Concussion-associated gene variants in elite rugby

M R ANTROBUS

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Mark Robert Antrobus

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Publications

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Abbreviation	Definition
Aβ	Amyloid plaques
AMPA	α -amino-3-hydroxy-5methyl-4-isoxazole-propionic acid
ANKK1	Ankyrin repeat and kinase domain containing 1
APOE	Apolipoprotein E
ATP	Adenosine triphosphate
AUC	Area under the curve
BDNF-AS	Brain derived neurotrophic factor antisense RNA
BH	Benjamini-Hochberg corrections
Ca^{2+}	Calcium ions
CI	Confidence intervals
COMT	Catechol-O-methyltransferase
chr	Chromosome
CTE	Chronic traumatic encephalopathy
DNA	Deoxyribonucleic acid
ε	Epsilon
EDTA	ethylenediamine tetra acetic acid
eNOS	Endothelial nitric oxide
FAM	Fluorescein dye
G	Gauge
Glut	Glutamate
GWAS	Genome-wide association studies
HWE	Hardy-Weinberg equilibrium
K^+	Potassium ions
MAPT	Microtubule associated protein tau
MDR	Multifactor dimensionality reduction
Met	Methionine
mRNA	Messenger ribonucleic acid
mTBI	Mild traumatic brain injury
NMDA	n-methyl-d aspartic acid
NO	Nitric oxide
NOS3	Endothelial nitric oxide synthase
OR	Odds ratio
p	Petit (short arm of chromosome)
PCR	Polymerase chain reaction
pro-BDNF	Precursor brain derived neurotrophic factor
q	Queue (tail/long arm of chromosome)
RL	Rugby league
ROC	Receiver operating characteristic curve
ROX	6-carboxy-X-rhodamine reference dye
rpm	Revolutions per minute
rs	Reference SNP cluster identification number
RU	Rugby union
SD	Standard deviation
SNP	Single nucleotide polymorphisms

TATA box	Type of promoter sequence of DNA that identifies where a genetic sequence can be read and decoded
TBI	Traumatic brain injury
TGS	Total genotype score
Val	Valine
VIC	Aequorea Victoria dye
w	Cohen's w effect size for Chi square tests
χ^2	Chi square

Abstract

Elite rugby league and union have some of the highest reported rates of concussion in professional sport due in part to their full-contact high velocity collision-based nature. Concussion is a complex phenotype, influenced by environmental factors and an individual's genetic predisposition. The overall aim of the current thesis was to investigate genetic risk factors for frequency and severity of concussion within an elite rugby athlete population. 1683 participants, consisting of 668 elite Caucasian male rugby athletes and 1015 non-athlete Caucasian men and women were recruited for this thesis. Genotype data were generated for eight suspected concussion-associated polymorphisms (*APOE* rs429358, rs7412 and rs405509, *ANKK1* rs1800497, *BDNF-AS* rs6265, *COMT* rs4680, *MAPT* rs10445337 and *NOS3* rs2070744). Only *COMT* rs4680 GG genotype was more common in elite rugby athletes than non-athletes (odds ratio (OR) 1.39, 95% confidence interval (CI) 1.04-1.86). The mean number of concussions experienced by athletes was 2.4 (standard deviation 3.1) concussions. The GG genotype of *APOE* rs405509 (OR = 2.10, 95% CI = 0.58-7.59), GG genotype of *BDNF-AS* rs6265 (OR = 17.75, 95% CI = 1.96-160.78) and AA genotype of *COMT* rs4680 (OR = 2.90, 95% CI = 0.84-10.04) were more common in elite rugby athletes with a history of concussion. In addition, the TT genotype of *APOE* rs405509 was more common in elite rugby athletes with a concussion recovery duration of <10 days (OR = 4.14, 95% CI = 1.14-15.06). Polygenic profile was quantified as a total genotype score, and suggested that elite rugby athletes do not have a more 'preferable' concussion-associated polygenic profile than non-athletes. Similarly, concussion-focused TGS algorithms were not effective in discriminating between elite rugby athletes with a history of concussion and those without. The novel findings presented in this thesis support the growing evidence that elite status, as well as incidence, severity and recovery from concussion, could be influenced by an athlete's genetic predisposition with respect to concussion injury. Nonetheless, genomic information could in the future potentially be used to inform individualised concussion injury management strategies for athletes in possession of risk genotypes.

Chapter 1

Introduction and Literature Review

A portion of this chapter is published in:

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1.1 Introduction to the thesis

Athletic performance is dependent upon nature and nurture [1]. The nurture component is concerned with environmental factors such as training, rest and recovery [2]. The nature component is concerned with the genetics of an athlete such as variations within an individual's DNA sequence. Indeed, genetic variation has been documented to substantially contribute to athletic performance and injury-related phenotypes [3–5]. As elite male rugby has one of the highest incidences of concussion in professional sport [6,7] it makes it an ideal population to investigate concussion-associated genetic variants. In fact, to achieve elite status in a high injury risk sport such as rugby, it is possible that a degree of inherited resistance to injury such as concussion is required. However, few studies have investigated genetic variants associated with sports-related concussion and most have not been in a homogenous cohort of elite athletes. To enhance the understanding of how suspected concussion-associated genetic variants relate to elite rugby athlete status and concussion risk is an attractive proposition. Rugby union (RU) and rugby league (RL) are both full contact collision-based codes of rugby, which have some of the highest reports of concussion in professional sports ("rugby" will be used to refer to both RU and RL). Rugby governing bodies have traditionally introduced safety measures to protect the ball carrier. However research has identified the tackler is at greater risk of concussion [8]. Recently, welfare-driven driven law changes to lower tackle height have been introduced within RU to reduce head impact incidences within the sport in conjunction with focus on coaching interventions to improve tackle technique [9]. Head injury assessments via use of video technology to identify potential concussions [10,11] and greater awareness of concussions by officials, coaches and athletes, have contributed to increased concussion incidence reporting [12]. Achieving elite status is a sport such as rugby (regularly playing (>5 matches) in Rugby Premiership, Super League and other highest-level leagues since the professionalism of RU in 1995) is a multifactorial accomplishment due to the interactions of nurture and the polygenic aspect of nature [13]. Elite male rugby athletes can be classified as a relatively homogenous group (with variations according to playing position) with quantified physical attributes, training loads and exposure to concussion risks, well-defined training and competition. Consequently, the purpose of this thesis is to investigate concussion-associated gene variants in elite rugby athletes.

1.1.1 Overview of thesis

Chapter 1 reviews concussion incidence within elite rugby, addresses the biomechanics and pathophysiology of concussion and how genetic predisposition may influence incidence, severity and outcome. Several likely genetic variants within or near candidate genes of interest, namely *APOE* rs429358, rs7412 and rs405509, *ANKK1* rs1800497, *BDNF-AS* rs6265, *COMT* rs4680*, MAPT* rs10445337 and *NOS3* rs2070744 are reviewed and evaluated, with a rationale for their inclusion in this thesis. Finally, the aims and specific objectives of the thesis are stated based on the review of prior concussion-related gene association evidence.

Chapter 2 is concerned with the general methodology adopted to investigate genotype frequencies of elite rugby athletes and non-athletes necessary for Chapters 3, 4 and 5. Chapter 2 details the participants recruited, DNA sample collection, DNA isolation techniques, genotyping protocols, athlete concussion history data collection and statistical analyses employed in the subsequent Chapters.

The main aim of Chapter 3 was to investigate whether genotype frequencies of the eight suspected concussion-associated polymorphisms differ between elite rugby athletes and non-athletes, and between RU playing positions. Based on the published associations of the polymorphisms with concussion risk and poorer outcome following brain injury outlined in the preceding paragraphs, and the interruption to competitive careers that could result [14], it was hypothesised that the concussion-associated risk genotypes and alleles would be underrepresented in elite rugby athletes compared to non-athletes. In other words, it was hypothesised that rugby athletes would have more genetic resistance to concussion than non-athletes, because it would have facilitated their prolonged participation in a high-risk environment.

The main aim of Chapter 4 was to investigate whether concussion-associated polygenic profiles differ between elite rugby athletes and non-athletes via the application of a total genotype score (TGS) algorithm. A secondary aim was to compare polygenic characteristics between RU forwards and RU backs. It was hypothesised that the elite rugby athletes would have a higher TGS than non-athletes indicating a more ‘preferable’ polygenic profile.

The aim of Chapter 5 was to investigate whether suspected concussion-associated polymorphisms are associated with history of previous concussion in elite rugby athletes. It was hypothesised that the concussion-associated risk genotypes and alleles would be overrepresented in elite rugby athletes with a history of previous concussion compared to those with no history of previous concussion. Finally, the aim of Chapter 6 was to collate and review the findings from the previous chapters and discuss the genetic factors that could affect concussion in elite rugby. In addition, this chapter details potential future practical implications and research directions.

1.2 Literature review

1.2.1 Introduction

Achieving elite status in rugby is a complex task, influenced by genetic and environmental factors. Anthropometric and physiological characteristics are often used as key discriminators of playing level within rugby. For example, elite forwards have higher body mass (RU ~111 kg, RL ~103 kg) compared to elite backs (RU ~93 kg, RL ~90 kg) [15]. Similarly, elite forwards are stronger (back squat: RU ~176 kg, RL ~188 kg; bench press: RU ~131 kg, RL ~122 kg) compared to elite backs (back squat: RU~157 kg, RL ~168 kg; bench press: RU ~118 kg, RL ~113 kg) [15]. Over a sport-specific sprint distance of 10 m, elite backs are faster (RU ~1.77 s, RL ~1.83 s) compared to elite forwards (RU ~1.87 s, RL ~1.9 s) [15].

Due to the contact nature of rugby, athletes are exposed to multiple collisions from defensive tackles, offensive hit-ups and from clearing rucks, mauling, mid-air contact and falls. In RL, forwards complete approximately twice the number of tackles per game than RL backs (24.6 vs 12.8) and all RL athletes complete an average of 32.7 collisions per match [16]. In RU, both forwards and backs perform approximately 23 tackles per game [17] and reported a mean of 137 collisions for forwards and 94 for backs [18]. The differing numbers of collisions between RL and RU indicate the varied styles of play between codes, and thus differing physiological requirements and injury risks.

Psychosocial factors also play an important role in achieving elite status as higher-level rugby athletes have greater positive perceptions (Cohen's *d'* >0.4) of team spirit, adapting to change, and fitting in with new team members, and demonstrate greater self-confidence and personal coping resources than lower-level counterparts [19]. While anthropometric, physiological and psychosocial factors are all important contributors to achieving elite status in rugby, another important determinant is also resistance to injury, such as concussion. Athletes with lower injury burden and greater match availability (~85%) are more likely to progress to elite status in their chosen sport [14].

Rugby-related concussions have been the focus of recent concern over the potential short and long-term neurodegenerative consequences. In addition, athletes who have had a prior concussion have a 3-8 fold [20–22] risk of repeated concussions and have a 60%

greater risk of subsequent time-loss injury [23]. There is a reported increased risk of potential short and long-term consequences associated with concussion such as: increased injury risk [23], cognitive impairment [24], forms of dementia [25], chronic post-concussion syndrome, migraines, sleep dysfunction, anxiety [26], post-traumatic stress disorder [27] and second impact syndrome [28]. These consequences could interrupt or terminate an athletic career, causing short- or long-term ill health.

Sport-related concussion has been defined as a traumatic brain injury (TBI) induced by biomechanical forces [26]. However, many factors contribute to concussion risk such as age, sex, neck strength, migraines, behaviour and sleep quality. For example, the relationship of age with risk of concussion is unclear, as the findings within the literature are mixed (probably due to the differences in research methods employed such as different sports, sexes, level of play and reporting measures). Gessel et al. [29] observed high school athletes reported a higher sport-related concussion incidence than collegiate athletes (8.9% vs. 5.8%). In contrast, Shields et al. [30] reported high school athletes were less likely to sustain a concussion than collegiate athletes. Smaller neck circumference, neck to head circumference ratio, and lower neck strength have been associated with concussion in youth athletes [31]. It appears neck strength and girth could play a role in preventing head and neck injuries, potentially mitigating concussion risk in youth athletes [32]. For every 0.5 kg increase in neck strength the odds of sustaining a concussion can be reduced by 5 % [31]. However, this study was carried out on student athletes of non-contact sports such as basketball and soccer, and findings have not been replicated in elite level sport where the margins for strength gain are smaller and impact forces higher. Currently, there are no studies regarding direct associations between neck strength and concussion in elite rugby [32].

Evidence supports the notion that female athletes have greater risk of concussion compared to male athletes [31,33], potentially due to lower neck strength [31] and differing physiology [34]. Pre-concussion migraine is reported to be a risk factor for prolonged recovery (up to 21 days) post-concussion, particularly in females, although the exact mechanisms are not fully understood [35]. However, it is difficult to make comparisons of concussion risk between sexes across the same sport as males are more likely to take risks within the same sport [36]. Behaviour appears to affect risk, as athletes

who participate in sports to relieve tension and aggressive are more likely to sustain a concussion than athletes with less aggressive tendencies [37]. Moderate-to-severe insomnia (Relative Risk (RR) = 3.13), and daytime sleepiness two or more times per month (RR = 2.86), are associated with increased risk of sustaining a sports-related concussion [38].

Concussion has been widely studied in relation to environmental factors, especially in rugby where factors considered include activity when concussion occurred (e.g. tackling/being tackled), playing experience, history of concussion, positional differences, use of protective equipment (e.g. headgear/mouth-guards) and return to play protocols and standard of competition [39,40]. However, a further step to better understanding inter-individual variability involves genetic variation and its association with concussion and related phenotypes. Evidence already exists suggesting an association between several genetic factors and inter-individual variability in traumatic brain injury incidence and severity [41]. For example, athletes carrying the *APOE* rs405509 T allele have an 8-fold greater risk of experiencing two or more concussions [20] and the TT genotype is associated with a 4-fold greater risk of a history of concussion with loss of consciousness [21]. Similarly, possession of the *MAPT* rs10445337 TT genotype has been associated with a history of one or more concussions (OR 2.1; 95% CI 0.3 to 14.5) [21]. *COMT* rs4680 Met carriers are ~3-fold more likely to have a history of concussion possibly as a result of increased impulsivity and risk taking behaviours [42]. Concussed *APOE* ε4 allele athlete carriers have been associated with prolonged post-concussive symptoms compared to non-ε4 allele carrying athletes with concussion [43].

Classical genetic studies (twin or family studies) quantify the heritability of phenotypic traits [44]. As concussion is only experienced by a small proportion of the population [45], recruiting a sufficient number of twins/family members who have experienced concussion is difficult (though not impossible) and has not been undertaken, to our knowledge. Consequently, a classical study on the inheritance of concussion risk, to elucidate the relative contribution of environmental versus genetic factors affecting inter-individual variability in concussion incidence, severity and outcome, would be extremely valuable. Many other sport-related injuries or risk factors for injury have substantial genetic contributions to their inter-individual variability, such as tennis elbow (epicondylitis) for

which heritability has been estimated at a substantial ~40% in women [46] and bone mineral density (a predictor of osteoporotic fracture) for which heritability is even greater at 50-85% [47]. Substantial heritability estimates for brain structure (~90%) and cognitive performance (~60%) have also been reported [48–51]. Given these and other observations of substantial genetic contributions to inter-individual variability in most human traits, it is likely that a substantial genetic component also applies to concussion.

Indeed, the substantial inter-individual variability in injury occurrence, and in outcomes following concussion, are probably due to the interaction of multiple genes in a polygenic manner that reflect the complex pathophysiology [52,53]. Prediction of recovery and future risk is therefore currently difficult [26]. This unexplained inter-individual variability could suggest a future role for genetic screening of concussion-associated risk polymorphisms in order to i) stratify potential risk of initial injury, for individuals ii) identify players with a greater risk of prolonged recovery and potential concussion-associated neurological issues, iii) identify those at risk of repeated concussions, iv) provide further insight into concussion pathophysiology, and v) inform concussion management strategies at a practical level in elite sport.

Within a sporting context, Heffernan et al. [54] previously reported single nucleotide polymorphism (SNP) frequency differences between elite rugby athletes and non-athletes, which could contribute towards achieving elite athlete status. Similarly, genetic predisposition to altered concussion risk could play an important role in attaining elite status and career longevity in a high-concussion risk sport such as rugby. Genetic analysis could provide information on genetic variability with elite rugby populations associated with risk, severity and recovery from concussion. Current research into concussion-associated gene variants in rugby is limited to four peer-reviewed articles, which were carried out on mixed cohorts (junior and senior [amateur and elite]) of rugby union athletes [42,55–57]. Consequently, there is a requirement for further research into the genetic basis of concussion within elite rugby athletes.

Therefore, the aims of this narrative review are to (1) describe the current data on incidence rates and severity of concussion in elite rugby; (2) provide an overview of the mechanisms and pathophysiology of concussion; (3) evaluate how genetic variation could

affect predisposition for and recovery from concussion; and (4) inform the future direction research regarding genetic aspects of concussion in rugby.

1.2.2 Incidence rate and severity of concussion in rugby

The professionalisation of rugby has resulted in alterations in the physical characteristics of players [15,58–60]. These alterations in physical characteristics such as body mass, strength, power and speed have increased the physical demands of modern rugby, such as more tackles and rucks per match [15,61–64]. This increased physicality has contributed to increased incidence rates of concussion in rugby [6,7].

There are many similarities in anthropometric and physiological characteristics of players in RU and RL that reflect comparable physical demands including frequent, heavy physical contact in both rugby codes [15]. Elite rugby (RU and RL) has been reported to have a concussion incidence of ~8–28 concussions per 1000 match hours [65,66], which is lower than sports such as horse racing (17–95) and boxing (13) but higher than sports such as soccer (0.4) [67–69]. Seventy percent of head injury assessments in elite RU as a result of a tackle are experienced by the tackler and 30% by the ball carrier [10]. In elite rugby league 63% of head injury assessments are experienced by the tackler [11]. This concussion risk is influenced by athlete speed, playing position, impacting force, body position, type of tackle, tackle technique [70], physiological [11,70,71] and anthropometric characteristics [11,70,71].

Recovery from concussion has been defined as a return to sport that encompasses a resolution of post-concussion-related symptoms and a return to clinically normal balance and cognitive functioning [26]. Within 7-10 days, 80-90% of adults with sport-related concussions could be clinically recovered and returned to play (Figure 1.1) [26,72,73].

Figure 1.1. Sequence of events and possible recovery durations post-concussion.

For 10-20% of concussion cases, symptoms can persist for >10 days [72]. Time taken to recover from a concussion differs for individuals, as 6.5% of concussed athletes have been reported to not return to play until 14 days post-concussion. For 1.6% of concussed athletes, recovery can take longer than 14 days and these individuals could have chronic post-concussion symptoms for up to 12 months [73,74].

Concussion and mild traumatic brain injury are terms often used interchangeably within a sporting context and the scientific literature. In many instances the term 'concussion' is used to describe mild traumatic brain injury as a transient disruption of brain function. Consensus statement definitions and diagnostic criteria produce an overlap of both terms [26]. The most recent definition of concussion during the Zurich 2016 Concussion Consensus Statement was 'sports-related concussions are a form of traumatic brain injury induced by biomechanical forces [26]. However, the same consensus group indicated that additional features can also be included in the definition for clinical purposes, such as 'functional disturbance rather than structural' and 'rapid on-set of short-lived impairment of neurological function that can resolve spontaneously or last' [26].

Following a concussive event, medical professionals are tasked with the diagnosis of concussion via the assessment of one or more signs and symptoms within the categories of physical, behavioural, cognitive and sleep related [26]. The consequences of concussion can include a variety of neurologic and cognitive symptoms termed post-concussion syndrome (Table 1.1) [26]. However, PCS has been debated in the literature in terms of an accurate definition and pathophysiology. Jotwani and Harmon [75] define PCS as concussion symptoms persisting >3-6 months. In contrast, Cantu *et al*. [76] (2010) described PCS as signs and symptoms which last >1 month. The pathophysiology of these symptoms is not fully understood and could be multifactorial and requires a multidisciplinary team to manage treatment [26]. The acute neurocognitive effects of concussion also include reductions in cognitive processing speed and efficiency, learning, executive function, working memory, attention and verbal fluency [26].

Table 1.1 Overview of signs and symptoms of concussion.

Physical	Behavioural	Cognitive	Sleep Related

Headache	Irritability	Reduced reaction times	Insomnia
Dizziness	Depressed mood	Fogginess feeling	Drowsiness
Fatigue	Emotional lability	Amnesia	Changes in sleep patterns
Nausea	Anxiety	Reduced concentration	
Reduced stability	Frustration	Loss of consciousness	
Vomiting	Observable disorientation	Dazed or 'dinged'	
Tonic Posturing	Confusion	Ataxia	
Concussive	Definite behavioural change		
Convulsions			
Visual disturbance			
Light sensitivity			
Sound sensitivity			

(Adapted from McCrory et al. [26]).

Concussion prevalence during the Rugby World Cups has seen a small increase from ~14% of all injuries in 2015 to ~16% in 2019 [77,78]. In the English Rugby Premiership (the top tier of competition in England), concussion incidence increased dramatically from 8 per 1000 match hours in the 2013-14 season to 22 in 2016-17, although this is thought to be largely due to increased awareness and reporting [79]. Concussion incidence within the English RU Premiership decreased to 18 concussions per 1000 match hours in 2017-2018 (~1 concussion per match) [66]. However, during the COVID-impacted season of 2019-20, concussion incidence rose to 20 per 1000 match hours [80]. In elite RL, concussion incidence in the National Rugby League (the top tier of competition in Australia) has ranged from ~9-28 concussions per 1000 player match hours over a 17-year period with a tendency to increase over time [81–83].

The incidence of concussions in RU is similar for forwards (4-19 per 1000 player match hours) and backs (5-18 per 1000 player match hours) [39,84]. However, this similar incidence of concussion in RU forwards and backs might not represent the exposure to concussion risk, because forwards have the highest involvement in defence and thus experience more collisions than backs (51 vs. 20 per 80 min) [85]. On the other hand, on average RU backs are lighter than RU forwards [15]. However, they reach higher sprint velocities than forwards [15] and thus could experience larger forces but less frequent

collisions during match play [85]. Consequently, it could be hypothesised that RU forwards and backs differ in terms of inherited genetic resistance to concussion.

In RL, incidence of concussions ranges from 12-48 per 1000 player match hours in forwards and a similar 14-44 per 1000 player match hours in backs [86]. Concussion incidence in both codes during training is much lower, accounting for only ~5% of concussions (0.03-0.07 per 1000 player training hours) [39,87]. Fluctuations in incidence over time could be attributed to developments in concussion education or operational strategies such as using 'Hawkeye' video analysis [66]. Increased awareness of players, support staff and coaches could account for the increased incidence of concussion reported in recent years [66]. Awareness is thought to be increased due to education initiatives by rugby governing bodies and player associations involving increased recent media attention [88].

The average range of concussion severity in RU ranges from 9-21 days absence (period from injury to availability for match selection) [23,66,79,84]. However, inter-individual variability means that severity can range from 2 days to >84 days absence [66]. Data from 2013-2015 Super League RL seasons suggest severity can range from 9 to 15 days absence [65].

As most concussions in rugby occur during tackles, governing bodies have trialled law changes as a potential mechanism to reduce injuries. Recently, World Rugby trialled law changes to lower tackle to the line of the ball carrier's shoulders, producing a 30% reduction of contact to the ball carrier's head and neck within the Championship Cup in England (one tier of competition below the Premiership Rugby competition in the UK) [89]. However, this lowered height did not influence the incidence of concussion [89]. In fact, lowering the tackle height further to below the ball carrier's armpit increased concussion incidence by 30% within the Championship Cup, potentially as a result of lowered and sub-optimal body position of the tackler [89]. Undoubtedly, rules will continue to be changed to protect the athletes in an athlete welfare driven approach adopted by the governing bodies. However, the effectiveness of such rule changes are still to be determined within the elite level of the sport.

1.2.3 Mechanisms of concussion

Rugby-related concussions can be the result of either direct head contact or inertial causes, but each concussion is a unique event. Contact injuries (e.g. from collisions) cause the brain to impact on the internal surfaces of the skull. Particularly injurious are incidents involving the frontal and temporal fossae regions due to ridges and bony protuberances that deform brain tissue [90]. Kinematic analysis indicates that inertial forces from direct or indirect impacts resulting in angular/linear acceleration/deceleration of the brain from head and neck motions can lead to concussion [91].

The contributions of angular or linear acceleration/deceleration to concussion is debated in the literature [92]. Linear acceleration is associated with changes in pressure gradients within the skull, compared to angular acceleration/deceleration that is associated with shear stresses on the brain forcing tissues to slide over one another and stretch [93]. Shear and stretch mechanical forces stretch axons to the point of axotomy (physical breaking) or partial breaking in areas, such as grey and white matter junctions, small blood vessels and axonal projections [90,94,95].

Concussions appear to vary in impact locations (front, top, back and sides of the head), linear acceleration/deceleration magnitude (61–169 g in collegiate American Football players, although there are concerns about the validity of those high values [96]) and clinical outcomes [97]. However, head impacts from high magnitude angular acceleration/deceleration result in more severe clinical outcomes due to the propensity of brain tissue to deform more readily from shear forces and is the predominant mechanism in multifocal concussion [91,95]. A tackle or collision may produce whiplash, which in turn produces both linear and angular acceleration/deceleration to the player's brain [97].

Quantitative measurement of head trauma has been investigated through the use of accelerometers. An instrumented mouthguard study has quantified mean levels of linear acceleration (22.2 ± 16.2 *g*) and mean angular acceleration (3903 ± 3949 rad/s^2) in premier club-level amateur RU, comparable to collegiate level American football [98]. Nominal data could be used to assess acceleration values, concussion events and accumulative values of an individual player, providing an injury risk profile [99]. However, Eckner et al. [100] could

not find evidence to support a concussion impact threshold or cumulative sub-concussive threshold from >100,000 impacts over a 4 year period. Limitations of accelerometers exist, such as: the all-in-mouth mouthguard can be ill-fitting, excess saliva can result in inactive contacts, and evidence indicates differing accelerometers (in mouth and helmets) have 10-15% error for both forms of acceleration [98].

Wearable head accelerometers are currently being used to provide insight into head impact accelerations during rugby (unpublished). However, evaluation studies have highlighted the over-prediction of acceleration magnitudes and dislocation of sensors producing errors in measurements [101]. Findings from head impact monitoring suggest that higher magnitudes of head impacts are more likely to cause concussion [101]. When and if this technology results in producing threshold data, probably combined with other methods of concussion assessment and genomic data, it could provide valuable information for personalised concussion management.

1.2.4 Pathophysiology of concussion

In rugby, the primary mechanical stress injury to neurons is likely the result of a collision that elicits a neuronal stretch. A stretch of ~10-20% of a neuron's resting length within 100 ms (sub-lethal axonal injury threshold) can trigger the secondary biochemical response of the neurometabolic cascade [102,103]. The resultant microstructural damage caused by the stretch is hypothesised to be the root cause of all forms of TBI [104–106]. The neurometabolic cascade following a concussive event (Figure 1.2) has been reviewed by Giza and Hovda [102,103].

The initial disturbance and stretch results in the release of depolarising extracellular K^+ due to voltage dependent channels opening in the neuronal membranes and can last up to 6 hours post-concussion [107,108]. Further K^+ flux is caused by the release of the excitatory amino acid glutamate [109]. Proteolytic digestion of the axon membrane skeleton occurs due to Ca^{2+} activation of cysteine proteases and apoptotic genetic signals [110]. Ca^{2+} influx has been reported to contribute to axonal microtubule breakdown 6-24 hours after a concussive event [107]. During smaller insults to the brain, surrounding glial cells remove extracellular K^+ in order to maintain homeostasis [111]. However, this cannot be achieved during larger concussive events and greater quantities of excitatory amino acids are

released resulting in 'spreading depression' [112]. Multiple mechanisms are responsible for elevated Ca^{2+} levels. Firstly, the physical disruption of membranes through primary injury [113]. Secondly, increased glutamate binds receptors such as n-methyl-d aspartic acid (NMDA) sub-unit NR2A, increasing Ca2+ influx through the NMDA channel, prolonging neuronal dysfunction [114].

Disruption of ionic homeostasis leads to an energy crisis within the injured brain. Re-establishment of ionic homeostasis is further attempted by the employment of ATP-fuelled membrane pumps, which results in increased glycolysis to meet energy requirements due to reduced activity of cerebral oxidative metabolism and reduced cerebral blood flow of up to 50% [115]. Increased intracellular Ca^{2+}, Na^{+} and K^{+} can result in swelling and contribute to further reduced cerebral blood flow [116]. Mitochondrial oxidative metabolism is impaired due to the influx of extracellular Ca^{2+}, thus contributing to the energy crisis [117]. As part of the neurometabolic cascade, pro- and anti-inflammatory cytokines are released [118]. Cytokines from this neuroimmune response can play both beneficial and detrimental roles in the neuroinflammatory response following a concussion [118].

Individual variability in concussion risk, severity and outcomes could be influenced by genomic modulation of molecular mechanisms described by the neurometabolic cascade [102,103]. For example, reduced cerebral blood flow post-concussion could be exacerbated by the possession of the *endothelial nitric oxide synthase* (rs2070744) C allele [119]. The concussive event triggers up-regulation of APOE in order to repair damaged neurons [120,121]. The Ca^{2+} influx has been observed to facilitate the proteolysis of the apolipoprotein ε4 isoform, which can lead to the formation of neurotoxic amyloid plaques (Aβ) that are associated with a negative impact on concussion recovery [122]. Gene variants that play a role in structural support and function of the axon, such as *microtubule associated protein tau* (rs10445337) and *brain derived neurotrophic factor* (rs6265) could impact microtubule repair, due to the effects of extracellular Ca^{2+} [123,124]. Similarly, inflammatory process-associated genetic variants such as *interleukin 6 receptor* can affect the severity of concussion, potentially via modulation of the inflammatory process and cognitive behavioural capacity post-concussion [41].

