagnostic tool for concussion shows promise, but further work to verify its validity is necessary. Concussion proves to be concerning, with too many links to neurodegenerative disease to ignore. Further

research could look at the potential of 24-HC as a return-to-play measure in contact athletes.

4.1.2. 25-Hydroxycholesterol

It was found that the levels of 25-HC were elevated in the serum of the retired rugby concussion group when compared to the levels of the healthy non-contact control group (**Figure 12**). This reduction was statistically significant (* = $P<0.01$). There was some statistical significance ($P<0.01$) found between 25-HC and four other biomarkers. A statistically significant positive correlation can be established between 25-HC and $A\beta_{42}$ (**Figure 13**). It helps consolidate these findings when P-Tau231 and P-Tau 181 also show statistically significant positive correlations (**Figure 14, Figure 15**). This is expected, as other research suggested that the levels of P-Tau are positively correlated to levels of $A\beta_{42}$.

These results are in keeping with other research, thus making them significant as they consolidate previous ideas of 25-HC as a potential biomarker for concussions and neurodegenerative disease. A further development for 25-HC could be to look at how a contact athlete's levels of 25-HC differ from a baseline when they experience a concussion.

4.1.3. 27-Hydroxycholesterol

In this research, it is found that the levels of 27-HC were increased in the serum of the retired rugby concussion group when compared to the levels of the healthy non-contact control group (**Figure 19**). This increase was statistically significant (** = $P<0.05$). There was some statistical significance ($P<0.05$) found between 27-HC and four other

biomarkers. There is evidence of a positive correlation with statistical significance between 27-HC and $A\beta_{42}$ (**Figure 20**). Statistically significant negative correlations are established between 27-HC and 24-HC (**Figure 21**), SAA (**Figure 22**) or P-Tau231 (**Figure 23**).

These results align with other research, making them significant in that they cement previous ideas of 27-HC as a potential biomarker for concussion and neurodegenerative disease. Research on 27-HC as a diagnostic tool for concussion is encouraging, but additional work towards validation is necessary. Future research could look at the impacts of 27-HC on the blood-brain barrier.

4.1.4. Apolipoprotein E4

In this research, it is found that the levels of APOE4 were increased in the serum of the retired rugby concussion group when compared to the levels of the healthy non-contact control group (**Figure 26**). This increase was statistically significant (** = $P<0.05$). There was some statistical significance ($P<0.05$) found between 24-HC and four other biomarkers. There is evidence of positive correlations with statistical significance between APOE4 and Exosome Size (**Figure 27**). There is a statistically significant negative correlation between APOE4 and 24-HC **(Figure 28).**

These findings are expected and in line with other research where there has been an increased concentration of APOE, especially when considering the influence APOE4 has on amyloid plaques and NFT formation. The results aligning in this research with previous research solidify a good foundation that APOE4 can be looked at as a biomarker for concussion and neurodegenerative disease. The research surrounding APOE4 as a

biomarker is strong, but it would still benefit from further authentication. Further research could look at measuring APOE4 over elongated periods and how this could correlate with neurodegenerative disease progression.

4.1.5. Triglycerides

In this research, it is found that the levels of Triglycerides were increased in the serum of the retired rugby concussion group when compared to the levels of the healthy non-contact control group (**Figure 30**). This increase was statistically significant (** = P<0.05). There was some statistical significance (P<0.05) found between Triglyceride and three other biomarkers. There is evidence of positive correlations with statistical significance between Triglyceride and T-Tau (**Figure 31**) or APOE4 (**Figure 32**). There is a statistically significant negative correlation between APOE4 and 24-HC (**Figure 33**).

These findings are expected and in line with other research where there has been an increased concentration of Triglycerides in study participants. One other study commented on the positive correlation between Triglyceride and Alzheimer's pathology, so it is good to see a statistically significant correlation between Triglyceride and T-Tau in this research to support this. Whilst the data in this research does not find significance between Triglyceride and $A\beta_{42}$, it does find significance with APOE. APOE4 also shows positive $A\beta_{42}$ and T-Tau, whilst being a standalone potential biomarker that has been linked to concussion and neurodegenerative disease. The results aligning in this research with previous research solidify a good foundation that Triglyceride can be looked at as a biomarker for concussion and neurodegenerative disease. The research surrounding Triglyceride builds a profile of how it relates to the symptoms of concussion, the symptoms of neurodegenerative disease, and the pathological hallmark of

Alzheimer's. Further research could be used to further solidify the links/relationship between Triglyceride concentration and neurodegenerative disease.

