GENETIC RISK FACTORS FOR ANTERIOR CRUCIATE LIGAMENT RUPTURES

By

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2. **Posthumus, M.** September, AV. O'Cuinneagain, D. van der Merwe, W. Schwellnus, MP. Collins, M. The *COL5A1* gene is associated with increased risk of anterior cruciate ligament ruptures in female participants. **American Journal of Sports Medicine,** In Press.
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4. **Posthumus, M.** September, AV. Keegan, M. O'Cuinneagain, D. van der Merwe, W. Schwellnus, MP. Collins, M. Genetic risk factors for anterior cruciate ligament ruptures: The *COL1A1* gene variant. **British Journal of Sport Medicine**, 2009. 43:352-356.
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ABSTRACTS IN INTERNATIONAL JOURNALS

1. **Posthumus, M.,** September, A.V., O'Cuinneagain, D., van der Merwe, W., Schwellnus, M.P., Collins, M. The type V collagen (*COL5A1*) gene is associated with anterior cruciate ligament injuries. **Archivos de Medicina del Deporte.** 2008. 25(6), 461.
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PRESENTATIONS AT INTERNATIONAL CONGRESSES

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1. **Posthumus, M.** September, A. Keegan, M. O'Cuinneagain, D. van der Merwe, W. Schwellnus, MP. Collins, M. The *COL1A1* gene and Anterior Cruciate Ligament injuries. 13th South African Society of Human Genetics Conference, Spier, Cape Town, 5-8th April 2009. (Poster Presentation)
2. **Posthumus, M.** September, A. O'Cuinneagain, D. van der Merwe, W. Schwellnus, MP. Collins, M. The *COL5A1* gene in associated with increased risk of anterior cruciate ligament ruptures in female participants.

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4. **Posthumus, M.,** September, A.V., Keegan, M., O'Cuinneagain, D., van der Merwe, W., Schwellnus, M.P., Collins, M. The *Bst*UI RFLP of *COL5A1* is associated with anterior cruciate ligament rupture. **3rd Annual Clinical Sports Medicine Conference**, Newlands, Cape Town, 8-10 October 2008. (Oral Presentation)

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ABBREVIATIONS

ACC	Accident compensatory commission
ACL	Anterior Cruciate Ligament
AFL	Australian Football League
ALL	All acute soft tissue injuries group
AMB	Anteromedial bundle
ANOVA	One-way analysis of variance
AUS	Australia
BMI	Body Mass Index
bp	Base pairs
CI	Confidence Interval
CL	Cruciate ligament ruptures group
CLSD	Cruciate ligament ruptures and shoulder dislocations group
COL12A1	The gene encoding for the α1 chain of type XII collagen
COL14A1	The gene encoding for the α1 chain of type XIV collagen
COL1A1	The gene encoding for the α1 chain of type I collagen
COL1A2	The gene encoding for the α2 chain of type I collagen
COL3A1	The gene encoding for the α1 chain of type III collagen
COL5A1	The gene encoding for the α1 chain of type V collagen
COL5A2	The gene encoding for the α2 chain of type V collagen
COL5A3	The gene encoding for the α3 chain of type V collagen
CON	Control group
DIR	Direct contact mechanism of injury sub-group
ECM	Extracellular matrix
FACIT	Fibril Associated Collagen with Interrupted Triple helixes
GJL	Generalised Joint Laxity
HWE	Hardy-Weinberg Equilibrium
IND	Indirect contact mechanism of injury sub-group
LCL	Lateral Collateral Ligament
MCL	Medial Collateral Ligament
MMP	Matrix metalloproteinsase
MMP3	The gene encoding for the Matrix metalloproteinase-3
NCAA	National Collegiate Athletic Association

NFL	National Football League
NON	Non-contact mechanism of injury sub-group
NWI	Notch Width Index
NZ	New Zealand
OR	Odds Ratio
PAGE	Polyacrylamide gel electrophoresis
PCL	Posterior Cruciate Ligament
PCR	Polymerase Chain Reaction
PLB	Posterolateral bundle
Q angle	Quadriceps angle
RFLP	Restriction Fragment Length Polymorphism
RUP	Achilles rupture group
SA	South Africa
SNPs	Single Nucleotide Polymorphism(s)
TEN	Achilles tendinopathy group
TNC	The gene encoding for tenacin-C
TNC	Tenacin-C protein
UK	United Kingdom
US	United States
UTR	Untranslated region
α1(I)	The α1 chain of type I collagen
α1(III)	The α1 chain of type III collagen
α1(V)	The α1 chain of type V collagen
α1(XII)	The α1 chain of type XII collagen
α2(I)	The α2 chain of type I collagen
α2(V)	The α2 chain of type V collagen
α3(V)	The α3 chain of type V collagen

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ABSTRACT

BACKGROUND

Participation in regular physical activity has numerous health benefits, but there is also a risk of musculoskeletal soft tissue injuries that may occur as a result of participation in physical exercise. The number of training and competition days lost due to anterior cruciate ligament (ACL) ruptures has been reported as the highest of all musculoskeletal soft tissue injuries, implying that ACL ruptures are among the most severe musculoskeletal soft tissue injuries that can occur during exercise. The exact aetiology and mechanisms that cause these ACL ruptures are still under investigation. A large number of research studies have been conducted to establish the potential extrinsic and intrinsic risk factors, as well as mechanisms for ACL ruptures. Specific intrinsic risk factors for ACL ruptures that have been reported include anatomical, hormonal, and neuromuscular factors. Recently, the possibility of genetic influence on the risk of ACL ruptures has been suggested, but to date, this area has received little attention.

In a single study a specific genetic sequence variation, namely the functional *COL1A1* Sp1 binding site polymorphism, has been associated with acute soft tissue injuries (cruciate ligament ruptures and shoulder dislocations). In this study, the rare TT genotype of this polymorphism was under-represented within the acute ligament injury groups. Since soft tissue injuries, including ACL

ruptures are multifactorial disorders, it is unlikely that only a single genetic variation is associated with an altered risk for ACL ruptures.

AIM OF THE THESIS

The primary aim of this thesis was to identify candidate genes that may be associated with ACL ruptures, and then use a genetic association approach following a case-control study design to identify specific sequence variants (single nucleotide polymorphisms, SNPs) within these candidate genes which may predispose individuals to ACL ruptures. Candidate genes (*COL1A1, COL5A1* and *COL12A1*) were selected based on the biological function of their encoded proteins (type I, type V and type XII collagen respectively) within the basic structural and functional unit of ligaments, namely the collagen microfibril. The objectives of the specific gene association studies which addressed the primary aim of this thesis were as follows:

- To determine if the rare TT genotype of the functional Sp1 binding site polymorphism within intron 1 of the *COL1A1* gene was associated specifically with ACL ruptures in an independent second South African Caucasian population with gender-matched controls. (**Study 1**)
- To determine if two sequence variants (*Bst*UI and *Dpn*II restriction fragment length polymorphisms, RFLPs) within the 3'-UTR of the *COL5A1* gene, which has previously been investigated in Achilles tendon injuries, were associated with an increased risk of ACL ruptures. Due to the reported increased risk of ACL ruptures in females, a secondary objective

of this study was to investigate if there were any gender-specific associations between the two *COL5A1* sequence variants and risk of ACL ruptures. (**Study 2**)

- To determine if two previously described non-synonymous genetic sequence variants, the *Alu*I and *Bsr*I RFLPs within exons 65 and exon 29 respectively, within the *COL12A1* gene, were associated with an increased risk of ACL ruptures. A secondary objective of this study was to investigate if there were any gender-specific associations between the two *COL12A1* sequence variants and risk of ACL ruptures. (**Study 3**)

The secondary aim of this thesis was to investigate the similarities and differences between the genetic risk factors for ACL ruptures and other soft tissue injuries. The genetic variants investigated in Study 2 and 3 of this thesis have previously been investigated as risk factors for other musculoskeletal soft tissue injuries (Achilles tendinopathy and/or Achilles tendon ruptures). However, the possible association of the functional Sp1 binding site polymorphism with Achilles tendon injuries has not been investigated. Therefore, the objectives of the studies which addressed the secondary aim of this thesis are as follows:

- To determine if the rare TT genotype of the functional Sp1 binding site polymorphism within intron 1 of the *COL1A1* gene was associated with other common acute (spontaneous Achilles tendon ruptures) and chronic (Achilles tendinopathy) injuries. (**Study 4**)

- To report the combined effect of the rare *COL1A1* TT genotype and the risk for acute soft tissue ruptures from the previously published study, and the results presented in this thesis (Study 1 and Study 4). (**Study 5**)
- To determine if there are any gender-specific *COL5A1 Bst*UI RFLP genotype effects in chronic Achilles tendinopathy when the two previously published studies were re-analysed. Another objective of this study was to investigate if the distribution of the *COL5A1 Bst*UI RFLP within the combined asymptomatic control participants from this thesis (Study 2), as well as the two previously published studies were age-dependant. **(Study 6)**

METHODS

One hundred and twenty nine Caucasian participants with surgically diagnosed ACL ruptures, and 310 apparently healthy, unrelated, physically active gender-matched Caucasian control (CON) participants were recruited for the studies that address the primary aim of this thesis (Study 1 – Study 3). Due to slight differences in design, only 130 of these CON participants, which had no history of ligament and/or tendon injury, were included in Study 1, while 217 CON participants, with detailed sports participation information, were included in Studies 2 and 3.

In addition, 85 participants diagnosed with chronic Achilles tendinopathy and 41 participants diagnosed with Achilles tendon ruptures, as well as an additional 126 apparently healthy, unrelated, Caucasian CON participants without any

history of symptomatic Achilles tendon injuries, which had previously been recruited for other published studies, were included in Study 4.

The participants included in each of the first four studies were genotyped foreither; (1) the functional *COL1A1* Sp1 binding site polymorphism, (2) the *COL5A1 Bst*UI and *Dpn*II RFLPs, and/or (3) the *COL12A1 Alu*I and *Bsr*I RFLPs.

The combined analyses of Study 5 and the comparative analysis of Study 6, which were done to answer the second aim of this thesis, included pooled data from previous studies of the thesis, as well as previous published papers.

RESULTS AND DISCUSSION

Primary aim: Genetic variants associated with risk for ACL ruptures

The rare TT genotype of the *COL1A1* Sp1 binding site polymorphism was significantly under-represented (OR=12.3; 95% CI 0.7 - 220.4; P=0.031) in participants with ACL ruptures, when compared to controls with no history of ligament or tendon injuries, in a second South African Caucasian population (Study 1). There was no evidence of a gender-specific genotype effect in this study. Furthermore, the *COL5A1 Bst*UI RFLP (Study 2) and the *COL12A1 Alu*I RFLP (Study 3) were significantly associated with ACL ruptures in female, but not male participants. The CC genotype of the *COL5A1 Bst*UI RFLP (OR=6.6, 95% CI 1.5 – 29.7; P=0.006) and the AA genotype of the *COL12A1 Alu*I RFLP

(OR=2.4, 95% CI 1.0 – 5.5; P=0.048) were significantly under- and over-represented, respectively, in the female ACL group, when compared to the female CON group. No significant genotype distributions between the CON and ACL groups were observed for the *COL5A1 Dpn*II and *COL12A1 Bsr*I RFLPs.

When compared to the CON group included in Study 1, the participants in the ACL group had a significantly higher family history of ligament injury (13.5% vs 39.6%, P<0.001). Similar findings were observed when the female ACL group was compared to the female CON group included in Studies 2 and 3 (21.5% vs. 50.0%, P=0.002). No significant difference was found when the male ACL group was compared to the male CON group included in Studies 2 and 3.

When all the ACL and CON participants within Study 2 were combined and analysed, the genotype distribution of the *COL5A1 Bst*UI RFLP was significantly different between participants with a family history of ligament injury, and participants without a family history of ligament injury (P=0.022). This finding remained significant when only female participants were analysed (P=0.005), but not when male participants were analysed (P=0.369). A similar finding was also observed in Study 3, where there was a trend (P=0.082) for the AA genotype of the *COL12A1 Alu*I RFLP to be over-represented in female participants with a family history of ligament injury, when compared to female participants without a family history of ligament injury.

Secondary aim: Genetic variants associated with other soft tissue injuries and traits

Although not statistically significant, the main finding from Study 4 was that the rare TT genotype of the *COL1A1* Sp1 binding site polymorphism was absent in participants with another acute soft tissue injury (spontaneous Achilles tendon ruptures). The TT genotype was however present in participants with chronic Achilles tendinopathy. When reported data for the *COL1A1* Sp1 binding site polymorphism from this thesis, and previously published data, were combined and analysed (Study 5), the TT genotype was shown to be associated with an 11.1 times reduced risk of acute musculoskeletal soft tissue ruptures (cruciate ligament ruptures, shoulder dislocations, and Achilles tendon ruptures). Furthermore, when only the risk of cruciate ligaments were analysed, the TT genotype was associated with a 15.1 times reduced risk of cruciate ligament rupture.

There was no evidence that the previously reported association of the *COL5A1* *Bst*UI RFLP with Achilles tendinopathy is gender-specific. There was however a significant age dependant increase in the CC genotype distribution (P=0.047) among the pooled male asymptomatic CON participants of Study 2 of this thesis, and previously published papers. A similar trend among the female participants was not observed.

CONCLUSION:

The novel findings of this thesis provide initial evidence that genetic elements within the genes which encode for structural components of the collagen microfibril are significant risk factors for ACL ruptures, in particular among females. Based on the research presented in this thesis, further research studies designed to identify genetic intrinsic risk factors for ACL ruptures are warranted. Once the findings of this thesis have been confirmed in other populations, and a stronger estimate of risk predicted, these genetic sequence variants should be included in multifactorial models developed to reduce the incidence of ACL ruptures.

CHAPTER 1

INTRODUCTION AND SCOPE OF THE THESIS

The regular participation in physical activity is an important component in the development and maintenance of a healthy lifestyle [1]. In spite of the numerous health benefits, there is however, an increased risk of injuries, particularly musculoskeletal soft tissue injuries, during participation in physical activity [2]. Among these injuries, anterior cruciate ligament (ACL) ruptures have been described as the most severe injury [3]. Although the incidence of ACL ruptures is relatively low in the general population, the devastating consequences that may result from such an injury highlight the importance to identify the risk factors that are associated with this injury by conducting scientific research studies.

The exact aetiology and mechanisms that cause this acute injury are largely unknown, however various intrinsic and extrinsic risk factors for ACL ruptures have been identified [4-6]. Research to date has primarily focused on identifying anatomical, hormonal and neuromuscular intrinsic risk factors for ACL ruptures [6]. In comparison there are only limited data suggesting genetic elements are also intrinsic risk factors for ACL ruptures [7-9]. Studies investigating a familial predisposition to ACL ruptures initially provided evidence that hereditary factors may play an important role in the development of these injuries [7;10]. More recently, a single study has found a specific genetic element, namely the

functional *COL1A1* Sp1 binding site polymorphism, to be associated with risk of acute ligament injuries, including cruciate ligament ruptures and shoulder dislocation [9].

The identification of additional intrinsic risk factors, specifically genetic risk factors, will further improve the understanding of the aetiology and mechanisms of ACL ruptures. As originally proposed by van Mechelen and colleagues [11] (Figure 1.1), understanding the aetiology and mechanisms of sport injuries is required to introduce appropriate evidence-based preventative measures which may result in a reduction in the incidence of injuries, such as ACL ruptures.

The primary objective of this thesis was therefore to identify specific genetic elements which may predispose an individual to ACL ruptures. Genetic association studies (case-control design) were used to test whether sequence variants (single nucleotide polymorphisms) within candidate genes modify the risk of ACL ruptures. Candidate genes were selected, based on their structural and biological function within the ligament. For the purpose of this thesis, only genes which encode for the basic structural and functional unit of ligaments, namely the collagen microfibril, were identified as candidate genes.

Certain genetic variants investigated in this thesis have previously been investigated as possible risk factors for Achilles tendon injuries, another common soft tissue injury that includes Achilles tendinopathy and/or spontaneous Achilles tendon ruptures. A second objective of this thesis was therefore to further investigate the similarities and differences between the

genetic risk factors for ACL ruptures, chronic Achilles tendinopathy and/or spontaneous Achilles tendon ruptures.

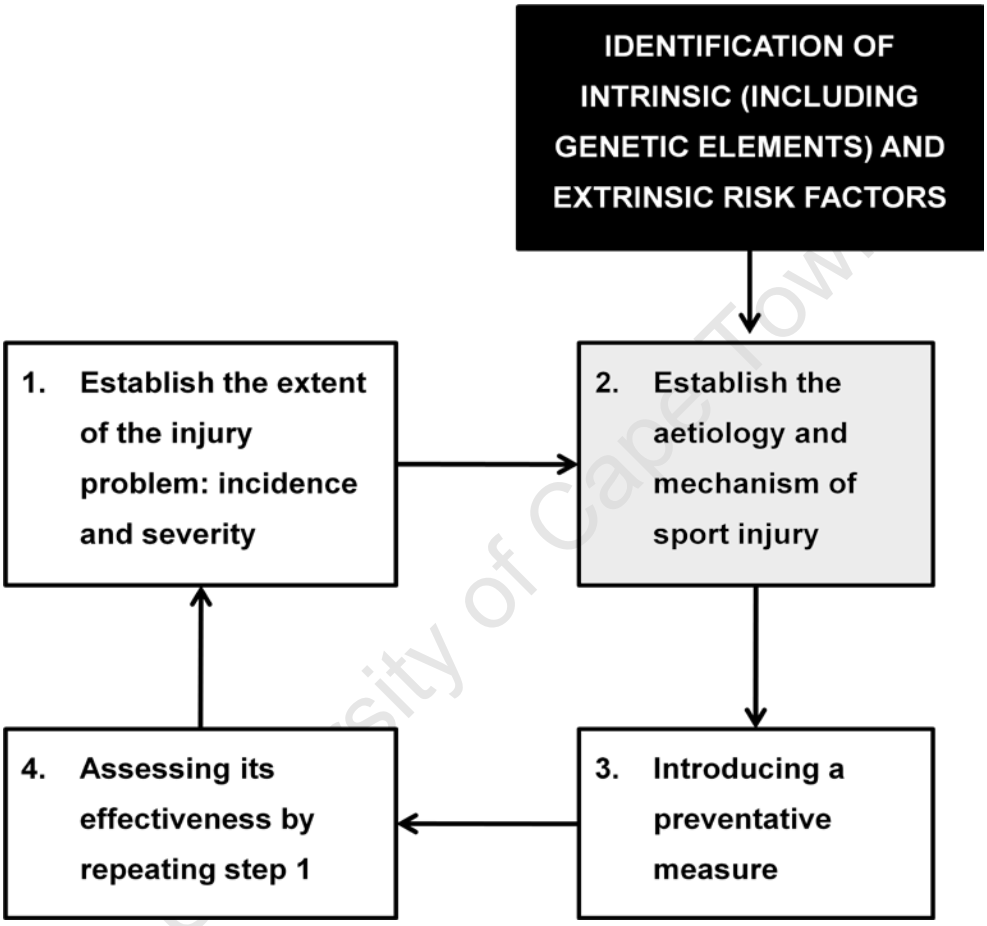

Figure 1.1: A modified four step model of sport injury prevention research as originally described by van Mechelen and colleagues [11]. The identification of intrinsic and extrinsic risk factors (black box) is essential in establishing the aetiology and mechanism of sport injuries (grey box).

In preparation for the exploration and further discussion of the experimental chapters of this thesis, Chapter 2 will provide a brief review of the gross anatomy of the ACL (Section 2.1), mechanisms of ACL ruptures (Section 2.2), the epidemiology of ACL ruptures (Section 2.3), the risk factors for ACL ruptures (Section 2.4), and the molecular structure of the ACL and selection of candidate genes for ACL ruptures (Section 2.5). Various comprehensive review articles covering similar topics have been published [4;6;12;13]. Therefore, this review will primarily focus on contemporary concepts, understanding and the evidence for identified extrinsic and intrinsic risk factors for ACL ruptures. Subsequent experimental chapters will use a candidate gene approach to achieve the primary (Chapters 3, 4 and 5) and the secondary (Chapters 6, 7 and 8) aims of this thesis.

CHAPTER 2

INTRINSIC RISK FOR ANTERIOR CRUCIATE LIGAMENT RUPTURES: A REVIEW

2.1 GROSS ANATOMY OF THE ACL

The anterior cruciate ligament (ACL) is an intra-articular ligament which connects the femur to the tibia (Figure 2.1). Together with the posterior cruciate ligament (PCL), the lateral collateral ligament (LCL) and the medial collateral ligament (MCL), the ACL guides the knee joint through its normal range of motion while a tensile load is applied [14]. This band-like structure of dense connective tissue runs from its femoral attachment on the posterior part of the inner surface of the lateral femoral condyle, medially, and distally to a fossa located anterior and lateral to the tibial spine [15].

Although not anatomically distinct, the ACL is commonly sub-divided into two functionally distinct bundles, the posterolateral bundle (PLB) and the anteromedial bundle (AMB) [15]. The AMB is slightly longer (34mm vs. 22.5mm) than the larger PLB and spirals around the rest of the ligament during flexion [16]. Both the PLB and the AMB consist of closely packed parallel bundles of collagens and other extracellular matrix (ECM) proteins [14]. The molecular structure, in particular the collagen component of the ECM is a key focus of this thesis, and will be further reviewed in more detail in section 2.5.1.

The specific function of the ACL is to resist anterior tibial translation and rotational loads over the range of motion of the knee [15]. The two functionally distinct bundles, the AMB and PLB, work synergistically with each other to optimise this restraining function of the ACL. These bundles experience different patterns of strain during passive knee flexion; the AMB lengthens and tightens during flexion, while the PLB shortens and becomes slack [17;18].

The collagen fibres, of the ACL are longitudinally arranged and display a crimp ("waviness"), which straightens when sufficient strain is applied to the ligament [19]. Once all the collagen fibres have been straightened, a sharp increase in stiffness is observed. The application of further strain at this point may result in ACL rupture [14]. Although there have been isolated reports of AMB and PBM ruptures, there is doubt whether a partial ruptures can actually occur [20]. The majority of studies classify significant injury to the ACL only as ruptures [4-6]. For the purpose of consistency in this thesis, all ACL injuries will be referred to as ACL ruptures.

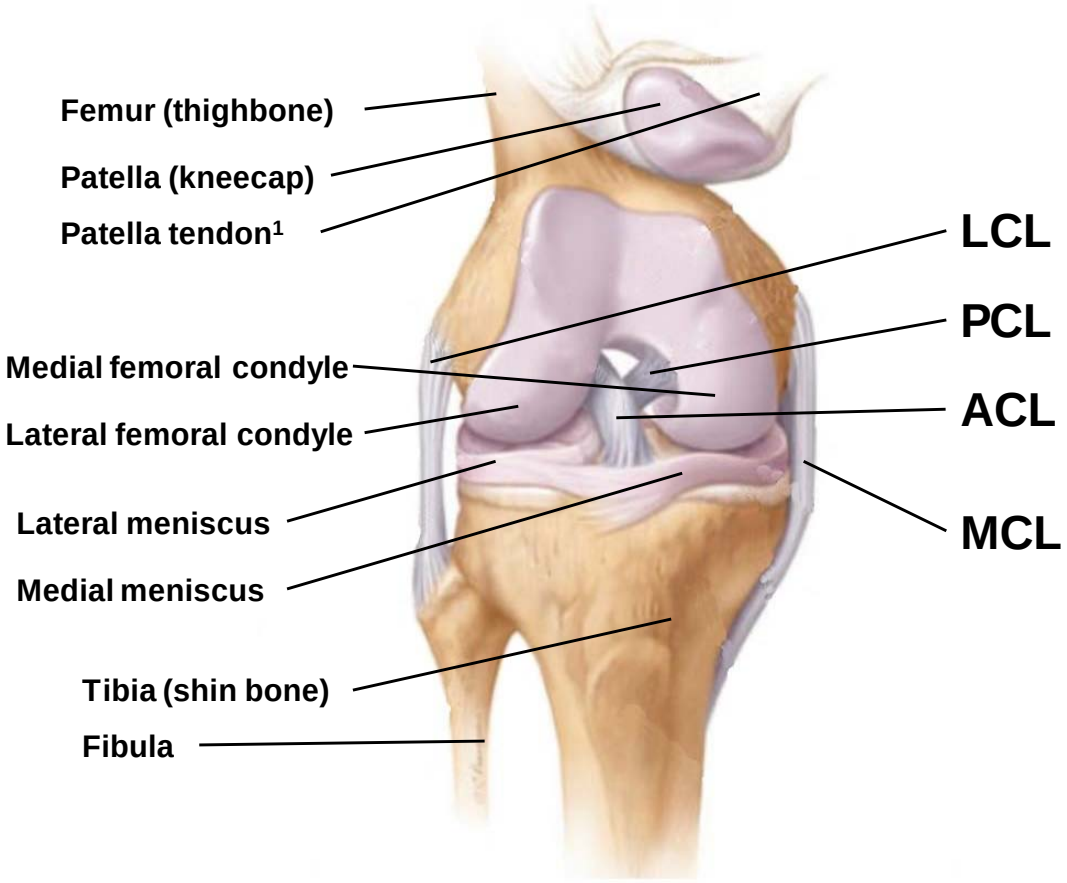

Figure 2.1: A schematic anterior view of the right knee. The patella and patella tendon are lifted to expose the inner structure of the knee joint. The locations of the primary ligaments (The anterior cruciate ligament (ACL), posterior cruciate ligament (PCL), the medial collateral ligament (MCL), and the lateral collateral ligament (LCL)) as well as other primary components of the knee joint and surrounding structures are indicated on the schematic diagram. [1] Although technically (by definition) a ligament, the structure which joins the patella bone to the tibia bone is commonly referred to as the patella tendon. Image adapted from Tandeter and Shvartzman [21].

2.2 THE MECHANISM OF ACL RUPTURES

2.2.1 The classification of mechanisms of injury

Although direct or indirect external forces to the knee can result in an ACL rupture, intrinsic or non-contact forces are also thought to play a critical role in the causation of ACL ruptures. Intrinsic forces occur as a result of the athlete's own movements [13], and not due to any external contact to the athlete. It may thus be hypothesised that intrinsic risk factors may have a greater contributing role in non-contact ACL ruptures, and it is therefore important to report the frequency of non-contact ACL ruptures separately from contact injuries.

There is however a lack of agreement regarding the classification and definition of ACL ruptures [13]. Although the majority of research has classified ACL ruptures as contact (direct contact to the knee) or non-contact (no direct contact to the knee); the lack of a common classification scheme has traditionally limited a researcher's ability to define non-contact injuries in a standardised manner. A standardised definition and classification scheme based on external force application was therefore recently proposed at the American Orthopaedic Society of Sports Medicine consensus conference on non-contact ACL ruptures (Atlanta, Georgia, "Hunt Valley II") [13]. This classification scheme divides ACL ruptures according to the degree of external force applied to the knee (Figure 2.2). Direct contact to the knee is classified as a direct contact mechanism of injury, whereas direct contact to the athlete, but not directly to the injured knee is classified as an indirect contact mechanism of injury [13]. A non-contact injury

is the result of the athlete's own movements and does not involve contact with another athlete or object [13]. For the purpose of this thesis, ACL ruptures will be classified using this scheme.

Direct contact

External force was directly applied to the injured knee as was probably the proximate cause of injury.

Example: Injured knee was forcefully struck by another player.

Indirect contact

External force was applied to the athlete but not directly to the injured knee. The force was involved in the injury process but was probably not the proximate cause.

Example: Injured athlete was struck and knocked off balance by an opponent in an area distal to the knee. Athlete's resultant movement led to the injury without direct contact to the knee.

Non-contact

Forces applied to the knee at the time of injury resulted from the athlete's own movements and did not involve contact with another athlete or object.

Example: Athlete landed from a jump and attempted to cut to one side.

Figure 2.2: Proposed classification scheme of the American Orthopaedic Society for sports medicine for ACL injuries by type of contact. This Figure was modified from Marshall et al. [13].

2.2.2 The mechanisms of non-contact ACL ruptures

ACL ruptures are unique when compared to other acute musculoskeletal soft tissue injuries, in that they are most commonly caused by non-contact mechanisms. The frequency of these non-contact injuries depends on the type of sports. Contact sports typically have a lower frequency of non-contact ACL ruptures, whereas sports which involve landing and pivoting have a much higher frequency of non-contact injuries [22]. The frequency of non-contact injuries thus varies from 21% during motor sports to 78% during netball [22]. The total frequency of all sports related ACL ruptures in a recent nationwide population based study in New Zealand, which were caused by a non-contact mechanism of injury, as previously defined [13], was 58.2% [22]. The population based study by Gianotti et al. [22] reported the mechanism of injury in 7375 cases of ACL ruptures. No other study has reported the mechanisms of injury in such a large cohort. Furthermore, Gianotti et al. [22] also used the proposed classification scheme (refer to Figure 2.2) which will be used in this thesis.

The mechanism of injury may also be explained by precise biomechanical descriptions at the time of injury, also referred to as the inciting event [23] (refer to Figure 2.6). Although various studies have attempted to describe the events which leads to non-contact ACL ruptures, the widely used terminology of injury mechanism has made interpretation of data difficult [23;24]. In spite of these difficulties it is generally accepted that non-contact ACL injuries typically occur with the knee in an extended position as the athlete lands from a jump, sidesteps or changes direction abruptly [23;24]. An expert panel of researchers

reviewing videos of ACL ruptures concluded that the ACL is likely to be ruptured if the following occurred: (1) the knee was less than 30º flexed, (2) the knee was in valgus, (3) the foot was in external rotation relative to the knee, and (4) the centre of gravity was behind the knee on landing a jump or stopping a run [25]. This biomechanical description is in agreement with the injury mechanism defined by Ireland [26] as the "position of no return", as described in Figure 2.3. In this position, failure of the muscles which normally protect the ACL, contribute to the rupture [24].

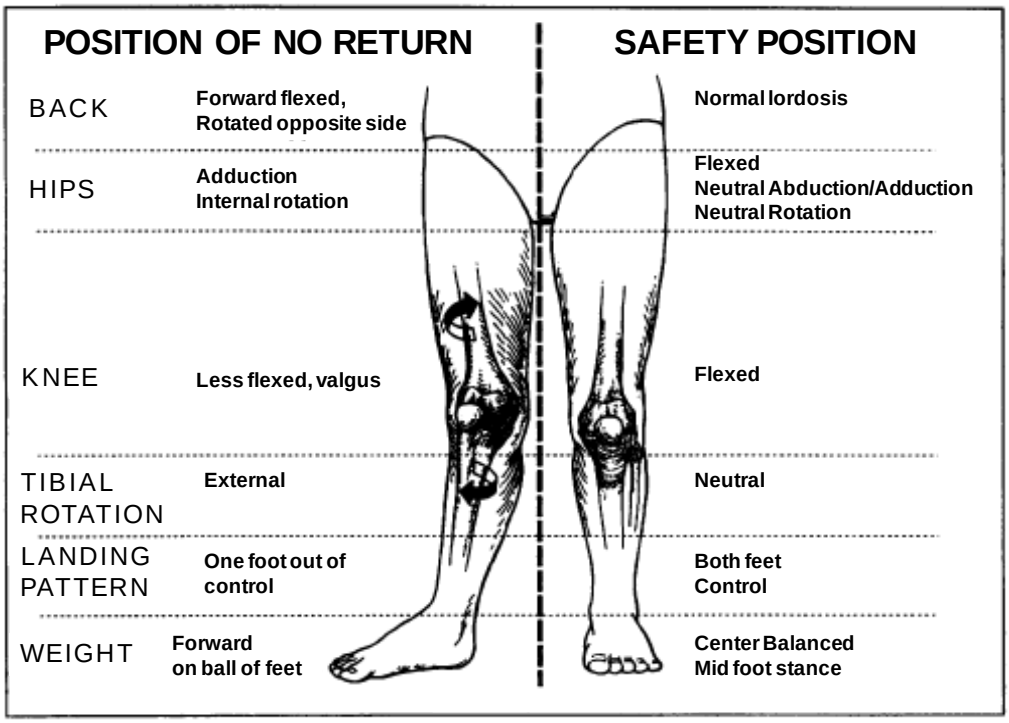

Figure 2.3: A schematic diagram showing the "position of no return". This term refers to a gross biomechanical description during landing. In this high risk landing position, the ACL is placed at increased risk, when compared to the safety position. This figure was modified from Ireland [26].

2.3 BRIEF OVERVIEW OF THE EPIDEMIOLOGY OF ACL RUPTURES

2.3.1 The incidence of ACL ruptures

2.3.1.1 The incidence in the general population

The incidence of injuries, such as ACL ruptures, are most commonly reported as an incidence rate, which is the number of new injuries per unit of time (years, hours or exposures) [13]. Determining the incidence of ACL ruptures in the general population is extremely difficult and is only possible in countries with comprehensive national injury registries. Within New Zealand (NZ), all citizens are covered by the government funded accident compensatory commission (ACC), and the ACC is thus uniquely positioned to provide detailed and accurate national epidemiological data. In a recent study, data from the ACC were used to provide a detailed descriptive epidemiology of knee ligament injuries, including injury to the ACL [22]. Seven thousand three hundred and seventy five claims for ACL surgery were accepted by the ACC over a 5 year period (2000 – 2005). The population-based incidence of ACL ruptures per 100,000 person-years was 37. The majority (65%) of ACL ruptures which required surgery occurred at a place of recreation or sport. These data by Gianotti et al. [22] is currently the largest measure of the general population incidence of ACL ruptures.

Research from Norway, which has recently introduced a national knee ligament registry, also provides useful and accurate incidence data [27]. Two thousand seven hundred and ninety three ACL ruptures were registered during the first 2 years of operation, and this equates to an incidence of 34 ACL ruptures per 100,000 citizens, and 85 per 100,000 citizens in the main at-risk age group, which is 16-39 years [27]. These data are remarkably similar to the data reported by the NZ ACC which, based on a population estimate of 4.1 million, reported 36 ACL ruptures per 100,000 citizens per year [22].

Although many studies have suggested that females are at greater risk of ACL ruptures (as will be discussed in subsequent sections of this review), it is however interesting to note that in both population based studies, the number of ACL ruptures in the general population was greater in males when compared to females. This may be explained by greater sports participation by males.

2.3.1.2 Variation in the incidence of ACL ruptures by age and gender

The mean age at which ACL ruptures occur are roughly 27 years [27;28], which is much younger than other common musculoskeletal soft tissue injuries [28]. In addition, various studies have shown that the incidence of ACL ruptures is greater during late adolescence and early adulthood [29;30]. The data from comprehensive population based New Zealand national injury registries (Figure 2.4) recently confirmed an increased rate of ACL surgery in late adolescent and early adulthood [22]. More specifically, it was shown that the highest number of

injuries occur between the ages of 16 and 18. This trend was observed in both males and females.

It has become widely accepted that the incidence of ACL ruptures in the general population is higher in males when compared to females [13;22;30]. This is most certainly due to a higher exposure in males to high risk athletic tasks such as landing, pivoting and cutting [31]. However, when comparing the incidence of ACL ruptures during specific sports where males and females are similarly represented, females consistently have a higher risk of ACL ruptures [29;31].

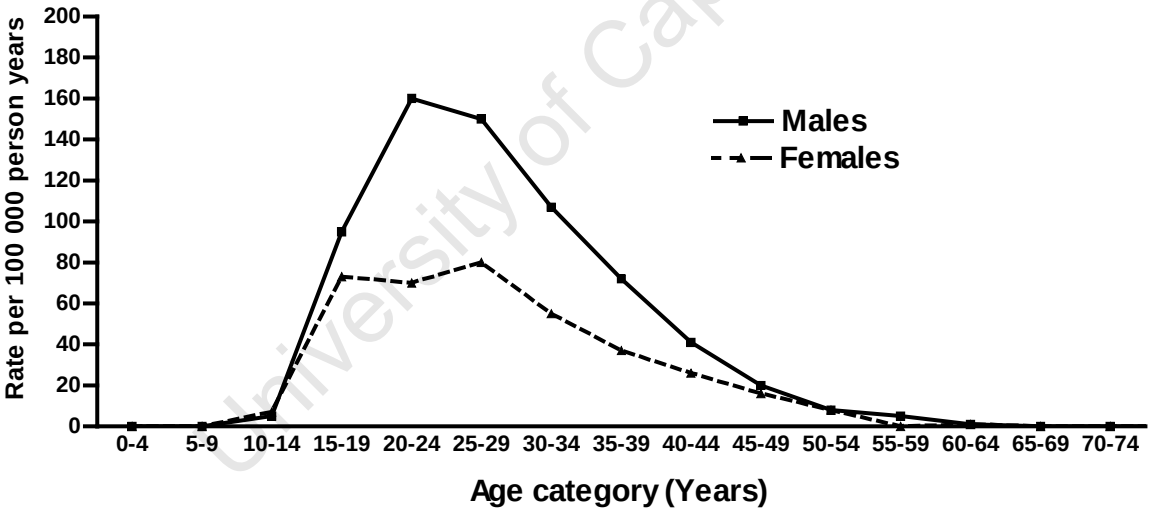

Figure 2.4: The incidence (number per 100,000 person-years) of ACL ruptures requiring surgery by age category amongst males and females. The data presented are from the New Zealand national injury registry [22]. The figure is adapted from Gianotti et al. [22].

2.3.1.3 Variation in the incidence of ACL ruptures by sport and gender

The gender-specific incidence of ACL ruptures in different sports have been published and extensively reviewed [13;32;33]. Although there is no consensus as to the magnitude, females have been shown to have an increased risk during most sporting activities.

It is important to briefly summarise some of the more recent studies investigating gender differences in incidence of ACL ruptures during specific sporting activities, however it is beyond the scope of this literature review to comprehensively review all available data. In a recent meta-analysis [32], studies reporting the incidence of ACL ruptures were combined and analysed to estimate the incidence of ACL ruptures as a function of gender and sport. The female:male ratios of ACL ruptures for various sports were found to be as follows: wrestling, 4.05; basketball, 3.5; indoor soccer, 2.77; soccer, 2.67; rugby, 1.94; lacrosse, 1.18; and alpine skiing, 1.00.

The most conclusive evidence for gender differences in the incidence of ACL ruptures in soccer and basketball are from data obtained through the North American National Collegiate Athletic Association (NCAA) injury surveillance system database. In this report, data were reviewed for all men's and women's basketball and soccer anterior cruciate ligament injuries between 1990 and 2002 [34]. Data from a total of 586 (394 Female, 192 Male) ACL injuries amongst soccer players and 682 (514 Female and 168 Male) ACL injuries amongst basketball players were analysed [34]. When comparing incidence of

ACL injury per 1000 athlete-exposures (an exposure was defined as a practice or match) by gender, the female to male ratio of ACL injuries was 2.8 (95% CI; 2.3 - 3.3) for soccer and 3.6 (95% CI 3.0 – 4.2) for basketball (Figure 2.5) [34]. Another noteworthy finding from this study was that in soccer, the frequency of non-contact injuries was significantly higher in females (58.3%) when compared to males (49.6%) [34]. A similar difference was however not found in basketball when comparing males (70.1%) and females (75.7%) [34].

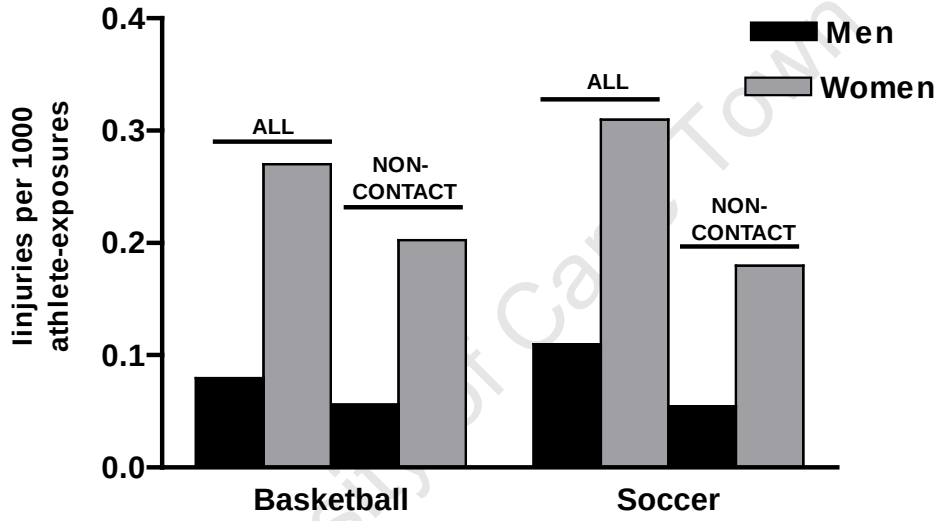

Figure 2.5: A summary of the data from Agel et al. [34], showing the incidence (number per 100,000 person-years) of ACL injuries occurring during men's and women's college basketball and soccer.

Although numerous other studies have been performed, the majority only report a small number of ACL ruptures. Further large scale studies, such as the study presented by Agel et al. [34], are required to determine the incidence of ACL ruptures as a function of gender for different sporting activities.

2.3.2 The consequence of ACL ruptures

ACL ruptures are one of the most severe injuries sustained during sports participation [3;31;35]. The time out of play due to ACL ruptures has been reported to be as high as 255 days [35]. For many athletes this also means a potential loss of sport participation for an entire season, loss of scholarship funding, lowered academic performance, long term disability, and a significantly greater risk of osteoarthritis of the knee may occur as a result of ACL ruptures [36;37].

Furthermore, the cost associated with the surgical repair of ACL (ACL reconstruction), and subsequent prolonged rehabilitation is extremely high. De Loes and colleagues [31] have shown that cost of an ACL rupture is the highest cost of medical care of any knee injury when this is calculated by cost per hour of participation. Conservative estimates of the cost of surgery and rehabilitation are between US$ 17,000 and US$ 25,000 per ACL rupture [31].

As previously mentioned, a rupture of the ACL significantly increases the risk of developing osteoarthritis of the knee joint [36;37]. In a analysis of 50 retired Australian rules footballers, Deacon at al. [38] showed that the risk of radiographically diagnosed osteoarthritis is 105 times greater in footballers who had sustained intra-articular ligamentous and/or meniscal injury when this was compared to a group who only injured their collateral ligaments or no ligament. In summary, due to the severity, cost and potential long term disability, ACL rupture is one of the most significant injuries sustained during sports.

2.4 FACTORS ASSOCIATED WITH INCREASED RISK OF ACL RUPTURE

Multiple factors have been shown to be associated with an increased risk for ACL ruptures. ACL ruptures are therefore, like most sports related injuries, considered multifactorial disorders. As a basis for understanding the multifactorial nature of sports injuries, such as ACL ruptures, Meeuwisse [39] developed a model to account for all factors involved in the causation of the injury (Figure 2.6). Risk factors for these multifactorial disorders are broadly divided into either intrinsic (within the body) or extrinsic (from outside the body). In the proposed model [39] the intrinsic risk factors predispose the individual or athlete to an increased risk of injury. Once predisposed, the susceptibility to injury is determined by the extrinsic risk factors. It is important to note that these intrinsic and extrinsic risk factors do not cause an injury. Rather, a specific inciting event has to occur and place the ACL under sufficient strain for it to rupture [39;40].

Although the exact aetiology and mechanism of ACL ruptures are poorly understood, various intrinsic and extrinsic risk factors have been indentified. As discussed in the introduction to this thesis (Chapter 1), the identification and characterisation of these risk factors are important in order to understand the aetiology and mechanism of these injuries (refer to Figure 1.1).

The primary objective of this thesis was to identify genetic sequence variants which may predispose an individual to ACL ruptures. To date there is only limited data that suggest that genetic elements are intrinsic risk factors for ACL

ruptures. In this section of the review, the genetic elements which have been associated with an increased risk of ACL ruptures, as well as the other risk factors for ACL ruptures will be discussed. In Sections 2.4.2 and Section 2.4.2 respectively the extrinsic and intrinsic risk factors for ACL ruptures will be reviewed.

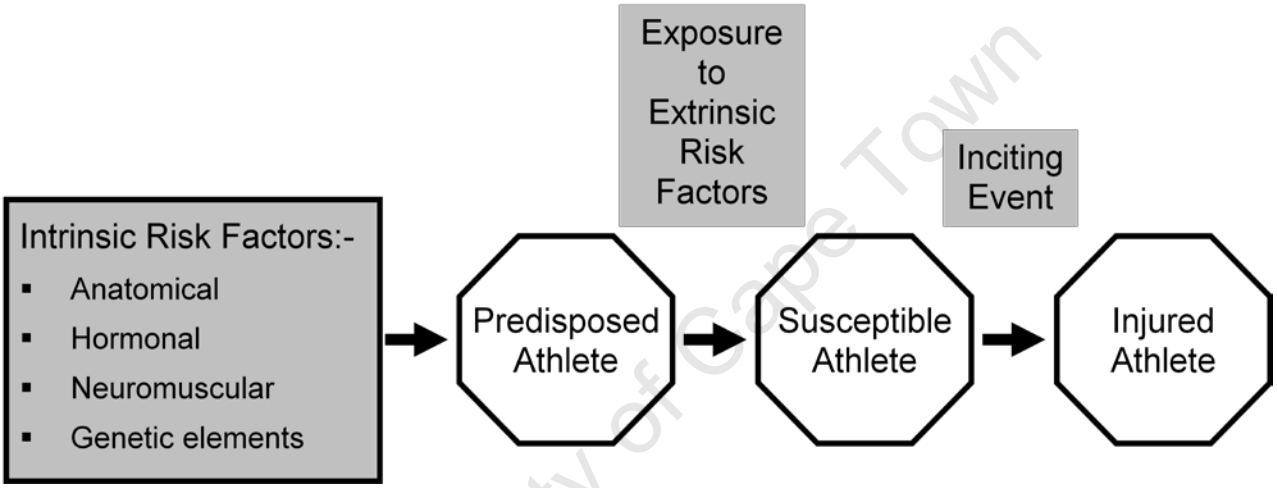

Figure 2.6: A schematic diagram, adapted from the original model proposed by Meeuwisse [39], illustrating the complex relationship between intrinsic risk factors, extrinsic risk factors and a specific inciting event in the causation of ACL ruptures. Several intrinsic risk factors, broadly classified as anatomical, hormonal, neuromuscular or genetic elements, have been identified. It is highly likely that these factors are not independent risk factors and that a relationship between them exists. With regards to genetic elements, while some sequence variants might alter the properties of ligaments, other genetic elements may be associated with phenotypes already identified as intrinsic risk factors.