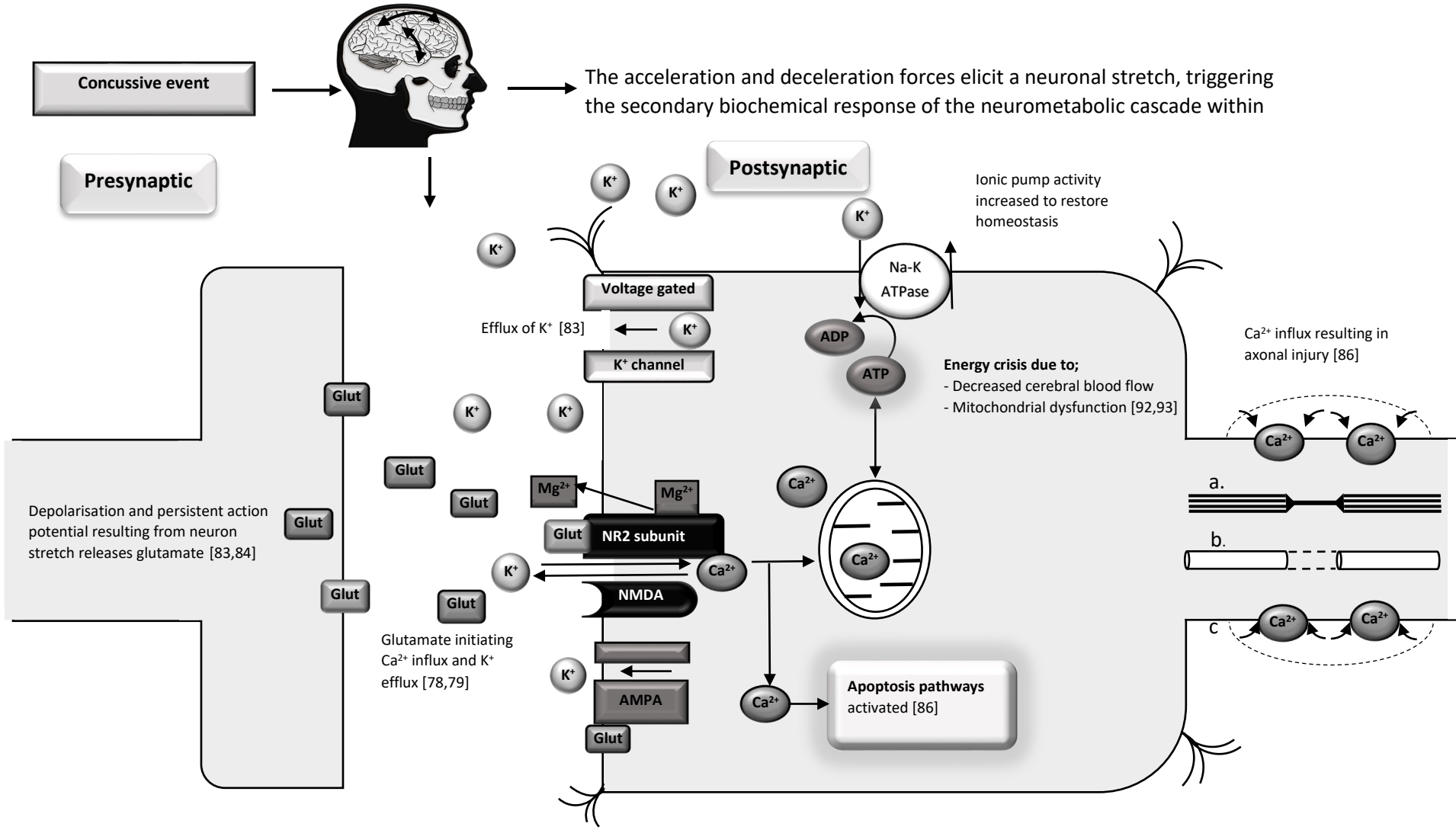


Figure 1.2. Concussive event leading to the neurometabolic cascade. Glut, glutamate; K^+, potassium; Ca^{2+}, calcium; Mg^{2+}, magnesium; AMPA, α -amino-3-hydroxy-5methyl-4-isoxazole-propionic acid.

1.2.5 Genetic associations with concussion

Genome-wide association studies (GWAS) enable the genome to be searched for unsuspected variations as opposed to candidate areas as in a gene association study [125,126]. In elite sport, however, the maximum number of individuals available for study is limited. For example, the English Rugby Premiership comprises ~600 players and Super League ~360 players. This limited sample size reduces the feasibility of GWAS, as considerably larger sample sizes are often required to meet the traditionally accepted significance value of $P < 5 \times 10^{-8}$. Genetic association studies utilising a candidate gene approach enable the study of genetic variance within a complex polygenic trait [127]. An advantage of the candidate gene approach is that genes are selected utilising an *a priori* hypothesis based on the biological function of a particular protein and the specific phenotype [127,128], and statistical power can be sufficient to test specific hypotheses using sample sizes available in elite sport. A disadvantage of the candidate gene approach is that only genes/variants already suspected are investigated, excluding the possibility of discovering hitherto unsuspected genes/variants that might be important.

Functionally, significant polymorphisms (single nucleotide polymorphisms (SNPs), repeat polymorphisms, insertions or deletions) used in the candidate gene approach are often selected based on the likeliness to affect gene function. Priority polymorphisms include those that alter an amino acid in a protein (missense variation) or produce a stop codon (nonsense variation) [127]. Polymorphisms in promoter and regulatory regions of a gene could also have functional consequences by influencing transcription rate [127].

Approximately 40 genetic variants have been associated with forms of traumatic brain injury [41]. It is hypothesised that rugby athletes would have more genetic resistance to concussion than non-athletes, because it would facilitate their prolonged participation in a high-risk environment. For example, possession of 'preferable' genetic variants such as *APOE ε2* and *ε3* and could enable elite rugby athletes to repair concussion-damaged neurons [120,121] and return to play within 7-10 days. In contrast, possession of the *APOE ε4* variant could result in the athlete experiencing a prolonged recovery [120,121]. Similarly, genetic variants that could influence behaviours such as *COMT* (rs4680), which has been associated with impulsivity and risk taking, could be preferable for rugby

performance behaviours, but also could place the athlete at increased risk of sustaining a concussion [129,130].

1.2.5.1 Candidate genetic variants

A complex array of physiological and psychological responses to concussion have been reported, so the proposed influencing genes have been categorised into four groups. These groups are based on current knowledge and some genes fit into more than one category due to the nature of their functions: 1. genes that affect the severity of concussion; 2. genes that affect repair and plasticity of the brain; 3. genes that affect post-concussion cognitive behavioural capacity; and 4. genes that affect personality traits and concussion risk. The genes are listed in Table 1.2 and the candidate genetic variants selected for this thesis are addressed in sections 1.2.5.2-1.2.5.7.

The SNPs involved in this thesis were in part selected via a systematic review of the relevant literature based on the strength of evidence from the literature and one or more of the following criteria: 1. previous association with a form of traumatic brain injury; 2. previous association with sports-related concussion; 3. biological function of the genetic variant was linked to pathophysiology of concussion or behavioural traits linked to concussion risk; 4. candidate SNPs had a minor allele frequency of > 1%; 5. financial and practical considerations of carrying out genotyping analysis for each SNP.

Table 1.2. Candidate genes linked to TBI.

Gene name	Gene abbreviation	Polymorphism identifier	Relevant effects associated with TBI
Apolipoprotein E	*APOE*	rs429358 rs7412 rs405509	Affects repair and plasticity of the brain. APOE isoforms have differing effects on neurite extension, which can influence ability to recover post-concussion. Associated with functional regulation of *APOE* transcription.
Microtubule associated protein tau	*MAPT*	rs10445337 rs2435211 rs2435200	Affects repair and plasticity of the brain via modulation of microtubule formation, structural stabilisation of the neuronal axons and drives growth of neurites.
Neurofilament heavy	*NEFH*	rs165602	Affects repair and plasticity of the brain via modulation of the neuronal cytoskeleton is to resist the resultant strain caused by biomechanical forces.
Membrane metalloendopeptidase	*MME*	GT repeat promoter polymorphism of neprilysin	Affects repair and plasticity of the brain as this gene encodes for the neprilysin protease which degrades Aβ proteins.
Brain derived neurotrophic factor	*BDNF*	rs6265	Affects repair and plasticity of the brain via strengthening existing synaptic connections and modulating the creation of new synapses.

Table 1.2. Continued.

Gene name	Gene abbreviation	Polymorphism identifier	Relevant effects associated with TBI
Glutamate ionotropic receptor NMDA type subunit 2A promoter	*GRIN2A*	rs3219790	Affects duration of concussion via potential modulation of glutamate-gated ion channel proteins.
Catechol-O-methyltransferase	*COMT*	rs4680	Affects cognitive behavioural capacity post-concussion and could increase impulsivity and risk taking.
Ankyrin repeat and kinase domain containing 1	*ANKK1*	rs1800497	Affects cognitive behavioural capacity via modulation of expression of D2 receptors.
Dopamine receptor D2	*DRD2*	rs12364283 rs1076560	Affects personality traits, associated with risk-taking behaviours (impulsivity, behavioural inhibition and novelty seeking).
Dopamine receptor D4	*DRD4*	rs1800955	
Catechol-O-methyltransferase	*COMT*	rs4680	Affects cognitive behavioural capacity post-concussion and could increase impulsivity and risk taking.

Table 1.2. Continued.

Gene name	Gene abbreviation	Polymorphism identifier	Relevant effects associated with TBI
Solute carrier family 6 member 4	*SLC6A4*	rs4795541 rs25531	Reported to play a role in personality and behavior via increased harm avoidance and impulsivity behaviours.
Endothelial nitric oxide synthase	*NOS3*	rs2070744	Could affect severity of concussion and cognitive behavioural capacity post-concussion via modulation of cerebral vasospasm.
Angiotensin I converting enzyme	*ACE*	rs4646994 rs7221780 rs8066276	Affects cognitive behavioural capacity post-concussion via modulation of cerebral blood flow.
Tumor necrosis factor	*TNF*	rs1800629 rs1800468 rs1800469	Could affect neuroinflammation and severity of concussion.
Transforming growth factor beta 1	*TGFB1*	rs1800468 rs1800469	Regulation of the anti-inflammatory mediator TGFB1 could affect severity of concussion.
Interleukin 1 alpha *interleukin 1 beta*	*IL1A* *IL1B*	rs1800587 rs16944 rs1143634	Affects severity of TBI via potential modulation of the inflammatory process and secondary conditions.
Interleukin 6 receptor	*IL6R*	rs2228145	Affects severity of concussion potential via modulation of the inflammatory process and cognitive behavioural capacity post-concussion.

(Adapted from Antrobus et al. [41])

1.2.5.2 Apolipoprotein E.

Apolipoprotein E (APOE) is the most researched gene in respect to TBI. APOE isoforms have both protective and detrimental effects (Figure 1.3). These effects are dependent upon which specific alleles an individual carries and thus gene expression after the TBI event. *APOE* has three common allelic isoforms ε2, ε3 and ε4 which differ by amino acid substitutions at residues 112 and 158 [131]. Two C/T SNPs at residues 112 (rs429358) and 158 (rs7412) result in amino acid substitutions of arginine (C) to cysteine (T) at each residue (Figure 1.3). The two nonsynonymous SNPs at residues 112 and 158 can produce the three isoforms of ε2, ε3, ε4 and six possible genotypes (Table 1.3) of relevance to concussion.

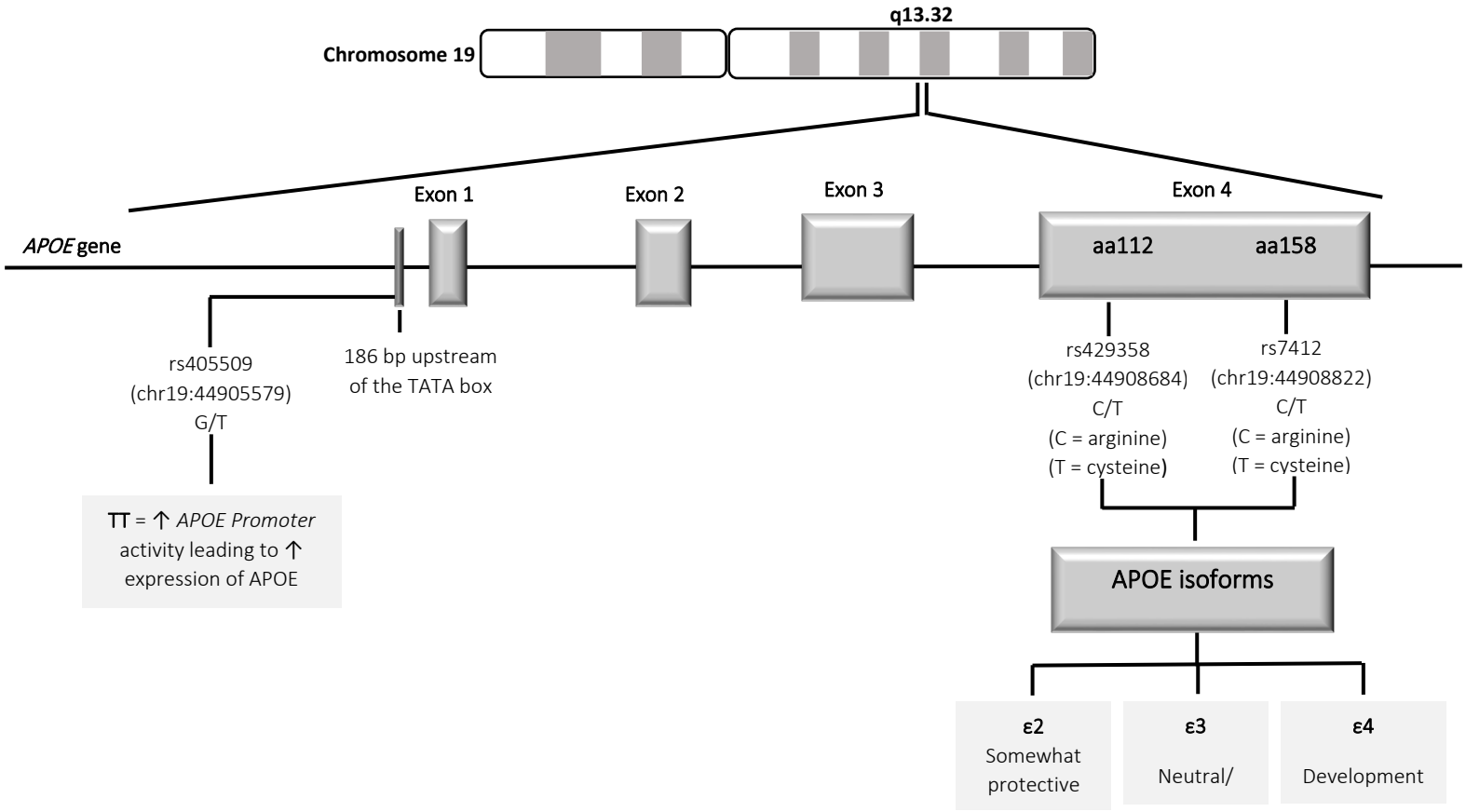


Figure 1.3. Schematic of *APOE* variants: The ε2 isoform binds with amyloid plaques (Aβ) and is removed enabling neuronal modelling and plasticity of neurons to be facilitated. The ε3 isoform binds with less infinity to Aβ, interacts with microtubules and is associated with neurite extension and branching. It also binds with tau to stabilise microtubules. The ε4 isoform does not bind with Aβ, meaning the activity of toxic cleaved APOE fragments can cause lysosomal leakage, leading to apoptosis and stimulation of tau and the formation of neurodegenerative neurofibrillary tangles

APOE isoforms have differing effects on neurite extension, which can influence ability to recover post-concussion. APOE ε3 stimulates neurite growth in cultured neuronal cells [120,121]. In contrast, APOE ε4 suppresses neurite growth [120,121]. These findings suggest that *APOE* ε2 and ε3 would provide more effective neuronal repair, such as proliferation of dendrites post-concussion compared to *APOE* ε4 [120,121]. In addition, the ε4 alleles have been associated with the formation of neurodegenerative Aβ and increased risk of Alzheimer's disease (AD) [132].

Table 1.3. Three isoforms and six possible genotypes of *APOE.*

APOE isoform	APOE genotype	rs429358	rs7412
ε2		T	T
ε3		T	C
ε4		C	C
	ε2/ε2	TT	TT
	ε2/ε3	TT	CT
	ε2/ε4	CT	CT
	ε3/ε3	TT	CC
	ε3/ε4	CT	CC
	ε4/ε4	CC	CC

There are many pathophysiological factors that have informed the hypothesis that *APOE* is associated with TBI. *APOE's* association with TBI was made due to the observations that AD may act synergistically to develop progressively over time in individuals with a history of TBI [132]. Also, autopsies on boxers with chronic traumatic encephalopathy have indicated similar pathophysiology to AD patients' brains [133]. Diffuse Aβ plaques have been observed in 30% of patients who have experienced a single TBI [133]. This Aβ accumulation has been observed in TBIs and differs in structure to age-related accumulation. These

diffuse Aβ plaques have been observed to form hours after TBIs [134]. APOE has both protective and detrimental effects (Figure 1.3). These effects are dependent upon which specific allele the individual carries and is expressed after the TBI event. The ε4 allele has been associated with an increased genetic risk of AD, compared to the somewhat neutral ε3 allele and the more protective ε2 allele [135].

The N-terminal domain of APOE interacts with APOE receptors and the C-terminal domain is the main lipid-binding region. It is this N-terminal domain that contains the 112 and 158 polymorphisms and produce differing structures and biological functions of the 3 common APOE isoforms (ε2, ε3 and ε4) [131]. It is hypothesised that ε4 has altered domain interaction and reduced protein stability (molten globule formation) resulting in the detrimental effects post-TBI [131]. This unstable molten globule formation leads to neurotoxic effects of the mis-folded ε4 protein [131].

APOE ε4 appears to reduce the stability of the cytoskeleton of neuronal cells, particularly effecting microtubule stability [136]. Strittmatter *et al*. [136] observed that tau protein binds more readily to APOE ε3 compared to APOE ε4. This suggests that APOE ε2 and ε3 can reduce the formation of paired helical filaments that can become hyperphosphorylated tau protein [122]. Neurotoxic hyperphosphorylated can occur via a mutation in *tau* [137]. Accumulation of hyperphosphorylated tau protein can produce neurotoxic neurofibrillary tangles a common marker of AD and other tauopathies [122].

APOE ε4 has been associated with causing neurodegeneration through differing mechanisms. Due to its differing structure APOE ε4 is more susceptible to proteolysis that APOE ε2 and ε3 isoforms [131]. Accumulation of APOE ε4 cleaved fragments have been associated with reduced learning and memory performance in animal models [122]. APOE fragments can translocate in to the cytosol. Within the cytosol fragments can interact with the cytoskeleton and the mitochondria [138]. Mitochondrial-apoptotic pathways can become activated via cleaved APOE ε4 fragments [138]. This is in contrast to the ε2 and ε3 variants that appear to protect cells from this process [138]. APOE ε4 is also associated with disruption of mitochondrial function and reduced glucose metabolism and reduced cognitive performance [139].

The full functions of *APOE* in relation to concussion are still not fully understood. However, the pathophysiology highlighted in the literature indicates that ε4 allele carriers could be at greater risk to a worse outcome and recovery post-concussion compared to ε2 and ε3 allele carriers.

Despite the pathophysiological roles that APOE ε4 plays in TBI, studies associating *APOE* ε4 and sport-related concussion are few and findings are conflicting. Kristman et al. [140] showed no association between *APOE* ε4 carriers and incidence of concussion in Varsity level athletes. These findings have been supported by Terrell et al. [2] and Tierney et al. [1], who also reported no association between concussion incidence and *APOE* genotypes in collegiate athletes. More recently, Abrahams [55] reported no association in *APOE* ε2, ε3 and ε4 genotypes and incidence of concussion in a mixed cohort of youth, amateur and professional South African RU players.

Early findings from Jordan et al. [141] indicated that *APOE* ε4 carrier boxers experiencing high-exposures (>12 professional bouts) had greater chronic brain injury scale scores than non-ε4 carrier high-exposure boxers. Indeed, it has been suggested that the *APOE* ε4 allele may be responsible for up to 64% of the 'hazardous influence' of TBI [142] and athletes who possess the ε4 allele suffer from prolonged physical (*d'* = 0.87) and cognitive (*d'* = 0.60) symptomatic responses to concussion [43].

Polymorphisms within the promoter region of *APOE* have been associated with functional regulation of *APOE* transcription and quantitative impacts on apolipoprotein E levels in brain tissue, as well as unfavourable outcomes post-TBI [143,144]. It has been hypothesised that the -219 T allele at rs405509 exacerbates the effects of the ε4 allele through upregulation of APOE gene transcription and increased Aβ plaque accumulation [143].

Lendon et al. [144] observed an association between individuals with rs405509 TT genotype and unfavourable outcomes post-TBI over a 6-month recovery period. Tierney et al. [20] reported carriers of the T allele had an 8-fold greater risk of experiencing two or more concussions. Similarly, Terrell et al. [21] suggest the TT genotype is associated with a 3-fold greater risk of previous concussion and a 4-fold greater risk of a history of concussion with loss of consciousness. In contrast, Abrahams et al. [47] reported TT genotype was

associated with a 45% reduced risk of concussion and the T allele was associated with a <1-week recovery period post-concussion in a mixed cohort of youth and professional South African RU players**.** These conflicting findings could be in part due to differences in sport and, in particular, geographic ancestry of the participants. Nevertheless, the plausible physiological mechanisms and the limited number of association studies warrants further investigation of this concussion-associated SNP.

1.2.5.3 Microtubule associated protein tau polymorphisms

The functions of *microtubule associated protein tau* (*MAPT*) include encoding the tau protein that modulates microtubule formation, structural stabilisation of the neuronal axons and driving growth of neurites [123,124]. Elevated post-TBI plasma levels of tau have been observed for up to 90 days [145]. Autopsies on American football players' brains who had experienced repetitive concussions indicate the presence of neurofibrillary tangles (aggregates of hyperphosphorylated tau protein) and neuropil filaments (abnormal neurite formations) [146]. These neurotoxic formations have been associated with neurodegenerative diseases such as Alzheimer's, chronic traumatic encephalopathy, Parkinson's, fronto-temporal dementia and a range of other neurodegenerative diseases under the term tauopathies [147–150]. The *MAPT* (rs10445337) T/C SNP is postulated to modulate the formation of neurotoxic-paired helical filaments composed of hyperphosphorylated tau [151,152] (Figure 1.4).

Tau is hypothesised to modulate concussion risk as APOE ε4 binds more weakly than the other APOE isoforms, enabling tau to be phosphorylated and reducing binding to the microtubules [153]. All SNPs within *MATP* can lead to the formation of neurotoxic-paired helical filaments composed of hyperphosphorylated tau [151,152]. It is hypothesised the rs10445337 TT genotype reduces ability to bind to microtubules leading to greater accumulation of paired helical filaments, NFT and neuritic plaques due to over expression of MATP and synergy with APOE ε4 [154].

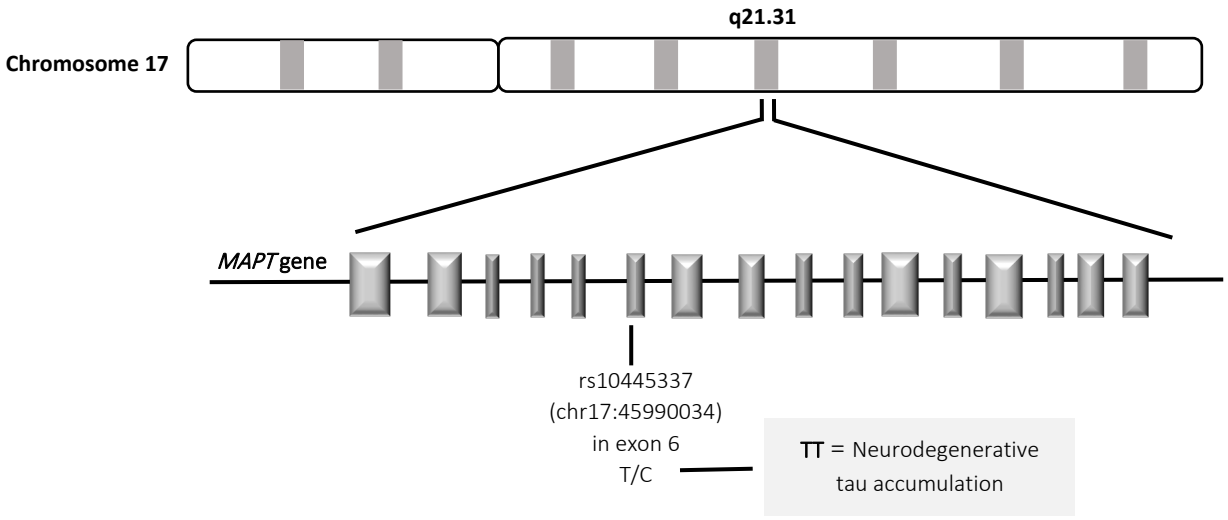


Figure 1.4. Schematic of *MAPT* polymorphisms associated with accumulation of tau clumps and the formation of neurofibrillary tangles and neuritic plaques.

Two studies have investigated cerebrospinal fluid tau levels in amateur boxers due to the repetitive blows to the head and associated high risk of concussion and neuronal damage. Neselius *et al*. [155] observed greater cerebrospinal fluid tau levels post-bout (449 pg/mL) in boxers compared to levels after 3 months of absence (306 pg/mL). Levels of cerebrospinal fluid tau were greater in individual boxers who experienced >15 hits or high impact hits compared to those who self-reported less hits. 3 months of absence from boxing indicated no difference in tau levels compared to a control group (325 pg/mL). Again, Neselius *et al*. [156] reported cerebrospinal fluid tau levels to be increased by >300% in a sample of amateur boxers post-concussion and >80% of the boxers had elevated total tau levels 6 days post-concussion.

Terrell et al. [21] reported a non-significant observation that the *MAPT* rs10445337 TT genotype was weakly associated with a history of one or more concussions (odds ratio, 2.1; 95% CI, 0.3 to 14.5). Similarly, in a later study no association was observed between concussion incidence and *MAPT* rs10445337 [157]. Recently, other MAPT SNPs (rs2435211 and rs2435200) have been implicated as potential pathophysiological mechanisms in RU players [29]. The AG genotype of rs2435200 has been associated with an increased risk of sustaining multiple concussions in senior (>18 years old) RU players [29]. In addition, the T-G haplotype (rs2435211 and rs2435200) has been associated with an increased risk of sustaining a concussion in senior amateur and elite RU players [29].

1.2.5.4 Brain derived neurotrophic factor polymorphism

Brain derived neurotrophic factor (BDNF) is a gene that affects the repair and plasticity of neurons. It is a member of the neurotrophin family, responsible for mediating neuronal plasticity [158,159]. Neurotrophins aid in the development, differentiation, proliferation and survival of neurons (dopaminergic, serotonergic and cholinergic) [158,160]. A widely studied SNP is the C to T missense variation at nucleotide 196 resulting in a valine to methionine (Val66Met) substitution at codon 66 [161] (Figure 1.5). BDNF mRNA is upregulated post-TBI event and can remain elevated for up to three days post-TBI [162–164]. *BDNF* plays an important role in strengthening existing synaptic connections and modulating the creation of new synapses [158]. The Met allele impairs intracellular tracking and packaging of precursor-BDNF (pro-BDNF) and activity-dependent secretion of BDNF [161].

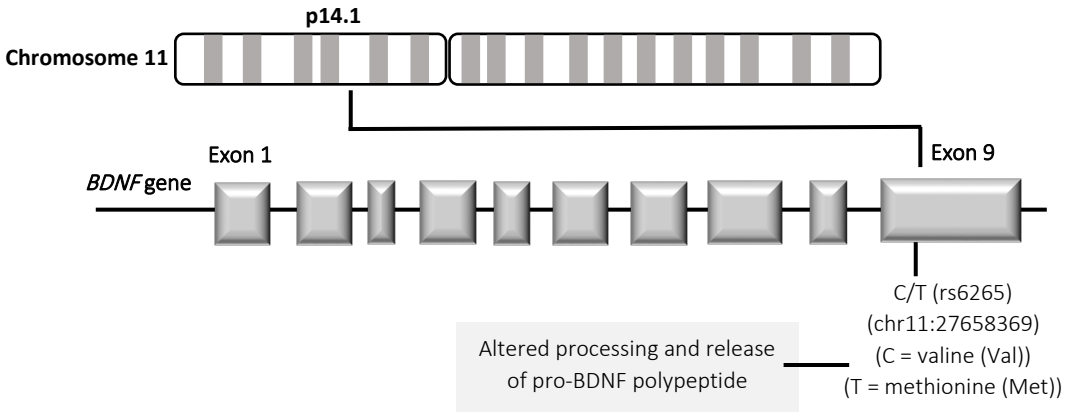


Figure 1.5. Schematic of *BDNF* (rs6265) Val to Met substitution results in poor packaging and intracellular protein trafficking of pro-BDNF polypeptide.

The associated effects of the rs6265 polymorphism within *BDNF* on neurocognitive performance such as memory and learning are well documented. The seminal paper by Egan *et al*. [161] indicated in a large sample (n = 641) Met allele carriers perform worse on episodic tests of memory (prose recall) and had reduced hippocampal activation during the testing compared to Val allele carriers. Through the use of functional magnetic resonance imaging (fMRI), Met allele carriers were observed to perform worse during a memory test compared to Val allele carriers in a small sample (n = 28 comprised of a range of geographic ancestries) [165]. Results from the fMRI also indicated Met allele carriers had less

engagement of the hippocampus during the memory tasks compared to the Val allele carriers [165].

Despite a large body of evidence supporting the role of the *BDNF* rs6265 polymorphism in neurocognitive performance, *BDNF* and concussion association studies are few. Due to *BDNF*'s role in development, modulating the differentiation, proliferation and survival of neurons, circulating serum levels of BDNF have been associated with TBI severity and post-TBI injury outcomes [158,166]. Korley et al. [166] suggested circulating serum BDNF levels on the day of injury to be a prognostic measure for identifying concussion patients likely to have persistent TBI-related symptoms 6 months post-injury. Korley et al.'s [166] findings indicated BDNF levels (on the day of injury) were higher in patients with concussion (8.3 ng/ml) compared to moderate TBI (4.3 ng/ml) and severe TBI patients (3.9 ng/ml). Findings support the concept that, as a neurotrophin, BDNF's role is protection of neurons and as the level of damage increases the amount of BDNF decreases [158,166].

