RISK FACTORS FOR ACHILLES TENDON INJURIES:
An emphasis on the identification of specific genetic factors

Gaonyadiwe George Mokone

Department of Human Biology
University of Cape Town
Cape Town
South Africa

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By

Gaonyadiwe George Mokone

Thesis Supervisors

Dr Malcolm Collins, BSc (Hons), PhD

Associate Professor Martin Schwellnus, MBChB, MSc, MD, FACSM

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Sports Science Institute of South Africa

Boundary Road

Newlands

7700

South Africa

Dedication

This thesis is dedicated to every teacher (both formal and informal) that has ever taught me something. I would also like to dedicate this work to my all-time greatest teacher, great grandmother MmaGaleboe, who is over 100 yrs old, still alive and kicking.

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DECLARATION

I, **Gaonyadiwe George Mokone**, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

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LIST OF PUBLICATIONS

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Gaonyadiwe G. Mokone, Mamta Gajjar, Alison V. September, Martin P. Schwellnus, Jacquie Greenberg, Timothy D. Noakes, and Malcolm Collins. The GT dinucleotide repeat polymorphism within the *Tenascin-C* gene is associated with Achilles tendon pathology. *Am J Sports Med.* **2005** July; *33(7):* 1016-1021

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ABSTRACT

Background

The Achilles tendon connects the calf muscles (gastrocnemius-soleus muscles) to the heel bone (calcaneus) and despite being the strongest and thickest tendon in the human body it is commonly injured in both recreational and professional sports. Although the mechanisms causing Achilles tendon injuries are poorly understood, both extrinsic and intrinsic risks factors have been implicated in the development of signs and symptoms of Achilles tendon ruptures (acute injuries) or Achilles tendinopathies (chronic overuse injuries). Although inconclusive, some investigators have shown that the ABO blood group system is associated with Achilles tendon ruptures and Achilles tendinopathies. This suggested that either the *ABO* gene or another closely linked gene(s) on the tip of the long arm of chromosome 9 (9q32-q34.3) could be associated with tendon injuries. Although a direct link between the *ABO* gene and tendon injury can not be excluded, it is more likely that another gene or several genes linked to the *ABO* gene on the 9q32-q34.3 locus, which encode for structural or functional components of tendons, would be directly linked to tendon pathology. It is possible that any protein found in the extracellular matrix (ECM) of a tendon regardless of its gene's chromosomal location is an ideal candidate for tendon pathology. However, in this thesis only genes located in the 9q32-q34.3 locus were investigated.

Aims and Objectives

This main purpose of this thesis was therefore to investigate whether any specific genes on the tip of the long arm of chromosome 9 were associated with Achilles tendon injuries, using a case-control study design. The specific objectives were: (i) to identify all genes located in the 9q32-q34.3 locus in close proximity to the *ABO* gene, that could be involved in tendon injuries (Chapter 2) and (ii) to investigate the

possible association of the identified candidate genes (*COL5A1* and *TNC*) with both Achilles tendon rupture and chronic Achilles tendinopathy (Chapter 3 and 4) and (iii) finally to investigate the possible interaction of these two genes with tendon function, namely the muscle-tendon unit flexibility (chapter 5) and structure, namely the morphological changes of the Achilles tendon (Chapter 6).

Methods

One-hundred and twenty-two physically active Caucasian patients with a current or past clinical history of Achilles tendon injury (ATI), including 79 with chronic tendinopathies (TEN) and 43 with tendon ruptures (RUP), were included in the thesis. Another group of 131 physically active Caucasian control (CON) subjects without any history of Achilles tendon injury symptoms were also included. The subjects were matched for age of onset of injury and gender. To avoid any possible effects of population stratification, the ATI and CON groups were also matched for their country of birth. Because of missing information on some of the measurement outcomes, the number of subjects reported in each section of the thesis differ slightly (Chapters 2, 3 and 4). In chapter 5, a sub-set of 71 male and female ATI subjects (designated ATI-F, F for flexibility) and a sub-set of 37 male and female CON subjects (designated CON-F) included in the CON were invited to participate in a sub-study to explore the *COL5A1* and *TNC* genotype effect on muscle tendon unit flexibility using the clinical measurement of range of motion (ROM). The ATI-F group consisted of 38 subjects diagnosed with chronic Achilles tendinopathy (TEN-F) and 33 with Achilles tendon rupture (RUP-F). In chapter 6, a sub-set of 36 male subjects diagnosed with chronic Achilles tendinopathy (designated TEN-M, M for morphology) and a sub-set of 22 male CON subjects (designated CON-M) were selected and also invited to participate in a sub-study to investigate the *COL5A1* and *TNC* genotype effect on Achilles tendon morphology using grey scale and colour Doppler ultrasonography.

An experienced clinician made the diagnosis of chronic Achilles tendinopathy in the TEN sub-group using clinical criteria. The diagnostic criteria for every subject were reviewed by a sports physician using validated clinical criteria. In addition to these clinical diagnostic criteria, soft tissue ultrasound examination was performed in a sub-group of subjects in both the CON and ATI groups (Chapter 6). The diagnosis of Achilles tendon rupture was made clinically using standard validated criteria and confirmed in all cases by examination at the time of surgery and/or an imaging technique. Subjects 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. Furthermore, subjects who had been diagnosed with any connective tissue disorders or any other systemic diseases believed to be associated with Achilles tendon pathology were also excluded from the study.

Approximately 4.5 ml of venous blood was collected from subjects and DNA was extracted and genotyped for polymorphisms within both *COL5A1* and *TNC* genes (Chapter 3 and 4) and ABO blood group determined (Chapter 2).

Genes that flank the *ABO* gene were identified by searching the OMIM™ (Online Mendelian Inheritance In Man), NCBI Map Viewer and Entrez Gene internet-based databases. Since the *ABO* gene has been mapped to the human chromosome 9q34, all genes mapped from 9q32 to the end of the chromosome 9 (q34.3) were initially identified. Genes were included as possible candidate genes for tendon pathology if they encoded for proteins that formed an integral structural component of the extracellular matrix (ECM). Since tenocytes have been shown to play an important role in tendon pathology, genes encoding for proteins involved in cell proliferation, cell death (specifically apoptosis) and other miscellaneous processes were also identified. The biological function of the proteins encoded for by the genes on the

telemetric end of the long arm of chromosome 9 were identified from their functional descriptions in the databases.

Results

Using internet-based genetic databases, a total of 404 genes located on the tip of the long arm of chromosome 9 (9q32-q34.3 locus) closely linked to the *ABO* gene were identified (chapter 2). After retrieving and interpreting all the summary information provided on the databases for each gene, 21 possible candidate genes were identified, which could theoretically be associated with Achilles tendon injuries. However, because the information for most genes was scanty it is very likely that more candidate genes in the 9q32-q34.2 locus could be identified in the future as the information and knowledge on these genes become available. Of the 21 genes, 2 coded for structural proteins found in tendons, namely the *COL5A1* and *TNC* genes. Therefore, in this thesis polymorphisms within these two genes were further investigated (chapters 3 and 4).

An additional objective of this thesis was to determine if the ABO blood group distribution was associated with Achilles tendon injuries in a South African Caucasian population. The percentage of individuals with blood group O and the A/O ratio were similarly distributed between the CON and ATI groups, as well as TEN and RUP sub-groups.

In chapter 3, 116 ATI and 129 CON subjects were genotyped for the *Bst*UI and *Dpn*II restriction fragment length polymorphisms (RLFPs) within the *COL5A1* gene, which encode for a component of type V collagen. There was a significant difference in the allele frequencies of the *COL5A1 Bst*UI RLFP between the ATI and CON subjects ($p=0.006$). The frequency of the A2 allele was significantly higher in the CON group (29.8%) than in the ATI group (18.0%) (odds ratio of 1.9; 95% CI 1.3-3.0; $p=0.004$).

The A2 allele was strongly over-represented in the CON group when only the 76 patients diagnosed with chronic Achilles tendinopathy were analyzed (odds ratio of 2.6; 95% CI 1.5-4.5).

In Chapter 4, 114 ATI and 127 CON subjects were genotyped for a GT dinucleotide repeat polymorphism within the *TNC* gene. The TNC gene encodes for a protein, tenascin-C, which is also a component of tendons. A significant difference in the allele frequencies of the GT dinucleotide repeat polymorphism was found between the CON and ATI groups (Chi square=51.0, p=0.001). The frequencies of the alleles containing 12 repeats (ATI, 18.9% vs CON, 10.2%) and 14 repeats (ATI, 9.2% vs CON, 0.8%) were significantly higher (over-represented, O) in the ATI group, while the frequencies of the alleles containing 13 repeats (ATI, 8.8% vs CON, 24.0%) and 17 repeats (ATI, 7.5% vs CON, 20.1%) were significantly lower (under-represented, U) in the ATI group. Subjects who were homozygous or heterozygous for the under-represented alleles (13 and 17 repeats), but who did not possess an over-represented allele (12 and 14 repeats) were therefore at a lower risk of developing Achilles tendon injuries (odds ratio, 6.2; 95% CI, 3.5-11.0; $p<0.001$).

The matching of subjects in chapters 3 and 4 was based on age-at-onset for Achilles tendon injury and therefore it was difficult to match for important variable such age, in the measurement of flexibility (Chapter 5) and morphological changes in the tendon (Chapter 6). To overcome this problem, both asymptomatic and symptomatic subjects were divided into the respective allele groups (over-represented and under-represented) for both *COL5A1* (A2 and A1/A3 alleles) and *TNC* genes (O and U alleles).

The range of motion (ROM) measurements of the upper and lower limb extremities as measured by a modified standard goniometer and the sit and reach test were used as proxy for muscle tendon unit flexibility for a particular joint motion. Except

for the non-dominant ankle dorsiflexion ROM in the ATI-F sub-group, there were no differences found in the lower limb flexibility measurements between the A2 and A1/A3 COL5A1 genotype groups of the CON-F as wells as the ATI-F subjects. The non-dominant ankle dorsiflexion (ND-ADF) was significantly greater in the ATI-F subjects with the A1/A3 allele (61 ± 9°) when compared to the ATI-F subjects with the A2 allele. (55 ± 9°) (p=0.046). Except for the dominant ankle dorsiflexion ROM in the ATI-F group, there were no significant differences in the lower limb flexibility measurements between the control (CON-F) subjects with under-represented, even and over-represented alleles. The dominant ankle dorsiflexion was significantly greater in the ATI-F subjects wit the under-represented alleles (61 ± 8°) when compared to ATI-F subjects with the over-represented allele (54 ± 11°) (p=0.047), but not different to the ATI subjects with evenly distributed alleles.

The mean age, weight and BMI were consistently higher in the Achilles tendinopathy group compared to the control group. Therefore, for the subsequent analysis of the *COL5A1* and *TNC* genotype effects on Achilles tendon morphology, both the CON-M and TEN-M groups were analysed separately to eliminate any possible effect these factors might have on flexibility. The mean pathology scores between the individuals without the A2 allele of the *COL5A1* gene were not significantly different from the mean pathology score for individuals containing the under-represented alleles (A2) in both the CON-M and TEN-M groups. However, it was interesting to note that in the whole CON-M group only individuals without the A2 alleles displayed some tendon pathology, while none of the 7 individuals with the A2 allele had an abnormal tendon morphological changes when examined using ultrasonography. This observation warrants further investigation. There were no significant differences in the mean pathology scores of the control subjects with over-represented (CON-M-O) and those under-represented (CON-M-U) as well as between the Achilles tendinopathy subjects with the overrepresented (TEN-M-O) and those with underrepresented (TEN-M-U) allele.

Conclusions

It has so far been suggested that several extrinsic and intrinsic risk factors, might play a role in tendon injuries. However to date no specific genetic factor(s) has been identified. Twenty-one genes closely linked to the *ABO* gene, located on the long arm of chromosome 9 (9q32-q34.2), which could theoretically be involved in tendon structure and/or function and possibly tendon pathology were identified in this thesis. Of the 21 genes, 2 genes that code for tendon structural proteins, namely the *COL5A1* and *TNC* genes were selected. The *Bst*UI RFLP within the *COL5A1* gene was associated with Achilles tendon injuries and more specifically, chronic Achilles tendinopathy. Individuals with an A2 allele of this gene were under-represented in the subjects with symptoms of chronic Achilles tendinopathy, while the A1/A3 alleles were over-represented in the control subjects. The GT dinucleotide repeat polymorphism within intron 17 of the *TNC* gene is associated with the symptoms of Achilles tendon injury. Alleles containing 12 and 14 GT repeats were over-represented in the subjects with Achilles tendon injuries, while the alleles containing 13 and 17 repeats were under-represented. The thesis found that the different polymorphisms of both *COL5A1* and *TNC* genes were not associated with measurements of range of motion in the lower limb joints tested. The results, therefore suggest that both the *COL5A1* and *TNC* genes do not influence the ROM measured. Although it was a pilot study, the use of grey scale and Doppler ultrasonography to detect tendon morphological changes appear to be highly correlated with tendon morphological changes, at least in the Achilles tendon. This study did not show that specific polymorphisms with the *COL5A1* and *TNC* genes were associated with Achilles tendon morphological changes. However, the fact that none of the CON-M subjects with the A2 allele of the *COL5A1* gene had pathological morphology while examined using ultrasonography warrants further investigation.

In conclusion, although it has been suggested for several years that there is a genetic risk factor (s) that may be related to Achilles tendon injuries, this is the first study to identify specific genetic elements, namely the *COL5A1* and *TNC* genes, to be associated with Achilles tendon injuries.

Abbreviations

ANOVA	analysis of variance
AT	Achilles tendon
ATI	Achilles tendon injury
BMI	body mass index
bp	base pair
DNA	deoxyribonucleic acid
dNTPs	2′-deoxy-nucleoside-5′-triphosphates
EDTA	ethylenediaminetetraacetic acid
HLA	human leucocyte antigens
HWE	Hardy-Weinberg equilibrium
ICC	intra-class coefficient
MRI	magnetic resonance imaging
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RCT	randomized controlled trial
RFLP	restriction fragment length polymorphism
ROM	range of motion
RUP	Achilles tendon rupture
SNP	single nucleotide polymorphism
TBE	tris-borate EDTA
TEN	Achilles tendinopathy
US	ultrasonography

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1.1 INTRODUCTION AND THE SCOPE OF THE THESIS

The Achilles tendon (AT) is the largest and strongest tendon in the human body (O'Brien, 1992) but is commonly injured in sports and physical activity related events (Kannus and Natri, 1997). Although details of the classification of AT injury will be discussed later in this thesis (section 1.2), broadly speaking, AT injury can present clinically as a gradual onset injury (chronic tendinopathy) or an acute onset injury (partial or complete AT rupture). Although many aspects of these injuries to the AT have been documented, the exact aetiology and pathogenesis leading to an AT injury is not fully understood (Kannus et al., 2004; Jarvinen et al., 2005; Riley, 2004). To date, research on AT ruptures has focused primarily on its management (surgical versus non-surgical) rather than on its aetiology. With regard to chronic Achilles tendinopathy, the debate is about the use of corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDS) in a condition that appears to be mainly degenerative in nature rather than inflammatory as initially thought, at least in its chronic stage (Khan et al., 2002; Kannus et al., 2004). Until recently, the role of other treatment modalities such as rest, physical modalities (ice, ultrasound, laser, electrical stimulation), stretching and eccentric strengthening exercises in chronic Achilles tendinopathy has not generated as much interest as the use of medication (corticosteroids and NSAIDS).

However, common to all researchers dealing with AT rupture and chronic Achilles tendinopathy is the fact that the number of patients seeking medical treatment as a result of Achilles tendon injuries appear to have increased since the early 1950s, more especially in the Western countries (Barfred, 1973; Jozsa et al., 1989; Sandelin et al., 1988). This is believed to be as a result of an increased availability of leisure time, increase in participation in recreational sport and physical activity, and an increase in the absolute time spent in training and intensity of training in the case of

professional athletes (Jarvinen, 1992). This increased participation in physical activity has been attributed partly to the jogging boom in the early 1970s and 1980s, resulting in even older individuals participating in recreational sports for many years (Smart et al., 1980). A higher number of older individuals generally lead sedentary lifestyles and tend to participate intermittently, but not regularly in recreational sports. This could also increase the risk for injuries, including AT injuries (Kvist, 1994). Furthermore, the increase commercialization of professional sport, places greater demands on the professional athlete's training habits. However, the increased number of Achilles tendon injuries could also be attributed to the absolute increase in population served by medical centers (Leppilahti and Orava, 1998). On the other hand, the increased number of these injuries could be as a consequence of increased research and publication on the subject. For example, Jozsa et al. (1991) reported that before 1929 there had only been 66 Achilles tendon ruptures reported in the medical literature.

Therefore, many reasons have been proposed for the perceived increase in the number of Achilles tendon injuries, particularly in Western countries. However, there is no doubt that the absolute number of these injuries is of concern to the medical profession. Because of (i) the accepted benefits of exercise towards health (Jarvinen, 1992; Jarvinen, 2000) and (ii) the increased economic benefits of sport, it is highly unlikely that there will be a decrease in participation in both recreational physical activity and professional sport. This will in turn result in more sports and physical activity related injuries, including Achilles tendon injuries.

There is therefore a need to define the risk factors associated with AT injuries so that preventive measures can be instituted and evidence-based advice may be offered to individuals wishing to participate in physical activity. The understanding of these risk factors will assist with the understanding of the aetiology and pathogenesis as well as possible treatment for AT injuries (Barfred, 1973).

Both extrinsic and intrinsic risk factors associated with either AT rupture or chronic Achilles tendinopathy have been widely reviewed (Kvist, 1994; Kainberger, 1997; Jarvinen, 1992; Jarvinen et al., 2001; Jarvinen et al., 2005; Maffulli et al., 2002; Paavola, 2000; Renstrom and Johnson, 1981; Riley, 2004). Extrinsic risk factors believed to be associated with AT injuries include occupation, physical activity, training errors, cold weather, running surface, shoes and other equipment. Intrinsic factors include previous injury, poor blood supply and nutrition, lower limb malalignment, reduced flexibility, male gender, systemic diseases, corticosteroids, quinolone antibiotics and genetic factors. Although both extrinsic and intrinsic risk factors will be discussed later (section 1.6), the broad focus of this thesis will be on the intrinsic factors that are believed to be associated with Achilles tendon injuries. The specific focus of this thesis will be to investigate the possible relationship between genetic factors and AT injuries. Genetic factors have been identified as one of the intrinsic risk factors for Achilles tendon injuries. However, to date no specific genes have been shown to be associated with these conditions. The main question that this thesis would like to answer is whether there are specific genetic elements associated with the development of AT injuries.

In this thesis, the reader is made aware of the following:

- It is believed that in many cases, AT rupture is the end stage of a degenerating tendon (Arner et al., 1959; Gibson, 2001; Kannus and Jozsa., 1991; Kainberger et al., 1997; Tallon et al., 2001), which has been exposed to mechanical loading but failed to adapt adequately, resulting in inadequate regeneration and repair. Tallon et al., 2001 have shown that ruptured tendons are more degenerated than tendinopathic tendons supporting the notion that tendinopathy precedes a tendon rupture. However, this is not the case for all tendon ruptures (see section 1.3). In a comprehensive study of 891

patients who had experienced various "spontaneous" tendon ruptures, including 397 Achilles tendon ruptures, the histopathology in the majority of the patient's tendon was commonly a degeneration of the tendon (Kannus and Jozsa, 1991).

- Therefore, in the tendon degeneration (due to mechanical load) model (see section 1.3), it is reasonable to assume that most risk factors associated with the development of AT rupture are also valid risk factors for Achilles tendinopathy.

- In this thesis, AT rupture and Achilles tendinopathy will be treated as two separate clinical entities, based mainly on the clinical diagnosis and supported by surgery and/or imaging results. Therefore, in this thesis, the term "Achilles tendon injuries" will include acute Achilles tendon ruptures and chronic Achilles tendinopathies (see section 1.2 for definitions).

- The main emphasis of the thesis is to identify the genetic factors associated with AT injury.

It is acknowledged that there are many aspects of Achilles tendon injury that the review can not cover as the subject is broad as evidenced by the number of recent reviews on the subject (Bramono et al., 2004; Jarvinen et al., 2005; Kjaer, 2004; Maganaris et al., 2004; Midwood et al., 2004; Riley, 2004; Riley, 2005a; Riley, 2005b). However, the review section of this thesis will focus the reader on three main areas that are important for the understanding of data and discussions in the subsequent chapters. Part one will include the classification of AT injuries (section 1.2), different hypotheses for the development of AT injury (section 1.3) and epidemiology of AT injuries (section 1.4). Part two will review the normal and pathological tendon structure and function, highlighting the specific features of the Achilles tendon

(section 1.5). The third part of the review will discuss the extrinsic (section 1.6.1) but mostly intrinsic (section 1.6.2) risk factors associated with Achilles tendon injuries.

1.2 CLASSIFICATION OF ACHILLES TENDON INJURIES

The literature concerning the classification or nomenclature of Achilles tendon pathology is not consistent (Lesic and Bumbasirevic, 2004; Paavola et al., 2002; Schepsis et al., 2002). However, it appears that most researchers in the field of tendon pathology seem to have adopted the classification by Puddu et al. (1976) with some modifications. With regard to the Achilles tendon injury classifications, the following terms and descriptions are currently generally accepted:

- **Injury** can be defined as "mechanical disruption of tissues resulting in pain" (Kumar, 2001)

- **Tendinitis** can be defined as an inflammation of a tendon. However, recent histopathological, biochemical and molecular studies show that true "tendinitis" is rare and the condition is perhaps more accurately described as a tendinosis (degeneration disorder) (Ashe et al., 2001; Astrom & Rausing, 1995; Khan et al., 2002; Movin et al., 1997; Paavola et al., 2002b), particularly if the condition is chronic (Alfredson et al., 2003). For acute tendinitis it is plausible that there is some transient inflammation, but that is yet to be shown (Kannus et al., 2004; Khan et al., 1999; Paavola et al., 2002). Investigations have failed to show an increased concentration of prostaglandin E_2 (a biochemical mediator of inflammation) in patients with chronic Achilles tendon pain compared to normal healthy controls (Alfredson et al., 1999). Furthermore, there is no agreed specific time criteria to classify the chronicity of tendinitis (Paavola et al., 2002), although it has been suggested that two weeks represents acute tendinitis, 2 to 6 weeks represents sub-acute tendinitis, while any duration greater than 6 weeks represents chronic tendinitis (El Hawary et al., 1997).

- **Tendinosis** can be defined as a degeneration of a tendon substance. Histologically, this degeneration can be classified as hypoxic, mucoid or myxoid, hyaline, lipoid, fibrinoid, calcific, and a combination of the two or more of the above may be found in a degenerate tendon (Jozsa and Kannus, 1997). Histopathological studies (Astrom and Rausing, 1995; Jozsa and Kannus, 1997) strongly suggest that the chronic tendon pain condition that was traditionally classified as tendinitis, is more accurately defined as tendinosis. However, this does not imply that the entity, tendinitis does not exist, at least in the acute stage of tendon adaptation to micro-injury or overloading (Kannus et. al., 2004). Clinically, tendinosis may be, and frequently is, asymptomatic (Maffuli et al., 1998). Furthermore, it is not clear that the disorder is purely degenerative in nature, and therefore some authors prefer the use of the term "tendinopathy" (Almekinders and Temple, 1998).