Although numerous articles have reviewed the available data on the extrinsic and intrinsic risk factors for ACL ruptures [4;12;41], this review will critically appraise the level of evidence for each study reviewed according to the classification adopted by the American edition of the Journal of Bone and Joint Surgery [42;43]. In the hierarchy of evidence, high quality prospective studies are level I studies, retrospective studies and lesser quality prospective studies are level II, case-control studies are level III, case series are level IV, and expert opinions are level V [42].

In addition, a level of certainty that the risk factor is associated with ACL ruptures, based upon the level of evidence for each individual study, will also be incorporated in this review. Based on previously definitions for the levels of certainty (High, moderate or low) by the US Preventative Services Task Force [44], the following descriptions were used for the purpose of this review.

i) **High Certainty**; The available evidence includes consistent results from level I studies. These studies provide a good estimate of risk and are unlikely to be strongly affected by future studies.

ii) **Moderate Certainty**; The available evidence includes sufficient evidence to determine that there is risk associated with the injury, but confidence in the estimate is constrained by factors such as the sample size and quality of studies, as well as inconsistency of findings across individual studies. As more information becomes available, the magnitude of risk could change or even alter the conclusion.

iii) **Low Certainty;** The available evidence is insufficient to assess risk. Evidence is insufficient because of the limited number or size of studies, and inconsistency of findings across individual studies. More information may allow an estimation of risk.

2.4.1 Extrinsic risk factors for ACL ruptures

Any external factor affecting the risk of ACL ruptures may be regarded as an extrinsic risk factor. Although previous reviews have only discussed the contribution of environmental risk factors, other extrinsic factors such as sports participation and type of sporting activity are often taken for granted.

In a large population based prospective cohort study with a 9 year follow up, Parkarri et al. [45], found that participation in organised sports resulted in a significantly increased risk of ACL ruptures. Furthermore, the frequency of participation was related to the degree of risk. Participation in organised sports > 3 times/week resulted in an 8.5 (95% CI 4.3 – 16.4) and a 4.0 (95% CI 2.7 – 6.1) times increased risk in females and males respectively. This study provides evidence that participation in organised sports is undoubtedly associated with ACL ruptures. As ACL ruptures are not able to occur without an inciting event (refer to Figure 2.6), which typically occurs during sports participation, sports participation will not be further discussed as a risk factor.

Environmental risk factors associated with ACL ruptures include, (1) meteorological conditions, (2) the type of surface, (3) footwear, and (4) protective bracing. Only a few studies have investigated the association of these environmental factors to risk of ACL ruptures, and therefore only a brief discussion for each factor will be included.

2.4.1.1 Environmental factors as extrinsic risk factors for ACL ruptures

2.4.1.1.1 *Weather conditions*

The environmental (weather) conditions have been investigated as a possible extrinsic risk factor for ACL ruptures in the Australian Football League (AFL). In this prospective cohort study, Orchard et al [46] reported an increased risk of ACL ruptures during periods of low rainfall and high water evaporation. In this study it was proposed that the likely mechanism involves an increased shoe-surface traction which occurs on dry hard fields, when compared to soft moist fields as a likely causative factor [46;47]. Similar findings were also observed in the National Football League (NFL), where more than 95% of all ACL ruptures occurred on a dry field [48].

Due to the evidence presented (Table 2.1), the certainty that meteorological conditions increase the risk of ACL ruptures are moderate and need to be further investigated. The number of studies investigating this risk factor is low and data are insufficient to accurately assess risk, especially among different sports.

2.4.1.1.2 *Type of playing surface*

As previously discussed (Section 2.4.1.1.1), increased shoe-surface traction has been implicated as a risk factor for ACL ruptures [47]. A prospective cohort study of 8 high schools NFL teams reported a 50% reduction in the rate of ACL ruptures with the use of artificial turf [49]. Similar results were also obtained in a retrospective analysis where a relationship between the type of floor in Norwegian team handball and risk of ACL ruptures was reported in females, but not in males. This study reported that females were at an increased risk of injury when playing on artificial floors, when compared to natural wooden floors [47]. In support of the hypothesis that increased shoe-surface traction increases the risk of ACL ruptures, friction tests demonstrated an increased coefficient of friction on the artificial compared to the natural wooden floor surface [47].

In summary (Table 2.1), based in the evidence presented (Table 2.2), the certainty that the type of surface is a risk factor for ACL ruptures is moderate in females, and low in males.

2.4.1.1.3 *Type of footwear*

As discussed, an increased shoe-surface friction is related to an increased risk of ACL ruptures. In support of this, there is evidence from a prospective cohort study that football cleat design may alter the risk of ACL ruptures [50]. Longer irregular cleats, which resulted in significantly higher torsional resistance when

compared to other cleat designs, were associated with a significantly higher risk of ACL ruptures. Although only one study investigated footwear as a risk factor for ACL ruptures, it appears that footwear with a greater shoe-surface friction increases the risk of ACL ruptures. However, the risk can not be accurately predicted from this single study and therefore the level of certainty is moderate (Table 2.1).

2.4.1.1.4 *Protective bracing*

To date, there is only one study where the effect of prophylactic knee bracing on the risk of ACL ruptures was investigated. The results from this randomised controlled study in 1396 cadets, playing tackle football at the US military academy, showed that the rate of ACL ruptures was 3.0 times higher in the unbraced compared to the braced group [51]. It is however important to note that only 16 ACL ruptures occurred (4 in the braced group, and 12 in the unbraced group) and this small sample size is a limitation in this study. In another large epidemiological study it was shown that the use of prophylactic knee braces did not reduce the risk of MCL injury [52;53]. In summary (Table 2.1), the role of prophylactic knee bracing on the risk of ACL ruptures still remain equivocal [6], and therefore the level of certainty is low.

Table 2.1: Summary of research studies investigating environmental risk factors for ACL ruptures, including the level of evidence of each individual study and the level of certainty that the risk factor is associated with risk of ACL ruptures.

Risk Factor	Study Details and References	Number of ACL ruptures	Level of evidence (I-V)[a]	Level of Certainty[b]
Weather conditions	**Positive Association**	59	I	**Moderate**
	Prospective cohort study – Australian Football league [46]	40	II	
	Retrospective study – National Football league [48]			
	No association - none			
Type of playing surface	**Positive Association**			**Moderate** (in females)
	Prospective cohort study – National Football league [49]	7	I	
		44	II	**Low** (in males)
	Retrospective study – Norwegian team handball [54] – Only females	9	II	
	No Association			
	Retrospective study – Norwegian team handball [54] – Only males			
Type of footwear	**Positive Association**			**Moderate**
	Prospective cohort study – National Football league [50]	42	I	
Protective bracing	**Positive Association**			**Low**
	Prospective cohort study – Cadets, National Football league [51]	16	I	

[a] The level of evidence according to evidence-based medicine criteria [42].

[b] The level of certainty, as described in section 2.4.

2.4.2 Intrinsic risk factors for ACL ruptures

There are different classification systems for the various intrinsic risk factors that have been associated with ACL ruptures. The system that is commonly used by physicians, physical therapists, athletic trainers, biomechanists, epidemiologists, and other scientists [6], sub-divides the intrinsic risk factors into four categories: (1) anatomical, (2) hormonal, (3) neuromuscular and (4) familial predisposition. Since the development of the above classification system, a specific genetic risk factor that is associated with cruciate ligament ruptures has been identified [9]. Therefore, for the purpose of this review, the intrinsic risk factors will be discussed under the following sub-divisions: (1) anatomical, (2) hormonal, (3) neuromuscular and (4) genetic intrinsic risk factors, which includes a familial predisposition.

As previously discussed, there is evidence from large scale prospective cohort studies that there is a greater risk of ACL ruptures in female athletes during specific sporting activities. These data indicate that there is strong evidence that female gender is an intrinsic risk factor for ACL ruptures [32;34;45;55]. However, it is likely that the increased risk of ACL ruptures in females is a result of various anatomical, hormonal and neuromuscular risk factors. Gender will therefore not be included as separate intrinsic risk factor for ACL ruptures in this review. Rather, when applicable, gender differences will be addressed within this review of the four categories of intrinsic risk factors.

2.4.2.1 Anatomical intrinsic risk factors for ACL ruptures

2.4.2.1.1 *The quadriceps (Q) angle*

Lower extremity alignment, specifically the quadriceps (Q) angle, has been related to an increased risk of ACL ruptures, possibly by altering lowed limb alignment and therefore the kinematics of the knee [56;57]. The Q angle is clinically defined as the angle in the frontal plane formed by intersecting lines from the centre of the patella to the anterior superior iliac spine and the centre of the patella to the tibial tubercle (Figure 2.7A) [58]. The Q angle is thought to reflect the pelvic angle, as well as hip rotation, tibial rotation, patella position, and foot position. This composite measure is larger in females when compared to males, and has therefore often been mentioned (anecdotally) as a possible reason for the increased risk of ACL ruptures in females [59;60]. It has been proposed that an increased Q angle may contribute to dynamic knee valgus, and thereby increase the risk of ACL ruptures [58].

In one study, where the relationship between lower extremity alignment and the risk of knee injury was reported, it was found that the Q angle of individuals who had injured their knee was greater than the Q angle of uninjured individuals (14º vs. 10º) [61]. However in two other studies where the Q angle was measured and compared between ACL injured and ACL non-injured individuals, no significant differences were observed [62;63]. In these two independent studies, where lower extremity alignment variables were measured in ACL injured and non-injured individuals, Q angle was not associated with injury risk [62;63].

In summary (Table 2.2), although an increased Q angle is commonly reported as a possible risk factor for ACL ruptures, there is a lack of clinical research to confirm that this is an independent risk factor for ACL ruptures. Therefore, the certainty that a large Q angle increases the risk for ACL ruptures is currently low.

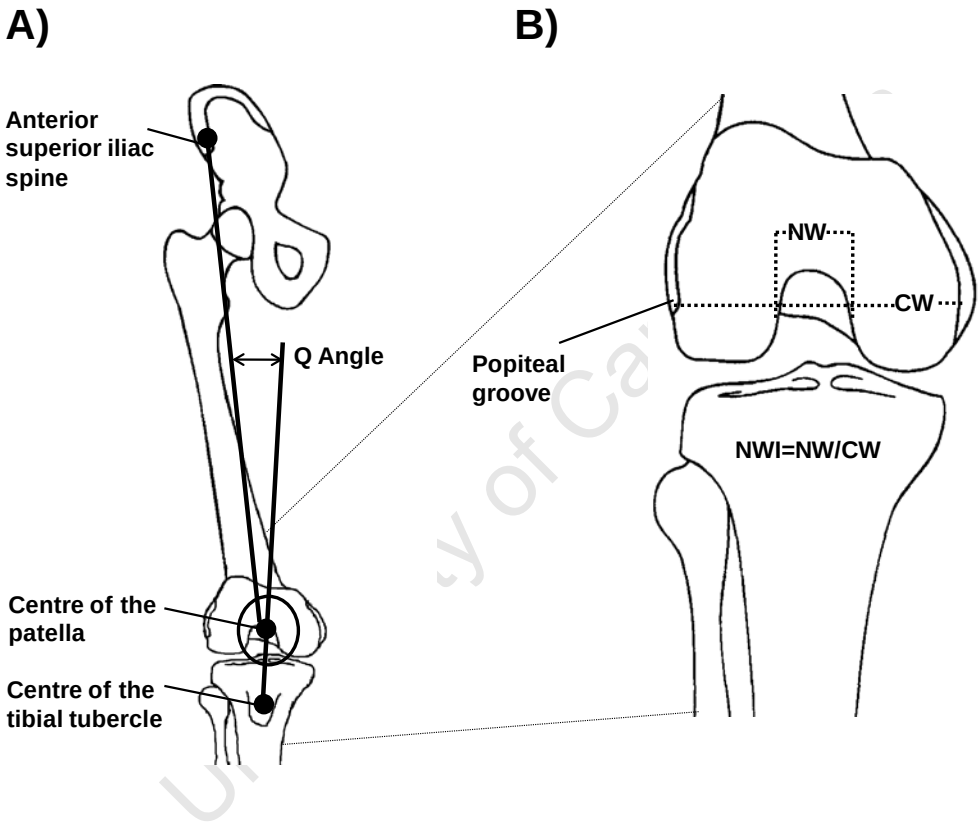

Figure 2.7: (A) The static Q angle is determined by measuring the acute angle produced by the two intersecting lines. The first line is drawn from the centre of the patella to the anterior superior iliac spine. The second line is drawn from the centre of the patella to the centre of the tibial tubercle. **(B)** The Notch width index (NWI) is the ratio of the width of the anterior outlet of the femoral intercondylar notch (NW) to the total condylar width (CW) at the level of the popiteal groove. Figure modified from Huston [64].

2.4.2.1.2 *Notch width*

Both a smaller femoral intercondylar notch width and a smaller femoral notch width index, (ratio of the width of the anterior outlet of the intercondylar femoral notch to the total condylar width at the level of the popiteal groove) (Figure 2.7B), have been shown to be associated with an increased risk of ACL ruptures [55;65-67]. A summary of the published literature has shown that the notch width of bilateral ACL injured knees are smaller than that of unilateral ACL injured knees [6], similarly, the notch width of unilateral ACL injured knees were smaller than the notch width of normal controls [6].

Various large cohort prospective studies have found association between (1) smaller notch width and (2) smaller notch width index, and increased risk of ACL ruptures [55;65-67]. In one prospective study of 902 high school athletes, Souryal and Freeman [65] showed that athletes with a small intercondylar notch width were at increased risk of ACL rupture. La Prade and Burnett [66] found similar results when 213 collegiate athletes were prospectively analysed. In addition, Shelbourne et al. [67] found a higher incidence of contra-lateral ACL rupture in individuals with narrower notches in a prospective study of 714 patients who underwent patella graft ACL reconstruction. In a recent published prospective study, Uhorchak et al.[55] investigated numerous risk factors, including notch width and notch width index in 859 cadets. In this study, both decreased notch width and notch width index were significantly associated with increased risk of ACL ruptures. Various retrospective case-control studies also

confirm the findings that notch width and notch width index is associated with increased risk of ACL ruptures [68;69].

In summary (Table 2.2), although some studies reported no association between notch width or notch width index and risk for ACL ruptures [70;71], several well controlled prospective studies and retrospective case-control studies have found a significant relationship. These studies provide a good estimate of risk and are unlikely to be affected by future studies. Therefore, the certainty that a decreased notch width and notch width index is associated with an increased risk of ACL ruptures is high.

2.4.2.1.3 *Tibial slope*

The tibial slope is defined as the angle between the line perpendicular to the tibial axis and the posterior inclination of the tibial plateau [72]. Tibial slope is commonly reported as an anatomical risk factor for ACL ruptures [73]. Dejour et al. [74] investigated the tibial slope in 281 knees and established a significant association between an increased tibial slope and anterior tibial translation. Increased anterior tibial translation, as a result of increased tibial slope, may increase the strain on the ACL. In addition, a recent study concluded that decreasing the tibial slope may be protective in the ACL-deficient knee [75].

In one recent case-control study (33 matched pairs of patients divided into two groups: a group with ACL ruptures and a control group which consisted of patients with patello-femoral pain) an increased tibial slope was documented in

the patients with ACL ruptures [72]. It was therefore suggested that an increased tibial slope is a risk factor for ACL ruptures. In contrast, findings from a similar case-control study failed to confirm an association between increased tibial slope and ACL ruptures [76].

In summary (Table 2.2), there are data from only one level III study that support the hypothesis that an increased tibial slope is a risk factor for ACL ruptures [43;72]. Therefore, due to the limited number of research studies and the lack of consensus among the published studies, the level of certainty that tibial slope is associated with risk of ACL ruptures is low.

2.4.2.1.4 *Foot pronation*

It has been suggested that increased sub-talar joint pronation (measured by greater navicular drop values) may be associated with an increased risk of ACL ruptures [77]. An increased pronation is correlated with a greater rotation moment at the knee joint [78]. In a number of studies significant differences in navicular drop values between ACL injured and ACL non-injured individuals were reported [62;79-81]. In the most recent published study, Hertel et al. [62] found that participants with a navicular drop greater than 8mm were 20 times more likely to rupture their ACL than subjects with a navicular drop of less than 6.3mm. However, in a similar case-control study, no significant differences in the navicular drop values between ACL injured and control subjects were observed [82].

In summary (Table 2.2), the majority of studies have shown that increased foot pronation (as measured by the navicular drop) is a significant predictor of ACL ruptures. However, there are few high quality prospective cohort studies: therefore the confidence in the estimate is constrained. The level of certainty that increased foot pronation, and more specifically navicular drop values, are associated with risk of ACL ruptures is therefore moderate.

2.4.2.1.5 *Pelvic tilt*

It has been hypothesised that an increased anterior pelvic tilt may be a predictor of ACL rupture due to its effects on lower extremity postural alignment [58]. Excessive anterior pelvic tilt of the pelvis may lead to an internal rotation and medial collapse of the lower extremities, specifically internal femoral rotation, genu valgus, genu recurvatum, and subtallar pronation [63;83]. A case-control study by Hertel et al. [62] found a significantly greater anterior pelvic tilt in male and female participants with a previous history of an ACL rupture compared to uninjured participants. In addition, a similar association was found when only females were analysed [63]. Therefore, data from two case-control studies have identified a univariate association between increased anterior pelvic tilt and ACL injury. However, it is not clear if the degree of anterior pelvic tilt itself or the lower extremity misalignments as a result of an increased anterior pelvic tilt are associated with increased risk of ACL ruptures. This highlights the fact that case-control studies do not provide evidence for a cause-effect relationship between two variables.

In summary (Table 2.2), there is some evidence that increased anterior pelvic tilt may be associated with an increased risk of ACL ruptures, but there is a lack of confidence in the estimate. Therefore the certainty that increased anterior pelvic tilt is associated with risk of ACL ruptures is at best, moderate.

2.4.2.1.6 *ACL geometry*

As previously reviewed, there is high certainty that a narrower notch width is a risk factor for ACL ruptures. However, there is no consensus in the literature over the possible mechanism by which narrower notches increase the risk of ACL ruptures [58]. One hypothesis is that a narrower intercondylar notch simply reflects a smaller ACL. It is generally stated that ACL size would be a risk factor for ACL ruptures because a smaller ACL has less material strength than a larger ACL and will rupture sooner under similar loading conditions. In support of a narrower notch reflecting a smaller, and therefore weaker ACL, Shelbourne et al. [67] showed that after reconstruction with a standardised 10mm autograft, the incidence of graft rupture is not dependant on notch width, whereas rupture of the contralateral ACL, as mentioned above, is associated with a reduced notch width.

In the prospective study of 859 cadets [55], eminence width and eminence width index, which are simple indirect measurements of the ACL diameter, were found to be significantly smaller in both the male and female participants with ACL ruptures, compared to uninjured controls. These measurements are based

on the assumption that the ACL diameter is the same as the tibial eminence width.

In summary (Table 2.2), it is generally stated that a smaller ACL size is at increased risk of rupture. However, there is little evidence from well conducted studies in support of this, particularly as no direct measurements of ACL size have been related to increased risk of ACL ruptures. Data from a single prospective study [55] has however found an association between an indirect measure of ACL geometry and risk of ACL ruptures. Therefore the certainty that a smaller ACL size is a risk factor for ACL ruptures is, at best, moderate.

2.4.2.1.7 *Increased body mass index*

An increased Body Mass Index (BMI) is often reported as a risk factor for lower extremity injuries in general, and ACL ruptures in particular [6]. It has been shown that an increased BMI results in a more extended knee position on landing, and increased knee extension during landing increases the risk of ACL ruptures [84]. In a recent prospective study [55], the US military academy found that BMI was a significant predictor of risk of ACL ruptures in female, but not male recruits. Female cadets with a BMI of greater than one standard deviation above the mean were at a 3.5 times greater risk of developing ACL ruptures. However, in another population based cohort study of 45 500 people, there was no significant association between being overweight (BMI > 25kg/m^2) and an increased risk of ACL ruptures in males (Hazard ratio = 1.1; 95% CI: 0.8 – 1.7) or females (Hazard ratio = 1.5; 95% CI: 0.8 - 3.1) [45]. However, the data from

this study were difficult to interpret due to the heterogeneity of the large population based cohort. Finally, in other studies that were primarily designed to investigate the relationship between BMI and training related injury risk, no relationship between BMI and an increased risk of training related injuries was observed [85].

In summary (Table 2.2), data from only one prospective study has found a relationship between increased BMI and risk of ACL ruptures in females [55]. Other prospective studies have however failed to show a similar association. Therefore, the certainty that increased BMI is a significant risk factor for ACL ruptures is low.

2.4.2.1.8 *Generalised joint laxity*

Generalised joint laxity (GJL), as measured by the Beighton score reflects an overall measure of whole body joint laxity [86]. The score ranges from 0 – 9 and is determined by assigning one point each for: (1) hyperextension of the metacarpophalangeal joint of each finger beyond 90º, (2) the ability to touch the volar surface of each forearm with the respective thumb, (3) hyperextension of each elbow, (4) hyperextension of each knee joint, and (5) the ability to place the palm of both hands on the floor by forward flexion with straight extended knees.

In general, females have a greater GJL when compared to males [87]. Although it has been suggested that increased GJL is a risk factor for lower extremity

injury [88], and ACL injury [89], there is limited research supporting an association between increased GJL and an increased risk of ACL ruptures [58]. In the military cadet prospective cohort study, that was previously mentioned, the association between GJL and the risk of ACL ruptures was investigated [55]. The results of this study showed that increased GJL was a significant predictor of ACL ruptures in both males and females. More specifically, cadets with a Beighton score of ≥ 5 regions were at 2.8 times greater risk of ACL ruptures. In a further case-control study, a significantly greater proportion of individuals with ACL ruptures had a GJL score of > 6 regions, when compared to an uninjured control group [63].

In certain studies, the association between individual components of the composite Beighton score and ACL ruptures were investigated. In one of these studies, increased knee hyperextension (genu recurvatum) greater than 10º, was significantly associated with risk of ACL ruptures [63]. Increased hamstring flexibility, another component of the Beighton score, has also been associated with an increased risk of ACL ruptures in a retrospective case-control study [90].

In summary (Table 2.2), the association between increased GJL and ACL injury has been reported in some retrospective and one prospective cohort study. The certainty that increased GJL is a risk factor for ACL ruptures is therefore moderate.

2.4.2.1.9 *Anterior knee laxity*

Increased anterior knee laxity, which refers to the increased anterior tibial translation, has often been cited as a risk factor for ACL ruptures [58]. It has been shown that females have a greater anterior knee laxity at comparable progressive forces when compared to their male counterparts [91]. However, to current knowledge, increased anterior knee laxity as a potential risk factor for ACL ruptures had only been investigated in two studies. Woodford-Rodgers et al. [80] investigated anterior knee laxity, as measured by an anthrometer, in 14 ACL injured males and 8 ACL injured females. In this retrospective case-control study, discriminate analysis and multiple regression showed that increased anterior knee laxity is an important factor in predicting ACL injury status in ACL ruptured and ACL intact individuals. In a prospective study, in which only 16 males and 8 females were investigated, increased anterior knee laxity, as measured on a KT-2000 arthrometer, was shown to increase the risk of ACL ruptures in females, but not in males [55]. In this study, the relative risk of sustaining an ACL rupture was increased by 2.7 times in female subjects who had increased knee laxity.

In summary (Table 2.2), there are data from two studies, a prospective and a retrospective case-control study, that support the hypothesis that increased anterior knee laxity is a risk factor for ACL ruptures. The results of these two studies suggest a moderate certainty that anterior knee laxity is associated with increased risk of ACL ruptures in females, but not males.

Table 2.2: Summary of research studies investigating anatomical intrinsic risk factors for ACL ruptures, including the level of evidence of each individual study and the level of certainty that the risk factor is associated with risk of ACL ruptures.

Risk Factor	Study Details, population studied, and References	Number of ACL ruptures	Level of evidence (I-V)[a]	Level of Certainty[b]
Q Angle	**Positive Association:**			**Low**
	Association with knee injuries [61]	N/A	V	
	No association:			
	Case-control studies			
	University students [62]	20	III	
	Female athletes [63]	20	III	
Notch Width	**Positive Association:**			**High**
	Prospective cohort studies –			
	High school athletes [65]	14	I	
	Intercollegiate athletes [66]	7	I	
	Military Cadets [55]	24	I	
	Case-control study –			
	ACL patients [69]	108	III	
	Female handball [68]	20	III	
	No Association			
	Case-control study –			
	Male national basketball	14	III	
Tibial Slope	**Positive Association**			**Low**
	Case-control study			
	Unspecified [72]	33	III	
	No Association			
	Case-control study –			
	Unspecified [76]	49	III	

Foot Pronation	**Positive Association**			**Moderate**
	Retrospective case-control			
	High School & College athletes [80]	22	II	
	Case-control study			
	University students [62]	20	III	
	ACL patients [79]	18	III	
	ACL patients [79]	50	III	
	No Association			
	Case-control study			
	Unspecified [82]	14	III	
Pelvic Tilt	**Positive Association**			**Moderate**
	Case-control study			
	University students [62]	20	III	
	ACL patients [63]	20	III	
	No Association - none			
ACL geometry	**Positive Association**			**Moderate**
	Prospective cohort study –			
	Military Cadets [55]	24	I	
	Case-series			
	Graft reconstruction patients [67]	714	IV	
	No Association - none			
BMI	**Positive Association**			**Low**
	Prospective cohort study –			
	Military Cadets [55] - Only females	8	I	
	No Association			
	Prospective cohort studies –			
	Military Cadets [55] - Only males	16	I	
	Finnish population [45]	265	I	

Generalised Joint Laxity	**Positive Association**			**Moderate**
	Prospective cohort study –			
	Military Cadets [55]	24	I	
	Case-control study-			
	ACL patients [92]			
	ACL patients [63]	169	I	
	Athletes [90]	20	III	
	No Association – none	89	III	
Anterior Knee Laxity	**Positive Association**			**Moderate** (in females) **Low** (in males)
	Prospective cohort study –			
	Military cadets [55] - Females only	8	I	
	Retrospective case-control			
	High School & College athletes [80]	22	II	
	No association			
	Military cadets [55] - Females only	16	I	

[a] The level of evidence according to evidence-based medicine criteria [42].

[b] The level of certainty, as described in section 2.4.

2.4.2.2 Hormonal factors as intrinsic risk factors for ACL ruptures

2.4.2.2.1 Phase of the menstrual cycle

There is evidence that oestrogen and progesterone receptors are found on the surface of cells within the ACL. More specifically, both oestrogen and progesterone receptors can be localised to the synoviocytes in the synovial

lining, fibroblasts in the ACL stroma and cells in the blood vessel walls of the ACL [93]. These findings suggest that these hormones may have a role in the biological processes of the ACL. In previous studies it has been shown that oestrogen reduces the rate of fibroblast proliferation and type I procollagen synthesis [94;95]. This inhibition is attenuated by progesterone, which is though to have the opposite effect on fibroblast proliferation and type I procollagen synthesis [94;95]. It therefore suggests that fluctuations in oestrogen and progesterone concentrations during the menstrual cycle may influence the material properties of the ACL, and that there may be an increased risk of ACL rupture during certain phases of the menstrual cycle. Numerous studies have attempted to investigate the relationship between the phase of the menstrual cycle and the risk of ACL ruptures [96-104].

The results of these studies are not consistent, but a consensus suggests that the risk of ACL ruptures does not remain constant during the menstrual cycle [105]. The two major problems with research in this field are (1) that it is difficult to accurately predict the phase of the cycle at the time of the injury, and (2) that authors have not used consistent classifications of the menstrual cycle. The perimenstrual [99;100;103], follicular [97], ovulatory [98;101;102] and pre-ovulatory [104] phases have been identified as possible high risk phases of the menstrual cycle. However, the results of the majority of studies suggest that the risk of ACL rupture is greater during the pre-ovulatory phase (first half of the menstrual cycle), compared to the post-ovulatory phase (second half of the menstrual cycle) [105]. In a systematic review, which combined all the published literature, Hewett et al. [105] established that both the follicular and ovulatory

phases, which together make up the first half of the menstrual cycle, was associated with an increased incidence of ACL ruptures when compared to expected values.

It is however important to note that only three studies have measured hormone levels in order to confirm the menstrual cycle phase at the time of injury. Wojtys et al. [102] measured the hormone levels of 69 females within 24 hours of sustaining an ACL injury. They found that women had significantly greater risk of ACL rupture during the ovulatory phase (mid-cycle) of the menstrual cycle. In a similar study, saliva samples were obtained from 38 females with ACL ruptures [103]. In this study, the frequency of ACL ruptures was greater in the days immediately after the onset of menses. Finally, in one further study, the serum concentrations of hormones were measured immediately after an ACL injury in 46 females [104]. The results of this study showed that females in the first half of the menstrual cycle had a significantly higher risk of ACL rupture.

In summary (Table 2.3), although there appears to be consensus that the majority of ACL ruptures occur during the pre-ovulatory phase of the menstrual cycle in females, further studies with greater sample sizes as well as a standardised classification system of the phases of the menstrual cycle are still required to confirm this. Due to this lack of consistency amongst the studies, the level of certainty that the first half of the menstrual cycle is a significant risk factor for ACL rupture is therefore moderate.

Table 2.3: Summary of research studies investigating hormonal intrinsic risk factors for ACL ruptures, including the level of evidence of each individual study and the level of certainty that the risk factor is associated with risk of ACL ruptures.

Risk Factor	Study Details and References	Number of ACL ruptures	Level of evidence (I-V)[a]	Level of Certainty[b]
Phase of the menstrual cycle (females only)	**Positive Associations**			**Moderate**
	Case-control studies:			
	Alpine skiers [104]	46	III	
	Systematic review			
	Level IV evidence studies	404	V	
	Case-series studies			
	ACL patients [101]	28	IV	
	Elite handball [99]	17	IV	
	Collegiate athletes [96]	28	IV	
	Collegiate athletes [97]	83	IV	
	Athletes [103]	68	IV	
	ACL patients [102]	69	IV	
	Elite handball [100]	64	IV	
	Athletes [98]	28	IV	

[a] The level of evidence according to evidence-based medicine criteria [42].

[b] The level of certainty, as described in section 2.4.

2.4.2.3 Neuromuscular intrinsic risk factors for ACL ruptures

A number of neuromuscular factors, including kinematic and kinetic differences during landing from a jump, cutting or pivoting, have been suggested as intrinsic risk factors for ACL ruptures [106]. These factors include the following: reduced hip and knee flexion angles, increased knee valgus, internal rotation of the

femur on the tibia, high quadriceps muscle activity relative to hamstring muscle activity and inadequate trans-knee muscle stiffness. Furthermore, gender differences in these factors have also been extensively researched [106]. However, there are limited studies where these factors have been studied as possible independent risk factors for ACL rupture. Therefore, for the purpose of this evidence-based review, only factors that have been evaluated as independent risk factors will be discussed.

2.4.2.3.1 Dynamic knee valgus

It is well established that landing from a jump is one of the primary non-contact mechanisms of ACL rupture [107]. During landing, the lower extremities absorb the forces through joint flexion. It had been widely reported that females land with greater knee valgus when compared to males [108;109]. In addition, it has been shown that valgus loading places increased strain on the ACL [26]. In one prospective cohort study, lower limb biomechanical data were collected from 205 female athletes during the execution of sports movements and then followed prospectively to determine if any measurable differences exists between ACL injured and non-injured individuals [110]. The results of this study show that females who developed non-contact ACL ruptures had a significantly increased lower extremity knee valgus and knee abduction loading during landing, before sustaining their injuries, when compared to the non-injured individuals. It is however important to note that only nine participants ruptured their ACL during the course of this prospective study. This is the only study, to current knowledge, where increased dynamic knee valgus as a possible risk

factor for ACL ruptures was investigated. Although the study by Hewett et al. [110] was a prospective study, further research incorporating a greater number of individuals with ACL ruptures is required. Therefore, the level of certainty that increased dynamic knee valgus is a risk factor for ACL ruptures is low (Table 2.4).

Table 2.4: Summary of research studies investigating neuromuscular intrinsic risk factors for ACL ruptures, including the level of evidence of each individual study and the level of certainty that the risk factor is associated with risk of ACL ruptures.

Risk Factor	Study Details and References	Number of ACL ruptures	Level of evidence (I-V)[a]	Level of Certainty[b]
Dynamic knee valgus	**Positive Associations** Prospective cohort study:- High risk sports [110]	9	II	**Low**

[a] The level of evidence according to evidence-based medicine criteria [42].

[b] The level of certainty, as described in section 2.4.

2.4.2.4 Genetic factors as intrinsic risk factors for ACL ruptures

One of the most recent additions to the list of possible risk factors for ACL ruptures is the possible genetic component. To date, there are data from only three studies which suggest that genetic factors are associated with ACL ruptures. Two of the studies have investigated a familial predisposition to ACL

ruptures and only a single study has shown that a specific genetic element is associated with an increased risk of ACL ruptures.

2.4.2.4.1 Familial predisposition

The first evidence of a genetic predisposition to ACL ruptures was presented in a study that was designed to investigate anatomical risk factors for ACL ruptures [10]. In this study, data obtained from personal information of the participants showed a highly significant difference in the frequency of ACL ruptures in immediate family members in patients with bilateral ACL ruptures compared with control subjects. Eleven of the 31 (35%) patients with bilateral ACL ruptures had a family history of ACL ruptures, in comparison to only 1 out of 23 (4%) of the control subjects.

In a more recent case-control study, the familial predisposition towards rupturing an ACL was investigated in 171 patients with ACL rupture and 171 matched controls [7]. Patients with an ACL rupture were twice as likely (OR = 2.00; 95% CI, 1.19 – 3.33) to have a relative (first, second or third degree) with an ACL rupture compared to participants without any history of ACL rupture. The risk was slightly increased (OR = 2.24; 95% CI, 1.24 – 4.00) when only first degree relatives were included in the study. The strength of this investigation was that data from a large number of participants were available for this study, which made matching of gender, age and primary sport possible. Of the 732 eligible subjects (348 cases and 384 controls), 171 matched pairs were achieved and used in the analysis. The percentage of cases with first, second

or third degree family history of ACL rupture was 31%, compared to only 19.3% amongst the control. Similarly, 23.4% of cases and 11.7% of control participants had a first degree family history of ACL rupture.

Although it appears that familial predisposition is a significant risk factor ACL rupture, the available evidence is insufficient to accurately predict risk. The level of certainty for this risk factor is therefore moderate (Table 2.5).

2.4.2.4.2 COL1A1 Sp1 binding site polymorphism

In a recently published study, the first genetic sequence variant to be associated with ACL ruptures was identified [9]. In this study, the TT genotype of the *COL1A1* (the gene encoding for the α1 chain of type I collagen) Sp1 binding site polymorphism was shown to be significantly under-represented in participants with cruciate ligament ruptures. Only 1 out of 233 participants with ACL rupture, compared to 6 out of 358 subjects control participants had a TT genotype at the Sp1 binding site within *COL1A1.*

However, due to the lack of data investigating this specific polymorphism and low frequency of the rare TT genotype, the level of certainty that the *COL1A1* Sp1 binding site polymorphism is a risk factor for ACL ruptures is low (Table 2.5).

Table 2.5: Summary of research studies investigating genetic risk factors for ACL ruptures, including the level of evidence of each individual study and the level of certainty that the risk factor is associated with risk of ACL ruptures.

Risk Factor	Study Details and References	Number of ACL ruptures	Level of evidence (I-V)[a]	Level of Certainty[b]
Familial predisposition	**Positive Associations**			**Moderate**
	Case-control studies:			
	ACL patients [10]	31[c]	III	
	ACL patients [104]	171	III	
***COL1A1* Sp1 binding site polymorphism**	**Positive Associations**			**Low**
	Case-control studies:			
	Khoschnau [9]	233[d]	III	

[a] The level of evidence according to evidence-based medicine criteria [42].

[b] The level of certainty, as described in section 2.4.

[c] All bilateral ACL ruptures

[d] Al cruciate ligament injuries, ACL and PCL.

2.4.3 Summary: Extrinsic and Intrinsic risk factors for ACL ruptures.

Both extrinsic (environmental) and intrinsic (anatomical, hormonal, neuromuscular and genetic) risk factors have been extensively reviewed according to evidence based guidelines. A summary of the specific risk factors, as well as the level of certainty (refer to Section 2.5) that each risk factor is associated with ACL ruptures is included (Table 2.6).

Table 2.6: Summary of the risk factors reviewed in this thesis. All risk factors are listed according to their level of certainty.

	Level of Certainty[a]		
Risk factors	**Low**	**Moderate**	**High**
Environmental	• Protective bracing • Type of playing surface (in males)	• Weather conditions • Type of playing surface (in females) • Footwear	
Anatomical	• Q angle • Tibial slope • BMI • Anterior knee laxity (in males)	• Foot pronation • Pelvic tilt • ACL geometry • GJL • Anterior knee laxity (in females)	• Notch Width
Hormonal		• Phase of the menstrual cycle (in females)	
Neuromuscular	• Dynamic knee valgus		
Genetic	• *COL1A1* Sp1 binding site polymorphism	• Familial predisposition	

[a] The level of certainty, as described in Section 2.4

As is evident from this summary (Table 2.6), the investigation of genetic risk factors of ACL ruptures is still in its infancy. Therefore, this thesis will use a candidate gene approach to identify further specific sequence variants which may alter the risk of ACL ruptures. A candidate gene approach requires basic knowledge of the molecular structure of the ACL. The following section will therefore review the molecular structure of the ACL, and the identification of candidate genes for ACL ruptures.

2.5 MOLECULAR STRUCTURE OF THE ACL AND IDENTIFICATION OF CANDIDATE GENES FOR ACL RUPTURES.

2.5.1 Molecular structure of the ACL

The major cell types within ligaments are the ligamentoblasts and ligamentocytes. These cells constitutes less than 5% of the total volume of the ligament [111]. Other minor cell types within ligament include osteocytes, synovial cells and vascular cells [111]. The remaining 95% of the tissue consists of the ECM, which consists of various collagen types, glycoproteins, proteoglycans and glycosaminoglycans [15;111]. A large proportion (55 - 70%) of the ligament consists of water [111].

Although there are important functional differences, ligaments and tendons have a very similar hierarchical structure and composition [111]. The collagen microfibril, the basic unit of the ligament, consists predominantly of the type I collagen molecules (also known as tropocollagen). Type I collagen constitutes 70% - 80% of the dry mass of ligaments and is responsible for the tensile strength of the ligament [14]. This protein is a member of the major fibrillar collagen sub-family and is a heterotrimeric triplehelix consisting of two α1(I) chains and one α2(I) chain. Other collagen types, such as types III, IV, V, VI, XII and XIV, are also structural components of the microfibrils and the surrounding ECM [112;113] (Figure 2.6). As illustrated in Figure 2.8 the collagen microfibrils assemble into subfibrils, which in turn form the fibrils. The

collagen based fibrils gather into fascicles, which are the largest subunits of ligaments [111].

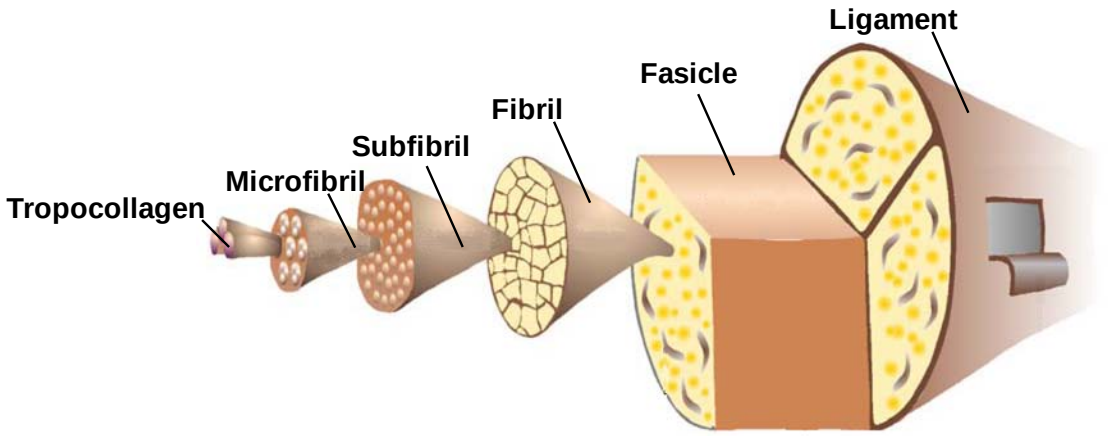

Figure 2.8: The hierarchical structure of ligaments. Figure adapted from Hoffman and and Gross [111].

It has been proposed that the quantitatively minor type III and type V fibrillar collagens, have significant functional roles within the collagen microfibril [114;115]. As illustrated in Figure 2.9, both type V and type III collagen are intercalated into the microfibril together with type I collagen. Type V and type III collagen have been shown to make up as much as 12% and 8% of the type I collagen content, respectively [114]. Although the specific role of type V and type III collagen has not been clearly defined, both collagens have been implicated in the formation of the fibril (fibrillogenesis) [114;116]. Type III collagen, a homotrimer consting of three α1(III) chains, is also a member of the major fibrillar collagen sub-family, while type V collagen is a member of the minor fibrillar collagen sub-family. The major isoform of type V collagen is a

heterotrimer consisting of two α1(V) and one α2(V) chains. Some of the minor isoforms of type V collagen contain an α3(V) chain. The major and minor fibrillar collagens are synthesised and secreted as procollagen molecules which contain globular amino- and carboxy-terminal domains. These domains are usually cleaved from the secreted procollagen molecules as a prerequiset for fibrillogenesis (Figure 2.10). The amino-terminal domain of type V collagen is however not removed.

The retained amino-terminal domain of type V collagen extends perpendicularly to the surface of the fibril, where it is believed to regulate microfibril diameter (Figure 2.9) [117]. In agreement with this proposed function, an age-dependant increase in the content of type V collagen of the rabbit patellar tendon has been shown to be associated with a decrease in fibril diameter [118]. The relative proportion of type V collagen also increases during ligament healing, this increase has been implicated in the poor mechanical properties of the healed ligament [114;119]. Furthermore, it has been proposed that reducing the synthesis of type V collagen through antisense gene therapy may be a viable option to improve the strength and mechanical properties of weak ligaments [119].

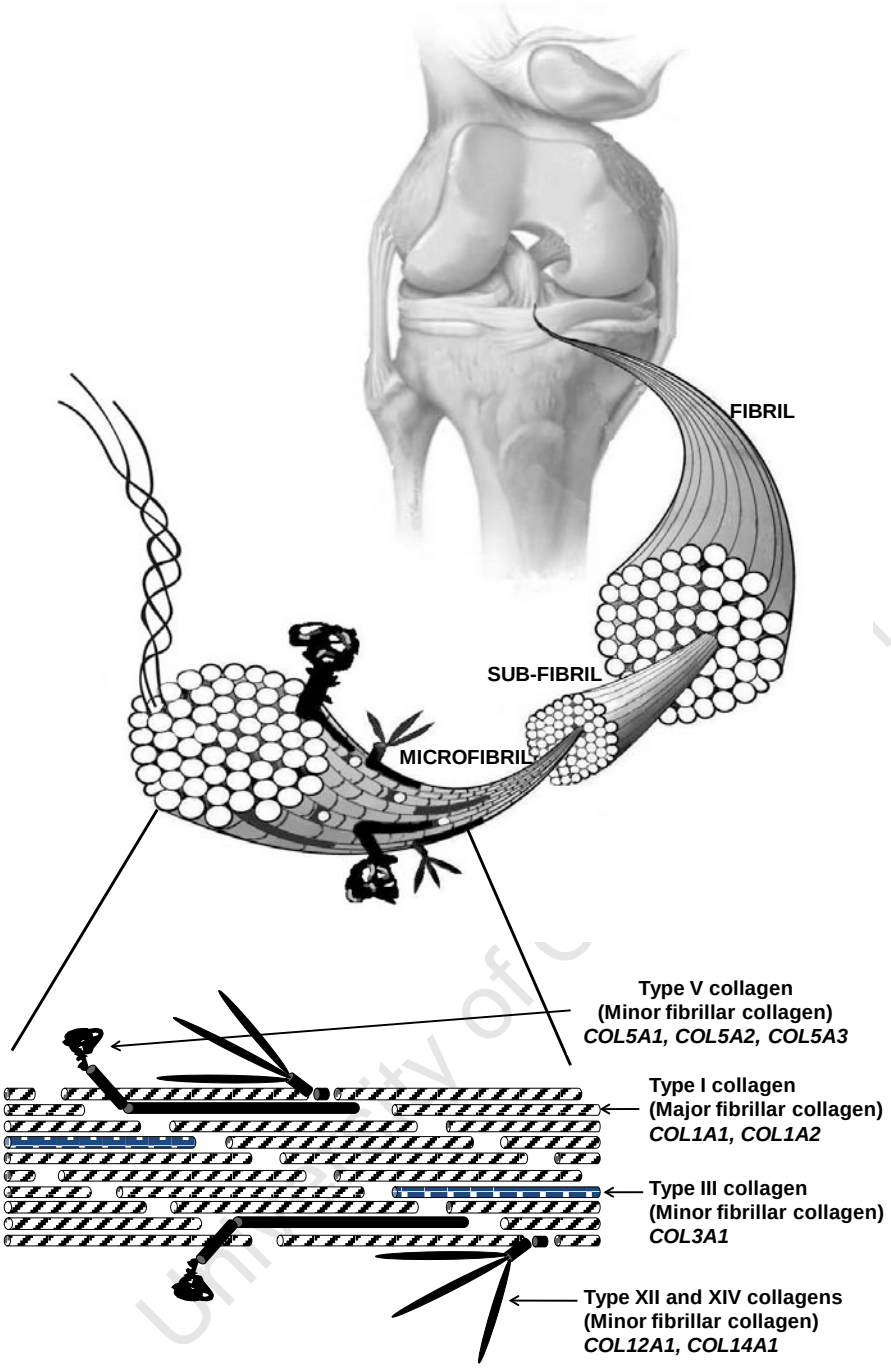

Figure 2.9: A schematic diagram of the basic structural unit of ligaments, the collagen microfibril. The microfibril consists predominately of type I collagen, a major fibrillar collagen. The microfibril also contains trace amounts of type III and type V collagen which belong to the sub-families of major and minor fibrillar collagens respectively. Types XII and XIV collagen are associated with the surface of the fibril and belong to the sub-family of fibril-associated collagens with interrupted triple helices (FACITs). Other collagen types, proteoglycans and glycoproteins which are associated with or structural components of the fibril are not shown**.** Genes that encode for type V collagen, type I collagen, type II collagen and type XII collagen, are italicised.