Dretsch et al. [136] reported that ~17% of Met/Met homozygotes suffered a concussion during military deployment compared to ~4% of Val carriers. Narayanan et al. [168] found that the rs6265 polymorphism was associated with neurocognitive performance in concussed individuals acutely and 6 months post-event, as Val/Val homozygotes performed better in measures of memory, executive function, attention and overall cognitive performance [168]. These findings are supported by McAllister [169] who observed Met/Met homozygotes had slower cognitive processing speeds compared to Val/Val homozygotes 1 month post-concussion. It was observed that Met allele carriers in the concussion group and the control group had slower cognitive processing speeds [169]. The literature supports the hypothesis that the *BDNF* rs6265 polymorphism is associated with influencing neurocognitive performance post-concussion.

1.2.5.5 Catechol-O-methyltransferase polymorphism

The *catechol-O-methyltransferase* (*COMT*) gene has been postulated to affect post-concussion cognitive behavioural capacity [170]. *COMT* encodes an enzyme that methylates and in turn deactivates catechol-based neurotransmitters such as synaptic

dopamine and noradrenaline [171] (Figure 1.6). Optimal cognitive function is affected by the prefrontal cortex's sensitivity to dopamine, which makes *COMT* an ideal candidate gene for influencing inter-individual variability in cognitive function post-concussion. A widely studied SNP within the *COMT* gene is the G to A missense variation at codon 158 resulting in a valine (Val) to methionine (Met) amino acid substitution. Val/Val carriers have greater COMT activity than Met/Met carriers [172].

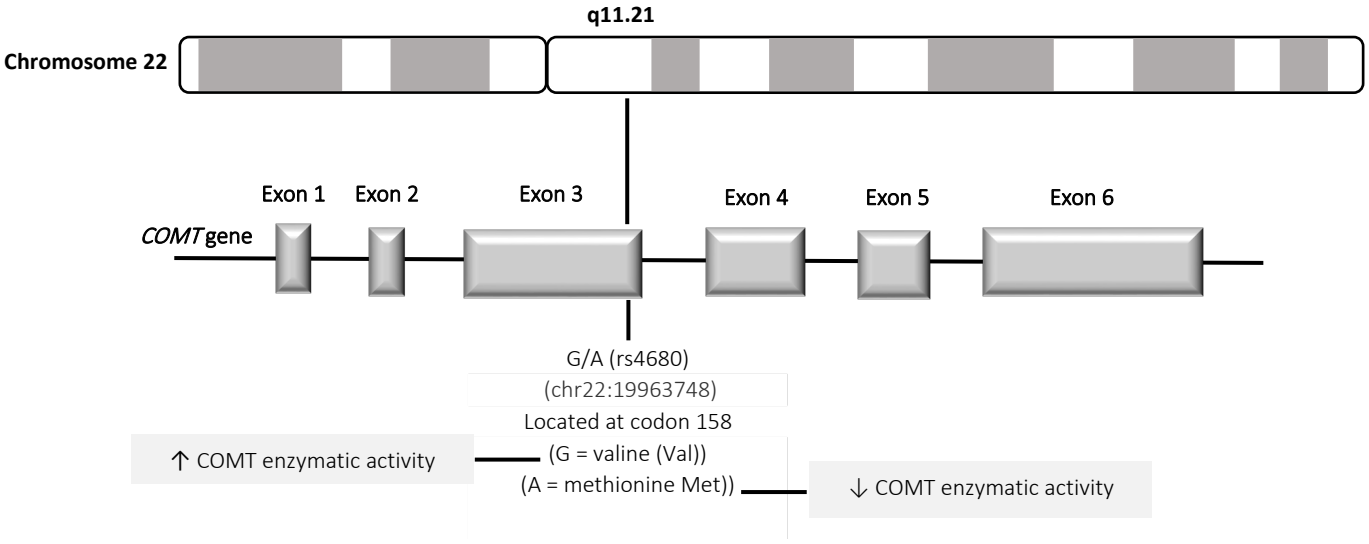


Figure 1.6. Schematic of rs4680 *Catechol-O-methyltransferase.*

The Val to Met substitution affects the thermostability of COMT. The Met allele has been observed to have lower thermostability and lower enzymatic activity at 37˚C [172]. Val/Val homozygotes have been observed to have greater COMT activity than Met/Met homozygotes [173]. This observation has been supported by Chen et al. [172], who noted Met/Met had ~33% decreased COMT activity than Val/Val. *COMT* (rs4680) alleles are co-dominant, resulting in heterozygous individuals producing intermediate levels of COMT [172].

COMT plays an important role in modulating dopamine in the prefrontal cortex [174]. The literature indicates that Val allele carriers have poorer cognitive performance. Val/Val homozygotes were observed to have poorer executive cognition during Wisconsin Card Sort Test [175]. Findings from Egan et al. [175] indicate that PFC cognitive performance was influenced by a Val dose response; Met/Met homozygotes had greater executive cognitive

performance and Val/Met heterozygotes had intermediate performance. These findings support the notion that *COMT* genotype can affect cognitive function.

Lipsky et al. [170] reported that Val allele carriers performed poorer on tests of executive function compared to Met allele carriers post-TBI. More recently and in contrast, Willmott et al. [176] reported no significant influence of *COMT* polymorphisms on cognitive performance in moderate to severe TBI patients. However, Lipsky et al. [170] employed a battery of executive function tests including the Wisconsin Card Sorting Test, while Willmott et al. [176] used the Glasgow Outcome Scale-Extended as a measure of functional outcome post-TBI. Mc Fie et al. [42] reported Met carriers in a cohort of youth and professional South African RU players were ~3-fold more likely to have a history of concussion and, accordingly, it has been postulated that elevated dopamine could increase impulsivity and risk taking meaning Met allele carriers could place themselves at increased risk of sustaining a concussion [129,130].

1.2.5.6 Ankyrin repeat and kinase domain containing 1 polymorphism

Ankyrin repeat and kinase domain containing 1 (*ANKK1*) is a dopaminergic gene known to affect working memory, reward and motivation [177,178]. *ANKK1* was originally referred to as *Taq1A* and is in linkage disequilibrium (D' >0.80) with the 10 kB downstream *dopamine receptor D2* (*DRD2*) gene [179]. The *ANKK1* C/T (rs1800497) SNP is hypothesised to be in a regulatory region within *DRD2* (Figure 1.7) [179]. ANKK1 is expressed in astroglial cells (a type of brain-derived glial cell), post-mitotic neurons and neural precursors from neurogenic niches and as a member of the serine/threonine receptor-interacting protein kinases is responsible for dopaminergic signal transduction and cellular response [179,180].

ANKK1 polymorphisms affect dopamine transporter densities within the striatum which influences working memory, reward and motivation [177,178]. The T allele of *ANKK1* has been associated with a 30-40% reduction in the expression of D2 receptors within the ventral striatum [181,182]. *ANKK1*'s polymorphic role in modulating working memory and cognitive performance vis-à-vis concussion/TBI is limited to three studies. McAllister et al. [183,184] observed concussed T allele carriers performed significantly worse in measures of learning, working memory and response latencies. Similarly, Yue et al.'s [185] findings

support McAllister et al. [183,184] and indicate a dose-dependent association with the T allele. Thus, this polymorphism could influence recovery from a concussive event.

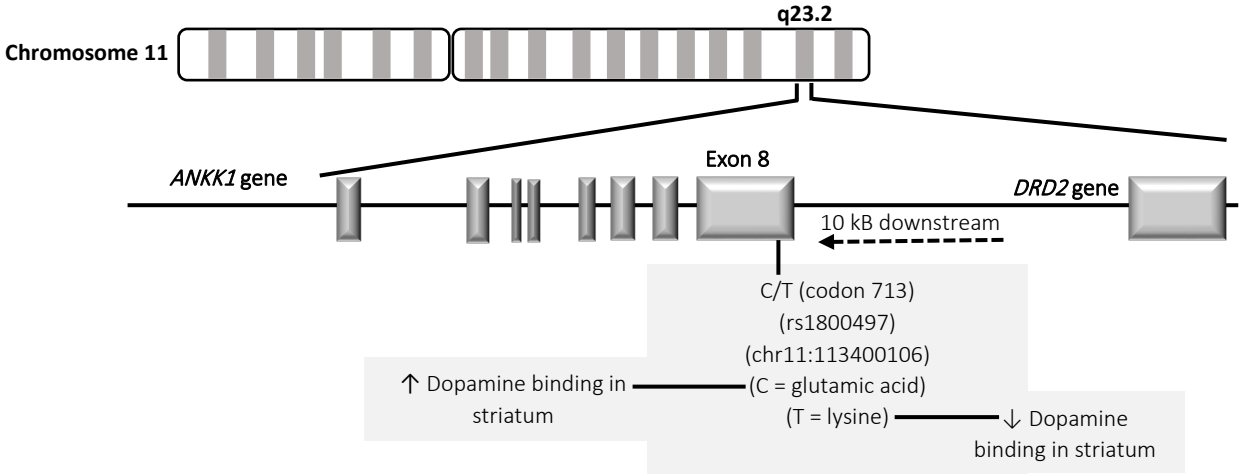


Figure 1.7. Schematic of *ankyrin repeat and kinase domain containing 1* (rs1800497). T allele of *ANKK1* has been associated with dopaminergic function.

1.2.5.7 Endothelial nitric oxide synthase polymorphism

Nitric oxide (NO) plays a major role in the maintenance of cerebral blood flow and is synthesised by three NO synthase isoforms; endothelial (eNOS), neuronal and inducible [186,187]. Nitric oxide is reduced post-TBI under experimental conditions [188,189] and the *NOS3* -786T/C (rs20707044) promoter polymorphism has been associated with promoter region activity, reduced NO synthesis and cerebral vasospasm (persistent contraction of the blood vessels) [190] (Figure 1.8). During the neurometabolic cascade, cerebral blood flow is reduced up to 50% [102,103], meaning possession of alleles that contribute to a greater ischemic cerebral environment could increase the risk of concussion incidence and poorer recovery.

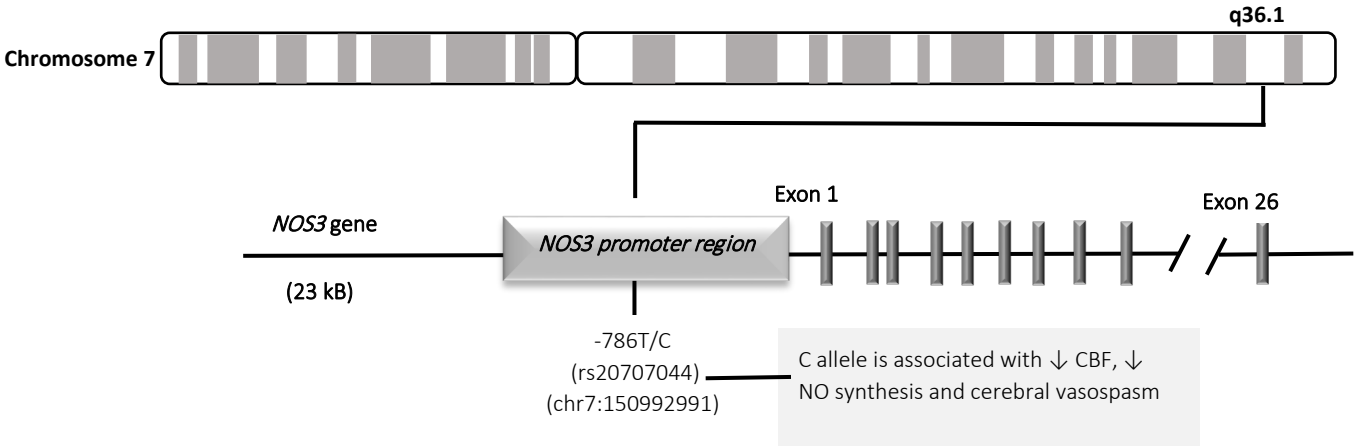


Figure 1.8 Schematic of rs2070744 *NOS3* C allele is associated with reduced cerebral blood flow in traumatic brain injury patients.

Robertson et al. [119] reported lower cerebral blood flow in -786 C allele (rs2070744) carrying patients with severe-TBI. Multifactorial pathophysiological mechanisms contribute to the reduction of cerebral blood flow as a result of sustaining a concussion [115]. Thus, it could be postulated that possession of a -786 C allele could negatively affect a concussed individual, due to further reduced cerebral blood flow and this warrants further investigation.

1.2.5.8 Conclusions and future directions

Elite rugby players are exposed to a higher risk of concussion during a playing career than athletes in many other sports. A critical step in better understanding inter-individual variability in the risk of sustaining a concussion and the duration of recovery following a concussion involves identifying genetic variations associated with those risks. The literature has already identified several genetic factors with inter-individual variability in concussion and TBI incidence, severity and recovery. The genes and polymorphisms reviewed here, along with many others, need to be investigated further in relation to incidence rates and recovery from concussion, particularly in a sport such as rugby with a relatively high concussion risk. In the foreseeable future, studies will identify new candidate polymorphisms and replication studies could indicate stronger associations between

existing polymorphisms and concussion risk, which could be used to increase the accuracy of a prediction model. For example, a recent genome-wide-association study has identified 2 novel SNPs (*SPATA5* rs144663795 and *PLXNA4* rs117985931) associated with concussion [191]. Further GWASs and replication studies of candidate gene approaches are needed to establish a reliable TGS for indicating concussion risk. The number of individuals competing in truly elite rugby is low, so highly collaborative research is required to achieve sample sizes sufficient for satisfactory statistical power.

The interindividual variation in outcomes following concussion makes predictions of recovery and future risk difficult. This variability could mean there is a future valuable role for genetic screening of concussion-associated risk polymorphisms to complement other data. Achieving elite status in a sport such as rugby is a multifactorial accomplishment due to the complex interactions of multiple environmental factors and the polygenic nature of inherited characteristics and predispositions. Epigenetic regulation of genome function in the context of particular environmental stimuli might also be important in modulating the risk of concussion injury and the rate of recovery. Elite rugby players are exposed to one of the highest risks of concussion in team sports, so distinctive genetic characteristics may exist in those athletes that offer advantages in resisting frequent or severe concussions, relative to those less successful in the sport. Athletes in other sports with a high risk of concussion are also particularly likely to benefit from this kind of genetic resistance to injury. The findings, however, could be applied to a wider range of sports, including those with a lower but still extant risk of concussion. Thus, future research that combines an individual's concussion history and other phenotypes with detailed genomic information could facilitate more personalised management of concussion, and eventually help protect athletes from unfavourable longer-term health outcomes. The RugbyGene project at Manchester Metropolitan University has one of the largest cohorts of elite rugby DNA samples in the world. An aim of the RugbyGene project is to investigate the role genetic variation plays in physical fitness, training, risk of injury and success in elite rugby. Access to this biobank and collaboration with RugbyGene researchers will support the aims of this thesis.

1.3 Aims and objectives

The overall aim of the current thesis was to investigate whether elite rugby athletes differ in terms of genetic risk factors for frequency and severity of concussion compared to non-athletes and within an elite rugby athlete population. More specifically, the objectives were:

1. Recruit additional participants for the ongoing RugbyGene project with the purpose of investigating molecular characteristics of elite rugby athletes.
2. Investigate whether genotype frequency of suspected concussion-associated polymorphisms (*ANKK1* rs1800497, *APOE* rs429358, rs7412 and rs405509, *BDNF-AS* rs6265, *COMT* rs4680, *MAPT* rs10445337 and *NOS3* rs2070744) differ between elite rugby athletes and non-athletes, and between RU playing positions.
3. Investigate if concussion associated polygenic profiles differ between elite rugby athletes compared to a non-athlete control population via the application of a total genotype score (TGS) algorithm. Secondly, to compare the polygenic difference between RU forwards and RU backs.
4. Investigate whether suspected concussion-associated polymorphisms are associated with history of previous concussion in elite rugby athletes.

General Methods

2.0 General methods

Extended details of the methods employed are described in this chapter, from which chapters 3, 4 and 5 use some or all of the participants and experimental procedures.

2.1 Participants

As part of the ongoing RugbyGene project [192], a total of 1683 individuals were recruited and gave written informed consent to participate in the present chapter. The total sample comprised elite Caucasian male rugby athletes (n = 668: mean (standard deviation) height 1.85 (0.07) m, mass 102 (12) kg, age 29 (7) yr) including 62.9% British, 13.8% South African, 10.8% Irish, 8.9% Italian, and 3.6% of other nationalities and non-athlete Caucasian control participants (n = 1015, 48% male, height 1.71 (0.11) m, mass 73 (13) kg, age 38 (22) yr) including 91.8% British, 6.7% South African, 1.5% other nationalities. Inclusion criteria for non-athletes included having no known inherited disease, and never being a competitive athlete in any sport at a level above 'community/recreational'.

Athletes were considered elite if they had competed regularly (> 5 matches) since 1995 in the highest professional league in the UK, Ireland or South Africa for RU, or the highest professional league in the UK for RL. 49.1% of the RU athletes had competed at international level for a "high performance union" (Regulation 16, http://www.worldrugby.org) and 42% of RL athletes had competed at international level. RU athletes were also sub-divided into the major positional groups of forwards and backs for comparison. Ethical approval was granted by the ethics committees of Manchester Metropolitan University (approval number 12.07.11), University of Glasgow, University of Cape Town and University of Northampton, and all experimental procedures complied with the Declaration of Helsinki [193]. The principle researcher contributed to increasing the sample size of the rugby athlete cohort by ~20% (190 elite rugby athlete samples).

2.2 DNA sample collection.

Description of all molecular procedures have previously been described by Heffernan et al. [54,194,195]. Blood (70.4% of all samples), buccal swabs (15.4%) or saliva (14.2%) samples were obtained via the following protocols. In accordance with the Human Tissue Act (2004),

all samples were coded and labelled to maintain participant anonymity. Phlebotomy training was completed at NHS Leighton Hospital, UK.

Blood was drawn from a superficial forearm vein (Figure 2.1, appendix 1) into a 5 mL ethylenediamine tetra acetic acid (EDTA) tube (BD Vacutainer Systems, Plymouth, UK) using a 19 G needle. The blood samples were aliquoted in to 2 mL sterile tubes (Eppendorf AG, Hamburg, Germany) and stored at -20°C until processing.

Saliva samples were collected into Oragene DNA OG-500 collection tubes (DNA Genotek, Ottawa, Ontario, Canada) according to the manufacturer's protocol (Figure 2.2, appendix 2) and stored at room temperature (15°C–30°C) until processing. Participants were instructed not to eat, drink, smoke or chew gum for 30 min before providing the saliva sample, which took 2-5 min.

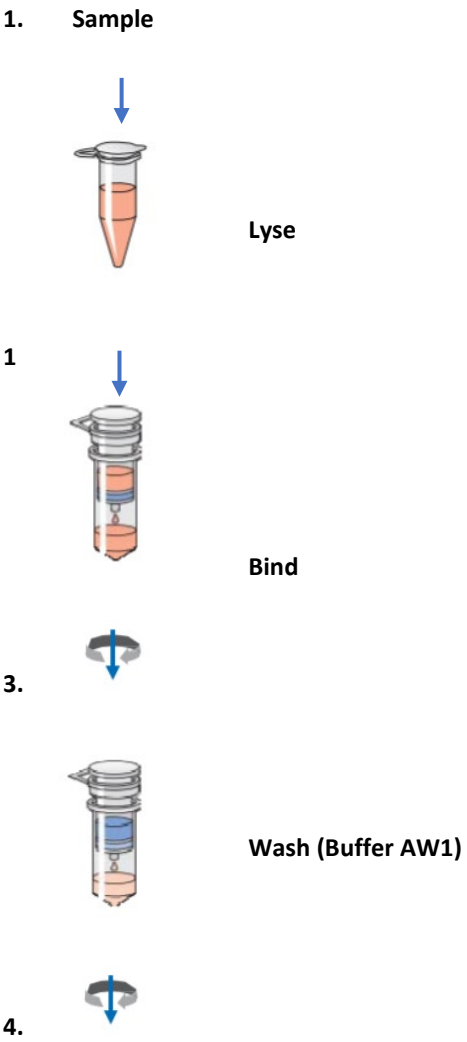
Sterile buccal swabs (OmniSwab; Whatman, Springfield Mill, UK) were rubbed against the buccal mucosa of the cheek for ∼30 s (Figure 2.3, appendix 3). Tips were ejected into sterile 2 mL tubes and stored at −20°C until processing.

2.3 DNA isolation

DNA isolation and genotyping were performed in the Manchester, Glasgow and Cape Town laboratories. The majority of samples were processed and genotyped in the Manchester laboratory. Three methods of extraction were performed due to samples being collected from different locations, transportation of whole samples and implications of not being a Human Tissue Authority licenced institution. There are some differences between protocols, described below.

In Manchester and Glasgow, DNA isolation was performed with the QIAamp DNA Blood Mini kit and standard spin column protocol, using the automated Qiagen QIAcube following the manufacturer's instructions (Qiagen, West Sussex, UK) (Figure 2.4). At Glasgow, the isolation was performed by a research team led by Professor Y. Pitsiladis. Whole blood samples were the preferred sample as they provide large quantities of cells containing DNA [196]. However, saliva and buccal collection methods were used as alternatives due to their non-invasive methods which also provide sufficient DNA for PCR [196]. 200 μL of whole

blood/saliva, or one buccal swab (600 μL of phosphate-buffered saline required for swab sample), were incubated at 56°C for 10 min and lysed with Qiagen protease. Ethanol (96%) was added to buffers and the resultant lysate and was centrifuged at 6000 *g* (8000 rpm) for 60 s at room temperature (15-25°C). During the centrifugation process, the DNA is absorbed by the spin column silica membrane (Figure 2.5). Two additional wash buffers were passed through the silica membrane during two additional centrifugation cycles removing residual contaminants (proteins, nucleases and other impurities), which can inhibit PCR [197]. This process improves the purity of the eluted DNA. The remaining purified DNA was eluted in to a low-salt pH-balanced buffer providing a 100 μL solution containing isolated DNA, which was stored at 4°C until further analysis. Typical yields from 200 μL whole blood range between 3-12 μg of DNA (mean yield of 10 μg), saliva yields of 5-15 μg and 0.5-3.5 μg from one buccal swab.



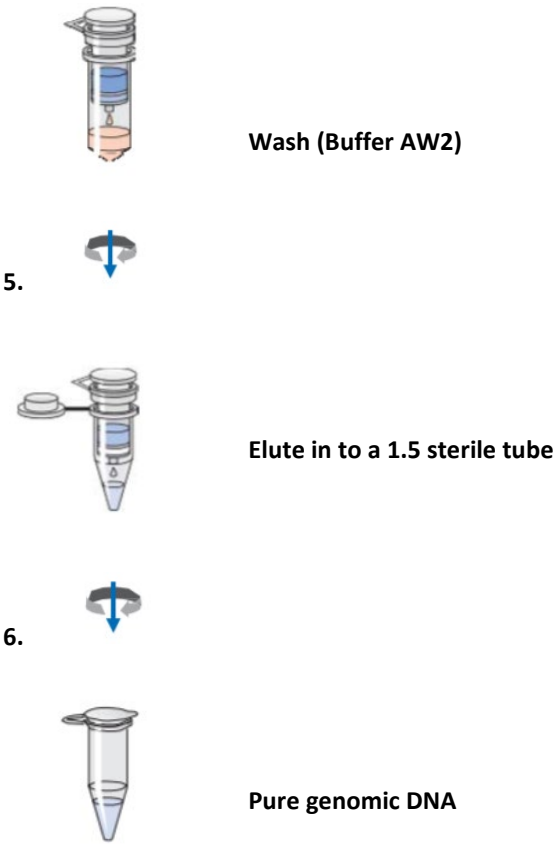


Figure 2.5. Automated QIAamp spin column protocol [198].

In Cape Town, DNA was isolated from whole blood using a different protocol [199] by a research team lead by Professor M. Collins. In brief, 15 mL whole blood samples were combined with low-salt buffer and Nonidet P-40 (NP-40, Sigma) to lyse the cells. Samples were centrifuged at 2200 rpm for 10 min at room temperature. The supernatant was removed, nuclear pellets were washed and resuspended in a high-salt buffer. Samples were mixed with a lysis buffer and were incubated at 55°C for 10 min and were centrifuged at 12000 rpm for 5 min. 100% ethanol was added to the supernatant and mixed until the DNA precipitated. The DNA sample was centrifuged at 12000 rpm for 5 min, washed with 70% ethanol and dried. Dehydrated DNA was resuspended in low-salt pH-balanced buffer and samples were stored at -20°C until subsequent analysis. Genotyping of DNA isolated in Cape Town was performed in Glasgow.

At Northampton, DNA was isolated from whole blood using FlexiGene kits (Qiagen) (Figure 2.6) by a research team lead by Dr S. Raleigh. Lysis buffer was added to each sample and

centrifuged for 5 min at 2000 *g* at room temperature. The supernatant was removed, nuclear pellets were washed and resuspended in denaturation buffer with Qiagen protease. Samples were incubated at 65°C for 10 min and were centrifuged at 6000 *g* for 5 min. DNA was precipitated and centrifuged for 3 min at 2000 *g*. The pelleted DNA was washed in 70% ethanol and dried. Dehydrated DNA was resuspended in low-salt pH-balanced buffer and samples were stored at -20°C until subsequent analysis (DNA yield 35 μg/mL of whole blood).

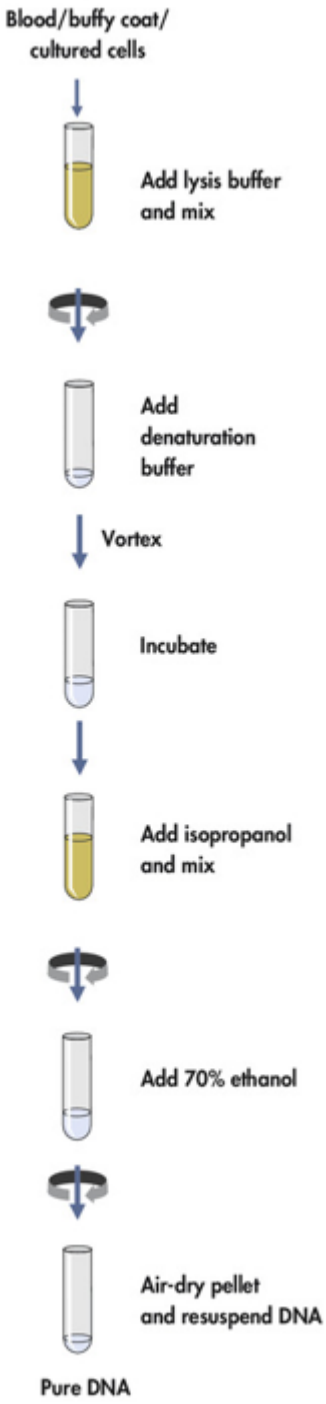


Figure 2.6. FlexiGene protocol [200].

2.4 Quantification of DNA

Yield and purity (removal of contaminants and inhibitors such as; heme, heparin, and mucus) [201,202] of the DNA samples were determined using a BioPhotometer Plus

(Eppendorf UK Limited, Stevenage, UK) at 260 and 280 nm. This was performed for a fraction of samples (from all extraction methods) periodically to check DNA obtained had similar and suitable quality and concentration when different batches of samples were isolated months or years apart. Ultraviolet (UV) spectrophotometry is a standard nucleic acid quantification method, where absorbance of UV light is measured at specified wavelengths. In brief ~12 μL of DNA was transferred in to a cuvette (UVettes, Eppendorf UK) with an optical light path of 10 mm for photometric determination. UV absorbance at 260 nm (A_{260}), is used to measure nucleic acid and absorbance at 280 nm (A280) measures protein contaminants in the sample [203]. An optical density A_{260}/A_{280} ratio of ~1.8 indicates 'pure' DNA [204]. A range of 1.6-2.0 (1.8 ± 0.2) is suggested as acceptable for PCR amplification [205]. Any samples that failed to meet the threshold optical density A_{260}/A_{280} ratio of 1.8 (± 0.2) underwent the DNA isolation process again using the spin column protocol. After quantification of DNA the sample was stored at 4°C until further analysis.

2.5 Genotyping

Genotyping for *ANKK1* (rs1800497), *APOE* (rs429358, rs7412 and rs405509), *BDNF-AS* (rs6265), *COMT* (rs4680), *MAPT* (rs10445337) and *NOS3* (rs2070744) was performed using two protocols in Manchester Protocol one: Approximately 40% of DNA samples were genotyped via real-time PCR using a StepOnePlus (Applied Biosystems, Paisley, UK) as previously described in detail by Heffernan et al. [194] with some slight variations to thermocycling conditions as GTXpress Master Mix (Applied Biosystems) was used for a minority (75 samples). Samples were genotyped by combining 5 μL Genotyping Master Mix, (Applied Biosystems, Paisley, UK) 4.3 μL H_2O, 0.5 μL SNP-specific TaqMan assay (Applied Biosystems), and 0.2 μL of purified DNA (~9 ng), for samples derived from blood, per reaction well. For samples derived from buccal cells 1 μL of DNA, 5 μL of TaqMan genotyping master mix, 3.5 μL of nuclease-free H_2O and 0.5 μL of TaqMan genotyping assay mix was used per reaction well. Negative controls of nuclease-free H_2O were included on each 96-well in place of the DNA sample. PCR was performed using a StepOnePlus real-time detector (Applied Biosystems). In brief, denaturation began at 95°C for 10 min, with 40 cycles of incubation at 92°C for 15 s and then annealing and extension at 60°C for 60 s.

Genotyping analysis was performed with StepOnePlus software version 2.3 (Figure 2.7). Duplicates of all samples were in 100% agreement.