- **Tendinopathy** is a non-specific encompassing term that implies that there is an underlying pathology in and around the tendon (Ashe et al., 2001). It is often due to tendinosis but can include paratenonitis. This is because it is often difficult to differentiate between tendinosis and paratenonitis on a clinical basis (Paavola et al., 2002). Furthermore, several pathologic conditions of the Achilles tendons may co-exist (Schepsis et al., 2002), the most common of which are paratenonitis and tendinosis. The cardinal feature in tendinopathy is chronic pain, which has been referred to as "Achillodynia" (Movin et al., 1997; reviewed in Paavola et al., 2002). However, some authors include tendon rupture within the overall diagnosis of tendinopathy (Koike et al., 2004; Riley, 2004). However, in this thesis Achilles tendon rupture and tendinopathy are treated as two separate conditions.

The chronicity of Achilles tendinopathy is also a subject of debate and uncertainity, with the duration of symptoms of more than 6 weeks (Kader et al., 2002; Schepsis et al., 2002) or more than 2 months (Blankestein et al, 2001) being regarded as chronic. However, it is generally accepted that it takes a long time (certainly longer than 6 weeks in the majority of cases) to treat chronic Achilles tendinopathy and many cases of painful tendinopathy persist for months, sometimes even for years (Cook et al., 2003; Riley, 2005; Schepsis et al., 2002). For the purposes of this thesis, the duration of chronic Achilles tendinopathy has been defined as symptoms lasting at least 6 months. It is important to note that tendinopathy is a clinical diagnosis which can be confirmed using imaging.

- **Tendon rupture** can be defined as a tear of a tendon, either partially or completely. However, Smart et al. (1980) suggested that partial ruptures do not exist. These authors and others are of the opinion that what most clinicians refer to as "partial ruptures" are actually areas of focal degeneration (Gibbon et al., 1999; Smart et al., 1980). However, it is possible that cross-sectional incomplete ruptures are true ruptures while longitudinal focal tears are not. However, clinically, a history of sudden onset of severe pain is indicative of an acute tendon rupture (partial or complete), while gradual onset of pain and discomfort is the most common clinical presentation of a tendinopathy (Leppilahti and Orava, 1998; Maffulli et al., 2003; Schepsis et al., 2002).

- **Paratenonitis** can be defined as an inflammation of the connective sheath surrounding the Achilles tendon, the paratenon, which may be accompanied by infiltration of inflammatory cells and blood vessels (neovascularization). Clinically, it presents as a gradual onset of pain and swelling (Lesic and Bumbasirec, 2004). It has been suggested that the pathogenesis of

paratenonitis includes 3 stages: acute (2 weeks), sub-acute (2-6 weeks) and chronic (more than 6 weeks) (Clancy et al., 1976).

- **Bursitis** refers to an inflammation in a bursa. In the case of the Achilles tendon this refers to inflammation of either the retrocalcaneal or the subcutaneous calcaneal bursae or both (Schepsis et al., 2002).

The focus of this thesis was patients diagnosed with an Achilles tendon rupture and chronic tendinopathy. Therefore, any patient diagnosed with either paratenonitis or bursitis was excluded from this study.

1.3 HYPOTHESES FOR THE DEVELOPMENT OF ACHILLES TENDON INJURIES

Although the exact mechanisms for Achilles tendon injuries are poorly understood, three main hypotheses for the development of Achilles tendon injuries have been proposed (Fig 1.1). These include the degeneration, pathological and failure of the neuro-inhibitory mechanism hypotheses.

1.3.1 Degeneration hypothesis

In the degeneration hypothesis, the tendon is believed to be exposed to repetitive mechanical loads, which result in microscopic damage. However, the tendon is unable to repair fully, leading to chronic tendinitis (an inflammatory process) or tendinosis (tendon degeneration). This results in a weak, inflexible and abnormal tendon, which can be painful and impair sports performance (Paavola et al., 2002). When this weak tendon is exposed to further repetitive mechanical load it fails to adapt and eventually ruptures. Thus, according to this hypothesis, the rupture is preceded by a structural weakening of the tendon. These ruptures are believed to contribute the majority of Achilles tendon injuries (Kannus and Jozsa, 1991). There is strong convincing evidence that the majority of Achilles tendon ruptures occur via this mechanism (Arner et al., 1959; Gibson, 2001; Kannus and Jozsa., 1991; Kainberger et al., 1997; Tallon et al., 2001). Tallon et al., 2001 have shown that ruptured tendons are more degenerated than tendinopathic tendons, supporting the notion that tendinopathy precedes a tendon rupture, although this is not the case for all tendon ruptures. Furthermore, it has been documented that energy-storing tendons such as the Achilles and patella tendons are more frequently injured than positional tendons such as tibialis anterior tendon (Smith et al., 2002). However, there have been some views not supporting this hypothesis. It has been

documented that most of the Achilles tendon ruptures and chronic tendon injuries are unilateral (Inglis et al., 1976; Williams, 1986). Also, it is not clear as to the reason why the majority of patients who had experienced an acute Achilles tendon rupture have had no previous signs and symptoms of Achilles tendon pathology (Inglis et al., 1976). It has been documented that the majority of individuals with AT rupture do not experience warning symptoms prior to the injury (Kannus and Jozsa, 1991).

It must be noted that most tendinopathies are probably a result of many factors, and the degenerative process that in most cases precedes tendon rupture may be a result of a variety of pathways and aetiological factors (Riley, 2004). Such pathways may include increased apoptosis (Murrell, 2002), tissue hypoxia (Arner et al., 1959), persistent release of various biochemical factors such as cytokines, growth factors, matrix degrading enzymes and their inhibitors, pain-inducing modulators (Riley, 2004). It has also been suggested that primary inflammatory insults may be present at the early phases of tendinopathy and may instigate a cascade of events leading to a degenerate tendon (Barr and Barbe, 2004; Kannus et al., 2004).

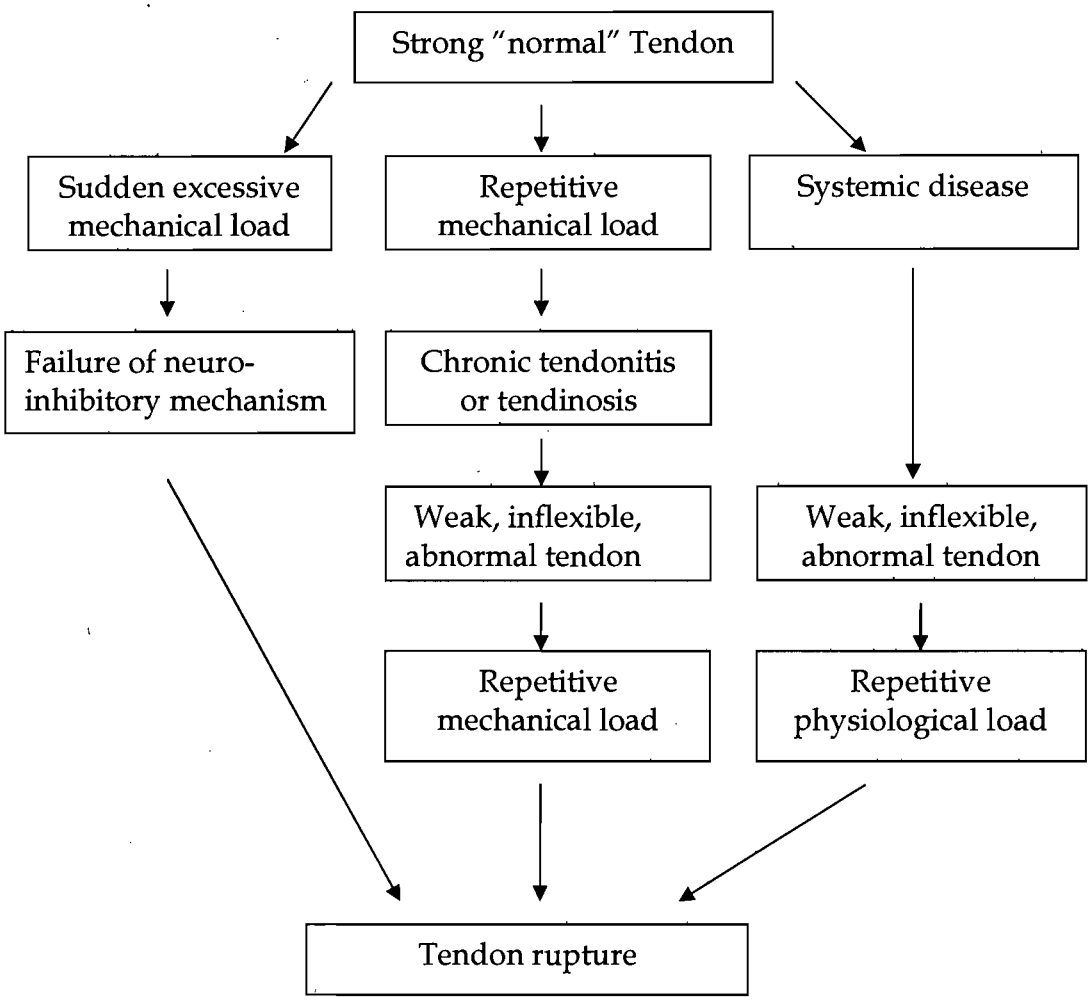

Fig. 1.1 Schematic diagram showing the three traditional hypotheses from left to right, (i) failure of the neuro-inhibitory mechanism, (ii) degeneration and (iii) pathological hypotheses, for the development of Achilles tendon injury

1.3.2 Pathological hypothesis

In the second hypothesis, systemic disease(s) through yet largely unknown pathological mechanism(s), weaken the tendon. When the tendon is then exposed to repetitive physiological mechanical load it can cause injury including rupture. A detailed list of systemic diseases that have been associated with the development of AT injuries have been extensively described (Josza and Kannus, 1997; Riley, 2004). These systemic diseases associated with the development of AT injuries can be broadly classified into inherited, endocrine, metabolic and rheumatoid diseases. Some of the most common inherited systemic diseases that may be associated with AT injuries include Marfan's syndrome, Ehler's Danlos syndrome, Osteogenesis imperfecta, and lipid storage diseases. Endocrine and metabolic diseases that may be associated with AT injuries include diabetes mellitus and renal diseases, while rheumatoid diseases include rheumatoid arthritis and gout (Jozsa and Kannus, 1997). The ruptures associated with systemic diseases are known as pathological ruptures. Pathological ruptures have been reported to account for only a small proportion of all Achilles tendon ruptures (Jozsa and Kannus, 1997; Kannus and Jozsa, 1991). In a comprehensive study of tendon ruptures, which included 292 individuals who had sustained Achilles tendon ruptures, only 23 (7.8%) had a previous history of systemic disease (Jozsa et al., 1989).

1.3.3 Failure of the Neuro-inhibitory mechanism hypothesis

In the third hypothesis, it is suggested that a normal tendon ruptures when it is exposed to a sudden excessive load that it cannot withstand (William, 1986). These are referred to as acute traumatic ruptures. In this case, prior weakening of the tendon due to degeneration is not a requirement. In addition, the inhibitory neuroprotective (golgi tendon organs) mechanisms are overcome by the higher than normal sudden and excessive loading on the tendon.

From the above hypotheses, it is generally assumed that the tendon is "normal" (starting point, see Fig. 1.1). However, in this thesis, the possibility that due to the genetic background, some individuals may have tendon structure and function that is not able to withstand repetitive or acute mechanical loads as a result of their genotype will be explored. This suggests that genetic factors may, at least in part, determine the adaptation to the different mechanical and other stimuli, making the susceptibility to tendon injury variable amongst individuals. This hypothesis will be further explored in this review of the literature and in this thesis.

1.4 BRIEF OVERVIEW OF THE EPIDEMIOLOGY OF ACHILLES TENDON INJURIES

It has been stated that between 30 to 50% of all sports injuries are overuse injuries (reviewed in Jarvinen, 1992), of which between 6 to 18% are overuse Achilles tendon injuries (Mazzone and McCue, 2002). Both acute and overuse tendon injuries are common in competitive and recreational sports (Jozsa et al., 1989). Amongst all tendon injuries, AT injuries are the most commonly associated with sports or physical activity (Jozsa et al., 1989). Individual national sports cultures and traditions can influence the nature and patterns of sports injuries in each country. For instance, in Canada and many European nations, soccer, badminton and skiing are responsible for the majority of all AT injuries (Cetti and Christensen, 1981; reviewed in Jarvinen, 1992; Jozsa et al., 1989; Kellam et al., 1985; Nillius et al., 1976;), while in the United States of America, basketball is associated with Achilles tendon ruptures (Inglis and Sculco, 1981). In South Africa, Pinshaw et al. (1984) showed in a case series study of 196 running injuries, that tendinopathy was the fifth most common injury among runners. Within the tendinopathy group, injury to the Achilles tendon was reported to be the most common (Pinshaw et al., 1984). The precise quantification of the occurrence of AT injuries has however not been well established. This is because the terms incidence (a descriptive measure of new occurrences during a specified period) and prevalence (a measure of the presence of disease or condition at a specified period or point) have frequently been used interchangeably.

The majority of the data on the epidemiology of Achilles tendon injury has been published specifically on Achilles tendon rupture. This is possibly because it was easier to document an acute rupture that in most cases mandated hospitalization as opposed to an Achilles tendinopathy. Therefore, the following sections will focus on

Achilles tendon rupture to demonstrate the epidemiology of Achilles tendon injury. There are similarities and differences in the nature of these two separate injuries that are highlighted on Table 1.1. Briefly, an Achilles tendon rupture has an acute onset, while an Achilles tendinopathy has a gradual onset (Schepsis et al., 2002). It has been suggested that a rupture is associated with ball games, while an Achilles tendinopathy is associated with running. Also, it has been suggested that Achilles tendon ruptures are mostly associated with extrinsic factors, while Achilles tendinopathy often involves the interaction of both intrinsic and extrinsic fators (Jarvinen et al., 2005).

It appears that there might be a stronger indication for involvement of genetic factors in Achilles tendinopathy than in ruptures. In a 8-year follow-up study of patients who had sustained an Achilles tendinopathy and operated on, it was found that unilateral injury strongly predicted future contralateral leg injury (Paavola et al., 2002). However, it was also found that previous unilateral rupture also predicted future rupture in the other leg (Aroen et al., 2004), but the proportion of individuals sustaining a bilateral Achilles tendinopathy is far larger than those sustaining Achiles tendon rupture. The majority of individuals sustaining an Achilles tendon injury are males.

Table 1.1 Comparison of the epidemiology of Achilles tendinopathy and Achilles tendon rupture

Factor	Achilles tendinopathy	Achilles tendon rupture
Onset	Gradual	Sudden
Activity	Extrinsic and intrinsic factors interact	Extrinsic factors predominate
Sports	Predominantly running	Predominantly ball games
Prevalence	55-65% of AT injury	3% of AT injury
Gender	Males predominate	Males predominate
Age	Peak 4th decade	Peak 4th decade
Genetic predisposition	Bilateral injury common	Bilateral injury rare
Genetic Predisposition	Unilateral injury may predict future injury in the contralateral leg[a]	Unilateral rupture may predict higher risk for future rupture in the contralateral leg[b]

[a]Paavola et al., 2002a; [b]Aroen et al., 2004

1.4.1 What is the incidence, prevalence and the trend of Achilles tendon injury?

It appears that the incidence of AT injuries has increased in European industrialised countries since the late 1950s. There is some evidence that the number and rate of Achilles tendon injuries has increased in the last few decades (Barfred, 1973; Jarvinen, 1992, Jozsa and Kannus, 1997; Leppilahti et. al., 1996; Maffulli et. al., 1999). In Hungary, the number of patients who sustained Achilles tendon ruptures increased 285% in males and 500% in women between two successive 7-year periods

(Jozsa and Kannus, 1997), while in Finland the incidence increased from 2 per 100,000 in 1986 to 12 per 100,000 on 1994 (Leppilahti et al., 1996). In Scotland, the overall incidence of AT rupture increased from 4.7 per 100,000 in 1981 to 6 per 100,000 in 1994 (Maffulli et al., 1999).

1.4.2 When does Achilles tendon rupture occur in life?

In the majority of studies, the peak incidence of Achilles tendon rupture occurs during the fourth decade of life (Table 1.1). However, two studies have reported another peak during the eighth decade (Maffulli et al., 1999; Moller et al., 1996). Therefore, this suggested that the peak incidence of Achilles tendon rupture is bimodal, with the first peak associated with overuse injuries while the latter peak is associated with ageing-induced degeneration (Maffulli et al., 1999; Moller et al., 1996).

1.4.3 Who is most affected by Achilles tendon ruptures?

Achilles tendon rupture affects predominantly middle aged people 30-50 years old (Mazzone and McCue, 2002; Nillius et al., 1976; Noble, 1991), mostly with white collar or sedentary occupations. Athletes in running sports predominantly experience overuse injuries (Mazzone and McCue, 2002), whereas sports or actions that includes abrupt repetitive jumping and sprinting, short runs and stops, pushing off and quick turns such as ball games, may result in acute injury such as Achilles tendon rupture (Jozsa et al., 1989; Leppilahti and Orava, 1998). Between 60 to 75% of all AT ruptures were related to sports in contrast to other tendon ruptures, which recorded only a 2% association (Kannus and Natri, 1997; Josza et al., 1989). The majority of AT injuries in sport were found to occur in males, presumably because higher numbers of males participate in sport compared to females (Kvist, 1994). However, it is possible that a gender difference plays a role in Achilles tendon

ruptures (Kvist, 1994). In the injury studies reviewed, the ratio of males to women has ranged from 2:1 to 18:1 (Table 1.1)

1.4.4 How does Achilles tendon injury occur?

Majority of Achilles tendon ruptures occur spontaneously during a sporting activity or through falling, tripping and push-off, with only very few resulting from trauma or violent forces (Jozsa et al., 1989). Many of these sports involve abrupt repetitive jumping and sprinting movements, such as downhill skiing, soccer, basketball, tennis, squash, badminton and tennis. In one study, the majority (88%) of acute AT ruptures had occurred in ball games (Leppilahti et al., 1996). In addition, the majority (70%) of athletes were recreational (Leppilahti et al., 1996). However, it must be noted that professional or competitive athletes constitutes a small number of participants in any sport. A large proportion (80-90%) of the affected individuals with Achilles tendon ruptures report no previous warning symptoms before the rupture (Jozsa et al., 1989; Beskin et al., 1987). About 83% of the Achilles tendon rupture occurs 2 to 6 cm above calcaneal insertion (Jozsa et al., 1989). The onset of an AT rupture is sudden, while in Achilles tendinopathy, the onset is gradual (Jozsa and Kannus, 1997).

1.4.5 Why does Achilles tendon injury occur?

The increase in AT ruptures has been attributed to (i) the increased popularity of recreational sports (Leppilahti and Orava, 1998) (ii) to the increase in sedentary lifestyle in these countries which is associated with irregular participation in physical activity or sport (Jozsa et al., 1989) and (iii) to the availability and the general awareness of health care in these countries, which in turn result in increase chance of case reporting (Beskin et al., 1987; Jarvinen, 2000; Leppilahti and Orava, 1998). Both extrinsic and intrinsic risk factors have been implicated in the aetiology

of AT rupture and chronic Achilles tendon injuries (Jarvinen et al., 2005; Riley, 2005b). However, the precise aetiogy and pathogenesis of AT rupture is still not well documented (Jarvinen et al., 2005; Riley, 2005b).

In summary and as shown in Table 1.1:

- The percentage of running-associated AT ruptures is only less than 8%
- The dominant peak incidence of AT ruptures occurs during the 4th decade of life
- Most AT ruptures are sustained by men
- Majority of AT ruptures are associated with sports
- The sporting culture of different nations influences the distribution of AT ruptures association with specific sports.

Table 1.2 Investigative studies on the epidemiology of Achilles tendon rupture[a]

Author	Male:Female ratio (n) $Year_0$ - $Year_f$	Peak Incidence[b] (mean age)	Incidence (per 10^5)	City, Country	Major Sport(s)	%Sport	%Running
Houshian et al., 1998	3:1 (718) **1984-1996**	4th (42.1)	37	Ribe County, Denmark	Badminton (46.3%)	74.2%	0.9%
Levi et al., 1997	2.9:1 (209) **1978-1995**	4th (41)	33	Copenhagen, Denmark	Badminton (49.7%)	N/A	2.1%
Maffulli et al., 1999	1.7:1 (4201) **1980-1995**	4th, 8th (30)	8	Scotland	N/A	N/A	N/A
Moller et al., 1996	6:1 (153) **1987-1991**	4th, 8th (37)	30	Malmo, Sweden	Badminton (50%), Soccer (24%)	64%	<4%
Leppilahti et al., 1996	5.5:1 (110) **1979-1994**	4th	18	Oulu, Finland	Volleyball (24%) Badminton (22%) Soccer (17%)	81%	6%
Jozsa et al., 1989	4:1 (292) **1972-1985**	4th (35.2)	N/A	Budapest, Hungary	Soccer (33.5%) Basketball (33%)	59.2%	8.1%
Nillius et al., 1976	7:1 (229) **1950-1973**	5th	10	Malmo, Sweden	Badminton (28%), Soccer (26%)	59%	4.4%

Table 1.2 (continued). Investigative studies on the epidemiology of Achilles tendon rupture[a]

Author	Male:Female ratio (n) $Year_0$ - $Year_f$	Peak Incidence[b] (mean age)	Incidence (per 10^5)	City, Country	Major Sport(s)	%Sport	%Running
Arner & Lindholm, 1959	6.1:1 (92) **1940-1957**	4th	N/A	Stockholm, Sweden	Running (N/A) Soccer (N/A) Handball (N/A)	N/A	N/A
Rantanen et al., 1993	3.9:1 (39) **1980-1991**	5th (46)	2	Salo, Finland	Volleyball (33%)	64%	N/A
Cretnik and Frank, 2004	18:1 (116) **1991-1996**	4th (37)	7	Maribor, Slovenia	Soccer (31%) Basketball (11%) Tennis (9%)	65%	N/A
Suchak et al., 2005		4th (41.4)	8	Edmonton, Canada	Soccer (12%) Volleyball (10%)	78.6%	N/A

[a]All the studies investigated Achilles tendon rupture only.

[b]Peak incidence is recorded in decades

N/A, data not available

$Year_0$ - $Year_f$, the duration of data collection from year 0 to the final year of study

%Sport, The percentage of subjects sustaining injury during sporting activity

%Running, The percentage of sports-related ruptures that occured during running.