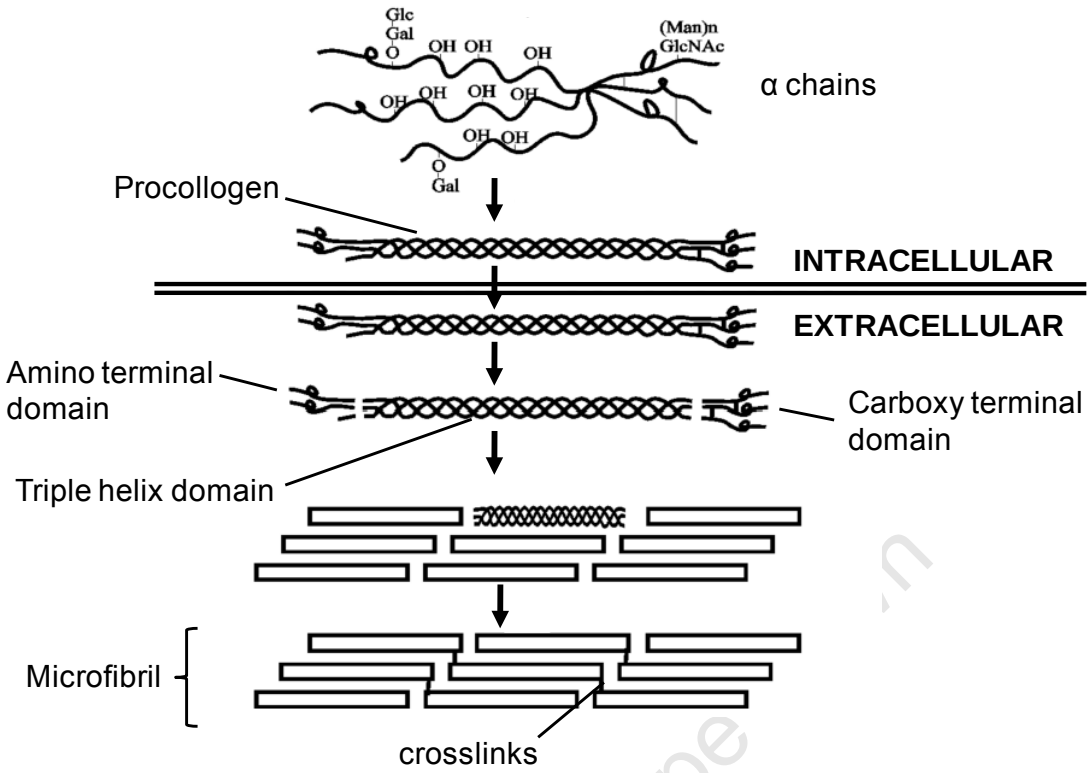

Figure 2.10: The synthesis of the major and minor fibrillar collagens. Procollagen polypeptides (α chains) are synthesised from the genes which for encode the respective chains of the specific collagen. A procollagen triple helix molecule is formed from the procollagen polypeptides within the cells (ligamentocytes), and then secreted into the extracellular space. Once in the extracellular space, the amino- and carboxy-terminal domains are cleaved and the newly formed collagen triple helix molecule is incorporated into the microfibril.

It has been proposed that type III collagen may regulate the diameter of type I collagen fibrils by coating the surface of the fibrils and thereby allowing tip growth and not lateral growth of the fibrils [120]. Similarly to type V collagen, type III collagen is also increased in content during healing, specifically during the early phase [115]. It is suspected that the increased type III content during healing results in smaller fibrils [116].

In addition to type III and type V collagen, other collagen types have also been implicated in fibrillogenesis. A sub-family of collagens, refered to as Fibril Associated Collagens with Interupted Triple helices (FACITs), which include type XII and type XIV collagen, associate with the surface of collagen microfibrils and are thus able to form interfibrillar connections and mediate fibril interaction with other extracellular and cell surface molecules within ligaments and tendons (refer to Figure 2.9) [121-125]. These fibril interactions may further influence the fibril and matrix density, which suggests that these quantitatively minor FACIT collagens may also be involved in fibrillogenesis [122;126;127]. In addition, the expression of these collagens is regulated by mechanical stretch [126;128].

As a common feature of FACITs, type XII collagen consists of two collagenous domains (COL1 and COL2), interrupted and flanked by three non-collagenous domains (NC1-NC3) [129]. Differential splicing within the NC3 domain, the largest of the NC domains, results in long (XIIA) and short (XIIB) isoforms [130]. One of the short isoforms, the XIIB-1 isoform is predominantly expressed in ligaments and tendons [130;131]. Furthermore, similarly to type V collagen, type XII collagen has also been shown to be increased during healing [132]. It can thereofre be proposed that these two collagens are at least partly involved in similar biological processes.

Other quantitatively minor components of the collagen fibril include type IV and type VI collagen [15;113]. Type IV collagen is a common component of the basement membranes, whereas type VI collagen serves as a gliding

component between fibril sub-units [15;113]. These genes that encode for these collagens were not considered as candidates in this thesis and the detailed functions of these proteins will therefore not be reviewed.

Although this section of the review focuses on collagens within the microfibril, it is important to mention that ligaments also contain non-collageneous proteins and other macromolecules. The ligamentous ground substance, which surrounds the collagen molecules and cells, consists of various proteoglycans (such as decorin, lumican and versican) and glycoproteins (such as elastin, tenacin C, fibrillin and Cartliage Oligomeric Matrix Protein (COMP)) [133]. The proteoglycans consists of a protein core which is covalently bound to glycosaminoglycans, a polysaccharide chain which has a consiberable water binding capacity, and therefore provide support and stability to the collagenous tissue [133]. Glycoproteins are macromolecules that consist of a large protein fraction and a small glycidic component, and are involved in many biological processes within the ligament [133]. Some of the functions of these protein molecules include providing elasticity, connecting cells to the ECM and binding collagens. Although many of the genes that encode for these non-collagenous molecules could also be considered as ideal candidate genes, such as for example tenacin-C, none were investigated in this thesis and therefore these molecules were not reviewed.

2.5.2 Genetic risk factors for soft tissue injuries

As discussed in Section 2.4.2.4, genetics elements, which include a familial predisposition and a specific genetic variation (the functional Sp1 binding site polymorphism), have been identified as possible risk factors to ACL ruptures. Recently, similar observations have also been made in other soft tissue injuries. Harvie et al. [134] demonstrated that the siblings of patients who had ruptured their rotator cuff were 4.7 times more likely to rupture their rotator cuff, when compared to controls. Similarly, Aroen et al. [135] also found that patients with Achilles tendon ruptures are at increased risk of a contralateral Achilles tendon rupture. These findings imply that genetic factors play a role in the development of acute soft tissue injuries in general.

Some investigators have also reported an association of the ABO blood group, a biochemical marker for genetic elements, with Achilles and other tendon injuries [136;137]. The O blood group has been shown to be associated with an increased risk of Achilles and other tendon ruptures [136;137]. A decreased A/O ratio was also reported to be associated with an increase risk of Achilles tendon ruptures and Achilles peritendinitis [138]. Other studies have however failed to show similar associations [139-142]. Since the ABO blood group is determined by a single gene (*ABO*) on the long arm of chromosome 9 (9q34), Mokone et al. investigated the association between two genes, namely *COL5A1* [143] and *TNC* [144], which neighbour the *ABO* gene. The *COL5A1* and *TNC* genes encode the previously discussed (Section2.5.1) α1 chain of type V collagen and glycoprotein tenacin-C, respectively [143;144].

Mokone et al. found that the *Bst*UI restriction fragment length polymorphism (RFLP) within the 3'-untranslated (UTR) region of the *COL5A1* gene was associated with Achilles tendinopathy in a South African Caucasian population [143;145]. The CC genotype of this RFLP was significantly over-represented in the asymptomatic control participants of the study [143;145]. Although only few participants (n=44) with Achilles tendon ruptures were investigated, this *Bst*UI RFLP does not seem to be associated with acute tendon ruptures [143]. In a recent repeat of this study in an independent Australian Caucasian population, the CC genotype of the *Bst*UI RFLP was also over-represented in the asymptomatic control participants when compared to those with Achilles tendinopathy [145].

In addition to the *COL5A1* gene, the *TNC* gene was also found to be associated with Achilles tendon injuries [144]. In this study the variants containing 12 and 14 GT repeats were over-represented in participants with Achilles tendinopathy and Achilles tendon rupture, while variants containing 13 and 17 repeats were under-represented [144]. Similar results were observed for the chronic Achilles tendinopathy and the Achilles tendon rupture groups.

Other genes which encode for the proteins involved in biological processes in the tendon are also likely to be associated with Achilles tendon injuries. The matrix metalloproteinases (MMPs) serve a functionally significant role within tendons, and one of the proteins within this family, MMP3, may catalytically degrade various collagen types and other ECM components [146]. For this

reason, Raleigh et al. [147], investigated the possible association of the *MMP3* gene and Achilles tendon injuries. In this study, participants who were homozygous for any of the identified risk alleles were at least twice as likely to develop Achilles tendinopathy [147]. Furthermore, when the genotypes of the one sequence variant within the *MMP-3* gene and the *Bst*UI RFLP within the *COL5A1* gene were combined and analysed, the risk of Achilles tendinopathy was increased [147].

Other genes which have also been investigated as candidate genes for Achilles tendon injuries include *COL12A1 and COL14A1* [148]. These two genes encode for the type XII and type XIV FACIT collagens, respectively. As previously discussed (Section 2.5.1), these two FACIT collagens are involved in similar biological processes as type V collagen and tenacin-C, and were therefore chosen as candidate genes. Sequence variants within either the *COL12A1* and *COL14A1* genes were however not associated with Achilles tendinopathy. Although not significant, the absence of both the rare CC and GG genotypes of the *COL12A1 Bsr*I and *Alu*I restriction fragment length polymorphisms, respectively, in the Achilles tendon rupture subjects warrants further research [148].

From the genetic-association studies discussed above, it has become evident that there is a genetic contribution to soft tissue injuries. To date, only one genetic sequence variant has however been investigated as risk factor for ACL ruptures.

2.5.3 Candidate genes for ACL ruptures

There are however more than 25 000 protein coding genes within the human genome, and many different strategies may be used to identify genetic elements that could be associated with or cause disease and injury. Genetic association studies remain the most widely used method to identify genetic risk factors that may predispose individuals to an increased risk of multifactorial conditions such as an injury [149]. The underlying genes may be identified either through whole genome screens, or by following a candidate gene approach. A large amount of genetic information may be obtained from genome wide screens however, these studies are expensive, require very large sample sizes, and often not hypothesis driven. The candidate gene approach requires the identification of candidate genes based on their biological function. Case-control studies, following a candidate gene approach, are more suited to initial exploratory genetic studies, and are less expensive than whole genome screens and was therefore the method used in this thesis. Potential candidate genes for ACL ruptures include any gene encoding proteins that are either directly or indirectly involved in the structure, degradation, remodelling and/or metabolism of ligaments.

To date, a single genetic variant, namely the *COL1A1* Sp1 binding site polymorphism, has been associated with risk of ACL ruptures (Section 2.4.2.4) [55]. However, it is unlikely that a single gene or a single sequence variant within that gene exclusively predisposes individuals to ACL ruptures. ACL

ruptures, similar to Achilles tendon injuries, are multifactorial conditions, and therefore more likely to be polygenic in nature.

Polymorphisms within the *COL1A1* and *COL5A1* genes have been associated with acute ligament injuries [9], and/or Achilles tendinopathy [143;145]. Therefore, it is reasonable that genes which encode for structural collagenous components of the ligament microfibril should be chosen for initial investigations. Although there are a large number of genes which encode for structural components of the ligament (Table 2.7), the previously investigated *COL1A1* [9]*, COL5A1* [143;145] *and COL12A1* [148] genes were selected as initial possible candidate genes that could be associated with an increased risk of ACL ruptures and will now be reviewed.

2.5.3.1 The *COL1A1* gene

Type I collagen, which constitutes up to 80% of the dry mass of the ligament [14], is a heterotrimer consisting of 2 α1(I) chains and 1 α2(I) chain. The α1(I) and α2(I) chains are encoded for by the *COL1A1* (17q21.33) and *COL1A2* (7q22.1) genes respectively.

The *COL1A1* gene, and specifically the functional Sp1 binding site polymorphism, has been associated with various complex disorders of connective tissue, including, myocardial infarction [151], lumbar disc disease [152] stress urinary incontinence [153], and most notably, osteoporosis [154;155].

Table 2.7: The genes encoding for the ligament and tendon extracellular matrix (ECM) collagens, proteoglycans and glycoproteins.

Protein	Structure/Type	Gene(s) and Chromosomal Location(s)
Collagen		
Type I	Fibril-forming	*COL1A1* (17q); *COL1A2* (7q)
Type III	Fibril-forming	*COL3A1* (2q)
Type IV	Forms meshwork	*COL4A1* & *COL4A2* (13q); *COL4A3* & *COL4A4* (2q); *COL4A5* & *COL4A6* (Xq)
Type V	Fibril-forming	*COL5A1* (9q); *COL5A2* (2q); *COL5A3* (19q)
Type VI	Forms beaded filaments	*COL6A1* & *COL6A2* (21q); *COL6A3* (20q)
Type XII	FACIT[a]	*COL12A1* (6q)
Type XIV	FACIT[a]	*COL14A1* (8q)
Proteoglycan		
Decorin	SLRP[b]	*DCN* (12q)
Fibromodullin	SRLP[b]	*FMOD* (7q)
Lumican	SLRP[b]	*LUM* (12q)
Versican	Hyalectan	*CSPG2* (5q)
Glycoprotein		
Elastin	Oligomeric network	*ELN* (7q)
Fibrillin	Linear arrays	*FBN1* (15q); *FBN2* (5q)
Tenascin-C	Oligomeric molecule	*TNC* (9q)
COMP[c]	Oligomeric molecule	*COMP* (19p)
Fibronectin	Modular protein	*FN1* (2q)
Laminin[d]	Modular protein	
Thrombospondin	Modular protein	*THBS1* (15q); *THBS2* (6q); *THBS3* (1q); THBS4 (5q)
Link protein	Globular protein	*CTR1* (5q)
Tenomodulin	Transmembrane protein	*TNMD* (Xq)

The fibrocartilagenous types II, IX, X and XI collagens, as well as biglycan and aggregan are not included in this table.

[a]FACIT, Fibril Associated Collagen with Interrupted Triple helix; [b]SLRP, small leucine-rich repeat proteoglycan; [c]COMP, cartilage oligomeric matrix protein; [d]Multiple genes coding for multiple proteins. Adapted from Riley [150] and September [163].

A recent study investigating the molecular mechanism of the functional *COL1A1* Sp1 binding site polymorphism, the most widely studied candidate gene for osteoporosis, demonstrated that the G to T substitution results in reduced bone quality [155]. In this study, it was shown, by in vitro gel shift assays, that the T allele had an increased binding affinity for the transcription factor Sp1, which was accompanied by an increase in *COL1A1* mRNA, and an altered production of the α1(I) chain relative to the α2(I) chain [155]. The mechanism whereby this altered protein ratio results in reduced bone quality and strength does however remain unknown. Although a $\alpha1(I)_3$ homotrimer is known to impair bone strength, as is seen in the classical Mendelian disorder osteogenesis imperfecta [113], no studies have formally demonstrated the presence of a $\alpha1(I)_3$ homotrimer in bone derived from patients with a T allele. In addition, the T allele was also associated with a reduced inorganic component and an increased organic component in bone [155].

In contrast to the effects in bone, the previously discussed Swedish study (Section 2.4.2.4.2) showed the TT genotype of the Sp1 binding site polymorphism to be significantly under-represented in participants with acute soft tissue injuries, including cruciate rupture and shoulder dislocations. These data suggests that the G to T substitution increases the tensile strength of ligaments. Further investigation is however required to confirm these findings in a second population, more specifically within participants with ACL ruptures and with gender-matched controls. Studies are also required to determine possible molecular mechanisms.

2.5.3.2 The *COL5A1* gene

The *COL5A1* gene encodes for the α1(V) chain of the fibril forming type V collagen. Type V collagen has been implicated in the regulation of fibril diameter (fibrillogenesis). Furthermore, type V collagen has also been shown to be the second largest collagen component of ligaments [114].

The *COL5A1* gene (as discussed in Section 2.5.2) has been shown to be associated with Achilles tendinopathy. More specifically the CC genotype of the *Bst*UI RFLP within the 3'-untranslated region (UTR) of *COL5A1* gene was significantly over-represented in the control group, when compared the Achilles tendinopathy groups within two independent populations [143;145]. It is however important to note that the biological mechanisms for these observations are currently unknown.

2.5.3.3 The *COL12A1* gene

Type XII collagen is a homotrimer consisting of 3 α1(XII) chains and is encoded for by a single gene, *COL12A1*, which has been mapped to chromosome 6q12-q13 [156]. Type XII is involved in a similar biological process than type V collagen, both collagens are believed to regulate microfibril diameter (fibrillogenesis) [125;127;157]. Furthermore, mechanical forces have been shown to regulate the production of type XII collagen. More specifically the *COL12A1* gene is directly activated by mechanical force [126;128].

As discussed (Section 2.5.2) the *COL12A1 Alu*I and *Bsr*I RFLPs, have been investigated as candidate genes for Achilles tendinopathy. Although not significant, the absence of both the rare CC and GG genotypes of the *COL12A1 Bsr*I and *Alu*I RFLPs, respectively, in participants with Achilles tendon ruptures (acute soft tissue injuries) warrants further research.

2.6 SUMMARY AND CONCLUSIONS OF THE LITERATURE REVIEW

Although there has been a considerable amount of research and development in the understanding of the factors which predisposed individuals to ACL ruptures. It is widely accepted that the exact aetiology and mechanism of these acute injuries are currently unknown. It is however clear that ACL ruptures are a multifactorial condition, which have been associated with various intrinsic and extrinsic risk factors. Numerous, extrinsic (environmental) and intrinsic (anatomical, hormonal, neuromuscular and genetic) risk factors have been extensively reviewed in this chapter according to evidence based guidelines. It is clear from the levels of certainty presented, that a greater amount of research is required for all, but one risk factor, notch width.

Furthermore, the identification of genetic risk factors for ACL ruptures are still in its infancy. Only two studies have identified a familial predisposition, and only a single study has identified a specific genetic element, the *COL1A1* Sp1 binding site polymorphism, which is associated with risk of ACL rupture. Due to the multifactorial nature of ACL ruptures, it is highly unlikely that this is the only genetic element associated with this injury. In support of this argument, various polymorphisms have been shown to be associated with Achilles tendon injuries.

In conclusion, novel genetic elements have recently been identified as possible intrinsic risk factors for ACL ruptures. The preliminary evidence in support of this has been reviewed. Candidate genes that may be associated with

increased risk of ACL ruptures have also been identified, and should be investigated.

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2.7 AIMS AND OBJECTIVES OF THE THESIS

The primary aim of this thesis was to identify candidate genes that may be associated with ACL ruptures, and then use a genetic association approach following a case-control study design to identify specific sequence variants (single nucleotide polymorphisms, SNPs) within these candidate genes which may predispose individuals to ACL ruptures. Candidate genes (*COL1A1, COL5A1* and *COL12A1*) were selected based on the biological function of their encoded proteins (type I, type V and type XII collagen respectively) within the basic structural and functional unit of ligaments, namely the collagen microfibril. The objectives of the specific gene association studies which addressed the primary aim of this thesis were as follows:

- To determine if the rare TT genotype of the functional Sp1 binding site polymorphism within intron 1 of the *COL1A1* gene was associated specifically with ACL ruptures in an independent second South African Caucasian population with gender-matched controls. (**Study 1**)
- To determine if two sequence variants (*Bst*UI and *Dpn*II restriction fragment length polymorphisms, RFLPs) within the 3'-UTR of the *COL5A1* gene, which has previously been investigated in Achilles tendon injuries, were associated with an increased risk of ACL ruptures. Due to the reported increased risk of ACL ruptures in females, a secondary objective of this study was to investigate if there were any gender-specific associations between the two *COL5A1* sequence variants and risk of ACL ruptures. (**Study 2**)

- To determine if two previously described non-synonymous genetic sequence variants, the *Alu*I and *Bsr*I RFLPs within exons 65 and exon 29 respectively, within the *COL12A1* gene, were associated with an increased risk of ACL ruptures. A secondary objective of this study was to investigate if there were any gender-specific associations between the two *COL12A1* sequence variants and risk of ACL ruptures. (**Study 3**)

The secondary aim of this thesis was to investigate the similarities and differences between the genetic risk factors for ACL ruptures and other soft tissue injuries. The genetic variants investigated in Study 2 and 3 of this thesis have previously been investigated as risk factors for other musculoskeletal soft tissue injuries (Achilles tendinopathy and/or Achilles tendon ruptures). However, the possible association of the functional Sp1 binding site polymorphism with Achilles tendon injuries has not been investigated. Therefore, the objectives of the studies which addressed the secondary aim of this thesis are as follows:

- To determine if the rare TT genotype of the functional Sp1 binding site polymorphism within intron 1 of the *COL1A1* gene was associated with other common acute (spontaneous Achilles tendon ruptures) and chronic (Achilles tendinopathy) injuries. (**Study 4**)
- To report the combined effect of the rare *COL1A1* TT genotype and the risk for acute soft tissue ruptures from the previously published study, and the results presented in this thesis (Study 1 and Study 4). (**Study 5**)
- To determine if there are any gender-specific *COL5A1 Bst*UI RFLP genotype effects in chronic Achilles tendinopathy when the two previously

published studies were re-analysed. Another objective of this study was to investigate if the distribution of the *COL5A1 Bst*UI RFLP within the combined asymptomatic control participants from this thesis (Study 2), as well as the two previously published studies were age-dependant. **(Study 6)**

CHAPTER 3

STUDY ONE

GENETIC RISK FACTORS FOR ANTERIOR CRUCIATE LIGAMENT RUPTURES: THE *COL1A1* SP1 BINDING SITE POLYMORPHISM

The data presented in this chapter was published in the following peer-reviewed article: Posthumus, M. September, AV. Keegan, M. O'Cuinneagain, D. van der Merwe, W. Schwellnus, MP. Collins, M. Genetic risk factors for anterior cruciate ligament ruptures: The *COL1A1* gene variant. ***British Journal of Sport Medicine***, 2009, 43:352-356.

3.1 INTRODUCTION

As discussed in the literature review (Section 2.5.3.1), the gene encoding for the α1 chain of type I collagen (α1(I)), *COL1A1*, was selected as a candidate gene for ACL ruptures for the following reasons: (1) type I collagen is the major protein component (70-80% of its dry weight) of the ACL, and (2) various other complex connective tissue disorders, including acute ligament injuries, have been shown to be associated with the Sp1 binding site polymorphism within the *COL1A1* gene.

The Sp1 binding site polymorphism is a functional polymorphism within intron 1 of *COL1A1*. It has been shown to alter the expression and translation of the α1(I) chain (Figure 1.1) [155]. This polymorphism is the most widely researched genetic variant within the *COL1A1* gene.

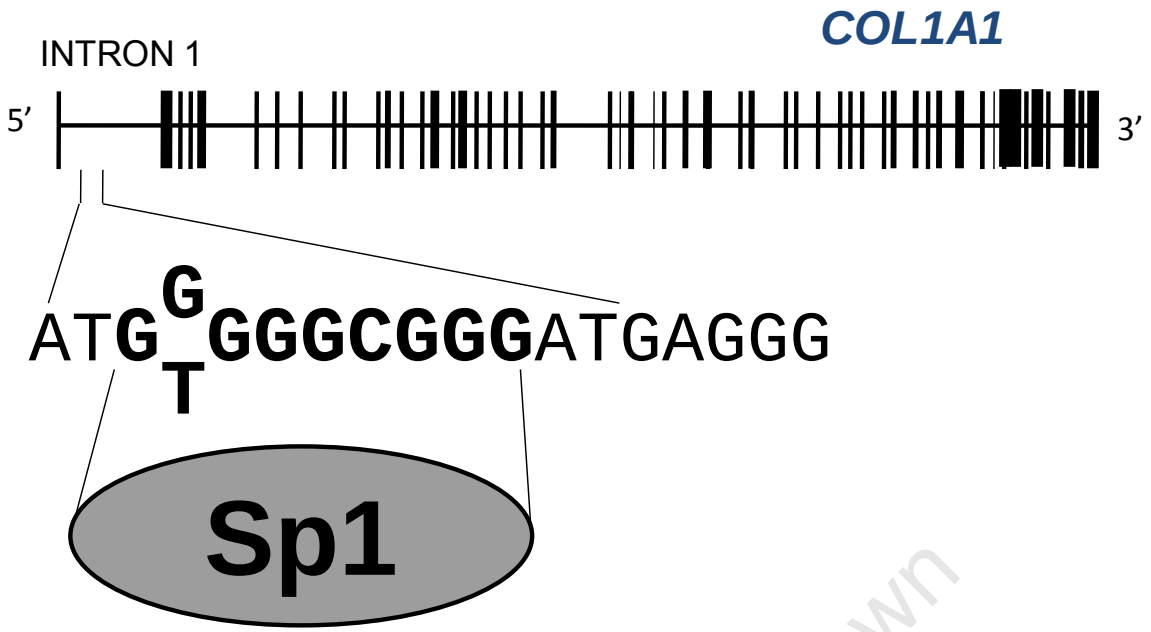

Figure 3.1: A schematic diagram showing the G>T substitution (SNP rs1800012) at nucleotide 1023 of intron 1 (IVS1) within the *COL1A1* gene. The relative sizes and positions of the exons (vertical lines) and the introns (horizontal lines) which make up the *COL1A1* gene are shown. Eighteen bases within intron 1, which contain the the Sp1 binding site (nucleotides in bold) are also shown. The binding of the Sp1 transcription factor (grey oval) is shown. The substitute of the wild type G nucleotide with T nucleotide increases the affinity of the Sp1 transcription factor, which in turn results in an increased expression of the *COL1A1* gene [155].

As previously discussed (Section 2.4.2.4.2), a single Swedish study has recently shown that the functional *COL1A1* Sp1 binding site polymorphism is associated with acute ligament injuries, including cruciate ligament ruptures and shoulder dislocations [9]. In this Swedish study, all cruciate ligament ruptures (not limited to ACL) were investigated. Additionally, the control group consisted of only females, while the injury group consisted of females and males [9]. Female gender is a significant risk factor for ACL ruptures (Section 2.3.1.3). In addition, it has been

reported that if a gender difference in the risk of a disorder exists, it might be expected that the at-risk allele or genotype is over-represented among participants at less risk [158]. Therefore, repeating the Swedish study in an independent population focusing on a specific clinical phenotype, such as ACL ruptures, with gender-matched controls, will provide further evidence to support the initial association.

The aim of this first study of this thesis was therefore to determine whether the functional Sp1 binding site polymorphism within intron 1 of the *COL1A1* gene is associated specifically with ACL ruptures in an independent second South African Caucasian population with gender-matched controls.

3.2 MATERIALS AND METHODS

3.2.1 Participants

One hundred and twenty nine Caucasian participants with surgically confirmed anterior cruciate ligament (ACL) ruptures were recruited by convenience sampling for this study, and subsequent studies of this thesis, from the Sports Science Orthopaedic and Sports Medicine Clinics in Cape Town, South Africa. These same participants were also studied in subsequent studies (Study 2 and 3) that are presented in this thesis.

Of the 129 participants with ACL ruptures, only 117 participants were genotyped in the current study. Eleven (9.4%) of the participants had a history of bilateral ACL ruptures. Twenty nine percent (31 of 106; detailed information was unknown in 11 participants) of all participants with ACL ruptures did not sustain any associated (concurrent) injuries. The most common associated injury was injury to the meniscus (49% of participants; 52 of 106) and injury to the MCL (20% of participants; 21 of 106). In addition, 130 apparently healthy, unrelated, physically active gender-matched Caucasians without any self-reported history of ligament or tendon injury were recruited as control (CON) participants from sports clubs and a wellness centre (SSISA) within the Southern Suburbs region of Cape Town, South Africa.

Participants which had detailed information available on the mechanism of ACL rupture, were subdivided into sub-groups consisting of those where the injury was sustained through direct contact (DIR, n=15), indirect contact (IND, n=18) or non-

contact (NON, n=50), using the American Orthopaedic Society for Sports Medicine classification system [13]. In 34 (29%) of the participants who had ruptured their ACL, no clear mechanism of injury could be identified and were not included in any of the sub-groups.

Although care was taken to ensure that all participants in the CON group were physically active, only the ACL group completed sports participation details for this particular study. A list of specific sports most often played by the ACL groups (male and female) are included in Appendix 1 (Additional material, Table A1.1). In summary, the sport most often played by males in the ACL group was rugby (65.6%), while the females most often played field hockey (60.0%). Further sports participation details are included in Table 4.3 (Study 2). Amongst the female ACL group, 57.1% (20 of 35) and 5.7% (2 of 35) participated in non-contact jumping sports and contact sports respectively. Amongst the male ACL group, 13.4% (11 of 82) and 87.8% (72 of 82) participated in non-contact jumping sports and contact sports respectively. Detailed lists of the sports within these categories are presented in Study 2 (Section 4.2.2).

Prior to participation in this study, all the participants gave informed written consent (Appendix 2). In addition, each participant completed personal details, injury details and medical history questionnaire forms (Appendix 3). This study was approved by the Research Ethics Committee of the Faculty of Health Sciences within the University of Cape Town, South Africa (reference number 164/2006; Appendix 4).

3.2.2 DNA extraction

Approximately 4 ml of venous blood was obtained from each participant by venipuncture of a forearm vein and collected into an EDTA vacutainer tube. Blood samples were stored at -20°C until total DNA extraction.

DNA was extracted using the procedure described by Lahiri and Nurnberg [159], with slight modification. Briefly, the blood samples were transferred to sterile 15 ml polypropylene tubes, to which 10 ml of TKM1 buffer (10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM $MgCl_2$ and 2 mM EDTA) containing 2.5 % Nonidet P-40 was added to lyse the red blood cells. After incubating at room temperature for 10 minutes, the white blood cells (WBC) were pelleted by centrifugation at 1200 X g at room temperature for 10 minutes and washed at least once with one volume of TKM1 buffer. The washed WBC pellets were resuspended in 800 µl of TKM2 buffer (10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 0.4 M $NaCl_2$ and 2 mM EDTA) containing 50 µl of 10 % SDS and incubated for at least 60 minutes at 55 ºC to lyse the WBC. One hundred and fifty µl of 5 M $NaClO_4$ and 500 µl of chloroform was added to each sample, which was then mixed thoroughly by vortexing for 15 - 20 seconds. The samples were transferred to 1.5 ml microfuge tubes and the protein precipitated by centrifugation at 15 000 X g (13 000 rpm) for 5 minutes at room temperature. Five hundred µl of the top aqueous phases were transferred to new microfuge tubes containing 1 ml of absolute ethanol, mixed and the DNA pelleted by centrifugation at 13 000 rpm for 2 minutes at room temperature. The precipitated DNA was air dried for at least 30 minutes and resuspended in 200 µl of TE buffer (10

mM Tris-HCl, 1 mM EDTA, pH 8.0). Each tube was incubated at 65 °C for 15 minutes before being stored 4°C until PCR analysis.

3.2.3 *COL1A1* genotyping

DNA samples were genotyped for the Sp1 binding site polymorphism (SNP rs1800012; IVS1+1023G>T) within intron 1 of the *COL1A1* gene using a nested polymerase chain reaction (PCR) amplification method as previously described [153] (Figure 3.2). The PCR reactions were performed in a final volume of 60 µl containing at least 200 ng genomic DNA (primary reaction) or 1 µl of PCR product (secondary reaction); 20 pmol of each primer; 2.0 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 200 µM each dATP , dCTP, dGTP, and dTTP and 0.5 U Taq DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA).

The following primer pairs were used: forward primer for the primary reaction, 5'-GGA AGA CCC GGG TTA TTG CT-3'; reverse primer for the primary reaction, 5'-CGC TGA AGC CAA GTG AAA TA-3'; forward primer for the secondary reaction, 5'-TAA CTT CTG GAC TAT TTG CGG ACT TTT TGG-3' and the reverse primer for the secondary reaction, 5'-GTC CAG CCC TCA TCC TGG CC-3'. The secondary reverse primer was designed to contain two mutated nucleotides (underlined in the primer sequence) which introduced a restriction site (TGG/CCA) for the restriction endonuclease *Msc*I at the 3' end of the 260 bp secondary PCR product when amplifying the T allele [153]. The conditions for the primary and secondary PCRs were as follows: (i) denaturing at 95°C for 15 minutes; (ii) 5 cycles of denaturation at 95°C for 25 seconds, annealing at 70°C for 45 seconds and extension at 72°C for 30

seconds; (iii) 27 cycles of denaturation at 95°C for 25 seconds, annealing at 58°C for 45 seconds and extension at 72°C for 30 seconds; and (iv) a final extension at 72°C for 10 minutes (*XP Thermal Cycler Block*; *Bioer technology Co*; Middlesex, UK).

Figure 3.2: A schematic diagram showing the G>T substitution (grey box in the genomic sequence; SNP rs1800012) at nucleotide 1023 of intron 1 (IVS1) within the *COL1A1* gene. The relative sizes and positions of the exons (vertical lines) and the introns (horizontal lines) within the *COL1A1* gene are shown. A 600 bp genomic sequence within intron 1 is shown to indicate the position of the outer forward (P1) and reverse (P2) primers (solid arrows) designed to amplify the primary 598 bp fragment, and the inner forward (P3) and reverse (P4) nested primers (dashed arrows) to amplify a 260 bp PCR fragment and introduce a *Msc*I restriction recognition site (TGG/CCA) for the T allele. The P4 primer partially spans the consensus binding site (boxed) for the Sp1 transcription factor.

The secondary PCR products were digested with the restriction endonuclease *Msc*I (New England Biolabs, Ipswich, MA, USA) for 3 hours at 37°C following the manufacturers instructions. The resultant fragments together with a 100 bp molecular weight marker (Promega Corporation, Madison, Wisconsin, USA), and *SYBER® Gold* nucleic acid gel stain (*Invitrogen Molecular Probes*™, Oregon, USA) were separated on 6% non-denaturing polyacrylamide gels. The gels were photographed under UV light using a Uvitec photodocumentation system (Uvitec Limited, Cambridge, UK) and the sizes of the DNA fragments determined. The G allele produces a 260 bp fragment while the T allele produces 242 bp and 18 bp fragments (Figure 3.3).

3.2.4 Statistical analysis

A one-way analysis of variance (ANOVA) was used to determine any significant differences between the characteristics of the CON and ACL groups, as well as the DIR, IND and NON sub-groups. A least squares difference (LSD) post-hoc test was used to identify specific differences when the overall F value was found to be significant. A chi-squared (χ^2) analysis or Fisher's exact test was used to analyse any differences in the genotype and allele frequencies, as well as family injury history between the groups. The sample sizes used for this study and subsequent studies within the thesis (Study 2 to Study 5) were based on those reported in previous published studies on the genetic basis of Achilles tendon injuries [143-145; 147] where associations were reported with an odds ratios >2 at a power of 80% and significance level of 5%. When appropriate, adjusted p-values are indicated.

Significance was accepted when $p<0.05$. Hardy-Weinberg equilibrium was established using the program Genepop web version 3.4 (http://genepop.curtin.edu.au/).

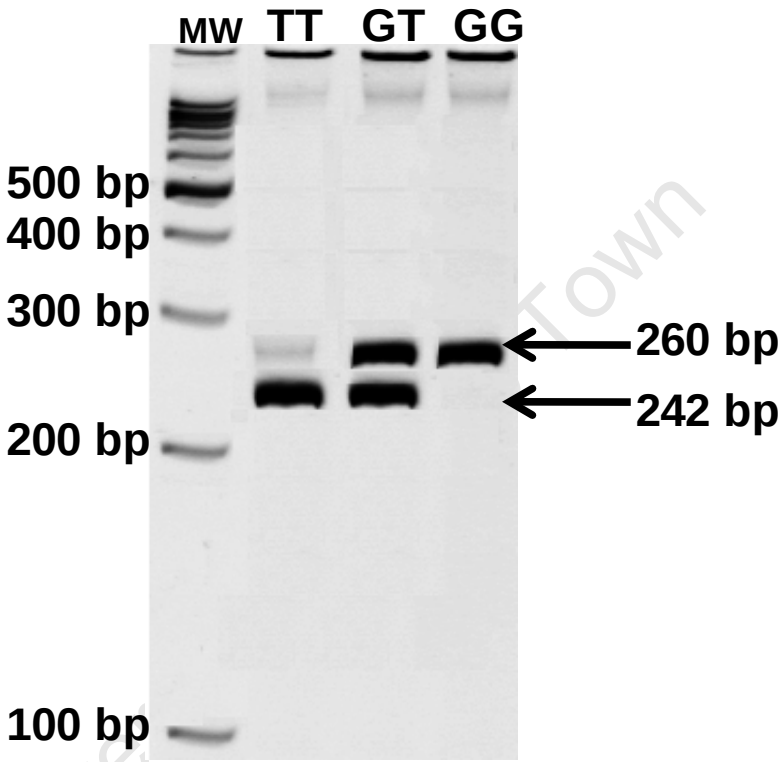

Figure 3.3: A typical 6% non-denaturing polyacrylamide gel showing the genotypes of the *COL1A1* Sp1 binding site polymorphism. The secondary PCR product (260 bp) is digested with *Msc*I to produce 242 bp and 18 bp fragments for the T allele. The small 18 bp fragment ran off the gel and can therefore not be seen in the figure. *Msc*I is not able to digest the secondary 260 bp PCR product when the G allele is present. The genotype of each sample is indicated at the top of each lane. The left lane contains the 100 bp molecular weight marker (MW) and the appropriate fragment sizes are given in base pairs (bp).

3.3 RESULTS

3.3.1 Participant characteristics

The participants within the CON and ACL groups, as well as the DIR, IND and NON sub-groups, were similarly matched for gender and height (Table 3.1). The age of the control group at recruitment was significantly older than the age of the ACL group, as well as the DIR, IND and NON sub-groups, at the time of first ACL rupture ($p<0.001$). The ACL participants were recruited on average 5.1 ± 8.7 years after their initial ACL rupture. Although there were no significant differences in weight between the CON and ACL groups after adjusting for age, the IND sub-group was significantly heavier at the time of injury when compared to the control group at the time of recruitment. The BMI of the ACL participants were significantly higher ($p=0.030$), when compared to the CON group, even after adjusting for age ($p=0.018$). The ACL participants were on average 1.3 ± 4.4 kg heavier, with a corresponding higher average BMI (0.4 ± 1.5 kg/cm^2), at recruitment when compared to the time of first ACL rupture. There were significantly less South African born participants in the CON group ($p<0.012$), when compared to the ACL group. Similar results were however obtained when only South African born participants were analysed (Appendix 1, Additional material, Table A1.2).

Table 3.1: General characteristics of the control (CON) and anterior cruciate ligament rupture (ACL) group, as well as the direct contact (DIR), indirect contact (IND) and non-contact (NON) ACL rupture sub-groups.

	CON	ACL	P-value [b]	DIR	IND	NON	P-value [c]
Age (years) [a]	37.7 ± 10.0 (125) [d,e]	29.0 ± 11.2 (114)	**<0.001**	26.3 ± 12.3 (15)[d]	32.8 ± 12.3 (18)	28.0 ± 10.9 (50) [e]	**<0.001**
Height (cm)	177.4 ± 9.6 (126)	176.7 ± 9.8 (104)	0.578	175.4 ± 8.2 (14)	180.9 ± 8.9 (17)	177.0 ± 9.5 (49)	0.418
Weight (kg) [a]	76.2 ± 13.1 (130) [f]	79.4 ± 17.3 (104)	0.094 [g]	81.4 ± 12.5 (14)	87.9 ± 15.9 (17) [f]	79.9 ± 17.9 (49)	**0.008** [g]
BMI (kg/cm^2) [a]	24.1 ± 3.3 (126)	25.1 ± 3.7 (100)	**0.018** [g]	26.2 ± 2.7 (14)	25.9 ± 2.8 (16)	25.3 ± 4.4 (47)	**0.014** [g]
Gender (% males)	74.6 (130)	69.0 (116)	0.325	93.3 (15)	88.9 (18)	66.0 (50)	n.d.
Country of birth (% South Africa)	71.1 (128)	85.1 (107)	0.012	92.9 (14)	83.3 (18)	84.0 (50)	n.d.

Gender and country of birth are represented as a frequency (%). The remaining variables are expressed as mean ± standard deviation. The number of subjects (n) for each variable is in parentheses.

[a] Age, weight and BMI are self-reported values at the time of the first ACL rupture for the ACL group, as well as the DIR, IND and NON sub-groups, and at recruitment for the CON group. For the ACL group the age, weight and BMI at recruitment were 5.1 ± 8.7 years (n=114), 1.3 ± 4.4 kg (n=103) and 0.4 ± 1.5 kg/cm^2 greater than at the time of the first ACL rupture.

[b] CON vs. ACL; [c] CON vs. DIR vs. IND vs. NON

Pairwise, post hoc significant differences: [d] CON vs. DIR ($p<0.001$); [e] CON vs. NON ($p<0.001$); [f] CON vs. IND ($p=0.010$)

[g] Adjusted for age

n.d. = not determined due to small sample sizes

Except for one CON participant, none of the CON participants reported a previous history of any ligament or tendon injuries (Table 3.2). The single CON participant had reported a previous finger ligament injury. Sixty (56.1%) of the ACL participants reported a previous history of any other ligament injury, of which 13 (12.3%) and 40 (37.7%) reported a history of other knee and/or ankle ligament injuries, respectively. The other knee ligament injuries included injury to the MCL (n=11), LCL (n=2) and the PCL (n=1). Ankle ligament injuries included injury to the lateral (n=39) and (n=5) medial ankle ligaments. Only 11 (10.4%) ACL participants reported a history of Achilles tendon injuries.

3.3.2 Family history

The family history of any ligament injury, which includes ligament injury to any blood relative as reported by the participant at the time of recruitment, was significantly higher (OR=4.2; 95% CI = 2.2 – 8.0; P<0.001) in the ACL group (39.6%; n=106) compared to the CON group (13.5%; n=126) (Table3.2). Within the ACL group there was a similar incidence in the family history of any ligament injury amongst the DIR (n=14, 35.7%), IND (n=18, 38.9%) and NON (n=50, 40.0%) sub-groups (p=0.981).

Table 3.2: Personal and family history of soft tissue injuries amongst the control (CON) and anterior cruciate ligament rupture (ACL) group, as well as the direct contact (DIR), indirect contact (IND) and non-contact (NON) sub-groups.

	CON	ACL	P-value[a]	DIR	IND	NON	P-value[b]
Previous ligament injury	0.8 (129)	56.1 (107)	n.d.	50.0 (14)	61.1 (18)	57.1 (49)	0.818
Previous Knee ligament[c]	0 (0)	12.3 (106)	n.d.	7.1 (14)	22.2 (18)	12.0 (50)	0.414
Previous Ankle Ligament sprain[d]	0 (0)	37.7 (106)	n.d.	35.7 (14)	55.6 (18)	36.0 (50)	0.325
Previous Achilles tendon injury	0 (0)	10.4 (106)	n.d.	0 (14)	5.6 (18)	18.4 (49)	0.112
Joint Capsule Disease	0 (129)	1.9 (103)	n.d.	7.7 (13)	0 (17)	2.1 (48)	n.d.
Family ligament injury	13.5 (126)	39.4 (104)	**P<0.001**	35.7 (14)	38.9 (18)	49.6 (48)	0.966
Family Achilles tendon injury	1.6 (127)	3.8 (106)	n.d.	0 (14)	0 (17)	4 (50)	n.d.