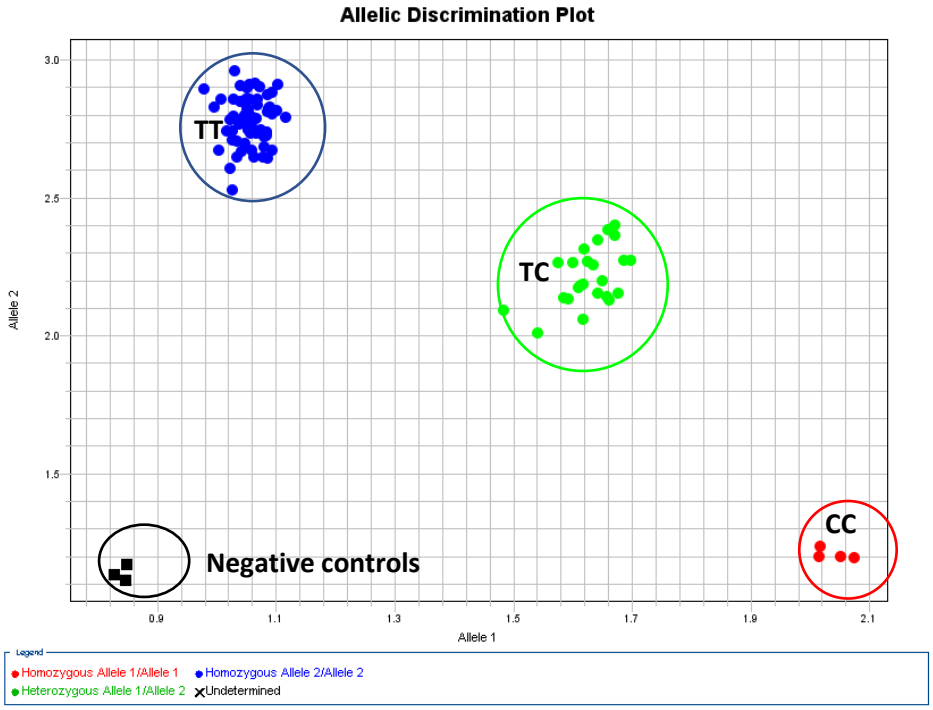


Figure 2.7. Example allelic discrimination plot for *MAPT* rs10445337 obtained using the StepOnePlus Real-Time PCR system.

Protocol two: Approximately 60% of DNA samples were genotyped using the Fluidigm EP1 PCR system (Fluidigm, Cambridge, UK) by combining 2 μL GTXpress Master Mix (2X) (Applied Biosystems), 0.2 μL 20X Fast GT Sample Loading Reagent (Fluidigm), 0.2 μL H_2O and 1.6 μL of purified DNA, for samples derived from blood, saliva and buccal swab. Furthermore, 1.78 μL assay (20X) (Applied Biosystems), 1.78 μL 2X Assay Loading Reagent (Fluidigm) and 0.18 μL ROX reference dye (Invitrogen, Paisley, UK) were combined per assay inlet on the 192X24 microchip plate. Negative controls of nuclease-free H_2O were included on each 96-well in place of the DNA sample. An integrated fluid circuit controller RX (Fluidigm) was used to mix samples and assays using a Load Mix (166x) script. PCR was performed using a real-time FC1 Cycler (Fluidigm) GT 192X24 Fast v1 protocol. In brief, denaturation began at 95°C for 120 s followed by 45 cycles of incubation at 95°C for 2 s and then annealing and extension at 60°C for 20 s. The 192X24 microchip plate was then placed into the EP1 Reader (Fluidigm) for end-point analysis. Genotyping analysis was performed with the Fluidigm SNP genotyping analysis software (Figure 2.8). Duplicates of all samples

were in 100% agreement.

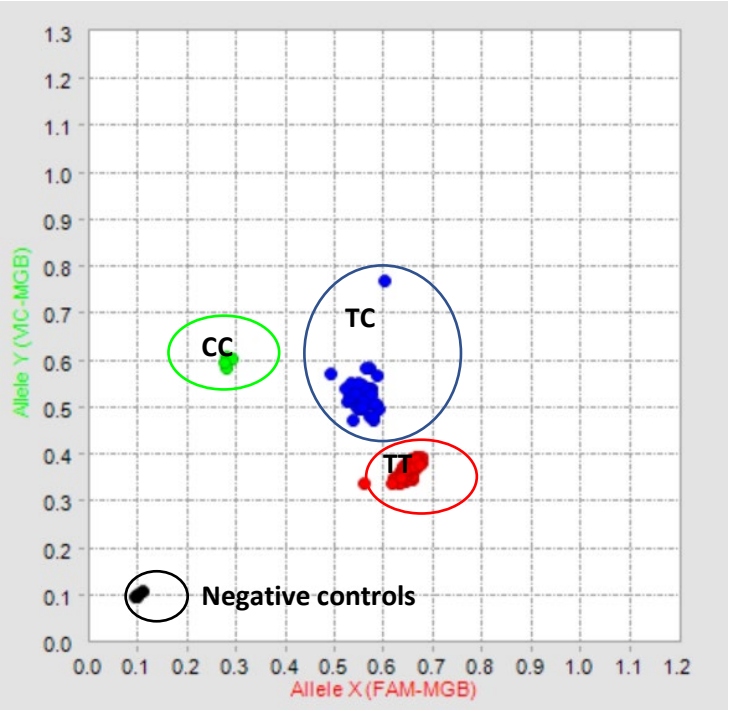


Figure 2.8. Example allelic discrimination plot for *MAPT* rs10445337 obtained using the Fluidigm EP1 PCR system.

2.6 Genotyping assays

Each participant was genotyped for eight polymorphisms: *ANKK1* (rs1800497), *APOE* (rs429358, rs7412 and rs405509), *BDNF-AS* (rs6265), *COMT* (rs4680), *MAPT* (rs10445337) and *NOS3* (rs2070744), where the appropriate TaqMan assays were utilised (Applied Biosystems) (Table 2.1). The *APOE* gene ε2/ε3/ε4 haplotype was derived from rs429358 and rs7412 producing six possible genotypes [ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, and ε4/ε4; [206]]. In brief, the 5'-nuclease genotyping TaqMan assays use fluorogenic probes during real-time polymerase chain reaction (PCR) to discriminate between two alleles of a specific SNP (Figure 2.9). During the amplification stages of PCR, the wild-type allele and alternative allele were amplified separately using biallelic-specific forward and reverse primers and two allele-specific probes. Each probe is labelled with a reporter dye (VIC or FAM, Applied Biosystems) and anneals to a complementary allele-specific sequence binding preferentially (Table 2.1). Throughout the amplification process, Taq polymerase cleaves bound probes, emitting a fluorescent signal detected by PCR instrumentation and software (Figures 2.7 and 2.8). Homozygosity was indicated by an increase in VIC or FAM

fluorescence and an increase in both reporter dyes indicated heterozygosity (Figures 2.7 and 2.8).

Table 2.1. TaqMan assay context sequence for each polymorphism with VIC/FAM, respectively (evidence for identifying the concussion-associated risk alleles is detailed in sections 1.2.5.2-1.2.5.7).

Polymorphism	VIC	FAM	Context sequence (5'-3')
ANKK1 (rs1800497)	**A** allele	**G** allele	TGGTC[**A/G**]AGGCA
APOE (rs429358)	**C** allele	**T** allele	ACGTG[**C/T**]GCGGC
APOE (rs7412)	**C** allele	**T** allele	AGAAG[**C/T**]GCCTG
APOE (rs405509)	**G** allele	**T** allele	GTCTG[**G/T**]ATTAC
BDNF-AS (rs6265)	**C** allele	**T** allele	TATCA[**C/T**]GTGTT
COMT (rs4680)	**A** allele	**G** allele	CTGGC[**A/G**]TGAAG
MAPT (rs10445337)	**C** allele	**T** allele	TCACT[**C/T**]CCCGA
NOS3 (rs2070744)	**C** allele	**T** allele	CTGGC[**C/T**]GGCTGA

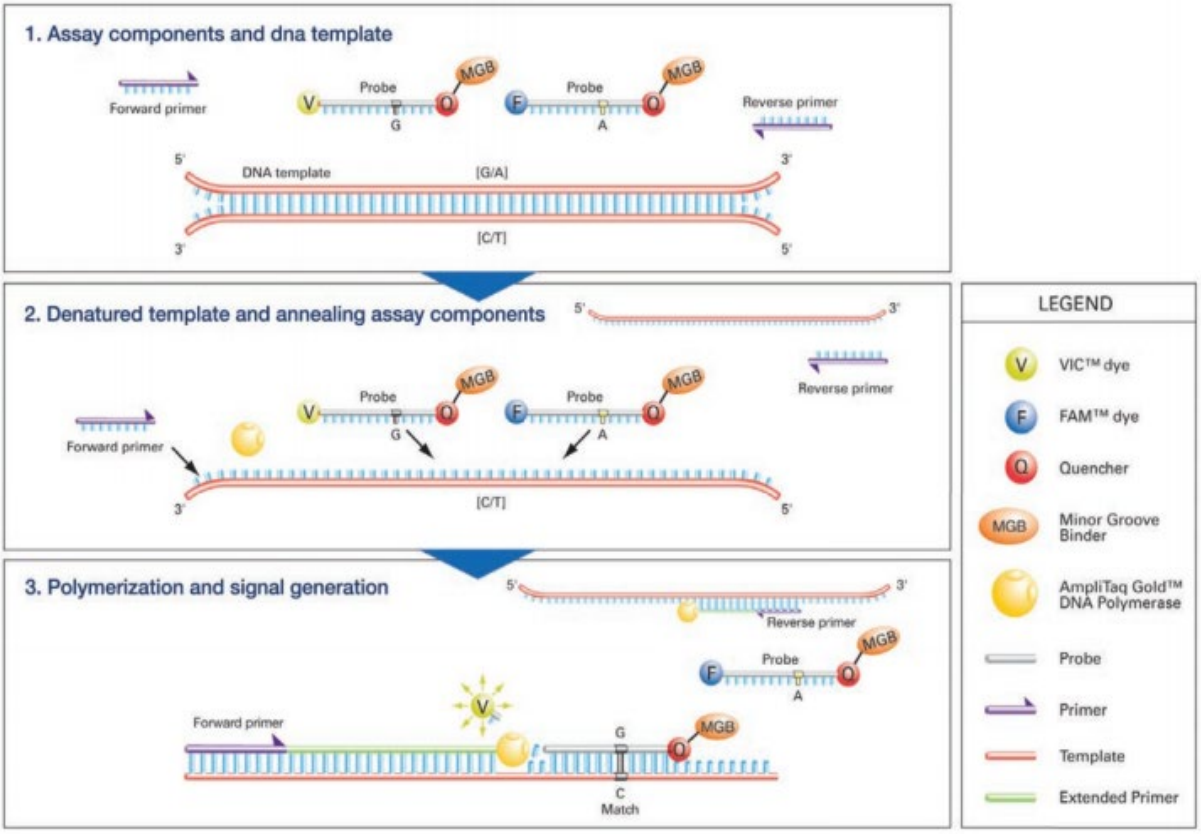


Figure 2.9 The TaqMan SNP genotyping Assay. (1) Reaction mixture containing TaqMan genotyping master mix, forward and reverse primers, TaqMan SNP probes and the target DNA template with the SNP alleles in parentheses. (2) The denatured DNA target and annealing of the assay components to a complementary sequence. (3) DNA polymerase cleaves probes on the target DNA template separating the reporter and quencher dye, generating a fluorescence signal leading to specific allele detection [207].

2.7 Positional groups

To assess genotype within the RU player group, athletes were allocated into subgroups: forwards (props, hookers, locks, flankers and number eights) and backs (scrum halves, fly halves, centres, wings and full backs) [15]. Due to the relatively small sample of RL players, comparisons of genotype frequency between RL subgroups were not carried out.

2.8 Concussion history of elite rugby players

Concussion history in elite rugby athletes was collected using a self-reported concussion history questionnaire (appendix 4). To limit athlete-response bias, coaches and medical staff were not present during completion of the questionnaires [208]. Athletes were asked

to provide details of their: geographic ancestry, playing position, playing history, concussion incidence, activity performed immediately before they were concussed, and duration of recovery. In addition, whether each concussion was medically diagnosed and family history of neurological disorders including dementia, Alzheimer's disease, chronic traumatic encephalopathy (CTE), cognitive impairment, movement disorders, psychiatric disorders and motor neuron disease were assessed. The concussion questionnaire took ~5 min to complete with an investigator present to assist participants to maximise honesty and accuracy. Once completed they were returned to the investigator. Recent changes in identification, management, awareness and education of concussion have been suggested to influence high rates of self-reported concussion within rugby [209]. However, a recent finding suggests professional rugby players underestimate the number of clinically diagnosed concussions by 30% during self-reporting [210]. This suggests the self-reported concussion history questionnaire data could underestimate the number of concussions experienced, although researchers were aware of this possibility and encouraged full disclosure and prompted athletes to recall their experiences over their full playing careers.

To maximise the reliability and validity of the self-report questionnaire, it was developed from previous studies that also assessed self-reported sports-related concussion history [20–22] and used rugby specific terms such as 'tackle' [211]. In addition, the questionnaire was sent to applied practitioners at a concussion clinic in the UK to review its suitability. As a form of validation, athletes disclosed if each concussion they sustained was diagnosed by a medical professional. Seventy-eight percent of athletes with a history of concussion who completed this self-reported questionnaire had their concussions confirmed by a medical professional.

2.9 Calculation of Total Genotype Score

To quantify the combined influence of the candidate polymorphisms (Table 2.1), an additive Total Genotype Score (TGS) algorithm was utilised [212], based on the assumption of codominance effects of the alleles. For bi-allelic polymorphisms, the homozygote genotypes with the 'preferable' concussion risk and outcome were allocated a 'genotype score' of 2, heterozygote genotypes were scored 1 and the other 'non-preferable' homozygote genotypes were scored 0. *APOE* is a tri-allelic (ε2, ε3, ε4) polymorphism giving

rise to six possible genotypes (ε2/ ε2, ε2/ ε3, ε2/ ε4, ε3/ ε3, ε3/ ε4, ε4/ ε4). A score of 0 was allocated for ε4 allele possession (ε4+) and a score of 2 was allocated for non-possession of a ε4 allele (ε4-) (there was no APOE group allocated a score of 1).

TSG algorithm: TGS = (100/14) * (*ANKK1*$_{rs1800497}$ + *APOE*$_{rs429358,\ rs7412}$ + *APOE*$_{rs405509}$ + *BDNF-AS*$_{rs6265}$, *COMT*$_{rs4680}$ + *MAPT*$_{rs10445337}$ + *NOS3*$_{rs2070744}$)

Using the TSG algorithm, a TGS of 100 represents the 'perfect' polygenic profile for concussion risk and outcome and 0 represents the 'worst' possible outcome for concussion risk and outcome for the candidate genes examined in this chapter.

2.10 Statistical analysis

Statistical Package for Social Sciences (SPSS) for Windows version 26 (SPSS, Chicago, IL) software was used for analysis. *A-priori* power analyses were computed using G*power 3.1.9.7 (Franz Faul, Universitat Kiel, Germany) to determine the statistical power to detect genetic associations between groups. Alpha was set at 0.05 and beta of 0.20 (a power of 0.80) [213,214]. Prior to completing any statistical analysis the data was tested for parametricity. Normal distribution of the non-athletes and athletes was identified using Shapiro-Wilks test of normality, skewness and kurtosis.

Hardy-Weinberg equilibrium (HWE) was used for quality control, to identify genotyping errors in unrelated individuals [215,216]. In large populations, allele frequencies and genotypes should distribute in accordance to the HWE principle [217]. Deviation from HWE could indicate errors such as sample mishandling or errors in the genotyping process [218,219], which could produce false conclusions [220].

Pearson's χ^2 tests were utilised to compare genotype and allele frequencies between rugby athletes and non-athletes and between positional subgroups. Two non-parametric χ^2 tests were utilised, the goodness-of-fit and the test of independence. Within this thesis, eight genetic variants have been investigated in relation to elite rugby athlete status. Throughout the experimental chapters (3,4 and 5), multiple comparison χ^2 tests have been applied to a large family of hypotheses, increasing the probability of making a false positives or Type I error beyond a 0.05 threshold with each additional test. For example, twenty-four tests per

SNP (18 tests for *APOE* haplotype) were subjected to Benjamini-Hochberg corrections [BH; 221,222] to control false discovery rate and corrected probability values are reported in the following chapters.

In Chapters 4 and 5, Pearson's χ^2 tests were utilised to compare genotype frequencies between rugby athletes and non-athletes, between RU positional subgroups and concussion history in the upper and lower 25% and 33% of TGS results. Bonferroni adjustment was utilised for each TGS model where appropriate to control for false discovery [223]. Means and kurtosis were calculated to examine the distribution of TGS within groups. Additionally, area under the receiver operating characteristic (ROC) curves (AUC) were used to estimate the sensitivity of TGS to detect differences between non-athletes, RU positional subgroups and concussion history using 95% confidence intervals (95% CI) [224]. Differences in TGS between athletes, non-athletes, RU positional subgroups and athletes with differing concussion history were analysed using unpaired t-tests. One-way Analysis of Variance (Kruskal-Wallis H test), was used to observe differences in age of athletes and number of concussions experienced.

Odds ratios (OR) were calculated to estimate effect size (where applicable) as a measure of strength of an association. Due to the selected sampling process the odds of possessing concussion associated genotypes/alleles are not directly measurable. The OR provides the odds (probability of possession genotype or allele compared with that it is absent) of the genotype or allele possession with athlete status or positional group comparted to non-athletes [225].

Concussion has a polygenic component, which traditional statistical methods are not suitable to handle high-dimensional data of epistasis (SNP-SNP interactions). The combinatorial-based data mining approach of multifactor dimensionality reduction (MDR) was used to detect SNP-SNP interactions using a non-parametric and genetic model-free data mining process [226]. Multifactor dimensionality reduction (https://sourceforge.net/projects/mdr/) software was used [226]. Testing accuracy was used to represent the model most likely to generalise to independent data [227] using 90% of the data set as training data. As with many machine learning methods, MDR was implemented with cross-validation using the remaining 10% of the data set to account for

potential overfitting and to evaluate the predictive ability of the model [228].

Concussion-associated gene variants and elite rugby athlete status

3.1 Introduction

Rugby is a full contact high velocity collision-based team sport comprised of two differing codes, RL and RU). Both are characterised by multiple high-intensity collisions (RL 24-47, RU 24-89 contact events per match) [70,229]. Contact events are responsible for the prevalence of concussion in both codes of rugby [40,66,77]. Sport-related concussion has been defined as a form of TBI induced by biomechanical forces [26]. In the 2017-18 season of the English RU Premiership (the top tier of competition in England) there was a reported incidence of ~18 concussions per 1000 match hours (~1 concussion per match) [66]. In elite RL, concussion incidence ranges from ~15-28 concussions per 1000 player match hours [39,40].

Potential short- and long-term neurodegenerative consequences associated with concussion include increased injury risk [23], cognitive impairment [24], forms of dementia [25], chronic post-concussion syndrome, migraines, sleep dysfunction, anxiety [26], post-traumatic stress disorder [27] and second impact syndrome [28]. These consequences impact on continuance of an athletic career, causing temporary suspension of play, early retirement and potential neuropathological consequences.

Concussion has a polygenic component due to the actions and interactions of multiple genes [230]. Two common C/T single nucleotide polymorphisms (SNPs) at residues 112 (rs429358) and 158 (rs7412) of the *apolipoprotein E* (*APOE*) gene have been associated with forms of TBI [142,231] and in combination are termed ε2/ε3/ε4. *APOE* ε4 allele could be responsible for up to 64% of the 'hazardous influence' of TBI [142] and athletes who possess the ε4 allele suffered from prolonged physical (Cohen's d = 0.87) and cognitive (d = 0.60) symptomatic responses to concussion [43]. A promoter region SNP of *APOE* (rs405509) has been associated with quantitative impacts on APOE levels in brain tissue [143]. Carriers of the T allele (rs405509) had a 3-8-fold greater risk of experiencing repeated concussions [20,21] and TT genotype carriers experienced lower Glasgow Outcome Scale scores post-TBI [144]. In contrast, Abrahams et al. [47] reported TT genotype (rs405509) was associated with a 45% reduced risk of concussion and the T allele was associated with a rapid recovery (< 1 week) post-concussion in RU players. *Microtubule associated protein tau* (*MAPT*) TT genotype (rs10445337) has been weakly associated with a greater risk of

repeated concussion [21,22]. Mutations in *MAPT* accelerate aggregation of markers of neurotoxic hyperphosphorylated tau in response to repetitive concussions by 20-60% in animal-based studies and are associated with neurodegenerative diseases in humans [152,232]. The *nitric oxide synthase 3* (*NOS3)* -786T/C polymorphism (rs2070744) has been associated with promoter region activity, reduced NO synthesis and cerebral vasospasm [190]. Approximately 20-35% lower cerebral blood flow has been reported in patients with severe TBI who carry the C allele [119].

The T allele (rs1800497) of the *ankyrin repeat and kinase domain-containing 1* (*ANKK1*) gene has been associated with a 30-40% reduction in the expression of D2 receptors within the ventral striatum [181,182]. Post-TBI T allele carriers perform worse in measures of learning, working memory and response latencies [183–185]. A polymorphism (rs6265) of the *brain derived neurotrophic factor (BDNF)* gene has been associated with neurocognitive performance post-concussion; G allele carriers performed approximately 2-6 times better in memory, executive function, attention and overall cognitive performance, both acutely and 6 months post-concussion compared to A allele carriers [168]. In addition, AA homozygotes appear to be at higher risk of sustaining a concussion than GG homozygotes (~17% of AA homozygotes suffered a concussion compared to ~4% of AG/GG) [167]. The G (Val) to A (Met) missense variation at codon 158 (rs4680) in the *catechol-O-methyltransferase* (*COMT*) gene appears to have multiple, pleiotropic effects. It is associated with behavioural traits and executive function [171], with Lipsky et al. [138] reporting that Val homozygotes performed 40% poorer on tests of executive function than Met homozygotes post-TBI. Significantly, Met-carrying RU players are reportedly ~3-fold more likely to have a history of concussion [30]. However, in addition, the *COMT* warrior/worrier theory describes the Val allele as advantageous for stress resilience (warrior) and the Met allele advantageous for cognitive function (worrier) [233,234]. Indeed, mixed martial arts professional fighter status is associated with Val/Val genotype [235], potentially due to better performance in threatening environments [236]. Thus, *COMT* could also influence rugby player behaviours, including those that affect risk of concussion.

Given the biological and clinical associations with the polymorphisms introduced here, possession of the risk alleles might limit an individual's ability to withstand exposure to the

environment of competitive rugby due to an elevated risk of repeated concussions and greater risk of delayed recovery and consequent neurological impairment. Such individuals would be more likely than their peers to miss training, selection and competitive events important for career progression [14]. Indeed, Heffernan et al. [54] previously reported an association between injury risk-associated *COL5A1* (rs12722 and rs3196378) polymorphisms and elite rugby status based on the same premise regarding career progression.

The primary aim of this chapter, therefore, was to investigate whether genotype frequency of suspected concussion-associated polymorphisms differed between elite rugby athletes and a non-athlete control population, and between RU playing positions. Based on published associations of the polymorphisms with concussion risk and poorer outcome following brain injury, and the interruption to competitive careers that could result, it was hypothesized that the concussion-associated risk genotypes and alleles would be underrepresented in elite rugby athletes compared to non-athletes. In other words, it was hypothesised that rugby athletes would have greater genetic resistance to concussion than non-athletes, because that would have facilitated their prolonged participation in a high-risk environment.

3.2 Methods

Participants

A total of 1683 individuals were recruited and gave written informed consent to participate in the present chapter. An *a priori* calculation for 80% power to detect a small effect size (w) of 0.1 required >785 participants and 0.12 required >546 participants. The total sample comprised 668 Caucasian elite male rugby athletes (mean (standard deviation) height 1.85 (0.07) m, mass 102 (12) kg, age 29 (7) yr) including 62.9% British, 13.8% South African, 10.8% Irish, 8.9% Italian, and 3.6% of other nationalities, and 1015 Caucasian non-athletes (48% male, height 1.71 (0.11) m, mass 73 (13) kg, age 38 (22) yr) including 91.8% British, 6.7% South African, 1.5% other nationalities. 49.1% of the RU athletes had competed at international level for a "high performance union" (Regulation 16, http://www.worldrugby.org) and 42% of RL athletes had competed internationally. As the majority of athletes competed in RU, they were also divided into forwards and backs for

comparison.

Procedures

Sample collection. Procedures were consistent with those described previously in Chapter 2.

DNA isolation and genotyping. DNA isolation and genotyping have previously been described in detail in Chapter 2.

Genotyping assays

For *ANKK1* (rs1800497), *APOE* (rs429358, rs7412 and rs405509), *BDNF-AS* (rs6265), *COMT* (rs4680), *MAPT* (rs10445337) and *NOS3* (rs2070744), the appropriate TaqMan assays were utilised (Applied Biosystems) (Chapter 2, Table 2.1). *APOE* ε2/ε3/ε4 data were derived from rs429358 and rs7412 [206] (Chapter 2, Table 2.1).

Data Analysis

SPSS for Windows version 26 (SPSS, Chicago, IL) software was used. Height and body mass were compared between athletes and non-athletes using independent t-tests. Pearson's χ^2 tests compared genotype and allele frequencies between athletes and non-athletes and between positional subgroups. Twenty-four comparisons per SNP (18 for *APOE* ε2/ε3/ε4) were subjected to Benjamini-Hochberg corrections [BH;221] to control false discovery rate and corrected probability values are reported. Odds ratios (OR) were calculated to estimate effect size. Alpha was set at 0.05.

3.3 Results

Genotype frequencies were in Hardy-Weinberg equilibrium for all polymorphisms in the non-athlete and athlete groups. Athletes (all male) were taller and heavier ($P < 0.05$) than the male non-athletes.

For *COMT* rs4680, the AA (Met/Met) genotype, proportion of A allele carriers and A allele were underrepresented in all athletes (24.8%, 74.6% and 49.7%, respectively) and RU athletes (24.3%, 73.1%, and 48.7%) compared with non-athletes (30.2%, 77.6%, and 54.0%, Table 3.1 and Fig. 3.1, $P \leq 0.05$). The GG (Val/Val) genotype was more common in all rugby

athletes than non-athletes (OR = 1.39, 95% confidence interval (CI) = 1.04-1.86), and more common in RU athletes than non-athletes (OR = 1.49, 95% CI = 1.10-2.03). The AA genotype was underrepresented in the subgroup of RU backs compared with the non-athletes (21.1% versus 30.3%, Table 3.1, $P \leq 0.05$), with the GG genotype more common in RU backs (OR = 1.62, 95% CI = 1.07-2.48). However, there was no difference in genotype frequency between RU backs and forwards ($P = 0.49$). In addition, there was no difference in genotype frequency between RL athletes and non-athletes ($P = 0.32$).

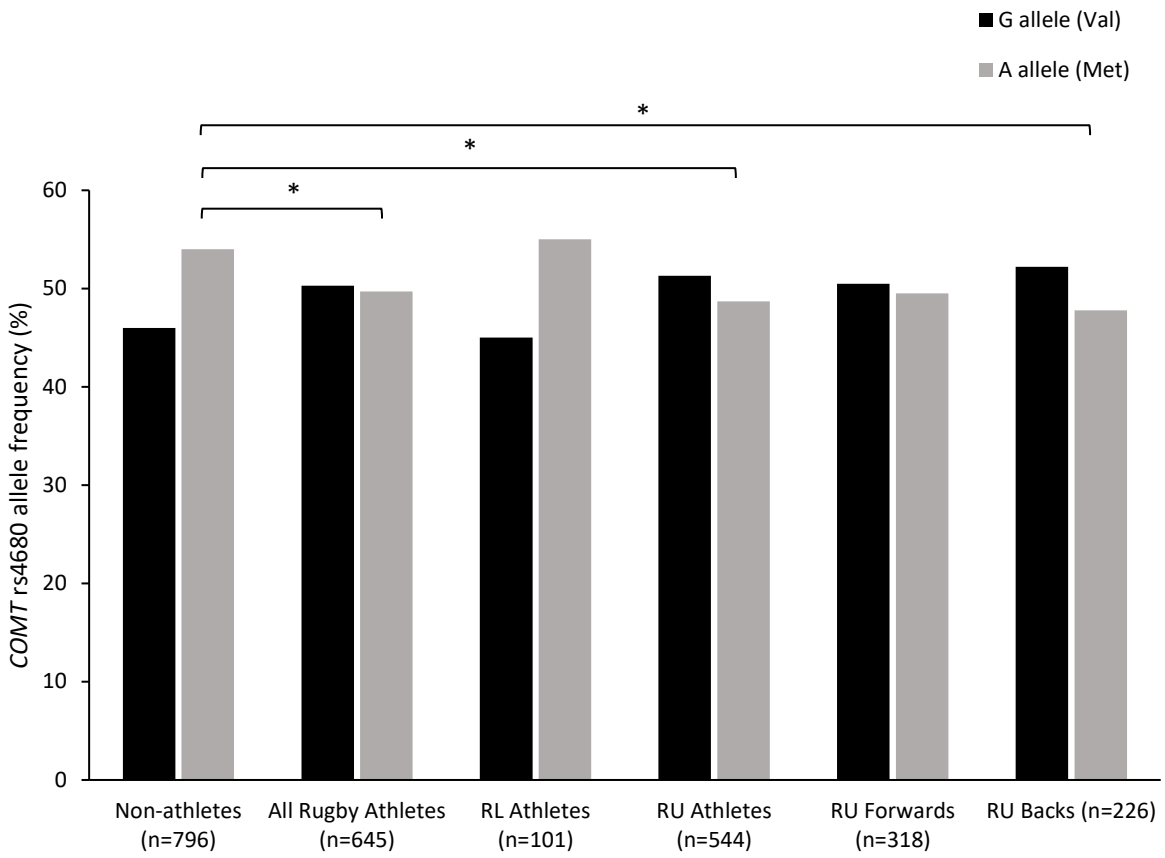


Figure 3.1. Allele frequency of *COMT* rs4680 for non-athletes and athlete groups. G allele = black, A allele = grey. Asterisks (*) indicate G (Val) allele more common and A (Met) allele less common in athletes than non-athletes ($P \leq 0.05$). RU, rugby union; RL, rugby league.

There were no differences in *APOE* ε2/ε3/ε4 genotype or ε4 allele possession frequency when comparing all ($P = 0.19$, $P = 0.71$), RU ($P = 0.28$, $P = 0.71$), RU forwards ($P = 0.62$, $P = 0.85$) and RU backs ($P = 0.62$, $P = 0.65$) with non-athletes (Table 3.1). Furthermore, no *APOE*

ε2/ε3/ε4 genotype frequency or ε4 allele possession differences were observed between RU backs and forwards (P = 0.87, P = 0.83, respectively). There were no differences in *APOE* rs405509 genotype or allele frequency when comparing all groups of athletes to non-athletes (Table 3.1). In addition, no *APOE* rs405509 differences in genotype or allele frequency were observed between RU backs and forwards.

Similarly, there were no differences in genotype or allele frequency when comparing all athletes with non-athletes for all other polymorphisms (*ANKK1* rs1800497, *BDNF-AS* rs6265, *MAPT* rs10445337 and *NOS3* rs2070744) (P > 0.05; Table 3.1). Furthermore, no genotype frequency or allele differences were observed between RU backs and forwards for all other polymorphisms analysed in this chapter (Table 3.1).