1.5 BRIEF OVERVIEW OF HUMAN TENDON STRUCTURE AND FUNCTION

The following section will review the macroscopic, microscopic, and biochemical structure and function of healthy and injured tendons. The specific features of the Achilles tendon such as blood supply and innervation will also be reviewed.

1.5.1 Macroscopic anatomy of normal human tendons

Tendons are anatomic structures joining muscles to bones and function as force transducers allowing joint movement (Benjamin and Ralphs, 1996). They are able to withstand large mechanical loads, in the form of tensile, compressive and shear stress (Kannus, 2000). The main body of the tendon is usually exposed to tension, while the sites where tendon pulleys or bony prominences are located also experience compression and shear forces. Therefore, the structural composition of a tendon at these sites differs slightly. In general the area experiencing tensile stress being more fibro-elastic (Kannus, 2000), while the area experiencing compression or shear stress being fibrocartilaginous (Smith et al., 2002). Because the focus of this thesis is to identify genetic factors which encode for proteins in the predominant fibro-elastic portion of the tendon, the structure and function of this portion will be emphasized in the review.

It should be noted that tendons are part of the complex muscle-tendon unit (MTU) and therefore their function are interlinked to the muscle function (Benjamin and Ralphs, 1996). Tendons are relatively non-elastic, but provide substantial contribution to the storage and release of passive elastic energy of the MTU (Fukunaga et al., 2002). Also, it should be borne in mind that although tendons are largely inextensible, they are able to stretch (Benjamin and Ralphs, 1996). At rest the tendon fibers exhibit a wavy configuration or crimp, but straighten under mechanical loading (Jozsa and Kannus, 1997).

The area where the muscle and tendon join is called the myotendinous junction (MTJ), while the area where the tendon joins the bone is called the osteotendinous junction (OTJ) (Jozsa and Kannus, 1997). The detailed structure of the MTJ and OTJ are specialized and beyond the scope of this review. However, the structure of MTJ and OTJ has been extensively reviewed (Jozsa and Kannus, 1997). Macroscopically, healthy tendons appear "brilliant white" in colour and are fibro-elastic in texture (Jozsa and Kannus, 1997; Kannus, 2000). They appear in many shapes and differ in the way they attach to bone (Kannus, 2000). They are covered by many layers, which may be fibrous sheaths or retinacula, reflection pulleys, synovial sheaths, paratenon and bursae (Kannus, 2000).

Specifically, the Achilles tendon is formed by the conjoined tendon of the gastrocnemius and soleus muscles (Maffulli, 1999). Its length of the gastrocnemius component of the Achilles tendon ranges from 11 to 26 cm and the soleus component ranges from 3 to 11 cm (Jones, 1998; reviewed in Maffulli, 1999). Unlike most tendons, which are enclosed in a sheaths, the AT is wrapped in a paratenon (which is not a true sheath) (Jozsa and Kannus, 1997). A retrocalcaneous bursa lies between the Achilles tendon and the calcaneus, while a subcutaneous bursa may be found between the tendon and the skin (Jozsa and Kannus, 1997). These bursae are useful in reducing the tendon friction as it glides during movement. Associated with the Achilles tendon is the plantaris tendon that lies between the gastrocnemius and soleous muscles (Invaco et al., 1987).

The blood supply to the Achilles tendon comes mainly from the posterior tibial artery and the vessels enter via three sites. These are the musculotendinous (MTJ) and oseteotendinous junctions (OTJ), and the paratenon (Carr and Norris, 1989).

The nerves to the Achilles tendon are received via fascicles occurring subcutaneously, mainly originating from the sural nerve, and via nerves supplying the attaching muscles (reviewed in Bjur et al., 2005). However, the exact pattern of distribution of nerve supply within the Achilles tendon is currently unknown (Bjur et al., 2005).

1.5.2 Microscopic anatomy of normal tendons

Tendons consist mainly of dense regular collagenous connective tissue (Jozsa and Kannus, 1997; Riley, 2005). Tendon cells, tenocytes and tenoblasts, are embedded and anchored in the collagenous tissue. The essential feature of connective tissue that distinguishes it from other tissues is that it consists of relatively fewer cells that are separated by an abundant extracellular matrix (ECM) (Junqueira et al., 1998). In fact, the major constituent of connective tissues is the ECM. The ECM consists of fibrous proteins (collagen and elastin), non-fibrous proteins (glycoproteins), ground substance and tissue fluid. The major macromolecules of the ground substance are proteoglycans and glycosaminoglycans (GAGs).

Tendons are made predominantly of type I collagen, which is organized in a complex hiearchical structure from soluble tropocollagen molecules, which aggregate to form insoluble collagen molecules (which are cross-linked tropocollagen molecules) (Jozsa and Kannus, 1997). The collagen molecules then form collagen fibrils (Figure 1.2). A group of collagen fibrils aggregate to form collagen fibers, which in turn are grouped to form primary (subfascicle), secondary (fascicle) and tertiary fiber bundles of tendon (Figure 1.2). The primary, secondary and tertiary fiber bundles are covered by a layer of connective tissue called endotendon, while the whole tendon is covered by another layer of connective tissue called epitenon (Jozsa and Kannus, 1997).

1.5.2.1 Tendon Cells

The main resident cell types found in tendons are a special type of fibroblasts known as tenoblasts (active) or tenocytes (quiescent) (Junqueira et al., 1998). These cells are responsible for the synthesis and degradation of the tendon matrix, a process called matrix turnover (Jozsa and Kannus, 1997). This is a normal process in response to tendon loading as a result of exercise, mechanical strain and injury. The matrix is constantly being remodeled to keep the balance between synthesis and degradation at homeostasis, therefore maintaining the correct matrix composition, tenocytes numbers and amount of tissue fluid.

The tenocytes are predominantly flat and elongated in shape and have elongated nuclei, just like other fibroblasts (Junqueira et al., 1998). They are usually found sparsely distributed in normal tendons, between the collagen fibrils (Jozsa and Kannus, 1997). Although sparsely distributed, they have been reported to communicate effectively via gap junctions (Banes et al., 1999; Mc Neilly et. al., 1996). These gap junctions are found at the end of the tenocytes cellular processes. The tenocytes use the gap junctions to communicate both longitudinally and laterally. Although relatively fewer (about 10%), other cell types such as chrondrocyte-like cells, capillary endothelial cells, smooth muscle cells, nerve cells and mesenchymal cells are also found in tendons (Jozsa and Kannus, 1997).

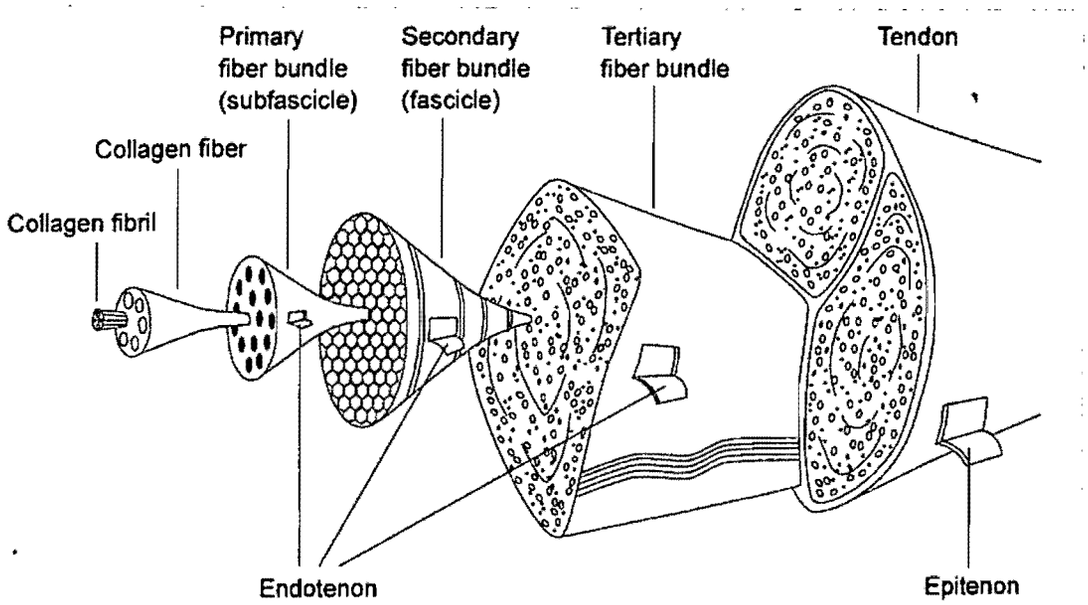

Figure 1.2 The hierarchical organization of a tendon structure (taken from Jozsa and Kannus, 1997).

1.5.2.2 Tendon Proteins

For the purpose of this thesis, the proteins found in tendons will be categorized into the collagen and non-collagen proteins. However, functional classifications will be highlighted where appropriate.

1.5.2.2.1 Collagen proteins in normal tendons

Tendons consist predominantly of the protein, collagen, constituting about 65-80% of the total dry mass of tendon protein content (Kannus, 2000). It has been stated that collagen is the most abundant protein found in most mammalian bodies (Fuller and Shields, 1998). Briefly, collagen is made up of three polypeptide alpha chains, which could all be identical (forming a homotrimer) or might be constituted from two or three different alpha chains (forming a heterotrimer). The collagen molecule formed from the 3 alpha chains is a right-handed triple helix.

To date, forty two different vertebrate alpha chains have been identified and it is these alpha chains that combine in different ways to produce the 27 different collagen molecules that have been identified to date (Riley, 2005b). Each alpha chain contains one or more typical repeated amino acid motif Gly-X-Y of variable length, where X and Y are usually proline and hydroxyproline. The collagens also contain globular, non-collagenous domains located at variable positions, of different sizes and numbers (Myllyharju and Kivirikko, 2001).

In tendons, the major collagen type is type I collagen, which is believed to constitute up to 95% of the total collagen in tendon (Riley et al., 1994; reviewed in Riley, 2004). The next most abundant collagen is type III, constituting approximately 3%, at least in human supraspinatus and biceps brachii tendons (Riley et al., 1994). Other collagens found in relatively smaller concentrations are types IV, V, VI, XII and XIV, but their exact proportions in tendons are not known (Riley, 2005b). In addition, collagen type II, IX, X and XI are found at the fibrocartilagenous tendon insertion and other areas subjected to compression forces such as pulleys and osteotendinous region (Waggett et al., 1998; Fukuta et al., 1998).

Table 1.2 shows the 11 different types of collagens found in tendons to date and their various functions. These collagens can be classified and have been grouped accordingly, into (i) fibril forming, (ii) fibril-associated collagens with interrupted triple helix (FACIT), (iii) meshwork forming and (iv) beaded filament forming (Myllyharju and Kivirikko, 2001; Riley, 2005). Types I, II, III, V and XI collagens are fibril-forming. The collagens found on the surface of fibrils (FACIT) are type XII, XIV. Types IV and X form extensive meshwork of fibers, while type VI forms beaded microfilaments (Riley, 2005). However, tendons require at least both fibril-forming collagens to maintain its structure and other types of collagens for other regulatory processes such as fibrillogenesis, ECM modulation and fibril

growth. Precisely which one collagen type dominates in content is dependent on its functional demands.

In the core of the tendon, which experiences predominantly tensile stress, the major fibril forming collagens are type I (about 95% of the total collagen and 60% of the dry mass of tendon tissue) and to a lesser extent types III (about 3%) and V (Riley, 2004). Type III collagen forms thinner fibrils, often associated with repair of damaged tendons (Jozsa and Kannus, 1997; reviewed in Riley, 2004). Type V forms heterotypic fibrils with type I collagen and is believed to regulate the diameter and tensile strength of fibrils (Birch et al., 1990; Birch, 2001), however, the detailed role of type V collagen will be discussed in Chapter 3. In the predominantly fibrocartilagenous insertion region the fibril forming collagen type II is the major collagen molecule, while type XI is a minor structural component (Waggett et al., 1998).

The collagens of the FACIT group, type IX, XII and XIV are found at the surface of the type I collagen fibrils, especially at the fibrocartilaginous insertion region (reviewed in Riley, 2005b). They are believed to mediate interactions between cells and matrix. Meshwork forming collagens, type IV and X are restricted to the basal membranes of the tendon cells, blood vessels and fibrocartilaginous portion of tendon (Fukuta et al., 1998; Myllyharju and Kivirikko, 2001). Type VI collagen is the only beaded filament forming collagen found in tendons (Riley, 2005b). It is found at both the core and fibrocartilagenous regions (Waggett et al., 1998). However, in the core region of the tendon it is associated with type I collagen fibrils, while in the fibrocartilagenous region it is predominantly found associated with cells (Waggett et al., 1998). Type VI collagen is believed to play a role in binding cells to components of the ECM such as hyalorunan, decorin and fibrillar collagens (reviewed in Waggett et al., 1998).

Table 1.3 Descriptive summary of the location and function of the 11 different collagen types found in a tendon

Collagen	Location	Function
I[a]	Mid-portion (about 95% of total collagen)	Forms larger and organized fibrils within the tensile region
II[a]	Insertion	Forms fibrils within the compressed region
III[a]	Mid-portion (about 3% of total collagen)	Forms thinner and less organized fibrils within the tensile region
V[a]	Mid-portion	Forms heterotypic fibrils with type I collagen, fibrillogenesis and modulation of fibril growth
XI[a]	Insertion within the core of type II fibrils	Forms heterotypic fibrils with type II collagen, fibrillogenesis
IX[b]	Insertion, associated with the surface of type II fibrils	Mediates cell-matrix interactions with type II collagen fibril surface
XII[b]	Insertion, associated with the surface of type I fibrils	Modulation of ECM interaction with type I fibrils
XIV[b]	Insertion, associated with the surface of type I fibrils	Modulation of ECM interaction with type I fibrils
IV[c]	Basement membrane of blood vessels	Forms meshwork
X[c]	Insertion, associated with the cells	Forms meshwork
VI[d]	Insertion, associated with cells as well as the core, associated with the type I fibrils	Forms beaded filaments (sheet-like structure)

[a]Fibril-forming collagens, [b]Fibril-associated collagens with interrupted triple helices (FACIT), [c]Hexagonal network (meshwork)-forming, and [d]Beaded filament-forming collagens. ECM, Extracellular matrix (adapted from Riley, 2005b).

1.5.2.2.2 Non-collagen proteins in normal tendons

Two major classes of non-collagen proteins are found in tendons. These are proteoglycans and glycoproteins. These proteins have greater turn-over rates than collagen proteins.

1.5.2.2.2.1 Proteoglycans and their associated glycosaminoglycans in normal tendons

Proteoglycans are composed of a core protein (about 5%) covalently bound to linear (unbranched) polysaccharide chains (glycosaminoglycans or GAGs) (95%) (Junqueira et al., 1998). The polysaccharides (GAGs) are made up of repeated chains of disaccharides, which are highly hydrophilic, enabling the ECM to absorb water and swell (Fuller and Shields, 1998). This hydrophilic property permits the ECM to withstand large compressive and shear forces (Kannus, 2000). The GAGs are also important for stabilizing the collagenous tissue and help maintain ionic homeostasis and collagen fibrillogenesis (Kannus, 2000).

The most common GAGs found in tendons include dermatan sulphate, chondroitin sulphate, heparan sulphate and hyaluronic acid (Koboyashi et al., 1999). The presence of keratin sulphate and heparin has not been demonstrated in tendon tissue (Kannus, 2000). The most abundant GAG in the fibro-elastic region of the tendon is dermatan sulphate (60% of the GAG content), while the insertion region is dominated by chondroitin sulphate (Kannus, 2000). Compared to other tissues such as cartilage, healthy tendons contain very little amount of GAGs (about 0.2% dry mass of tissue versus 3.5 - 5% in cartilage) (Kannus, 2000).

There are two groups of extracellular matrix (ECM) proteoglycans; the small leucine-rich repeat proteoglycans (SLRPs) and the large modular proteoglycans

(Riley, 2005a). The large modular proteoglycans are sub-divided into the hyalectans (as they can bind both hyaluronan and lectin) and those that do not bind hyaluronan (Riley, 2005a). In tendons, proteoglycans include hyalectans such as aggrecan and versican as well as the SLRPs, which include decorin, biglycan, fibromodullin and lumican. The distribution of these proteoglycan in tendons varies according to the tendon function and region. The anatomical sites experiencing compression contain large proteoglycans such as aggrecan and biglycan, which have fibrocartilageneous characteristics (Waggett et al., 1998; Thomopoulos et al., 2003). On the other hand, the tensile regions contain small proteoglycans, which are leucine-rich such as decorin and fibromodulin as well as the large proteoglycan versican (Riley, 2004; Samiric et al., 2004; Wagett et al., 1998). Within the proteoglycan family, the turnover rate of hylectans is much greater than that of SLRPs (Samiric et al., 2004).

In human Achilles tendons, both mRNA and protein expression of decorin, biglycan, lumican and fibromodulin has been detected in both the mid-substance and fibrocartilagenous regions (Waggett et al., 1998). It has also been observed that versican is the major large proteoglycan found in the mid-substance of the Achilles tendon, with virtually no aggrecan, while a reciprocal observation has been made at the insertion region, which contains abundant aggrecan and very little versican (Waggett et al., 1998). Of the SLRPs, decorin is the most abundant proteoglycan in the mid-substance of the Achilles tendon (Riley, 2005a).

Proteoglycans play a role in holding water in tissues, helping tissues resist compression, lubricating fiber bundles during movement and in the sequesteration of growth factors, mediate cell-matrix interactions and enzymes in the ECM (Hardigham and Fosang, 1992; reviewed in Riley, 2005a). It has also been shown using knockout animal models of these molecules that they may be important in fibrollogenesis (Ameye et al., 2002; Jepsen et al., 2002; Vij et al., 2004).

1.5.2.2.2.2 Glycoproteins in normal tendons

Glycoproteins are protein molecules to which branched chains of monosaccharides are covalently attached (Junqueira et al., 1998). The glycoproteins found in tendons include elastin, fibrillin, tenascin-C, cartilage oligomeric matrix protein (COMP), fibronectin, laminin, thrombospondin, link protein (Riley, 2005b) and tenomodulin (Docheva et al., 2005).

Table 1.3 summarizes the functions of the different proteoglycans and glycoproteins found in tendons. Briefly, elastin is a fibrous glycoprotein found in small quantities (1-2% of the dry mass) in tendon and together with fibrillin they form intergral part of elastic fibers (Midwood and Schwarzbauer, 2002). They are believed to be responsible for providing some elasticity to tendons, therefore allowing tendon tissue to stretch and recoil without damage (Midwood and Schwarzbauer, 2002). The glycoproteins tenascin-C, COMP, fibronectin and thrombospondin function to attach cells to the matrix (reviewed in Riley, 2005b). The detailed role of Tenascin-C will be discussed further in Chapter 4. Laminin is a modular protein found in the basement membrane of cells and walls of blood vessels (Sasaki et al., 2004). Laminin is also found in large amounts at the myotendinous junction (Kannus et al., 1998) and helps in anchoring cells to the matrix mainly via intergrin connections (Sasaki et al., 2004). The interaction between proteoglycans and hyaluronan is stabilized by link protein (Jozsa and Kannus, 1997).

A new transmembrane glycoprotein, tenomodulin has been identified and characterized (Brandau et al., 2001). Tenomodulin was found to modulate tenocyte proliferation and collagen fibril maturation (Docheva et al., 2005).

Table 1.4 The non-collagen proteins found in a tendon

	Protein	Description	Function
Proteoglycan	Decorin	SLRP	Binds collagen
	Biglycan	SLRP	Binds collagen
	Fibromodullin	SLRP	Binds collagen
	Lumican	SLRP	Binds collagen
	Aggrecan	Hyalectan	Resist compression
	Versican	Hyalectan	Lubricates fibrils
Glycoprotein	Elastin	Branched network	Provides elasticity
	Fibrillin	Linear arrays	Provides elasticity
	Tenascin-C	Branched molecule	Attach cells to matrix
	COMP	Branched molecule	Attach cells to matrix
	Fibronectin	Modular protein	Attach cells to matrix
	Laminin	Modular protein	Basement membrane constituent
	Thrombospondin	Modular protein	Attach cells to matrix
	Link protein	Globular protein	Stabilizes proteoglycan-hyaluronan interaction
	Tenomodulin	Transmembrane Protein	Tenocyte proliferation and collagen fibril maturation

Legend: **COMP**, cartilage oligomeric matrix protein; **SLRP**, small leucine-rich repeat proteoglycan (Adapted from Riley, 2005b).

1.5.2.2.3 Tendon matrix regulatory enzymes in normal tendons

Although cell-mediated synthesis of collagen and non-collagen fibers is crucial for the maintenance of tendon proper structure and function, degradation of any damaged proteins in tendon tissue is equally important. Although beyond the scope of this thesis detailed reviews of regulatory enzyme found in ECM have been published (Kaushal and Shah, 2000; Riley, 2005a; Riley, 2005b). The degradation of connective tissue proteins is achieved by two families of enzymes, also produced by tenocytes, collectively known as (i) matrix metalloproteinases (MMPs) and (ii) a disintegrin and metalloproteinase (ADAM)-thrombospondin (TS) family (ADAM-TS) (reviewed in Riley, 2005a; Riley, 2005b).

The MMPs can proteolytically cleave a wide range of substrates, including collagens, proteoglycans and glycoproteins (Bramono et al., 2004). They can also act on cell surface receptors and growth factors, regulate cell proliferation, cell death and cell migration (reviewed in Riley, 2005a). There are currently 23 classified human MMPs (Riley, 2005a), which can be grouped into (i) collagenases (cleave native collagen type I, II and III), (ii) gelatinases (cleave denatured collagens and type IV collagen), (iii) steromelysins (cleave proteoglycans, type III, IV, and V collagens, fibronectin) and (iv) the membrane type MMPs (MT-MMPs) (Bramono et al., 2004). Their function is influenced by growth factor and cytokine activity (Riley, 2005a). They are also tightly regulated *in vivo* at the levels of messenger RNA and protein synthesis, activation and inhibition (Riley, 2005a). Generally, the activity and expression of MMPs is low in normal tissue and have been detected in ligaments, tendons and cartilage (Bramono et al., 2004). MMP-1 is found present in normal patellar tendon (Fu et al., 2002), while in normal supraspinatus and biceps brachii tendons MMP-1, 2 and 3 are expressed (Riley et al., 2002). However, the expression of all these MMPs was higher in supraspinatus tendons compared to biceps brachii tendons, emphasizing the different demands of these tendons (Riley et al., 2002). MMP-2,

3, 14, 16, 19 have been shown to be expressed in normal Achilles tendons using cDNA arrays (Ireland et al., 2001). Surprisingly, MMP-1 expression was not detected in normal Achilles tendon tissues using this technique (Ireland et al., 2001).