All values are represented as frequency (%) of subjects, or the frequency (%) of subjects' relatives whom have presented with the relevant connective tissue pathology. The number of subjects (n) for each variable is in parentheses.

n.d.= non-determined P-values. [a]= CON vs. ACL. [b]= DIR vs. IND vs. NON. [c]= Includes the lateral and medial ankle ligaments

[d]= Includes the posterior cruciate ligament (PCL), the lateral collateral ligament (LCL), and the medial collateral ligament (MCL).

3.3.3 *COL1A1* genotype frequencies

There were no significant differences in the distribution of the genotype (GG vs. GT + TT, p=0.890) or allele (p=0.745) frequencies of the *COL1A1* Sp1 binding site polymorphism between the ACL and CON groups, as well the genotype (GG vs. GT + TT, p=0.548) or allele (p=0.935) frequencies between the CON and NON sub-groups (Table 3.3). Although the sample size of the DIR and IND sub-groups were too small for formal analysis, theses sub-groups appeared to have similar genotype distributions to the NON sub-group. It is important to note that although there were no differences in the genotype distribution, none of the ACL participants were homozygous for the minor T allele. The rare TT genotype was significantly under-represented in the ACL group when compared to the CON group (OR=12.3; 95% CI 0.7 – 220.4; P=0.031) (Figure 3.4).

The *COL1A1* genotype distribution of the CON (p=0.219) and ACL (p=0.075) groups were in Hardy-Weinberg equilibrium. More females have a TT genotype (4 of 6; 66.7%) when compared to the GT (18 of 70; 25.7%) and GG (47 of 125; 27.3%) genotypes. There were however no *COL1A1* genotype effects on any of the other physical characteristics of the participants when adjusted for gender (Table 3.4). Similar results were obtained when only the SA born subjects were analysed (Appendix 1, Additional material, Table A1.3). The genotype distribution between the male and female ACL participants was similar (P=0.523; male 54 GG, 66.7%; 27 GT, 33.3%; female 26 GG, 72.2%; 10 GT, 27.8%).

Table 3.3: Relative genotype and allele frequencies of the *COL1A1* Sp1 binding site polymorphism within the control (CON) and anterior cruciate ligament rupture (ACL) group, as well as the direct contact (DIR), indirect contact (IND) and non-contact (NON) ACL rupture sub-groups.

	CON (n=130)	**ACL** (n=117)	**DIR** (n=15)	**IND** (n=18)	**NON** (n=50)
GG genotype (%)	70.0 (91)	68.4 (80)	80.0 (12)	66.7 (12)	72.0 (36)
GT genotype (%)	25.4 (33)	31.6 (37)	20.0 (3)	33.3 (6)	28.0 (14)
TT genotype (%) [a]	4.6 (6)	0 (0)	0 (0)	0 (0)	0 (0)
G allele (%)	82.7 (215)	84.2 (197)	90.0 (27)	83.3 (30)	86.0 (86)
T allele (%)	17.3 (45)	15.8 (37)	10.0 (3)	16.7 (6)	14.0 (14)

The values are expressed as a percentage with the number of subjects (n) in parentheses.
[a] Due to the absence of participants with a TT genotype in the ACL group and three sub-groups, the GT and TT participants were combined and compared to the GG participants. CON vs. ACL genotypes, p=0.890. CON vs. ACL, TT genotype vs. GG + GT genotypes, p=0.031, OR=0.08, 95% CI <0.01 to 1.46. CON vs. NON genotypes, p=0.935. CON vs. ACL alleles, p=0.745. CON vs. NON alleles, p=0.548.

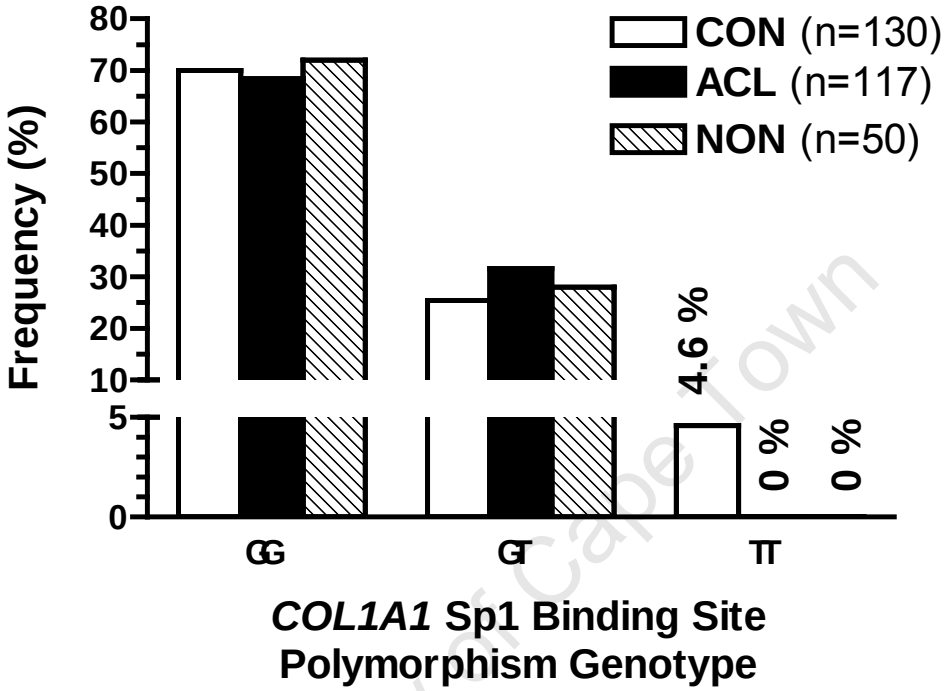

Figure 3.4: Relative genotype frequencies of the *COL1A1* Sp1 binding site polymorphism (SNP rs1800012; IVS1+1023G>T) of the asymptomatic control group (CON, clear bars), the anterior cruciate ligament rupture group (ACL, black bars), and the non-contact mechanism of rupture sub-group (NON, hatched bars).

Table 3.4: Genotype effects of the Sp1 binding site polymorphism within the *COL1A1* gene on the characteristics of the combined control (CON) and anterior cruciate ligament ruptured (ACL) participants.

	GG	GT	TT	P-value [a]
Age (years)[b]	33.2 ± 11.3 (165)	33.8 ± 11.7 (68)	39.3 ± 10.2 (6)	0.425
Height (cm)[b]	176.9 ± 9.9 (158)	177.9 ± 9.1 (66)	172.8 ± 9.4 (6)	0.750
Weight (kg)[b]	77.7 ± 15.8 (162)	78.9 ± 13.1 (66)	62.7 ± 8.5 (6)	0.334
BMI (kg/cm^2)[b]	24.6 ± 3.8 (155)	24.9 ± 2.7 (65)	20.9 ± 1.3 (6)	0.152

The values are expressed as a mean ± standard deviation, with the number of subjects (n) for each variable is in parentheses. [a] Except for age, P values are co-varied for gender. [b] All values are self self-reported values at the time of the first ACL rupture for the ACL injured participants, and at recruitment for the CON participants.

3.4 DISCUSSION

The main finding of this study was that the rare TT genotype of the functional *COL1A1* Sp1 binding site polymorphism was significantly under-represented (OR=12.3; 95% CI 0.7 – 220.4; P=0.031) among participants with ACL ruptures, indicating a possible protective role of this genotype for these injuries. The second finding of this study was that participants with an ACL rupture were more than four times as likely (OR=4.2; 95% CI = 2.2 – 8.0; P<0.001) of reporting that a blood relative had a history of any ligament injury when compared to the control participants. The third finding was that the mechanism of the majority of ACL ruptures in our population was as a result of a non-contact event, and that the genotype distribution in this sub-group was similar to that of the indirect and direct injury sub-groups.

The finding of an under-representation of the TT genotype in the ACL participants is in agreement with what was previously reported in a Swedish population [9]. Furthermore, the genotype distribution of the control participants in this study were similar to the Swedish study [9] and other larger cohorts [155;160]. Of specific clinical importance is that the TT genotype was absent from all 117 participants with ACL ruptures in our study, while only one individual out of 233 participants (0.4%) with a cruciate ligament rupture in the Swedish study had the TT genotype [9]. The combined results from these two studies provide strong evidence that the TT genotype of the Sp1 binding site polymorphism within the *COL1A1* gene has a protective role in anterior cruciate ligament ruptures, as will be further analysed and discussed in Study 5 of this thesis.

Previous investigations have proposed that the increased Sp1 transcription factor binding, which occurs when a G nucleotide is substituted for a T at the Sp1 binding site, leads to increased expression of the α1(I) chain [155]. Although the consequence is not yet fully understood, the increased expression of the α1(I) chain has been shown to reduce the quality and strength of bone [155]. There is no known previous study that has investigated the effect of this polymorphism on other type I collagen containing connective tissues such as ligaments. It is however interesting to note that the TT genotype of the Sp1 binding site polymorphism is not always associated with increased risk of pathology, and has also been shown to be associated with reduced risk of radiographic osteoarthritis of the hip characterised by joint space narrowing [160]. No studies, to date, have investigated changes in *COL1A1* RNA or type I collagen protein levels in ruptured ACL material. Differential expression of the type I collagen genes has however been shown to be associated with the degree of healing after ACL rupture [161].

The second finding from this study, that participants with an ACL rupture were more than four times as likely to have a blood relative with a ligament injury, also supports a genetic risk for ligament injury. Despite the fact that it was not possible to control for exposure to non-genetic factors in family members of our ACL ruptured participants, the results are similar to that reported by Flynn et al. [7], where siblings of individuals whom had an ACL tear were at a two times greater risk of also tearing their ACL.

The third finding of this study was that the majority (57.7%) of ACL ruptures occurred during a non-contact event compared to direct contact (19.2%) or indirect contact (23.0%) events. Non-contact ACL ruptures occur from the athletes own movements, and not as result of contact with another athlete or objects [13], it may thus be hypothesised that intrinsic risk factors, in particular genetic elements, may have a greater contributing role in non-contact ACL ruptures. Even though the direct and indirect contact sub-groups did involve the application of an external force, it might be of clinical relevance to note that the TT genotypes were also absent in these sub-groups.

It is important to note that there are strengths and limitations to the study design presented in this chapter. A strength of this study was that the injury population consisted of a homogenous group of participants with confirmed diagnosis of ACL ruptures at surgery. Furthermore, our injured population and controls included both genders and were suitably matched. One of the limitations of this study was that the exposure to extrinsic risk factors could not be well documented in all the control participants. However, as previously mentioned, the genotype frequencies of the control participants in this study was similar to other much larger cohorts [155;160]. Although care was taken to recruit physically active individuals, participation in high risk sports such as sports involving cutting, pivoting and landing was not known in all the control participants. Additional physically active control participants, with known sports participation history, were therefore recruited for the subsequent studies of this thesis. Sample sizes in these sub-groups (according to injury mechanism) were small and therefore separate analysis of genotype frequencies need to be interpreted with caution.

The main findings of this study support the hypothesis that genetic factors are associated with the risk of ACL ruptures. However, it is important to emphasise that ACL rupture is a multifactorial disorder and is therefore caused by a complex interaction of a number of different intrinsic and extrinsic risk factors [162]. It is therefore highly likely that sequence variants within other genes may also be associated with ACL rupture. As previously described, the aim of this thesis is to test whether genes which encode for the collagen microfibril are associated with ACL ruptures. This will be further explored in subsequent chapters of the thesis.

In conclusion, this study found that the rare TT genotype of the functional Sp1 binding site polymorphism within intron 1 of *COL1A1* was significantly under-represented in participants with ACL ruptures, when compared to gender-matched controls, in an independent second population (Figure 3.5).

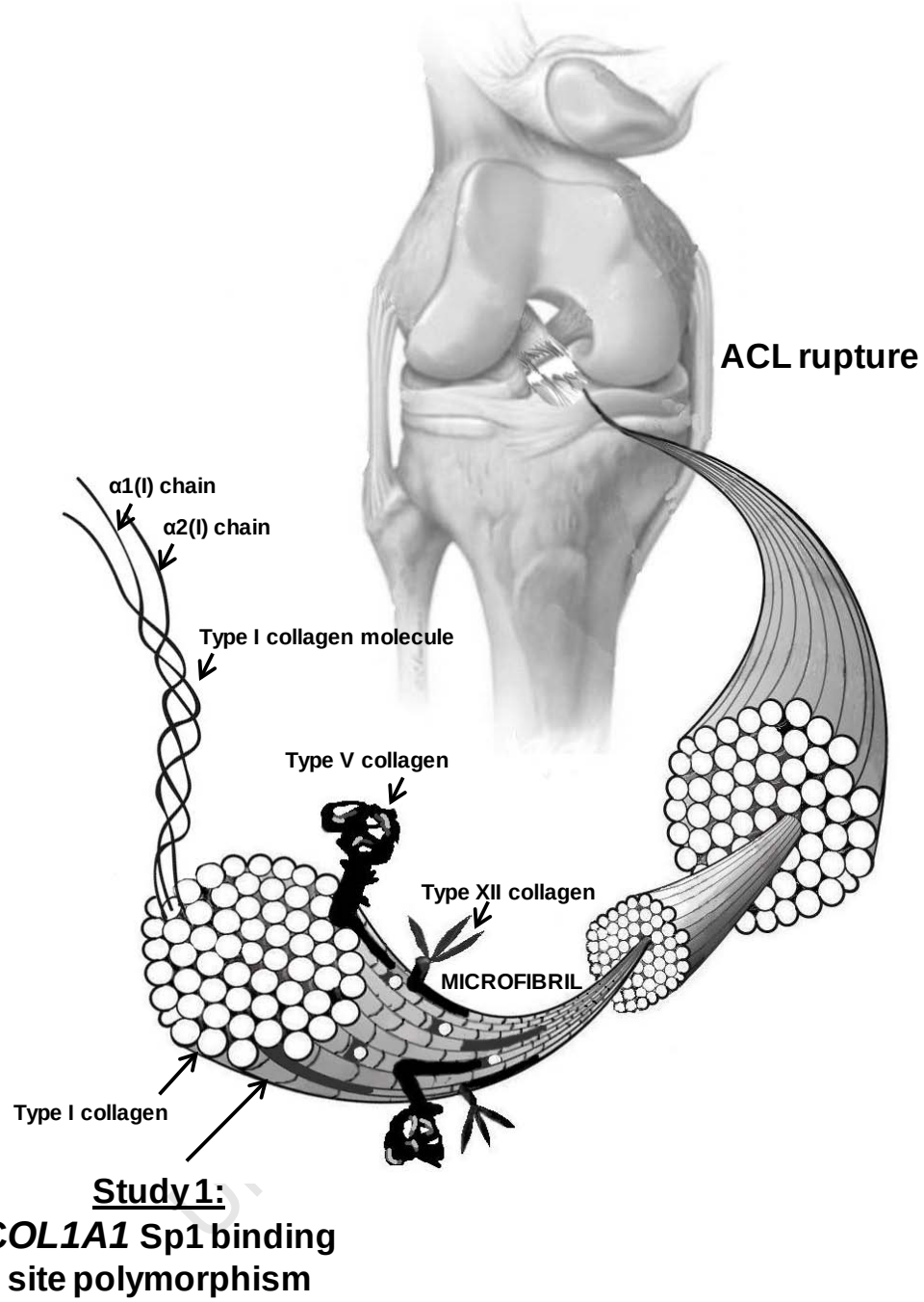

Figure 3.5: A schematic presentation of the primary finding from this study. The *COL1A1* Sp1 binding site polymorphism was associated with risk of ACL ruptures. The *COL1A1* gene encodes for the α1 chain of type I collagen, the major component of the collagen microfibril fibril. Refer to Section 2.5.1 for further detail regarding the structure of the collagen microfibril.

CHAPTER 4

STUDY TWO

THE *COL5A1* GENE IS ASSOCIATED WITH ANTERIOR CRUCIATE LIGAMENT RUPTURES IN FEMALE PARTICIPANTS

The data presented in this chapter was published in the following peer-reviewed article: Posthumus, M. September, AV. O'Cuinneagain, D. van der Merwe, W. Schwellnus, MP. Collins, M. The *COL5A1* gene is associated with increased risk of anterior cruciate ligament ruptures in female participants. ***American Journal of Sports Medicine*****,** In Press.

4.1 INTRODUCTION

The primary objective of this thesis is to identify genetic factors which predispose individuals to ACL ruptures. As discussed (Section 2.4.2.4.2), the rare TT genotype of the functional Sp1 binding site polymorphism within the first intron of the *COL1A1* gene, which encodes for the α1 chain of type I collagen, was shown to be under-represented in Swedish patients with cruciate ligament ruptures and shoulder dislocations [9]. Study 1 of this thesis provides further evidence that this genetic variant within the *COL1A1* gene is also associated with risk of ACL ruptures in a second independent South African Caucasian population. Since ACL rupture is a complex disorder, it is unlikely that this is the only genetic variant predisposing or protecting individuals from ACL ruptures [163]. Therefore, variants within other genes that also encode for structural components of the most basic unit of ligaments, the collagen microfibril, may also be associated with these injuries.

As reviewed (Section 2.5.3.2), the gene encoding for the α1 chain of type V collagen, *COL5A1*, was selected as a candidate gene for ACL ruptures for the following reasons: (1) the *Bst*UI RFLP within the gene has been shown to be associated with Achilles tendinopathy in two independent populations, and (2) type V collagen was shown to be the second largest component in ligaments (Section 2.5.1), and regulates the diameter of the collagen microfibril [114;157].

The primary aim of this study was therefore to determine if the two sequence variants (*Bst*UI and *Dpn*II RFLPs) within the 3'-UTR of the *COL5A1* gene are associated with an increased risk of ACL ruptures. Although a relatively larger proportion of ACL ruptures occur in males, females have a 4.6 times increased risk of ACL ruptures [13]. Specific intrinsic risk factors have been implicated in the increased predisposition to ACL ruptures among females, however, the underlying etiology of the observed increased risk associated with females is still unknown. A secondary aim of this study was therefore to determine if there were any possible gender-specific associations between the two *COL5A1* sequence variants and increased risk of ACL ruptures.

4.2 MATERIALS AND METHODS

4.2.1 Participants

As described in Study 1, 129 (38 females and 91 males) Caucasian participants with surgically confirmed ACL ruptures were recruited for this study as described in Study 1 (Section 3.2.1). All 129 ACL participants were successfully genotyped and included in this study. In addition, 217 (84 females and 133 males) apparently healthy, unrelated, physically active Caucasian participants, without any self-reported history of ACL injury, and detailed sports participation information, were recruited as control (CON) participants for this study and the next study (Study 3) from sport clubs and a wellness centre (SSISA) within the Southern Suburbs region of Cape Town, South Africa. During the current study only 216 CON participants (84 females and 132 males) were successfully genotyped, and therefore included in the study.

Prior to participation in this study, all participants gave informed written consent (Appendix 2) and completed the questionnaire (Appendix 3), as detailed in Study 1 (Section 3.2.1). The CON group for the current study and the next study (Study 3) of this thesis is different to the control participants included in Study 1. As discussed (Section 3.2.1), not all the control participants in Study 1 completed detailed sports participation information in the questionnaire (Appendix 3). This was a limitation of study one and therefore 179 additional CON participants were recruited. It is however important to mention that 37 of the 130 CON participants which were included in Study 1, did complete the detailed sports participation information and were thus also included in the current study.

Sports participation was categorised into contact sports, non-contact jumping sports, non-contact non-jumping sports and skiing sports, as previously defined [7], with slight modification. Contact sports included soccer, rugby, touch rugby, Gaelic football, muay thai, hurling, Australian football league (AFL) and boxing. Non-contact jumping sports included netball, basketball, volleyball, gymnastics, ballet, motorcross, skateboarding, paragliding, handball and sky diving. Non-contact non-jumping sports included field hockey, cricket, tennis, horseback riding, running, bicycling, spinning, squash, swimming, aerobics, yachting, athletics (excluding long and triple jump), golf, dancing, tennis, canoeing, waterpolo, surfing, windsurfing, badminton, gym training, bowls, triathlon, softball and lifesaving. Skiing sports included any mode of water or snow skiing.

The exact mechanism of injury could only be identified in 21 female (55.7%) and 67 male (73.6%) participants. Participants who had ruptured their ACL via a non-contact (NON) mechanism, as previously defined [13], were identified and analysed in this study as a separate sub-group. Thirty-six (53.7%) male participants and 18 (85.7%) female participants ruptured their ACL through a non-contact mechanism.

This study was approved by the Research Ethics Committee of the Faculty of Health Sciences within the University of Cape Town, South Africa (reference number 164/2006; Appendix 4).

4.2.2 DNA extraction and *COL5A1* genotyping

Approximately 4.5 ml of venous blood was obtained from each participant by venipuncture of a forearm vein and collected into an EDTA vacutainer tube. Blood samples were stored at 4°C until total DNA extraction. DNA was extracted using the procedure described by Lahiri and Nurnberg [159], with slight modification, as described in Study 1.

A 667 bp fragment containing the *Dpn*II (single nucleotide polymorphism, SNP rs13946) and *Bst*UI (SNP rs12722) RFLPs within the 3'-UTR of the *COL5A1* gene was PCR amplified as described by Greenspan and Pasquinelli [164] and modified by Mokone et al. [143] (Figure 4.1). Briefly, the PCR reaction was performed in a final volume of 60 μl containing at least 100 ng of DNA, 20 pmol of the forward (5'-GAA GAC GTT TCT GGA GGA TC-3') and reverse (5'-GGA GGC ACC TGC AGA ATG AC-3') primers, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each dNTP (dATP, dTTP, dCTP and dGTP) and 2.5 units of DNA *Taq* polymerase (New England Biolabs, Ipswich, Massachusetts, USA). The amplification was performed with an initial denaturing step at 94°C for 3 minutes, followed by 35 cycles of denaturing at 94°C for 1 minute, annealing at 53°C for 1 minute, extension at 72°C for 1.5 minutes, and a final extension step at 72°C for 8 minutes (*XP Thermal Cycler Block*; *Bioer technology Co*; Middlesex, UK).

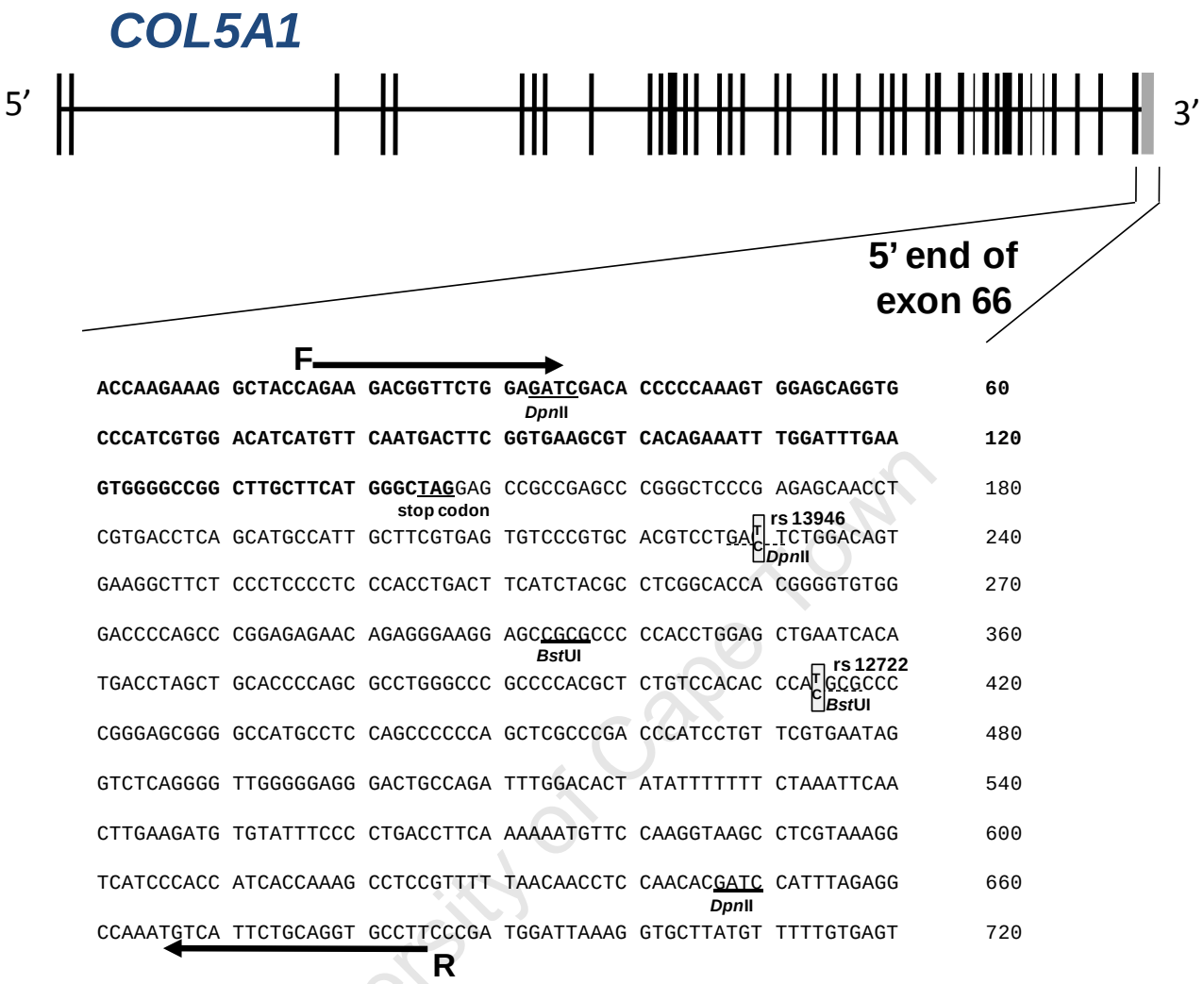

Figure 4.1: A schematic representation of exon (vertical lines) and intron (horizontal lines) boundaries of the the *COL5A1* gene, as well as a 720 bp genomic sequence at the 5'-end of exon 66, which contains the start (stop codon, underlined TAG sequence) of the 3'-UTR. The binding position of the forward (F) and reverse (R) primers (solid arrows) designed to amplify a 667 bp PCR fragment containing the *Dpn*II (rs 13946) and *Bst*UI (rs 12722) restriction fragment length polymorphisms (RFLPs; grey boxes in the genomic sequence). The recognition (undelined) sequences of the *DpnII* (/GATC) and BstUI (CG/CG) restriction enzymes are indicated on the genomic sequence. The T to C substitution for the *Bst*UI RFLP and the T to C substitution for the *Dpn*II RFLP causes a recognition sequence to be destroyed.

The C and T alleles of the two polymorphisms were identified by digesting the PCR products with the restriction endonucleases, *Bst*UI or *Dpn*II, as previously described [143;145]. Digestion of the 667 bp PCR fragment with *Dpn*II produced 612 bp, 40 bp and 15 bp fragments for the C allele and 418 bp, 194 bp, 40 bp and 15 bp fragments for the T allele. Digestion of the same 667 bp PCR fragment with *Bst*UI produced 351 bp and 316 bp fragments for the T allele, and 316 bp, 271 bp and 80 bp fragments for the C allele. The resultant fragments were separated, together with a 100 bp DNA ladder of known size markers (Promega Corporation, Madison, Wisconsin, USA) and *SYBER® Gold* nucleic acid gel stain (*Invitrogen Molecular Probes*™, Oregon, USA), on 6% non-denaturing polyacrylamide gels (Figure 4.2). The gels were photographed under UV light using a Uvitec photodocumentation system (Uvitec Limited, Cambridge, UK) and genotypes were determined based on the sizes of the DNA fragments.

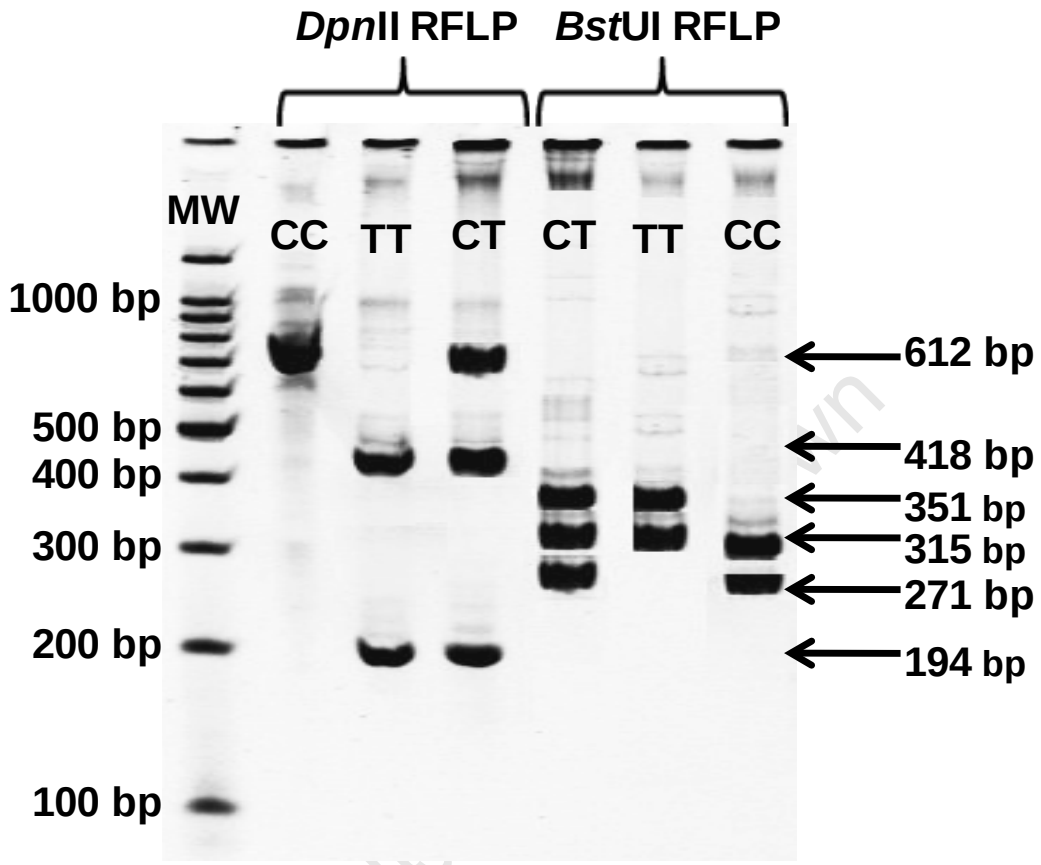

Figure 4.2: A typical 6% non-denaturing polyacrylamide gel showing the genotypes of the *COL5A1 Dpn*II and *Bst*UI restriction fragment length polymorphisms (RFLPs). Digestion of the 667 bp PCR product with *Dpn*II produced 612 bp, 40 bp and 15 bp fragments for the C allele and 418 bp, 194 bp, 40 bp and 15 bp fragments for the T allele. Digestion of the same 667 bp fragment with *Bst*UI produced 351 bp and 316 bp fragments for the T allele, and 316 bp, 271 bp and 80 bp fragments for the C allele. The 80 bp, 40 bp and 15 bp fragments ran off the gel and are therefore not visible on the Figure. The left lane contains the 100 bp molecular weight marker (MW) with the appropriate fragment sizes given in base pairs (bp).

4.2.3 Statistical analysis

Data were analysed using STATISTICA Version 8.0 (Statsoft Inc., Tulsa, Oklahoma, USA) and Graphpad InStat Version 3 (Graphpad Software, San Diego, California, USA) statistical programs. The data were initially analysed for the whole group and then separately according to gender. Because the sample consisted predominately of males the whole group results were similar to the male participants. Only the separate male and female results are reported in this chapter, while the combined results, where appropriate, are reported in Appendix 1 (additional material). A one-way analysis of variance (ANOVA) was used to determine any significant difference between the characteristics of the ACL and CON group, as well as the CON group and NON sub-group. The direct (DIR) and indirect (IND) mechanisms of ACL ruptures sub-groups were not included in this and subsequent chapters of the thesis due to small sample sizes. A chi-squared (χ^2) analysis or Fisher's exact test was used to analyse any differences in the genotype and allele frequencies, as well as other categorical data between the groups. Significance was accepted when $P<0.05$. Hardy-Weinberg equilibrium was established using the program Genepop web version 3.4 (http://genepop.curtin.edu.au/).

4.3 RESULTS

4.3.1 Participant characteristics

There were no significant differences in the proportion of female participants within the CON (n=84, 38.9%) and ACL (n=38, 29.5%) groups (P=0.260), as well as between the CON group (P=0.715) and the NON sub-group (n=18, 33.3%). The female and male participants within the CON and ACL groups, as well as the NON sub-group, were matched for age, height and country of birth (Table 4.1). The female CON and ACL groups, as well as the NON sub-group were also matched for weight and BMI. The male participants within the ACL group and NON sub-group were however significantly heavier and had a significantly higher BMI than the CON group. Within the ACL group, the female participants self-reported age, weight and BMI at recruitment were 6.4 ± 9.9 years (n=37), 0.7 ± 4.5 kg (n=34) and 0.4 ± 1.5 kg/m^2 (n=33), greater than at the time of the first ACL rupture. The males self-reported age, weight and BMI at recruitment were 4.3 ± 7.7 years (n=83), 1.6 ± 4.7 kg (n=76) and 0.5 ± 1.5 kg/m^2 (n=73), greater than at the time of the first ACL rupture. The combined analysis of the male and female participant characteristics are included in the additional material (Appendix 1, Table A1.4).

The relative frequency of the self-reported history of any other (excluding ACL) ligament (P=0.003) and other knee ligament (P=0.029) injuries were significantly higher in the female ACL group when compared to the female CON group (Table 4.2). The previous other knee ligament injuries included injury to either the posterior cruciate ligament (PCL), the lateral collateral ligament (LCL), or the medial collateral

ligament (MCL). Similarly, the female participants within the NON sub-group also reported significantly greater history of any other previous ligament (P=0.014) and knee ligament (P=0.018) injuries compared to the CON group.

4.3.2 Family history

It is interesting to note that the self-reported family history of any ligament injury at the time of recruitment, was significantly higher in the female ACL group (P=0.002), as well as the NON sub-group (P=0.014), when compared to the female CON group. Except for a significant difference in the frequency of other knee ligament injuries (P=0.047) between the male ACL and CON groups, there were no significant differences between the ACL and CON groups, as well as between the CON group and NON sub-group for a history of Achilles tendon injury or any other category of personal or family history of ligament injury.

Table 4.1: Characteristics of the female and male participants within the asymptomatic control (CON) group, the anterior cruciate ligament rupture (ACL) group and the ACL sub-group with a non-contact (NON) mechanism of injury.

	CON	ACL	P-Value [b]	NON	P-value [c]
Female participants:-	n=84	n=38		n=18	
Age [a] (years)	28.2 ± 10.0 (83)	29.8 ± 12.1 (37)	0.453	28.6 ± 13.1 (18)	0.896
Height (cm)	166.2 ± 5.8 (210)	166.4 ± 6.8 (34)	0.929	167.8 ± 6.7 (17)	0.325
Weight [a] (kg)	61.9 ± 8.3 (82)	62.2 ± 7.5 (34)	0.864	63.2 ± 7.8 (17)	0.570
BMI [a] (kg/m^2)	22.4 ± 2.7 (79)	22.3 ± 2.1 (33)	0.940	22.1 ± 2.0 (16)	0.727
Country of birth (% South Africa)	82.5 (80)	80.0 (35)	0.955	77.8 (18)	0.737
Male participants:-	n=132	n=91		n=36	
Age [a] (years)	29.0 ± 12.2 (132)	28.1 ± 10.5 (87)	0.584	27.6 ± 9.7 (36)	0.512
Height (cm)	180.4 ± 6.3 (131)	181.1 ± 6.6 (79)	0.450	181.8 ± 6.6 (35)	0.436
Weight [a] (kg)	82.0 ± 13.9 (130)	87.3 ± 14.3 (80)	**0.008**	87.5 ± 14.7 (36)	**0.040**
BMI [a] (kg/m^2)	25.1 ± 3.8 (129)	26.6 ± 3.7 (76)	**0.010**	26.7 ± 4.2 (34)	**0.037**
Country of birth (% South Africa)	89.0 (127)	85.4 (82)	0.577	88.9 (36)	1.000

Gender and country of birth are represented as a frequency (%). The remaining variables are expressed as a mean ± standard deviation. The number of subjects (n) for each variable is in parentheses.

[a] Age, weight and body mass index (BMI) are self-reported values at the time of the first ACL rupture for the ACL group, as well as the NON sub-group, and at recruitment for the control group. For the ACL group, age, weight and BMI at recruitment were 6.4 ± 9.9 years (n=37), 0.7 ± 4.5 kg (n=34) and 0.4 ± 1.5 kg/m^2 (n=33), greater than at the time of the first ACL rupture for the female participants, and 4.3 ± 7.7 years (n=83), 1.6 ± 4.7 kg (n=76) and 0.5 ± 1.5 kg/m^2 (n=73), greater than at the time of the first ACL rupture for the male participants.

[b] CON vs. ACL. [c] CON vs. NON.

Table 4.2: Self-reported personal and family (blood relative) history of soft tissue injuries within the asymptomatic control (CON) group, the anterior cruciate ligament rupture (ACL) group and non-contact (NON) mechanism of ACL injury sub-group in the female and male participants.

	CON	ACL	P-value [c]	NON	P-value [d]
Female Participants:-	n=84	n=38		n=18	
Any other ligament injury [a]	25.9 (81)	54.3 (35)	**0.003**	55.6 (18)	**0.014**
Knee ligament injury [b]	1.2 (81)	11.4 (35)	**0.029**	16.7 (18)	**0.018**
Achilles tendon injury	4.9 (81)	5.7 (35)	1.000	11.1 (18)	0.299
Family ligament injury	21.5 (79)	50.0 (34)	**0.002**	52.9 (17)	**0.014**
Male Participants:-	n=132	n=91		n=36	
Any other ligament injury [a]	43.6 (124)	57.5 (80)	0.051	54.3 (35)	0.260
Knee ligament injury [b]	4.8 (124)	12.5 (80)	**0.047**	8.6 (35)	0.414
Achilles tendon injury	10.5 (124)	11.1 (81)	0.887	20.0 (35)	0.134
Family ligament injury	27.1 (122)	33.8 (80)	0.308	34.3 (35)	0.404

Values are represented as frequencies (%) with the number of subjects (n) is in parentheses.

[a] Excluding ACL injuries

[b] Includes the posterior cruciate ligament (PCL), the lateral collateral ligament (LCL), and the medial collateral ligament (MCL)

[c] CON vs. ACL

[d] CON vs. NON

4.3.3 Sports participation

When compared to all the male participants, significantly more females participated in non-contact jumping sports (P<0.001) (Table 4.3). The female and male participants however participated for a similar number of years in non-contact jumping sports (P=0.918). The female and male participants were similarly matched for proportion and duration of participation in non-contact non-jumping and skiing sports. More males participated in contact sports (P<0.001) for significantly more years (P=0.001), when compared to the female participants.

When only the female participants were analysed, similar proportions of participants within the CON and ACL groups participated in contact, non-contact non-jumping and skiing sports. In contrast, the female ACL group however reported a greater participation in non-contact jumping sports (P=0.035). The ACL and CON female participants reportedly participated for a similar number of years in contact sports, non-contact jumping sports and non-contact non-jumping sports. Similar proportions of male participants within the CON and ACL groups participated in non-contact jumping, non-contact non-jumping and skiing sports for a similar number of years. Significantly more male participants within the ACL group participated in contact sports when compared to the CON group (P<0.001). There were however no significant differences in the duration of participation (P=0.374).

Table 4.3: Sports participation according to type of sport within the asymptomatic control (CON) groups, the symptomatic anterior cruciate ligament rupture (ACL) groups, as well as the symptomatic noncontact (NON) mechanism of injury subgroups for all female and male participants.

	Female Participants			Male Participants			
	CON (n=82)	**ACL** (n=35)	**P-Value** [e]	**CON** (n=126)	**ACL** (n=82)	**P-Value** [e]	**P-Value** [f]
Contact sports [a]							
Participants (%)	13.4 (11)	5.7 (2)	0.339	65.9 (83)	87.8 (72)	**<0.001**	**<0.001**
Participation (years)	5.7 ± 3.6	1.0 ± 0.0	0.101	12.6 ± 7.6	11.5 ± 7.9	0.374	**0.001**
Non-contact jumping sports [b]							
Participants (%)	34.1 (28)	57.1 (20)	**0.035**	5.6 (7)	13.4 (11)	0.086	**<0.001**
Participation (years)	8.2 ± 5.2	9.3 ± 5.8	0.493	10.9 ± 5.2	7.0 ± 5.4	0.152	0.918
Non-contact non-jumping sports [c]							
Participants (%)	93.9 (77)	94.3 (33)	1.000	94.4 (119)	92.7 (76)	0.826	0.923
Participation (years)	28.4 ± 20.8	28.2 ± 22.6	0.953	25.4 ± 17.9	24.4 ± 22.2	0.729	0.169
Skiing sports [d]							
Participants (%)	3.7 (3)	14.3 (5)	0.051	5.6 (7)	9.8 (8)	0.384	0.900

The legend is on the following page.

(Legend from the previous page) The number of participants is represented as a frequency (%), while the years of participation is represented as a mean ± standard deviation. The number of participants (n) is in parentheses.

[a] Soccer, rugby, touch rugby, Gaelic football, muay thai, hurling, Australian football league (AFL) and boxing.

[b] Netball, basketball, volleyball, gymnastics, ballet, motorcross, skateboarding, paragliding, handball and sky diving.

[c] Field hockey, cricket, tennis, horseback riding, running, bicycling, spinning, squash, swimming, aerobics, yachting, athletics (excluding long and triple jump), golf, dancing, tennis, canoeing, waterpolo, surfing, windsurfing, badminton, gym training, bowls, triathlon, softball and lifesaving.

[d] Any mode of water or snow skiing.

[e] CON vs. ACL

[f] male (CON + ACL) vs. female (CON + ACL) participants.

4.3.4 *COL5A1* genotype and allele frequencies

4.3.4.1 The *Bst*UI RFLP genotyping

When the female and male participants were analysed together, there were no significant differences in genotype or allele frequencies between the CON and ACL groups, nor the CON group and NON sub-group for the *COL5A1 Bst*UI RFLP (data presented in Appendix 1, Additional material, Table A1.5). However when the female and male participants were analysed separately, the *Bst*UI RFLP CC genotype was over-represented in the control participants within the female (Figure 4.3A) (OR=6.6; 95% CI 1.5 - 29.7; P=0.006), but not the male (Figure 4.3B) participants, when the ACL group were compared to the CON group. The allele frequencies between the CON and ACL groups for the *Bst*UI RFLP was also significantly different within the

female participants (OR=2.2; 95% CI 1.2 - 4.0; P=0.010)(Figure 4.4A), but not the male participants (Figure 4.4B). The T allele was significantly over-represented among the female ACL group. Similarly, the T allele of the *Bst*UI RFLP was also significantly over-represented in the female NON sub-group (OR= 2.6; 95% CI; 1.2 - 5.7; P=0.016) when compared to the CON group (Figure 4.4A). This data should be interpreted with caution because of the small sample size, however the genotype frequency of the *Bst*UI RFLP was not significantly different when the female CON group and NON sub-group were analysed (Figure 3.1A), although a trend existed for the CC to be over-represented in the control population (P=0.065). There were no further significant *Bst*UI genotype (Figure 4.3B) or allele (Figure 4.4B) frequency differences when the male CON group and NON sub-group were analysed.

Similar *Bst*UI RFLP genotype distributions were obtained when a sub-group of 105 (48.6%) control participants without a self-reported history of any ligament or tendon injuries were analysed separately (females 15 TT, 31.2%; 21 TC, 43.8% and 12 CC, 25.0%; males 17 TT, 29.8%; 32 TC, 56.1% and 8 CC, 14.04%). The genotype distribution of all groups was in Hardy-Weinberg equilibrium for the *Bst*UI RFLP (Table 4.4).

4.3.4.2 The *Dpn*II RFLP genotyping

There were no significant differences in the *COL5A1 Dpn*II RFLP genotype distribution when male and female participants were analysed together (data presented in Appendix 1, Additional material, Table A1.5), or separately between the female (Figure 4.3C and Figure 4.4C) or male (Figure 4.3D and Figure 4.4D) CON and ACL groups, as well as the NON sub-groups.

Furthermore, there were also no significant differences in allele frequency distributions between the groups (Figure 4.4 C and D). The genotype distribution of all groups was in Hardy-Weinberg equilibrium for the *Dpn*II RFLP (Table 4.4).