Table 3.1. Genotype and allele distribution of non-athletes and athletes, including athletes separated by code (RL and RU) and into positional groups for RU. Data are genotype/allele count followed by percentage in parentheses.

Polymorphism	Genotype/ Hardy-Weinberg Equilibrium (HWE)	All Rugby Athletes	RL Athletes	RU Athletes	RU Forwards	RU Backs	Non-athletes
***ANKK1* rs1800497**	GG	417 (65.2)	59 (58.4)	358 (66.5)	208 (66.1)	150 (66.1)	475 (65.2)
	GA	198 (31.0)	37 (36.6)	161 (29.9)	99 (31.4)	64 (28.2)	223 (30.6)
	AA	24 (3.8)	5 (5.0)	19 (3.5)	8 (2.5)	13 (5.7)	31 (4.2)
	Total	639	101	538	315	227	729
	G allele	1032 (80.8)	155 (76.7)	877 (81.5)	515 (81.7)	364 (80.2)	1173 (80.5)
	A allele	258 (20.2)	47 (23.3)	199 (18.5)	115 (18.3)	90 (19.8)	285 (19.5)
	G allele carriers	615 (96.2)	96 (95.1)	519 (96.5)	307 (97.5)	214 (94.3)	698 (95.7)
	A allele carriers	222 (34.7)	42 (41.6)	180 (33.5)	107 (34.0)	77 (33.9)	254 (34.8)
***APOE* rs405509**	GG	163 (25.8)	23 (23.0)	140 (26.3)	75 (24.4)	66 (28.8)	191 (26.2)
	GT	308 (48.7)	51 (51.0)	257 (48.3)	154 (50.2)	105 (45.9)	344 (47.3)
	TT	161 (25.5)	26 (26.0)	135 (25.4)	78 (25.4)	58 (25.3)	193 (26.5)
	Total	632	100	532	307	229	728
	G allele	634 (50.2)	97 (48.5)	537 (50.5)	304 (49.5)	237 (51.7)	726 (49.9)
	T allele	630 (49.8)	103 (51.5)	527 (49.5)	310 (50.5)	221 (48.3)	730 (50.1)
	G allele carriers	471 (74.5)	74 (74.0)	397 (74.6)	229 (74.6)	171 (74.7)	535 (73.5)
	T allele carriers	469 (74.2)	77 (77.0)	392 (73.7)	232 (75.6)	163 (71.2)	537 (73.8)
***APOE* ε2/ε3/ε4**							
	ε2/ε2	1 (0.2)	0 (0.0)	1 (0.2)	1 (0.3)	0 (0.0)	4 (0.6)
	ε2/ε3	74 (11.2)	14 (13.7)	60 (10.8)	32 (10.0)	28 (11.8)	88 (12.7)
	ε2/ε4	11 (1.7)	1 (1.0)	10 (1.8)	7 (2.2)	3 (1.3)	19 (2.7)
	ε3/ε3	393 (59.7)	51 (50)	342 (61.5)	199 (62.0)	146 (61.3)	404 (58.5)
	ε3/ε4	159 (24.2)	32 (31.4)	127 (22.8)	73 (22.7)	54 (22.7)	159 (23.0)
	ε4/ε4	20 (3.0)	4 (3.9)	16 (2.9)	9 (2.8)	7 (2.9)	17 (2.5)
	Total	658	102	556	321	238	691
	ε4 allele carriers	190 (28.9)	37 (36.3)	153 (27.5)	89 (27.7)	64 (26.9)	195 (28.2)
	Non- ε4 allele carriers	468 (71.1)	65 (63.7)	403 (72.5)	232 (72.3)	174 (73.1)	496 (71.8)

Table 3.1. Continued

Polymorphism	Genotype/ Hardy-Weinberg Equilibrium (HWE)	All Rugby Athletes	RL Athletes	RU Athletes	RU Forwards	RU Backs	Non-athletes
***BDNF-AS* rs6265**							
	GG	432 (67.5)	74 (73.3)	358 (66.4)	206 (65.4)	154 (67.6)	530 (66.3)
	GA	185 (28.9)	23 (22.7)	162 (30.1)	98 (31.1)	66 (28.9)	241 (30.1)
	AA	23 (3.6)	4 (4.0)	19 (3.5)	11 (3.5)	8 (3.5)	29 (3.6)
	Total	640	101	539	315	228	800
	G allele	1049 (82.0)	171 (84.7)	878 (81.4)	510 (81.0)	374 (82.0)	1301 (81.3)
	A allele	231 (18.0)	31 (15.3)	200 (18.6)	120 (19.0)	82 (18.0)	299 (18.7)
	G allele carriers	617 (96.4)	97 (96.0)	520 (96.5)	304 (96.5)	220 (96.5)	771 (96.4)
	A allele carriers	208 (32.5)	27 (26.7)	181 (33.6)	109 (34.6)	74 (32.5)	270 (33.8)
***COMT* rs4680**							
	GG	164 (25.4)	18 (17.8)	146 (26.8)	86 (27.0)	60 (26.5)	178 (22.4)
	GA	321 (49.8)	55 (54.5)	266 (48.9)	149 (46.9)	116 (51.3)	377 (47.4)
	AA	160 (24.8)*	28 (27.7)	132 (24.3)*	83 (26.1)	50 (22.1)*	241 (30.2)
	Total	645	101	544	318	226	796
	G allele	649 (50.3)	91 (45.0)	558 (51.3)	321 (50.5)	236 (52.2)	733 (46.0)
	A allele	641 (49.7)*	111 (55.0)	530 (48.7)*	315 (49.5)	216 (47.8)*	859 (54.0)
	G allele carriers	485 (75.2)	73 (72.3)	412 (75.7)	235 (73.9)	176 (77.9)	555 (69.7)
	A allele carriers	478 (74.6)	83 (82.2)	398 (73.1)	232 (73.0)	166 (73.5)	618 (77.6)
***MAPT* rs10445337**							
	TT	384 (59.6)	54 (53.5)	330 (60.8)	201 (63.8)	133 (57.4)	465 (63.9)
	TC	230 (35.7)	40 (39.6)	190 (35.0)	102 (32.4)	88 (37.9)	229 (31.5)
	CC	30 (4.7)	7 (6.9)	23 (4.2)	12 (3.8)	11 (4.7)	34 (4.7)
	Total	644	101	543	315	232	728
	T allele	998 (77.5)	148 (73.3)	850 (78.3)	504 (80.0)	354 (76.3)	1159 (79.6)
	C allele	290 (22.5)	54 (26.7)	236 (21.7)	126 (20.0)	110 (23.7)	297 (20.4)
	T allele carriers	614 (95.3)	94 (93.1)	520 (95.8)	303 (96.2)	221 (95.3)	694 (95.3)
	C allele carriers	260 (40.4)	47 (46.5)	213 (39.2)	114 (36.2)	99 (42.7)	263 (36.1)

Table 3.1. Continued

Polymorphism	Genotype/ Hardy-Weinberg Equilibrium (HWE)	All Rugby Athletes	RL Athletes	RU Athletes	RU Forwards	RU Backs	Non-athletes
NOS3 **rs2070744**							
	TT	239 (37.6)	36 (35.6)	203 (37.9)	115 (37.0)	91 (39.9)	282 (38.7)
	CT	303 (47.6)	50 (49.5)	251 (46.9)	145 (46.6)	106 (46.5)	323 (44.3)
	CC	94 (14.8)	15 (14.9)	81 (15.2)	51 (16.4)	31 (13.6)	124 (17.0)
	Total	636	101	535	311	228	729
	T allele	781 (61.4)	122 (60.4)	657 (61.4)	375 (60.3)	288 (63.2)	887 (60.8)
	C allele	491 (38.6)	80 (39.6)	413 (38.6)	247 (39.7)	168 (36.8)	571 (39.2)
	T allele carriers	542 (85.2)	86 (85.1)	454 (84.9)	260 (83.6)	197 (86.4)	605 (83.0)
	C allele carriers	397 (62.4)	65 (64.4)	332 (62.1)	196 (63.0)	137 (60.1)	447 (61.3)

The genotype and allele carrier data represent the additive, dominant and recessive models, respectively. Asterisks (*) indicate lower frequency than non-athletes ($P \leq 0.05$).

3.4 Discussion

The aim of this chapter was to investigate whether genotype frequency of eight suspected concussion-associated polymorphisms differed between elite rugby athletes and a non-athlete control population, and between RU playing positions. It was hypothesised that the concussion-associated risk genotypes and alleles would be underrepresented in elite rugby athletes compared to non-athletes, because of the interruption to competitive careers that could result. The main finding was that *COMT* rs4680 genotype was associated with elite rugby athlete status. However, the elite rugby athletes had ~1.4 times the odds of being Val/Val (GG) genotype (previously associated with poorer cognitive function post-concussion) than non-athletes, contradicting this chapter's original hypothesis. Nevertheless, *COMT* rs4680 has pleiotropic effects as the two alleles have varying

associations with history of concussion and behavioural traits [42,235,236] some compatible with observations from this chapter.

Previously, *COMT* Val/Val (GG) homozygotes have been associated with poorer cognitive function post-TBI than Met/Met (AA) homozygotes [170,237], which led postulation that possessing the Met allele would contribute to the attainment of elite status via quicker or more complete recovery following TBI after the inevitable high-intensity contacts that occur during rugby. Thus, better cognitive function post-concussion would facilitate rugby athletes' prolonged participation in the high concussion-risk environment of competitive rugby. However, the Val/Val genotype was overrepresented in elite rugby athletes (25.4%), and RU athletes separately (26.8%), compared to non-athletes (22.4%) (Figure 1). *COMT* encodes an enzyme that methylates and in turn deactivates catechol-based neurotransmitters such as synaptic dopamine [170]. Optimal cognitive function is affected by the prefrontal cortex's (PFC) sensitivity to dopamine [238], which makes *COMT* a strong candidate to influence inter-individual variability in cognitive function post-concussion. Chen *et al*. [172] noted Met/Met carriers had ~33% decreased COMT activity (higher dopamine activity) compared to Val/Val carriers (lower dopamine activity); heterozygotes had intermediate activity.

Furthermore, Mc Fie at al. [42] recently reported Met carriers in a cohort of youth and professional South African RU players were ~3-fold more likely to have a history of concussion. Elevated dopamine could increase impulsivity and risk taking, meaning Met allele carriers could place themselves at increased risk of sustaining a concussion [129,130]. We found the Met allele was underrepresented in elite rugby athletes (49.7%), RU athletes (48.7%), RU forwards (49.5%) and RU backs (47.8%) compared to non-athletes (54.0%).

These findings are therefore compatible with Mc Fie et al. [42], because lower risk of concussion via the Val allele would provide less disruption to rugby training and selection, increasing the chance of long-term career success. However, further replication studies are warranted to support this hypothesis. Interestingly, *COMT* genotype frequency did not differ between RL athletes and non-athletes. This different result from RU could be attributed to differing genetic predisposition and requirements between the differing codes, or more likely due to the smaller cohort of RL athletes analysed (n = 101). Future larger scale RL replication studies are needed to confirm this.

Professional fighters have been reported to have a higher frequency of Val/Val genotype (52%) than non-athletes (20%) [235]. The higher COMT activity in the PFC of Val/Val carrying professional fighters (compared to the MET carriers) is in line with the COMT warrior/worrier U-shaped curve theory of excessive or insufficient dopamine in the PFC impairing cognitive performance [233,234]. Previous findings indicate that Val/Val carriers performing under stressful conditions would have an increased resilience to stress and be more able to cope with perceived threats [233,235]. Met allele carriers have an increased reactivity to aversive stimuli and relative greater cognitive performance capacity than Val carriers [233,234,239,240]. This greater executive function and working memory of Met allele carriers is attributed to the effects of higher levels of extracellular dopamine in the PFC [233,234]. In addition, the Met allele has been associated with experiencing anxiety and pain sensitivity [241,242], characteristics unfavourable for elite rugby competition.

For *APOE* ε2/ε3/ε4 there were no differences in ε4/ε4 genotype or ε4 allele frequency between elite rugby athletes and non-athletes (Table 1). Previous findings indicate that ε4 allele carriers experience more severe cognitive and physical symptoms following TBI

[43,142,231], so it was hypothesised that the ε4 allele would be underrepresented in elite rugby athletes compared to non-athletes. However, the present data do not support that hypothesis, despite elite rugby being an environment of high risk of concussion [39,40,66,77,84,86]. Nevertheless, it is noteworthy that 28.9% of elite rugby athletes were ε4 carriers, including several of ε4/ε4 genotype (3.0% of all athletes), who may be at elevated risk of cognitive and physical impairments post-concussion compared to non-carriers [43,142,231].

Similarly, this chapter found no association between any other polymorphism examined in this chapter and elite rugby athlete status. However, based on previous biological and clinical data regarding those polymorphisms, this chapter confirms the existence of a considerable number of athletes who appear to have a genetic predisposition for sustaining repetitive concussions and/or poorer outcomes post-concussion. For example, ~20% of elite rugby athletes could be at risk of poorer cognitive performance post-concussion due to possession of either the A allele of *ANNK1* rs1800497 or the A allele of *BDNF* rs6265 [168,183–185]. In addition, 60% of elite rugby athletes possess the *MAPT* rs10445337 TT genotype which could suggest a greater risk of repeated concussion [21,22] and potential risk of neurodegenerative disease [152,232]. Similarly, 60% of elite rugby athletes could experience reduced cerebral blood flow post-concussion due to possession of the *NOS3* rs20707044 C allele [119,190].

The limitations of this chapter include the analysis of populations that have a potential of population stratification via genetic drift. Subjects were all Caucasian, but geographic ancestry varied slightly. For example, ~14% of rugby athletes were South African while only ~1% of non-athletes were from countries other than the UK. Also, self-identification of

geographic ancestry is an imperfect means to identify a homogenous group, but does provide some data regarding geographic ancestry, especially if four generations of family ancestry are recorded, as done in this thesis.

3.5 Conclusion

A considerable number of elite rugby athletes possess several concussion-associated risk alleles that should be explored further in conjunction with concussion injury data. In addition, the Val allele and Val/Val genotype of the *COMT* rs4680 polymorphism were more common in elite rugby athletes than non-athletes, suggesting an advantage for attaining elite competitive status. Based on this observation and prior literature, it is proposed that elite rugby athletes possessing the Val allele of *COMT* (rs4680) could be at lower risk of experiencing concussions, potentially due to greater stress resilience and reduced anxiety in threatening competitive environments. However, they might also be at increased risk of poorer cognitive function post-concussion. Consequently, it is recommend continued careful monitoring of brain injury in rugby, tight adherence to return-to-play procedures, and the development of more sensitive methods for early detection of neurodegeneration, particularly in those athletes potentially at higher risk.

Due to the likely polygenic nature of concussion, combining multiple genetic variants suspected to be associated with concussion, such as those presented in this chapter, could provide a concussion-associated polygenic profile of elite rugby athletes. Concussion-associated polygenic profiles could help explain the inter-individual variability in injury occurrence and outcomes following concussion.

Concussion-associated polygenic profiles of elite male rugby athletes.

4.1 Introduction

It has been reported that over a playing career ~80% of rugby (league and union) players will experience at least one concussion [243]. In male elite RU, concussion has been the most common injury in the English Premiership since 2011 (accounting for 21% of all injuries from 2014-2019 seasons) [244]. In elite male RL, concussion accounted for 29% of all injuries in illegal play and 9% of all injuries in legal play [40]. Sustaining a prior concussion increases the risk of subsequent time-loss injuries and repeated concussions [20–23]. There is growing concern about the potential short and long-term neurodegenerative consequences associated with concussion, such as chronic post-concussion syndrome, cognitive impairment, forms of dementia, migraines, sleep dysfunction and anxiety [24,26,245–248].

Inter-individual variability means that severity of concussion in rugby can range from 2 days to >84 days absence (period from injury to availability for match selection), but typically ranges from ranges 9-21 days [23,66,79,84,246]. To better understand and manage the inter-individual variability in injury occurrence and outcomes following concussion, the main risk factors must be identified. One such factor is genetic predisposition, as the interaction of multiple genes in a polygenic manner could reflect the complex pathophysiology of incidence and recovery from concussion [230]. Heritability of concussion has not been determined, but it is likely that a substantial genetic component exists for concussion risk and recovery, as heritability of brain structure is shown to be ~90% and cognitive performance ~60% [48–51]. Previous candidate gene studies have identified potential genetic risk factors associated with risk of concussion and recovery [41]. Those genetic variants influencing concussion risk and recovery may confer an advantage/disadvantage for rugby athletes by affecting the ability to train and compete and thus advance their careers, and investigating this could provide additional information to support the management of the cumulative effects of concussions [41].

The ε4 allele of a*polipoprotein* (*APOE*) gene could be responsible for up to 64% of the 'hazardous influence' of TBI [142] and athletes who possess the ε4 allele suffered prolonged physical and cognitive symptomatic responses to concussion [43]. Carriers of the *APOE* promoter T allele have a 3-8-fold greater risk of experiencing repeated concussions [20,21] and TT genotype carriers were observed to experience unfavourable outcomes

post-TBI [144]. From the *microtubule associated protein tau* (*MAPT*) gene the TT genotype has been weakly associated with a greater risk of repeated concussion [21,22]. The *Nitric oxide synthase* (*NOS3)* gene C allele has been associated with lower cerebral blood flow in patients with severe TBI [119]. The T allele of *ankyrin repeat and kinase domain-containing 1* (*ANKK1*) gene has been associated with worse measures of learning, working memory and response latencies post-TBI [183–185]. In addition, *brain derived neurotrophic factor (BDNF)* gene Met/Met homozygotes have been reported to be at a higher risk of sustaining a concussion than Val/Val homozygotes [167]. *Catechol-O-methyltransferase* (*COMT*) gene rs4680 Val allele carriers performed poorer on tests of executive function post-TBI [170] and Met carrying RU players have been reported ~3-fold more likely to have a history of concussion [30]. Indeed, elite rugby athletes have ~1.4 times the odds of being Val/Val (GG) genotype compared to non-athletes (described in Chapter 3), while other SNP variants related to different injury types are also more frequent in elite rugby athletes [54]. However, for all other suspected concussion-associated variants studied in Chapter 3 the risk-associated genotypes were equally prevalent in rugby athletes and non-athletes.

The Total Genotype Score (TGS) has been used to indicate the extent of an individual's genetic predisposition for athletic performance, muscle damage and disease risk [212,249–254]. Based on a genetic algorithm proposed by Williams and Folland [212], TGS can range from 0-100 and represents the number of 'preferable' genotypes an individual possesses for the phenotype in question. Previous hypothetical and experimental TGS studies indicate that athletes have higher TGS scores, thus possessing more 'preferable' polygenic profiles than non-athletes for performance, injury and potential disease [212,249–257]. The potential applications of this approach to concussion are attractive, but have not yet been explored.

Therefore, the primary objective of this chapter was to investigate if concussion-associated polygenic profiles differ between elite rugby athletes and non-athletes. Based on prior literature, it was hypothesised that the elite rugby athletes would have a higher TGS than non-athletes indicating a more 'preferable' polygenic profile with respect to concussion, and/or display gene-gene interactions that differ from non-athletes.

4.2 Methods

The procedures used in Chapter 4 have already been described in detail in Chapters 2 and 3, thus, only a brief description of these methods is provided below.

Participants

A total of 1357 individuals were recruited and gave written informed consent to participate in the present chapter. An *a priori* calculation for 80% power to detect a small effect size (w) of 0.1 indicated >785 participants were required. The total sample comprised elite 635 Caucasian male rugby athletes (mean (standard deviation) height 1.85 (0.07) m, mass 102 (12) kg, age 29 (7) yr) including 66.4% British, 11.4% Irish, 9.5% Italian, 8.9% South African, and 3.8% of other nationalities, and 722 Caucasian non-athletes (48% male, mean (standard deviation) height 1.70 (0.10) m, mass 73 (13) kg, age 41 (23) yr) including 97.6% British and 2.4% other nationalities. 53.5% of the RU athletes had competed at international level for a "high performance union" (Regulation 16, http://www.worldrugby.org) and 45.8% of RL athletes had competed at international level. As the majority of athletes (534) competed in RU, they were also divided into forwards (304) and backs (230) for comparison (to detect an effect size (w) of 0.13 required >464 participants).

Procedures

Sample collection. Procedures were consistent with those described previously in Chapter 2.

DNA isolation and genotyping. DNA isolation and genotyping have previously been described in detail in Chapter 2.

Calculation of TGS

To quantify the combined influence of the candidate polymorphisms, an additive TGS algorithm was utilised [212] based on the assumption of codominant allele effects. For bi-allelic polymorphisms, the homozygote genotypes with the 'preferable' concussion risk and outcome according to prior literature were allocated a genotype score of 2, heterozygote genotypes scored 1 and the other 'non-preferable' homozygote genotypes scored 0 (Table

4.1). *APOE* is a tri-allelic (ε2, ε3, ε4) polymorphism - two C/T SNPs at residues 112 (rs429358) and 158 (rs7412) produce six possible genotypes (ε2/ ε2, ε2/ ε3, ε2/ ε4, ε3/ ε3, ε3/ ε4, ε4/ ε4). A score of 0 was allocated for ε4 allele possession (ε4+) and a score of 2 was allocated for non-possession of a ε4 allele (ε4-) (no score of 1 allocated). A TGS of 100 represents the 'perfect' polygenic profile for low concussion risk and favourable outcome, for the SNPs examined, while 0 represents the 'worst' possible profile for concussion risk and outcome.

Model 1: TGS = (100/14) * (*ANKK1*$_{rs1800497}$ + *APOE*$_{rs429358,\ rs7412}$ + *APOE*$_{rs405509}$ + *BDNF-AS*$_{rs6265}$, *COMT*$_{rs4680}$ + *MAPT*$_{rs10445337}$ + *NOS3*$_{rs2070744}$)

In addition, Model 2 TGS algorithm determined only by observed genotype frequencies in elite rugby athletes (Chapter 3) was also calculated, wherein three of the eight genotype scores differed (*APOE* rs405509 TT = 2, GT = 1, GG = 0, *COMT* rs4680 GG = 2, GA = 1, AA = 0 and *NOS3* rs2070744 TT = 2, TC = 1, CC = 0).

Table 4.1. Genotype score of each polymorphism and genotype frequencies in elite rugby athletes and in non-athletes [from Chapter 3].

Gene name	Gene abbreviation	Polymorphism	Alleles	Genotype score	Frequency in elite rugby athletes (%)	Frequency in non-athletes (%)
Ankyrin repeat and kinase domain containing 1	*ANKK1*	rs1800497	A/G	GG = 2, GA = 1, AA = 0	65.2, 31.0, 3.8	65.2, 30.6, 4.2
Apolipoprotein E	*APOE*	rs429358 and rs7412	ε4+/ ε4-	ε4- = 2, ε4+ = 0	28.9, 71.1	28.2, 71.8
		rs405509	G/T	GG = 2, GT = 1, TT = 0	25.8, 48.7, 25.5	26.2, 47.3, 26.5
Brain derived neurotrophic factor antisense RNA	*BDNF-AS*	rs6265	C/T	CC = 2, CT = 1, TT = 0	67.5, 28.9, 3.6	66.3, 30.1, 3.6
Catechol-O-methyltransferase	*COMT*	rs4680	A/G	AA = 2, GA = 1, GG = 0	24.8, 49.8, 25.4	30.2, 47.4, 22.4
Microtubule associated protein tau	*MAPT*	rs10445337	C/T	CC = 2, TC = 1, TT = 0	4.7, 35.7, 59.6	4.7, 31.4, 63.9
Endothelial nitric oxide synthase	*NOS3*	rs2070744	C/T	CC = 2, TC = 1, TT = 0	14.8, 47.6, 37.6	17.0, 44.3, 38.7

Alleles previously associated with traumatic brain injury are underlined. ε4+ = ε4 allele possession, ε4- = absence of ε4 allele.

Data Analysis

SPSS for Windows version 26 (SPSS, Chicago, IL) software was used for analysis. TGSs of athletes and non-athletes were compared using independent t-tests, as were height and body mass. Pearson's χ^2 tests were utilised to compare genotype frequencies in upper and lower TGS quartiles of all rugby athletes vs. non-athletes, RU athletes vs. non-athletes, RL athletes vs. non-athletes, RU Forwards vs. non-athletes, RU Backs vs. non-athletes, and RU Forwards vs. RU Backs. Additionally, receiver operating characteristic (ROC) curves and area under the curve (AUC) were used to evaluate the ability of the TGS to correctly distinguish between athletes (including positional groups) and non-athletes [224]. Multifactor dimensionality reduction (https://sourceforge.net/projects/mdr/) software was used to identify SNP-SNP epistasis interactions [226]. Alpha was set at 0.05.

4.3 Results

TGS

Genotype frequencies were in Hardy-Weinberg equilibrium for all polymorphisms in the non-athlete and athlete groups. Athletes (all male) were taller and heavier ($P < 0.001$) than male non-athletes. No participant had a TGS of zero or 100 (range was 21.4-92.9). Only one rugby athlete and one non-athlete possessed the highest observed TGS of 92.9. Similarly, five rugby athletes (0.8%) and just one non-athlete possessed the lowest observed TGS of 21.4. Collectively, 76.7% of rugby athletes and 77.2% of non-athletes had a TGS >50.

There was no difference in Model 1 TGS between any rugby athlete group (all rugby athletes, RU, RL, RU forwards, RU backs) and the non-athlete group. Mean (SD) and kurtosis statistics of TGSs are reported in Table 4.2 and frequency distributions of rugby athletes and non-athletes are shown in Figure 4.1 (Panel A). Similarly, there was no difference in TGS between RU forwards and backs ($P = 0.842$).

When the numbers of athletes (including discrete groups) and non-athletes in the upper and lower 25% of TGS were compared, no significant differences were found (Table 4.2). Similarly, there were no differences between RU forwards and backs in terms of their presence in the upper and lower quartiles of TGS ($P = 0.668$). ROC AUC analysis confirmed

the TGS algorithm could not identify elite rugby athlete status (AUC = 0.504; 95% CI = 0.470-0.538; *P* = 0.800; Figure 4.1 (Panel B)). There was also no ability to distinguish between athletes and non-athletes when discrete groups of athletes were considered (RU vs. non-athletes, RL vs. non-athletes, RU Forwards vs. non-athletes, RU Backs vs. non-athletes; Table 4.2).

Even when using the Model 2 TGS algorithm using genotype scores determined solely by genotype frequencies observed in elite rugby athletes (Chapter 3), there was no difference between all rugby athletes and non-athletes (*P* = 0.065; Figure 4.2). Neither was there a difference between the numbers of athletes and non-athletes in the upper and lower TGS quartiles (*P* = 0.144). ROC AUC analysis again demonstrated that the data could not correctly distinguish elite rugby athletes from non-athletes (AUC = 0.532; 95% CI = 0.497-0.567, *P* = 0.067).

Table 4.2. Prior literature-based TGS (Model 1) with kurtosis statistics, and group comparisons via independent t-test, top quartile vs. bottom quartile comparisons via χ^2, and ROC curve analysis AUC.

Group	**Mean (SD) TGS**	**Mean (SE) kurtosis**	***P*-value athlete group vs. non-athletes**	***P*-value top quartile vs. bottom quartile TGS**	**ROC curve analysis AUC (95% CI)**	***P*-value AUC**
Non-athletes	56.4 (12.8)	-0.403 (0.217)				
All Rugby Athletes	56.5 (13.6)	-0.506 (0.198)	0.797	0.349	0.504 (0.470-0.538)	0.800
RU Athletes	56.4 (13.4)	-0.490 (0.215)	0.828	0.415	0.504 (0.468-0.539)	0.830
RL Athletes	56.9 (14.7)	-0.617 (0.488)	0.821	0.444	0.507 (0.440-0.575)	0.823
RU Forwards	56.3 (13.3)	-0.384 (0.283)	0.934	0.678	0.502 (0.460-0.544)	0.935
RU Backs	56.5 (13.5)	-0.613 (0.328)	0.769	0.326	0.507 (0.460-0.554)	0.772

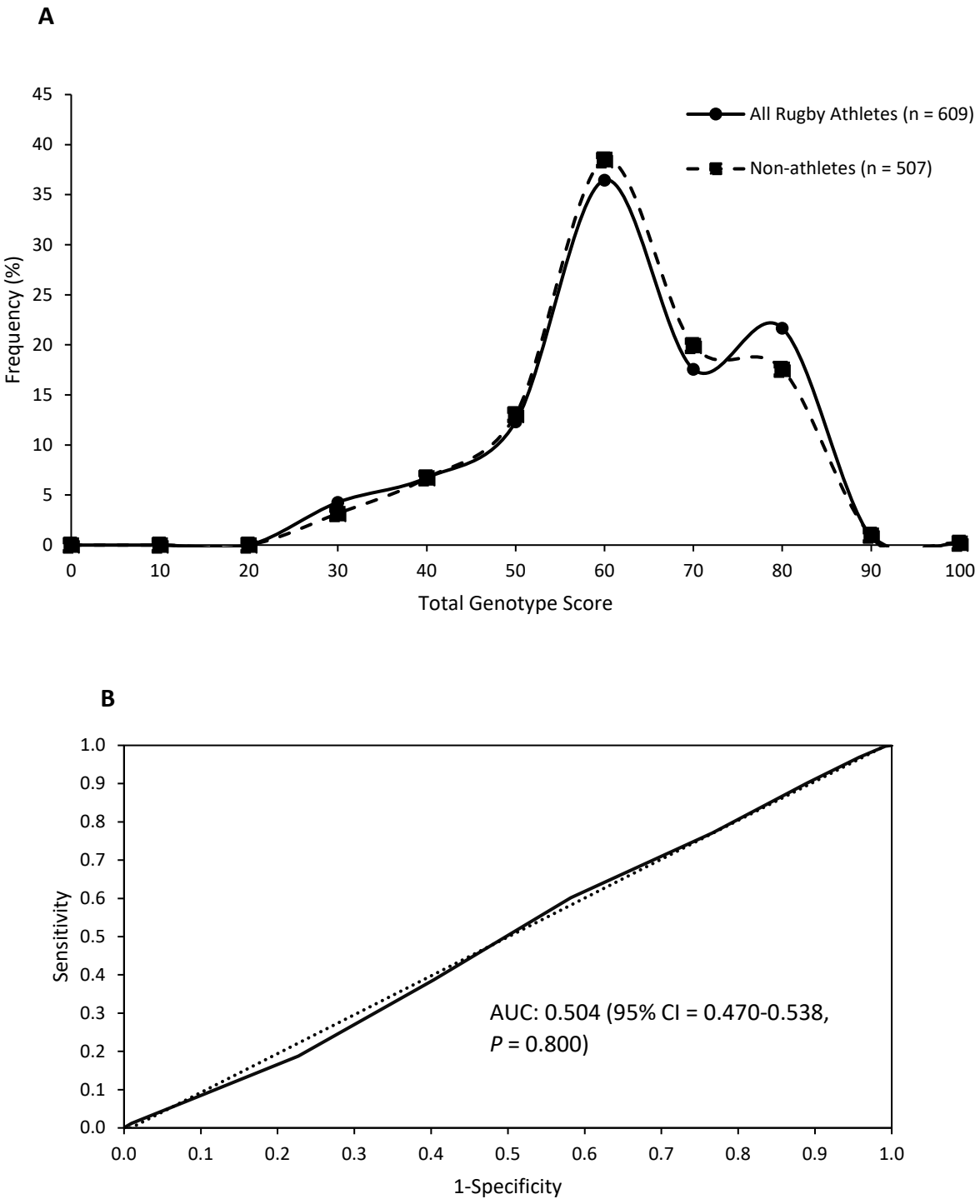


Figure 4.1. Panel A: No difference in frequency distributions of the Model 1 TGS of all athletes and non-athletes (P = 0.797 for comparison of means). Panel B: Receiver operating characteristic curve displays the inability of the TGS to discriminate elite rugby athletes from non-athletes. Dotted line = no discrimination. AUC; area under the curve.