As it can be imagined the continued degradation of the matrix can be detrimental to tissues, therefore the MMPs activity has to be regulated. The specific inhibitors of MMP activity are enzymes (also produced by resident tendon cells) known as tissue inhibitors of metalloproteinases (TIMPs) (Murphy et al., 1995). To date, four TIMPs, namely TIMP-1 to TIMP-4 have been identified (Riley, 2005a). The expression of three (TIMP-1 to TIMP-3) of the four TIMPs in normal Achilles tendon tissue sample has been detected using cDNA arrays (Ireland et al., 2001).

Another group of enzymes with proteolytic activity predominantly for non-collagen proteins (proteoglycans and glycoproteins) in connective tissue are known as a disintegrin and metalloproteinase (ADAM)-thrombospondin (TS) family, of which 19 have been classified (reviewed in Riley, 2005a). The most commonly studied members of this group are the aggrecanases, known for cleaving the proteoglycan aggrecan (reviewed in Riley, 2005a, Riley, 2005b).

1.5.2.3 Tendon Tissue Fluid

Normal tendon contains about 55-70% water (Kjaer, 2004), which is essential in the hydration of the ECM and serves as a medium for water-soluble substrates providing nutrients to cells. Tissue fluid also contains inorganic compounds constituting less than 0.2% of the dry mass of the tendon (Kannus, 2000). The most abundant inorganic compound in the tendon is calcium, which is higher at the insertion area compared to the tensile region of the tendon. Other inorganic components include magnesium, manganese, cadmium, cobalt, copper, zinc, nickel, lithium, lead, fluoride, phosphor and silicon (reviewed in Kannus, 2000).

These molecules are important in growth, development and normal tendon metabolism. For instance, the activity of the MMPs requires the presence of bivalent cations (Bramono et al., 2004). Collagen cross-linking requires copper, while manganese is required for a variety of enzymatic reactions (reviewed in Kannus, 2000). However, the detailed function of the inorganic component of the tendon matrix is relatively under-investigated (Kannus, 2000).

1.5.3 Pathology of injured tendons

1.5.3.1 Macroscopic anatomy of injured tendons

Through a naked eye observation, a tendinopathic tendon appears grey and armophous, having lost their glistening white colour (Kannus and Jozsa, 1991; Jozsa and Kannus, 1997). When palpated, the tendon feels tender, appears swollen and thicker (Kannus and Jozsa, 1991). A summarized comparative description of both a normal and injured tendon is showed in Table 1.5.

1.5.3.2 Microscopic anatomy of injured tendons

The characteristic histopathologic features of Achilles tendinopathy are tendon degeneration, disordered arrangements of collagen fibers, increased vascularity together (Astrom and Rausing, 1995). Further review of Achilles tendon imaging will be discussed in chapter 6. The histopathology of Achilles tendon rupture is similar to Achilles tendinopathy (Tallon et al., 2001). However a recent study has shown that ruptured Achilles tendons exhibit more degenerate features than tendinopathic tendons (Tallon et. al., 2001). This suggests that Achilles tendinopathy may preceed a rupture.

Achilles tendon degenerative change exhibits increased signal intensity on magnetic resonance imaging (MRI) and hypoechoic areas under soft tissue

diagnostic ultrasonography (Astrom et al., 1996). It is believed that these areas of abnormal imaging correspond to areas of disorganized collagen fibers and increased ground substance (Jozsa and Kannus, 1997). Light microscopy confirms that collagen fibers that are thinner than normal (Josza and Kannus, 1997). There is also visible evidence of longitudinal splitting of collagen fibers while some of the fibers show angulation (Kannus and Jozsa, 1991). Intrafibrillar mucoid patches and vacuoles are also present (Kannus and Jozsa, 1991). In tendinopathy, mitochondria and the nuclei of tenocytes both show altered size and shape. The tenocytes and mitochondria accumulate calcium deposits internally (Kannus and Jozsa, 1991). There is pronounced rough endoplasmic reticulum (RER) presence, indicating active protein production/synthesis (Kannus and Jozsa, 1991). Markers of an inflammatory process could not be found samples obtained from subjects with chronic Achilles tendinopathy (Ireland et al., 2001).

1.5.3.2.1 Tendon cells in injured tendons

It has been demonstrated that in chronic painful tendinopathy, there is an increased number of cells (Jarvinen et al., 1997; Leadbetter, 1992). The majority of the cells at the site of tendinopathy are tenoblasts, which have clearly changed their morphological appearance from elongated to rounded or ovoid shape (Jozsa and Kannus, 1997; Puddu et al., 1976). Many investigators have not been able to find inflammatory cells within tissue samples obtained from subjects with chronic tendinopathy (Arner et al., 1959; Astrom and Rausing, 1995; Kannus and Jozsa, 1991). However, some have observed inflammatory cells in the paratenon but not in tendon substance, when tendinopathy was accompanied by paratenonitis (Astrom and Rausing, 1995). It has also been shown that tendinopathic tendons exhibit increased number of apoptotic tenocytes compared to normal tendons in the human rotator cuff tendon and horse

superficial digital flexor tendon (Hosaka et al., 2005; Murrel, 2002; Yuan et al., 2002; Yuan et al., 2003).

Table 1.5 Summary of contrasting characteristics of a normal healthy versus a symptomatic tendinopathic tendon (adapted from Khan et al., 1999)

Technique	Normal healthy tendon	Symptomatic tendinopathic tendons
Naked eye	Appear glistening white	Appear grey and armophous
Colour Doppler Ultrasound	Inconspicuous vasculature	Increased vasculature
Microscopy	Hierarchical arrangement of tightly packed, parallel bundle of collagen fibers	Discontinuous and disorganized collagen fibers
Microscopy and polarized light	Fibers have a characteristic reflectivity	Fibers lack specific reflectivity
Microscopy and Alcian blue staining	Very little stainable ground substance	Increased stainable mucoid ground substance
Microscopy/Histology	•Inconspicuous tenocytes	•Increased number of tenocytes
	•Absence of inflammatory cells	•Absence of inflammatory cells
Avidin biotin	fibroblasts and myofibroblasts absent	Increased presence of fibroblasts and myofibroblasts

1.5.3.2.2 Tendon proteins in injured tendons

1.5.3.2.2.1 Collagen proteins in injured tendons

In spontaneously ruptured Achilles tendons the degeneration of collagen fibrils is present and has been shown to be more severe than in chronic tendinopathy (Tallon et al., 2001). There is also an increased type I and III collagen in tendinopathy, although the ratio of type I to III collagen is decreased, indicating that although both type I and III collagen content increase, the amount of type III increase is much more than type I (Jozsa and Kannus, 1997). This has been postulated to indicate a reparative process of the collagen matrix (Riley, 2004). Maffulli et al. (2000), has shown that cultured tenocytes from ruptured Achilles tendons produce more type III collagen compared to those from normal tendons. It has been shown that type III fibrils are thinner than type I fibrils and it has been shown that the thickness of collagen fibrils is associated with the tendon tensile strength (Jozsa and Kannus, 1997). Also, morphologically larger tendons consist of larger collagen fibrils in keeping with their function (Jozsa and Kannus, 1997). Type V collagen has been shown to be up-regulated in biopsy samples of degenerate human posterior degenerate tendons (Goncalves-Neto et al., 2002). Type XII collagen is increased, while the expression of type X collagen is decreased in rat healing supraspinatus tendons as detected by *in situ* hybridization (Thomopoulos et al., 2002).

1.5.3.2.2.2 Non-collagen proteins in injured tendons

1.5.3.2.2.2.1 Proteoglycans in injured tendons

The expression of four of the proteoglycans found in tendons has recently been examined in fresh human cadaver samples (Corps et al., 2006). In tendinopathy, the level of aggrecan and biglycan mRNA increased 10-fold and 5-fold,

respectively, while the level of decorin and versican did not change. In ruptured tendons, the level of decorin mRNA decreased, while aggrecan, biglycan and versican mRNA expression remained unchanged. An increase in GAG concentration has been detected in Achilles tendinopathy (Movin et al., 1997), ruptured Achilles tendons (Kannus and Jozsa, 1991; Maffulli et al., 2002), and patellar tendinopathy (Khan et al., 1996). It has also been shown that there is an increased hyalaronan and other GAGs in tendinopathy of other human tendons (Riley et al., 1996). Increased expression of versican, biglycan and perlecan, with no change in decorin has been shown using cDNA arrays (Ireland et al., 2001). In support of the human studies, changes in the expression of proteoglycans and their associated GAGs has been detected in injured rat and exercising chicken tendons (Thomopoulos et al., 2002; Yoon et al., 2003).

1.5.3.2.2.2.2 Glycoproteins in injured tendons

The distribution of elastin and fibrillin in tendon injury has not fully studied, however it has been suggested that tendons become less elastic and stiffer with aging (reviewed in Riley, 2004). Fibronectin has been found to be up-regulated in injured human and horse tendons (Williams et al., 1984; Jozsa et al., 1989; Tillander et al., 2002). However, data have showed using cDNA arrays that fibronectin messenger RNA was down-regulated in Achilles tendinopathy but up-regulated in Achilles tendon rupture (Ireland et al., 2001). In another study using cDNA arrays, up-regulation of the mRNA fibronectin subunit B (FNRB) in tissues samples obtained from subjects with chronic Achilles tendinopathy was detected (Alfredson et al., 2003). A mutation in the COMP gene result in short individuals exhibiting lax joints and early-onset osteoarthritis (Briggs et al., 1990). Laminin α4 has been shown to be up-regulated in both Achilles tendinopathy and rupture samples, while laminin β1 and laminin 37kDa types are only up-regulated in Achilles tendon rupture samples (Ireland et al., 2001). Thrombospondin 1 was not detected in both normal and abnormal (rupture and

tendinopathy) samples, while thrombospondin 2 was found to be up-regulated in both Achilles tendinopathy and rupture samples using cDNA arrays (Ireland et al., 2001). TNC has also been shown to be up-regulated in Achilles tendinopathy (Jarvinen et al., 2000) and rotator cuff tendinopathy (Riley et al., 1996). This up-regulation of TNC in degenerate tendons has been supported by cDNA arrays (Ireland et al., 2001). The glycoprotein SPARC was also found to be up-regulated in degenerated tendons using cDNA arrays (Ireland et al., 2001).

1.5.3.2.3 Tendon tissue fluid in injured tendons

Because of the increased GAG content associated with tendinopathy and rupture, there is an increase in tissue fluid resulting in swelling which is often visible to the naked eye. Also, increased calcium deposits have been detected in between or on the degenerated collagen fibrils in 3%, 7%, 5% and 4% of subjects with ruptured Achilles, biceps brachii, extensor policis longus and quadriceps group tendons, respectively, and the process leading to this deposition has been termed calcifying tendinopathy (Kannus and Jozsa, 1991). Other components of the inorganic material found in tendons have not been fully investigated.

1.5.3.3 Tendon enzymes and their inhibitors in injured tendon

In patellar tendinosis, increased MMP-1 has been detected (Fu et al., 2002). However, in ruptured tendons increased activity of MMP-1 and decreased activities of MMP-2 and -3 have been recorded (Riley et al., 2002). It was shown that MMP-3 expression was decreased in Achilles tendinopathy and almost absent in ruptured tendon tissue using cDNA arrays (Ireland et al., 2001). Surprisingly, the cDNA arrays could not detect MMP-1 expression in abnormal Achilles tendon tissues. The mRNA expression levels of the three TIMPs detected in normal Achilles tendons showed no changes in tissues samples

obtained from both Achilles tendinopathy and rupture tissue subjects (Ireland et al., 2001).

In summary, tendons contain many molecules, including collagen and non-collagen proteins. There proteins serve diverse functions within the tendon. Therefore it can be appreciated that a search for candidate genes for a disturbance in protein synthesis and degradation could involve any or several of these molecules. The expression of these proteins may differ according to the tendon function, such as at the insertion and mid-portion of the Achilles tendon. The recent molecular studies of tissues obtained normal, degenerated and ruptured tendons are continuing to enhance the available knowledge on tendon pathology. However, the limitation with samples obtained from ruptured tendons is that it is not clear whether the observed changes are the result or the cause of a rupture (Riley, 2004).

1.6. RISK FACTORS ASSOCIATED WITH ACHILLES TENDON INJURIES

As previously mentioned, the exact aetiology and pathogenesis of Achilles tendon injuries is still not fully understood. The main aetiologic factors (both extrinsic and intrinsic) associated with Achilles tendon acute and overuse injuries will be briefly reviewed and are summarized in Tables 1.6 and 1.8, respectively (as reviewed in Jarvinen, 1992; Jarvinen et al., 2001; Jarvinen et al., 2005; Kainberger et al., 1997; Maffulli et al., 2002; Paavola, 2000; Renstrom and Johnson, 1981; Riley, 2004). It is important to note that extrinsic and intrinsic factors associated with AT injuries have generally not been systematically studied. There is a distinct lack of well conducted prospective cohort studies or randomized controlled trials (RCTs) that confirm risk factors for injuries (Jarvinen et al., 2005).

In the following section, key studies that focused on identifying extrinsic and intrinsic risk factors for AT injuries will be discussed. More importantly, the level of evidence for the risk factor associated with AT injuries will be explored. For the purpose of this thesis, three categories will be used broadly to indicate the level of evidence for the association between a specific risk factor and AT injury (Wright et al., 2003; refer to Appendix 1.1).

i) **Strong evidence** will be defined as evidence from well designed RCTs, prospective cohort studies with low risk of bias, systematic reviews of RCTs and well-conducted meta-analyses of level I studies. These are level I studies.

ii) **Limited evidence** will be defined as evidence from retrospective studies, cross-sectional studies, well designed RCTs and prospective cohort studies but with high probability of bias and confounding (all level II studies), case-control studies (level III studies) and meta-analyses of level II and III studies.

iii) **Weak evidence** will be defined as evidence from case series, case reports (level IV studies) and expert opinion (level V studies).

The extrinsic and intrinsic risk factors for AT injury will now be reviewed and interpreted according to the level of evidence. Evidence for risk factors that are documented only in animal models will be classified as weak.

1.6.1 Extrinsic Risk Factors for Achilles tendon injuries

1.6.1.1 Physical activity

Anecdotal practical examples of the role of physical activity or mechanical loading as possibly the single most instigating stimulus for connective tissue homeostasis and adaptation throughout life include immobilization on the one extreme and chronic repetitive loading of the musculotendinous unit on the other (reviewed in Wren et al., 2001). Results from case series have suggested that the majority of Achilles tendon injuries (both ruptures and tendinopathies) are associated with participation in sporting activities (Arner and Lindhlom, 1959; Cretnik and Frank, 2004; Houshian et al., 1998; Jozsa et al., 1989; Kvist, 1994; Levi, 1997; Moller et al., 1996; Nillius et al., 1976; Rantanen et al., 1993; Suchak et al., 2005). The proportion of Achilles tendon injuries associated with sports participation ranged from 59 to 81% (Cretnik and Frank, 2004; Houshian et al., 1998; Jozsa et al., 1989; Moller et al., 1996; Nillius et al., 1976; Rantanen et al., 1993; Suchak et al., 2005).

The type of physical activity could also be related to the type of AT injury. In most case series, it has been suggested that Achilles tendon rupture is associated with ball and racket games (Leppilahti and Orava, 1998), while Achilles tendinopathy is more common in long distance running (Kvist, 1994). In one

study, 53% of the subjects with Achilles tendinopathy were runners (Kvist, 1994). It has also been suggested from case series that non-sports related Achilles tendon ruptures occur at a later age compared to sports-related Achilles tendon ruptures (Leppilahti et al., 1996; Houshian et al., 1998; Nillius et al., 1976). This example illustrates the possible interaction between increased age (an intrinsic factor) and physical activity (an extrinsic factor) in the development of Achilles tendon injury. The implication is that the mean age of sedentary people who experience Achilles tendon injury is higher compared to the mean age of sports people experiencing AT injuries.

The amount of physical activity is crucial in the induction of appropriate adaptation that the musculoskeletal tissue can withstand without injury. It has been shown that physical activity in the form of strenuous, non-damaging exercise results in increased collagen protein synthesis (Miller et al., 2005), while an earlier study had showed that physical activity can lead to an increase in tendon width in runners (Rosager et al., 2002). On the other hand, immobilization has been shown to result in a reduction in tendon size (Akeson et al., 1987). In an in vitro study, using canine patellar tenocytes, sustained cyclic mechanical stress induced apoptosis via loading- and calcium-dependent mechanism (Arnoczky et al., 2002). This study showed that the frequency of mechanical loading had no effect on apoptosis. Therefore, this *in vitro* study demonstrated that the amount and duration rather than the frequency of mechanical load were important in the intracellular activation of stress kinase-induced apoptosis of tenocytes. More importantly, the study showed that physical activity plays a role in cell-mediated tendon degeneration as increased apoptosis has been shown in chronic rotator cuff tendinopathy (Yuan et al., 2002).

In an animal (rat) model that mimics human rotator cuff injury, a strong causal relationship between direct exposure to physical activity and rotator cuff injury has been demonstrated (Soslowsky et al., 2000). However, the results obtained

from this model can not be directly translated and interpreted to the study of human Achilles tendon.

In one case-control study, it was shown that orienteering athletes were 10 times more likely to sustain Achilles tendinopathy than controls, supporting the notion that exercise or physical activity contributes to the development of Achilles tendon injury (Kujala et al., 1999). However, in a prospective cohort study of 69 male military recruits (mean age of 20 yrs) undergoing a 6-week basic military training, physical activity was not associated with the development of Achilles tendon injury (Mahieu et al., 2006). It must be noted that although this is a prospective study, the results may only be generalized to military training studies as they tend to recruit young and homogenous groups. The mean age of onset for chronic Achilles tendinopathy in the general population is about 40 yrs. The chronicity of the Achilles tendinopathy is also a debatable issue in the military study.

There is a general lack of RCTs and prospective cohort studies investigating increased and decreased physical activity as an independent risk factor for Achilles tendon injury in humans. Therefore, the strength of evidence that increased or decreased physical activity is an independent risk factor for Achilles tendon injury is currently limited.

1.6.1.2 Training errors

Training errors can be defined as sudden increases in training load, frequency or intensity or excessive hill training (James et al., 1978; Renstrom and Johnson, 1985; Smart et al., 1980; Kvist, 1994). Other training errors could possibly include excessive interval training and resumption of training activity after a long period of inactivity. All of these errors imply that excessive mechanical loading or improper loading technique may be a major factor in the development of Achilles

tendon injury. Kvist and Kvist (1980) suggested that training errors were the most common cause of overuse Achilles tendon injuries. However, it is not clear why only one leg usually gets injured (Inglis et al., 1987) and why only certain individuals rather than all individuals exposed to similar training loads sustain the injuries. Furthermore, the percentage of subjects who suffer from Achilles tendon injuries amongst all physically active individuals is relatively low, considering that the majority of physically active individuals are recreational athletes. It has been reported that the prevalence of major injuries is very low, while a large percentage of all sports related injuries are minor injuries (Stevenson et al., 2003).

It has also been shown that novices are more likely to get injured than experienced athletes (Stevenson et al., 2003), possibly because there is higher probability of training errors amongst novice compared to experienced athletes. Alternatively, it could be that muscle-tendon unit adaptation requires time, and novices are more likely to load the muscle-tendon unit relatively quicker than expected, without the knowledge and experience. Additionally, it is not clear why in the majority of patients there are no prodromal symptoms before the occurrence of an Achilles tendon rupture (Soldatis et al., 1997).

Although intuitive, the different training errors as risk factors for Achilles tendon injury have not been properly investigated using prospective cohort studies. The strength of evidence for training errors as independent risk factors for AT injury is largely based on the experience of medical personnel and athletes and is therefore weak.

1.6.1.3 Training surfaces

It has been suggested that running on very hard, slippery, cambered or sloping surfaces is associated with chronic Achilles tendinopathy (Kannus et al, 1989;

Schepsis et al., 2002). It has been shown that the soccer players who played more frequently on artificial turf experienced more injuries, including Achilles tendon injuries, compared to those playing on grass (Ekstrand and Nigg, 1989). However, no studies have directly linked the type of running surface to the development of Achilles tendon injuries. Although both uphill and downhill running have been implicated in the development of Achilles tendon injuries, there are no controlled studies that have investigated the association of either uphill or downhill running with Achilles tendon injury. Therefore, the level of evidence for training surface as an independent risk factor for Achilles tendon injury is weak.

1.6.1.4 Environmental conditions

Poor environmental conditions such as darkness, too cold or hot weather, high or low humidity, high altitude and strong wind have all been implicated in the development of injuries (reviewed in Jozsa and Kannus, 1997). Cold weather may be associated with an increase in local tissue viscosity and therefore may induce tissue injury, especially when the athletes do not warm up adequately before vigorous exercise. Darkness may become important in a sport such as orienteering. In his retrospective study, Kvist (1994) attributed only 2% of Achilles tendon injuries to poor environmental conditions. However, none of these environmental factors have been systematically investigated. Therefore, the level of evidence for poor environmental conditions as independent risk factors for Achilles tendon injury is also weak.

1.6.1.5 Footwear

Shoes are used to reduce impact forces, and in running, could provide mediolateral stability preventing excessive subtalar joint pronation and supination (reviewed in Jozsa and Kannus, 1997). It has been suggested that an

inadequate heel wedge may contribute to compensatory over-pronation, which is believed to be associated with Achilles tendon injuries (reviewed in Mc Crory et al., 1999). In a review article, Kvist (1994) suggested that worn midsoles, insufficient heel height, too rigid soles, shoe's lack of cushioning and inability to twist may predispose athletes to Achilles tendon injury. However, there are currently no controlled studies investigating the role of footwear on the development of Achilles tendon injury. Therefore, the level of evidence for footwear as an independent risk factor for this injury is weak.

1.6.1.6 Smoking

It has been suggested that smoking might be associated with tendon ruptures. In one retrospective study, Safran and Graham (2002) studied medical records collected over a 5-year period and found 14 patients (13 men; 1 woman) who had sustained a distal biceps tendon rupture. The investigators found that 6 of 14 (43%) patients were cigarette smokers compared to 9% in the general reference population. Although the study sample size was small, the researchers concluded that individuals who smoke were at a 7.5 times higher risk of developing a distal biceps tendon rupture. In another retrospective study of 23 patients who had sustained an acute rupture of flexor tendon, there was no association between smoking and rupture (Harris et al., 1999).

In one prospective cohort study it was documented that the risk of lower extremity injury, which included Achilles tendinopathy, was increased in smokers than non-smokers during a 12-week military training (Jones et al., 1993). However, the specific relationship between smoking cigarettes as a risk factor for Achilles tendon injury has not been investigated.

Because of the small sample sizes in both retrospective studies and the lack of prospective studies specific for Achilles tendon injury, causality between

cigarette smoking and Achilles tendon injury could not be established. Therefore the level of evidence for cigarette smoking as an independent risk factor for Achilles tendon injury is limited.