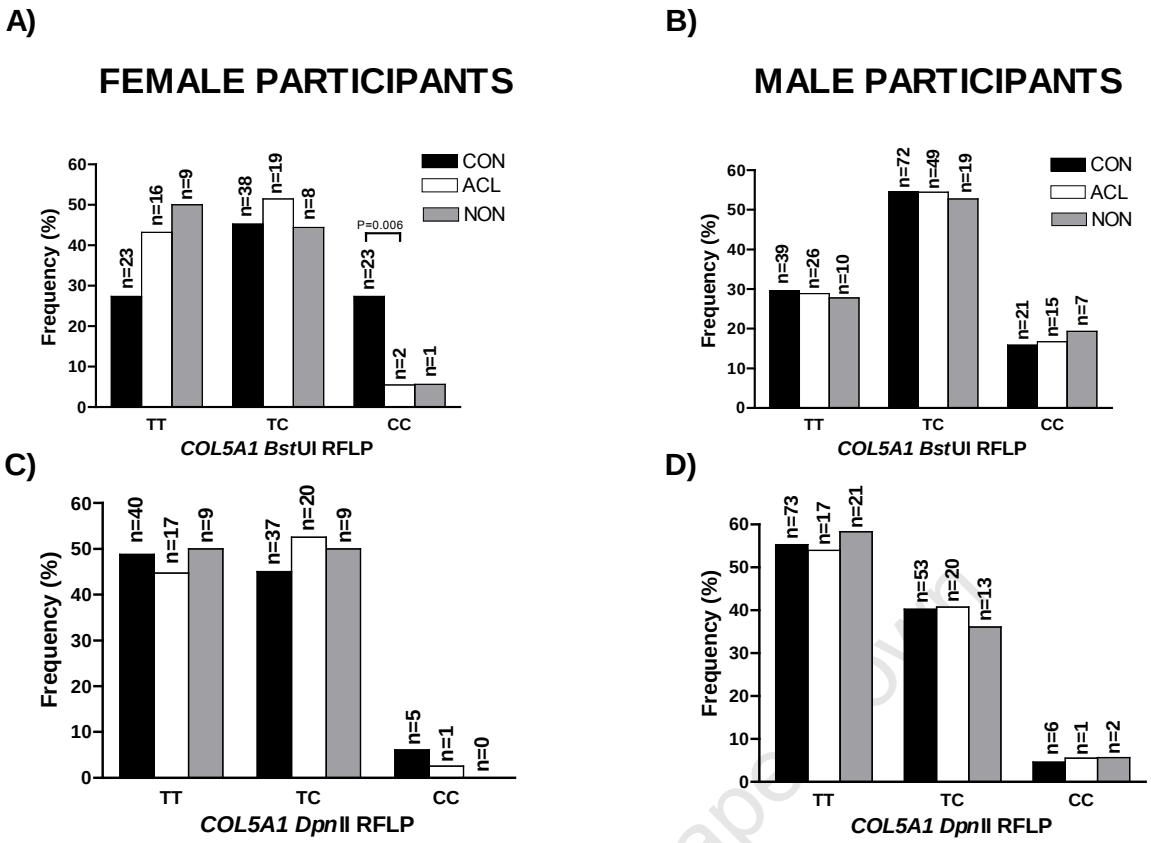

Figure 4.3: The relative genotype frequency of the *COL5A1* gene *Bst*UI and *Dpn*II restriction fragment length polymorphisms (RFLP) within the asymptomatic control (CON; black bars) groups, the anterior cruciate ligament rupture groups (ACL; white bars) and the non-contact mechanism of injury sub-group (NON; grey bars) in all female (A and C) and male (B and D) participants. The number of participants (n) within each group is shown in parentheses. Because of the small sample size of the *Bst*UI RFLP CC genotype in the female ACL and NON groups (A), the CC genotype was compared to the combined TC and TT genotypes. The TT genotype was also compared to the combined TC and CC genotypes. Similarly, because of the small sample size of the rare *Dpn*II RFLP CC genotype (C and D), it was combined with the TC genotype and compared to the TT genotype. **(A)** The *Bst*UI RFLP genotype frequency distributions within the female participants. CON vs. ACL, P=0.006 (CC vs. TT+TC) and P=0.131 (TT vs. TC+CC); CON vs. NON, P=0.065 (CC vs. TT+TC) and P=0.110 (TT vs. TC+CC). **(B)** The *Bst*UI RFLP genotype frequency distributions within the male participants. CON vs. ACL, P=0.987; CON vs. NON, P=0.879. **(C)** The *Dpn*II RFLP genotype frequency distributions within the female participants. CON vs. ACL, P=0.829 (TT vs. TC+CC); CON vs. NON, P=0.925 (TT vs. TC+CC). **(D)** The *Dpn*II RFLP genotype frequency distributions within the male participants; CON vs. ACL, P=0.938 (TT vs. TC+CC); CON vs. NON, P=0.892 (TT vs. TC + CC).

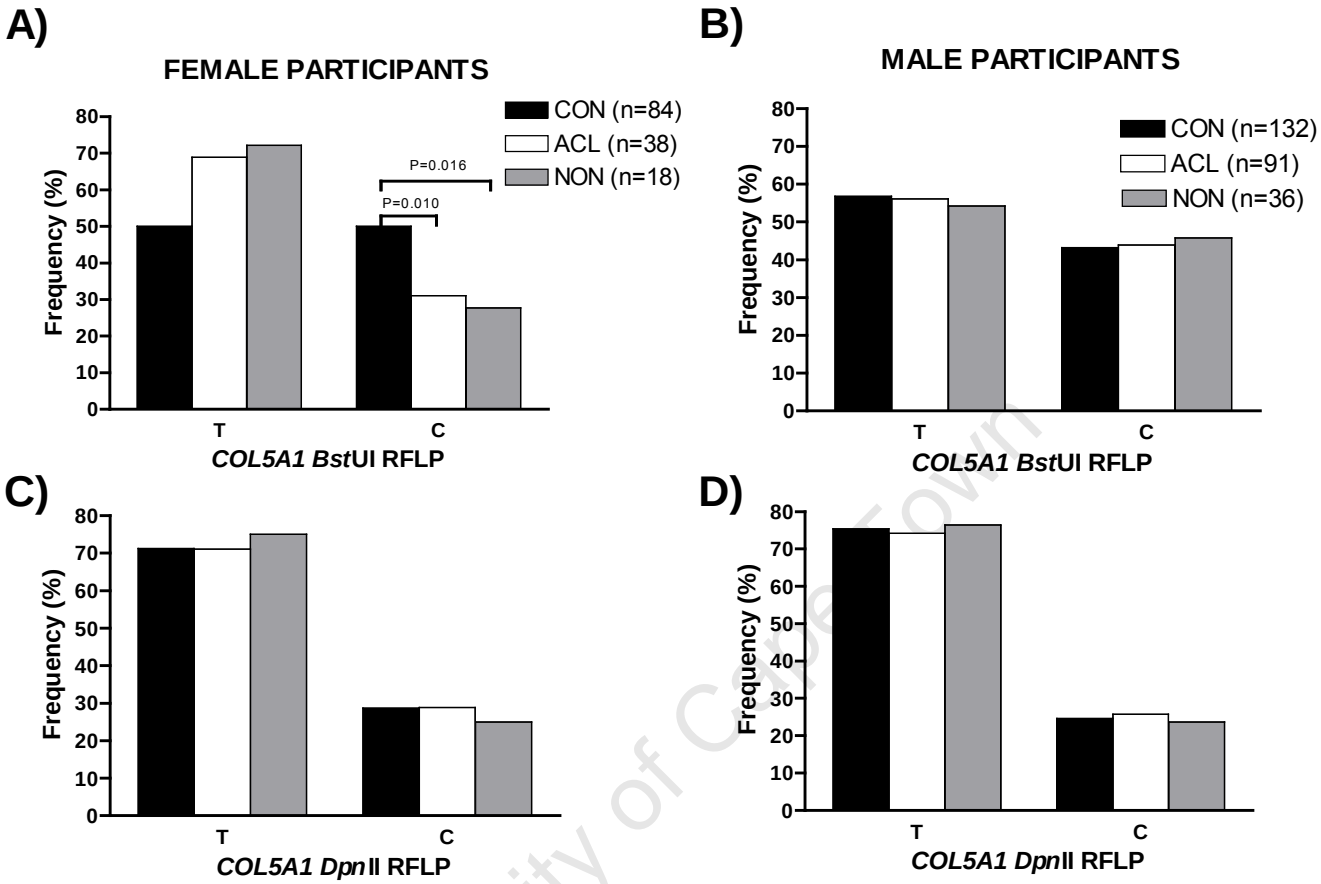

Figure 4.4: The relative allele frequency of the *COL5A1* gene *Bst*UI and *Dpn*II restriction fragment length polymorphisms (RFLP) within the asymptomatic control (CON; black bars) groups, the anterior cruciate ligament rupture groups (ACL; white bars) and the non-contact mechanism of injury sub-group (NON; grey bars) in all female (A and C) and male (B and D) participants. **(A)** The *Bst*UI RFLP allele frequency distributions within the female participants. CON vs. ACL, P=0.010; CON vs. NON, P=0.016. **(B)** The *Bst*UI RFLP allele frequency distributions within the male participants. CON vs. ACL, P=0.960; CON vs. NON, P=0.789. **(C)** The *Dpn*II RFLP allele frequency distributions within the female participants. CON vs. ACL, P=0.860; CON vs. NON, P=0.982. **(D)** The *Dpn*II RFLP allele frequency distributions within the male participants; CON vs. ACL, P=0.860; CON vs. NON, P=0.982.

Table 4.4: Hardy-Weinberg Equilibrium (HWE) P-values of all separately analysed groups for the *Dpn*II and *Alu*I restriction fragment length polymorphisms (RFLP).

	***Dpn*II RFLP HWE P-Value**	***Bst*UI RFLP HWE P-value**
Female ACL group	0.439	0.132
Male ACL group	0.391	0.785
Male + Female ACL groups	0.273	0.336
Female CON group	0.385	0.429
Male CON group	0.283	0.480
Male + Female CON groups	0.784	0.742
All participants	0.382	0.405

4.3.4.3 *BstUI* RFLP Genotype effect of family history of ligament injury

Lastly, it is interesting to note that there was a significant difference in the *Bst*UI RFLP genotype distribution when all (ACL and CON) participants in the study were divided into those with and without a family history of ligament injuries (P=0.022). This association was also significant when only the female participants (Figure 4.5A)(TT vs. TC + CC, P=0.005), but not the male participants (Figure 4.5B)(P=0.396) were analysed. In the female participants the TT genotype was significantly over-represented in those with a family history of ligament injury (n=34; TT, 52.9%) when compared to those without a previous family history of ligament injury (n=79; TT, 24.1%) (OR=3.6; 95% CI 1.5 - 8.3; P=0.005). The CC genotype was however not significantly under-represented in those with a family history of ligament injury (n=34; CC, 11.8%) when compared to those without a previous family history of ligament injury (n=79; CC, 24.1%) (P=0.203).

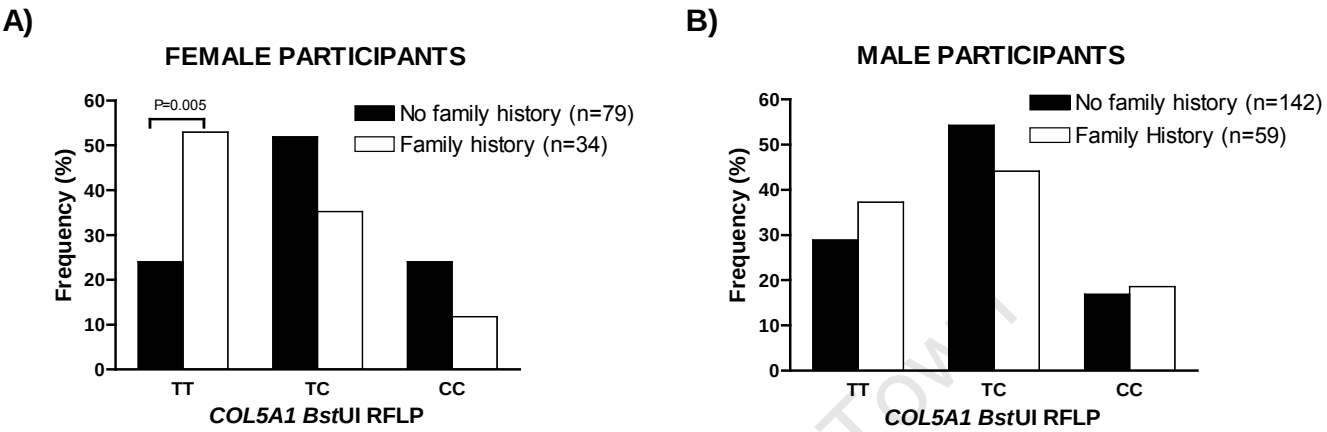

Figure 4.5: The relative genotype frequency of the *COL5A1* gene *Bst*UI and *Dpn*II restriction fragment length polymorphisms (RFLP) when **(A)** female **(B)** and male participants in the study were divided into those with and without a family history of ligament injuries. (A) The *Bst*UI RFLP genotype distributions within the female participants; TT vs. TC + CC, P=0.005; CC vs. TC + TT, P=0.203. (B) The *Bst*UI RFLP genotype distributions within the male participants; P=0.396.

4.4 DISCUSSION

The main finding of this study was that the CC genotype of the one variant (the *Bst*UI RFLP) within the 3'-untranslated region of the *COL5A1* gene was associated with a 6.6 times decreased risk (OR 6.6; 95% CI 1.5 - 29.7; P=0.006) of ACL ruptures in female, but not in male, participants. The second variant (the *Dpn*II RFLP) was not associated with ACL ruptures in either the female or the male participants. An additional finding of this study was that the female, but not male, participants within the ACL group reported a significantly higher family history of ligament injuries (OR=3.6; 95% CI 1.5 - 8.3; P=0.005). The *Bst*UI RFLP was also associated with a family history of ligament injuries among the female participants.

The novel finding, that females with a CC genotype of the *COL5A1 Bst*UI RFLP had a decreased risk of ACL ruptures, has not been previously reported. The *COL5A1* gene encodes for the α1 chain in type V collagen which is an important structural constituent of both ligaments and tendons [165]. It is therefore of interest to note that a previous study from this research unit has found the same CC genotype to be associated with a decreased risk of chronic Achilles tendinopathy in males and females [143;145]. The observation that the *Dpn*II RFLP within the *COL5A1* gene was not associated with ACL ruptures in this study is in agreement with the previously published study investigating chronic Achilles tendinopathy [143;145]. It is important to mention that the findings of this study did not change when participants who reported a past history of Achilles tendon injury, were excluded from the analysis (Data in Appendix 1, Additional material, Table A1.6).

The additional finding of this study, that the ACL group reported a significantly higher family history of ligament injuries when compared to the CON group, is in agreement with the results from Study 1 of this thesis, even though two different control groups were used. However, when the male and female participants were analysed separately, this finding was only present among the female, and not the male participants. The reason for this gender-specific association is unknown. The previous studies which found a familial predisposition to ACL ruptures did not analyse male and female participants separately [7;10]. Although it is possible that the family members of the ACL participants, especially the females, in the current study were exposed to a greater amount of contact sports, or other extrinsic risk factors for ligament injuries, this observation is however consistent with a familial predisposition to an increased risk of ligament injuries. In support of this genetic predisposition, our study found that the *Bst*UI RFLP within *COL5A1* was also associated with a self-reported family history of ligament injuries in the female, but not male, participants when both symptomatic and asymptomatic groups were combined. However, it is not apparently obvious why this association was only present in females.

Although various intrinsic risk factors have been proposed to be associated with female ACL ruptures [4;12], the exact mechanism by which these factors contribute to an increased risk of ACL ruptures in females remain unknown. Certain intrinsic risk factors broadly classified as, anatomical, hormonal or neuromuscular, might be specific or exaggerated in females [12]. One hypothesis may be that gene-hormone interactions exist which makes this genetic association specific to females. Sex hormones are known to exert their biological effects on the ACL through regulating

gene expression, especially many of the matrix metalloproteinases (MMPs) [147]. Although no previous research has investigated the effect of the female sex hormones on the regulation of *COL5A1* gene expression, previous research has however shown that relative to *COL1A1* gene expression, *MMP3* and *MMP1* gene expression is higher in the ACL of females when compared to males [166]. It is therefore interesting to note that Raleigh et al. [147] recently reported an interaction between a sequence variant within the *MMP3* gene and the *COL5A1 Bst*UI RFLP that modifies the risk of Achilles tendinopathy. Further studies are required to determine whether variants within the *MMP3* gene are associated with ACL ruptures, particularly within females.

Since ACL ruptures are complex disorders, some non-genetic factors, which are potential confounding variables in injury risk, need to be discussed. In this study, the female participants within the CON and ACL groups were matched for age, height, body weight, BMI and country of birth. It has previously been shown that body weight and BMI are risk factors for ACL ruptures in females, but not males [55]. Female participants were also matched for participation in contact sports, non-contact non-jumping sports and skiing sports. Significantly more females within the ACL group did however participate in non-contact jumping sports when compared to the control group. Since participation in non-contact jumping sports does increase the risk of ACL ruptures, this is a potential limitation of this study. There was however no genotype effects with sports participation in this study (data not shown). It is important to note that significantly more non-contact ACL ruptures were observed in the female group (85.7%) when compared to the male group (53.7%). This may be

expected, as it is widely reported that females are at a greater risk than males to develop non-contact ACL ruptures [4;12].

Among the male participants in the current study, the ACL group and NON sub-group were significantly heavier, and had a significantly higher BMI than the CON group. Although the possibility cannot be excluded, the level of certainty that BMI is a risk factor is weak (Section 2.4.2.1.7). The ACL group had however reported playing a significantly greater amount of contact sports. The fact that the male controls were not matched for participation in contact sports is a limitation of this study. It must however be noted that weight, BMI or exposure to contact sports had no effects on genotype distributions. Furthermore, no genotype associations were observed when a sub-group of the male participants were matched according to weight and exposure to contact sports (data not shown).

Another limitation of this study was the relatively small sample size of the female participants. The primary aim of our study was not designed to investigate gender specific genetic risk factors and therefore further research is required to confirm this finding in larger female cohorts.

In conclusion, the study presented in this chapter found that the CC genotype of the *Bst*UI RFLP within the 3'-UTR of the *COL5A1* gene is associated with reduced risk of ACL rupture in female participants (Figure 4.6).

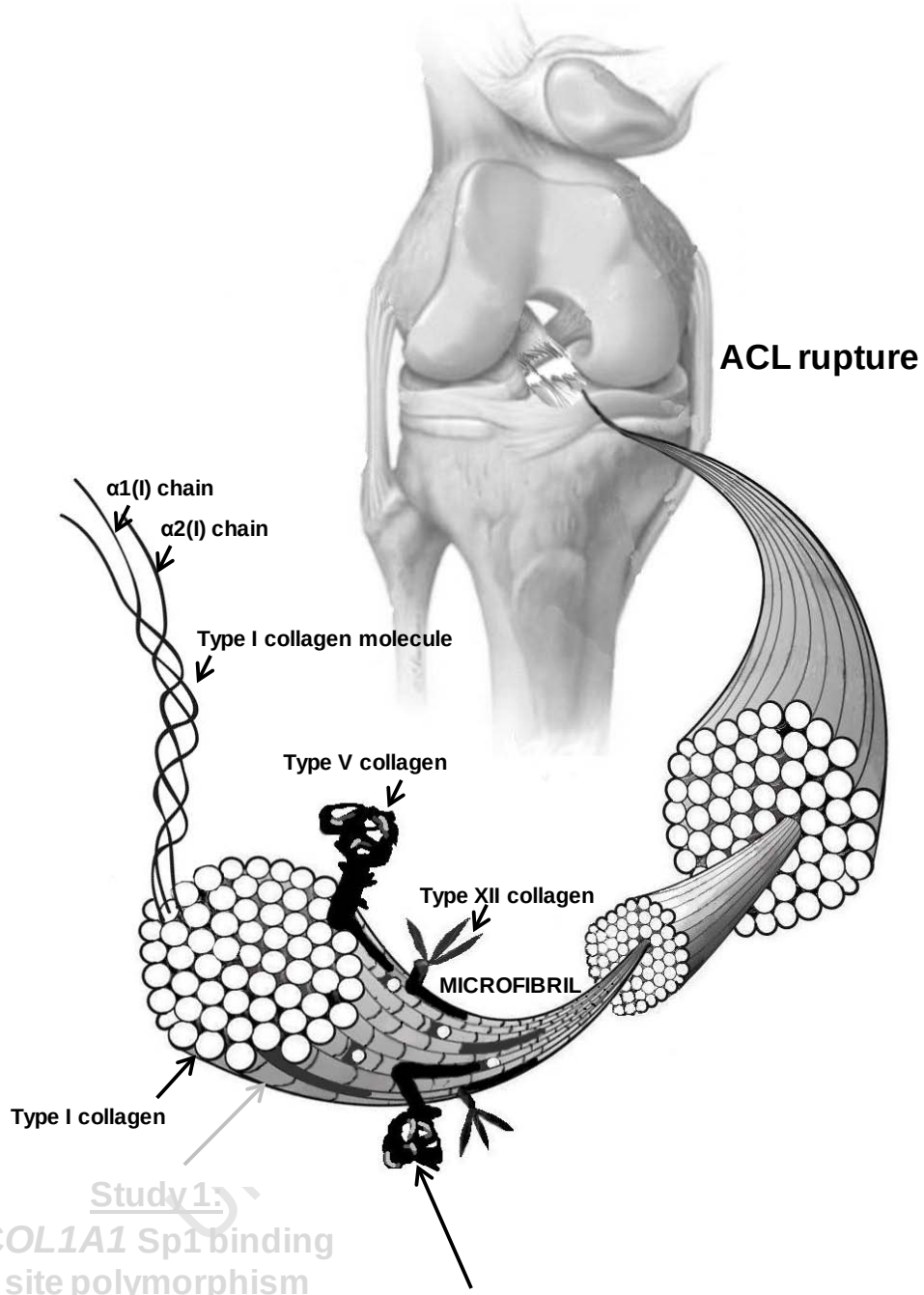

Figure 4.6: A schematic presentation of the primary finding from this study. The *COL5A1* *Bst*UI RFLP was associated with risk of ACL ruptures in female participants. The *COL5A1* gene encodes for the α1 chain of type V collagen, a member of the family of minor fibrillar collagens. Refer to Section 2.5.1 for further detail regarding the structure of the collagen microfibril.

CHAPTER 5

STUDY THREE

THE ASSOCIATION BETWEEN THE *COL12A1* GENE AND ANTERIOR CRUCIATE LIGAMENT RUPTURES

The data presented in this chapter was published in the following peer-reviewed article: Posthumus, M. September, AV. O'Cuinneagain, D. van der Merwe, W. Schwellnus, MP. Collins, M. The *COL12A1* gene and risk of anterior cruciate ligament ruptures. ***British Journal of Sports Medicine***, In Press.

5.1 INTRODUCTION

In the previous study (Study 2) of this thesis, the gene encoding the α1 chain of type V collagen, *COL5A1*, was associated with risk of ACL ruptures in female participants. This finding provides further evidence that it is likely that multiple genetic sequence variants alter the risk of developing ACL ruptures. This result also provides additional reasoning for the selection of the gene encoding for type XII collagen, *COL12A1*, as a candidate gene for ACL ruptures. As discussed in Section 2.5, the *COL12A1* gene was selected as a candidate gene for the following reasons; (1) The *COL12A1* and *COL5A1* genes encode for quantitatively minor components of the collagen microfibril which are involved in a similar biological process (fibrillogenesis), and (2) both the GG and CC genotypes of the *Alu*I and *Bsr*I restriction fragment length polymorphisms (RFLPs) respectively, within this gene, were not present in participants with another acute soft tissue injury (Achilles tendon rupture) [148].

The primary objective of this study was to determine if two selected non-synonymous exonic single nucleotide polymorphisms (SNPs), namely the *Alu*I (rs240736) and *Bsr*I (rs970547) RFLPs, within the *COL12A1* gene are associated with ACL ruptures. A secondary objective of this study is to investigate if a similar gender-specific association, as shown in Study 2, is also present between the two *COL12A1* sequence variants and increased risk of ACL ruptures.

5.2 MATERIALS AND METHODS

5.2.1 Participants

One hundred and twenty nine (38 Females and 91 Males) Caucasian participants with surgically diagnosed ACL ruptures and 217 (84 females and 133 males) asymptomatic control (CON) participants were recruited as previously described in Study 2 (Section 4.2.1). Of the 217 CON participants only 216 (83 females and 133 males) were successfully genotyped for the selected SNPs within this study. Except for this minor difference in the CON groups (one additional male CON participant and one less female CON participant), the CON and ACL groups are identical to what was described in Study 2 of this thesis. Prior to participation in this study, all participants gave informed written consent (Appendix 2) and completed the questionnaire (Appendix 3), as detailed in Study 2. As previously described (Study 2), sports participation was categorised into contact sports, non-contact jumping sports, non-contact non-jumping sports and skiing sports [7]. Participants who had ruptured their ACL via a non-contact (NON) mechanism [13] were identified and analysed in this study as a separate sub-group. Thirty-six (53.7%) male participants and 18 (85.7%) female participants ruptured their ACL through a non-contact mechanism.

This study was approved by the Research Ethics Committee of the Faculty of Health Sciences within the University of Cape Town, South Africa (reference number 164/2006; Appendix 4).

5.2.2 *COL12A1* genotyping

From databases hosted by the National Centre for Biotechnology Information (NCBI) and the Ensembl Genome Data Centre, two single nucleotide polymorphisms (SNPs) were selected for the *COL12A1* gene [148]. SNPs with the highest heterozygous frequencies were selected and preference was given to non-synonymous coding variants (SNPs which change the amino acid sequence in the gene product). From the NCBI database, only two non-synonymous exonic SNPs, (1) a C/T transition at position 116 of exon 29 (5326 C/T; T1738I; rs240736) and (2) a A/G transition at position 162 of exon 65 (9285 A/G; S3058G; rs970547), were identified in *COL12A1*. These two SNPs, 50 Kb apart, are located within the region of the *COL12A1* gene which encodes for the short XIIB-1 isoform predominantly expressed in tendons and ligaments [130], and were therefore selected for this study (Figure 5.1).

Genomic DNA was extracted from blood samples donated from each participant as previously described in the Study 1 of this thesis. Primer pairs were designed to amplify the DNA fragments containing each of the two selected SNPs. Polymerase chain reaction (PCR) amplification of these fragments was performed in 60 µl volumes containing 200 ng genomic DNA; 20 pmol of each primer; 2.0mM MgCl2; 50mM KCl; 10mM Tris-HCl (pH 8.3); 200 µMdATP, dCTP, dGTP, and dTTP and 0.5 U Taq DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA). Amplifications were conducted by denaturing for 1 cycle at 94ºC for 3 minutes, followed by 30 cycles at 94ºC for 30 seconds, 55ºC for 30 seconds, and 72ºC for 1

minute, followed by a final extension of 1 cycle at 72ºC for 5 minutes on a thermal cycler (Hybaid; PCR Express, Middlesex, UK).

The 673 bp fragment (containing SNP rs240736) generated from primer pairs COL12-2F 5'-GAG CTC ATG ACA TGC ATC AG-3' and COL12-2R 5'-GTC TTG GAC TTC TCA GCC TC-3' was digested with restriction endonuclease *Bsr*I to produce 57 bp, 616 bp sized fragments for the T allele and 57 bp, 366 bp, 250 bp sized fragments for the C allele (Figure 5.2). The 615 bp fragment (containing SNP rs970547) generated from primer pairs COL12-1F 5'-GAG AAT CCA GAA CAG CTC CAC CAG-3' and COL12-1R 5'-CAT GGC TAG TAT GGG ACA G-3' was digested with restriction endonuclease *Alu*I to produce 16 bp, 599 bp sized fragments for the G allele and 16 bp, 139 bp, 460 bp sized fragments for the A allele. The COL12-1F primer was designed to contain a mutated nucleotide (underlined in the primer sequence) which introduces and additional restriction site (AG/CT) for the *Alu*I restriction endonuclease. The resultant fragments were separated, together with a 100 bp DNA ladder of known size markers (Promega Corporation, Madison, Wisconsin, USA) and *SYBER® Gold* nucleic acid gel stain (*Invitrogen Molecular Probes*™, Oregon, USA), on 6% non-denaturing polyacrylamide gels. The gels were photographed under UV light using a Uvitec photodocumentation system (Uvitec Limited, Cambridge, UK) and genotypes were determined based on the sizes of the DNA fragments.

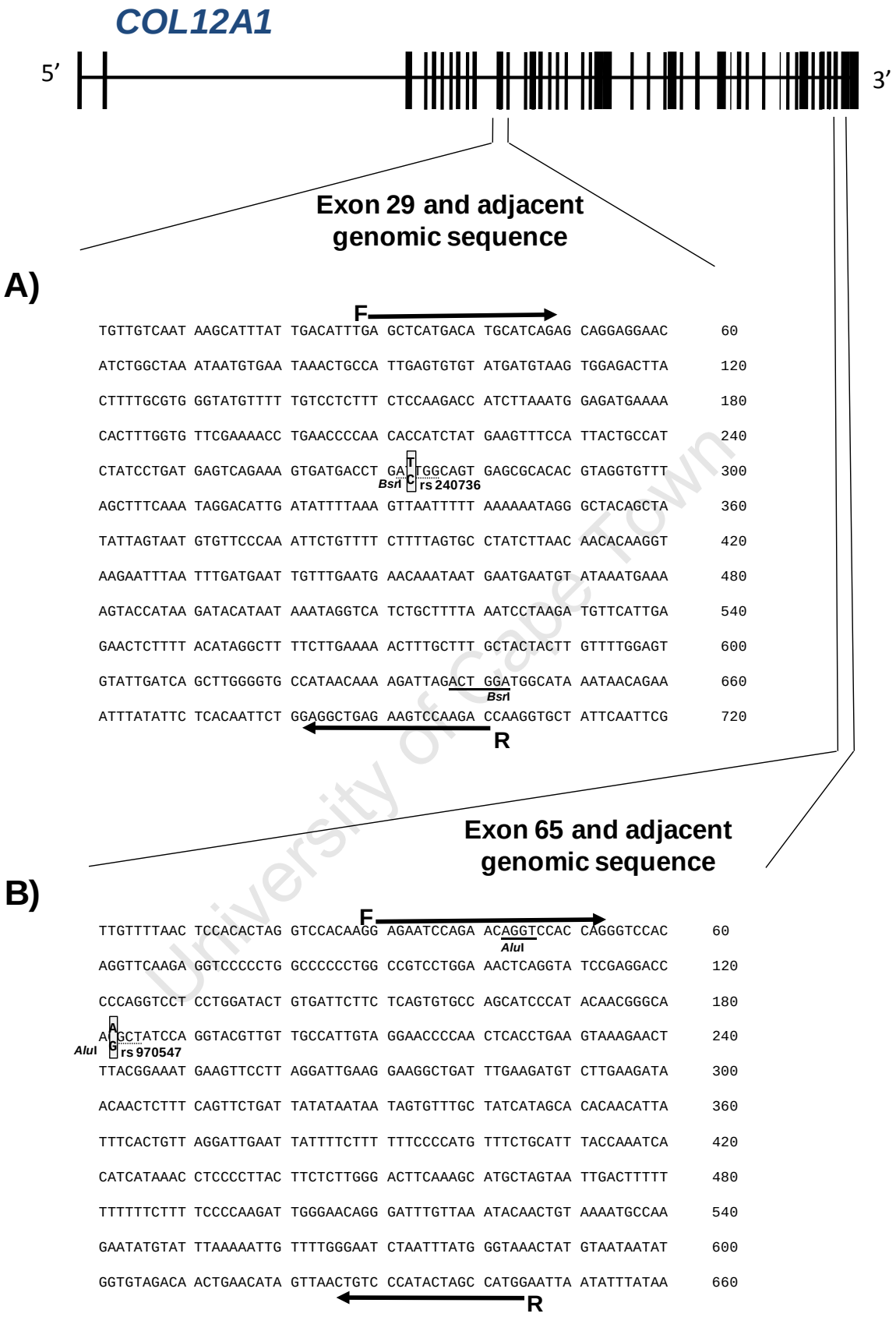

Figure 5.1: Legend on the following page

(Legend from the previous page) A schematic representation of exon (vertical lines) and intron (horizontal lines) boundaries of the short variant of *COL12A1*, as well as the genomic sequence surrounding **A)** the *Bsr*I restriction fragment length polymorphism (RFLP) within exon 29, and **B)** the *Alu*I RFLP within exon 65 of the short variant of *COL12A1.* The exact binding position of the forward (F) and reverse (R) primers are shown with solid arrows. The selected SNPs are boxed within the genomic sequence. The T>C transition (rs240736) within intron 29 changes the recognition sequence (A**C**TGGN/) of the restriction endonuclease *Bsr*I, and therefore only digests the 673 bp DNA fragment when the C allele is present. Similarly, the A>G transition (rs970547) within exon 65 changes the recognition sequence (**A**G/CT) of the restriction endonuclease *Alu*I, and therefore only digests the 615 bp DNA fragment when the A allele is present. The *Bsr*I RFLP sequence included an additional recognition sequence which is underlined in Figure. The forward primer of the *Alu*I RFLP was designed to contain a mutated nucleotide which introduces and additional restriction site (underlined in the genomic sequence) for the *Alu*I restriction endonuclease.

5.2.3 Statistical analysis

Data were analysed using STATISTICA Version 8.0 (Statsoft Inc., Tulsa, Oklahoma, USA) and Graphpad InStat Version 3 (Graphpad Software, San Diego, California, USA) statistical programs. The data were initially analysed for the whole group and then separately according to gender. The sample consisted predominately of males and therefore the whole group results were similar to the male participants. Only the separate male and female results are reported in this chapter, while the combined results, where appropriate, are reported in Appendix 1 (additional material). A one-way analysis of variance (ANOVA) was used to determine any significant differences between the characteristics of the ACL and CON group, as well as the CON group and NON sub-group. A chi-squared (χ^2) analysis or Fisher's exact test was used to analyse any differences in the genotype and allele frequencies, as well as other categorical data between the groups. Significance was accepted when $P<0.05$.

Hardy-Weinberg equilibrium was established using the program Genepop web version 3.4 (http://genepop.curtin.edu.au/). In addition, gene-gene interactions between the *COL12A1 Alu*I RFLP and the *COL5A1 Bst*UI RFLP were assesed. Allele combinations consisting of the markers on the two different genes were constructed and their association with case-control status was tested. For the gene-gene interactions, significance was accepted when P<0.025.

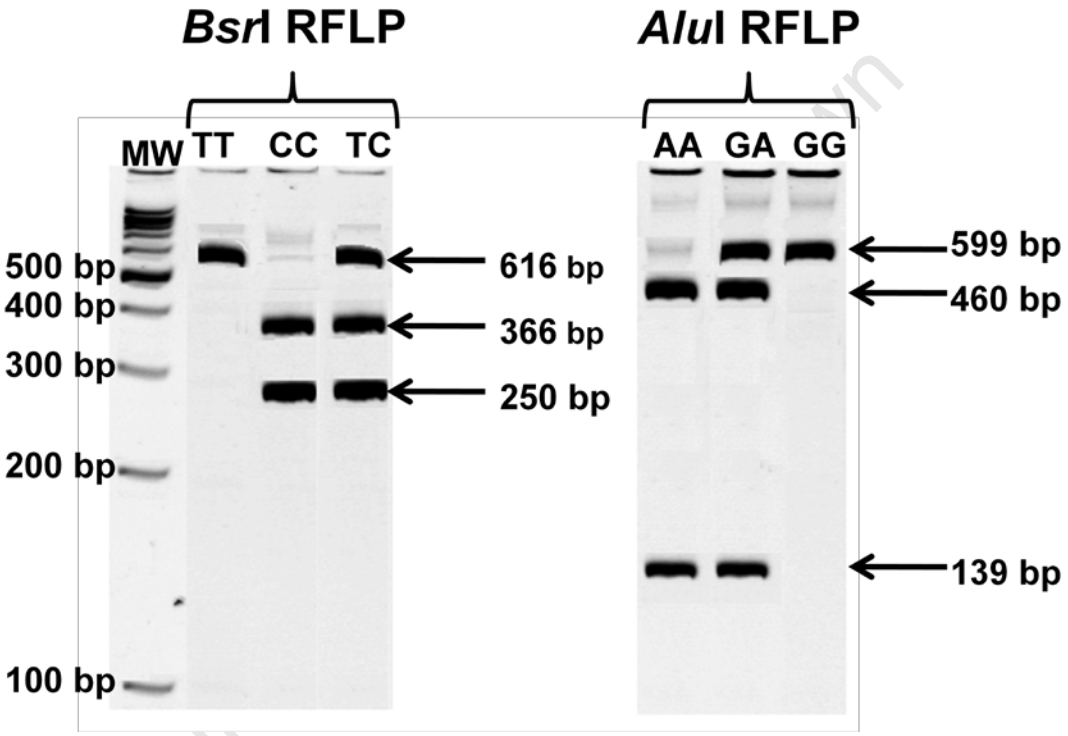

Figure 5.2: Typical 6% non-denaturing polyacrylamide gels showing the genotypes of the *COL12A1 Bsr*I (Left panel) and *Alu*I (right panel) restriction fragment length polymorphisms (RFLPs). Left Panel: Digestion of the 673 bp PCR product with *Bsr*I produce 616 bp and 57 bp fragments for the T allele and 366 bp, 250 bp and 15 bp fragments for the C allele. The 57 bp and 15 bp fragments ran off the gel and are therefore not visible on the Figure. Right Panel: Digestion of the 615 bp PCR product with *Alu*I produce 599 bp and 16 bp fragments for the G allele and 460 bp, 139 bp and 16 bp fragments for the A allele. The 16 bp fragments ran off the gel and is therefore not visible on the Figure. The left lane of the gel on the left shows the 100bp molecular weight (MW) marker with the appropriate fragment sizes given in base pairs (bp).

5.3 RESULTS

5.3.1 Participant characteristics

Although there were slight differences in the number of CON participants included in this study, when compared to Study 2, there were still no significant differences in the proportion of female participants within the CON (n=83, 38.4%) and ACL (n=38, 29.5%) groups (P=0.116), as well as between the CON group and NON sub-group (n=18, 33.3%)(P=0.593). The female and male participants within the CON and ACL groups, as well as the NON sub-group, were also still matched for age, height and country of birth (Table 5.1). The female CON and ACL groups, as well as the NON sub-group were also still matched for weight and BMI. In addition, the male participants within the ACL group were significantly heavier, with a significantly higher BMI when compared to the CON group. Contrary to what was reported in the previous chapter, the male NON group was not significantly heavier (P=0.123). However, the BMI in this group was still significantly higher when compared to the CON participants. Within the ACL group, the mean ± standard deviation of the difference between the self reported age, weight and BMI at the time of first ACL rupture and that at recruitment, has been reported in the previous study (Chapter 4) of the thesis.

Table 5.1: Characteristics of the female and male participants within the asymptomatic control (CON) group, the anterior cruciate ligament ruptures (ACL) group and the ACL sub-group with a non-contact (NON) mechanism of injury.

	CON	ACL	P-Value [b]	NON	P-value [c]
Female participants:-	n=83	n=38		n=18	
Age [a] (years)	28.2 ± 10.0 (82)	29.8 ± 12.1 (37)	0.480	28.6 ± 13.1 (18)	0.921
Height (cm)	166.4 ± 5.8 (78)	166.4 ± 6.8 (34)	0.931	167.8 ± 6.7 (17)	0.329
Weight [a] (kg)	62.0 ± 8.4 (81)	62.2 ± 7.5 (34)	0.880	63.2 ± 7.8 (17)	0.583
BMI [a] (kg/m^2)	22.4 ± 2.7 (78)	22.3 ± 2.1 (33)	0.923	22.1 ± 2.0 (16)	0.716
Country of birth (% South Africa)	82.3 (79)	80.0 (35)	0.955	77.8 (18)	0.915
Male participants:-	n=133	n=91		n=36	
Age [a] (years)	29.0 ± 12.1 (133)	28.1 ± 10.5 (87)	0.596	27.6 ± 9.7 (36)	0.519
Height (cm)	180.5 ± 6.3 (132)	181.1 ± 6.6 (79)	0.472	181.4 ± 6.6 (35)	0.452
Weight [a] (kg)	82.8 ± 16.5 (131)	87.3 ± 14.3 (80)	**0.045**	87.5 ± 14.7 (36)	0.123
BMI [a] (kg/m^2)	25.2 ± 3.8 (130)	26.6 ± 3.7 (76)	**0.012**	26.7 ± 4.2 (34)	**0.043**
Country of birth (% South Africa)	89.1 (128)	85.4 (82)	0.577	88.9 (36)	1.000

Gender and country of birth are represented as a frequency (%). The remaining variables are expressed as a mean ± standard deviation. The number of subjects (n) for each variable is in parentheses.

[a] Age, weight and body mass index (BMI) are self-reported values at the time of the first ACL rupture for the ACL group, as well as the NON sub-group, and at recruitment for the control group. For the ACL group, age, weight and BMI at recruitment were 6.4 ± 9.9 years (n=37), 0.7 ± 4.5 kg (n=34) and 0.4 ± 1.5 kg/m^2 (n=33), greater than at the time of the first ACL rupture for the female participants, and 4.3 ± 7.7 years (n=83), 1.6 ± 4.7 kg (n=76) and 0.5 ± 1.5 kg/m^2 (n=73), greater than at the time of the first ACL rupture for the male participants.

[b] CON vs. ACL. [c] CON vs. NON.

Although the relative frequencies of the male and female CON participants are slightly different to what was reported in the previous study (chapter 4) of the thesis, it did not change the previously reported findings (Table 5.2). Briefly, amongst the female participants the relative frequency of the self-reported history of any other (excluding ACL) ligament (CON vs. ACL, P=0.004; CON vs. NON, P=0.016) and other knee ligament (CON vs. ACL, P=0.029; CON vs. NON, P=0.019) injuries were also still significantly higher in the female ACL group and NON sub-group, when compared to the female CON group. A history of previous other knee ligament injuries included injury to either the posterior cruciate ligament (PCL), the lateral collateral ligament (LCL), or the medial collateral ligament (MCL). In addition, the frequency (%) of a self-reported family history of any ligament injury at the time of recruitment was still significantly higher in the female ACL group (P=0.003), as well as the NON sub-group (P=0.021), when compared to the female CON group. Amongst the male participants, the frequency of other knee ligament injuries (P=0.045) between the male ACL and CON groups was still the only significantly different reported frequency.

Table 5.2: Self-reported personal and family (blood relative) history of soft tissue injuries within the asymptomatic control (CON) group, the anterior cruciate ligament ruptures (ACL) group and non-contact (NON) mechanism of ACL injury sub-group in the female and male participants.

	CON	ACL	P-value [c]	NON	P-value [d]
Female Participants:-	n=83	n=38		n=18	
Any other ligament injury [a]	26.3 (80)	54.3 (35)	**0.004**	55.6 (18)	**0.016**
Knee ligament injury [b]	1.3 (80)	11.4 (35)	**0.029**	16.7 (18)	**0.019**
Achilles tendon injury	5.0 (80)	5.7 (35)	1.000	11.1 (18)	0.303
Family ligament injury	21.8 (78)	50.0 (34)	**0.003**	52.9 (17)	**0.021**
Male Participants:-	n=133	n=91		n=36	
Any other ligament injury [a]	44.4 (126)	57.5 (80)	0.067	54.3 (35)	0.302
Knee ligament injury [b]	4.8 (125)	12.5 (80)	**0.045**	8.6 (35)	0.412
Achilles tendon injury	10.4 (125)	11.1 (81)	0.871	20.0 (35)	0.129
Family ligament injury	27.6 (123)	33.8 (80)	0.353	34.3 (35)	0.445

Values are represented as frequencies (%) and the number of participantss (n) are in parentheses.

[a] Excluding ACL injuries

[b] Includes the posterior cruciate ligament (PCL), the lateral collateral ligament (LCL), and the medial collateral ligament (MCL)

[c] CON vs. ACL

[d] CON vs. NON

5.3.2 Sports participation

The small change in relative frequency of the CON groups in this study did not change the results found for sports participation (Table 5.3) between the groups in the previous study, Study 2, of the thesis. Briefly, significantly more female ACL and male ACL participants had participated in non-contact jumping (P=0.035) and contact sports (P=0.001) respectively. In addition, significantly more males had participated in contact sports for a significantly greater number of years (P<0.001). A significantly greater relative number of females had also participated in non-contact jumping sports (P<0.001) in this study.

Table 5.3: Sports participation according to type of sport within the asymptomatic control (CON) groups, the symptomatic anterior cruciate ligament ruptures (ACL) groups, as well as the symptomatic noncontact (NON) mechanism of injury subgroups for all female and male participants.

	Female Participants			Male Participants			
	CON (n=81)	**ACL** (n=35)	**P-Value** [e]	**CON** (n=127)	**ACL** (n=82)	**P-Value** [e]	**P-Value** [f]
Contact sports [a]							
Participants (%)	13.6 (11)	5.7 (2)	0.339	65.9 (84)	87.8 (72)	**0.001**	**<0.001**
Participation (years)	5.7 ± 3.6	1.0 ± 0.0	0.101	12.7 ± 7.6	11.5 ± 7.9	0.340	**0.001**
Non-contact jumping sports [b]							
Participants (%)	34.6 (28)	57.1 (20)	**0.035**	5.6 (7)	13.4 (11)	0.083	**<0.001**
Participation (years)	8.2 ± 5.2	9.3 ± 5.8	0.493	10.9 ± 5.2	7.0 ± 5.4	0.152	0.918
Non-contact non-jumping sports [c]							
Participants (%)	95.1 (77)	94.3 (33)	1.000	94.4 (120)	92.7 (76)	0.815	0.890
Participation (years)	28.4 ± 20.8	28.2 ± 22.6	0.953	25.3 ± 17.9	24.4 ± 22.2	0.762	0.169
Skiing sports [d]							
Participants (%)	3.7 (3)	14.3 (5)	0.051	5.6 (7)	9.8 (8)	0.375	0.925

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(Legend from the previous page) The number of participants is represented as a frequency (%), while the years of participation is represented as a mean ± standard deviation. The number of participants (n) is in parentheses.

[a] Soccer, rugby, touch rugby, Gaelic football, muay thai, hurling, Australian football league (AFL) and boxing.

[b] Netball, basketball, volleyball, gymnastics, ballet, motorcross, skateboarding, paragliding, handball and sky diving.