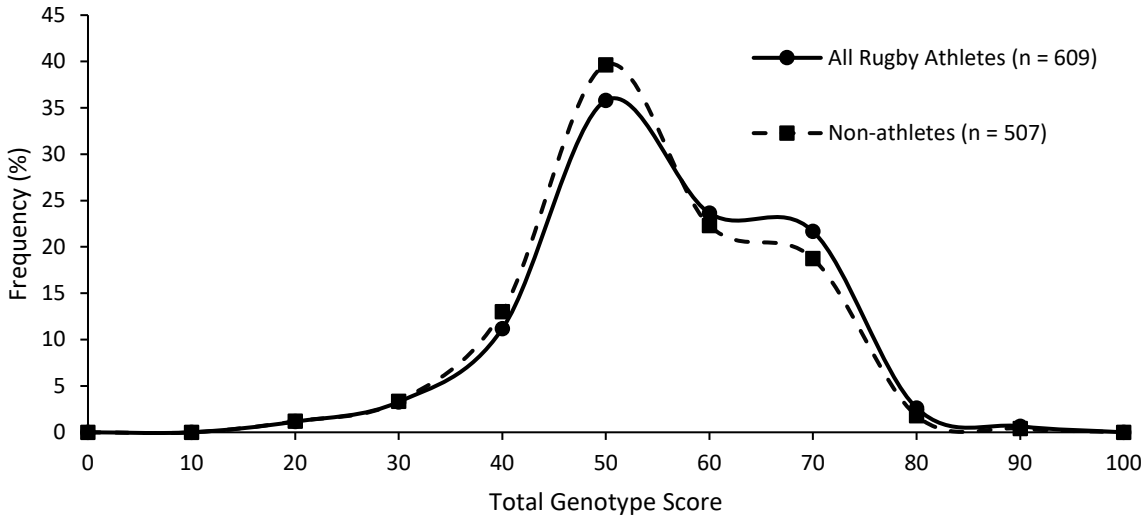


Figure 4.2. Similar frequency distribution of the data-led TGS for all athletes and non-athletes; *P* = 0.065 for difference in mean (SD) between all athletes (59.6 (12.4)) and non-athletes (58.4 (12.1)).

SNP epistasis

Multifactor dimensionality reduction analysis identified a SNP-SNP interaction of *COMT* rs4680 and *MAPT* rs10445337 polymorphisms that best predicted elite athlete status (testing accuracy 0.531; cross-validation consistency 9/10). There was a greater frequency of the *COMT-MAPT* G-C allele combination in all rugby athletes (31.7%; OR = 1.43, 95% CI = 1.12-1.81) and RU athletes (31.8%; OR = 1.44, 95% CI = 1.12-1.84) than non-athletes (24.5%; both comparisons $P < 0.001$) (Figure 4.3).

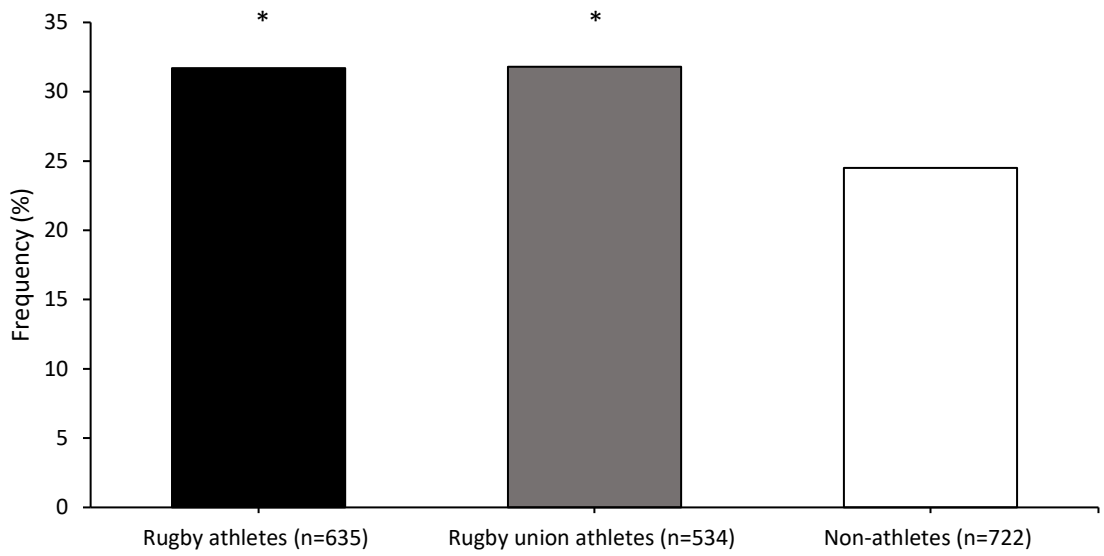


Figure 4.3. *COMT* (rs4680) and *MAPT* (rs10445337) G-C allele combination frequencies. *different from non-athletes ($P < 0.001$).

4.4 Discussion

This present chapter investigated suspected concussion-associated polygenic profiles for determining elite status in rugby. This was the first use of elite rugby athlete data, TGS models and SNP-SNP epistasis interactions to evaluate whether a concussion-associated polygenic profile is more suitable for achieving elite status in the high concussion risk environment of rugby. For the eight suspected concussion-associated genetic variants used in the TGS algorithm, there was no difference in elite rugby athlete and positional subgroup TGSs compared to non-athletes. However, multifactor dimensionality reduction analysis found a 2-SNP model of *COMT* (rs4680) and *MAPT* (rs10445337) G-C allele combination produced the best model for predicting elite athlete status (testing accuracy 0.531; cross-validation consistency 9/10).

Data shows that mean concussion-associated TGS is approximately 56-57 for both rugby athletes and non-athletes, based on the eight SNPs studied. This finding indicates that, for these eight SNPs previously associated with concussion incidence and/or severity, elite rugby athletes do not tend to have a more 'preferable' polygenic concussion associated profile than non-athletes, thus not supporting the hypothesis. Previous evidence has shown that elite athlete status of track and field athletes, rowers, cyclists and soccer athletes can

be reflected in a higher mean and more 'preferable' performance-associated TGS than non-athletes in Caucasian populations [250,252,253,256,258].

It was anticipated that the TGS distributions of athletes and non-athletes might differ at their extremes. However, there was no difference between the upper and lower TGS quartiles in terms of the proportion of rugby athletes and non-athletes. The same large interindividual variability (TGS range = 21-93) was observed for both elite rugby athletes and non-athletes. This wide distribution of scores highlights the variable genetic potential with respect to concussion in the general population. Nevertheless, ~23% of elite rugby players possessed a concussion-associated TGS of 50 or less, which could indicate those athletes are more at risk of concussion and/or poorer outcome post-concussion due to possession of a 'less preferable' polygenic concussion profile.

Separately no differences in genotype frequencies between elite rugby athlete and non-athletes for seven out of eight concussion-associated genetic variants were found (Chapter 3). That might have been because individual variants cannot represent the complexity of concussion risk and do not reflect SNP-SNP interactions, known as non-linear interaction or epistasis [226]. In this chapter, the 2-SNP model of the *COMT* (rs4680) and *MAPT* (rs10445337) polymorphisms produced the best model to predict elite athlete status. The G-C allele combination was more common in rugby athletes (~32%) than non-athletes (~25%). Previously, GG (Val/Val) carriers of *COMT* (rs4680) have been observed to have ~33% increased COMT activity than AA (Met/Met) carriers, thus reducing dopamine levels in the prefrontal cortex region of the brain [172]. Lipsky et al. [138] observed that GG carriers had 40% poorer executive function than AA carriers post-TBI. Recently, it has been observed that elite rugby athletes have 1.4 times the odds of possessing the GG genotype of *COMT* (rs4680) compared to non-athletes (Chapter 3). In addition, Mc Fie at al. [42] observed that A allele carriers in a cohort of youth and professional South African RU players were ~3-fold more likely to have a history of concussion. Considering the pleiotropic nature of *COMT* (rs4680), G carriers could possess greater stress resilience and reduced anxiety in competitive environments and be at lower risk of experiencing concussions, but also be at risk of poorer cognitive function post-concussion (Chapter 3, 129,130]). The *MAPT* TT genotype (rs10445337) has been weakly associated with a greater risk of repeated concussion [21,22]. Mutations in *MAPT* have been shown to accelerate

aggregation of markers of neurotoxic hyperphosphorylated tau in response to repetitive concussions by 20-60% in animal studies and associated with neurodegenerative diseases in humans [152,232]. Elite rugby athletes who possess the C allele could have a reduced risk of repeated concussion and potential neurodegenerative diseases. The G-C allele combination could reduce the risk of experiencing concussions and provide a small advantage for attaining elite competitive status in the high concussion risk environment of competitive rugby. Dopamine has previously been associated with acute increases in tau phosphorylation [259]. The increased activity of the *COMT* G allele, associated with reduced dopamine [172], and reduced tau protein expression in the presence of the C allele of *MAPT* [152,232], could suggest a potential concussion resistance mechanism. It should be noted that the SNP-SNP interaction analysis relies on data mining to identify the best genetic model to fit the data, potentially leading to overfitting. Cross-validation was utilised to compensate, although the 2-SNP model we identified should be investigated in other cohorts to confirm it.

The discriminatory power of the TGS is dependent upon the polymorphisms included and the mathematical model utilised [260]. In both athletes and non-athletes, Ben-Zaken et al. [256] observed a higher mean TGS in a 2-SNP model than a 5-SNP model. However, the 5-SNP model provided greater discriminatory accuracy between groups [256]. In contrast, including many SNPs in a TGS model could reduce the explained variance. Thomaes et al. [261] reported that a 54-SNP model probably increased 'background noise' as all alleles were weighted equally, whereas in reality some variants will have larger effects on a phenotype than others. Adjustments to the weightings applied to each genetic variant in the algorithm could compensate, but a more extensive body of literature is required to apply relative weightings to different SNPs with confidence. The polymorphisms included in the TGS are all reported to be associated with concussion (incidence, severity or recovery) or its related biological mechanisms [41]. In addition, the TGS gave all the SNPs equal weighting as was assumed that allelic effects are codominant and each SNP to have an equal additional effect, which may not uniformly be the case with respect to the pathophysiology of concussion in elite rugby athletes. The addition of polymorphisms in the TGS that do not influence the phenotype in question can decrease discriminatory accuracy of the model [262]. In this chapter, only *COMT* (rs4680) has individually been

previously associated with elite rugby athlete status (Chapter 3). Indeed, using a data-led TGS algorithm determined from previous observed genotype frequencies in elite rugby athletes (Chapter 3), the eight suspected concussion-associated genetic variants examined here were still unable to collectively distinguish athletes from non-athletes. Therefore, it cannot be excluded that there is possibility that the TGS included polymorphisms that potentially do not influence concussion risk in elite rugby athletes. Future studies will no doubt identify new candidate polymorphisms and replication studies could indicate stronger associations between existing polymorphisms and concussion, which could be used to increase the accuracy of the algorithm. For example, a recent genome-wide-association study has identified 2 novel SNPs (*SPATA5* rs144663795 and *PLXNA4* rs117985931) associated with concussion [191]. Further GWASs and further replication studies of candidate gene approaches are needed to establish a TGS that quantifies estimated concussion risk effectively.

Limitations of this chapter include the previously discussed population stratification issues (latter part of Section 3.4), the inclusion of unweighted polymorphisms and the inclusion of polymorphisms that potentially do not influence concussion risk in elite rugby athletes. In addition, it is not fully understood how concussion-associated alleles affect physiological mechanisms related to the pathophysiology of concussion and associated-traits that could affect incidence, severity and risk of concussion.

4.5 Conclusion

Concussion is a complex phenotype influenced by environmental factors and an individual's genetic predisposition, and in the high concussion risk environment of elite rugby, genetically-mediated resistance to aspects of concussion could be advantageous for career success and longevity. However, in contrast to this chapter's original hypothesis, a concussion-focused polygenic model could not discriminate between elite rugby athletes and non-athletes, although the large range of TGS scores could underpin the inter-individual variability in injury occurrence and outcomes following concussion. Nevertheless, epistasis analysis identified a genetic interaction of *COMT* (rs4680) and *MAPT* (rs10445337) G-C alleles as more common in elite rugby athletes and carriage of these variants may affect stress resilience, behavioural traits and altered risk of concussion incidence and severity. It is possible that combining genetic data from multiple concussion-

associated gene variants such as these could inform risk assessment and recovery from concussion in the future. Future studies should include polymorphisms for which strong associations with concussion have been newly observed, to increase the accuracy of the model, and potentially build towards a practical tool for concussion screening and management strategies in high concussion risk sports such as rugby.

Concussion-associated gene variants and history of concussion in elite rugby athletes

5.1 Introduction

Over the duration of a professional rugby career, elite rugby athletes could sustain 14-28 concussions [263,264]. In addition, previously concussed RU players have a 38-60% greater injury risk of subsequent injury than non-concussed athletes [23,79] and it is reasonable to suggest previously concussed RL athletes could experience similar subsequent risk.

Typically, concussions result in short-term impairment of neurological function, although in some cases symptoms may be prolonged [26]. Inter-individual variability is evident in recovery as 80-90% of adult athletes with sport-related concussions are considered to be clinically recovered and return to play within 7-10 days [26,72,73]. However, for 10-20% of concussion cases, symptoms can persist for >10 days [72] and for 1.6% of concussed athletes post-concussion symptoms can persist for 12 months [73,74].

The potential short and long-term risks [24,26,245–248] to athletes sustaining concussions over their careers has been a well-publicised concern. There is a growing body of evidence to support a 'dose-response' relationship between a history of concussion and risk of sustaining future concussions. Athletes who have sustained >3 concussions are at 5-fold greater risk of cognitive impairment and are 3-fold more likely to sustain another concussion within the same season [208,265].

Incidence of and recovery from concussion have a substantial genetic component that probably involves the interaction of multiple genes in a polygenic manner [41,230]. Genetic variation could affect predisposition for and recovery from concussion [41,230], thus impacting time loss injuries, early retirement and potential neuropathological consequences. Indeed, previous observations from Chapter 4 demonstrates that 23% of elite rugby players possess a concussion-associated total genotype score (TGS) of 50 or less, which could indicate those athletes are more at risk of concussion and/or poorer outcome post-concussion due to possession of a 'less preferable' polygenic concussion profile.

Each sport has its own set of concussion risk factors, such as physical characteristics of athletes, demands of the sport and rules of the sport influencing behaviours of athletes that contribute to the incidence of concussion [266,267]. However, there is debate within the literature in relation to suspected concussion-associated gene variants (reviewed in Chapter 1) and inter-individual variability in traumatic brain injury incidence and severity.

The conflicting evidence from the literature could be partially accounted for by the diverse range of methods employed within studies, such as measurement of concussion and type of participants (level of athletes, differing ethnicities, sexes, and severity of TBI).

Within RU, higher incidences of concussion have been observed at sub-elite level than at youth and elite level [39]. Elite and sub-elite RL athletes have similar incidences of concussion [87]. One explanation for the RU observations is that injury risk at sub-elite level is increased due to high match intensity combined with lower skill [87]. Some conflicting findings in the literature regarding concussion risk could be due to variations in methods that make comparisons between differing levels of rugby athletes difficult. Recent RU investigations of concussion-associated gene variants and incidence of concussion have used mixed cohorts of youth, amateur and professional South African RU athletes, of differing ethnicities [42,55–57]. Age is a modifying risk factor for concussion, as athletes younger than 18 yr are at higher risk of concussion than adults [268], and 18-22 yr athletes recovery quicker than 13-16 yr athletes [269] potentially due to differences in maturation rates of physical qualities [270]. In addition, it has been reported that collegiate female athletes can experience concussions at a higher rate than males (11% and 7%, respectively) [271]. These modifying risk factors should considered when reviewing evidence regarding concussion-associated gene variants and sports-related concussion.

There has been no consistent approach to concussion assessment and concussion history data collection techniques in the literature. Some studies have used differing forms of self-reported concussion history questionnaires and others have used ‘clinical diagnosis’ by a medical professional [43,55]. Again, differences in assessment methods employed in studies could account for differences in findings and each is not without its limitations. For instance, the timing of a ‘clinical diagnosis’ Glasgow Coma Scale assessment has been reported to affect apparent severity of TBI [272]. It is recommended that a GCS score is recorded 30 min post-injury or later for an accurate diagnosis of concussion [272]. However, protocols differ within the literature diagnoses would be less accurate if a recording is taken too soon post-injury [272]. Professional rugby players underestimate the number of clinically diagnosed concussions by 30% during self-reporting [210], adversely affecting data accuracy. Indeed, players have been reported to not disclose accurate information in order to return to play, potentially due to pressure to perform [273], lack of

concussion education [274], environment and policy issues [40]. Fuller et al. [275] indicated that in elite RU the head injury assessment protocol has strong predictive values for concussion confirmation (88.1% for negative prediction and 67.6% for positive prediction). However, it is dependent upon the experience of the medical professional conducting the assessment and true baseline concussion data relating to each athlete [275].

Some previous studies have investigated concussion risk and genetic predisposition in athletic cohorts of mixed ethnicities. For example, Terrell et al.'s [21] athletic cohort was comprised of a mixture of ethnic groups (described as 54% white, 40% black and 6% other). However, differences in allele frequency between populations of differing ethnicity/geographic ancestry should be considered because they can confound findings and conclusions [276]. In contrast to mixed cohorts of athletes, elite male Caucasians are the largest group in RugbyGene and provide a relatively homogenous group with which to investigate genetic associations [15].

Consequently, the first aim of this chapter was to investigate whether suspected concussion-associated polymorphisms are associated with history of previous concussion in elite Caucasian rugby athletes. It was hypothesised that the concussion-associated risk genotypes and alleles would be overrepresented in elite rugby athletes with a history of previous concussion compared to those with no history of previous concussion. The second aim was to compare the polygenic profile of elite rugby athletes with a history of concussion to those with no history of concussion. It was hypothesised that athletes with no history of concussion would have a higher TGS than previously concussed elite rugby athletes, indicating a more 'preferable' polygenic profile.

5.2 Methods

The participants and procedures used in Chapter 5 have already been described in detail in Chapter 2, thus, only a brief description is provided below.

Participants

A total of 141 individuals were recruited and gave written informed consent to participate in the present chapter. An *a priori* calculation for 80% power to detect a medium effect

size (w) of 0.3 required >108 participants to analyse genotype frequencies — for some SNP-group analyses the total sample was slightly above or below this sample size. A medium effect size (*d*) of 0.5 required a sample of >128 participants to compare TGS algorithms. The total sample comprised elite Caucasian male rugby athletes (121 RU and 20 RL: mean (standard deviation) height 1.86 (0.07) m, mass 102 (12) kg, age 26 (5) yr) including 41.8% Italian, 25.5% Irish, 23.4% British, 5.0% South African, and 4.3% of other nationalities. Fifty-nine percent of the RU athletes had competed at international level for a "high performance union" (Regulation 16, http://www.worldrugby.org) and 52.6% of RL athletes had competed at international level.

Procedures

Concussion history. Concussion history in elite rugby athletes was collected using a self-reported concussion history questionnaire previously described in Chapter 2 (Appendix 7). The concussion questionnaire took ~5 min to complete, and an investigator assisted participants to maximise honesty and accuracy.

Sample collection, DNA isolation and genotyping. Saliva (74.5% of all samples) or blood (25.5%) samples were obtained via the procedures previously described in Chapter 2.DNA isolation and genotyping are also described in detail in Chapter 2.

Calculation of TGS

To quantify the combined influence of the candidate polymorphisms (Table 3.1, Chapter 3), the two additive TGS algorithms previously described in Chapter 4 were utilised, plus a third version:

Model 1: TGS = (100/14) * (*ANKK1*$_{rs1800497}$ + *APOE*$_{rs429358,\ rs7412}$ + *APOE*$_{rs405509}$ + *BDNF-AS*$_{rs6265}$ + *COMT*$_{rs4680}$ + *MAPT*$_{rs10445337}$ + *NOS3*$_{rs2070744}$)

Model 2 used the same eight polymorphisms as Model 1, but with genotype scores allocated according to the data presented in the current chapter (Table 5.1), regardless of statistical significance. Higher scores were thus allocated to genotypes more common in athletes with no history of concussion, wherein seven of the eight genotype scores differed

to Model 1 (*APOE* rs405509 TT = 2, GT = 1, GG = 0; *APOE* rs429358, rs7412 ε4+ = 2, ε4- = 0; *BDNF-AS* rs6265 AA = 2, AG = 1, GG = 0; *COMT* rs4680 GG = 2, GA = 1, AA = 0; *MAPT* rs10445337 TT = 2, TC = 1, CC = 0 and *NOS3* rs2070744 TT = 2, TC = 1, CC = 0).

Model 3 only included polymorphisms that showed statistically significant differences in frequency between elite rugby athletes with a history of at least one concussion and those with no history in the current chapter (as detailed in Table 5.1, there were three: *APOE* rs405509 TT = 2, GT = 1, GG = 0; *BDNF-AS* rs6265 AA = 2, AG = 1, GG = 0 and *COMT* rs4680 GG = 2, GA = 1, AA = 0).

Data Analysis

SPSS for Windows version 26 (SPSS, Chicago, IL) software was used. Pearson's χ^2 tests were used to compare genotype and allele frequencies between elite rugby athlete groups in four separate analyses; 1. Athletes with no history of concussion were compared to athletes with a history of at least one concussion, 2. Athletes with no history of concussion vs. athletes with a history of multiple concussions, 3. Athletes who recovered within 10 days vs. those whose recovery took >10 days, 4. Athletes with no history of concussion and no family history of neurological conditions vs. athletes with a history of concussion and family history of neurological conditions. Sixteen comparisons per SNP (12 for *APOE* ε2/ε3/ε4) were subjected to Benjamini-Hochberg corrections [BH;221] to control false discovery rate and corrected probability values are reported. Odds ratios (OR) were calculated to estimate effect size. One-way Analysis of Variance (Kruskal-Wallis H test), was used to compare number of concussions experienced by different age groups of athletes. TGSs of athletes with and without a history of concussion were compared using independent t-tests. Pearson's χ^2 compared the numbers of athletes with and without a history of concussion in the upper and lower thirds of TGS. Bonferroni adjustment was utilised where appropriate to control for false discovery when comparing TGS scores. Additionally, receiver operating characteristic (ROC) curves and area under the curve (AUC) were used to evaluate the ability of the TGS to correctly distinguish between athletes with a history of at least one concussion versus those with no such history [224]. Multifactor dimensionality reduction (https://sourceforge.net/projects/mdr/) software was used to identify SNP-SNP epistasis interactions [226]. Alpha was set at 0.05.

5.3 Results

Genotype and allele frequencies

Genotype frequencies were in Hardy-Weinberg equilibrium for all polymorphisms in the athlete groups, apart from *BDNF-AS* rs6265 in athletes with no history of concussion (P <0.05) (Table 5.1). Seventy-six percent of athletes reported a history of sustaining at least one concussion and 52% of athletes reported sustaining multiple (≥2) concussions from rugby. Seventy-five percent of athletes with a history of concussion had a recovery duration of <10 days. The mean number of concussions experienced by athletes increased with age (19-23 yr = 1.44 concussions, 24-28 yr = 2.43 concussions, 29-33 yr =3.23 concussions and 34-38 yr = 3.29 concussions (P = 0.007).

For *APOE* rs405509, the GG genotype, proportion of G allele carriers and G allele were underrepresented in athletes with no history of concussion (12.5%, 45.1% and 42.2%, respectively) compared with athletes with a history of at least one concussion (26.7%, 49.3%, and 49.0%) and athletes with a history of multiple concussions (24.7%, 47.1% and 45.9%, respectively, Table 5.1 and Figure 5.1, $P \leq 0.001$). The GG genotype was more common in athletes with a history of at least one concussion than athletes with no history of concussion (OR = 2.10, 95% confidence interval (CI) = 0.58-7.59), and more common in athletes with a history of multiple concussions than those with no history of concussion (OR = 1.69, 95% CI = 0.45-6.36). The TT genotype was underrepresented in the >10 days recovery group compared with the <10 days recovery group (11.5% vs. 10.0%, Table 5.1 and Figure 5.1, P = 0.036), with the TT more common in the <10 days recovery group (OR = 4.14, 95% CI = 1.14-15.06). However, there was no difference in genotype frequency between athletes with no history of concussion and no family history of neurological conditions compared to athletes with a history of concussion and family history of neurological conditions (P = 0.536).

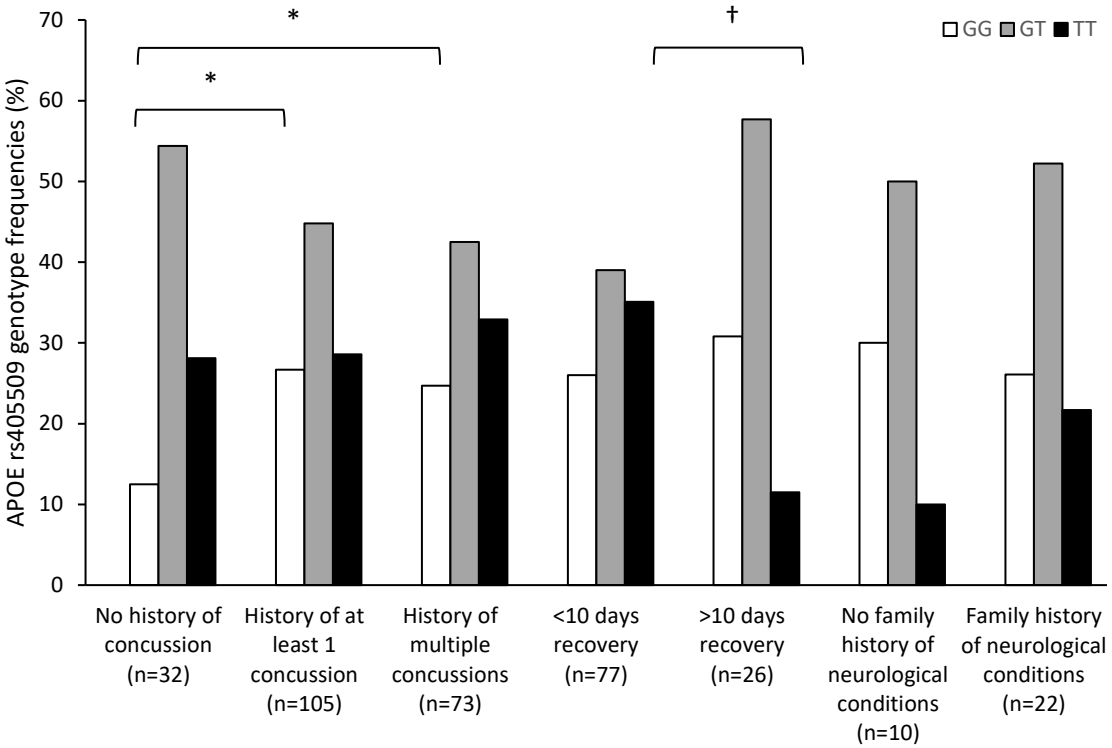


Figure 5.1. Genotype frequency of *APOE* rs405509 for athletes. * GG less common in athletes with no history of concussion ($P \leq 0.01$). † TT more common in athletes with a shorter recovery period ($P = 0.036$).

Table 5.1. Genotype and allele distribution of athletes with different histories of concussion, recovery durations and family histories of neurological conditions. Data are genotype/allele count followed by percentage in parentheses.