1.6.1.7 Medication and drug use

In recent years, quinolones especially fluoroquinolones antibiotics, which are used in the treatment of bacterial infections, have been found to be associated with Achilles tendon ruptures (van der Linden et al., 2003; van der Linden et al., 2002; van der Linden et al., 2001; van der Linden et al., 1999). These antibiotics are known to be toxic to tenocytes and inhibit matrix formation (Mc Garvey et al., 1996; Movin et al., 1997; Movin et al., 1998) and have been recently shown to induce apoptosis in human tendon cells in vitro (Sendzik et al., 2005). A population-based case-control study from the United Kingdom showed that between 2-6% of Achilles tendon ruptures in individuals older than 60 years were associated with quionolones (van der Linden, 2003). Therefore the level of evidence for quinolone use as an independent risk factor for Achilles tendon injuries is present but limited.

It has been suggested that the use of corticosteroids for the alleviation of pain in Achilles tendinopathy, is associated an increased risk of AT ruptures (Mahler and Fritschy, 1992; Speed, 2001; Read and Motto, 1992; Astrom and Rausing, 1995; Westlin and Astrom, 1994; Inglis et al., 1976). In animal studies corticosteroids have been shown to induce collagen breakdown (Balasubramaniam et al., 1972), although the doses used have been high compared to those use in humans (Mahler and Fritschy, 1992). Recently, it has been shown that low doses of corticosteroids were harmless to human tendon structure using flouriscopically guided injection (Gill et al., 2004). Although it has been shown *in vitro* that corticosteroids result in collagen disruption and tendon matrix degeneration, the association of corticosteroids with tendon injuries is still highly controversial

(Leppilahti et al., 1998; Kannus and Jozsa, 1997). The continued use of corticosteroids may be because of the pain-reducing effect of corticosteroids in tendinopathy and other conditions. However, Shier et al., 1996 conducted a meta-analysis of studies that investigated the association of corticosteroids and Achilles tendon injuries. These investigators concluded that there was insufficient evidence with regard to human studies to make definite conclusions. Therefore, the strength of evidence for corticosteroids as independent risk factors for the development of Achilles tendon injury is present but limited.

Several case reports have also suggested that anabolic steroids are associated with Achilles tendon injuries (reviewed in Battista et al., 2003). A case report of a 35-year old male bodybuilder who experienced asynchronous bilateral Achilles tendon rupture after 1 month of androstenediol use has been reported (Battista et al., 2003). In a randomized controlled study in rats, Inhofe et al. (1995) showed that the quality of tendon that was injected with anabolic steroids was compromised after 12 weeks of follow up. Based on the lack of controlled studies in humans, the evidence for anabolic steroid use as an independent risk factor for the development of an Achilles tendon injury is weak.

1.6.1.8 Psychological factors

It has been suggested that the likelihood of Achilles tendon injury in exercising subjects may increase during fatigue. This has been attributed to the decreased ability for coordination (Gilcreest, 1933). However, this assertion was based on the author's opinion and there have been no studies to investigate fatigue as specific risk factor for Achilles tendon rupture. Other psychological factors that have been associated with musculoskeletal injuries include being less tough-minded, decreased cognitive abilities, and life stresses (reviewed in Jozsa and Kannus, 1997; Kvist, 1994). However, there have been no controlled studies to investigate the relationship between psychological factors and tendon injuries

(Jozsa and Kannus, 1997). Therefore the level of evidence for psychological factors as independent risk factors for Achilles tendon injury is weak.

1.6.1.9 Nutrition

Because of the perceived decreased blood flow in the area 2 to 6 cm above the calcaneal insertion, it was thought that this area will be predisposed to lack of nutrients (Schmidt-Rolfing et al., 1992). The other possible alternative is the requirements for vitamin C as a cofactor for proline hydroxylase, the enzyme required for the hydroxylation of proline, an essential step in the formation of collagen cross-links (Junqueira et al., 1998). However, lack of vitamin C causes scurvy, which is characterized by ulceration of gums and haemorrhages. There has been no study investigating the possible role of nutrition on the development of Achilles tendon injury. Therefore, the evidence for nutrition as an independent risk factor for Achilles tendon injuries is weak.

1.6.1.10 Occupation

In a case series, it has been shown that the majority of patients with Achilles tendon injuries have white collar jobs and lead sedentary lifestyles (Jozsa et al., 1989). Also the majority of Achilles tendon injuries have been associated with recreational sports (Jozsa et al., 1989). However, the proportion of white collar, manual labourer was not stated so that the true prevalence could be determined. Therefore, the strength of evidence for occupation as an independent risk factor for Achilles tendon injury is weak.

Table 1.6 Extrinsic risk factors associated with acute and overuse Achilles tendon injuries

Extrinsic factors	Level of evidence	Key references
1. Physical activity	Limited	Jozsa et al., 1989
2. Training errors	Weak	Kvist, 1994
3. Surfaces	Weak	Ekstrand and Nigg, 1998
4. Environmental conditions	Weak	Jozsa and Kannus, 1997
5. Footwear	Weak	Jozsa and Kannus, 1997
6. Smoking	Weak	Safran and Graham, 2002 Harris et al., 1999
7. Medication use	Limited (Quinolones)	van Der Linden et al., 2003
	Limited (Corticosteroids)	Shrier et al., 1996
	Weak (Anabolic steroids)	Inhofe et al., 1995
8. Psychological factors	Weak	Gilcreest, 1933
9. Nutrition	Weak	Schmidt-Rolfing et al., 1992
10. Occupation	Weak	Jozsa et al., 1989

1.6.2 Intrinsic Risk Factors for Achilles tendon injuries

1.6.2.1 Age

In a number of case series it has been reported that the majority of Achilles tendon injuries, in particular ruptures, occur in individuals aged between 30 and 50 years old, with the mean age around 40 years of age (Leppilahti et al., 1996; Levi, 1997; Arner and Lindholm, 1959; Jozsa et al., 1989; Houshian et al., 1998; Maffulli et al., 1999; Moller et al., 1996; Cretnik and Frank, 2004; Suchak et al., 2005). As previously mentioned, the mean age at which AT injuries occurs is predominantly sports-related (Leppilahti et al., 1996). In non-sporting individuals, the age at which AT injuries occur is higher (Houshian et al., 1998; Moller et al., 1996). Epidemiological data show that both physical activity (exercise) and increased age are strongly associated with strain-induced tendinopathy (Gibbon et.al., 1999; Houshian et al., 1998; Nillius et al., 1976; Moller et al., 1996). Therefore, effects of both aging and exercise may be synergistic.

Also, reduced blood flow has been reported in older healthy individuals compared to younger individuals and it has been linked by implication to a reduced nutrient supply to tissues including tendons (Schmidit-Rolfing et al., 1992; Hastad et al., 1958). However, it was shown, in a case-control study that during exercise the blood flow to the Achilles tendon was not different between young, middle and old age subjects, suggesting that the role of increased age and decreased blood flow in tendons may be insignificant in the development of Achilles tendon injury (Langberg et al, 1999).

Ageing is also associated with many changes in the tendon matrix. These changes include decreased water content in the tendon (Hess et al., 1989),

increased collagen degeneration, decreased elastin and proteoglycan matrix, especially the GAG content (Astrom and Rausing, 1995). In normal tendons it is known that tenocyte activity and number as well as collagen turnover decline with age (reviewed in Riley, 1994).

Although a discussion of advanced glycation end-products (AGEs), markers of non-enzymatic collagen cross-linking, is beyond the scope of this thesis, it is important to note that blood circulating reducing sugars are able to bind to collagen molecule, resulting in irreversible cross-links along the length of collagen molecule (reviewed in Riley, 2004). As the individual age increases, these AGEs have been shown accumulate along the length of the collagen molecule (Riley, 2004). In a cross-sectional study of 28 patients who had undergone surgery for Achilles tendon rupture, tendon biopsy samples showed that the mean collagen fiber diameter was smaller in patients older than 30 years and younger than 20 years compared to the 20-29 year-old group (Sargon et al., 2005). It was also shown in another cross-sectional study that elderly women had an increased (22%) cross-sectional area compared to younger women, although the younger women had greater moment of force around the ankle joint (Magnusson et al., 2003).

The above biochemical changes in the tendon structure associated with ageing and maturity could lead to decreased tendon tensile strength, decreased flexibility and therefore increased stiffness (Clement et al., 1984; James et al., 1978). The reduced tensile strength has been linked to an increased type III to type I collagen ratio, as type III collagen forms relatively smaller diameter fibrils compared to type I fibrils (Jozsa and Kannus, 1997). In a case-control study, Chard et al. (1994) obtained biopsy samples from cadavers of supraspinatus and common extensor tendons and found that there were increased features of degenerative changes with age when compared to uninjured common extensor tendon samples. In another case-control study, Maffulli et al. (2000) has however

shown using light microscopy, that the number of degenerative changes associated with increased age in normal cadavers were much lower compared to frequency of abnormal degenerative changes found in a biopsies obtained from individuals who had sustained an Achilles tendon rupture.

It has also been noted that unlike adults, young athletes are unlikely to sustain Achilles tendon overuse injuries but they are more prone to develop growth plate injuries such as calcaneal apophysitis (Sever's disease) (Noble, 1991). It has been suggested that energy-storing tendons are able to adapt to tendon loading during growth but has little ability to adapt after skeletal maturity, hence the increased incidence of tendon injury with aging (Smith et al., 1994).

Because of the many biochemical changes that have been documented with increased age, it is intuitively reasonable that increased age should be associated with increased risk for the development of any injury. However, so far there are no prospective cohort studies in humans that can document age as an independent risk factor for Achilles tendon injury. Because the majority of the studies that suggested that age was a risk factor for Achilles tendon injury were case series and one case-control study could not establish age as a risk factor, the level of evidence for increased age as an independent risk factor for Achilles tendon injury is weak.

1.6.2.2 Gender

As shown in several case series investigations (Table 1.1), the male to female ratio of patients who have sustained an Achilles tendon rupture ranged from 2:1 to 18:1 (Houshian et al., 1998; Levi et al., 1997; Maffulli et al., 1999; Moller et al., 1996; Leppilahti et al., 1996; Nillius et al., 1976; Kvist, 1994; Arner and Lindlohm, 1959; Jozsa et.al., 1989; Astrom and Rausing, 1995; Leppilahti et al., 1996; Rantanen et al., 1993; Cretnik and Frank, 2004; Fahlstrom et al., 2002).

Furthermore, case-control studies have also shown that chronic Achilles tendinopathy is more frequent in males compared to females (Kvist, 1994).

In a retrospective study of subjects who had previously sustained an Achilles tendon rupture it was found that the peak incidence of Achilles tendon rupture for men was in the 4th decade, while it was in the 5th decade for women (Suchak et al., 2005). The difference in the onset of the rupture was statistically significant, suggesting that perhaps there was some other factor(s) protecting women from earlier Achilles tendon injury. However another retrospective study, showed that in 32 badminton players who had sustained an Achilles tendon rupture within the past 5 years, 25 (78%) were males (Fahlstrom et al., 2002). The limitation with the methodology in the latter study is that it does not normalize the proportion to actual number of males and females participating in badminton.

It has been suggested that males and females are likely to participate equally in conditioning sports, but that males are much more likely to participate in vigorous activities (Stephens et al., 1985). This implies that males may have a greater chance for exposure to injury. However, most studies do not take into account the relative number of males and females participating in both recreational and professional sport. It has been shown that a greater number of men participate in sport from adolescence (Dovey et al., 1998). In South Africa, where distance running is very popular, the majority of participants are males. In two of the most popular ultra-marathon race events, namely the 56 km Two Oceans and the 90 km Comrades ultra-marathons, the number of finishers in 2005 were 5474 (78.5%) males/1502 (21.5%) females and 8209 (70%) males/3519 (30%) females, respectively (Unpublished data, 2005). It is therefore suggested that the number of males and females taking part in any one sport such as distance running should be taken into account to determine the true meaning of gender as a risk factor for Achilles tendon injuries.

Interestingly, few prospective studies where exposure to the amount of training is similar between men and women have documented, an increased risk of injury of lower extremities in women compared to men taking part in basic military training (Bell et al., 2000; Altarac et al., 2000). However, these military studies did not investigate Achilles tendon injuries specifically. There have been two prospective cohort studies that investigated patellar and Achilles tendon injuries in civilian and military populations, respectively. However these studies did not identify gender as a risk factor for tendon injury (Witvrouw et al., 2003; Mahieu et al., 2006). The association of male gender as a risk factor for Achilles tendon injury is limited as there are limited prospective cohort studies for this condition.

1.6.2.3 Previous tendon injury

In a large case series investigating 891 patients who had sustained various tendon ruptures, including Achilles tendon ruptures, the number of previous symptoms of tendinopathy was 34%, suggesting that Achilles tendon rupture, may in some cases be preceded by symptomatic injury (Kannus and Jozsa, 1991). Also, 97% of the 891 patients showed a degenerative tendon change compared to 34% in the 445 cadaver controls (Kannus and Jozsa, 1991). Other case series have shown similar results in the Achilles tendon (Arner et al., 1959) as well as other tendon injuries (Bank et al., 1999).

There is some evidence that both Achilles tendon rupture and tendinopathy are associated with abnormal tissue morphology, suggesting that the tendon matrix has been degenerated prior to the development of the injury. In a case-control study, it has been shown that patients with Achilles tendon injuries have more degenerate tendons than asymptomatic subjects (Maffulli et al., 2002). In another case-control study, it was also shown that biopsy samples obtained from human Achilles tendon tissue from patients who had sustained a rupture showed increased pathology score compared to control tendon samples (Tallon et al.,

2001). Furthermore, it was shown that individuals with Achilles tendinopathy had less degenerated tendons than those with ruptured tendons, but both ruptured and tendinopathic tissues were more abnormal than tissues obtained from control subjects (Tallon et al., 2001). This may indicate that in most cases, tendinopathy precedes a rupture. The above case-control studies provided limited evidence for previous injury as a risk factor for Achilles tendon injury, although it has been reported that many cases of painful tendinopathy usually persist for months or even years without rupture (Riley, 2005).

In a prospective cohort study, Fredberg and Bolvig (2002), studied 54 asymptomatic soccer players over a period of 1 year soccer season. They measured, using utrasonography (US), the morphological characteristics of both the Achilles and patellar tendons pre and post the soccer season. The outcome measures were a spindle shape thickening (fusiform) greater than 1 mm in the Achilles tendon and a hypoechoic area greater than 1mm diameter for the patellar tendon. The study result were that soccer players with preseason US features of abnormality had increased risk for developing tendinopathy during the season. From this prospective cohort study the strength of evidence for previous injury as an independent risk factor for Achilles tendon injuries is strong. However, it must be borne in mind that there is still a proportion of individuals with abnormal features as shown by US who are asymptomatic and do not sustain a tendon injury (Khan et al., 1999). This observation might be explained by the multifactorial aetiological nature of tendon injury.

From the above review it can be concluded that there is strong association between previous injury of the Achilles tendon and future Achilles tendon injury. This conclusion is largely based on the results of the prospective cohort study, but it also supported by the limited evidence from the case-control studies as well as some evidence provided from case series. Therefore, the strength of evidence

for previous injury as a likely independent risk factor for Achilles tendon injury is strong.

1.6.2.4 Body mass and size

Although increased body weight and size have been cited as predisposing to Achilles tendon rupture (Kvist, 1994), no study has been published that specifically investigated the role of body weight in tendon ruptures (Kannus and Natri, 1997; Leppilahti et al., 1998). In one prospective study, Jones et al. (1993) found low and high BMI and high body fat content for men and shorter height for women to be risk factors for sustaining lower extremity injuries among military recruits. However, the latter study was not specific for Achilles tendon injuries. Therefore, the evidence for increased body mass and size as independent risk factors for Achilles tendon injury is weak.

1.6.2.5 Blood supply

In several case series investigations of cadavers, obtained from healthy individuals before death, using different blood flow measurement techniques, it was demonstrated that within the length of the Achilles tendon, the area with the least amount of blood perfusion is the region that is 2-6 cm proximal to the insertion (Lagergren and Lindholm, 1959; Carr and Norris, 1989; Stein et al., 2000; Schmidt-Rolfing et al., 1992; Zantop et al., 2003). However, in one case series of 12 cadavers, also obtained from healthy individuals before death, it was shown using angiography and histology that the Achilles tendon had poor blood supply throughout its entire length and that there was no difference in the number of blood vessels per cross-sectional area of the tendon (Ahmed et al., 1998).

There were limitations with the cadaver studies as the measurement of blood flow was carried on tissue that was not live which may not have reflected live

mechanically loaded tendons. It has also been suggested that the use of dye techniques may not reliable (Lagergren and Lindholm, 1959; Theobald et al., 2005). Therefore, there was a need for better designed studies using live tissue in control and symptomatic subjects to establish the reduced blood flow and investigate the association between blood flow in the mid-portion of the Achilles tendon and Achilles tendon injury.

However, case-control studies of healthy and symptomatic subjects did not confirm the reduced blood flow in the mid-portion of the normal Achilles tendon using more reliable and advanced blood flow measurement techniques, such as laser Doppler flowmetry and ^{133}Xenon technique (Astrom and Westlin, 1994; Astrom, 2000; Langberg et al., 2001; Knobloch et al., 2006).

Most recently, it was shown that there was no difference in tissue oxygen saturation in all tendon regions (Knobloch et al., 2006). It was also shown that there was no difference in the blood flow during exercise in the Achilles tendons of young subjects compared to middle-aged subjects using a radioactively labelled Xenon-133 technique (Langberg et al., 2001). Furthermore, degenerate tendons have been shown to have increased blood vessels compared to normal tendons (Kannus and Jozsa, 1991; Astrom and Rausing, 1995; Knobloch et al., 2006). Table 1.7 shows studies that investigated the blood flow in the different regions of the Achilles tendon.

In general, in these studies using cadavers and different techniques to yield contrasting results but seem to confirm that the Achilles tendon has fewer blood vessels as a whole compared to muscle tissue. Studies using human subjects found that during exercise the blood flow in the tendon increased sufficiently when young and old subjects were compared (Langberg et al., 2001). It appears to be true that the region most affected by injury is the mid-portion but a direct link between poor blood supply in the area and Achilles tendon injury has not

been established. It may well be that other factors such as tensile mechanical loading play more important role than blood flow. Therefore, the level of evidence for reduced blood flow as an independent risk factor for Achilles tendon injury is weak as no case-control study supported the results of case series observations.

Table 1.7 Studies investigating blood flow in the human Achilles tendon

Author	Subjects[a]	Methods	Outcome
Stein et al., 2000	10 legs from Cadavers mean age, 67	Cadavers, vascular injection into Achilles tendon	Blood flow was lowest in the middle (3-6cm above calcaneous) part of the tendon
Astrom and Westlin, 1994*	40 healthy subjects & 26 patients	Laser Doppler Flowmetry (LDF)	Blood flow lowest in the area near the calcaneous, but evenly distributed in the rest of the tendon
Ahmed et al., 1998	12 Cadavers	1.Angiography 2. Histology	No difference in the number of vessels per cross-sectional area of tendon. The Achilles tendon has poor blood supply throughout its entire length.
Carr and Norris, 1989	Mean age, 62 16 Cadavers	Angiography (qualitative)	Decreased blood supply in the paratenon in the area 4cm above calcaneous
Langberg et al, 2001	3 small groups; young, middle- and old-aged	^{133}Xenon technique	Blood flow not different between young and middle-aged at rest and during exercise, but lower in old-aged subjects
Astrom, 2000	40 healthy subjects & 35 patients	Laser Doppler Flowmetry (LDF)	*Simillar to the 1994 study. Patient numbers have been increased.

Table 1.7. Studies investigating blood flow in the human Achilles tendon (continued)

Author	Subjects	Methods	Outcome
Schmidt-Rolfing et al.,1992	Cadavers (n=8)	Epoxy resin injection	Poor blood flow in the midportion and posterior distal part of the tendon
Largergren and Lindholm, 1959	Cadavers	Angiography of the paratenon (qualitative)	Decreased blood flow in the midportion of the tendon
Zantop et al., 2003	Cadavers, 10 legs, freshly frozen Mean age, 67	1.Injection technique 2.Immunohistochemistry	Decreased number of vessels in the midportion of the tendon
Knobloch et al., 2006	66 subjects (132 tendons), 3 groups (healthy Achilles tendons, Insertional pain, midportion pain)	Laser Doppler Flowmetry (LDF) combined with oxygen-to-see (O2C) system (quantitative)	Increased blood flow in both insertional and mid-portion tendons, but blood flow similar in uninjured legs compared to healthy tendons Postcapillary venous filling pressure was higher in both insertional and mid-portion tendons Tissue oxygen saturation similar in all tendons

[a]All humans

1.6.2.6 Increased tendon temperature

It has been hypothesized that tendon tissue may be compromised by repeated and prolonged loading associated with exercise which theoretically could cause an increase in temperature of the cells, therefore affecting tenocyte function (Wilson and Goodship, 1994). In equine superficial digital flexor tendon (SDFT), which has the same function in horses as the Achilles tendon does in humans, the threshold for some type of fibroblasts viability has been reported to be 42.5 °C. In four horses that were made to gallop for a total of 7 minutes on a treadmill it was determined that the temperature measured in the centre of the SDFT rose to a mean of 43.3 °C (range 40-45 °C). The normal horse rectal temperature is 37-38 °C.

In an in vitro study, fibroblasts derived from equine SDFT were shown to be resistant to exposure to temperature below 45 °C, with cell viability sharply declining at a peak temperature of 46 °C (Birch et al., 1997). It was concluded that it was unlikely that temperatures experienced in the central core of SDFT in vivo caused cell death, although other cell functions such as synthesis of matrix products, might be compromised. Furthermore, it was shown in vitro that SDFT cultured tenocytes were more resistant to heat stress compared to equine dermal fibroblasts and a commercially available fibroblasts cell line (Birch et al., 1997).

Although not yet determined, it is likely that during repetitive tendon loading such as in running in humans, the temperature of the Achilles tendon may rise to levels high enough to affect tenocyte function. However, the evidence directly linking the increase in tendon temperature and tenocyte function is lacking in both equine and human tendons. Therefore, the evidence for increased tendon temperature as an independent risk factor for Achilles tendon injury is weak.

1.6.2.7 Biomechanical and Alignment Factors

A number of lower limb biomechanical and alignment factors have been associated with Achilles tendon injuries. These include muscle-tendon unit range of motion (ROM) (or flexibility), muscle-tendon unit stiffness, leg length inequality, muscle strength, muscle imbalance and leg dominance, plantaris tendon presence and foot morphological characteristics such as cavus foot, flat foot, genu varum (Kvist, 1994; Schepsis et al., 2002).

1.6.2.7.1 Musculotendinous Joint Range of Motion and Stiffness

A decreased or increased ankle dorsiflexion range of motion (ROM) or flexibility has been associated with decreased incidence of musculotendinous injuries. It should be noted that until a few years ago, studies investigating the specific relationship between tendon flexibility and injury occurrence were not available (Almekinders and Temple, 1998). However, in one retrospective study it has been reported that patients with Achilles tendon injuries frequently presented with laxity of ligaments, joint tightness and hyperflexibility (Kvist, 1994).