[c] Field hockey, cricket, tennis, horseback riding, running, bicycling, spinning, squash, swimming, aerobics, yachting, athletics (excluding long and triple jump), golf, dancing, tennis, canoeing, waterpolo, surfing, windsurfing, badminton, gym training, bowls, triathlon, softball and lifesaving.

[d] Any mode of water or snow skiing.

[e] CON vs. ACL

[f] male (CON + ACL) vs. female (CON + ACL) participants.

5.3.3 *COL12A1* genotype and allele frequencies

5.3.3.1 The *Alu*I RFLP

When the female and male participants were analysed together, there were no significant differences in genotype or allele frequencies between the CON and ACL groups (genotype, P=0.067; allele, P=0.122, nor the CON group and NON sub-group (genotype, P=0.208; allele, P=0.433) for the *Alu*I RFLP (Appendix 1, Additional material, Table A1.7).

However, when the female participants were analysed separately, the *Alu*I RFLP AA genotype was significantly over-represented (AA vs. GT + GG; OR=2.4; 95% CI 1.0 - 5.5; P=0.048) in the ACL group when compared to the CON group (Figure 5.3A).

There were however no *Alu*I RFLP genotype association (AA vs. GT + GG; P=0.359) when the male ACL group was compared to the male CON group (Figure 5.3B).

Furthermore, the genotype frequencies between the CON groups and NON sub-groups for the *Alu*I RFLP was not significantly different within the female participants or the male participants (Figure 5.3A and B). However, it must be noted that the sample size of the female NON group was too small (n=18).

The allele frequencies between the CON and ACL groups for the *Alu*I RFLP was not significantly different within the female participants, or the male participants (Figure 5.4A and B). As reported in Table 5.4, all the groups were in Hardy-Weinberg equilibrium.

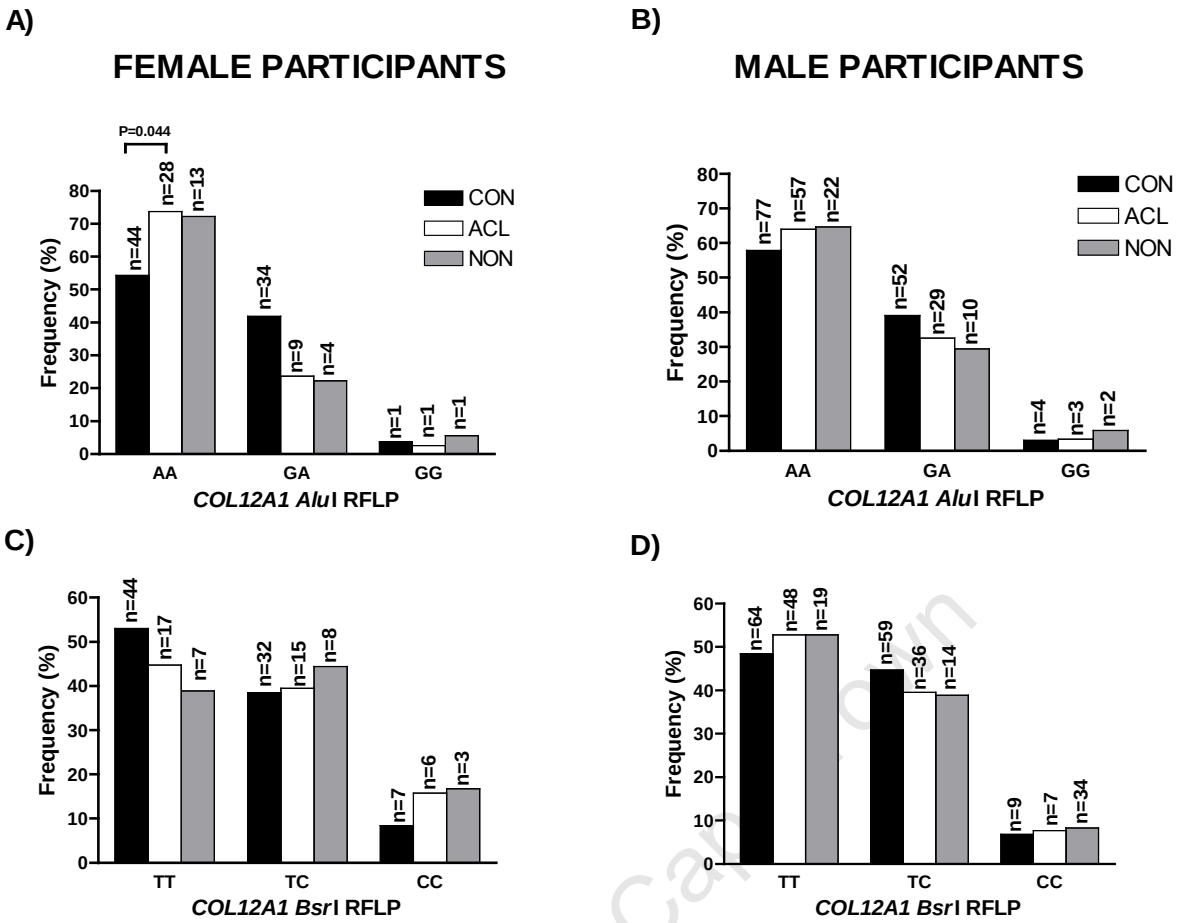

Figure 5.3: The relative genotype frequency of the *COL12A1* gene *Alu*I and *Bsr*I restriction fragment length polymorphisms (RFLP) within the asymptomatic control (CON; black bars) groups, the anterior cruciate ligament rupture groups (ACL; white bars) and the non-contact mechanism of injury sub-group (NON; grey bars) in all female (A and C) and male (B and D) participants. The number of participants (n) within each group is shown in parentheses. Because of the small sample size of the *Alu*I RFLP GG genotype (A and B), the AA genotype was compared to the combined GA and GG genotypes. Similarly, because of the small sample size of the *Bsr*I RFLP CC genotype in the female and male NON groups (C and D), the CON vs. NON genotype data was analysed by comparing the TT genotype to the combined TC and CC genotypes. **(A)** The *Alu*I RFLP genotype distributions within the female participants. CON vs. ACL, P=0.044 (AA vs. GA + GG); CON vs. NON, P=0.180 (AA vs. GA + GG) **(B)** The *Alu*I RFLP genotype distributions within the male participants. CON vs. ACL, P=0.359 (AA vs. GA + GG); CON vs. NON, P=0.471 (AA vs. GA + GG). **(C)** The *Bsr*I RFLP genotype distributions within the female participants. CON vs. ACL, P=0.433; CON vs. NON, P=0.643 (TT vs. TC+CC). **(D)** The *Bsr*I RFLP genotype distributions within the male participants; CON vs. ACL, P=0.746; CON vs. NON, P=0.533 (TT vs. TC + CC).

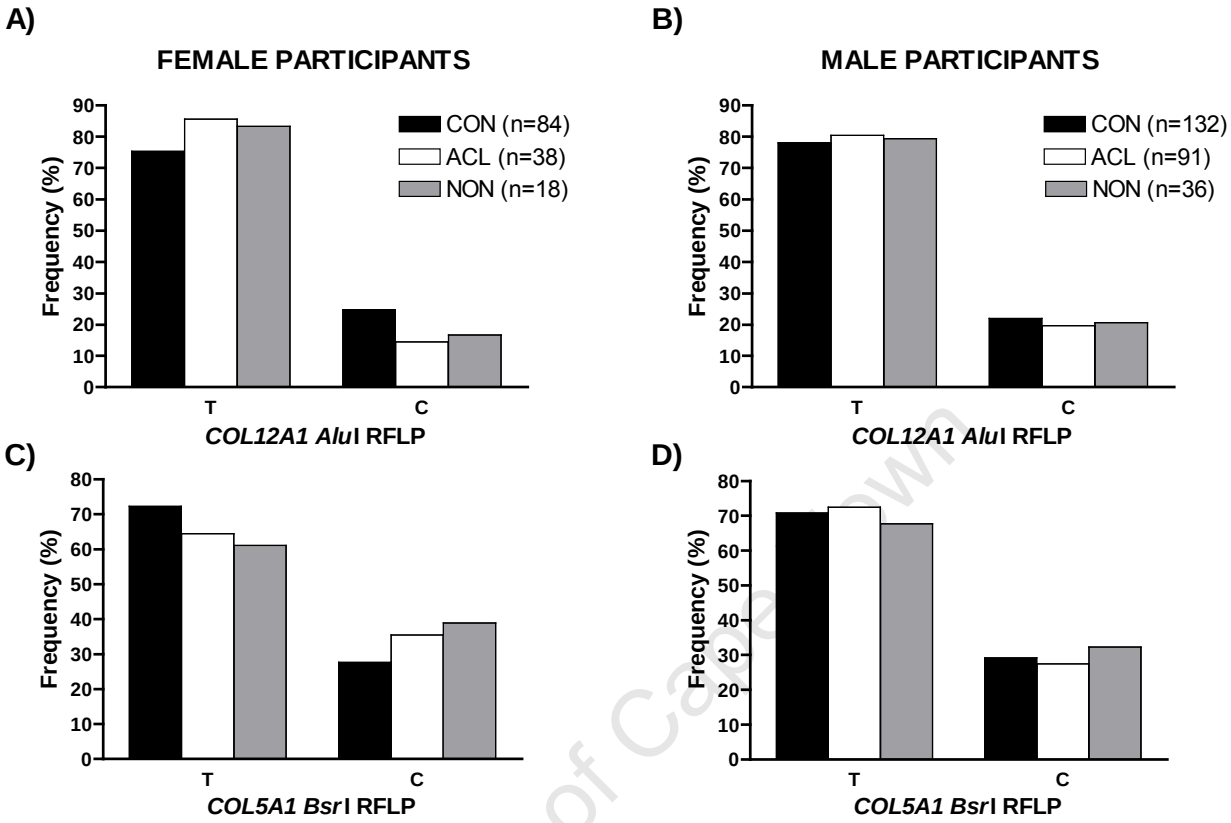

Figure 5.4: The relative allele frequency of the *COL12A1* gene *Alu*I and *Bsr*I restriction fragment length polymorphisms (RFLP) within the asymptomatic control (CON; black bars) groups, the anterior cruciate ligament rupture groups (ACL; white bars) and the non-contact mechanism of injury sub-group (NON; grey bars) in all female (A and C) and male (B and D) participants. **(A)** The *Alu*I RFL1P allele distributions within the female participants. CON vs. ACL, P=0.090; CON vs. NON, P=0.385. **(B)** The *Alu*I RFLP allele distributions within the male participants. CON vs. ACL, P=0.634; CON vs. NON, P=0.870. **(C)** The *Bsr*I RFLP allele distributions within the female participants. CON vs. ACL, P=0.230; CON vs. NON, P=0.227. **(D)** The *Bsr*I RFLP allele distributions within the male participants; CON vs. ACL, P=0.749; CON vs. NON, P=0.645.

Table 5.4: Hardy-Weinberg P-values of all separately analysed groups for the *Alu*I and *Bsr*I restriction fragment length polymorphisms (RFLP).

	***Alu*I RFLP P-value**	***Bsr*I RFLP P-Value**
Female ACL group	≥0.572	≥0.489
Male ACL group	1.000	1.000
Male + Female ACL groups	1.000	≥ 0.534
Female CON group	0.372	≥ 0.783
Male CON group	0.217	≥ 0.405
Male + Female CON groups	0.088	≥ 0.737
All participants	0.195	1.000

5.3.3.2 The *Bsr*I RFLP

When the female and male participants were analysed together, there were no significant differences in genotype or allele frequencies between the CON and ACL groups (genotype, P=0.665; allele, P=0.730), nor the CON group and NON sub-group (genotype, P=0.679; allele, P=0.556) for the *Bsr*I RFLP. (Appendix 1, Additional material, Table A1.7).

There were also no significant differences in the *COL12A1 Bsr*I RFLP genotype (Figure 5.3 C and D) or allele (Figure 5.4 C and D) frequency distributions between the female or male CON and ACL groups. Similarly, there were also no significant genotype (Figure 5.3 C and D) or allele (Figure 5.4 C and D) frequency distibutions between the CON groups and NON sub-groups. As reported in Table 5.4 all the groups were in Hardy-Weinberg equilibrium.

5.3.3.3 *Alu*I RFLP genotype effects on family history of ligament injury

Although there were no genotype differences between the female and male participants with and without a previous history of ligament injury, there is a trend for the AA genotype to be over-represented (AA vs. GA + GG; P=0.082) in female participants with a family history of ligament injury (Figure 5.5). This finding is similar to the association noted between with the *COL5A1 Bst*UI RFLP and family history in Study 2.

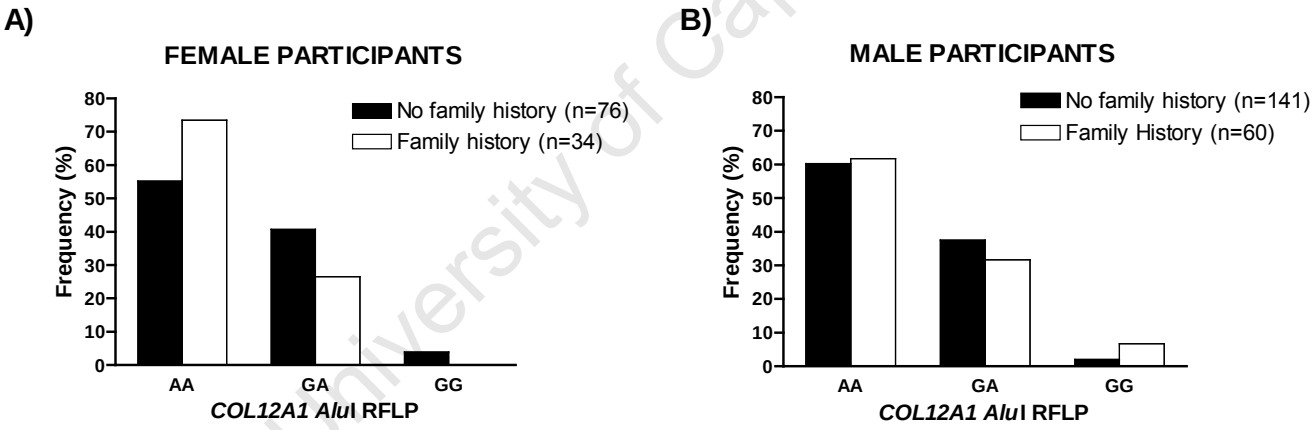

Figure 5.5: The relative genotype frequency of the *COL12A1* gene *Alu*I restriction fragment length polymorphism (RFLP) when all female (A) and male (B) participants in the study were divided into those with and without a family history of ligament injuries. **(A)** The *Alu*I RFLP genotype distribution within the female participants; AA vs. GA + CC, P=0.082; **(B)** The *Alu*I RFLP genotype distribution within the male participants; P=0.854.

5.3.3.4 Combined analysis of the *COL12A1 Alu*I and *COL5A1 Bst*UI RFLPs.

It was shown in the previous study of the thesis that the CC genotype of the *COL5A1 Bst*UI RFLP is over-represented in the female CON group when compared to a female CON group. The combined analysis of the *Alu*I RFLP and the *Bst*UI RFLP within the male and female participants are therefore reported in Table 5.5. Although this data should not be over interpreted, the best estimates of risk of the combined genotype for ACL ruptures resulted from comparing the female CON and ACL groups with a TT genotype for the *Bst*UI RFLP and a AA genotype for the *Alu*I RFLP (OR = 2.43; 95% CI 0.95 – 6.19; P=0.079), as well as the female CON and ACL participants with a TC genotype for the *Bst*UI RFLP and a AA genotype for the *Alu*I RFLP (OR = 1.83; 95% CI 0.81 – 4.15; P=0.199)(Table 5.4). Of interest, when the above mentioned genotype combinations of highest risk were combined, a T allele (TT or TC genotype) for the *Bst*UI RFLP and a AA genotype for the *Alu*I RFLP was significantly over-represented (OR=3.62; 95% CI 1.57 – 8.34; P=0.003) in the ACL group (70.3%), when compared to the CON group (39.5%).

Table 5.5: The genotype pairs of the *COL12A1 Alu*I and *COL5A1* BstUI RFLPs, together with their frequencies within the anterior cruciate ligament rupture (ACL) and control (CON) groups, as well as their estimated risk (OR).

		Female Participants			Male Participants		
COL12A1	***COL5A1***	**CON** (N=81)	**ACL** (N=37)	**OR**	**CON** (N=133)	**ACL** (N=88)	**OR**
AA	**TT**	14.8 (12)	29.7 (11)	2.43	16.7 (22)	20.5 (18)	1.30
AA	**TC**	27.2 (22)	40.5 (15)	1.83	34.9 (46)	33.0 (29)	0.93
AA	**CC**	12.4 (10)	2.7 (1)	n.d.	6.8 (9)	10.2 (9)	1.57
AG	**TT**	12.4 (10)	13.5 (5)	1.11	12.1 (16)	5.7 (5)	0.44
AG	**TC**	14.8 (12)	10.8 (4)	0.70	18.1 (24)	21.6 (19)	1.25
AG	**CC**	14.8 (12)	0 (0)	n.d.	8.3 (11)	5.7 (5)	0.67
GG	**TT**	0 (0)	0 (0)	n.d.	0.8 (1)	2.3 (2)	n.d.
GG	**TC**	3.7 (3)	0 (0)	n.d.	1.5 (2)	0 (0)	n.d.
GG	**CC**	0 (0)	2.7 (1)	n.d.	0.8 (1)	1.1 (1)	n.d.

The CON and ACL values are represented as a frequency (%) with the number of subjects (N) in parenthesis.

n.d. = not determined due to small sample sizes.

5.4 DISCUSSION

The main finding of this study was that the AA genotype of the *Alu*I RFLP within the terminal exon 65 of the *COL12A1* gene was associated with a 2.4 times increased risk (OR=2.4; 95% CI 1.0 - 5.5; P=0.048) of ACL ruptures in female, but not male participants. The upstream *Bsr*I RFLP, within exon 29, was however not associated with ACL ruptures in neither the females nor the male participants. The finding of an increased frequency of family history of ligament injury amongst participants in the ACL group was reported and discussed in the previous Study 2. An interesting finding of this study was that there was a trend (not statistically significant) for the AA genotype of the *Alu*I RFLP to be over-represented in female participants with a family history of ligament injury. An additional finding of this study was that female participants with an AA genotype for the *COL12A1 Alu*I RFLP and a T allele (TC or TT genotype) for the *COL5A1 Bst*UI RFLP were at 3.6 times greater risk (OR=3.6; 95% CI 1.6 – 8.3; P=0.003) of ACL ruptures, when the results from this study were combined with the results from the study reported in Study 2 of this thesis.

The novel finding, that females with an AA genotype for the *Alu*I RFLP had a increased risk of ACL ruptures, has not been previously reported. The *COL12A1* gene, which has been mapped to 6q12-q13, and encodes both the various long (XIIA) and short (XIIB) isoforms of type XII collagen, is a homotrimer consisting of 3 α1(XII) chains [130]. The short XIIB-1 isoform is predominantly expressed in both tendons and ligaments in response to mechanical loading [130]. The *Alu*I RFLP within exon 65, is a non-synonymous coding variant, which changes the amino acid at position 3058 from a serine to a glycine. It is interesting to note that the wild type

amino acid, serine, is a neutral polar amino acid with a larger side chain than the non-polar neutral glycine amino acid; this change in amino acid sequence has no known function. The findings from this study do however suggest that the A>G substitution of this variant results in an altered type XII collagen protein, and, although speculative, this may alter the biomechanical properties of the collagen fibril. Further research is therefore required.

The *Bsr*I RFLP of type XII collagen, which changes the amino acid at position 1738 from isoleucine to threonine, was however not associated with ACL ruptures in male or females participants. Although the genotype frequencies of the *Alu*I and *Bsr*I RFLPs in the current study are similar to what has previously been reported [148], it is interesting to note that the *Alu*I RFLP GG genotype and the *Bsr*I RFLP CC genotypes were not indentified in patients with Achilles tendon ruptures. The significance of this finding is however not currently known [148]. Furthermore, the *Alu*I and *Bsr*I RFLPs were previously shown not to be in linkage disequilibrium [148].

The additional finding of this study, that female participants with an AA genotype for the *Alu*I RFLP and either a TC or TT genotype for the *Bst*UI RFLP within the *COL5A1* were at 3.6 times greater risk of ACL ruptures. These results should however be interpreted with caution due to the small sample size. This finding provides useful information to direct future research, and in particular, to the design of appropriate multifactorial models that may be developed to reduce the incidence of ACL ruptures in genetically predisposed individuals. Interestingly, previous data have also demonstrated that a significant interaction exists between the *Bst*UI RFLP within *COL5A1* and the *MMP-3* gene and the risk of Achilles tendinopathy [147].

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The main finding of this study is remarkably similar to the main finding of Study 2 of this thesis. In this previous study the *Bst*UI RFLP within *COL5A1* was also only associated with ACL rupture in female participants. As discussed in Study 2, the reasons for these gender-specific associations are unknown. Specific to the gender-association of the *Alu*I RFLP within *COL12A1* and ACL ruptures, there are no known previous reports of the effects of the female sex hormones on the regulation of *COL12A1* or any gene-gene interactions which could possibly provide a possible explanation. Both genes however encode for quantitatively minor structural components of the collagen fibril, the major building block of the ligament [14;112].

It has been widely reported that females are at increased risk of ACL rupture when compared to males (refer to Section 2.3.1.3). A number of intrinsic anatomical risk factors classified as either anatomical, hormonal, or neuromuscular, have been linked to this phenomenon [4;12]. The gender-specific association found in this study (as well as the results reported in Study 2), may indicate that genetic variants alter structure and/or biomechanical function of the ACL which may compromise this ligament. Therefore it remains possible that even though the biomechanical properties of the ACL were influenced by the *Alu*I RFLP in male and females participants, it did not alter the risk of ACL rupture in the male participants significantly enough to demonstrate an association in this study. For this reason, a larger group of homogenous male participants with non-contact ACL ruptures and exposure matched controls are required for further investigation.

The previous discussion of the non-genetic factors which may be possible confounding variables in determining injury risk, as described in Study 2 is also valid for the current study. Briefly, the fact that females were not matched for participation in non-contact jumping sports, and males were not matched for contact sports, is a limitation of this study. Furthermore, the BMI of the male ACL group and NON sub-group were both significantly higher than the CON group, and is thus another limitation of the study. A third limitation of this study, as previously discussed (Study 2), was the relatively small sample size of the female participants. The primary aim of the thesis was not designed to investigate gender specific genetic risk factors and therefore further research is required to confirm this finding in larger female cohorts.

In conclusion, the AA genotype of the *Alu*I RFLP within exon 65 of the *COL12A1* gene is associated with an increased risk of ACL rupture in female participants (Figure 5.6).

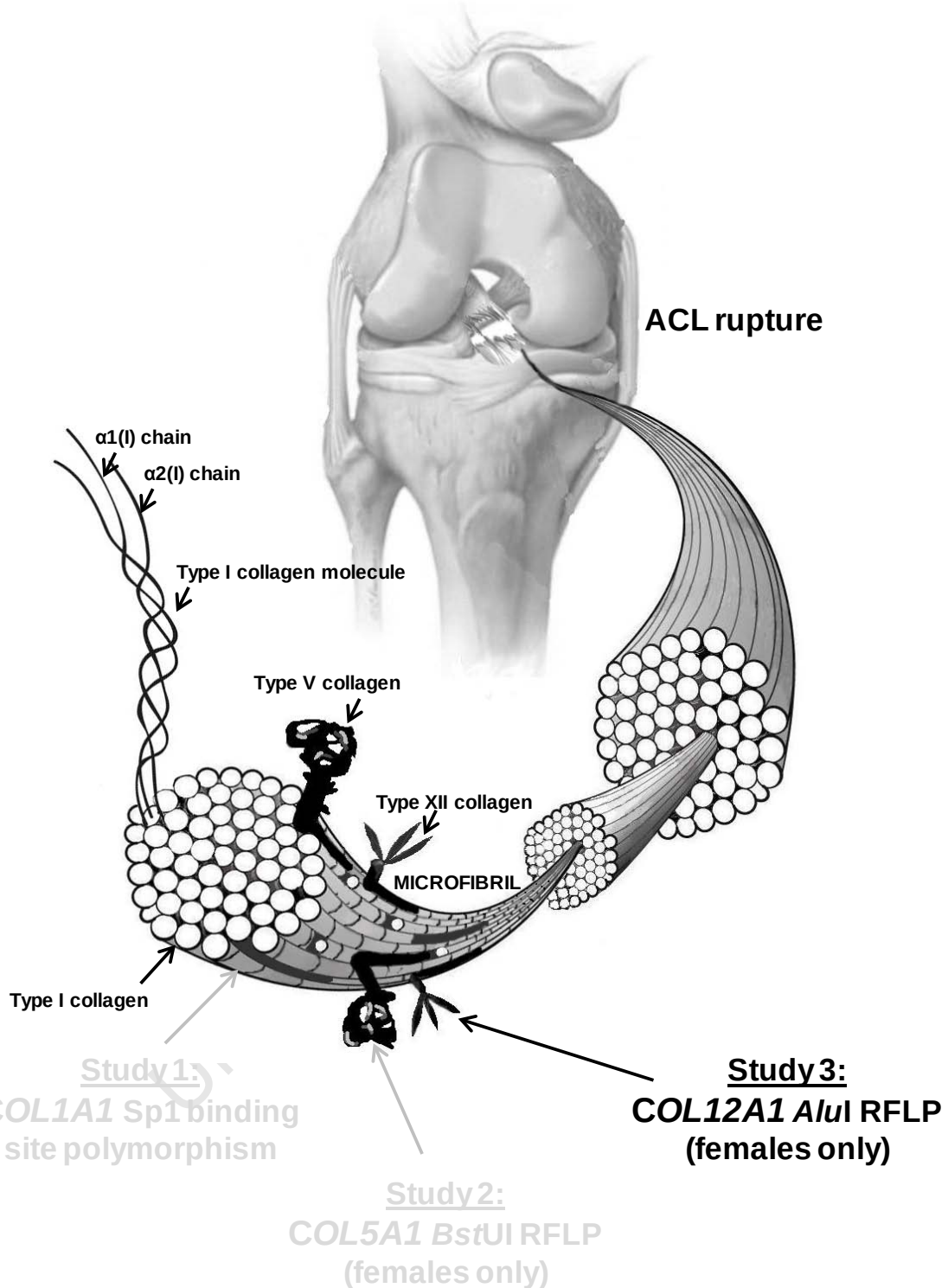

Figure 5.6: A schematic presentation of the primary finding from this study. The *COL12A1* *Alu*I RFLP was associated with risk of ACL ruptures in female participants. The *COL12A1* gene encodes for type XII collagen, a member of the FACIT collagens commonly found within the microfibril. Refer to Section 2.5.1 for further detail regarding the structure of the collagen microfibril.

CHAPTER 6

STUDY FOUR

THE *COL1A1* SP1 BINDING SITE POLYMORPHISM: RISK FOR OTHER ACUTE AND CHRONIC SOFT TISSUE INJURIES

The data presented in this chapter was published in the following peer-reviewed article: Posthumus, M., September, A.V., Schwellnus, M., Collins, M. Investigation of the Sp1 binding site polymorphism within the *COL1A1* gene in participants with Achilles tendon injuries and controls. ***Journal of Science and Medicine in Sport***, 2009. 12(1); 184-189

6.1 INTRODUCTION

The first three experimental chapters (Studies 1 to 3) of this thesis investigated the association of variants within three candidate genes (*COL1A1*, *COL5A1* and *COL12A1*) and the risk of ACL ruptures. Previous research studies from this laboratory have also investigated whether the same variants within the *COL5A1* [143;145] and *COL12A1* [148] genes are associated with another common chronic (Achilles tendinopathy) and acute (Achilles tendon rupture) soft tissue injury. Although some interesting similarities were found between the genetic risk factors for ACL ruptures, chronic Achilles tendinopathy and/or spontaneous Achilles tendon ruptures, there were also some notable differences. These findings do however provide initial evidence that there are, in part, some potential common underlying genetic predispositions to these acute and chronic soft tissue injuries.

Among the three candidate genes investigated in the first three studies of this thesis, only the *COL1A1* variant has not been investigated as a candidate gene for other chronic and acute musculoskeletal soft tissue injuries.

The aim of this study was therefore to determine whether the functional Sp1 binding site polymorphism within intron 1 of the *COL1A1* gene is also associated with acute (spontaneous Achilles tendon ruptures) and chronic (Achilles tendinopathy) injuries of another soft tissue, namely the Achilles tendon.

6.2 MATERIALS AND METHODS

6.2.1 Participants

Eighty five Caucasian participants diagnosed with chronic Achilles tendinopathy (TEN), and 41 Caucasian participants diagnosed with partial (n=3) or complete (n=38) Achilles tendon ruptures (RUP) were previously recruited from the medical practice at the Sports Science Institute of South Africa and other clinical practices within the greater Cape Town area of South Africa (as previously described [143;144]). In addition, 126 apparently healthy, unrelated, Caucasian participants without any history of symptomatic Achilles tendon injuries (CON) were also included in this study. The TEN, RUP and CON participants compared in this study were the same cohort that was previously used to identify candidate genes for Achilles tendon injuries [143-145;147;148].

An experienced clinician made the diagnosis of chronic Achilles tendinopathy using clinical criteria. The diagnostic criteria for every subject were reviewed by an experienced Sports Physician (MS). The clinical diagnostic criteria for chronic Achilles tendinopathy were gradual progressive pain over the posterior lower limb in the Achilles tendon area for greater than 6 months, together with at least one out of the following six criteria: (1) early morning pain over the Achilles tendon area, (2) early morning stiffness over the Achilles tendon area, (3) a history of swelling over the Achilles tendon area, (4) tenderness to palpation over the Achilles tendon, (5) palpable nodular thickening over the affected Achilles, or (6) movement of the painful area in the Achilles tendon with plantar-dorsi-flexion (positive “shift” test) [167;168].

[168;168;168] In addition to these clinical diagnostic criteria, soft tissue ultrasound examination was performed in a sub-group (n=36) of participants to confirm the diagnosis of the affected Achilles tendon.

The diagnosis of Achilles tendon rupture was made clinically using standard validated criteria [167] [142;169;169]and confirmed in all cases by examination at the time of surgery (33 of 41, 80.5%s) or by ultrasound imaging (5 of 41, 12.2%), MRI imaging (2 of 41, 4.9%) or computer tomography (CT) scan (1 of 41, n=2.4%). Participants who had a history of current or past fluoroquinolone antibiotic use or previous local corticosteroids injection in the Achilles tendon or the area surrounding the Achilles tendon were excluded from the study. This was necessary because of the known association between fluoroquinolone antibiotic [170] or possibly corticosteroids use, and an increased risk of Achilles tendon rupture [171]. Furthermore, participants who had been diagnosed with any connective tissue disorders or any other systemic diseases believed to be associated with Achilles tendon pathology, such as, but not limited to, Ehlers-Danlos syndrome, benign hypermobility joint syndrome, rheumatoid arthritis, systemic lupus erythematosus, hyperparathyroidism, renal insufficiency, diabetes mellitus and familial hypercholesterolaemia were also excluded from the study [171].

Multiple (greater than one) injuries to the same Achilles tendon were documented in 17 (20.0%) and 14 (34.1%) of the TEN and RUP subjects respectively. Forty four percent of the TEN (37 of 85) and 34% (14 of 41) the RUP subjects reported either a bilateral and/or multiple Achilles tendon injuries.

Prior to participation in this study, all the participants gave informed written consent. In addition, each subject completed medical history questionnaire forms (Appendix 5). This study was approved by the Research Ethics Committee of the Faculty of Health Sciences within the University of Cape Town, South Africa (reference number 170/2005, Appendix 6).

6.2.2 DNA extraction and *COL1A1* genotyping

Approximately 4.5 ml of venous blood was collected from each subject into EDTA vacutainer tubes by venipuncture of a forearm vein and stored at 4°C until DNA extraction. DNA was extracted using the procedure described in Study 1 and modified by Mokone et al. [143]. DNA samples were genotyped for the functional Sp1 binding site polymorphism (SNP rs1800012; IVS1+1023G>T) within intron 1 of the *COL1A1* gene using a nested polymerase chain reaction (PCR) as previously described in the first study of the thesis (Section 3.2.3).

The resultant fragments together with 100 bp molecular weight marker (Promega Corporation, Madison, Wisconsin, USA), were separated on 6% non-denaturing polyacrylamide gels and visualised by ethidium bromide staining. The gels were photographed under UV light using a Uvitec photodocumentation system (Uvitec Limited, Cambridge, UK) and the sizes of the DNA fragments determined. The G allele produces a 260 bp fragment while the T allele produces 242 bp and 18 bp fragments (Figure 3.3, chapter 3).

6.2.3 Statistical analysis

Data was analysed using STATISTICA Version 7 (Statsoft Inc., Tulsa, Oklahoma, USA) and Graphpad InStat Version 3 (Graphpad Software, San Diego, California, USA) statistical programs. A one-way analysis of variance (ANOVA) was used to determine any significant differences between the characteristics of the TEN, RUP and CON groups. A least squares difference (LSD) post-hoc test was used to identify specific differences when the overall F value was found to be significant. A chi-squared (χ^2) analysis was used to analyse any differences in the genotype frequencies and allele frequencies between the three groups. Significance was accepted when $P<0.05$. Hardy-Weinberg equilibrium was established using the program Genepop web version 3.4 (http://genepop.curtin.edu.au/).

6.3 RESULTS

6.3.1 Participant Characteristics

The TEN, RUP and CON groups were similarly matched for age, height, gender and country of birth (Table 6.1). The age of the TEN and RUP groups are the age of initial onset of the Achilles tendon injury, which were on average 7.9 ± 9.3 and 7.6 ± 8.7 years after their initial symptoms respectively. The TEN and RUP groups were on average significantly heavier (P<0.001) with corresponding higher body mass indexes (P<0.001) than the CON group. There were however no *COL1A1* genotype effects on weight and BMI (Table 6.2).

6.3.2 *COL1A1* genotype and allele frequencies.

There were no significant differences in the distribution of the genotype (P=0.602) or allele (P=0.578) frequencies of the *COL1A1* Sp1 binding site polymorphism between the CON, TEN and RUP groups (Figure 6.1). The rare TT genotype was however not present in the RUP group. Due to the small sample size this finding was not statistically significant (TT vs. GG + GT; P=0.338).

The *COL1A1* genotype distribution of the CON (P=0.198), TEN (P=1.000) and RUP (P=1.000) groups were in Hardy-Weinberg equilibrium.

Table 6.1: Characteristics of the control (CON), Achilles tendinopathy (TEN) and Achilles rupture (RUP) participants.

	CON (n)	**TEN** (n)	**RUP** (n)	**P value**[b]
Age (years)[a]	37.1 ± 10.6 (119)	39.4 ± 14.7 (79)	40.2 ± 11.3 (40)	0.241
Height (cm)	174.8 ± 9.3 (121)	176.2 ± 9.3 (78)	175.5 ± 8.4 (41)	0.554
Weight (kg)	71.3 ± 12.0 (124)[c,d]	78.0 ± 14.0 (80)[c,e]	86.4 ± 14.3 (41)[d,e]	<0.001
BMI (kg/cm^2)	23.3 ± 2.6 (121)[c,d]	24.9 ± 3.4 (78)[c,f]	28.0 ± 3.7 (41)[d,f]	<0.001
Gender (% males)	64.0 (125)	71.8 (85)	75.61 (43)	0.279
Country of birth (% South Africa)	72.6 (124)	71.6 (81)	80.49 (43)	0.504

Gender and country of birth are represented as a frequency (%), while the remaining variables are expressed as mean ± standard deviation.

BMI – body mass index.

[a]The age of the TEN and RUP groups are the age of the onset of the initial symptoms of Achilles tendon pathology, which were on average 7.9 ± 9.3 and 7.6 ± 8.7 years after their initial symptoms respectively.

[b] P value = CON vs. TEN vs. RUP; [c] CON vs. TEN (P<0.001); [d] CON vs. RUP (P<0.001); [e] TEN vs. RUP (P=0.002); [f] TEN vs. RUP (P<0.001)

Table 6.2: Genotype effects of the Sp1-binding site polymorphism (SNP rs1800012; IVS1+1023G>T) within the *COL1A1* gene on the characteristics of the combined control (CON), Achilles tendinopathy (TEN) and Achilles rupture (RUP) participants.

	GG genotype (n)	**GT genotype** (n)	**TT Genotype** (n)	**p value**[b]
Age (years)[a]	38.2 ± 11.8 (169)	38.0 ± 13.7 (61)	45.4 ± 8.58 (8)	0.262
Height (cm)	174.7 ± 9.5 (172)	177.6 ± 7.5 (60)	174.2 ± 10.7 (8)	0.096
Weight (kg)	75.4 ± 14.8 (175)	78.4 ± 11.9 (61)	71.3 ± 15.3 (8)	0.232
BMI (kg/cm^2)	24.6 ± 3.7 (172)	24.7 ± 3.0 (60)	23.2 ± 2.4 (8)	0.540
Gender (% males)	65.9 (179)	76.6 (64)	62.5 (8)	0.342
Country of birth (% South Africa)	71.59 (176)	80.65 (62)	62.5 (8)	0.296

Gender and country of birth are represented as a frequency (%), while the remaining variables are expressed as mean ± standard deviation.

BMI – body mass index.

[a] The age of the TEN and RUP groups are the age of the onset of the initial symptoms of Achilles tendon pathology, which were on average 7.9 ± 9.3 and 7.6 ± 8.7 years after their initial symptoms respectively .

[b] P value = CON vs. TEN vs. RUP.

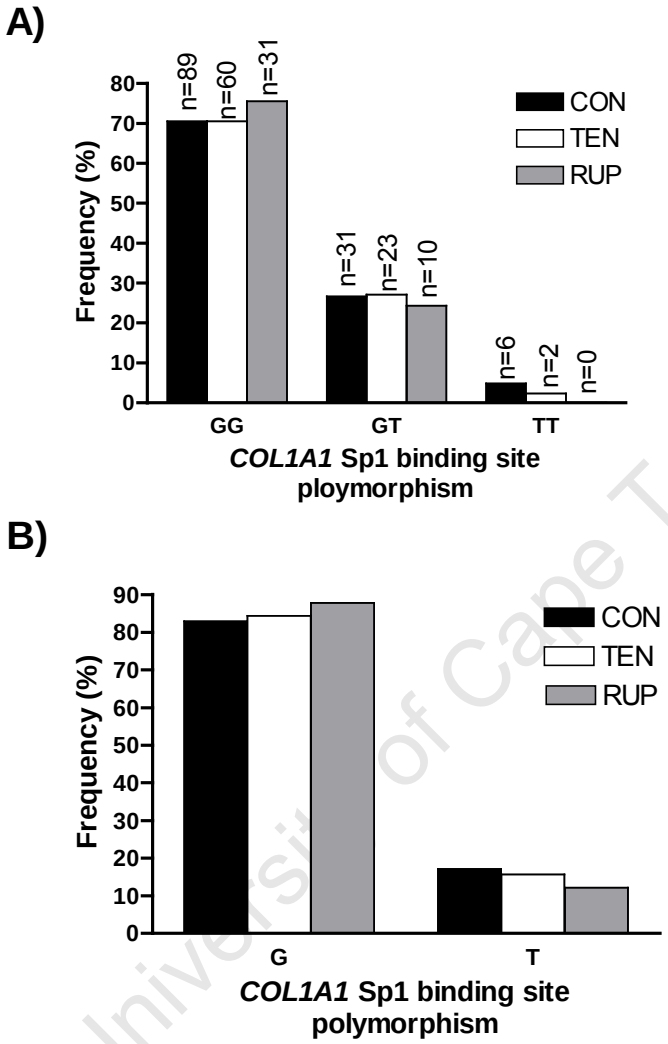

Figure 6.1: Relative genotype **(A)** and allele **(B)** frequencies of the *COL1A1* Sp1 binding site polymorphism (SNP rs1800012; IVS1+1023G>T) of the asymptomatic control (CON, solid bars), the chronic Achilles tendinopathy (TEN, clear bars) and Achilles tendon rupture (RUP, grey bars) participants. There were no significant differences in the distribution of the genotype (P=0.602) or allele (P=0.578) frequencies of the *COL1A1* Sp1 binding site polymorphism between the three groups.

6.4 DISCUSSION

The purpose of this study was to investigate whether the functional Sp1 binding site polymorphism within intron 1 of the gene encoding for the α1 chain of type I collagen (*COL1A1*) was associated with other soft tissue injuries, namely spontaneous Achilles tendon rupture and/or chronic Achilles tendinopathy. The main finding of this study was that there was no association between this functional *COL1A1* polymorphism and any of the studied Achilles tendon injuries. The rare TT genotype was however not present in the small group of participants with Achilles tendon rupture. This observation was similar to the findings presented in Study 1, which found no TT genotypes in participants with ACL ruptures, and the findings of the previous Swedish study [9], which found the TT genotype to be under-represented in participants with acute ligament injuries. When combined, the observation that the TT genotype is rare, suggests that the Sp1 binding site polymorphism is a common genetic risk factor for several different acute soft tissue injuries. This observation will be further explored in the next chapter of this thesis.

The genotype frequencies of the CON group in this study were notably similar to the genotype distribution of the independent control group used in Study 1 of this thesis, the Swedish study [9] reporting an association with acute ligament injuries, and other very large cohorts [154;155] (Table 6.3).

Table 6.3: The genotype frequencies of the Sp1 binding site polymorphism, and the sample size (N) of the control groups of the current study (Study 4), the first study of the thesis (Study 1), the study by Khoschnau [9], and other very large cohort studies[155;160].

	Posthumus (PhD thesis 2009 - Study 4)	Posthumus (PhD thesis 2009 – Study 1)	Khoschnau [9]	Mann [155]*	Lian [160]
N	126	130	325	4733	4175
GG (%)	70.6	70.0	70.8	66.1	65.3
GT (%)	24.6	25.5	25.5	29.5	30.9
TT (%)	4.8	4.6	3.7	4.4	3.8

Although it has been reported that the T allele of the functional Sp1 binding site polymorphism alters the α1(I) collagen to α2(I) collagen protein ratio [155], no statistically significant association with Achilles tendinopathy was observed. It is however interesting to note that only 2 participants (2 of 85; 2.4%) within the Achilles tendinopathy group had a TT genotype. Further research with a larger sample size will be required to determine if there is any minor affect of this polymorphism on Achilles tendinopathy.

CHAPTER 7

STUDY FIVE

THE *COL1A1* GENE AND ACUTE SOFT TISSUE RUPTURES

The data presented in this chapter was published in the following peer-reviewed article: Collins, M. **Posthumus, M.** Schwellnus, M. The *COL1A1* gene and acute soft tissue ruptures. ***British Journal of Sports Medicine.*** In Press.

7.1 INTRODUCTION

Acute soft tissue injuries, such as Achilles tendon ruptures and anterior cruciate ligament ruptures, are not only common, but also amongst the most severe injuries sustained in recreational and competitive athletes [3;35]. A critical step in an injury prevention model, as discussed in the first chapter of this thesis, is understanding the aetiology and mechanisms of injury [11]. The aetiology and mechanisms may be described by a complex interaction between intrinsic risk factors, extrinsic risk factors, and a specific inciting event [39]. As reviewed (Section 2.4.2), intrinsic risk factors, which include a genetic component, may influence the risk of sustaining a soft tissue rupture, predisposing an individual to injury. Furthermore, the addition of extrinsic risk factors may render the individual susceptible to injury. It is important to note that the mere presence of these risk factors is not sufficient to result in injuries, they merely "prepare" the athlete for an injury to occur in a given situation, termed the "inciting event". However, understanding these risk factors is important for the development of injury prevention models.

The functional Sp1 binding site polymorphism within the first intron of the *COL1A1* (as discussed in Section 2.5.3.1), has recently been associated with cruciate ligament ruptures [9] and shoulder dislocations [9]. In addition this polymorphism was shown in Study 1 of this thesis to be associated with ACL ruptures in a South African Caucasian population. In this study the rare TT genotype for this polymorphism was not present in participants with ACL ruptures. Similarly, the previous study (Study 4) of this thesis also found no TT genotypes in a small group of participants with Achilles tendon ruptures.

As shown in Table 6.3 of the previous chapter, the genotype distribution of this polymorphism was similar within the asymptomatic 256 South African Caucasian (4.7% TT genotype) (Study 1 and Study 4) and 325 Swedish (3.7% TT genotype) [9] control populations. It is important to mention that the Swedish control population consisted of only female participants [9], while only 36% (Study 4) and 25% (Study 1) of the South African studies consisted of female subjects. Similarly, the genotype distribution within the South African and Swedish control populations were similar to the distributions reported in larger control cohorts consisting of 4175 and 4733 asymptomatic subjects, which reported a TT genotype frequency of 3.8% and 4.4%, respectively (refer to Table 6.3) [155;160].

By combining the similar results that were observed in the independent South African (Studies 1 and 4) and Swedish [9] studies, the association between acute soft tissue ruptures and a specific genotype could perhaps be strengthened. Stronger evidence that the *COL1A1* TT genotype potentially protects an athlete from

an acute musculoskeletal soft tissue rupture would have significant clinical relevance in any injury risk model.

Therefore, the aim of this chapter is to report the combined effect, from the published Swedish study [9], and the results presented in this thesis, of the rare TT genotype of the *COL1A1* Sp1 binding site polymorphism on the risk of acute soft tissue ruptures. A Fisher's exact test was used to analyse any differences in the genotype frequencies (TT vs. GT and GG) of the 581 combined control (CON) and injured groups in the three combined studies. The injured groups were analysed as (1) 350 cruciate ligament ruptures (CL), (2) 476 cruciate ligament ruptures and shoulder dislocations (CLSD), and (3) all 517 soft tissue ruptures (ALL; cruciate ligament, shoulder dislocations and Achilles tendon).