Polymorphism	Genotype	No history of concussion	History of at least one concussion	History of multiple concussions	<10 days recovery	>10 days recovery	No family history of neurological conditions	Family history of neurological conditions
***ANKK1* rs1800497**	GG	22 (66.7)	71 (67.0)	43 (59.7)	53 (67.9)	16 (61.5)	7 (70.0)	16 (72.7)
	GA	11 (33.3)	32 (30.2)	26 (36.1)	23 (29.5)	9 (34.6)	3 (30.0)	5 (22.7)
	AA	0 (0.0)	3 (2.8)	3 (4.2)	2 (2.6)	1 (3.8)	0 (0.0)	1 (4.6)
	Total	33	106	72	78	26	10	22
	G allele	55 (83.3)	174 (82.1)	112 (79.2)	129 (82.7)	41 (78.8)	17 (85.0)	37 (84.1)
	A allele	11 (16.7)	38 (17.9)	32 (20.8)	27 (17.3)	11 (21.2)	3 (15.0)	7 (15.9)
	G allele carriers	33 (100.0)	103 (74.6)	69 (70.4)	76 (75.2)	25 (71.4)	10 (70.0)	21 (77.8)
	A allele carriers	11 (33.3)	35 (25.4)	29 (29.6)	25 (24.8)	10 (28.6)	3 (30.0)	6 (22.2)
***APOE* rs405509**	GG	4 (12.5)	28 (26.7)*	18 (24.7)*	20 (26.0)	8 (30.8)*	3 (30.0)	6 (26.1)
	GT	19 (54.4)	47 (44.8)	31 (42.5)	30 (39.0)	15 (57.7)	5 (50.0)	12 (52.2)
	TT	9 (28.1)	30 (28.6)	24 (32.9)	27 (35.1)	3 (11.5)	2 (10.0)	5 (21.7)
	Total	32	105	73	77	26	10	23
	G allele	27 (42.2)	103 (49.0)*	67 (45.9)	70 (45.5)	31 (59.6)	11 (45.0)	24 (52.2)
	T allele	37 (57.8)	107 (51.0)	79 (54.1)	84 (54.5)	21 (40.4)	9 (55.0)	22 (47.8)
	G allele carriers	23 (45.1)	75 (49.3)	49 (47.1)	50 (46.7)	23 (56.1)	8 (53.3)	18 (51.4)
	T allele carriers	28 (54.9)	77 (50.7)	55 (52.9)	57 (53.3)	18 (43.9)	7 (46.7)	17 (48.6)

Table 5.1. Continued

Polymorphism	Genotype	No history of concussion	History of at least one concussion	History of multiple concussions	<10 days recovery	>10 days recovery	No family history of neurological conditions	Family history of neurological conditions
***APOE* ε2/ε3/ε4**	ε2/ε2	0 (0.0)	3 (2.8)	2 (2.7)	2 (2.6)	1 (3.7)	0 (0.0)	1 (4.3)
	ε2/ε3	2 (6.1)	8 (7.5)	5 (6.8)	4 (5.1)	4 (14.8)	0 (0.0)	2 (8.7)
	ε2/ε4	0 (0.0)	1 (0.9)	0 (0.0)	0 (0.0)	1 (3.7)	0 (0.0)	0 (0.0)
	ε3/ε3	22 (66.7)	74 (69.2)	49 (67.1)	56 (71.8)	16 (59.3)	8 (80.0)	17 (73.9)
	ε3/ε4	9 (27.3)	19 (17.8)	17 (23.3)	14 (17.9)	5 (18.5)	2 (20.0)	3 (13.0)
	ε4/ε4	0 (0.0)	2 (1.9)	0 (0.0)	2 (2.6)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	33	107	73	78	27	10	23
	ε4 allele carriers	9 (27.3)	22 (20.6)	17 (23.3)	16 (20.5)	6 (22.2)	2 (20.0)	3 (13.0)
	Non-ε4 allele carriers	24 (72.7)	85 (79.4)	56 (76.7)	62 (79.5)	21 (77.8)	8 (80.0)	20 (87.0)
***BDNF-AS* rs6265**	GG	20 (60.6)	71 (66.4)	50 (68.5)	51 (65.4)	19 (70.4)	7 (70.0)	15 (65.2)
	GA	8 (24.2)	35 (32.7)	22 (30.1)	26 (33.3)	8 (29.6)	3 (30.0)	7 (30.4)
	AA	5 (15.2)	1 (0.9)*	1 (1.4)*	1 (1.3)	0 (0.0)	0 (0.0)	1 (4.3)
	Total	33	107	73	78	27	10	23
	G allele	48 (72.7)	177 (82.7)	122 (83.6)	128 (82.1)	46 (85.2)	17 (85.0)	37 (80.4)
	A allele	18 (27.2)	37 (17.3)*	24 (16.4)*	28 (17.9)	8 (14.8)	3 (15.0)	9 (19.6)
	G allele carriers	28 (68.3)	106 (74.6)	72 (75.8)	77 (66.2)	27 (77.1)	10 (76.9)	22 (73.3)
	A allele carriers	13 (31.7)	36 (25.4)	23 (24.2)	27 (33.8)	8 (22.9)	3 (23.1)	8 (26.7)

Table 5.1. Continued

Polymorphism	Genotype	No history of concussion	History of at least one concussion	History of multiple concussions	<10 days recovery	>10 days recovery	No family history of neurological conditions	Family history of neurological conditions
***COMT* rs4680**	GG	12 (37.5)	30 (28.3)	20 (27.4)	19 (24.4)	10 (38.5)	3 (30.0)	5 (21.7)
	GA	16 (50.0)	47 (44.3)	33 (45.2)	38 (48.7)	9 (34.6)	5 (50.0)	11 (47.8)
	AA	4 (12.5)	29 (27.4)*	20 (27.4)*	21 (26.9)	7 (26.9)	2 (20.0)	7 (30.4)
	Total	32	106	73	78	26	10	23
	G allele	40 (62.5)	107 (50.5)	73 (50.0)	76 (48.7)	29 (55.8)	11 (55.0)	21 (45.7)
	A allele	24 (37.5)	105 (49.5)*	73 (50.0)*	80 (51.3)	23 (44.2)	9 (45.0)	25 (54.3)
	G allele carriers	28 (58.3)	77 (50.3)	53 (50.0)	57 (49.1)	19 (54.3)	8 (53.3)	16 (47.1)
	A allele carriers	20 (41.7)	76 (49.7)	53 (50.0)	59 (50.9)	16 (45.7)	7 (46.7)	18 (52.9)
***MAPT* rs10445337**	TT	23 (69.7)	66 (61.7)	43 (58.9)	47 (60.3)	19 (70.4)	8 (80.0)	14 (60.9)
	TC	9 (27.3)	38 (35.5)	28 (38.4)	28 (35.9)	8 (29.6)	2 (20.0)	8 (34.8)
	CC	1 (3.0)	3 (2.8)	2 (2.7)	3 (3.8)	0 (0.0)	0 (0.0)	1 (4.3)
	Total	33	107	73	78	27	10	23
	T allele	55 (83.3)	170 (79.4)	114 (78.1)	122 (78.2)	46 (85.2)	18 (90.0)	36 (78.3)
	C allele	11 (16.7)	44 (20.6)	32 (21.9)	34 (21.8)	8 (14.8)	2 (10.0)	10 (21.7)
	T allele carriers	32 (80)	104 (71.7)	61 (67.0)	75 (70.8)	27 (77.1)	10 (83.3)	22 (71.0)
	C allele carriers	10 (20)	41 (28.3)	30 (33.0)	31 (29.2)	8 (22.9)	2 (16.7)	9 (29.0)
***NOS3* rs2070744**	TT	9 (28.1)	31 (29.5)	23 (31.5)	21 (27.3)	10 (26.9)	4 (40.0)	8 (34.8)
	CT	19 (59.4)	55 (52.4)	37 (50.7)	42 (54.5)	11 (53.8)	4 (40.0)	11 (47.8)
	CC	4 (12.5)	19 (18.1)	13 (17.8)	14 (18.2)	5 (17.9)	2 (20.0)	4 (17.4)
	Total	32	105	73	77	26	10	23
	T allele	37 (57.8)	117 (55.7)	83 (56.8)	84 (54.5)	31 (59.6)	12 (60.0)	27 (58.7)
	C allele	27 (42.2)	93 (44.3)	63 (43.2)	70 (45.5)	21 (40.4)	8 (40.0)	19 (41.3)
	T allele carriers	28 (54.9)	86 (53.8)	60 (54.5)	63 (52.9)	21 (56.8)	8 (57.1)	19 (55.9)
	C allele carriers	23 (45.1)	74 (46.2)	50 (45.5)	56 (47.1)	16 (43.2)	6 (42.9)	15 (44.1)

The genotype and allele carrier data represent the additive, dominant and recessive models, respectively. * lower frequency than non-athletes ($P \leq 0.001$).

For BDNF-AS rs6265, the AA genotype, proportion of A allele carriers and A allele were overrepresented in athletes with no history of concussion (15.2%, 31.7% and 27.2%, respectively; Table 5.1) compared with athletes with a history of at least one concussion (0.9%, 25.4%, and 17.3%) and athletes with a history of multiple concussions (1.4%, 24.2% and 16.4%, respectively, $P \leq 0.001$). The GG genotype was more common in athletes with a history of at least one concussion than athletes with no history of concussion (OR = 17.75, 95% confidence interval (CI) = 1.96-160.78), and more common in athletes with a history of multiple concussions compared with no history of concussion (OR = 12.50, 95% CI = 1.37-113.81). However, there was no difference in *BDNF-AS* rs6265 genotype frequencies between athletes with different durations of recovery from concussion ($P = 0.649$), nor between those with no history of concussion and no family history of neurological conditions compared to athletes with a history of concussion and family history of neurological conditions ($P = 0.852$).

For *COMT* rs4680, the AA genotype, proportion of A allele carriers and A allele were underrepresented in athletes with no history of concussion (12.5%, 41.7% and 37.5%, respectively; Table 5.1) compared with athletes with a history of at least one concussion (27.4%, 49.7%, and 49.5%) and athletes with a history of multiple concussions (27.4%, 50.0% and 50.0%, respectively); Table 5.1, $P \leq 0.001$). The AA genotype was more common in athletes with a history of at least one concussion than athletes with no history of concussion (OR = 2.90, 95% confidence interval (CI) = 0.84-10.04), and more common in athletes with a history of multiple concussions compared with no history of concussion (OR = 3.00, 95% confidence interval (CI) = 0.83-10.90). However, there was no difference in *COMT* rs4680 genotype frequencies between athletes with different durations of recovery from concussion ($P = 0.203$), nor between those with no history of concussion and no family history of neurological conditions compared to athletes with a history of concussion and family history of neurological conditions ($P = 0.407$).

For *ANKK1* rs1800497, *APOE* rs429358 and rs7412, *MAPT* rs10445337 and *NOS3* rs2070744 there were no differences in genotype or allele frequencies between any groups ($P \geq 0.055$; Table 5.1).

Total genotype score

Using TGS algorithm Model 1 (based on prior literature), there was no difference between athletes with no history of concussion (TGS 52.9) and athletes with a history of at least one concussion (TGS 58.1) after Bonferroni adjustment ($P > 0.018$), nor when compared to athletes with a history of multiple concussions (TGS 56.9) ($P = 0.120$). There was no difference in TGS between the <10 days recovery group and the >10 days recovery group ($P = 0.087$). Similarly, there was no difference in TGS between athletes with and without a family history of neurological conditions ($P = 0.381$). Mean (SD) and kurtosis statistics of TGSs are reported in Table 5.2.

When the upper and lower thirds of the TGS algorithm Model 1 were compared, athletes with a history of at least one concussion were overrepresented in the upper third (90.0%) and underrepresented in the lower third (76.3%) when taking into account athletes with no history of concussion (respective values in upper third 10.0%, lower third 23.7%; $P < 0.01$). Similarly, athletes with history of multiple concussions were overrepresented in the upper third (87.5%) and underrepresented in the lower third (65.1%) when taking into account athletes with no history of concussion (respective values 12.5% and 34.9%; $P = 5.6 \times 10^{-5}$). ROC AUC analysis confirmed the TGS algorithm could identify history of at least one concussion (AUC = 0.622; 95% CI = 0.520-0724; $P = 0.038$). When the numbers of athletes in the upper and lower thirds of the <10 days recovery group TGS were compared to the >10 days recovery group, no significant differences were found ($P = 0.326$). Similarly, there were no differences between athletes with and without a family history of neurological conditions in the upper and lower TGS thirds ($P = 0.559$).

Using TGS algorithm Model 2 (based on elite rugby athlete genotype frequencies observed in this chapter), there was no difference in TGS between athletes with no history of concussion (TGS 56.5) and those with a history of at least one concussion (TGS 51.0) ($P = 0.054$) or multiple concussions ($P = 0.120$) (Table 5.2). Neither was there a difference in TGS between the <10 days recovery group and the >10 days recovery group ($P = 0.981$) and between athletes with and without a family history of neurological conditions ($P = 0.512$).

When the upper and lower thirds of TGS algorithm Model 2 were compared, athletes with

a history of at least one concussion were underrepresented in the upper third (70.0%) and overrepresented in the lower third (88.6%) when taking into account athletes with no history of concussion (respective values in upper third 30.0%, lower third 11.4%; $P = 0.8 \times 10^{-5}$). Similarly, athletes with history of multiple concussions were underrepresented in the upper third (59.1%) and overrepresented in the lower third (78.1%) when taking into account athletes with no history of concussion (respective values 40.9% and 21.9%; $P = 0.012$). ROC AUC analysis demonstrated that the data could not correctly distinguish athletes without a history of concussion from those with a history of at least one concussion (AUC = 0.389; 95% CI = 0.280-0.497, $P = 0.058$). When the numbers of athletes in the upper and lower thirds of the <10 days recovery group TGS were compared to the >10 days recovery group, no significant differences were found ($P = 0.405$). Similarly, there were no differences between athletes with and without a family history of neurological conditions in the upper and lower TGS thirds ($P = 0.083$).

Table 5.2. Mean and kurtosis statistics for the three TGS models.

Group	Mean (SD) TGS Model 1	Mean (SE) kurtosis
No history of concussion	52.9 (10.7)	0.283 (0.809)
History of at least 1 concussion	58.1 (13.3)	-0.347 (0.472)
History of multiple concussions	56.9 (13.1)	-0.131 (0.559)
<10 days recovery	58.3 (13.9)	-0.375 (0.545)
>10 days recovery	56.9 (11.2)	-0.466 (0.902)
No family history of neurological conditions	59.7 (11.6)	-0.262 (0.566)
Family history of neurological conditions	59.7 (13.6)	-0.470 (0.953)
Group	**Mean (SD) TGS Model 2**	**Mean (SE) kurtosis**
No history of concussion	56.5 (12.9)	-2.292 (0.809)
History of at least 1 concussion	51.0 (13.2)	-0.111 (0.472)
History of multiple concussions	51.1 (13.3)	0.134 (0.559)
<10 days recovery	51.1 (13.3)	-0.72 (0.545)
>10 days recovery	51.1 (13.2)	0.040 (0.902)
No family history of neurological conditions	51.6 (13.3)	-0.282 (0.566)
Family history of neurological conditions	50.0 (10.3)	1.315 (0.953)
Group	**Mean (SD) TGS Model 3**	**Mean (SE) kurtosis**
No history of concussion	49.0 (18.9)	-2.654 (0.809)
History of at least 1 concussion	39.3 (20.5)	-0.577 (0.472)
History of multiple concussions	40.1 (20.1)	-0.208 (0.559)
<10 days recovery	40.4 (20.8)	-0.531 (0.545)
>10 days recovery	36.0 (19.1)	-0.323 (0.902)
No family history of neurological conditions	40.2 (20.0)	-0.462 (0.566)
Family history of neurological conditions	37.1 (21.2)	-1.148 (0.953)

For the TGS algorithm Model 3 (based on statistically significant differences in elite rugby athlete genotype frequencies observed in this chapter) there was no difference in TGS between athletes with no history of concussion (49.0) and athletes with a history of at least one concussion (39.3) after Bonferroni adjustment ($P > 0.015$), as shown in Figure 5.2 (Panel

A). In addition, there was no difference in TGS between athletes with no history of concussion and athletes with a history of multiple concussions (TGS 40.1) after Bonferroni adjustment ($P > 0.025$) (Table 5.2). There was no difference in TGS between the <10 days recovery group and the >10 days recovery group ($P = 0.327$). Similarly, there was no difference in TGS between athletes with and without family history of neurological conditions ($P = 0.658$).

When the upper and lower thirds of the TGS algorithm Model 3 were compared, athletes with a history of at least one concussion were underrepresented in the upper third (65.5%) and overrepresented in the lower third (89.7%) when taking into account athletes with no history of concussion (respective values in upper third 34.5%, lower third 10.3%; $P = 1.3 \times 10^{-7}$). Similarly, athletes with history of multiple concussions were underrepresented in the upper third (54.5%) and overrepresented in the lower third (83.3%) when taking into account athletes with no history of concussion (respective values 45.5% and 16.7%; $P = 12.4 \times 10^{-5}$). ROC AUC analysis confirmed the TGS algorithm could identify history of at least one concussion (AUC = 0.376; 95% CI = 0.269-0.483; $P = 0.034$), as shown in Figure 5.2 (Panel B). When the numbers of athletes in the upper and lower thirds of the <10 days recovery group TGS were compared to the >10 days recovery group, no differences were found ($P = 0.527$). Similarly, there were no differences between athletes with and without a family history of neurological conditions in the upper and lower TGS thirds ($P = 0.117$).

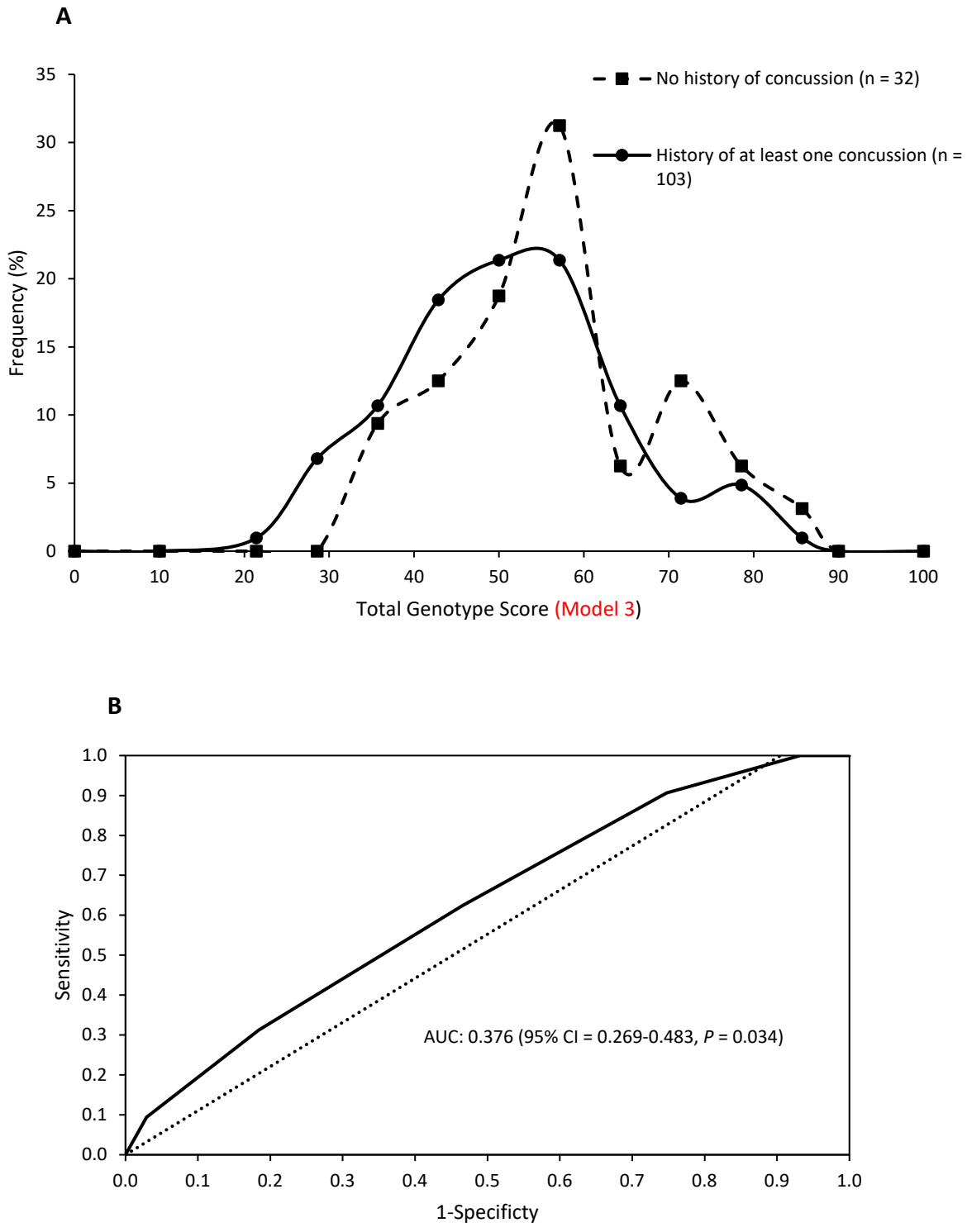


Figure 5.2. Panel A: Difference in frequency distributions of the TGS based solely on SNPs associated with a history of at least one concussion from elite rugby athletes with no history of concussion (*P* > 0.015 for comparison of means). Panel B: Receiver operating characteristic curve displays the ability of the significant data TGS to discriminate elite rugby athletes with a history of at least 1 concussion from elite rugby athletes with no history of concussion. Dotted line = no discrimination. AUC; area under the curve.

SNP epistasis

Multifactor dimensionality reduction (MDR) analysis identified a 3-SNP interaction of *APOE* rs405509, *COMT* rs4680 and *MAPT* rs10445337 that best predicted family history of neurological conditions (testing accuracy 0.55; cross-validation consistency 10/10). In addition, a single SNP MDR model of *APOE* rs405509 best predicted concussion recovery duration (testing accuracy 0.618; cross-validation consistency 10/10). However, MDR could not identify a model to predict concussion history with a sufficiently powerful cross-validation statistic ($P \leq 0.050$).

5.4 Discussion

The aims of this chapter were firstly, to investigate whether suspected concussion-associated polymorphisms are associated with history of previous concussion in elite rugby athletes. It was hypothesised that the concussion-associated risk genotypes and alleles would be overrepresented in elite rugby athletes with a history of previous concussion compared to those with no history of previous concussion. Secondly, to compare the polygenic characteristics of athletes with a history of concussion to those with no history of concussion. It was hypothesised that previously concussed elite rugby athletes would have a lower TGS than athletes with no history of concussion indicating a less 'preferable' polygenic profile.

The main findings from this chapter are that *APOE* rs405509, *BDNF-AS* rs6265 and *COMT* rs4680 are associated with concussion history in elite rugby athletes. For *APOE* rs405509, athletes with a history of at least one concussion had 2.10 times the odds of being GG genotype and athletes with a history of multiple concussions had 1.69 times the odds of being GG genotype. However, athletes with a history of concussion recovery periods of <10 days had 4.14 times the odds of being TT genotype. There are conflicting findings regarding *APOE* rs405509 in the literature. Abrahams et al. [55] similarly reported the *APOE* rs405509 TT genotype was associated with a 45% reduced risk of and concussion and the T allele was associated with a <1-week recovery period post-concussion, in a mixed cohort of youth and professional South African RU players. However, the T allele and TT genotype of *APOE* rs405509 have also been associated with greater risk of concussion [20]. Our findings are therefore compatible with Abrahams et al. [55], because lower risk of concussion via the

TT genotype would provide less disruption to rugby training and selection and potentially influence recovery duration post-concussion. However, further replication studies are warranted to support this observation.

For *BDNF-AS* rs6265, athletes with a history of at least one concussion had 17.75 times the odds of being GG (Val/Val) genotype and athletes with a history of multiple concussions had 12.5 times the odds of being GG genotype. Our findings are in contrast to those of Dretsch et al. [167], who reported that 17% of AA (Met/Met) carriers suffered a concussion compared to 4% of GG carriers during military deployment. However, the A allele has been previously associated with 2-6 times poorer neurocognitive performance post-concussion compared to G allele carriers [168]. Therefore, GG carrier athletes could perform better in measures of memory, executive function, attention and overall cognitive performance post-concussion compared to AA carrier athletes post-concussion.

For *COMT* rs4680, athletes with a history of at least one concussion had 2.90 times the odds of being AA (Met/Met) genotype and athletes with a history of multiple concussions had 3.00 times the odds of being AA genotype. Similarly, Mc Fie at al. [42] recently reported A allele carriers in a cohort of youth and professional South African RU players were ~3-fold more likely to have a history of concussion. The A allele carriers could place themselves at increased risk of sustaining a concussion due to the altered behavioural traits of impulsivity and risk taking, resultant from elevated dopamine [129,130]. This in part supports the previous findings that elite rugby players have ~1.4 times the odds of being GG genotype (Chapter 3), i.e. hypothetically, those less likely to place themselves at even greater (unnecessary) risk than is required for effective competitive rugby (GG genotype) are ultimately more successful in the sport than alternative genotypes at the same locus, perhaps via avoidance of additional (potentially concussion-inducing) collisions.

There were no associations between any other polymorphism examined in this chapter and concussion history. However, based on previous biological and clinical data regarding those polymorphisms, this chapter confirms the existence of a considerable number of athletes who appear to have some genetic predisposition for sustaining repetitive concussions and/or poorer outcomes post-concussion [41,230].

Multifactor dimensionality reduction analysis found a 3-SNP model of *APOE* rs405509,

COMT rs4680 and *MAPT* rs10445337 produced the best model for predicting concussion risk (testing accuracy 0.55; cross-validation consistency 10/10). In addition, a single SNP MDR model of *APOE* rs405509 polymorphism best predicted concussion recovery duration (testing accuracy 0.618; cross-validation consistency 10/10). Elite rugby athletes who possess the T allele and TT genotype of *APOE* rs405509 could have a decreased risk of concussion and shorter recovery duration post-concussion [55]. Due to the pleiotropic nature of *COMT* (rs4680), G carriers could possess greater stress resilience and reduced anxiety in competitive environments and be at lower risk of experiencing concussions, but also be at risk of poorer cognitive function post-concussion (Chapter 3, 129,130]). The *MAPT* TT genotype (rs10445337) has been weakly associated with a greater risk of repeated concussion [21,22], potentially as a result of aggregation of neurotoxic hyperphosphorylated tau in response to repetitive concussions [152,232]. It should be noted that SNP-SNP interaction analyses rely on data mining to identify the best genetic model to fit the data, potentially leading to overfitting. Cross-validation was utilised to compensate, however the 3-SNP and 1-SNP model identified in this chapter should be investigated in other cohorts to confirm.

TGS Models 2 and 3 which used only observed data from this chapter, suggested mean TGS for athletes with no history of concussion was higher than athletes with previous concussion history. Thus, supporting the chapter's hypothesis. This was in contrast to TGS Model 1 (based on prior literature) which indicated mean TGS for athletes with a history of concussion was higher than athletes without. However, for all three TGS algorithms there was no significant difference in athletes with no history of concussion TGSs compared to athletes with a history of at least one concussion and those with a history of multiple concussions. Only *APOE* rs405509, *BDNF-AS* rs6265 and *COMT* rs4680 were associated with concussion history in elite rugby athletes. Therefore, it is possible that the TGSs Models 1 and 2 included polymorphisms that potentially do not influence concussion risk in elite rugby athletes and therefore decrease discriminatory accuracy of these TGS Models [262]. All the TGS algorithms gave all the SNPs equal weighting as it was assumed that the allelic effects are codominant and each SNP had an equal additional effect, whereas in reality some variants will have larger effects on a phenotype than others [261].

As anticipated, TGS distributions of athletes with a history of concussion compared to no

history did differ at their extremes. The prior literature-based TGS algorithm Model 1 demonstrated athletes with history of previous concussions had a higher frequency in the top third compared to athletes with no history, which is in contrast to the chapter's hypothesis. However, TGS algorithm Models 2 and 3 demonstrated that athletes with history of previous concussions had a lower frequency in the top third compared to athletes with no history of concussion. Existing evidence supporting sports-related concussion gene associations is limited and few findings have been replicated. The lack of empirical evidence supporting allocations of TGS SNP scores could account for the lack of sensitivity of the prior literature-based approach used in this chapter. Findings from the present chapter indicate allocation of SNP TGS scores based on observations from this chapter (admittedly not all significant) produced TGS models (2 and 3) that could differentiate between TGS distributions and concussion history in elite rugby athletes. ROC analyses found the prior literature-based TGS Model 1 and TGS Model 3 algorithm based solely on SNPs associated with history of concussion in elite rugby athletes to have significant discriminating accuracy in identifying concussion risk in elite rugby athletes, which does enable a distinction between groups athletes with a history of at least one concussion and those without. However, no differences in TGS for all algorithms were observed between no history of concussion and history of multiple concussion, athletes with different durations of recovery from concussion and family history of neurological disorders groups. These findings indicate that elite rugby athletes with no history of concussion tend to have a different polygenic concussion-associated profile than athletes with a previous history of concussion. Further research is warranted to investigate gene-associations and recovery and long-term neurological disorders in high concussion risk sports.

The limitations of this study were the inclusion of self-reported ancestry and history of concussions. While sport related-concussion diagnosis often depends on self-reported concussion symptoms by athletes [20–22], recall bias may reduce the accuracy of concussion history data [210].

5.5 Conclusion

The novel findings presented in this chapter, although preliminary, support the growing evidence that concussion incidence and recovery in elite rugby athletes could be partly

affected by an individual's genetic predisposition. The GG genotype of *APOE* rs405509, GG genotype of *BDNF-AS* rs6265 and AA genotype of *COMT* rs4680 were more common in elite rugby athletes with a concussion of history, suggesting higher risk of experiencing a concussion for carriers of these genotypes. In addition, the TT genotype of *APOE* rs405509 was more common in elite rugby athletes with a concussion recovery duration of <10 days. Allocation of TGS SNP scores based on observations of athletes with a history of concussion compared to no history can be used to identify differences in TGS distributions. Epistasis analysis identified a 3-SNP genetic interaction model of *APOE* rs405509, *COMT* rs4680 and *MAPT* rs10445337 for altered risk of concussion incidence and severity in elite rugby athletes. The novel findings presented in this chapter support the growing evidence that incidence, severity and recovery from concussion could be influenced by an athlete's genetic predisposition. Such knowledge could be used, in future and when additional relevant variants have been identified, to inform individualised management strategies for athletes in possession of risk genotypes.

General discussion

6.1 Overview of thesis

Rugby (league and union) has one of the highest reports of concussion in male professional sports. Concussion is a complex phenotype, influenced by environmental factors and an individual's genetic predisposition. It was postulated that to achieve elite athlete status in rugby, athletes could possess genetic resistance to concussion to facilitate prolonged participation in a high concussion-risk environment. Indeed, Heffernan et al. [54] reported associations between injury risk-associated polymorphisms (*COL5A1* rs12722 and rs3196378; associated with musculoskeletal soft tissue injury) and elite rugby athlete status. However, there is limited evidence of association between specific gene variants and concussion in elite rugby. The work presented in this thesis is concerned with investigating the genetic influence of concussion related polymorphisms within a population of elite rugby players.