In a prospective cohort study, an increased tightness of the gastrocnemius muscle was found to be a risk factor for Achilles tendinopathy (Kaufman et al., 1999). In contrast, a recent 6-week prospective cohort study of basic military recruits has identified an ankle dorsiflexion greater than 9 degrees as a risk factor for the development of Achilles tendon overuse injuries (Mahieu et al., 2006). Interestingly, this latter study did not identify Achilles tendon stiffness as a risk factor for Achilles tendon injury. In another two-year prospective cohort study, but of an athletic population, decreased hamstring and quadriceps muscle flexibility were shown to be significantly associated with the development of patellar tendinopathy (Witvrouw et al., 2001). However, it has been reported that both reduced and

excessive range of motion around a joint can predispose a joint to injury (Jones et al., 1993).

Based on the lack of prospective cohort studies in the civilian population and conflicting results of the two prospective cohort studies, the level of evidence for joint range of motion as an independent risk factor for Achilles tendon injury is at best limited.

1.6.2.7.2 Leg length inequality

Anatomical or structural leg length inequality can be defined as a bilateral difference in the length of the bony components of the lower limb (McCaw, 1992). Two other types, functional and environmental leg length inequality can exit. For instance, if there is muscular weakness or inflexibility on one side of the body this would create a functional leg length inequality, when camber on the road would create an environmental leg length inequality (McCaw, 1992). However, in most cases, structural leg length inequality is the measured and analyzed parameter. In a retrospective study, leg length inequality of greater than 1 cm has been detected in 15% of patients with Achilles tendon injuries (Kvist, 1994). However the prevalence of leg length inequality in the general population was not stated to make a valid conclusion regarding leg length as a risk factor for Achilles tendon injuries. It has been suggested that the size of the population with a characteristic of interest need to be known to make valid comparison (Powell et al., 1986).

In one case-control study of 101 patients who had sustained an Achilles tendon rupture and 87 healthy subjects, Leppilahti et al. (1998) suggested that leg length inequality was not an important risk factor for Achilles tendon rupture. It was interesting to note that only 10% of the patients who had sustained a rupture had no leg inequality, with the remainder displaying either a longer left (59%) or a longer

right (31%) leg. Therefore, the level of evidence for leg length inequality as an independent risk factor for Achilles tendon injury is weak.

1.6.2.7.3 Abnormal foot morphology

It has been suggested that pes planus (flat foot), pes cavus (high-arched foot), increased talocrural or subtalar joint mobility may predispose to Achilles tendon injuries (Smart et. al., 1980). Retrospective studies have reported that individuals with pes cavus, pes planus as well as forefoot "over-pronation" may expose the Achilles tendon to an excessive "whipping action", creating shear forces across the Achilles tendon, presumably by placing high eccentric stresses on the medial side of tendon (Clement et al., 1981; Kvist, 1994; Leppilahti et al., 1998). In one retrospective study, it was shown that 99 (91%) of the 109 legs of subjects with Achilles tendinopathy in the proximal two thirds of the Achilles tendon displayed abnormal sonographic features (hypoechoic) (Gibbon et al., 2000). This further supported the whipping action mechanism of the Achilles tendon as the degeneration was also dominant on the medial side of the Achilles tendon. The medial side of the Achilles should the mostly highly degenerated if the whipping action hypothesis is true (Gibbon et al., 2000). However, the Achilles tendon "whipping action" hypothesis needs to be further investigated.

In a case-control study, a high longitudinal arch was reported in 37% of Achilles tendon rupture patients compared to 29% of controls ($p<0.001$) (Leppilahti et al., 1998). It was also found that 21% of the patients had underpronation of the foot compared to 5% of the controls ($p<0.001$), while 32% of patients had overpronation of foot compared to 45% of the controls. However, only 10% of the patients had both high longitudinal arch and an underpronation of the foot compared to 1% of the control subjects ($p<0.001$).

In retrospective study, it has been reported that forefoot varus may predispose to Achilles tendon overuse injuries (Kvist, 1994). However, there were no statistical differences in the amount of forefoot varus and valgus in Leppilahti et al. (1998) control and symptomatic subjects. Anomalies to calcaneal bone may also predispose to Achilles tendon injuries (Kvist, 1994). Other factors that may be associated with Achilles tendon injuries include bowed legs (genu varum or tibia varum) (Schepsis et al., 2002). Studies reporting on abnormal foot morphology are based on case series, retrospective and case-control studies at best. It is not clear whether the observed foot abnormality in cases is a cause or effect of the injury. Therefore, the level of evidence for abnormal foot morphology as an independent risk factor for AT injury is weak to limited.

1.6.2.7.4 Leg dominance

A few case series have reported that a greater number of individuals experience Achilles tendon injuries on their left leg more often than in the right leg (Cetti et al., 1993, Levi, 1997; Jozsa et al., 1989; Cretnik and Frank, 2004; Leppilahti et al., 1996). It has been suggested that the reason for this finding is the need to push off with the left leg more often as majority of people are right-foot dominant (Maffulli et al., 1999). It has been reported that the tendon on the left leg was ruptured in 57% of the cases, while the tendon on the right leg was ruptured in 43% and this difference was statistically significant (Levi, 1997). The same trend has been reported by Jozsa et al. (1989) (59% left Achilles tendon ruptures), Cretnik and Frank (2004) (53% left Achilles tendon ruptures) and Leppilahti et al. (1996) (56% left Achilles tendon ruptures). Although there have been several case series identifying the left leg as the most vulnerable to an Achilles tendon injury, the evidence for leg dominance as an independent risk factor for Achilles tendon injury remains weak.

1.6.2.7.5 Associated Plantaris tendon

The plantaris muscle and tendon, if present, lies between and runs parallel to the gastrocnemius and soleus muscles (Daseler and Anson, 1943; Invaco et al., 1987). It is regarded as a vestigial structure (Daseler and Anson, 1943). In one case-control study it was found that 16 (40%) of the 40 subjects with Achilles tendon rupture had the plantaris tendon compared to 1295 (92.5%) of 1400 cadavers known to have no previous AT injury obtained from the general population (Incavo et al., 1987). Another relatively small case series, using a qualitative US examination found that 9 of the 15 subjects (60%) who had experienced a complete Achilles tendon rupture had an intact plantaris tendon as shown by ultrasonography (Gibson, 1998). In a case series of 750 human Achilles tendon specimens, it was found that only 6-8% of the general population has no plantaris tendon (Daseler and Anson, 1943).

The above studies have been on case series and case-control studies, therefore the evidence for the absence of the plantaris tendon as an independent risk factor for Achilles tendon injury is weak.

1.6.2.7.6 Muscle strength

It well documented that the majority of Achilles tendon injuries occur during the 4th decade of life (Jozsa and Kannus, 1997). Furthermore, muscle strength is decreased in older individuals. Also, the lower extremity muscle strength is important in coordination of movement and posture. It is therefore reasonable to assume that the muscle strength will be important during tendon loading. However, until recently, the association of muscle strength and tendon injury has not been investigated (Almekinders and Temple, 1998). However, in a recent prospective cohort study it was shown that lower plantarflexor muscle strength but not explosive strength was a significant predictor of an Achilles tendon overuse injury in 18 year old male

military recruits (Mahieu at al., 2006). Because so far this is the only prospective study that have shown this relationship, the level of evidence for plantar flexor muscle strength as an independent risk factor for Achilles tendon injury is limited.

1.6.2.8 Systemic diseases

It has been suggested that a variety of systemic medical conditions may play a role in the pathogenesis of Achilles tendinopathy and rupture (reviewed in Riley, 2004; Jozsa and Kannus, 1997; Jozsa et al., 1989). Some of the most common inherited systemic diseases that may be associated with AT injuries include Marfan's syndrome, Ehler's Danlos syndrome, Osteogenesis imperfecta, lipid storage diseases (Jozsa and Kannus, 1997; reviewed in Riley, 2004). Endocrinologic and metabolic diseases include diabetes mellitus, renal diseases and disorders of thyroid, parathyroid, adrenocorticcotrophic and adrenocortical hormone systems (Jozsa and Kannus, 1997). Rheumatoid diseases such as rheumatoid arthritis, gout and ankylosing spondylitis have also been associated with Achilles tendon injuries (Jozsa and Kannus, 1997). For instance, in one case series 14.3% of the patients who had experienced acute Achilles tendon rupture also had gout (Beskin et al., 1987). Furthermore, it has been reported that in Caucasian patients with ankylosing spondylitis the occurrence of the human leucocyte antigen type B27 (HLA-B27) is found in 89% of the cases as opposed to 9% in controls (Naik, 2003; Klein and Sato, 2000).

The association between high blood cholesterol or lipids and Achilles tendon injury has also been reported (Kannus and Jozsa, 1991). However, to date only two studies have documented an association between high serum lipid concentration and Achilles tendon injury (Mathiak et al., 1999; Ozgurtas et al., 2003). In a case series investigation, Mathiak et al. (1999) measured serum cholesterol amongst 41 patients with Achilles tendon ruptures and found cholesterol levels to be elevated in 83% of

the patients. However, this study did not have an age, gender and physical-activity matched control group to make a valid conclusion. In a case-control study of 47 subjects who had sustained an Achilles tendon rupture and 26 control subjects, the mean total cholesterol, low-density lipoprotein cholesterol and triglycerides were found to be higher in the symptomatic compared to the control subjects. The mean high-density cholesterol was lower in the Achilles tendon rupture group compared to the control group (Ozgurtas et al., 2003). It is also known that lipid deposition on the tendon is associated with some reported cases of Achilles tendon ruptures (Kannus and Jozsa, 1991) and has been linked with longitudinal collagen fiber separation associated with tendinopathy.

In a large case series of patients with Achilles tendinopathy, it was shown that the proportion of subjects with previous systemic disease was quite small (Jozsa et al., 1989). Although hyperlipidemia, hypercholesterolemia and other systemic diseases may be risk factors for Achilles tendon injuries, the strength of evidence for a systemic disease as independent risk factor for Achilles tendon injury is weak.

1.6.2.9 Genetic factors

Ethnicity, the human leucocyte antigen (HLA) system and the ABO blood groups have been the most commonly cited genetic risk factors associated with Achilles tendon injuries.

1.6.2.9.1 Ethnic differences

A 3 year retrospective study of 865 military personnel showed that black Americans were at higher risk of sustaining an Achilles tendon rupture than non-blacks (relative risk=4.15, 95%CI 3.63-4.74) (Davis et al., 1999). Interestingly, 53% of these injuries occurred while playing basketball, however 65% of the injuries were

basketball-related in blacks and 34% were in non-blacks. The authors reported that among the injured subjects, blacks had a greater risk of injury related to basketball than non-blacks (relative risk=1.82, 95% CI 1.58-2.10). It is clear from the study that basketball is a risk factor for Achilles tendon rupture in both groups. However, the relative proportions of blacks and non-blacks who played basketball on a regular basis as well as the amount of play or training were not stated.

Other authors have reported on the lack of data published on Achilles tendon injuries from the developing world, except for China and Saudi Arabia, suggesting that perhaps these injuries were less common or given less priority in the developing world (reviewed in Jozsa and Kannus, 1997). Therefore, the strength of evidence for ethnicity as an independent risk factor for Achilles tendon injury is weak.

1.6.2.9.2 Human Leucocyte Antigen (HLA) system

There have been two case reports of patients with Achilles tendinopathy who tested positive for the HLA-B27 (Olivieri et al., 1987; Olivieri et al., 1992). Therefore, the HLA system has been cited as a possible genetic risk factor for Achilles tendinopathy. However, Achilles tendon ruptures have not been associated yet with polymorphisms in the HLA markers (Kannus and Natri, 1997), however both the A2 and BW 35 HLA antigens have been associated with tendon pathology (Amor et.al., 1977; Gibson et. al., 1975). Because of the two case reports of Achilles tendinopathy associated with a HLA-B27, the strength of evidence for the HLA system as an independent risk factor in the in the development of Achilles tendon injury is weak.

1.6.2.9.3 ABO blood groups

A possible association between the ABO blood group and AT ruptures or chronic Achilles "peritendinitis" has been reported in some retrospective studies (Josza et al. 1989; Kujala et al., 1992; Kannus and Natri, 1997), but not in all studies (Mahrlein et al., 1995; Leppilahti et al., 1996; Maffulli et al., 2000; Aroen et al., 2004; Suchak et al., 2006). The first study to report a possible association between the ABO blood group and Achilles tendon injury showed that 53% of individuals who had sustained an Achilles tendon injury were of ABO blood group O which was higher than the 31% found in the general Hungarian population (Jozsa et al., 1989). However, a detailed discussion of the ABO blood group and its possible role in Achilles injury will be provided in Chapter 2 of this thesis. Nonetheless, the evidence for ABO blood group as an independent risk factor for Achilles tendon injury is weak.

1.6.3 Summary of the extrinsic and intrinsic factors

Several extrinsic and intrinsic factors for Achilles tendon injuries have been cited, but the majority of them were shown to have weak level of evidence. Only previous injury was classified as strongly associated with Achilles tendon injuries. None of the extrinsic factors were strongly associated with Achilles tendon injuries. Three extrinsic risk factors, namely physical activity, use of quinolones and corticosteroids showed limited level of evidence. The intrinsic factors gender, flexibility, muscle strength and hyperlipidemia showed limited level of evidence. It is clear that many of the risk factors for Achilles tendon injury have not been investigated properly using well designed studies. It is important to note that classification of a possible risk factor for Achilles tendon injury as weak level of evidence does not mean it is not a risk factor. It only means that currently the only evidence available dictates its status, according to the level of evidence rating system.

Table 1.8 Intrinsic risk factors associated with acute and overuse Achilles tendon injuries

Intrinsic risk factor	Level of evidence	Key references
1. Age	Weak	Leppilahti et al., 1996
2. Gender	Limited	Jozsa et al., 1989
3. Body mass and size	Weak	Kannus and Natri, 1997
4. Previous injury	Strong	Fredberg and Bolvig, 2002
5. Tendon blood supply	Weak	Knobloch et al., 2006
6. Tendon temperature	Weak	Wilson and Goodship, 1994
7. Biomechanical factors:		
• Flexibility	Limited	Mahieu et al., 2006
• Leg length inequality	Weak	Leppilahti et al., 1998
• Leg dominance	Weak	Levi, 1997
• Foot morphology	Weak	Kvist, 1994; Leppilahti et al., 1998
• Muscle strength	Limited	Mahieu et al., 2005; Kvist, 1994
• Associated Plantaris	Weak	Daseler and Anson, 1943
8. Genetic factors		
• ABO blood group	Weak	Jozsa et al., 1989
• HLA	Weak	Olivieri et al., 1987
• Ethnic group	Weak	Davis et al., 1999
9. Systemic diseases	Weak	Jozsa et al., 1989
• Hyperlipidemia	Limited	Ozgurtas et al., 2003

1.7. SUMMARY AND CONCLUSIONS OF THE LITERATURE REVIEW

Although there have been advancement in the study of tendon pathology, it is widely accepted that the aetiology and pathogenesis of Achilles tendon injuries and other tendon injuries is multifactorial and still poorly understood. Both extrinsic and intrinsic risk factors for Achilles tendon injuries have been implicated in the development of these injuries. Amongst the intrinsic factors, genetic factors have been implicated, as some but not all studies that have shown an association of the ABO blood group with AT injuries, suggesting that genes on the same locus (telomeric end of the long arm of Chromosome 9) as the ABO gene might be involved in the aetiology of AT injuries. More recently, studies of the rotator cuff tendon (Harvie et al, 2004) and anterior cruciate ligament (Flynn et al., 2005) have provided further support to the possible involvement of genetic factors in the development of connective tissue injury. However, to date no study has identified specific gene (s) involved in tendon pathology.

1.8. AIMS OF THE THESIS

The main aim of the thesis was therefore to investigate whether certain individuals are more prone to Achilles tendon injuries by posing the following specific aims:

1. To identify any candidate gene(s) that are located on the tip of the long arm of chromosome 9 (more specifically the 9q32-q34 locus) closely linked to the *ABO* gene which could theoretically be associated with Achilles tendon overuse injuries. In addition, the association of ABO blood group system with Achilles tendon rupture or chronic tendinopathy in a South African Caucasian population was investigated (Chapter 2),

2. To determine whether one of these identified genes, the *COL5A1* gene (which encodes an alpha1 chain of type V collagen) is associated with Achilles tendon injury (Chapter 3),

3. To investigate whether a second candidate gene, the *TNC* gene (which encodes an extracellular matrix glycoprotein found in tendons) is associated with Achilles tendon injury (Chapter 4),

4. To investigate whether there was any *COL5A1* or *TNC* genotype effect on the muscle-tendon unit range of motion (ROM) (Chapter 5), and finally

5. To investigate whether there was any *COL5A1* or *TNC* genotype effect on the morphological changes associated with Achilles tendon injury by grey scale and colour Doppler ultrasonography (US) (Chapter 6).

2.1 INTRODUCTION

As previously discussed in the previous chapter, although there is a reported increase in the incidence of Achilles tendon overuse injuries (Leppilahti et al., 1996a), the mechanisms responsible for these pathologies are poorly understood (Riley, 2005a). Several extrinsic and intrinsic factors have nevertheless been shown to be associated with Achilles tendon pathology (reviewed in Riley, 2004; Kvist, 1994). The ABO blood group has been identified as one of the intrinsic factors shown to be associated with Achilles tendon ruptures or chronic Achilles "peritendinitis" (Josza et al. 1989; Kujala et al., 1992; Kannus and Natri, 1997).

In a retrospective study, Josza et al. (1989) compared the frequencies of the ABO and Rh blood groups of 292 Hungarian patients with primary Achilles tendon ruptures or re-ruptures and 540 patients with various other tendon ruptures with a control group consisting of 1 200 000 subjects. These researchers showed that the frequency of blood group O was significantly higher in the patients with Achilles tendon ruptures (53.1%) than for the general Hungarian population (31.1%). The O blood group was also more frequently observed in all the other investigated tendon ruptures, which included rupture of the long head of biceps (48.2%), extensor pollicis longus (53.9%) and the quadriceps (49.0%). Interestingly, 68.7% of the patients with multiple tendon ruptures (n=48) or re-ruptures (n=35) were of blood group O. No association was found in this study between the Rh group and any of the tendon rupture groups. Blood group O was still significantly higher in the combined tendon rupture group (54%) when 443 additional subjects were recruited and analyzed (Kannus and Natri, 1997).

In another study, Kujala et al. (1992) described a group of 917 Finnish patients diagnosed with a variety of musculoskeletal soft tissue injuries. This group included 86 patients diagnosed with Achilles tendon ruptures and 63 with chronic Achilles peritendinitis. The frequency of the blood group O among the patients diagnosed with Achilles tendon ruptures (31.2%) was not significantly

higher than in the control group (31.4%). The frequency of blood group O was however higher in the patients diagnosed with Achilles peritendinitis (42.9%). The A/O ratios, however, for the groups with rupture (1.0) or peritendinitis (0.7) of the Achilles tendon were lower than the control population (1.42). The ABO blood group was not associated with any of the other soft tissues injuries, including rotator cuff impingement (n=142), patellar dislocation (n=92), anterior cruciate ligament (ACL) rupture (n=205), spondylolithesis (n=177), and intervertebral disc herniation (n=152).

More recently, Aroen et al. (2004) have suggested that there may be a genetic predisposition towards tendon ruptures as they observed that nine of the ten subjects who suffered a contralateral Achilles tendon rupture were of blood group B. The investigators, however, did not put forward suggestions why blood group B instead of blood group O was associated with Achilles tendon rupture in their study.

Contrary to these findings, other studies investigating Finish, German and Scottish populations (summarized in Table 2.1), did not show an association between the ABO blood groups and Achilles tendon ruptures (Leppilahti et al., 1996b; Mahrlein et al., 1995; Maffulli et al., 2000). The differences in the result of the various studies on the association of ABO blood distribution with Achilles tendon injuries could be due to variations in the sample sizes and differences in the distribution of the ABO blood group in genetically segregated ethnic groups (population stratification) (Maffulli et al., 2003).

The ABO blood group system is one of approximately 400 blood antigens that have been described and together with the Rhesus blood group system is the most commonly used clinically. Antigenic surface proteins found on red blood cells determine the various blood group systems. In the ABO blood group system, 99.99% of individuals express the H antigen on their red blood cells (reviewed in Harmerning-Pittiglio, 1989; Suzuki, 2005).

Table 2.1. Summary of the studies investigating the association between ABO blood groups and Achilles tendon pathology.

Reference	Population group	Pathology group(s) sample size	Control sample size	Outcome
Jozsa et al., 1989	Hungarian	ATR=292 TR=540	1,200,000	Blood group O associated
Kujala et al.,1992	Finnish	Achilles Peritendinitis & ATR= 149	5,536	Blood group distribution different
Kannus and Natri, 1997	Hungarian	ATR & TR =1275	1,200,000	Blood group O associated
Leppilahti et al., 1996	Finnish	ATR =215	5,536	No association
Mahrlein et al., 1995	German	ATR =531	Unknown	No association
Maffulli et al., 2000	Scottish	ATR=78	24,501	No association
Aroen et al., 2004	Norwegian	ATR=10	None	9/10 re-ruptures were B

ATR, Achilles tendon ruptures and **TR**, various other tendon ruptures (excluding Achilles tendon ruptures)

As shown in Figure 2.1, different glycosyltransferases are able to modify the H antigen to produce the 3 major antigens found in the ABO blood group system. Two of the enzymes are encoded by a single gene, the *ABO* gene, which has been mapped to human chromosome 9q32-q34 (Bennet et al., 1995). Different alleles of this gene have been identified; allele A encodes for an α1-3-acetylgalactosaminyltransferase (transferase A), while allele B encodes for a α1-3-galactosyltransferase (transferase B). A third allele (O) encodes for a non-

functional protein, leaving the H antigen unchanged. Further discussion of the ABO antigens is beyond the scope of this introduction, but it is important to note that these three products of the *ABO* gene determine an individual's ABO blood group type (Figure 2.1).