7.2 RESULTS

The rare TT genotype was significantly under-represented in the CL group (0.3% TT genotype, n=1) when compared to the CON group (4.1% TT genotype, n=24) of all three published studies (OR=15.1; 95% CI 2.0 - 111.7; P=0.0002). Similar results were obtained when the CLSD group (0.4% TT genotype, n=2; OR=10.2; 95% CI 2.4 - 43.4, P<0.0001) or the ALL group (0.4% TT genotype, n=2; OR=11.1; 95% CI 2.6 - 47.2; P<0.0001) were compared to the CON group (Figure 7.1).

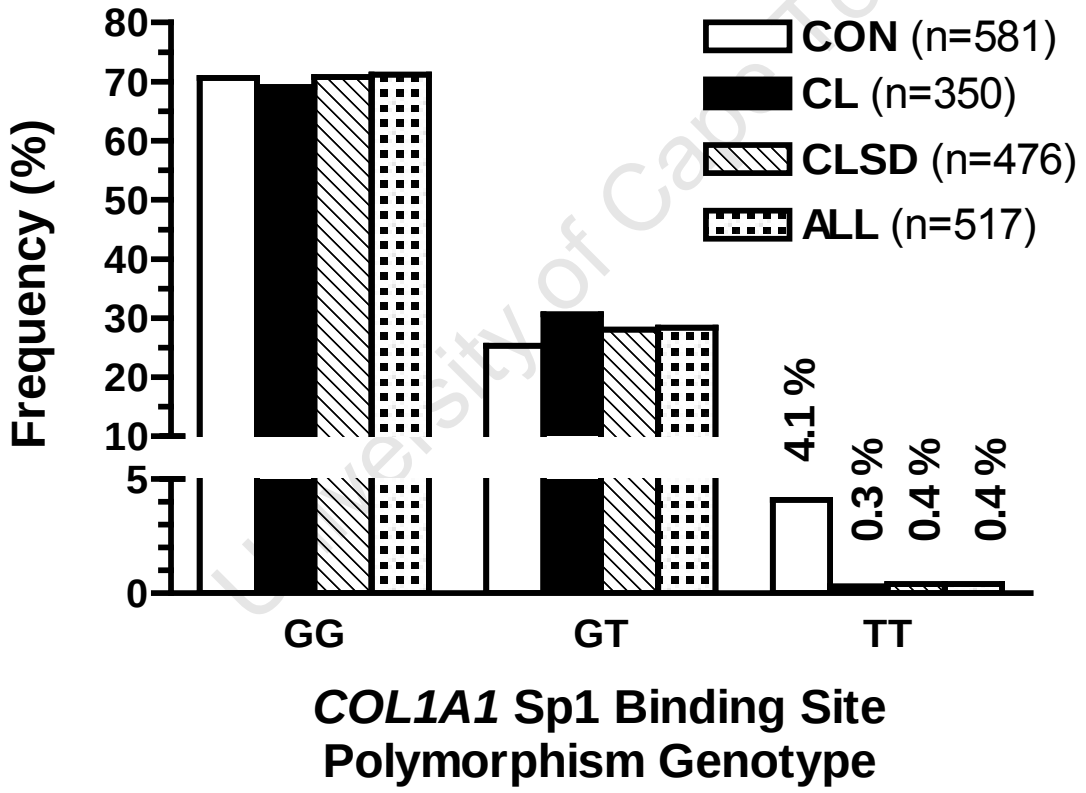

Figure 7.1: The relative genotype frequencies of the functional Sp1 binding site polymorphism within intron 1 of the *COL1A1* gene of the combined Swedish [9] and South African (Study 1 and 4) for the (1) asymptomatic control (CON), (2) cruciate ligament ruptures (CL), (3) cruciate ligament ruptures and shoulder dislocations (CLSD), and the, (4) soft tissue ruptures (cruciate ligament, shoulder dislocations Achilles tendon) (ALL). The data from both the South African Caucasian control populations were from unrelated individuals.

7.3 DISCUSSION

In summary, the combined results from the recently published Swedish study [9], and the data that was presented in this thesis (Study 1 and 4), show that the TT genotype of the *COL1A1* Sp1 binding site polymorphism is associated with an 11.1 times reduced risk (OR=11.1; 95% CI 2.6 – 47.2; P<0.001) of acute musculoskeletal soft tissue ruptures. Furthermore, when only the risk of cruciate ligaments were analysed, the TT genotype was associated with a 15.1 times reduced risk (OR=15.1; 95% CI 2.0 – 111.7; P<0.001) of cruciate ligament rupture.

The biological mechanism/s to explain this finding is not known. Furthermore, these data should be interpreted with some caution due to the low frequency of the rare TT genotype.

In conclusion, this *COL1A1* gene polymorphism is the first variant to be associated with risk of acute soft tissue rupture when data from three independent studies were combined. The clinical relevance of this finding is that the *COL1A1* TT genotype protects an athlete from acute soft tissue ruptures and should be included in future risk models for acute soft tissue ruptures.

CHAPTER 8

STUDY SIX

THE *Bst*UI RFLP WITHIN THE *COL5A1* GENE: FURTHER UNDERSTANDING

8.1 INTRODUCTION

The previous study (Study 5) of this thesis reported the combined results of three independent studies on the *COL1A1* Sp1 binding site polymorphism as a risk factor for acute musculoskeletal soft tissue ruptures. This combined analysis further strengthened the evidence that the *COL1A1* TT genotype appear to protect individuals from acute musculoskeletal soft tissue ruptures. Similarly to the *COL1A1* Sp1 binding site polymorphism, the *Bst*UI RFLP within the *COL5A1*, which was shown to be associated with ACL ruptures in female participants in Study 2 of this thesis, has also been associated with other musculoskeletal soft tissue injuries [143;145]. For this reason, performing a comparative analysis of the results presented in Study 2, and the results from the two previously published studies [143;145], may further strengthen our understanding of the association between the *Bst*UI RFLP and the risk of musculoskeletal soft tissue injuries.

Initial studies performed by this laboratory demonstrated that the *Bst*UI RFLP within the 3'-UTR of the *COL5A1* gene was associated with chronic Achilles tendinopathy in a South African (SA) Caucasian population [143;145]. The main finding from this initial investigation was that the CC genotype of this polymorphism was significantly

over-represented in an age-matched asymptomatic control group (28%) when compared to the group with Achilles tendinopathy (13%) (Figure 8.1). In a repeat of the initial study in an Australian (AUS) Caucasian population, the CC genotype of the *COL5A1 Bst*UI RFLP was also significantly over-represented in an age-matched asymptomatic control group (24%), when compared to the Achilles tendinopathy group (12%) [145]. The frequency of the CC genotype within the asymptomatic control and symptomatic Achilles tendinopathy groups were similar in both the SA and AUS studies (Figure 8.1).

Based on the findings of these two independent Achilles tendinopathy studies, the association of the *COL5A1 Bst*UI RFLP with ACL ruptures was investigated, and this was reported in Study 2 of this thesis. Due to the reported increased risk of ACL ruptures in females (Section 2.4.2), the male and females participants were analysed separately. In this, the third study investigating the *Bst*UI RFLP as a risk factor for musculoskeletal soft tissue injuries, it was reported that the CC genotype of this polymorphism was significantly over-represented in the female age-matched asymptomatic control participants (27%), when compared to the female participants with ACL ruptures (5%). The female control group had a similar CC genotype frequency to the combined male and female control participants of the previous two SA and AUS Achilles tendinopathy studies (Figure 8.1). It is however interesting to note that this association was not present when the male participants in the SA ACL study were analysed. The frequency of the CC genotype of the age-matched asymptomatic male control participants (16%) where distinctly different (Figure 8.1) to the frequency of the previously reported combined male and female asymptomatic

control participants in the Achilles tendon studies, as well as the females participants in the ACL study (24-27%).

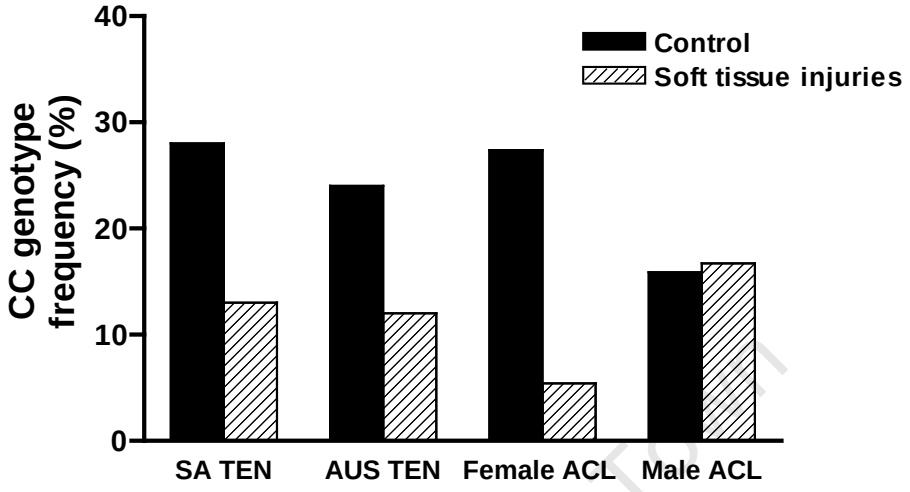

Figure 8.1: The CC genotype frequency within the South African chronic Achilles tendinopathy study (SA TEN) [143], the Australian chronic Achilles tendinopathy study (AUS TEN) [145], and the South African ACL study, presented in study two of this thesis, with females (Female ACL) and males (Male ACL) analysed separately. The soft tissue injury and control groups are shown as hatched and solid bars respectively. The SA TEN and AUS TEN studies consisted of both male and females participants.

Considered together, the results of these three studies highlight two important aspects which need further investigation. Firstly, based on the gender-specific association of the *COL5A1 Bst*UI RFLP in the ACL study, it may retrospectively be seen as a limitation that the male and female participants were not analysed separately in the SA and AUS Achilles tendinopathy studies. Secondly, although the asymptomatic control participants in these case-control studies were all matched for age of their respective symptomatic groups, the age of the male and female

participants within the ACL study was about 10 years younger than the combined male and female control participants in the two Achilles tendon studies (Figure 8.2).

Therefore, the first objective of this study was to determine whether there are any gender-specific *COL5A1* *Bst*UI RFLP genotype effects on chronic Achilles tendinopathy. The younger male control group within the ACL study had a lower CC genotype frequency, and therefore the second objective was to investigate whether the distribution of this *COL5A1* polymorphism within the combined asymptomatic control participants is age-dependent, particularly among the males.

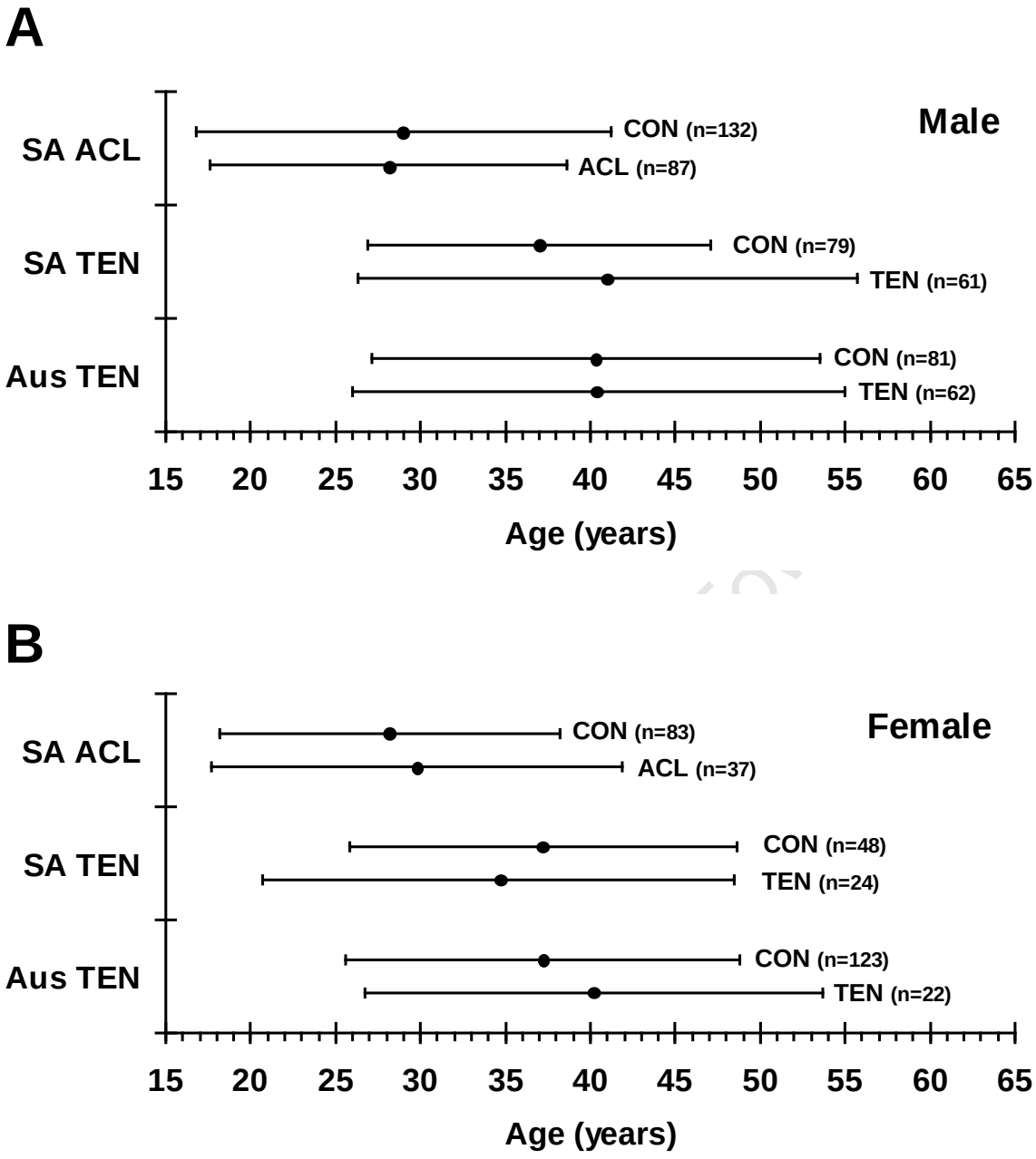

Figure 8.2: The mean (black dots) and standard deviation (horizontal error bars) of the age of the **(A)** male and **(B)** female CON (asymptomatic control participants), ACL (participants with ACL rupture) and TEN (participants with Achilles tendinopathy) groups within the South African ACL study (SA ACL), the South African Achilles tendinopathy study (SA TEN), and the Australian Achilles tendinopathy study (AUS TEN). The number of participants (n) in each group is shown in parentheses.

8.2 RESULTS

8.2.1 GENDER-SPECIFIC *COL5A1* BSTUI RFLP GENOTYPE EFFECTS IN CHRONIC ACHILLES TENDINOPATHY.

When re-analysed, there was no observed evidence that the *COL5A1* CC genotype was only associated with Achilles tendinopathy in females (Figure 8.3 B,C,E and F). Although the relative distribution of the *Bst*UI RFLP CC genotypes within the SA CON participants (CC vs. TT + TC, P=0.085)(Figure 8.3 B), as well as the AUS male CON participants (CC vs. TT + TC, P=0.055)(Figure 8.3 C) were not significantly over-represented when compared to the TEN groups, the genotype distributions of the male participants were similar to the genotype distributions of the female participants (Figure 8.3 E and F). Furthermore, the CC genotype was only significantly over-represented within the female CON participants of the SA TEN study (CC vs. TT + TC, P=0.013), and not within the female CON participants of the AUS TEN study (CC vs. TT + TC, P=0.291).

It is interesting to note that certain groups (refer to Figure 8.3) were not in Hardy-Weinberg equilibrium (HWE), and the relevance of this finding will be addressed in the following discussion.

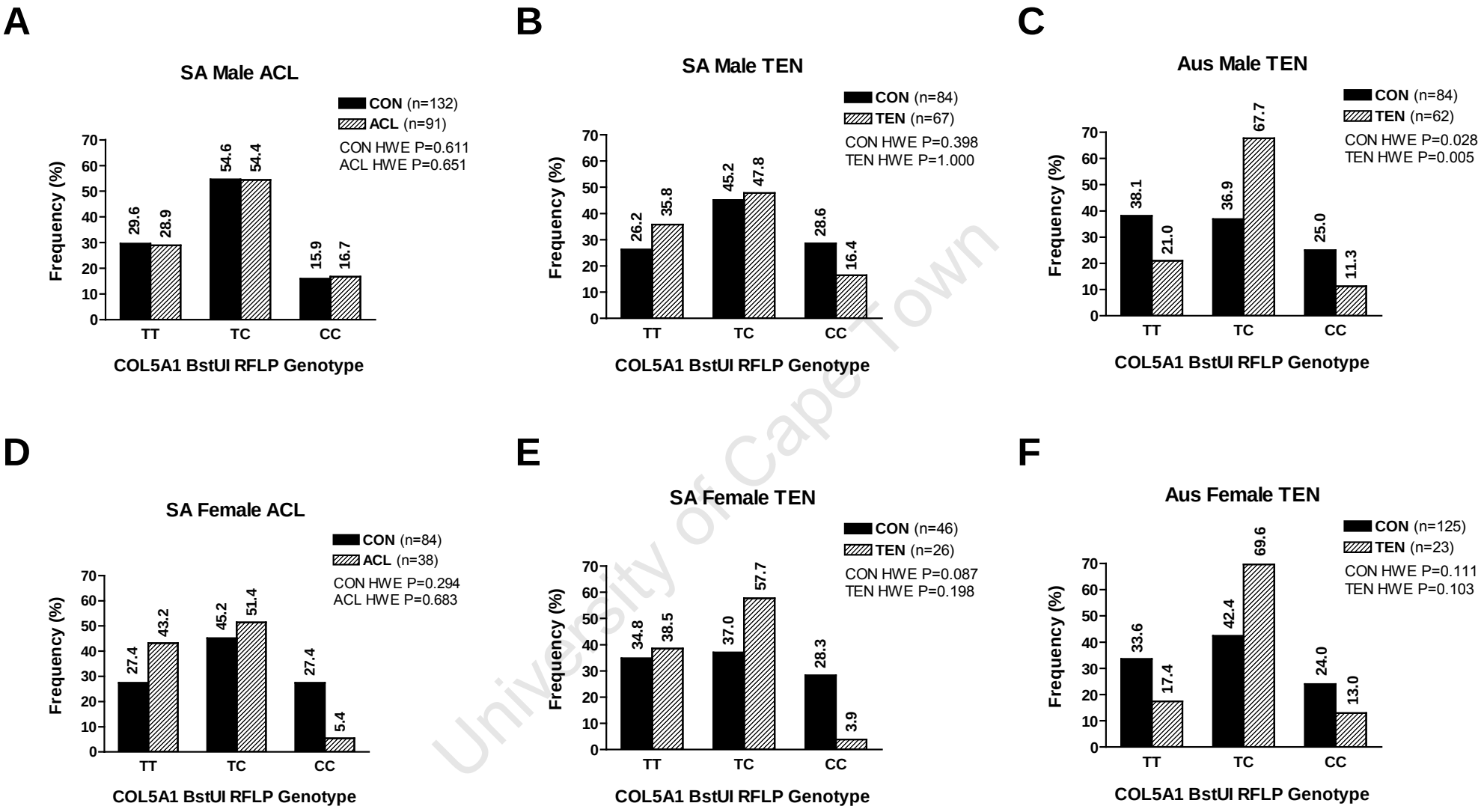

Figure 8.3: Legend on the following page.

(Legend from the previous page) The genotype frequencies of the *Bst*UI RFLP within the 3'-UTR of the *COL5A1* gene among, (**A**) South African (SA) male participants with ACL ruptures (ACL) and controls (CON) (**B**) South African (SA) male participants with Achilles tendinopathy (TEN) and controls (CON) (**C**) Australian (AUS) male participants with Achilles tendinopathy (TEN) and controls (CON) (**D**) South African (SA) female participants with ACL ruptures (ACL) and controls (CON) (**E**) South African (SA) female participants with Achilles tendinopathy (TEN) and controls (CON) (**F**) Australian (AUS) female participants with Achilles tendinopathy (TEN) and controls (CON). The number of participants (n) within each groups is shown in parentheses. The Hardy-Weinberg Equilibrium (HWE) P-values for each group is also shown.

8.2.2 AGE-DEPENDANT DISTRIBUTION OF THE *COL5A1 BST*UI RFLP GENOTYPE WITHIN ASYMPTOMATIC CONTROL PARTICIPANTS

To investigate whether the genotype distribution of the *COL5A1 Bst*UI RFLP within the asymptomatic control participants is age-dependent, all 550 (299 male, age range 18-77 years and 251 female, age range 18-72 years) participants from the three studies (SA ACL, 131 male and 83 female; SA TEN, 84 male and 46 female; and AUS TEN, 84 male and 123 female) were combined and divided into three male and three female age groups; namely (1) <25 years old (male 21.8 ± 1.4 years and female 22.1 ± 1.5 years), (2) 25 to 41 years old (male 31.5 ± 4.9 years and female 31.6 ± 4.7 years), and (3) >41 years old (male 51.7 ± 8.3 years and female 52.0 ± 6.8 years). The average age of onset of injury in the SA ACL study (29.8 ± 12.1 years, as reported in Study 2 and Study 3 of this thesis), the SA TEN study (39.4 ± 14.7 years) [143], and the AUS TEN study (40.8 ± 14.2 years) [145], as well as the final number of samples in each group was used to determine the age ranges of the three groups.

As shown in Figure 8.4A there was a similar CC genotype content in all three female age groups (23 to 26%). There was however a significant linear trend (P=0.047) for the CC genotype frequency amongst the male age groups, where the youngest group had the lowest CC frequency (17%), and the oldest group the highest CC frequency (29%) (Figure 8.4 B). It is interesting to note that the genotype distribution within certain age categories were not in Hardy-Weinberg equilibrium (HWE) (Figure 8.4 A and 8.4 B).

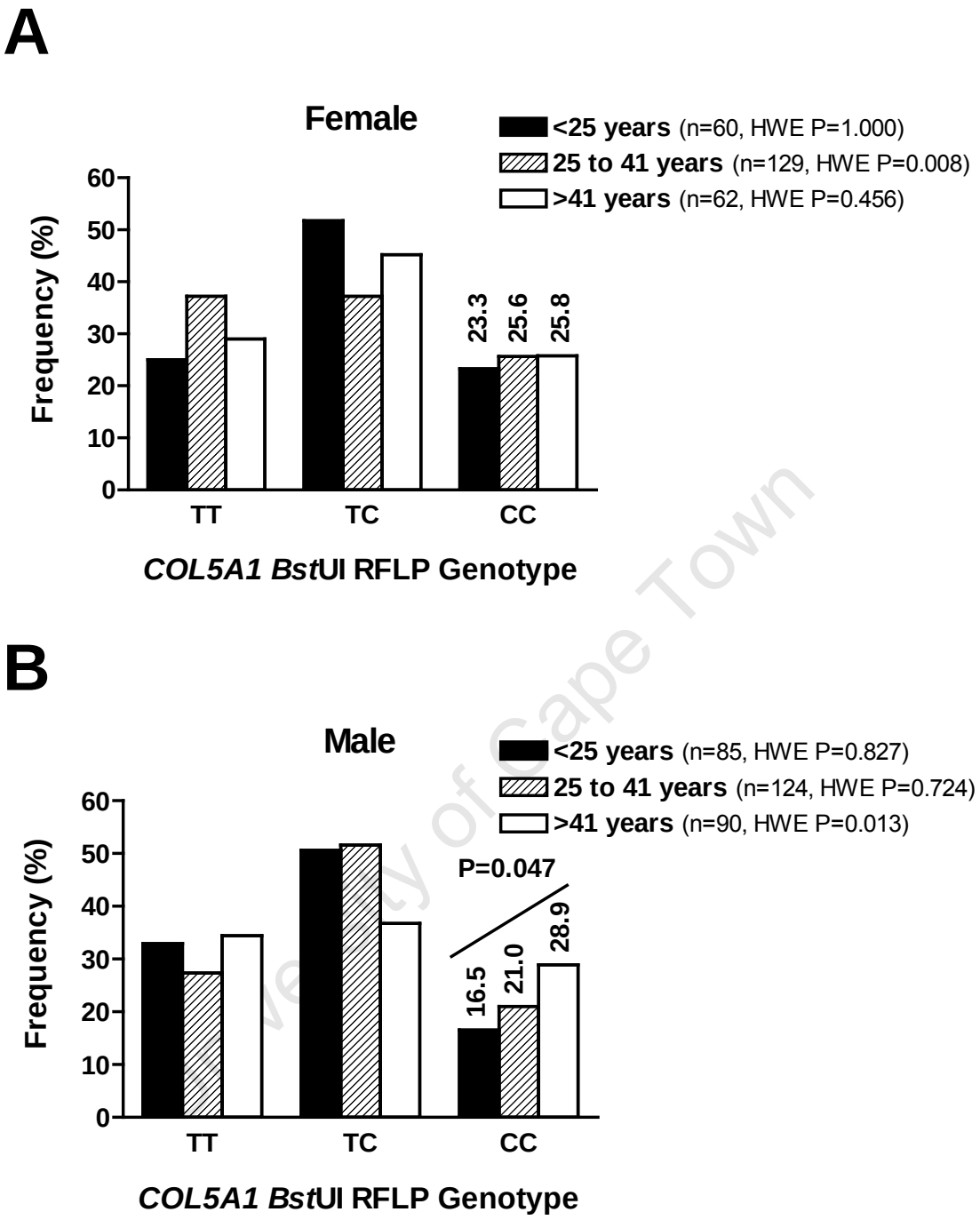

Figure 8.4: The genotype frequency of the *COL5A1 Bst*UI restriction fragment length polymorphism (RFLP) within all (**A**) female and (**B**) male asymptomatic control participants divided according to age, into participants less than 25 years old (black bars), between 25 and 41 years old (thatched bars), and greater than 41 years old (clear bars). A significant linear trend (P=0.047) for the CC genotype content amongst the male age groups was found. The number of participants (n) within each category, as well as the Hardy-Weinberg Equilibrium (HWE) P-values are shown in parentheses.

8.3 DISCUSSION

The main finding of this study was that there is no observed evidence that the associations of the *COL5A1 Bst*UI RFLP with Achilles tendinopathy in the two previously published studies were gender-specific. An additional finding was that there is an age-dependant distribution of the *COL5A1 Bst*UI RFLP CC genotype within the pooled asymptomatic male control participants of the three studies which investigated this polymorphism as a possible risk factor for soft tissue injury. These two findings provide additional information which may assist in understanding the observations presented in Study 2 of this thesis, and the two previously published studies [143;145].

A possible explanation for the age-dependant increase in the frequency of the CC genotype of the *COL5A1 Bst*UI RFLP within the pooled asymptomatic male control participants is illustrated in Figure 8.5. The youngest group of control participants will most likely consists of a mixture of individuals who are at a low and high risk for ACL ruptures and/or Achilles tendinopathy, due to the fact that they haven't been exposed to a sufficient number of extrinsic risk factors and/or an inciting event (as described in Section 2.4 of the literature review). However, with increasing chronological age, and thus an increased likelihood of sufficient exposure to extrinsic factors and an inciting event, more individuals with an inherent genetic predisposition in this asymptomatic group will become injured, and less subjects will remain asymptomatic. This could explain the observed increased frequency of participants at low risk of ACL and Achilles tendinopathy within the older groups. It is evident

from this explanation that a well selected asymptomatic control group is also highly selective, especially with increasing age, similar to the symptomatic injury group.

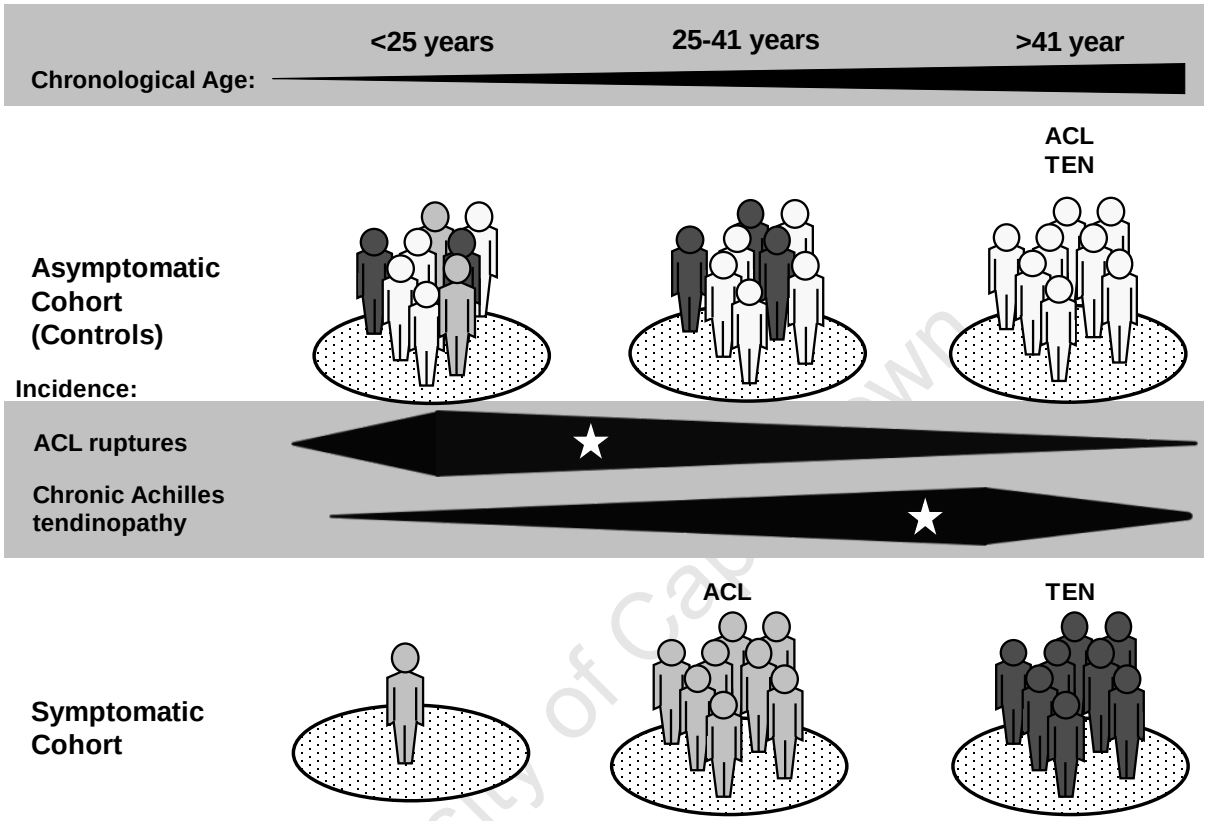

Figure 8.5: A proposed explanation for the significant linear trend in CC genotype frequency among the asymptomatic male participants when divided into the 3 age groups (<25 year, 25 – 41 year, and >41 years). It is proposed that asymptomatic participants in the age category, <25 years, will most likely include participants at high risk of ACL rupture (grey individuals), those at high risk of Achilles tendinopathy (black individuals), and those with low risk of ACL ruptures and/or Achilles tendinopathy (light individuals). Increasing chronological age is graphically represented in the top grey box. The age-specific incidence (age at which individuals are most likely to incur an injury) of ACL ruptures and Achilles tendinopathy [28] are graphically represented within the bottom grey box, and the mean age of onset (mean age of all injuries) of ACL and TEN injuries are shown with a white start. The age-specific incidence and the mean age of onset are indications of the age at which individuals are exposed to sufficient extrinsic factors and/or an inciting event to develop the injury. Individuals are more accurately selected for either the asymptomatic cohort or symptomatic cohort once they have been exposed to sufficient extrinsic factors.

The selection which occurs when recruiting symptomatic injury and asymptomatic control participants may be observed as a departure from Hardy-Weinberg equilibrium (HWE), which, amongst other possible explanations, can also be an indication of a natural selection for a particular genotype [158]. Due to the selective nature of the symptomatic and asymptomatic injury groups, especially the older asymptomatic control groups, not all groups from the previous studies were in Hardy Weinberg equilibrium (HWE) (refer to Figure 8.3). However, when all the asymptomatic and symptomatic participants were combined and analysed, the genotype distributions of all participants in the study were in HWE (SA ACL study p=0.911, SA TEN study p=0.276, and AUS TEN study p=0.724) [145;172].

Furthermore, it is interesting to note that the frequency of the CC genotype in the young female asymptomatic control participants with ACL ruptures, were similar to the older AUS TEN and SA TEN studies. This could be explained by the fact that it has been widely reported, as previously discussed (Section 2.4.2), that females are at greater risk of ACL rupture compared to their male counterparts. Therefore it remains possible that, among already predisposed female participants, the asymptomatic participants will become more selected at an earlier age, when compared to male asymptomatic participants.

A limitation of this study was that it was not possible to analyse the SA and AUS data separately due to small sample sizes and uneven genotype distribution. Forty-three percent (n=39 of 90) of the oldest male age group for example were from the AUS study, while 87% (n=74 of 85) of the youngest male age group were from the

SA studies. Another limitation of this study was that although all the control participants were asymptomatic for ACL and/or Achilles tendon injuries, as discussed in chapter 4, and in the previously published original articles [143;145], not all of the participants were free of self-reported tendon and ligament injuries.

In conclusion, although the CC genotype of the *COL5A1 Bst*UI RFLP was significantly under-represented in females, but not males with ACL ruptures (refer to Study 2), there appears to be no gender-specific under-representation of this genotype within chronic tendinopathy. Consequently, there is an age-dependant increase in the CC genotype of this sequence variant within a pooled group of asymptomatic controls.

The practical implication of this study is that the selection of control groups is of critical importance when future studies of this nature are designed. Future research investigating this genetic variant as a risk factor for soft tissue injuries should consider the findings of this study when selecting a control group.

CHAPTER 9

SUMMARY AND CONCLUSIONS

The exact aetiology of and risk factors for ruptures of the anterior cruciate ligament (ACL) in the knee are not yet fully understood. This is despite a large volume of published research in this area. However, in reviewing the existing literature (Chapter 2), it is clear that ACL rupture is a multifactorial condition, for which various extrinsic and intrinsic risk factors have been identified [6]. Recently, among the intrinsic risk factors, certain genetic elements have been suggested to predispose individuals to ACL ruptures. To date, only two studies have investigated a possible familial predisposition for ACL ruptures [7;10], while only a single study has investigated the association of a specific sequence variant, namely the functional *COL1A1* Sp1 binding site polymorphism with acute ligament injuries [9]. Of further interest, genetic sequence variants within the *COL5A1* [143;145], *TNC* [144] and *MMP-3* [147] genes have on the other hand been shown to be associated with another soft tissue injury, Achilles tendinopathy. Previous research from this laboratory has also suggested that variants within the *COL12A1* gene might specifically be associated with Achilles tendon ruptures [148]. These observations suggest that soft tissue injuries such as Achilles tendon injuries and ACL ruptures might be polygenic in nature. Therefore, an approach to 1) identify possible candidate genes that are associated with these soft tissue injuries, and 2) conduct studies to determine a possible association between the candidate genes and these injuries, is logical. Data from these studies will increase the understanding of possible intrinsic risk factors for these injuries and may ultimately influence prevention, treatment and rehabilitation strategies for these injuries.

The *COL1A1, COL5A1* and *COL12A1* genes encode for structural collagenous components of the microfibril, the basic building block of ligaments. Since variants within these three genes have been shown or suggested to be associated with other soft tissue injuries, they were selected as candidate genes for the genetic association studies that were presented in this thesis. In this concluding chapter of the thesis, the aims will be-restated, and then a summary of the results from the studies that were conducted to address the aims will be presented.

1. **Primary aim: To identify whether the selected candidate genes (*COL1A1, COL5A1* and *COL12A1*) predispose individuals to ACL ruptures.**

As previously mentioned, the rare TT genotype of the functional *COL1A1* Sp1 binding site polymorphism was previously reported to be under-represented in Swedish participants with acute ligament injuries (cruciate ligament ruptures and shoulder dislocations) [9]. In support of this finding, this polymorphism was shown, in this thesis, to be significantly under-represented (OR=12.3; CI 0.7 - 220.4; P=0.031) in participants with ACL ruptures, when compared to controls with no history of ligament or tendon injuries, in a South African Caucasian population. In addition, there was no evidence of a gender-specific effect of this variant on ACL ruptures.

The novel findings of this thesis were that the *COL5A1 Bst*UI RFLP and the *COL12A1 Alu*I RFLP were significantly associated with ACL ruptures in female, but not male participants. The CC genotype of the *COL5A1 Bst*UI RFLP (OR=6.6, 95%

CI 1.5 – 29.7; P=0.006) and the AA genotype of the *COL12A1 Alu*I RFLP (OR=2.4, 95% CI 1.0 – 5.5; P=0.048) were significantly under- and over-represented, respectively, in the female ACL group, when compared to the female CON group. No significant genotype distributions between the CON and ACL groups were however observed for the *COL5A1 Dpn*II and *COL12A1 Bsr*I RFLPs.

These findings only provide initial evidence that sequence variants within genes which encode for structural collagenous components of the ligament microfibril are significant risk factors for ACL ruptures, especially among females. The possible reasons for the gender-specific associations of the *COL5A1 Bst*UI and *COL12A1 Alu*I RFLPs are not known. It is however not a unique observation that certain intrinsic risk factors for ACL ruptures are only associated with females. It has been documented that other intrinsic risk factors, such as BMI [55] and anterior knee laxity [55] are also risk factors which have only been found to be associated with ACL ruptures among female, but not the male participants. In addition, certain proposed anatomical risk factors such a ACL geometry, generalised joint laxity and lower extremity alignment have been shown to be significantly different between males and females. More specifically, females have a smaller ACL when normalised for bodyweight [71], increased generalised joint laxity [87] and an increased Q angle [59;60].

Besides the specific intrinsic risk factors which predispose females to ACL ruptures, the type of playing surface, an extrinsic risk factor, has also been shown to be associated with female, but not male participants [54]. It was proposed that greater traction may interact with the more prevalent intrinsic risk factors in women, when

compared to men, and thereby only increase the risk of ACL ruptures in females [54]. Similarly to this proposed mechanism, the gender-specific genetic associations in this thesis may also be a result of the interaction between the *COL5A1 Bst*UI and *COL12A1 Alu*I RFLPs and the additional intrinsic risk factors within females. Based on the gender-specific associations found in this thesis, it is proposed that separate models are developed to understand the causation of ACL ruptures in males and females (Figure 9.1).

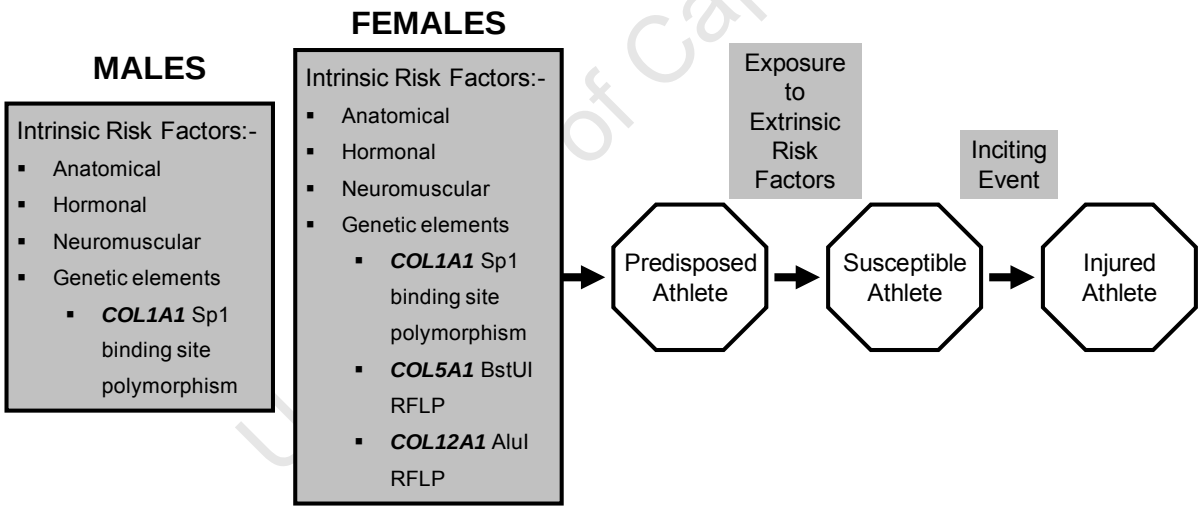

Figure 9.1: A schematic diagram, adapted from the original model proposed my Meeuwisse [39], illustrating the complex relationship between intrinsic risk factors, extrinsic risk factors and a specific inciting event in the causation of ACL ruptures. Several intrinsic risk factors, broadly classified as either anatomical, hormonal, neuromuscular or genetic elements, have been identified. The findings from this thesis are incorporated into the model. Different genetic sequence variants (the *COL1A1* Sp1 binding site polymorphism, the *COL5A1 Bst*UI RFLP and the *COL12A1 Alu*I RFLP) have been identified for males and females, and it is therefore proposed that separate injury causation models are produced.

2. Secondary aim: To further investigate the similarities and differences between the genetic risk factors for ACL ruptures and other soft tissue injuries.

Due to the similar structure of tendons and ligaments, it has been proposed that injuries to these tissues might share common genetic risk factors [163]. On the other hand, tendons and ligaments are functionally different, which implies that there might also be specific genetic sequence variants only associated with a specific injury [173]. The primary findings that the *COL5A1 Bst*UI *and COL12A1 Alu*I RFLPs are associated with ACL ruptures in females provide initial evidence that there are, in part, some potential common underlying genetic predispositions to ACL ruptures and Achilles tendinopathy and/or Achilles tendon ruptures. Furthermore, among the candidate genes investigated in this thesis, only the *COL1A1* variant has not been investigated as a candidate gene for other chronic and acute musculoskeletal soft tissue injuries (Achilles tendinopathy and/or Achilles tendon ruptures).

COL1A1 Sp1 binding site polymorphism

Although not statistically significant the rare TT genotype of the *COL1A1* Sp1 binding site polymorphism was absent in participants with another acute soft tissue injury (spontaneous Achilles tendon ruptures). The TT genotype was however present in participants with chronic Achilles tendinopathy. When the data for the *COL1A1* Sp1 binding site polymorphism from this thesis, and previously published data, were combined and analysed, the TT genotype was shown to be associated with an 11.1

times reduced risk of acute musculoskeletal soft tissue ruptures (cruciate ligament ruptures, shoulder dislocations, and Achilles tendon ruptures). Furthermore, when only the risk of cruciate ligaments were analysed, the TT genotype was associated with a 15.1 times reduced risk of cruciate ligament ruptures.

The T allele of the functional Sp1 binding site polymorphism has been shown to be associated with an increased binding affinity for the transcription factor Sp1, which was accompanied by an increase in *COL1A1* mRNA, and an altered production of the α1(I) chain relative to the α2(I) chain [155]. The relative increase in the α1(I) chain production is believed to result in a homotrimeric type I collagen molecule, consisting of 3 α1(I) chains, which has been proposed as a possible explanation for reduced bone quality and strength, that is seen in osteoporosis [155]. From the findings presented in this thesis, and the previous Swedish study [9], the *COL1A1* Sp1 binding site polymorphism does however seem to have the opposite effect within soft tissues. The previously proposed increased *COL1A1* mRNA which results from a TT genotype [155], may increase the tensile strength of ligaments, thereby reducing the risk of ACL rupture.

*COL5A1 Bst*UI *RFLP*

Furthermore, there was no evidence that the previously reported association of the *COL5A1 Bst*UI RFLP with Achilles tendinopathy was gender-specific. There was however a significant age dependant increase in the CC genotype distribution (P=0.047) among the pooled male asymptomatic CON participants studied in this thesis, and previously published papers [143;145]. A similar trend among the female participants was not observed. It was proposed that the younger male control

participants will most likely consist of a mixture of individuals who are at low and high risk of ACL ruptures and/or Achilles tendinopathy, due to the fact that they have not been exposed to a sufficient number of extrinsic risk factors and/or an inciting event. However, with increasing chronological age, and thus an increased likelihood of sufficient exposure to extrinsic factors and an inciting event, more individuals will become injured, and less individuals will remain asymptomatic. This comparative analysis highlights the importance of control groups recruitment and selection.

As proposed in this thesis, it is important to mention that unlike the functional *COL1A1* Sp1 binding site polymorphism, the exact functions of the *COL5A1 Bst*UI and *COL12A1 Alu*I RFLPs are unknown. Although a priori hypothesis was used in selection of the candidate genes, the function of the variants used within these genes were not necessarily known. The nature of genetic association studies do not exclude the fact that the associated sequence variant is in linkage disequilibrium with the functional polymorphism within the same gene or a neighbouring gene. This possibility can however not be excluded. The *Bst*UI RFLP within the 3'-UTR of the *COL5A1* gene and the *Alu*I RFLP within exon 65 of the *COL12A1* do however have interesting features which warrant further discussion.

The 3'-UTR of a gene has been described as a zone rich in translational control mechanisms [174]. Regions within the 3'-UTR have been proposed to regulate (1) mRNA stability, (2) subcellular localisation of transcripts, (3) termination of transcription, and (4) the stabilisation of specific transcripts [174]. Moreover, miRNA recognition sequences [145], expressed sequence tags (ESTs) [145] and different polyadenylation signals (N, Laguette, personal communication) have been identified

within the 3'-UTR of the *COL5A1* gene. Further studies, using molecular and cell biological techniques are required to establish whether any of these features contribute to altered transcription and/or translation of the *COL5A1* gene.