4.1.6. Ceramide

In this research, it is found that the levels of Ceramides were increased in the serum of the retired rugby concussion group when compared to the levels of the healthy non-contact control group (**Figure 35**). This increase was statistically significant (** = $P<0.05$). There was some statistical significance ($P<0.05$) found between Ceramide and four other biomarkers. There is evidence of a positive correlation with statistical significance between Ceramide and $A\beta_{42}$ (**Figure 36**). There were no other statistically significant findings with the Ceramide data.

These findings are expected and in line with other research where there has been an increased concentration of Ceramides in study participants. The results aligning in this research with previous research solidify a good foundation that Ceramides can be looked at as a biomarker for concussion and neurodegenerative disease. The research surrounding Ceramides is like the research that surrounds Triglyceride in that it builds a profile of how it relates to the symptoms of concussion, the symptoms of neurodegenerative disease, and the pathological hallmark of Alzheimer's. Further research could be used to further solidify the links/relationship between Triglyceride concentration and neurodegenerative disease.

4.2. Neurocognitive Parameters

Cognitive parameters are important for diagnosing potential neurodegenerative disease. Whilst they are of importance when used as a diagnostic tool, they have not been looked at in conjunction with any other diagnostic measures. Clinical testing is used mostly in hospital and care settings to look toward a neurodegenerative diagnosis. This part of the research acknowledges that this typically happens in mid-late life, whilst there may be preclinical markers of neurodegenerative disease.

Commented [PC2]: Insert the cognitive data comparing control to high concussed group and discuss first evidence for relationship between cognitive data and concussion, before you do correlations

Commented [TR3R2]: i dont think I ever did this but I will have a go at doing it this week

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The P-value returned from the comparison (**Table 2**) reports significance when $P<0.05$. Neurocognitive index, cognitive flexibility and executive function all returned significant scores for the P-value.

In this research, Cognitive scores from 11 categories (memory, verbal memory, visual memory, psychomotor speed, reaction time, complex attention, cognitive flexibility, processing speed, executive function, simple attention and motor speed) were tested against the biomarkers 24-HC, 25-HC, 27-HC and APOE4. There was some statistical significance ($P<0.05$) found between some of the pairings. Statistically significant negative correlations were found between 25-HC and Raw Memory (**Figure 37**), and 25-HC and Verbal Memory (**Figure 38**). Statistically significant positive correlations were found between 27-HC and Psychomotor Speed (**Figure 39**) and 27-HC and Processing Speed (**Figure 40**). No correlations were found with statistical significance between any neurocognitive parameters and 24-HC or APOE4.

Whilst this area is not heavily researched, and the idea of linking cognitive parameters to levels of biomarkers is new, some reasoning can be given to support the results. In the

cases of the negative correlation of 25-HC and Raw memory, or Verbal Memory, when the levels of 25-HC are increased, the levels of inflammatory markers and the impairment of neuronal signalling increase correspondingly. A raised level of both inflammatory markers and impaired neuronal signalling is known to impair memory, so there is a reason for these results. In the other cases of 27-HC and Psychomotor Speed or Processing Speed when the levels of 27-HC are increased, synaptic function is also increased. This is explanatory of improvement in Psychomotor and Processing Speed. This research is quite new and seems to be quite controversial. Some biomarkers have dual effects that enhance cognitive function in the short term but contribute to neurodegeneration in the long run. More research is needed to try to understand how biomarkers have a unique effect on different cognitive parameters.

One treatment approach for Parkinson's, ALS and CTE has looked at the use of Ambroxol. Whilst typically used in cough medicines, studies show that Ambroxol is effective in restoring lipid metabolism, which is disturbed in people with neurodegenerative disease (Cure Parkinsons, 2025). There is currently a clinical trial taking place in Australia, that aims to take newly diagnosed ALS patients and treat them with ambroxol for 24 weeks (MND Australia, 2024). Participants are randomly assigned the drug, and disease progression will be monitored and compared between the placebo and drug-receiving groups. The research around this drug seems promising, and in growing abundance, but more trials like the one in Australia are needed to solidify findings and help develop an understanding of the treatment of neurodegenerative disease.