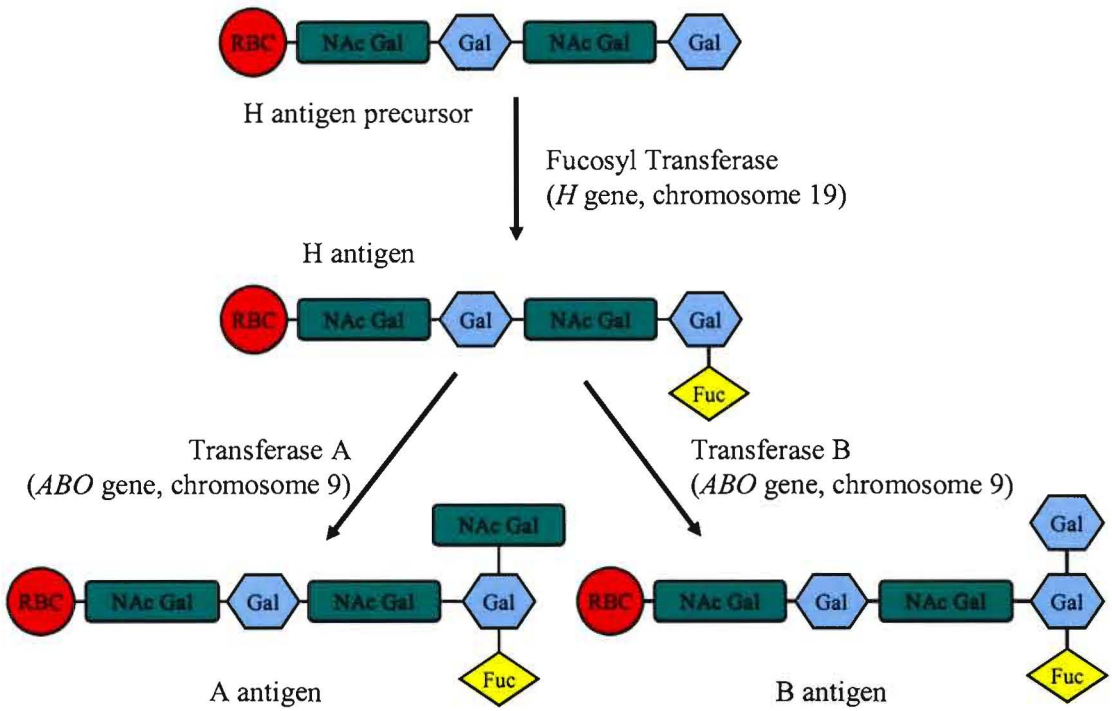

Figure 2.1. The A and B antigens produced by the A and B transferase activities respectively on the H antigen. Individuals with an O blood group have an unmodified H antigen because a non-functional enzyme is produced from the ABO gene. The names and chromosomal locations of the genes encoding the enzymes that modify the H antigen precursor are shown (adapted and modified from Daniels, 2005). RBC, red blood cell; Gal, galactose; Fuc, fucose; Nac Gal, N-acetyl glucosamine.

ABO antigens are also found on most other body cells, including white blood cells, platelets, epithelial and endothelial cells (Harmerning-Pittiglio, 1989). The relationship of an individual's ABO genotype and ABO blood group is summarized in table 2.2.

Table 2.2. The relationship of the ABO genotype with the ABO blood group

Possible *ABO* Genotype	Functional enzyme	ABO phenotype	A and B antigens	Serum antibody
AA or AO	Transferase A	A	A	Anti-B
BB or BO	Transferase B	B	B	Anti-A
OO	None	O	None	Anti-A, Anti-B
AB	Transferase A, Transferase B	AB	A & B	None

Since the *ABO* gene encodes for transferases, some investigators have suggested that the different enzymes produced by the *ABO* gene, not only determines the structure of the glycoprotein antigens on the red blood cells, but also the structure of some of the glycoproteins found in the ground substance of tendons (Jozsa et al., 1989). Others have however suggested that the association of the *ABO* gene with tendon injuries is not directly linked to the ABO blood group antigens (Kujala et al., 1992; Kannus and Natri, 1997; Maffulli et al., 2002). These investigators have proposed that other genes, closely linked to the *ABO* gene on the tip of the long arm of chromosome 9 (9q34) (Bennet et al., 1995), which encode for components of the extracellular matrix are more likely to be associated with Achilles tendon pathology (Kujala et al., 1992; Kannus and Natri, 1997). No other candidate genes have to date been identified within this locus.

The main aim of this chapter of the thesis was to identify any candidate gene(s) that are located on the tip of the long arm of chromosome 9 (more specifically the 9q32-q34 locus) closely linked to the *ABO* gene which could theoretically be

associated with Achilles tendon overuse injuries. In addition, the association of ABO blood group system with Achilles tendon rupture or chronic tendinopathy in a South African Caucasian population was investigated.

2.2 METHODS

2.2.1 *Subjects*

One-hundred and twenty-two physically active Caucasian patients with a current or past clinical history of Achilles tendon injury (ATI), including 79 with chronic tendinopathies (TEN) and 43 with tendon ruptures (RUP), were recruited from the Medical Practice at the Sport Science Institute of South Africa and other clinical practices within the greater Cape Town area in South Africa. The clinical practices invited their eligible patients to participate in the study (Appendix 2.1). Subjects were requested to contact the investigators if they were interested in volunteering for the study.

One-hundred and thirty-one physically active Caucasian control (CON) subjects without any history of Achilles tendon pathology were also recruited for this study. The subjects were matched for age and gender. To avoid any possible effects of population stratification, the ATI and CON groups were also similarly matched for their country of birth. An additional 8 non-Caucasian asymptomatic control and 21 non-Caucasian ATI subjects were also recruited but were not included in this study.

Approval for this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences within the University of Cape Town (Appendix 2.2). Once recruited, the subjects were required to complete an informed consent form (Appendix 2.3) and personal particulars, physical activity and medical history questionnaires prior to participation (Appendix 2.4).

2.2.2 *Diagnosis of Achilles tendon injury*

An experienced clinician made the diagnosis of chronic Achilles tendinopathy (n=73) using clinical criteria. The diagnostic criteria for every subject were reviewed by a sports physician (Associate Professor Martin Schwellnus). The clinical diagnostic criteria for chronic Achilles tendinopathy (Appendix 2.5) 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) (Schepsis et al., 2002; Kader et al., 2002). In addition to these clinical diagnostic criteria, soft tissue ultrasound examination was performed in a sub-group (n=36) of subjects to confirm the diagnosis of the affected Achilles tendon.

The diagnosis of Achilles tendon rupture (Appendix 2.6) was made clinically using standard validated criteria (Schepsis et al., 2002) and confirmed in all cases by examination at the time of surgery (n=35) or by ultrasound imaging (n=5), MRI imaging (n=2) or computer tomography (CT) scan (n=1). Subjects 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 (van der Linden et al., 2001) or possibly corticosteroids use, and an increased risk of Achilles tendon rupture (Leppilahti and Orava, 1998). Furthermore, subjects 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 (Leppilahti and Orava, 1998). A total of 43 subjects (this value was not included in the reported value of 122 ATI subjects in section 2.2.1) with unconfirmed diagnoses or did not meet all the inclusion criteria were excluded from the study.

2.2.3 *Sample collection and ABO blood grouping analysis*

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 (Chapter 3) and ABO blood group determination. A small quantity of the blood sample was aliquoted to determine the ABO blood group in 118 ATI and 131 CON subjects using standard (forward and reverse) agglutination techniques (Western Province Blood Transfussion Services, Cape Town, South Africa). Since the reverse test confirmed all the forward tests (n=22), the subsequent samples were only ABO typed using the forward test. In addition the forward test agreed with 94% (120 of 127) of the available self-reported ABO blood group types.

For the forward test, 10 µl of total blood was diluted with 190 µl of physiological saline. A single drop of this suspension was added to 3 separate 5mL Kimble test tubes containing 1 drop of anti-A, anti-B or anti-AB antibody suspension. The contents of the tube were mixed and centrifuged at 1200xg for 2 min at room temperature. The test tubes were tapped gently at the bottom to resuspend the contents to record if any agglutination occurred.

For the reverse test, about 4.5ml of blood was centrifuged for 10 min at 1650xg, then 100 µl of the subject's serum with unknown antibodies was aliquoted from the tube, from which 1 or 2 drops of the serum was added to 3 separate Kimble test tubes containing 1 or 2 drops of test cells (i.e. known antigens). The contents of the tube were then mixed and centrifuged for 15 seconds at 2100xg. Likewise,

macroscopic agglutination was recorded after gently resuspending the contents of the tube.

Individual Caucasian ABO blood group types (WC POP, n=62,216) were obtained from the Western Province Blood Transfusion Organization (the official blood transfusion center for the greater Cape Town area).

2.2.4 *Identification of candidate genes*

Genes that flank the *ABO* gene were identified by searching the OMIM™ (Online Mendelian Inheritance In Man) (www.ncbi.nlm.nih.gov/entrez/) and NCBI Map Viewer (www.ncbi.nlm.gov/mapview/) databases. Since the *ABO* gene has been mapped to the human chromosome 9q34, all genes mapped from 9q32 to the end of the chromosome q34.3 were initially identified. Additional information on the identified genes and the proteins for which they encode was obtained from the Entrez Gene (www.ncbi.nlm.nih.gov/entrez/) and OMIM™ databases. Genes were included as possible candidate genes for tendon pathology if they encoded for proteins that formed an integral structural component of the extracellular matrix (ECM). Since tenocytes have been shown to play an important role in tendon pathology (Kannus and Jozsa, 1991; Yuan et al., 2002; Murrell, 2002), genes encoding for proteins involved in cell proliferation, cell death (specifically apoptosis) and other miscellaneous processes were also identified. The biological function of the proteins encoded for by the genes on the telemetric end of the long arm of chromosome 9 were identified from their functional descriptions in the databases.

2.2.5 *Statistical analyses*

Data were analyzed using the STATISTICA version 7 (StatSoft Inc., Tulsa, OK, USA) and GraphPad InStat version 3 (GraphPad Software, San Diego, CA, USA) statistical programs. A one-way analysis of variance (ANOVA) was used to

determine any significant differences between the characteristics of the ATI and CON groups, as well as the TEN and RUP sub-groups. When the overall F value was significant, a Tukey's HSD post hoc test was used to identify specific differences. Statistical significance was accepted when $p<0.05$. Data are presented as means ± standard deviations (SD) or frequencies with the number of subjects in parentheses. Chi-square analysis (Graphpad Instat version 3) was used to analyze differences in the ABO blood group frequencies between the CON, ATI, TEN, RUP and WC POP groups as well as to analyze differences in the country of birth and gender frequencies between the CON and ATI groups, as well as the TEN and RUP subgroups. Chi-square analysis (Graphpad Instat version 3) was also used to compare number of people smoking, number of injuries experienced, and the limb injured. The Fisher's Exact Test (Graphpad Instat version 3) was used to compare the differences in the dominant hand and the position of the Achilles tendon injured.

2.3 RESULTS

2.3.1 *Subjects Characteristics*

A total of 118 Caucasian subjects with a current or past clinical history of Achilles tendon injury (ATI) and 131 apparently healthy asymptomatic Caucasian controls (CON) were included in the ABO blood typing study. The ATI group consisted of 75 patients diagnosed with Achilles Tendinopathy (TEN) and 43 with either a complete or partial rupture of the Achilles tendon (RUP). It is important to note that these numbers may be slightly lower in the subsequent tables since data such as age, height, weight, BMI, gender, country of birth and other variables were missing in some subjects. An additional 62,216 individual Caucasian ABO blood group types were obtained from the Western Province Blood Transfusion Organization (the official blood transfusion center for the greater Cape Town area). The larger group (WC POP) represented the general population in the study area.

As shown in table 2.3, the CON, ATI, TEN and RUP groups were similarly matched for age, height, gender and country of birth. The ATI, TEN and ATR groups were however significantly heavier, with the corresponding higher BMI, than the CON group. The RUP sub-group was also significantly heavier with a higher BMI than the TEN sub-group. The ATI subjects were recruited for the present study on average 7.4 ± 8.5 years, ranging from 0 to 39 years, after the onset of their initial symptoms. Although most of the subjects in each group were right handed, significantly more individuals were right handed in the CON group (n=114, 91.9 %) than in the ATI (n=88, 82.2 %, p=0.030, odds ratio of 2.5; 95% confidence interval 1.1 to 5.6) or in the TEN (n=55, 78.6 %, p=0.013, odds ratio of 3.1; 95% confidence interval 1.3 to 7.4). There was however no significant difference between the CON and RUP (n=33, 89.2 %, p=0.739, odds ratio of 1.4; 95% confidence interval 0.4 to 4.7) groups (Table 2.3). There were no reported ambidextrous individuals in the study. Significantly fewer ATI (n=70, 61.4%,

Chi-square=6.2, p=0.046) and RUP (n=21, 50.0 %, Chi-square=10.8, p=0.005) subjects have never smoked when compared to CON subjects (n=95, 75.4%). There were no differences when the CON and TEN (n=49, 68.1 %) groups were compared (Chi-square=1.5, p=0.480) (Table 2.3).

Table 2.3. Characteristics of the control (CON) and Achilles tendon injury (ATI) groups, as well as the Achilles tendinopathy (TEN) and Achilles rupture (RUP) sub-groups.

	CON (n=131)	ATI (n=118)	TEN (n=75)	RUP (n=43)
Age (yrs)[1]	41.3 ± 10.9 (124)	40.2 ± 13.6 (118)	40.0 ± 15.1 (75)	40.7± 11.1 (43)
Height (cm)	174.7 ± 9.3 (123)	176.0 ± 9.0 (118)	176.1 ± 9.3 (75)	175.8 ± 8.3 (43)
Weight (kg)	71.1 ± 12.0 (127)[a,b,d]	80.6 ± 14.0 (118)[a]	77.2 ± 14.0 (75)[b,e]	86.4 ± 15.2 (43)[d,e]
BMI (kg/cm^2)	23.2 ± 2.7 (123)[a,c,d]	25.9 ± 3.9 (118)[a]	24.8 ± 3.4 (75)[c,e]	27.9 ± 4.0 (43)[d,e]
Gender (% males)	62.3 (130)	73.8 (122)	72.2 (79)	76.7 (43)
Country of Birth (% South Africa)	70.6 (126)	76.9 (117)	74.3 (74)	81.4 (43)
Right Handed (%)	91.9 (124)[f,g]	82.0 (111)[f]	78.1 (73)[g]	91.9 (38)
Current Smoker (%)	3.2 (126)[h,i]	7.7 (117)[h]	5.4 (74)	11.6 (43)[i]
Ex-smoker (%)	21.4 (126) [h,i]	29.9 (117)[h]	25.7 (74)	37.2 (43)[i]

Values are expressed as mean ± standard deviation or a frequency (%) where applicable. Number of subjects (n) is in parentheses. [1]The age of the ATI group as well as the TEN and RUP sub-groups are the age of onset of the symptoms of Achilles tendon pathology. [a]CON vs ATI (p<0.001), [b]CON vs TEN (p=0.005), [c]CON vs TEN (p=0.002), [d]CON vs RUP (p<0.001), [e]TEN vs RUP (p<0.001), [f]CON vs ATI (p=0.030), [g]CON vs TEN (p=0.008), [h]CON vs ATI for smoking (p=0.063) and [i]CON vs RUP for smoking (p=0.006).

In the TEN group, the additional documented clinical criteria to confirm the diagnosis were tenderness to palpation (63 of 75), early morning stiffness (43 of 75), a history of swelling (26 of 75), early morning pain (19 of 75), palpable thickening (17 of 75) and a positive "shift" test (11 of 75). In 36 of the 75 subjects, the diagnosis was confirmed by soft tissue ultrasound examination of the affected Achilles tendon. There were 31 of the 75 subjects with confirmed bilateral Achilles tendinopathy.

In the RUP group, 39 of 43 subjects experienced acute severe pain in the posterior lower leg as the main presenting symptom. The diagnosis was confirmed in all these subjects by either direct examination at the time of surgery (38 of 43) or by imaging (8 of 43) (soft tissue ultrasound, Magnetic Resonance Imaging or Computerized Tomography). Four of the 43 subjects had confirmed bilateral ruptures of the Achilles tendon, and 16 of 43 subjects had a history of tendinopathy prior to rupture.

Table 2.4. The distribution of subjects with Achilles tendon injury (ATI), Achilles tendon rupture (RUP) and Achilles tendinopathy (TEN) according to the region of the Achilles tendon injured.

Position	ATI (n=101)	TEN (n=62)	RUP (n=39)
Lower Third	38.6 (39)	27.4 (17)	56.1 (22)
Middle Third	60.4 (61)	71.0 (44)	43.6 (17)
Upper Third	1.0 (1)	1.7 (1)	0 (0)

Values are expressed as percentage with the number of subjects (n) in parenthesis.
TEN vs RUP, Fisher's Exact test, p=0.007.

As shown in table 2.4 , 38.6 %, 60.4 % and 1.0 % of the ATI subjects injured the lower, middle and upper third of their Achilles tendon, respectively. Significantly more TEN subjects (71.0 %) injured the middle third of the Achilles tendon when compared to the RUP subjects (43.6 %, Fisher's exact test, p=0.007).

There were no significant differences in the distribution of number of subjects with a left or right leg injury within ATI (33.3 vs 35.1%), TEN (27.9 vs 26.7 %) and RUP (41.9 vs 48.8%) groups (Table 2.5). However, a greater number of subjects in the TEN group injured both legs (45.6%) compared to the RUP group (9.3%). The majority of the subjects in ATI (75.2%), TEN (81.4%) and RUP (65.1%) groups had sustained only 1 injury to the Achilles tendon (Table 2.6).

Table 2.5. The distribution of subjects with Achilles tendon injury (ATI) group, as well as Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP) sub-groups according to the injured leg.

Leg	ATI (n=111)	TEN (n=68)	RUP (n=43)
Right	35.1 (39)	26.7 (18)	48.8 (21)
Left	33.3 (37)	27.9 (19)	41.9 (18)
Both	31.5 (35)	45.6 (31)	9.3 (4)

Values are expressed as percentage with the number of subjects (n) in parenthesis.
TEN vs RUP, Chi-square=16.3, p<0.001.

Table 2.6. The distribution of subjects with Achilles tendon injury (ATI) group, as well as Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP) sub-groups according to the number of injuries experienced.

Injuries	ATI (n=113)	TEN (n=70)	RUP (n=43)
3 or more	8.9 (10)	8.6 (6)	9.3 (4)
2	15.9 (18)	10.0 (7)	25.6 (11)
1	75.2 (85)	81.4 (57)	65.1 (28)

Values are expressed as percentage with the number of subjects (n) in parenthesis.
TEN vs RUP, Chi-sqaure=5.0, p=0.081.

2.3.2 *Mode of Injury*

The activity resulting in injury in the majority of the ATI subjects was running (50 of 107, 46.7%) or playing squash (20 of 107, 18.7%), while the remaining injuries occurred as a result of participating in a variety of sports and activities (Table 2.7). When the ATI group was divided into TEN and RUP, the distribution of injuries associated with running and squash changes, showing more clearly that Achilles tendinopathy was associated more with running (43 of 65, 66.2%) while Achilles tendon rupture was more associated with squash (17 of 42, 40.5%).

2.3.3 *Physical activity history*

As shown in Table 2.8, all the subject groups and sub-groups were matched for the number of years participated in running. Seventy-four percent, 56%, 61% and 46% of the CON, ATI, TEN and RUP subjects respectively, participated in running. Over the last two years, both the ATI and RUP groups, but not the TEN group, trained significantly less than the CON group. The three symptomatic Achilles pathology groups, however, participated for significantly more years in

high impact sports than the CON group. There were no significant differences in the hours of training in high impact sports over the last two years between the groups. There were also no significant differences in years of participation and the level of training over the last two years when only the squash players were analysed (data not shown).

Table 2.7. The distribution of subjects with Achilles tendon injury (ATI) as well as the Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP) according to the activity of injury.

Activity	ATI (n=107)	TEN (n=65)	RUP (n=42)
Running	46.7 (50)	66.2 (43)	16.7 (7)
Squash	18.6 (20)	4.6 (3)	40.5 (17)
Tennis	4.7 (5)	1.5 (1)	9.5 (4)
Rugby	2.8 (3)	3.1 (2)	2.4 (1)
Touch Rugby	1.9 (2)	0.0 (0)	4.8 (2)
Hockey	0.9 (1)	0.0 (0)	2.4 (1)
Soccer (jogging)	0.9 (1)	0.0 (0)	2.4 (1)
Walking	0.9 (1)	1.5 (1)	0.0 (0)
Gymnastics	0.9 (1)	0.0 (0)	2.4 (1)
Unknown	1.9 (2)	3.1 (2)	0.0(0)
Other#	19.6 (21)	20.0 (13)	19.0 (8)

Values are expressed as percentage with the number of subjects (n) in parenthesis.
#Other activities included activities such as house work (1), horse riding (1), running upstairs (1), digging in the garden (1), getting off a flight (1), sudden crossing of a road (1), descending stairways (1), pushing a car (1), slipping down the road (1) and dismantling the antenna (1).

Table 2.8. Participation in physical activity and training of the control (CON) and Achilles tendon injury (ATI) groups.

	CON (n=127)	ATI (n=114)
Running (yrs)	7.9 ± 8.1 (127)	8.5 ± 10.6 (114)
Running in the past 2 yrs (h/wk)	3.3 ± 2.9 (123)[a]	2.0 ± 2.6 (102)[a]
High Impact Sports (yrs)	11.5 ± 8.6 (127)[a]	19.6 ± 13.0 (114)[a]
High Impact Sports in the past 2 yrs (h/wk)	5.1 ± 4.9 (127)	3.9 ± 5.0 (114)

Values are expressed as mean ± standard deviation. Numbers of subjects are in parentheses.

[a]CON vs ATI, $p<0.001$

2.3.4 *Blood group distribution*

As shown in Table 2.9, the overall ABO blood group distribution between the 5 groups was significantly different (Chi Square=32.1, p=0.001). Interestingly, the ABO blood group frequency distribution between the CON and WC POP groups were significantly different (Chi square=9.2, p=0.027). The CON and ATI ABO blood group distribution was also significantly different (Chi square=10.6, p=0.014). However, there were no significant differences in the ABO blood group distribution between the CON vs TEN, the CON vs RUP or the TEN vs RUP groups. The percentage of individuals with blood group O was similarly distributed between the CON, ATI, TEN and RUP groups (Chi-square=0.2, p=0.983). The A/O ratio of the CON (1.0) , ATI (1.0), TEN (1.1), and RUP (1.0) groups were also similar. The A/O ratio of the WC POP group was however lower (0.7). The ABO blood group distribution of the individuals with bilateral

injuries were also similar (data not shown) to those of the CON group (Chi square=0.4, p=0.795)

Table 2.9. ABO blood group relative frequencies of the control (CON), Achilles tendon injury (ATI), Achilles tendinopathy (TEN), Achilles tendon rupture (RUP) and the Western Cape Caucasian population (WC POP) subgroups.

	CON (n=131)	ATI (n=118)	TEN (n=75)	RUP (n=43)	WC POP (n=62,216)
A (%)	39.7 (52)	40.7 (48)	41.3 (31)	39.5 (17)	34.7 (21,564)
O (%)	41.2 (54)	39.8 (47)	38.7 (29)	41.9 (18)	48.9 (30,448)
B (%)	18.3 (24)	11.0 (13)	13.3(10)	7.0 (3)	12.6 (7,825)
AB (%)	0.8 (1)	8.5 (10)	6.7 (5)	11.6 (5)	3.8 (2,379)

Values are expressed as percentage with the number of subjects (n) in parenthesis.
CON vs WC POP Chi-square=9.2, p=0.027; CON vs ATI Chi-square=10.6, p=0.014; CON vs TEN Chi-square=0.1, p=0.937; CON vs RUP Chi-square=0.0, p=0.996 and TEN vs RUP Chi-square=0.1, p=0.942.