*COL12A1 Alu*I *RFLP*

The *COL12A1 Alu*I *RFLP* was not investigated as an additional combined analysis, it is however important to note that the findings of this thesis were consistent to the previous observations in Achilles tendon ruptures [148]. Although the GG genotype of the *COL12A1 Alu*I RFLP which was absent in participants with Achilles tendon ruptures, was present in ACL participants, the opposite AA genotype was over-represented in participants with ACL ruptures. Furthermore, the *Alu*I RFLP is a non-synonomous polymorphism, which changes the amino acid at position 3058 from a serine to a glycine. Although it is interesting to note that glycine is a smaller amino acid than serine, the exact function of this change in amino acid sequence is not known. Further research is therefore required to establish the function of this polymorphism, and more specifically, the function of this amino acid substitution on the protein.

*

In the process of answering the primary and secondary aims of this thesis, other noteworthy observations were made. In agreement with the previous findings of a familial predisposition to ACL ruptures [7;10], the participants in the ACL group had a significantly higher family history of ligament injury (13.5% vs. 39.6%, P<0.001), when compared to the CON participants in Study 1. When the CON group of Study 1 was separated by gender, both the male and female participants of the ACL group

had a significantly greater family history of ligament injuries. Similar findings were observed when the female ACL group was compared to the female CON group included in Studies 2 and 3 (21.5% vs. 50.0%, P=0.002). It is interesting to note that no significant difference was found when the male ACL group was compared to the male CON group included in Studies 2 and 3. The reasons for these differences are not apparently obvious. It is however important to note that there were differences between the CON groups of Study 1, 2 and 3. The CON group in Study 1 had no previous history of any ligament and/or tendon history, whereas the CON participants in Studies 2 and 3 had no previous history of only ACL ruptures.

These data further suggest that the genetic risk of ACL ruptures may be pronounced in females. As mentioned, only two studies have previously reported a familial predisposition to ACL ruptures, and therefore the data presented in this thesis contribute to the body of evidence suggesting a genetic predisposition as a risk factor for ACL ruptures (Table 9.1). As discussed in section 2.4.2.4.1, the level of certainty from the two previous studies, that familial predisposition is risk factor for ACL ruptures is moderate. However, the previous studies which investigated a familial predisposition did not analyse the male and female participants separately. When data from previous studies, and the data from the current thesis were combined, the level of certainty that familial predisposition is a risk factor for ACL ruptures is moderate among females, and low among males, due to the conflicting results (Table 9.1).

Table 9.1: Summary of research studies, from previous research and the results of this thesis, investigating genetic risk factors for ACL ruptures, including the level of evidence of each individual study and the level of certainty that the risk factor is associated with risk of ACL ruptures.

Risk Factor	Study Details and References	Number of ACL ruptures	Level of evidence (I-IV)[a]	Level of Certainty[b]
Familial predisposition	**Positive Associations**			**Moderate** (in females)
	Case-control studies:			
	ACL patients [10]	31[c]	III	**Low** (in males)
	ACL patients [104]	171	III	
	ACL patients			
	- Study 1 of this thesis	117	III	
	- Study 2&3 of this thesis – females only	38	III	
	No Associations			
	Case-control studies:			
	ACL patients			
	- Study 2&3 of this thesis - males only	91	III	
***COL1A1* Sp1 binding site polymorphism**	**Positive Associations**			**Moderate**
	Case-control studies:			
	ACL patients [9]	233[d]	III	
	ACL patients			
	- Study 1 of this thesis	117	III	
***COL5A1 Bst*UI RFLP**	**Positive Associations**			**Low**
	Case-control studies:			
	ACL patients			
	- Study 2 of this thesis – only females	129	III	
***COL12A1 Alu*I RFLP**	**Positive Associations**			**Low**
	Case-control studies:			
	ACL patients			
	- Study 3 of this thesis – only females	129	III	

The findings from this thesis are underlined. [a] The level of evidence according to evidence-based medicine criteria [42]. [b] The level of certainty, as described in section 2.4. [c] All bilateral ACL ruptures. [d] All cruciate ligament injuries, ACL and PCL.

Additionally, it was observed that when all the ACL and CON participants were combined and analysed, the genotype distribution of the *COL5A1 Bst*UI RFLP was significantly different between participants with a family history of ligament injury, and participants without a family history of ligament injury (P=0.022). This finding remained significant when only female participants were analysed (P=0.005), but not when male participants were analysed (P=0.369). A similar finding was also observed for the *COL12A1 Alu*I RFLP and family history of ligament injuries, where there was a trend (P=0.082) for the AA genotype of the *COL12A1 Alu*I RFLP to be over-represented in female participants with a family history of ligament injury, when compared to female participants without a family history of ligament injury. These findings provide further support that the *Bst*UI and the *COL12A1 Alu*I RFLPs are associated with an increased risk of ACL ruptures in females.

It is important to note that there are strengths and limitations to the study design presented in this thesis. A strength of this thesis is that the primary studies investigated a single well defined injury, namely ACL ruptures. All ACL ruptures were confirmed at the time of surgery. Moreover, all Achilles tendon injuries (Achilles tendinopathy and/or Achilles tendon ruptures) were either clinically diagnosed or diagnosed at the time of surgery. Furthermore, attempts were made to match the ACL, TEN and RUP groups to their respective CON groups, however, it might be seen as a limitation that there were certain differences. In particular, there were some differences in sports participation between the ACL groups and their respective CON groups. Another limitation of this thesis was the relatively small sample size of the female participants with ACL ruptures and the small sample size

of participants with Achilles tendon ruptures. The primary aim of the thesis was not to investigate gender-specific genetic risk factors and therefore future studies should recruit a larger group of either males and/or females and determine sample sizes for each group separately. Furthermore, there are also differences between chronic Achilles tendinopathy and acute Achilles tendon ruptures, future studies should also investigate these injuries separately.

It is important to emphasise that the findings of this thesis, particularly for the novel associations of the *COL5A1 Bst*UI and *COL12A1 Alu*I RFLPs, be confirmed in future studies within independent populations. The field would be advanced if international consortia collaborated to use high throughput genome wide association or sequencing technologies on large cohorts. Proposed molecular mechanisms from these association studies should be tested using various cell and molecular biology techniques. These experiments would assist to eventually determine a cause and effect relationships between polymorphisms and specific soft tissue injuries. Eventually, prospective cohort studies should be designed to confirm the clinical significance and relevance of these associations. Nevertheless, this thesis does provide very useful initial evidence that genetic elements within genes which encode for structural components of the ligament microfibril are significant risk factors for ACL ruptures, in particular among females. As reviewed (Section 2.4.2.4), prior to this thesis only a few studies have investigated genetic factors as risk factors for ACL ruptures (Refer to Table 2.5). The additional contribution this thesis makes to the body of research investigating genetic risk factors to ACL ruptures is significant (Table 9.1). In addition, this thesis has implicated specific genes which

require further investigation to assist our understanding of the aetiology of ACL ruptures.

Furthermore, the data presented in this thesis has potential significant clinical implications. Identifying individuals at an increased risk of ACL rupture may have significant application in the reduction of these severe injuries. It is recommended that the findings of this thesis be incorporated into multifactorial models developed to reduce the incidence of ACL ruptures among predisposed individuals.

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APPENDIX 1

ADDITIONAL INFORMATION

Table A1.1: The frequencies of the most common self reported form of sports participation in the female and male anterior cruciate ligament rupture (ACL) group.

Female participants (n=35)		Male participants (n=82)	
Sports[a]	Frequency (%)	Sports[b]	Frequency (%)
Field hockey	60.0	Rugby	75.6
Tennis	42.5	Cricket	36.6
Netball	42.9	Squash	29.3
Squash	25.7	Soccer	24.4
Swimming	22.9	Tennis	22.0
Athletics	17.1	Field hockey	15.9
Road running	14.3	Athletics	14.6
		Road running	12.2
		Swimming	12.2
		Golf	11.0

[a] Only sports which had been reportedly played by greater than 10% of all female participants were included in the table. Other minor sports not listed included aerobics, badmington, ballet, cross country, dancing, equestrian, golf, gymnastics, hiking, modern dancing, motorcross, paragliding, rugby, snow skiing, surfing, touch rugby, waterpolo and waterskiing.

[b] Only sports which had been reportedly played by greater than 10% of all male participants were included in the table. Other minor sports not listed include action cricket, badminton, baseball, basketball, BMX, canoeing, cross country, cycling, equestrian, Gaelic football, hurling, kite boarding, martial arts, mountain biking, motocross, paragliding, rowing, sailing, skateboarding, snow skiing, surfing, touch rugby, triathlon, volleyball, wakeboarding, waterpolo, water skiing and yachting.

Table A1.2: General characteristics of the South African born participants in the respective control (CON) and anterior cruciate ligament rupture (ACL) groups, as well as the direct contact (DIR), indirect contact (IND) and non-contact (NON) ACL rupture sub-groups.

	CON	ACL	P-value [b]	DIR	IND	NON	P-value [c]
Age (years) [a]	36.1 ± 9.2 (86) [d,e]	29.2 ± 11.7 (90)	**<0.001**	25.1 ± 12.2 (13) [d,f]	32.7 ± 12.8 (15) [f]	27.8 ± 11.4 (42) [e]	**<0.001**
Height (cm)	177.6 ± 10.4 (90)	176.7 ± 9.8 (88)	0.580	175.4 ± 8.2 (13)	179.4 ±9.0 (14)	1178.0 ± 9.2 (41)	0.781
Weight (kg) [a]	76.8 ± 14.2 (91)	79.5 ± 16.2 (88)	0.244	80.5 ± 12.5 (13)	87.7 ± 15.7 (14)	80.6 ± 16.4 (41)	0.080
BMI (kg/cm^2) [a]	24.2 ± 3.5 (90)	25.1 ± 3.2 (85)	0.078	26.1 ± 2.7 (13)	25.5 ± 2.7 (13)	25.2 ± 3.5 (40)	0.203
Gender (% males)	72.5 (91)	71.4 (91)	0.869	92.3 (13)	86.7 (15)	72.5 (42)	n.d.

Gender and country of birth are represented as a frequency (%). The remaining variables are expressed as mean ± standard deviation. The number of subjects (n) for each variable is in parentheses.

[a] Age, weight and BMI are self-reported values at the time of the first ACL rupture for the ACL group, as well as the DIR, IND and NON sub-groups, and at recruitment for the CON group. For the ACL group the age, weight and BMI at recruitment were 5.0 ± 8.7 years (n=90), 1.1 ± 4.5 kg (n=88) and 0.4 ± 1.5 kg/cm^2 (n=85) greater than at the time of the first ACL rupture.

[b] CON vs ACL; [c] CON vs DIR vs IND vs NON

Pairwise, post hoc significant differences: [d] CON vs DIR ($p<0.001$); [e] CON vs NON ($p<0.001$); [f] DIR vs IND ($p=0.032$)

n.d. = not determined due to small sample sizes

Table A1.3: Relative genotype and allele frequencies of the *COL1A1* Sp1 binding site polymorphism within the South African born control (CON) and anterior cruciate ligament rupture (ACL) groups, as well as the direct contact (DIR), indirect contact (IND) and non-contact (NON) ACL rupture sub-groups .

	CON (n=91)	**ACL** (n=91)	**DIR**[b] (n=13)	**IND**[b] (n=15)	**NON** (n=42)
GG genotype (%)	71.4 (65)	65.9 (60)	76.9 (10)	73.3 (11)	69.1 (29)
GT genotype (%)	24.2 (22)	34.1 (31)	23.1 (3)	26.7 (4)	31.0 (13)
TT genotype (%) [a]	4.4 (4)	0 (0)	0 (0)	0 (0)	0 (0)
G allele (%)	83.5 (152)	83.0 (151)	88.5 (23)	86.7 (26)	84.5 (71)
T allele (%)	16.5 (30)	17.0 (31)	11.5 (3)	13.3 (4)	15.5 (13)

The values are expressed as a percentage with the number of subjects (n) in parentheses.

[a] Due to the absence of participants with a TT genotype in the ACL group and three sub-groups, the GT and TT participants were combined and compared to the GG participants. CON vs ACL genotypes, p=0.523. CON vs NON genotypes, p=0.839. CON vs ACL alleles, p=1.000. CON vs NON alleles, p=1.000

[b] due the small sample size, the DIR and IND groups were not further analysed.

Table A1.4: Characteristics of all participants (combined male and female) within the asymptomatic control (CON) group, the anterior cruciate ligament rupture (ACL) group and the ACL sub-group with a non-contact (NON) mechanism of injury.

	CON	ACL	P-Value [b]	NON	P-value [c]
Age [a] (years)	28.7 ± 11.4 (215)	28.6 ± 11.0 (124)	0.958	27.9 ± 10.8 (54)	0.641
Height (cm)	175.1 ± 9.2 (210)	176.7 ± 9.5 (113)	0.145	177.0 ± 9.2 (52)	0.195
Weight [a] (kg)	74.2 ± 15.5 (212)[d]	79.8 ± 17.1 (114)	**0.003**	79.7 ± 17.2 (53)	**0.026**
BMI [a] (kg/m^2)	24.1 ± 3.7 (208)	25.3 ± 3.8 (109)	**0.007**	25.2 ± 4.3 (50)	0.054
Gender (% male)	61.1 (216)	70.5 (129)	0.076	66.7 (54)	0.451
Country of birth (% South Africa)	86.5 (207)	86.3 (117)	0.970	85.2 (54)	0.826

Gender and country of birth are represented as a frequency (%). The remaining variables are expressed as a mean ± standard deviation. The number of subjects (n) for each variable is in parentheses.

[a] Age, weight and body mass index (BMI) are self-reported values at the time of the first ACL rupture for the ACL group, as well as the NON sub-group, and at recruitment for the control group. For the ACL group, age, weight and BMI at recruitment were 5.0 ± 8.5 years (n=120), 1.3 ± 4.6 kg (n=110) and 0.5 ± 1.5 kg/m^2 (n=106), greater than at the time of the first ACL rupture.

[b] CON vs ACL. [c] CON vs NON.

Table A1.5: Relative genotype and allele frequencies of the *Bst*UI and *Dpn*II restriction fragment length polymorphisms (RFLPs) within the 3'-UTR of the *COL5A1* gene in all (males and females combined) control group, anterior cruciate ligament rupture (ACL) group and the non-contact mechanism of ACL rupture sub-group.

	CON (n=215)	**ACL** (n=129)	**P-Value**[a]	**NON** (n=54)	**P-Value**[b]
***Bst*UI RFLP**					
TT genotype (%)	28.8 (62)	33.1 (42)		35.2 (19)	
TC genotype (%)	51.2 (110)	53.5 (68)		50.0 (27)	
CC genotype (%)	20.0 (43)	13.4 (17)	0.280	14.8 (8)	0.549
T allele (%)	(234)	(152)		(65)	
C allele (%)	(196)	(102)	0.199	(43)	0.332
***Dpn*II RFLP**					
TT genotype (%)	52.8 (113)	51.2 (66)		55.6 (30)	
TC genotype (%)	42.1 (90)	44.2 (57)		40.7 (22)	
CC genotype (%)	5.1 (11)	4.7 (6)	0.921	3.7 (2)	0.717[c]
T allele (%)	(316)	(189)		(82)	
C allele (%)	(112)	(69)	0.939	(26)	0.748

The values are expressed as a percentage with the number of subjects (n) in parentheses.

[a] P values for CON vs ACL genotype and allele frequencies.

[b] P values for CON vs NON genotype and allele frequencies.

[c] Due to the small sample size of the *Dpn*II RFLP CC genotype, the CC and TC participants were combined and compared to the TT participants for the CON vs NON analysis.

Table A1.6: Relative genotype and allele frequencies of the *Bst*UI restriction fragment length polymorphisms (RFLPs) within the 3'-UTR of the *COL5A1* gene in the male and female participants of the control (CON) group, anterior cruciate ligament rupture (ACL) group and the non-contact mechanism of ACL rupture sub-group after all participants with a history of Achilles tendon injuries were excluded.

	CON (n=215)	**ACL** (n=129)	**P-Value**[a]	**NON** (n=54)	**P-Value**[b]
Male participants					
TT genotype (%)	27.0 (30)	29.6 (21)		28.6 (8)	
TC genotype (%)	55.9 (62)	52.1 (37)		50.0 (14)	
CC genotype (%)	17.1 (19)	18.3 (13)	0.884	21.4 (6)	0.822
T allele (%)	55.0 (122)	55.6 (79)		53.6 (30)	
C allele (%)	45.0 (100)	44.4 (63)	0.914	46.4 (26)	0.881
Female participants					
TT genotype (%)	29.0 (22)	45.5 (15)		50.0 (22)	
TC genotype (%)	46.1 (35)	48.5 (16)		43.8 (7)	
CC genotype (%)	25.0 (19)	6.1 (2)	**0.021**[c]	6.3 (1)	0.098[d]
T allele (%)	51.2 (79)	69.7 (46)		85.2 (51)	
C allele (%)	48.8 (73)	30.3 (20)	**0.017**	14.8 (9)	**<0.001**

The values are expressed as a percentage with the number of subjects (n) in parentheses.

[a] P values for CON vs ACL genotype and allele frequencies.

[b] P values for CON vs NON genotype and allele frequencies.

[c] The CC genotype is significantly under-represented in the female ACL group (CC genotype vs GG + GT genotypes).

[d] CC genotype vs GG +GT.

Table A1.7: Relative genotype and allele frequencies of the *Alu*I and *Bsr*I restriction fragment length polymorphisms (RFLPs) within the *COL12A1* gene in all (males and females combined) control group, anterior cruciate ligament rupture (ACL) group and the non-contact mechanism of ACL rupture sub-group.

	CON (n=215)	**ACL** (n=129)	**P-Value**[a]	**NON** (n=54)	**P-Value**[b]
***Alu*I RFLP**					
AA genotype (%)	56.5 (121)	66.9 (85)		67.3 (35)	
GA genotype (%)	40.2 (86)	29.9 (38)		26.9 (14)	
GG genotype (%)	3.3 (7)	3.15 (4)	0.067[c]	5.8 (3)	0.208[c]
A allele (%)	76.6 (328)	81.9 (208)		80.8 (84)	
G allele (%)	23.4 (100)	18.1 (46)	0.122	19.2 (20)	0.433
***Bsr*I RFLP**					
TT genotype (%)	50.2 (108)	50.4 (65)		48.2 (26)	
TC genotype (%)	42.3 (91)	39.5 (51)		40.7 (22)	
CC genotype (%)	7.4 (16)	10.1 (13)	0.665	11.1 (6)	0.679
T allele (%)	71.4 (307)	70.2 (181)		68.5 (74)	
C allele (%)	28.6 (123)	29.8 (77)	0.730	31.5 (34)	0.556

The values are expressed as a percentage with the number of subjects (n) in parentheses.

[a] P values for CON vs ACL genotype and allele frequencies.

[b] P values for CON vs NON genotype and allele frequencies.

[c] Due to the small sample size of the *Alu*I RFLP GG genotype, the GG and GA participants were combined and compared to the TT participants.

APPENDIX 2

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GENETIC BASIS OF EXERCISE-INDUCED LIGAMENT INUJURY

INFORMED CONSENT

I, the undersigned, have been fully informed about the UCT/MRC Research Unit for Exercise Science and Sports Medicine within the Department of Human Biology of the University of Cape Town's study on the genetic basis of exercise induced chronic ligament pathology. I have agreed to donate five millilitres of venous blood or a Buccal mouthwash sample, which will be used for the extraction and analysis of genetic material (DNA). I have also agreed to complete personal particulars, sporting participation, medical history, stretching and warm up questionnaires and understand that all the information that is collected during the study will be treated with the strictest confidentiality and will only be used for scientific research purposes. I also understand that my name and personal particulars will be not released under any circumstances and that all data will be analysed anonymously.

I agree to participate in the study and I have been informed that I will be free to withdraw from the study at any time if I so wish. I understand that my DNA sample will be destroyed on completion of the study on the genetic basis of ligament pathology. I also understand that I will be free to request that my DNA sample be destroyed before the completion of the study.

I understand that the DNA will be genotyped (analysed) for variations (polymorphisms) within genes relating to the genetic basis of ligament injuries. I understand that whilst there is no direct benefit to myself, if a genetic predisposition for ligament injuries can be established, then future generations will be able to establish their risk for this condition. This may allow better prevention and treatment options in the future. I understand that I will receive the overall results of the study. I have read (or where appropriate, have had read to me) and understand the information about this study, and any questions I have asked have been answered to my satisfaction. I agree to participate in the study, realising that I have the right to request that my DNA sample be destroyed at any time. I agree that research data provided by me or with my permission during the project may be included in a thesis, presented at conferences and published in journals on the condition that either my name not any other identifying information is used.

FULL NAME OF SUBJECT: _____

SUBJECT'S SIGNATURE: _____

DATE: _____

INVESTIGATOR : _____

INVESTIGATOR'S SIGNATURE: _____

APPENDIX 3

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GENETIC BASIS OF LIGAMENT INJURY QUESTIONNAIRES

A. PERSONAL PARTICULARS			
Surname			
First Name			
Postal Address		Code	
E-mail address		Phone (day time)	
Date of birth	Y Y Y Y / M M / D D	Cell	
Height (cm)		Gender	Male ☐ Female ☐
Weight (kg)	Pre-Injury:	Current:	
Ethnic group (Only Required and Used for Research Purposes)	Black/African ☐ White ☐ Indian ☐ Mixed Ancestry (Coloured) ☐ Asian ☐ Other ☐		
Ancestry: Tribal or national background (eg Xhosa, Dutch, Zulu, German, Italian)	Father	Unknown ☐	
	Mother	Unknown ☐	
Country of Birth			
Dominant Hand	Left ☐ Right ☐ Ambi ☐	Dominant Leg	Left ☐ Right ☐ Ambi ☐
Smoker	Yes (Current) ☐	Yes (Ex smoker) ☐	No, never ☐
	If yes, Number of years _____	If stopped, when _____	
	If yes, number per day _____		

(If you participate or have participated in more than 6 sports, please complete additional Sporting Details Questionnaires, Part B)

B. SPORTING DETAILS **Please record your sporting activities in order of importance**			
Type of sport(s) you have participated in (please name)	**Main sport 1**	**Other sport 2**	**Other sport 3**
Current or past participation	Current ☐ Past ☐	Current ☐ Past ☐	Current ☐ Past ☐
Year started participation			
Number of years involved in the sport			
Position played prior to injury (if appropriate)			
Playing level prior to injury (if appropriate)			
Number of years played prior to the injury.			

Type of sport(s) you have participated in (please name)	**Other sport 4**	**Other sport 5**	**Other sport 6**
Current or past participation	Current ☐ Past ☐	Current ☐ Past ☐	Current ☐ Past ☐
Year started participation			
Number of years involved in the sport			
Position played prior to injury (if appropriate)			
Playing level prior to injury (if appropriate)			
Number of years played prior to the injury.			

C. ANTERIOR CRUTIATE LIGAMENT INJURY DETAILS

Date of ACL injury?	
Which side was injured?	☐ Left ☐ Right ☐ Both
To what extent was your ligament ruptured?	☐ Complete ☐ Partial ☐ None ☐ Unknown
Investigation done to confirm the diagnosis	☐ MRI ☐ Surgery
How bad is your pain today? (mark line: e.g.I----------I----------I)	I----------I No pain Pain as bad as it can be
How was the ACL ruptured? **(please also explain exactly how the injury occurred)**	☐ Direct impact ☐ Twisting and bending with contact ☐ Twisting and bending without contact ☐ Other non-contact ☐ Other……
What was the initial treatment? (You may tick more than one block.)	☐ Ice application ☐ Compresion ☐ Immobilisation ☐ Medication ☐ Other……
What was the final treatment?	☐ Surgery ☐ Reabilitation ☐ Other……..
What are your current symptoms? (You may tick more than one block.)	☐ Pain ☐ Swelling ☐ Instability ☐ Weakness ☐ Other……
What is your current sports participation?	☐ None ☐ Limited to non-weight bearing exercise ☐ Limited, not to same level as pre-injury ☐ Full participation

If you are able to recall, what were the weather and pitch conditions like at the time of injury?	☐ Wet and soft ground ☐ Dry, but soft ground ☐ Dry and firm ground ☐ Wet, but firm ground ☐ Other……..
Associated injuries?	☐ Meniscal tear ☐ MCL tear ☐ Other ligament tear ☐ Bone bruising ☐ Other……..

D. HISTORY OF OTHER LIGAMENT AND TENDON INJURIES IN THE PAST					
Have you ever injured a ligament in the past?	Yes ☐ No ☐				
If yes, please specify which ligaments? (You may tick more than one block, please select either L (left) or R (right))		L	R		L R
	Knee (ACL)	☐	☐	Wrist ligaments	☐ ☐
	Knee (MCL)	☐	☐	Finger ligaments	☐ ☐
	Ankle lateral ligaments	☐	☐	Knee (PCL)	☐ ☐
	Spinal ligaments	☐	☐	Knee (LCL)	☐ ☐
	Shoulder ligaments	☐	☐	Ankle medial ligaments	☐ ☐
	Elbow ligaments	☐	☐	Other ligaments	☐ ☐
To your knowledge, have any other members of your family suffered from any ligament injury?	Yes ☐ No ☐	If Yes, please specify the family member ☐ Mother ☐ Father ☐ Sibling ☐ Son / daughter ☐ Other family member…….. and condition: Please choose ligament injury from the list above ……			
Have you ever injured a tendon in the past?	Yes ☐ No ☐				

<table>
<tr><td rowspan="10">If yes, please specify which tendon?
(You may tick more than one block, please select either L (left) or R (right))</td><td rowspan="3">Foot and ankle:</td><td></td><td>L R</td></tr>
<tr><td></td><td>Achilles tendon ☐ ☐</td></tr>
<tr><td></td><td>Tibialis posterior ☐ ☐</td></tr>
<tr><td></td><td>Plantar fascia ☐ ☐</td></tr>
<tr><td>Knee:</td><td></td><td>Patellar tendon ☐ ☐</td></tr>
<tr><td>Elbow and wrist:</td><td></td><td>Wrist extensor tendons ☐ ☐</td></tr>
<tr><td rowspan="4">Shoulder:</td><td></td><td>Subscapularis ☐ ☐</td></tr>
<tr><td></td><td>Supraspinatus ☐ ☐</td></tr>
<tr><td></td><td>Infraspinatus ☐ ☐</td></tr>
<tr><td></td><td>Teres minor ☐ ☐</td></tr>
<tr><td colspan="3">Other:……..</td></tr>
<tr><td>To your knowledge, have any other members of your family suffered from any tendon pathology?</td><td>Yes ☐ No ☐</td><td colspan="2">If Yes, please specify the family member
☐ Mother
☐ Father
☐ Sibling
☐ Son / daughter
☐ Other family member:……
Condition: Please choose tendon injury from the list above
……</td></tr>
<tr><td>Have you ever suffered from any of the following joint capsule injuries?</td><td colspan="3">☐ Acute shoulder dislocation
☐ Chronic shoulder instability
☐ Chronic ankle instability
☐ Other: _____
_____</td></tr>
</table>

E. MEDICAL HISTORY

Do you currently suffer from any of these medical conditions:

☐ High Blood Pressure	☐ Angina/Heart Attack	☐ Asthma
☐ Emphysema	☐ Rheumatoid arthritis	☐ Osteoarthritis (wear & tear)
☐ Malignant disease (cancer)	☐ Elevated Blood Cholesterol	☐ Adrenal disorders
If Yes, what type? _____	☐ Diabetes mellitus	☐ Thyroid disorders
	☐ Renal disease	☐ Amyloidosis

Do you currently suffer from any other Connective Tissue & Rheumatological Diseases & Disorders?	Yes ☐ No ☐	If Yes, please select from the list below

List of some Connective Tissue and/or Rheumatic Diseases and Disorders

☐ Ankylosing Spondylitis	☐ Lipid Storage Diseases	☐ Pseudogout
☐ Aspartylglycosaminuria (AGU)	☐ Marfan Syndrome	☐ Reactive Arthritis
☐ Behcet's Syndrome	☐ Menkes Kinky Hair Syndrome	☐ Reiter's Syndrome
☐ Crohn's Disease	☐ Mucopolysaccharidoses	☐ Relapsing Polychrondritis
☐ Discoid Lupus Erythematosus	☐ Myopathies and Dystrophies	☐ Scleroderma
☐ Ehlers-Danlos syndrome (EDS)	☐ Ochronosis (Homocystinuria)	☐ Sjogren's Syndrome
☐ Eosinophilic Fascitis	☐ Osteogenesis imperfecta (OI)	☐ Systemic Lupus Erythematosus (SLE)
☐ Giant Cell (Temporal) Arthritis	☐ Polyarteritis Nodosa	☐ Systemic Sclerosis
☐ Gout	☐ Polymyalgia Rheumatica	☐ Wegener's Granulomatosis
☐ Hypersentive Vasulatis	☐ Polymyositis & Dermatomyositis	☐ Other_____

	Operation	Date
What surgical operations have you had? (please list and give dates)		

If female:

At what age did you start menstruating? (years)	
Are you currently using any type of contraception?	☐ Yes ☐ No
If Yes, what type of contraception are you using?	☐ Pill ☐ Injection ☐ IUD

Are you currently?	☐ Pre-menopausal (±12 cycles per year at intervals of 23–33 days & bleeding lasts 3-7 days) ☐ Menopausal (cycles are irregular and less frequent) ☐ Post-menopausal (no longer menstruating)	
Family History		
Do any other members of your family suffer from elevated blood cholesterol?	Yes ☐ No ☐	If Yes, which relative? ☐ Mother ☐ Father ☐ Sibling ☐ Son / daughter ☐ Other relative:……
Is there any history of arthritis in your family?	Yes ☐ No ☐	If Yes, which relative? ☐ Mother ☐ Father ☐ Sibling ☐ Son / daughter ☐ Other relative:…… & What type of aethritis? Rheumatoid ☐ Osteoarthritis ☐ Other ☐

Drug and Allergy History	If yes, how long ago (or how many times, where applicable) did you use the medication?	
Have you ever used oral corticosteroids (cortisone tablets)?	Yes ☐ No ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months
Have you ever been given an injection with corticosteroids?	Yes ☐ No ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months
Have you ever been given an injection of corticosteroids in or around a tendon?	Yes ☐ No ☐	☐ Once ☐ Twice ☐ 3 times ☐ >3 times
Have you ever used anabolic steroids?	Yes ☐ No ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months
Have you ever used fluoroquinoline antibiotics?	Yes ☐ No ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months

If yes, please select from the list below:		
☐ ADCO-CIPRIN	☐ CIPROBAY	☐ SANDOZ CIPROFLOXACIN
☐ AVELON	☐ CIPROGEN	☐ TAFLOC
☐ BACTIDRON	☐ CPL ALLIANCE CIPROFLOXACIN	☐ TARIVID
☐ CIFLOC	☐ DYNAFLOC	☐ TAVANIC
☐ CIFRAN	☐ FLOXIN	☐ TEQUIN
☐ CIPLA-CIPROFLOXACIN	☐ MAXAQUIN	☐ UNIQUIN
☐ CIPLOXX	☐ NOROXIN	☐ UTN-400
☐ CIPRO-HEXAL	☐ ORPIC	☐ ZANOCIN
☐ Other_____		

What medication, if any, are you currently using? (please list)	
What allergies do you have? (please list)	

F. OCCUPATIONAL DETAILS	
What is your current occupation?	
What was your occupation prior to injuring your ligament?	
Prior to injury, did your occupation involve lower limb activity?	☐ Yes ☐ No
If yes please indicate which legs.	Right leg ☐ Both legs ☐ Left leg ☐ None ☐

APPENDIX 4:

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • Facsimile [021] 406 6411
e-mail: prewardf@curie.uct...ac.za

26 April 2006

REC REF: 164/2006

Dr M Collins
Human Biology

Dear Dr Collins

PROJECT TITLE: THE COL5A1 AND TNC GENES AND THEIR ASSOCIATION WITH ANTERIOR CRUCIATE LIGAMENT INJURIES

Thank you for submitting your study to the Research Ethics Committee for review.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study on the 21 April 2006.

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

Please quote the REC. REF in all your correspondence.

Yours sincerely

DR. M. BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
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16 September 2008

REC REF: 164/2006

Assoc Prof M Collins
Sports Science Centre

Dear Prof Collins

PROJECT TITLE: THE COL5A1 AND TNC GENES AND THEIR ASSOCIATION WITH ANTERIOR CRUCIATE LIGAMENT INJURIES

Thank you for your letter to the Research Ethics Committee dated 12th September 2008.

It is a pleasure to inform you that the Ethics Committee has approved the amendment described in your letter dated 12 September 2008.

Based on the information provided in the addendum, approval is also granted to continue the study for a further 12 months until 21st September 2009.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

APPENDIX 5

Department of Human Biology
UCT/MRC RESEARCH UNIT FOR EXERCISE SCIENCE & SPORTS MEDICINE
Faculty of Health Sciences, University of Cape Town
Private Bag, Rondebosch 7700, South Africa
Tel: + 27 21 650 4561
Fax: + 27 21 686 7530

GENETIC BASIS OF EXERCISE-INDUCED CHRONIC TENDON PATHOLOGY

INFORMED CONSENT

I, the undersigned, have been fully informed about the UCT/MRC Research Unit for Exercise Science and Sports Medicine within the Department of Human Biology and the Division of Human Genetics within the Department of Clinical Laboratory Sciences at the University of Cape Town's study on the genetic basis of exercise induced chronic tendon pathology. I have agreed to donate five millilitres of venous blood or a Buccal mouthwash sample, which will be used for the extraction and analysis of genetic material (DNA). I have also agreed to complete personal particulars, sporting participation, medical history, stretching and warm up questionnaires and understand that all the information that is collected during the study will be treated with the strictest confidentiality and will only be used for scientific research purposes. I also understand that my name and personal particulars will be not released under any circumstances and that all data will be analysed anonymously. I have agreed that my blood sample can also be used to determine my ABO blood group type. I have also agreed to allow my general flexibility and ankle dorsiflexion to be determined.

If requested, I am also prepared to visit SSISA early in the morning for a second visit in an overnight fasted state to donate another 5 ml blood sample for a total blood cholesterol test (please delete this sentence if not applicable). If requested, I am also prepared to visit a doctor (radiologist) at a later stage for a tendon scan at no cost to myself (please delete this sentence if not applicable). If requested, I am also prepared to visit the SSISA for measurements to determine musculo-tendinous stiffness.

I agree to participate in the study and I have been informed that I will be free to withdraw from the study at any time if I so wish. I understand that my DNA sample will be destroyed on completion of the study on the genetic basis of tendon pathology. I also understand that I will be free to request that my DNA sample be destroyed before the completion of the study.

FULL NAME OF SUBJECT: _____

SUBJECT'S SIGNATURE: _____

DATE: _____

INVESTIGATOR : _____

INVESTIGATOR'S SIGNATURE: _____

APPENDIX 6:

Department of Human Biology
UCT/MRC RESEARCH UNIT FOR EXERCISE SCIENCE & SPORTS MEDICINE
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Fax: + 27 21 686 7530

GENETIC BASIS OF TENDON INJURY QUESTIONNAIRES

A. PERSONAL PARTICULARS			
Surname			
First Name			
Postal Address		Code	
E-mail address		Phone (day time)	
Date of birth	Y Y Y Y / M M / D D	Cell	
Height (cm)		Gender	Male ☐ Female ☐
Weight (kg)			
Ethnic group (Only Required and Used for Research Purposes)	Black/African ☐ White ☐ Indian ☐ Mixed Ancestry (Coloured) ☐ Asian ☐ Other ☐		
Ancestry: Tribal or national background (eg Xhosa, Dutch, Zulu, German, Italian)	Father Unknown ☐ Mother Unknown ☐		
Country of Birth		Nationality	
Dominant Hand	Left ☐ Right ☐ Both ☐	Dominant Leg	Left ☐ Right ☐ Both ☐
Smoker	Yes (Current) ☐ Yes (Ex smoker) ☐ No, never ☐ If yes, Number of years _____ If stopped, when _____ If yes, number per day _____		
Do you know your blood group?	Yes ☐ A ☐ B ☐ AB ☐ O ☐ No ☐ Rh Pos ☐ Rh Neg ☐		

(If you participate or have participated in more than 6 sports, please complete additional Sporting Details Questionnaires, Part B)

B. SPORTING DETAILS **Please record your sporting activities in order of importance**			
Type of sport(s) you have participated in	**Sport 1**	**Sport 2**	**Sport 3**
Current or past participation	Current ☐ Past ☐	Current ☐ Past ☐	Current ☐ Past ☐
Year started participation			
Years involved in the sport			
Current hours of training per week (1-12 months)			
Current hours of training per week (13-24 months)			
Hours of training per week prior to first Injury (1-12 months)			
Hours of training per week prior to first Injury (13-24 months)			

Type of sport(s) you have participated in	**Sport 4**	**Sport 5**	**Sport 6**
Current or past participation	Current ☐ Past ☐	Current ☐ Past ☐	Current ☐ Past ☐
Year started participation			
Years involved in the sport			
Current hours of training per week (1-12 months)			
Current hours of training per week (13-24 months)			
Hours of training per week prior to first Injury (1-12 months)			
Hours of training per week prior to first Injury (13-24 months)			

C. GENERAL MEDICAL DETAILS		If Yes, How long ago or how often
Have you ever used oral corticosteroids (cortisone tablets)?	Yes ☐ No ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months
Have you ever been given an injection with corticosteroids?	Yes ☐ No ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months
Have you ever been given an injection of corticosteroids in or around a tendon?	Yes ☐ No ☐	☐ Once ☐ Twice ☐ 3 times ☐ >3 times
Have you ever used anabolic steroids?	Yes ☐ No ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months
Have you ever used fluoroquinoline antibiotics?	Yes ☐ No ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months
Do you suffer from any Connective Tissue and Rheumatological Diseases and Disorders?	Yes ☐ No ☐	**If Yes, please select from the list below**

List of some Connective Tissue and/or Rheumatic Diseases and Disorders

☐ Ankylosing Spondylitis	☐ Marfan Syndrome	☐ Pseudogout
☐ Aspartylglycosaminuria (AGU)	☐ Menkes Kinky Hair Syndrome	☐ Reactive Arthritis
☐ Behcet's Syndrome	☐ Mucopolysaccharidoses	☐ Reiter's Syndrome
☐ Crohn's Disease	☐ Myopathies and Dystrophies	☐ Relapsing Polychrondritis
☐ Discoid Lupus Erythematosus	☐ Ochronosis (Homocystinuria)	☐ Rheumatoid Arthrits
☐ Ehlers-Danlos syndrome (EDS)	☐ Osteoarthritis	☐ Scleroderma
☐ Eosinophilic Fascitis	☐ Osteogenesis imperfecta (OI)	☐ Sjogren's Syndrome
☐ Giant Cell (Temporal) Arthritis	☐ Polyarteritis Nodosa	☐ Systemic Lupus Erythematosus (SLE)
☐ Gout	☐ Polymyalgia Rheumatica	☐ Systemic Sclerosis
☐ Hypersentive Vasulatis	☐ Polymyositis & Dermatomyositis	☐ Wegener's Granulomatosis
☐ Lipid Storage Diseases		

Have any other members of your family suffered from any tendon pathology?	Yes ☐ No ☐	If Yes, please specify the family member _____ (eg Mother, Son) and type of injury Acute Injury ☐ Chronic Pain and Swelling ☐ Other ☐
Do you suffer from elevated blood cholesterol?	Yes ☐ No ☐	Do any other members of your family suffer from elevated blood cholesterol? Yes ☐ No ☐
Have you been diagnosed with any of the following systemic diseases?	☐ No systemic disease ☐ Diabetes mellitus ☐ Adrenal disorders ☐ Thyroid disorders ☐ Amyloidosis ☐ Renal disease ☐ Other endocrine and metabolic disease (Specify _____ _____)	

D. TENDON INJURY - MEDICAL DETAILS				
Symptoms				
How many times have you had tendon injuries? [1]Sudden onset is within a few seconds or minutes [2]Gradual onset is over days or weeks	Tendon Injured	Date of Injury	Acute or Chronic Injury	Sudden[1] or Gradual[2] Onset
	1			
	2			
	3			
	4			
	5			

Please complete a **separate form**, Part D only, for each Tendon Injury you have had	
Injury Number (1,2,3,4,or 5)	☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ _____
Which tendon did you injure?	Rotator cuff tendon ☐ • Supraspinatus ☐ • Infraspinatus ☐ • teres minor ☐ Patellar tendon ☐ Wrist extensor tendons ☐ Achilles tendon ☐
Which side was injured?	☐ Left ☐ Right ☐ Both
Which region of your tendon was injured? Please indicate on a diagram. (Only if applicable)	☐ Upper 1/3 ☐ Middle 1/3 ☐ Lower 1/3
To what extent was your Tendon ruptured?	☐ Complete ☐ Partial ☐ None
How were you injured? (e.g. sport, walking)	
Grade of injury **at the time** of injury	☐ pain only after exercise ☐ pain during exercise, but did not cause you to alter training ☐ pain during exercise, which causes you to alter training ☐ pain which causes you to stop training ☐ no pain ☐ not sure ☐ Other (Specify _____)
Grade of injury **currently**	☐ pain only after exercise ☐ pain during exercise, but did not cause you to alter training. ☐ pain during exercise, which causes you to alter training ☐ pain which causes you to stop training ☐ no pain ☐ not sure ☐ Other (Specify _____)

Which of the following symptoms were present **before** the injury	☐ Pain (less than 1 week) ☐ Pain (1-4 weeks) ☐ Pain (> 4 weeks)	☐ Stiffness ☐ Swelling ☐ None
Which of the following symptoms were present **after** the injury	☐ Pain (less than 1 week) ☐ Pain (1-4 weeks) ☐ Pain (> 4 weeks)	☐ Stiffness ☐ Swelling ☐ None
If you have or had chronic tendon pain, what seems to alleviate the pain?		

Diagnosis

Which type of Tendon Disease were you diagnosed with e.g. Rupture, Tendinitis, etc.	
Diagnosed by (Please indicate the name and contact number of the clinician who diagnosed you)	☐ Doctor _____ ☐ Physiotherapist _____ ☐ Biokineticist _____ ☐ Podiatrist _____ ☐ Other _____
If you had a tendon rupture. How was it treated?	☐ Surgically ☐ Non-surgically
If applicable, who was the surgeon?	Surgeon _____ Phone _____
If applicable, what diagnostic imaging was performed?	☐ Ultrasound ☐ MRI ☐ CT Other _____
If applicable, who did the imaging?	Clinician _____ Phone _____

General Information		**If Yes, How long ago or how often**	
Have you ever used oral corticosteroids **prior** to your symptoms of tendon pathology (cortisone tablets)?	Yes ☐ No ☐	☐ 3 months ☐ 12 months	☐ 6 months ☐ 24 or more months
Have you ever been given an injection with corticosteroids **prior** to your symptoms of tendon pathology?	Yes ☐ No ☐	☐ 3 months ☐ 12 months	☐ 6 months ☐ 24 or more months
Have you ever been given an injection of corticosteroids in or around the injured tendon **prior** to your symptoms of tendon pathology?	Yes ☐ No ☐	☐ Once ☐ 3 times	☐ Twice ☐ >3 times
Have you ever used anabolic steroids **prior** to your symptoms of tendon pathology?	Yes ☐ No ☐	☐ 3 months ☐ 12 months	☐ 6 months ☐ 24 or more months
Have you ever used fluoroquinoline antibiotics **prior** to your symptoms of tendon pathology?	Yes ☐ No ☐	☐ 3 months ☐ 12 months	☐ 6 months ☐ 24 or more months

E. STRETCHING AND WARM UP (PRIOR TO YOUR INJURY)					
I usually stretch each week as follows: (Please tick ALL the appropriate boxes)		☐	Never		
		☐	Occasionally		
		☐	Before sport		
		☐	After sport		
		☐	Once daily		
		☐	Twice daily		
		☐	More than twice daily		
Which of these muscle groups do you stretch?	Lower Back	☐	Always	☐	Occasionally
	Buttock	☐	Always	☐	Occasionally
	Hip Flexors	☐	Always	☐	Occasionally
	Quads	☐	Always	☐	Occasionally
	Hamstrings	☐	Always	☐	Occasionally
	Calf Muscles	☐	Always	☐	Occasionally
How many times do you stretch per week?		☐	Never		
		☐	< 5 min		
		☐	5 min		
		☐	10 min		
		☐	15 min		
		☐	20 min		
		☐	25 min		
		☐	> 30 min		
Do you warm up before exercise?		Yes ☐ No ☐			
If yes, for how many minutes and how?					
Do you cool down after exercise?		Yes ☐ No ☐			
If yes, for how many minutes and how?					

APPENDIX 7:

UNIVERSITY OF CAPE TOWN

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08 May 2008

REC REF: 086/2005

A/Prof M Collins
Human Biology
Sports Science Institute

Dear A/Prof Collins

PROJECT TITLE: THE GENETIC BASIS OF TENDINOPATHY

Thank you for your letter to the Research Ethics Committee dated 24 April 2008.

Addendum to undertake further genetic analysis is approved.
We note that future analysis will be performed on de-identified samples.

Please would you submit an annual progress report which includes a description of the current status of the research and your publication as outlined in this letter.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

Wesley Henley

PROFESSOR M BLOCKMAN
pp **CHAIRPERSON, HSF HUMAN ETHICS**