4.3. Strengths and Limitations

This study provides a new exploration of multiple serum biomarkers potentially associated with neuronal damage and cognitive function in individuals at risk of brain trauma. By looking at APOE4, ceramides, triglycerides and p-tau simultaneously, it offers a more comprehensive understanding than single-marker studies. It highlights potential avenues for early detection and intervention. It is good to contextualize how the findings fit into the broader objectives of this research, specifically, understanding how these biomarkers can be reflective of underlying neuronal damage and an unbalanced cholesterol metabolism.

Serum levels of APOE4, ceramides, triglycerides, and other markers can fluctuate within the same individual over time, influenced by factors such as diet, metabolic state, and the circadian cycle. These temporal variations highlight the importance of considering sampling time and individual physiological context when interpreting results.

Phosphorylated tau (p-tau), while a promising serum biomarker for neurodegenerative processes, may be less robust than other markers due to the labile nature of phosphorylation, which can be rapidly modified or degraded post-translationally, potentially affecting its stability and detectability in serum.

4.4. Future Implications

Overall, these findings provide preliminary support that monitoring serum biomarkers may reflect underlying neuronal damage and altered lipid metabolism in individuals at risk of brain trauma. Some correlations were made between cognitive parameters and the potential lipid biomarkers explored in this research, but more studies are needed to make

clear associations between these parameters. Further research should look at individual biomarkers and how they associate with specific components of cognition, i.e. 24-HC and Memory or APOE4 and Reaction time. The research could then go on to explain potential relationships by understanding the mechanistic links between the biomarker and the cognitive parameter.

In addition to the suggested developments for this research, a further avenue for this research could look at how hydroxycholesterols could be used as a concussion-based neurodegenerative disease therapy. For example, where reduced levels of 24-HC are shown to be potentially diagnostic of concussion-based neurodegenerative diseases, increasing these levels to investigate whether they reduce symptoms may be an option.

These findings matter because they highlight the potential of serum biomarkers as tools to monitor brain health and possibly prevent neurodegenerative diseases. By identifying changes early, interventions could be targeted before cognitive decline becomes significant, emphasizing the clinical and research relevance of this study.

5. Conclusion

This study is the first to comprehensively explore the relationship between serum lipid-based biomarkers and cognitive performance in retired UK rugby players with a history of multiple concussions, compared to non-contact sport controls. The rationale was to investigate whether repetitive concussive events alter lipid metabolism in a way that could serve as an early indicator of neurodegenerative processes.

Key findings show that concussed rugby players had reduced serum concentrations of 24-HC and elevated levels of 25-HC 27-HC APOE4, triglycerides, and ceramides. The results suggest a dysregulation of cholesterol metabolism and lipid signalling following repeated head injuries. Expected correlations were found between Aβ42 and 25-HC, 27-HC, and ceramide. Positive correlations between these biomarkers and the neurodegenerative markers, Aβ42 and T-Tau, indicate that upregulation of these lipids may promote amyloid plaque and neurofibrillary tangle formation, key pathological hallmarks of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and chronic traumatic encephalopathy (CTE).

The pattern of reduced 24-HC and elevated 25-HC and 27-HC observed here is consistent with previous studies linking repeated traumatic brain injury to oxidative stress, neuroinflammation, and impaired cholesterol metabolism across the blood–brain barrier. Similar findings were reported by Hind et al. (2021) in the UK Rugby Health Study, which identified long-term neurocognitive impairment in concussed rugby players, and by Killen et al. (2019), who demonstrated that repetitive trauma disrupts cholesterol metabolism and contributes to neuroinflammatory cascades in the brain.

The increased levels of APOE4, ceramides, and triglycerides in this study further support pathways involving neuronal damage, inflammation, and altered lipid transportation.

These findings have important implications for rugby players and other contact-sport athletes. Repeated concussions, particularly without adequate recovery, appear to trigger persistent metabolic and neurochemical changes that may predispose individuals to long-term neurodegenerative disease. Identifying these lipid biomarkers could therefore aid early detection, guide player monitoring, and inform strategies aimed at reducing concussion frequency whilst improving post-injury recovery protocols for rugby and other contact sports.

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