The biological and clinical associations of suspected concussion-associated polymorphisms (reviewed in Chapter 1) suggest possession of the risk alleles might limit an individual's ability to withstand exposure to the environment of competitive rugby, due to an elevated risk of repeated concussions and greater risk of delayed recovery and consequent neurological impairment. Such individuals would be more likely than their peers to miss training, selection and competitive events important for career progression [14]. As such, the overall aim of the current thesis was to investigate whether elite rugby athletes differ in terms of genetic risk factors for frequency and severity of concussion compared to non-athletes. More specifically, the objectives were:

1. Recruit additional participants for the ongoing RugbyGene project with the purpose of investigating molecular characteristics of elite rugby athletes.
2. Investigate whether genotype frequency of suspected concussion-associated polymorphisms (*ANKK1* rs1800497, *APOE* rs429358, rs7412 and rs405509, *BDNF-AS* rs6265, *COMT* rs4680, *MAPT* rs10445337 and *NOS3* rs2070744) differ between elite rugby athletes and non-athletes, and between RU playing positions.
3. Investigate if concussion associated polygenic profiles differ between elite rugby athletes compared to a non-athlete control population via the application of a total

genotype score (TGS) algorithm. Secondly, to compare the polygenic difference between RU forwards and RU backs.

4. Investigate whether suspected concussion-associated polymorphisms are associated with history of previous concussion in elite rugby athletes.

6.2 Main experimental findings

This thesis used a prioritised selection of SNPs based on empirically evidenced biological pathways of TBI, providing strength to the candidate gene approach utilised in this thesis [41,127]. The effect of suspected concussion-associated alleles on concussion risk is expected to be small, which affected the sample size required. In contrast to other methods of genetic variance such as GWAS, candidate gene approach sample sizes can be smaller [277], which is compatible with a study of elite rugby athletes, who are rare by definition. Nevertheless, by increasing the size of the ongoing RugbyGene project [192] cohort of elite male rugby athletes (Objective 1), sample size was increased to detect a 'small' effect size using *a-priori* calculation for 80% power.

Briefly, elite rugby athlete status is associated with the GG (Val/Val) genotype of *COMT* rs4680 that, acting pleiotropically, could affect stress resilience and behavioural traits during competition, concussion risk and/or recovery from concussion (Chapter 3, Objective 2). Consequently, assessing *COMT* rs4680 genotype might aid future individualised management of concussion risk amongst athletes. However, there were no differences in genotype or allele frequency when comparing all athletes with non-athletes for all other polymorphisms (*APOE* rs429358, rs7412 and rs405509, *ANKK1* rs1800497, *BDNF-AS* rs6265, *MAPT* rs10445337 and *NOS3* rs2070744). In addition, there was no difference in genotype frequency between RU backs and forwards for any polymorphism studied (Chapter 3, Objective 2).

Occurrence of and outcomes following concussion are probably affected by the interaction of multiple genes in a polygenic manner. Contrary to the hypothesis, the findings in Chapter 4 (Objective 3) demonstrated that the TGS based on prior literature (Model 1) did not differ between any elite rugby athlete group and non-athletes, nor between rugby union forwards and backs. Accordingly, the TGS could not discriminate between elite rugby athletes and non-athletes, suggesting that for the eight polymorphisms investigated elite

rugby athletes do not have a more 'preferable' concussion-associated polygenic profile than non-athletes. However, the *COMT* (rs4680) and *MAPT* (rs10445337) G-C allele combination was more common in rugby athletes and rugby union athletes than non-athletes. These findings suggest a genetic interaction between *COMT* (rs4680) and *MAPT* (rs10445337) could assist rugby athletes to achieve elite status.

Finally, Chapter 5 investigated whether suspected concussion-associated polymorphisms are associated with history of previous concussion in elite rugby athletes (Objective 4). The main findings from Chapter 5 are that *APOE* rs405509, *BDNF-AS* rs6265 and *COMT* rs4680 are associated with concussion history in elite rugby athletes. However, there was no association between any other polymorphism examined in this chapter and concussion history. Based on previous biological and clinical data regarding those polymorphisms, data from Chapter 5 confirms the existence of a considerable number of elite rugby athletes who appear to have a genetic predisposition for sustaining repetitive concussions and/or poorer outcomes post-concussion, which is similar to the proportion of non-athletes. A 3-SNP model of *APOE* rs405509, *COMT* rs4680 and *MAPT* rs10445337 produced the best model for predicting concussion risk and a single SNP model of *APOE* rs405509 polymorphism best predicted concussion recovery duration. Three TGS algorithms (Model 1 based on prior literature, Model 2 based on elite rugby athlete genotype frequencies observed in Chapter 5, Model 3 based on SNPs associated with history of concussion in elite rugby athletes from chapter 5) were used to quantify the combined influence of the candidate polymorphisms in elite rugby athletes. For all three TGS algorithms, there was no significant difference in TGS between athletes with no history of concussion and those with a history of at least one concussion or those with a history of multiple concussions. However, TGS distributions of athletes with and without concussion history did differ at their extremes, indicating that elite rugby athletes with no history of concussion tend to have a different polygenic concussion associated profile than athletes with previous history of concussion.

6.2.1 *COMT* rs4680

COMT rs4680 was investigated for associations with elite athlete status and concussion history, having been previously associated with behavioural traits[42,170], history of previous concussions [171] and executive function post-concussion [233–236]. In Chapter

3, elite rugby athletes had a higher frequency of GG (Val/Val) genotype than non-athletes. This suggests that achievement of elite status in rugby could be influenced by possession of the GG genotype, acting pleiotropically, potentially affecting stress resilience and behavioural traits during competition, concussion risk and/or recovery from concussion. In contrast, in Chapter 5, the AA (Met/Met) genotype of *COMT* rs4680 was more common in elite rugby athletes with a history of concussion. These findings are supported by Mc Fie at al. [42], as South African RU players (youth and professional) possessing the A allele were ~3-fold more likely to have a history of concussion. It is postulated that the effects of elevated dopamine in A allele carriers could increase their risk of sustaining a concussion due to the altered behavioural traits of impulsivity and risk taking [129,130]. However, the pleiotropic effects of *COMT* rs4680 could suggest that the AA athletes with a history of concussion could have better cognitive function post-concussion than GG homozygotes [170,237].

The prefrontal cortex's sensitivity to dopamine affects cognitive performance and behavioural traits thus influencing inter-individual variability in concussion risk and cognitive function post-concussion [238]. High activity of COMT reduces the quantity of dopamine in the prefrontal cortex [238]. Indeed, AA genotype carriers have a ~33% decreased COMT activity compared to GG genotype carriers [278]. Reduced or excessive dopamine could impact a range of cognitive functions and behavioural traits in a pleiotropic manner pre-and post-concussion, which makes it an ideal candidate gene for further sport-related concussion research. In addition, dopamine appears to modulate the function of other concussion-associated SNPs such as those in *MAPT* [172]. Further investigation into the role dopamine has on concussion risk, recovery and interaction with other concussion-associated genetic variants is warranted.

6.2.2 *APOE* variants (rs429358, rs7412 and rs405509) and *MAPT* rs10445337

APOE variants rs429358, rs7412 and rs405509 were investigated for associations with elite athlete status and concussion history, having been previously associated with increased risk of concussion [20,21], poorer outcome post-concussion [43,55] and neurodegenerative disease [142,143]. In Chapter 3, there were no differences in *APOE* ε2/ε3/ε4 genotype or ε4 allele possession frequency when comparing elite rugby athletes to non-athletes. Similarly, there were no differences in *APOE* rs405509 genotype or allele frequency

between elite rugby athletes and non-athletes. However, similar to the non-athlete cohort ~30% of elite rugby athletes were ε4 carriers. APOE ε4 suppresses neurite growth [120,121] and has been associated with the formation of neurodegenerative amyloid plaques [132], which could influence concussion risk and post-concussion recovery. Possession of an ε4 allele could increase the risk of cognitive and physical impairments post-concussion compared to non-carriers [43,142,231]. However, in Chapter 5 there was no difference in *APOE* ε2/ε3/ε4 genotype or ε4 allele possession frequency when comparing athletes with a history of concussion and those with no history of concussion. Studies associating *APOE* ε4 and sport-related concussion are still few and findings are conflicting [20,21,43,55,140], so further replication studies are warranted to determine the association of *APOE* and concussion risk. In addition, to the author's knowledge there have been no long-term investigations of concussion and *APOE* genotypes in sports, which could reveal potential neurodegenerative consequences of concussion.

For *APOE* (rs405509) ~50% of the elite rugby athletes were carriers of the T allele and, of those, ~25% possessed the TT genotype that could put them at altered risk of experiencing repeated concussion and altered recovery durations post-concussion [20,21,55,144]. However, in Chapter 5, athletes with a history of concussion had a higher frequency of GG genotype than elite rugby athletes with no history of concussion, which is consistent with the findings of Abrahams et al. [47] as the TT genotype was associated with a reduced risk of concussion. In addition, athletes with concussion recovery <10 days had 4 times the odds of being TT genotype than athletes with concussion recovery >10 days. *APOE* (rs405509) has been previously associated with quantitative impacts on APOE levels in brain tissue, which could influence recovery duration post-concussion [143].

MAPT rs10445337 was investigated for associations with elite athlete status and concussion history, having been previously been weakly associated with greater risk of repeated concussion risk [21,22], while mutations in *MAPT* have been associated with neurodegenerative tauopathy in response to repetitive concussions [152,232]. In Chapter 3, there were no differences in *MAPT* rs10445337 genotype or allele frequency when comparing all athletes with non-athletes. Similarly, in Chapter 5, there were no differences in *MAPT* rs10445337 genotype or allele frequency when comparing athletes with and without a history of concussion. Recently, other *MAPT* SNPs (rs2435211 and rs2435200)

have been associated with risk of sustaining concussions and future replication studies are warranted to verify this data [29].

6.2.3 *ANKK1* rs1800497, *BDNF-AS* rs6265 and *NOS3* rs2070744

ANKK1 rs1800497 was investigated for associations with elite athlete status and concussion history, having been associated with influencing recovery from a TBI event by modulating working memory and cognitive performance post-TBI [183–185]. In Chapter 3, there were no differences in *ANKK1* rs1800497 genotype or allele frequency when comparing all athletes with non-athletes. Similarly, in Chapter 5 there were no differences in *ANKK1* rs1800497 genotype or allele frequency when comparing athletes with and without a history of concussion. Future studies could investigate the role of *ANKK1* and other dopaminergic genetic variants known to affect cognitive performance in post-concussion recovery.

BDNF-AS rs6265 was investigated for associations with elite athlete status and concussion history, having been associated with risk of sustaining a concussion [167] and neurocognitive performance post-concussion [168]. In Chapter 3, there were no differences in *BDNF-AS* rs6265 genotype or allele frequency when comparing all athletes with non-athletes. However, in Chapter 5, athletes with a history of concussion had a higher frequency of GG (Val/Val) genotype than athletes with no history of concussion. These findings are not in agreement with previous ones by Dretsch et al. [167], however, who reported that AA (Met/Met) carriers were more likely to suffer a concussion that GG carriers. Incongruent findings could be due methodological differences in study designs such as sample size (141 vs. 24), procedure of assessing concussion history (self-reported questionnaire vs. brief TBI screen), and participants (elite rugby athletes vs. military personnel of differing geographic ancestry, sex, age and severity of TBI). In addition, in Chapter 5, the genotype distribution of *BDNF-AS* rs6265 in athletes with no history of concussion was not in Hardy-Weinberg equilibrium, which could be a result of type I experimental errors [218,219] and produce false conclusions [220].

NOS3 rs2070744 was investigated for associations with elite athlete status and concussion history, having been associated with cerebral vasospasm [190] and ~20-35% lower cerebral

blood flow in patients with severe TBI who carry the C allele [119]. In Chapters 3 and 5, there were no differences in *NOS3* rs2070744 genotype or allele frequency when comparing all athletes with non-athletes. The reduced cerebral blood flow described in the neurometabolic cascade following a concussive event (Chapter 1) [102,103] provides a physiological rationale for further investigation of genetic variants that could alter cerebral blood flow, and consequently affect post-concussion recovery.

6.2.4 RU forwards and backs

For all candidate gene variants studied in this thesis there were no differences in genotype or allele frequency between RU backs and forwards. These findings suggest that RU forwards and backs do not have a different genetic predisposition for sustaining repetitive concussions and/or poorer outcomes post-concussion.

6.2.5 Concussion-associated polygenic profiles

Polygenic profiling was conducted to investigate whether suspected concussion-associated polygenic profiles differed between elite rugby athletes and non-athletes, between RU forwards and backs and between athletes with and without a history of concussion. To date, this is the first such analysis of a concussion-associated polygenic profile in any population. In Chapter 4, neither Model 1 TGS algorithm based on prior literature and Model 2 TGS algorithm based on genotype frequencies observed in elite rugby athletes in Chapter 3 demonstrated a difference between any rugby athlete group (all rugby athletes, RU, RL, RU forwards, RU backs) and the non-athletes. Thus, Chapter 4 suggests that elite rugby athletes do not have a more 'preferable' polygenic concussion associated profile than non-athletes, at least for the SNPs we investigated. This is in contrast to previous performance-related TGS studies, where athlete groups have demonstrated a higher mean and more 'preferable' performance-associated TGS than non-athletes [250,252,253,256,258]. However, the variable genetic potential with respect to concussion was observed through large interindividual variability (TGS range = 21-93).

Similarly, for the three TGS algorithms used in Chapter 5, there were no significant difference in TGS between athletes with no history of concussion and those with a history of at least one concussion or multiple concussions. These findings could be used to identify those individuals who possess a less 'protective' polygenic concussion profile and thus at

risk of concussion and/or recovery. In fact, TGS distributions did differ at their extremes, indicating that elite rugby athletes with no history of concussion tend to have a different polygenic concussion associated profile than athletes with previous history of concussion. In this present thesis, only *APOE* rs405509, *BDNF-AS* rs6265 and *COMT* rs4680 were associated with concussion history in elite rugby athletes. Therefore, there is a possibility that the TGS algorithms included polymorphisms that potentially do not influence concussion risk in elite rugby athletes and thus decrease discriminatory accuracy of the TGS algorithm [262]. Accuracy of the algorithm can be increased with the emergence of new candidate genetic variants, and future studies confirming and quantifying the magnitude of effect of existing known associated polymorphisms.

The complex pathophysiology of concussion could be due to epistasis and account for the likely limited influence of a single genetic variant [226]. In Chapter 4, elite rugby athletes had a higher frequency of a *COMT* (rs4680) and *MAPT* (rs10445337) G-C allele combination than non-athletes. This G-C allele combination could reduce the risk of experiencing concussions, thus lowering the injury burden for athletes and increasing their availability for training and selection opportunities. In Chapter 5, a 3-SNP genetic interaction model of *APOE* rs405509, *COMT* rs4680 and *MAPT* rs10445337 altered risk of concussion incidence and severity in elite rugby athletes. These interactions should be investigated in other cohorts to confirm them.

6.3 Methodological considerations and limitations

To date ~40 genetic variants have been investigated in relation to forms of TBI in a range of populations and many more are probably yet to be identified. However, only eight suspected concussion-associated genetic variants were investigated within this thesis, primarily due to limits on financial resources and time, as well as only recent emergence of some of the relevant literature (see Chapter 1).

A criticism of the candidate gene approach is lack of replication of findings [279,280]. Explanations for lack of replication might include differing populations and phenotype definitions. Nevertheless, prioritised selection of SNPs based on empirically evidenced biological pathways of a complex phenotype provides strength to the candidate gene approach studies [127]. Conversely, an advantage of the candidate gene approach is that

genes are selected utilising an *a priori* hypothesis based on the biological function of a particular protein and the specific phenotype [127,128]. However, it should be noted that association and causation are different concepts [281]. Causal inferences cannot be drawn from the findings of this thesis as to the mechanisms each genetic variant has in relation to concussion. In contrast to other research approaches, sample size can be smaller in the candidate gene approach [277], which suits the study of elite rugby players who are, by definition, rare. In fact, the present sample is to the author's knowledge the largest elite rugby cohort (n = 668) studied in this field to date - sufficient to detect a small effect size. However, only a considerably smaller sample (n = 141) was available with data regarding concussion history (Chapter 5), only sufficient to detect a moderate effect size. As is a common observation in this field, increasing sample sizes for all types of study presented in this thesis would increase statistical power and thus confidence in results.

A GWAS would enable the entire genome to be searched for common variations, as opposed to candidate areas only in a candidate gene approach. To date, only one concussion-associated GWAS has been conducted, which included ~4000 concussion cases (no information provided on athlete/non-athlete status of participants and aetiology of concussions) and ~290,000 controls [191]. By the nature of elite sport, the number of eligible individuals is low, for example the English Rugby Premiership is comprised of ~600 players, reducing the feasibility of GWAS. In addition, the GWAS approach only considers common genetic variants, rare genetic variants that could have much larger individual effects are excluded [191]. An alternative approach could be whole exome or whole genome sequencing as these approaches would include rare genetic variants [282]. However, currently financial costs are prohibitive ($1000 per genome [282]).Nevertheless, continued collaborative efforts to recruit more elite rugby athletes should, in time, enable a hypothesis-free approach to identify genetic markers for concussion risk to be adopted with a reasonable chance of success.

Retrospective concussion history data were collected using a self-reported questionnaire in Chapter 5. However, it is likely that data accuracy could be enhanced using prospective methods, as professional rugby players can underestimate the number of clinically diagnosed concussions by up to 30% during retrospective self-reporting [210]. Future collaborations with the Professional Rugby Injury Surveillance Project or Rugby World Cup

research teams, who have collected and continue to collect injury data in a well-defined and systematic fashion would enable a more detailed investigation of the potential genetic predisposition to concussion risk and/or poorer outcomes post-concussion in elite rugby athletes.

6.4 Conclusion

The current thesis investigated eight suspected concussion-associated genetic variants in a cohort of elite male rugby athletes in relation to elite status and history of sustaining previous concussions. Results suggest that genetic predisposition and genetic interaction between suspected concussion-associated genetic variants (*COMT* rs4680 and *MAPT* rs10445337) could assist rugby athletes to achieve elite status compared to non-athletes. A considerable number of elite rugby athletes possess several concussion-associated risk alleles and the inter-individual variability in injury occurrence and outcomes following concussion could be partially explained by the large range of TGS scores in elite rugby athletes. However, a concussion-focused polygenic model could not discriminate between elite rugby athletes and non-athletes. In contrast, concussion incidence and post-concussion recovery in elite rugby athletes appears to be partially influenced by an individual's genetic predisposition in a polygenic manner. Further advancements on the initial findings from this thesis could be used to inform individualised management strategies for athletes in possession of risk genotypes.

Genetic testing for concussion incidence, severity and outcome is currently not at the practical applications stage; this field of research is still in its preliminary findings stage. While it is possible to now obtain cheap and non-invasive genetic tests that could be tailored to include suspected concussion-associated variants, there is still a relative paucity of concussion-associated genetic research in sports. Indeed, more research is required to identify genetic variants that are significantly associated with concussion incidence, severity and outcome. To the author's knowledge, the participants described in this thesis are the largest homogenous cohort of elite athletes studied in relation to concussion-associated genomics. Nevertheless, the findings from the experimental Chapters within this thesis cannot yet, alone, be used to inform practitioners on how to better manage concussion within a sporting environment.

To strengthen the evidence base towards translation to practical application, increasing the sample size from current 141 concussion-related injury data points to at least >600 is required. This would allow detection of a small effect size, as opposed to only a moderate size (see Chapter 5). If the pattern of data presented in Chapter 5 continues in the future larger sample, this should also narrow the confidence intervals of future effect size estimates. However, not only 'small' but also 'very small' effect sizes are likely to be common, which would mean a further increase in sample size will be required to capture and accumulate a satisfactorily large proportion of the genetic component that is believed to exist, before arguments for translation to practical application becoming convincing.

In addition, mechanistic studies should be conducted to better understand the biological pathways of newly identified genetic variants and gene-gene interactions, to develop understanding of the complex pathophysiology of concussion. Future studies should be conducted with new cohorts of athletes in different sports, because some gene-injury associations are likely to be sport-specific.

6.5 Directions for future research

Within the studies documented in Chapters 3, 4 and 5 eight suspected concussion-associated SNPs (*ANKK1* rs1800497, *APOE* rs429358, rs7412, *and* rs405509, *BDNF-AS* rs6265, *COMT* rs4680, *MAPT* rs10445337 and *NOS3* rs2070744) were selected to investigate genetic aspects of concussion within elite rugby athletes and non-athletes. Only *COMT* rs4680 and *MAPT* rs10445337 were associated with elite status and *APOE* rs405509, *BDNF-AS* rs6265 and *COMT* rs4680 were associated with concussion history in a smaller cohort. Approximately 40 SNPs have been investigated in relation to concussion to date and many more are probably yet to be identified. Therefore, existing associations could be strengthened via replication studies, and/or new candidate genetic variants could be investigated in relation to sport-related concussion in elite and non-elite populations. In addition, only Caucasian elite rugby athletes were investigated in this thesis, so future studies should investigate concussion-associated candidate genes in those with different geographic ancestry, where genotype-phenotype associations may differ. For example, currently ~45% of National Rugby League athletes are of Pasifika heritage [283], and rugby athletes who have recent geographic ancestry in the Pacific Islands are probably the second

most common geographic ancestry group found in elite rugby generally, making studies more feasible involving that group than others.

Important mechanisms regarding concussion injury risk and recovery could be epigenetic ones. Modifications to gene expression can occur as a result of epigenetic mechanisms such as DNA methylation, histone modifications and post-transcriptional mechanisms which regulate non-coding microRNAs [284]. The study of epigenetics and concussion within elite rugby athletes was not the focus of this thesis, but could provide a future area of study to aid in better understanding concussion mechanisms and management of sport-related concussions.

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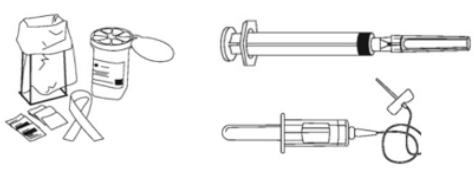
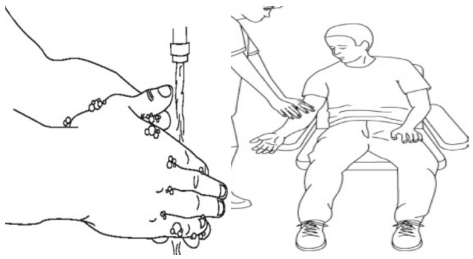
Appendix

1.



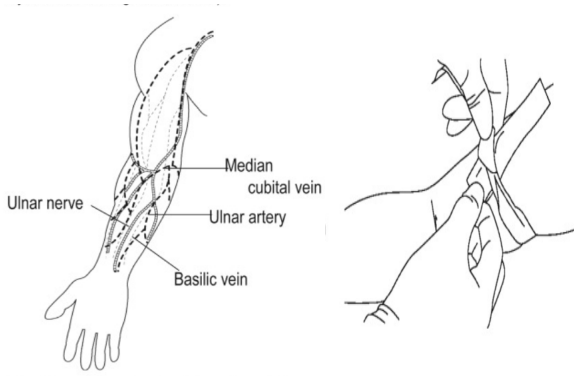
- Assemble equipment and include needle and syringe or vacuum tube dependent on which is used. Check forms of participant and label tubes.

2.



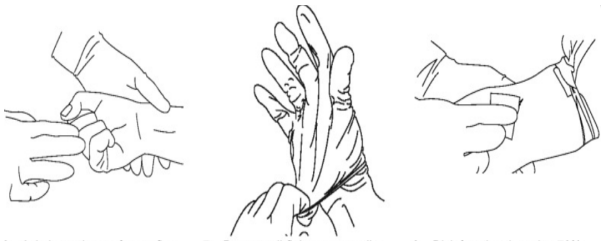
- Perform hand hygiene.
- Identify and prepare the patient.

3.



- Select the site at the antecubital fossa area. Palpate area to locate anatomic landmarks. Do not touch the site once antiseptic has been applied.
- Apply a tourniquet above the selected site.

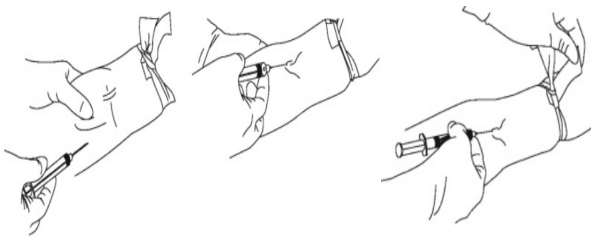
4.



- Ask participant to form a fist so that veins are more prominent.
- Ensure non-sterile gloves are worn.
- Disinfect the site and allow to dry completely.

5.

- Anchor the vein below the venepuncture site.
- Enter the vein swiftly at a 30^{o} angle.
- Once sufficient blood has been collected, release the tourniquet before withdrawing the needle



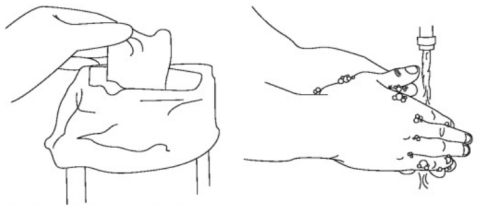
6.

- Withdraw the needle gently and provide the participant a clean gauze or dry cotton-wool ball to apply to the site with gentle pressure. Discard the needle or blood sampling device in to a sharps bin.
- Check the label and forms for accuracy.

7.

- If transferring blood to a EDTA tube, press the plunger slowly to reduce haemolysis.
- Place stopper in the tube.
- Follow laboratory instructions, invert the sample gently to mix EDTA additives with the blood before storage.

8.



- Discard sharps in a suitable sharps bin. Place items that can drip blood into the infectious waste bin.
- Remove gloves and place in contaminated waste bin.
- Perform hand hygiene.

Figure 2.1. Adult venipuncture instructions [adapted from [285]].

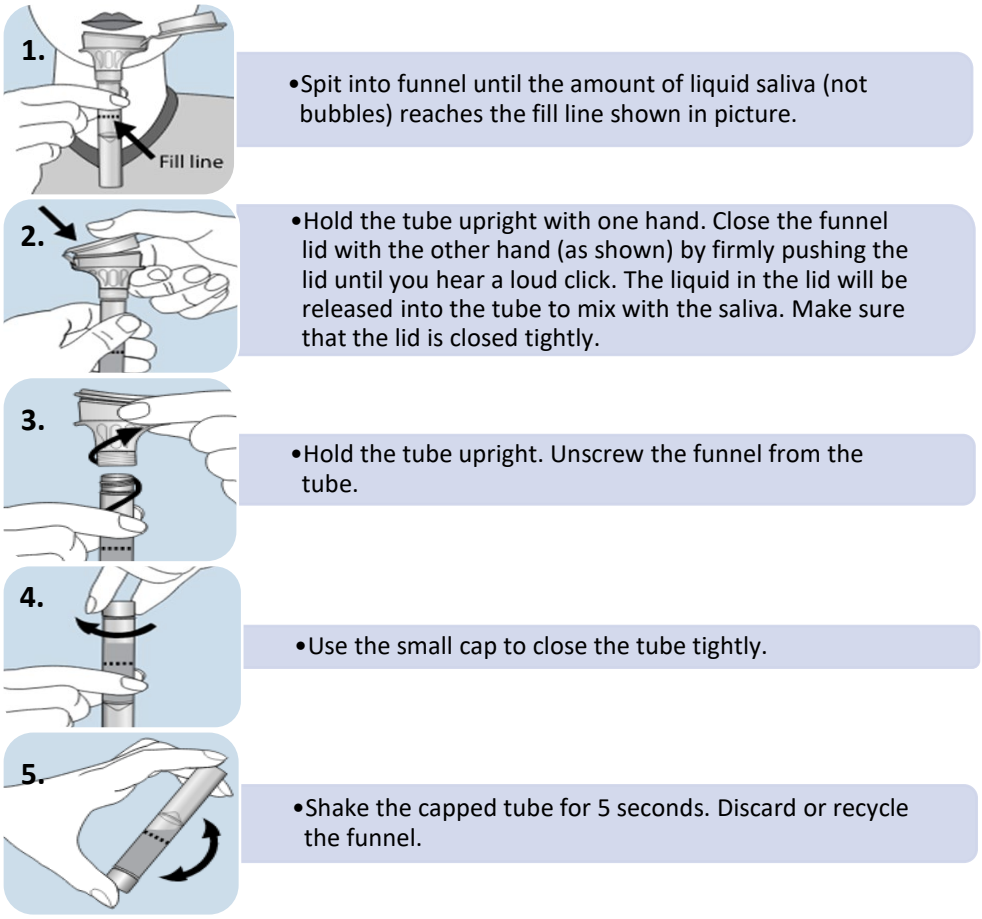
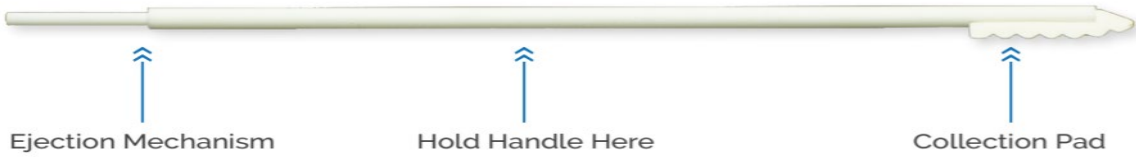


Figure 2.2. Oragene DNA OG-500 collection instructions [286].



- Instruct participants not eat or drink 1 hour prior to giving the sample.
- Remove swab from packaging and do not touch the collection pad of the swab.
- Holding the handle swab, scrape the collection pad firmly against the inside of the cheek for ~30 s, a second swab was collected from the opposite cheek.
- Avoid soaking the collection pad with saliva.
- Unscrew the labelled tube, insert the pad into a labelled sterile tube and push the end of the handle to eject collection pad into tube.

Figure 2.3. OmniSwab instructions.

The Genetic Profile of Elite Athletes: Concussion Questionnaire

22. Have you ever been concussed or knocked out? Yes ☐ No ☐

23. If Yes, how many times have you been concussed or knocked out?

_____ times

24. What were you doing at the time of the injury(ies)? E.g. rugby tackle, boxing, road accident.

_____
_____

25. If Yes, how long was your recovery period, until the day when you had no signs and symptoms and were free to train and play fully? (tick, multiple times if necessary, any recovery periods that apply for the different occasions)

<7 days ☐ 7-10 days ☐ 10-20 days ☐

20-40 days ☐ 40-60 days ☐ >60 days ☐

26. If Yes, was/were your concussion(s) or knock-out(s) diagnosed by a medical Yes ☐ No ☐

professional? (tick, multiple times if necessary, any that apply)

27. Does anyone in your close family (parents, siblings or grandparents) suffer from a neurological condition, such as:

    Dementia, Alzheimer's disease, chronic traumatic encephalopathy (CTE), cognitive impairment, movement disorders, psychiatric disorders, motor neuron disease

Yes ☐ No ☐ Don't know ☐

Who and which condition(s)?

e.g. grandfather, dementia

_____

_____