2.3.5 *Identification of genes on chromosome 9q32-q34*

One thousand and sixty-two genes have been mapped to chromosome 9 were initially identified by searching the OMIM™ (Online Mendelian Inheritance In Man) (www.ncbi.nlm.nih.gov/entrenz/) and NCBI Map Viewer (www.ncbi.nlm.gov/mapview/) databases. Of these, 404 have been mapped to the same locus as the *ABO* gene between 9q32-q34 (Appendix 2.7). Seven genes were identified as pseudogenes and were therefore excluded as candidate genes. Twenty-one of the remaining genes were identified as possible candidate genes for Achilles tendon injuries (Table 2.10). Four of the 21 genes, *COL5A1*, *TNC*,

COL27A1 and *LAMC3* encode for components of the extracellular matrix. Nine of the candidate genes, *TNFSF15*, *TRAF1*, *GSN*, *DAPK1*, *PTGES*, *WDR31*, *WDR34*, *CARD9* and *TRAF2*, encode for proteins involved in cell death. Four of the genes, *PPP2R4*, *ABL1*, *PRRX2* and *EDG2*, encode for proteins involved in cell growth, while the remaining 4 genes encode encode for proteins involved in a variety of processes ranging from angiogensis (*PTGS1*), signal transduction (*RAPGEF1*), cell interactions (*NOTCH1*) and a proteasome component (*PSMB7*). The relative location of these genes within the 9q32-9q34 region is shown in figure 2.2.

Table 2.10. Possible candidate genes for Achilles Tendon Injury, identified in the 9q32-34 locus flanking the *ABO* gene

Candidate Gene No	Cytogenetic Location	Gene Name	Short Description
1	9q34.2-q34.3	*COL5A1*	Encodes for the α1 chain of type V collagen
2	9q33	*TNC*	Encodes for a matricellular protein found in connective tissues, including tendons
3	9q33.1	*COL27A1*	Encodes for a fibrillar collagen found in a number of tissues
4	9q31-q34	*LAMC3*	Encodes for the gamma 3 chain of the extracellular matrix protein laminin 12
5	9q32	*TNFSF15*	Encodes for a cytokine expressed in endothelial cells and is capable of inducing apoptosis in endothelial cells
6	9q33-q34	*TRAF1*	Encodes for a protein that mediates signal transduction and anti-apoptotic signals
7	9q33	*GSN*	Encodes for a protein that is involved in apoptosis
8	9q34.1	*DAPK1*	Encodes for a kinase which mediates gamma-interferon induced cell death
9	9q34.3	*PTGES*	Encodes for a protein involved in apoptosis and inflammation

Table 2.10. Possible candidate genes for Achilles Tendon Injury, identified in the 9q32-34 locus flanking the *ABO* gene (Continued)

Candidate Gene No.	Cytogenetic Location	Gene Name	Short Description
10	9q32	*WDR31*	Encodes for protein involved in apoptosis and cell signaling
11	9q34.11	*WDR34*	Encodes for protein involved in apoptosis and cell signaling
12	9q34.3	*CARD9*	Encodes for a protein involved in cell apoptosis
13	9q34	*TRAF2*	Encodes for a protein that mediates signal transduction and anti-apoptotic signals.
14	9q34	*PPP2R4*	Encodes for a phosphatase involved in cell division and growth
15	9q34.1	*ABL1*	Encodes for a kinase involved in cell division, differentiation and adhesion and stress response
16	9q34.1	*PRRX2*	Encodes for a protein whose expression is localized to proliferating fetal fibroblasts
17	9q32	*EDG2*	Encodes for a protein involved in cell proliferation and signaling
18	9q32-q33.3	*PTGS1*	Encodes for an enzyme prostaglandin-endoperoxide synthase, which regulates angiogenesis
19	9q34.3	*RAPGEF1*	Encodes for a globular protein that mediates signal transduction from cell surface to the nucleus
20	9q34.3	*NOTCH1*	Encodes for a transmembrane protein which is thought to play a role in cell interaction
21	9q34.11-q34.12	*PSMB7*	Encodes for a core beta subunit in proteasome B-type family

Candidate genes number 1 to 4 encode for components of the extracellular matrix, while the proteins encoded for by candidate genes number 5 to 13 are involved in apoptosis. Proteins encoded for candidate genes number 14 to 17 are involved in cell growth. The remaining 4 candidate genes encode for proteins involved in a variety of biological process including angiogensis (18), signal transduction (19), cell interactions (20) and a proteasome component (21).

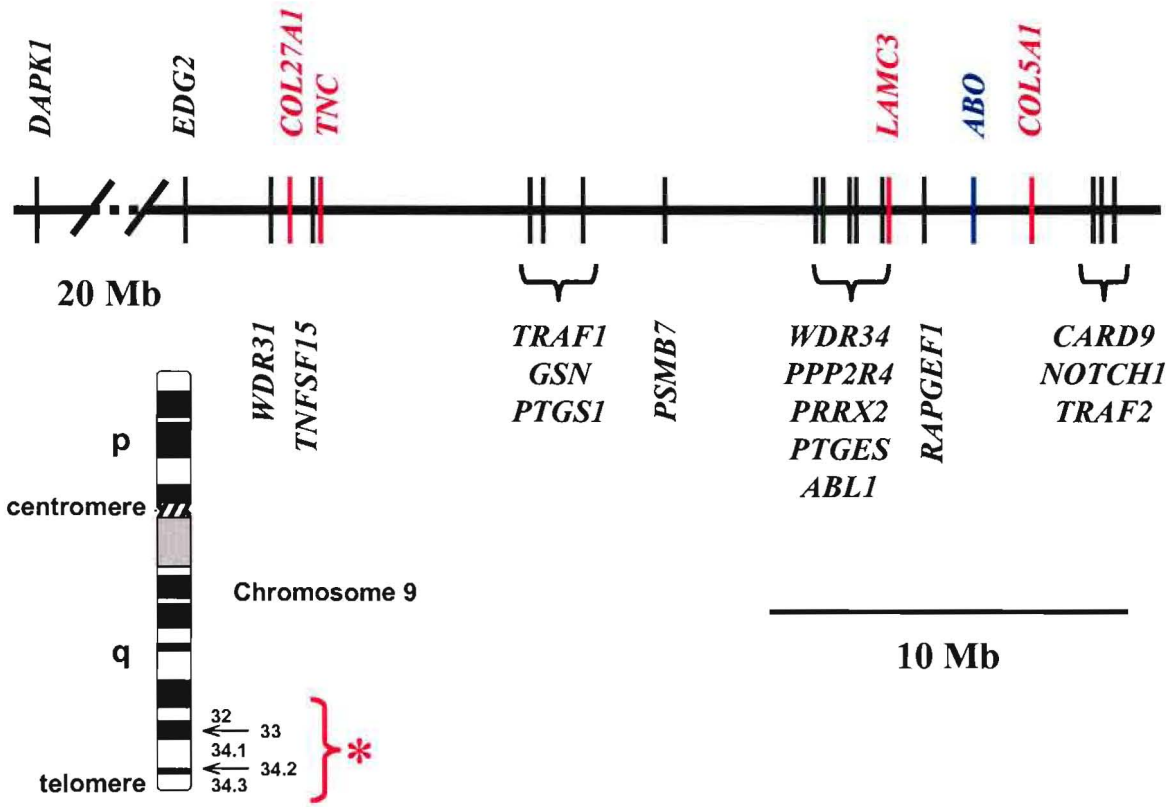

Figure 2.2. Schematic diagram depicting the relative positions of the genes within the chromosome 9q32-q34.3 locus (*). The position of the *ABO* gene is shown in blue. The genes which encode for components of the extracellular matrix are shown in red, while the remaining genes are shown in black. The dashed line represents 20 Mb of DNA not shown.

2.4 DISCUSSION

The ABO blood group was evenly distributed between the asymptomatic control subjects and the subjects with clinical symptoms of Achilles tendon injuries. There were also similar distributions of the ABO blood group when the Achilles tendon injury subjects were divided into the rupture and tendinopathy sub-groups. The ABO blood group distribution in the control and the Achilles tendon injury groups, as well as the Achilles tendon rupture and Achilles tendinopathy sub-groups, were however significantly different than the general Caucasian population in the greater Cape Town area. The O blood group and the A/O ratio were however not over-represented in the Achilles tendon injury group or any of the sub-groups when compared to the control group or the general population. The reason for differences in the blood group distribution of the general population when compared to the recruited subjects is not clear. The values for the general population are however similar to published values for other Caucasian populations (Maffulli et al., 2000; Garratty et al., 2004).

Since the ABO antigens are ubiquitous glycoproteins found throughout the body, some scientists have speculated that the different transferases encoded for by the *ABO* gene might play a role in tendon extracellular matrix function (Maffulli et al, 2000; Jozsa et al., 1989). A more plausible explanation for the reported association of the ABO blood group and Achilles tendon pathology would be that another gene(s) closely linked to the *ABO* gene encodes for a protein(s) which is directly involved in the aetiology of the pathology (Kujala et al., 1992). The *ABO* gene has been mapped to the telomeric end of the long arm of chromosome 9 (9q34). It could be that another gene(s) in linkage disequilibrium with the *ABO* gene encode for protein(s) either directly or indirectly involved in the synthesis, degradation, remodelling and/or metabolism of the tendon matrix. Nail Patella Syndrome (NPS), for example, was initially shown to be linked to the ABO blood group (reviewed in McIntosh et al., 2005). The gene causing NPS, *LMX1B*, was

identified and mapped 40 years later to the same locus as the *ABO* gene on chromosome 9q34 (Riddle et al., 1995; Chen et al., 1998). This supports the hypothesis that the reported association of the ABO blood group with Achilles tendon pathology is suggesting that other genes mapped to the long arm of chromosome 9q34 are involved in this pathology.

The novel component of the present study was to identify possible candidate genes that have been mapped to the same locus as the *ABO* gene on the tip of the long arm of chromosome 9 which could encode for proteins that theoretically could be involved in Achilles tendon pathology. Four hundred and four genes were initially identified within the same locus as the *ABO* gene between 9q32-q34. This region was calculated to be about 31 Mb or 31 centiMorgans (cM). One centimorgan in linkage terms represents about 1 million base pairs and is usually equivalent to 1% recombination in the offspring (ghr.nlm.gov/ghr/glossary). Twenty one candidate genes were identified from the 404 genes. The 21 candidate genes were divided into four groups according to the function of the proteins they code for, namely (i) structural proteins of connective tissue (4 of 21), (ii) proteins involved in cell proliferation (4 of the 21), (iii) proteins involved in regulatory processes (4 of the 21) (iv) and finally proteins involved in programmed cell death (apoptosis) (9 of the 21). Although four of the identified genes encode for structural components of the extracellular matrix, none of the genes on the tip of the long arm of chromosome 9 appeared to encode for enzymes and regulatory proteins, such as the matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin-like repeats) enzymes, involved in the remodeling of the extracellular matrix. However, these class of proteins would also be ideal candidate genes for tendinopathy (reviewed in Riley et al., 2005a). Information on the gene function for almost half of the 404 genes found in the 9q32-q34 locus was either unavailable or scanty. There may, therefore, be

additional candidate genes in this region, which will only be identified once their function has been elucidated.

When the ABO blood group was first shown to be associated with Achilles tendon injuries (Jozsa et al., 1989), researchers speculated that one of the collagen genes might be linked to the *ABO* gene on chromosome 9 (Maffulli et al., 2000; Kannus and Natri, 1997). It was reasoned that because the main structural component of tendons are collagen fibrils, consisting predominately of type I and III collagens, any gene encoding for a collagen α chain which mapped to the 9q32-q34 locus would be a very strong candidate gene for Achilles tendon injury. However, in 1992 it was erroneously stated that none of the collagen genes have been mapped to this region of chromosome 9 (cited in Kannus and Natri, 1997). Two of the identified candidate genes in this study, namely the *COL5A1* and *COL27A1* genes, encode for α-chains of type V and XXVII collagens, respectively.

The *COL5A1* gene encodes for the α1 chain (pro- α1(V) chain) of the low abundance heterotrimeric type V fibrillar collagen (Smith and Simpson, 1989; Kujala et al., 1989; Greenspan et al., 1992). The pro- α1(V) chain is found in most of the isoforms of type V collagen (reviewed in Ristiniemi and Oikarinen, 1989). Type V collagen is found in tissues, including tendons, containing type I collagen and appears to regulate the assembly (fibrillogenesis) of heterotypic fibers composed of both type I and type V collagen (Birk et al., 1990). It is thought that the proportion of type V collagen in tendons may influence the diameter of the fibre. Increases in type V collagen content have been reported with age in the rabbit patellar tendon and in biopsy samples of degenerative tendons (Dressler et al., 2002; Goncalves-Neto et al., 2002). In healing tendons and ligaments of rabbits, increases in type V collagen content relative to type I collagen are associated with increased proportion of smaller fibril diameters (Dressler et al., 2002). Recently, it has been demonstrated that type V collagen deficient mice could not form collagen fibrils (Wenstrup et al., 2004), emphasizing the role of

type V collagen in fibrillogenesis. The authors proposed that no other collagen studied so far, including type I, has such major control in fibril initiation as type V collagen. It was therefore reasonable that the *COL5A1* gene was identified as a strong candidate gene for this study, even though type V collagen is only a minor fibrillar collagen in tendons. The association of polymorphisms with the *COL5A1* gene and Achilles tendon injuries were investigated in this thesis. The results of this investigation are presented in the following chapter (Chapter 3).

The *COL27A1* gene, on the other hand, encodes for the homotrimeric type XXVII collagen, which is the latest fibrillar collagen to be characterized to date (Boot-Handford et al., 2003; Pace et al., 2003). Fibrillar collagens, which also include type I, II, III, V and XI collagens, form structural elements in extracellular matrices of tendons as well as other tissues. Type XXVII collagen is predominantly expressed in cartilage, eye, ear, lung and colon of the mouse and its precise function within these tissues is unknown (Pace et al., 2003). Although no investigators have to our knowledge reported that the gene is expressed in tendons, it is conceivable that it plays a role in tendon connective tissue. Since the expression and function, if any, of the *COL27A1* gene in tendons is currently unknown, the association of this gene with Achilles tendon injuries was not investigated in this study.

In addition to the two collagenous genes, two other genes, *TNC* and *LAMC3*, which encode for non-collagenous components of the ECM, were also mapped to the tip of the long arm of chromosome 9. The *TNC* gene encodes for tenascin C, which is a glycoprotein abundantly found in tissues subjected to high tensile and compressive stress such as tendons. In tendons the protein is found predominantly in the myotendinous and osteotendinous junctions, as well as in tendon substance (Jarvinen, 2000). The protein is believed to regulate cell-matrix interactions due to its ability to bind various cell surface receptors, such as integrins, and other components of the ECM (reviewed in Jones and Jones, 2000).

In addition, it has also been shown that expression of *TNC* gene is regulated in a dose-response manner by mechanical loading in tendons (Jarvinen et al., 1999; Jarvinen et al., 2003). In addition it has also been suggested that tenascin C might play an important role in providing elasticity to musculoskeletal tissues (Jarvinen, 2000). *TNC* gene expression has been shown to be up-regulated in Achilles tendinopathy (Jarvinen et al., 1999), suggesting the protein involvement in tendinopathy. The *TNC* gene is therefore also a strong candidate gene for Achilles tendon injuries due to its chromosomal location, its regulation by mechanical loading and its possible association with musculoskeletal elasticity. The association of polymorphisms within this gene and Achilles tendon injuries are presented in Chapter 4 of this thesis.

The *LAMC3* gene, which encodes for the γ-chain of laminin 12, is expressed in lung, reproductive tract, brain and retina (Koch et al., 1999, Gerhalmi-Friedman et al., 2001). To our knowledge it is not expressed in tendons. The laminins are a family of heterotrimeric glycoproteins consisting of an α-, β- and γ-chain to form a characteristic cruciform structure, which are the major non-collagenous constituent of basement membranes (Fuller and Shields, 1998). They have been implicated in a wide variety of biological processes including cell adhesion, differentiation, migration and signaling (Fuller and Shields, 1998). Although some laminin are found in human myotendinous junctions (Pedrosa-Domellof et al., 2000), none of the genes that encode for their α-, β- and γ-chains have not been mapped to the tip of the long arm of chromosome 9. The association of this gene with Achilles tendon pathology was therefore not investigated in this thesis.

The primary focus of this thesis was to identify genes located on the telomeric end of chromosome 9q that encode for structural components of the ECM and in particular tendons of which four and two were identified respectively. Since other biological processes, such as but not limited to apoptosis of tenocytes and neovascularization, have been proposed to be involved in tendon overuse

injuries, candidate genes encoding for proteins that, at least theoretically, could be involved in these biological processes were also identified. There associations with Achilles overuse injuries were however not investigated in this thesis.

As mentioned above, apoptosis (programmed cell death) has received some attention as one possible mechanism associated with mechanical loading-induced tendinopathy (Murrell, 2002). Nine candidate genes, *TNFSF15*, *TRAF1*, *TRAF2*, *GSN*, *DAPK1*, *PTGES*, *WDR31*, *WDR34* and *CARD9*, mapped to the tip of chromosome 9q that encode for proteins believed to be involved in the apoptosis pathway were identified in the study.

The protein encoded for by the *TNFSF15* gene is a cytokine, which is abundantly expressed in endothelial cells (Zhai et al., 1999). It can activate NF-κB and MAP kinases in an autocrine manner to induce apoptosis of endothelial cells (Yue et al., 1999). This cytokine is also found to inhibit endothelial cell proliferation, and thus may function as an angiogenesis inhibitor (Zhai et al., 1999). It is known that formation of new vessels in the tendon is associated with Achilles tendinopathy (Ohberg et al., 2001). The proteins encoded for by the *TRAF1* (TNF receptor-associated factor 1) and the *TRAF2* genes mediate the anti-apoptotic signals from TNF receptors (Tsitsikov et al., 2001). The two proteins form a complex, which activates the stress activated protein kinase (c-Jun N-terminal kinase, JNK) as well as the MAPK8 and NF-κB. TRAF2 also act indirectly to inhibit caspase activity, a process vital in apoptosis.

The GSN gene encodes a protein gelsolin, which has been shown to inhibit apoptosis via a reduction of caspase-3 activation as well as inhibiting cytochrome c release (Kamada et al., 1998). Both caspase activation and cytochrome c release are important steps in apoptosis (Kamada et al., 1998). Furthermore, caspase-3 has been shown to be responsible for cleavage of gelsolin (Kamada et al, 1998). It is therefore likely that during chronic tendinopathy there is an imbalance

between gelsolin cleavage and caspase-3 activity, which might be associated with the increased apoptosis during tendinopathy. The death-associated protein kinase 1 (*DAPK1*) gene is a positive mediator of programmed cell death which is initiated by either γ-interferon, TNF-α, activated Fas or detachment of the cell from the extracellular matrix (Cohen et al., 1997; Cohen et al., 1999). The *DAPK1* gene encodes a calcium/calmodulin dependent serine-threonine kinase, which is localized to the cytoskeleton (Cohen et al., 1997).

The protein encoded by the prostaglandin E sythase (*PTGES*) gene is a glutathione-dependent prostaglandin E synthase. The expression of *PTGES* has been shown to be induced by interleukin 1β (IL-1β) and p53 tumour suppressor protein (TP53) (Jakobsson et al., 1999a; Jakobsson et al., 1999b). It has been suggested that prostaglandin synthase E may be involved in TP53 induced apoptosis (Polyak et al., 1999). Knockout studies in mice suggest that this gene may contribute to the pathogenesis of collagen-induced arthritis and mediate acute pain during inflammatory responses (Trebino et al., 2003).

Both the *WDR31* and *WDR34* genes encode proteins which may facilitate formation of heterotrimeric or multiprotein complexes involved in a variety of cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation (reviewed in Lin et al., 2003). It is not clear whether these two proteins are found in tendons.

The CARD9 protein is encoded by the caspase-associated recruitment domain (*CARD9*) gene, which like all CARD family members, is defined by the presence of a caspase-associated recruitment domain (CARD) (Bertin et al., 2000). It is likely that the caspase-associated recruitment domain participates in the activation or suppression of CARD containing members of the caspase family, and thus plays an important regulatory role in cell apoptosis, via the activation of

NF-κB (Bertin et al., 2000). It is also not clear whether this protein is found in tendons.

It is important to note that although connective tissues, such as tendons, are made up predominantly of the ECM, the synthesis, degradation and maintenance of the ECM depends on the presence of cells within it. Piez (1997) summed this up beautifully by stating that "there can not be any ECM without cells". The cells possess membrane surface receptors such as integrins, which are crucial for cell-matrix interaction in many biological processes, such as signaling and detection of external stimuli such as mechanical loading in the case of tenocytes. The cells also contain autocrine and paracrine factors that are responsible for cell-cell communication (Piez, 1997). Four of the candidate genes, *PPP2R4*, *ABL1*, *PRRX2* and *EDG2*, identified in this study encode for proteins involved in cell growth, while the remaining 4 genes encode for proteins involved in a variety of processes ranging from angiogensis (*PTGS1*), signal transduction (*RAPGEF1*), cell interactions (*NOTCH1*) and a proteasome component (*PSMB7*).

The *PPP2R4* gene encodes a protein that catalyses the conversion of a known inactive Serine/Threonine phosphatase (protein phosphates 2A or PP2A) to an active form. PP2A is implicated in the negative control of cell growth and division and other cellular processes (reviewed in Janssens et al., 2005; Gallego and Virshup, 2005). The *ABL1* gene is an oncogene that encodes a tyrosine kinase that has been implicated in processes of cell differentiation, cell division, cell adhesion, and stress response in normal cells (reviewed in Cejkova et al., 2005). The *PRRX2* gene encodes a protein that is expressed predominantly in proliferating foetal fibroblasts and the developing dermal layer (reviewed in White et al., 2003). Protein expression is however down-regulated in the adult skin. Increased expression of this gene during foetal but not adult wound healing suggest a possible role in mechanisms that control mammalian dermal regeneration and prevent formation of scar tissue during foetal growth. Wound

healing by scar formation is the most prominent healing response during adult tendon repair, whereas foetal tendons heal without scar formation (reviewed in White et al., 2003). Interestingly, the *tenascin C* gene promoter has a PRRX2 binding site about 20 bp upstream of the TATA box (Norris and Kern, 2001). Blood borne lysophospholipids, Sphingosine-1-phophate (S1P) and Lysophosphatic acid (LPA) require receptors to mediate their varied functions, which include modulation of cell shape, cell migration, cell invasion, stimulation of cell growth, prevention of apoptosis, regulation of actin cytoskeleton (Takuwa et al., 2002). One of the receptors is encoded by the *EDG2* gene, modulates the function of LPA and is involved in cell signaling and other diverse biologic functions, including proliferation and chemotaxis (Takuwa et al., 2002). EDG2 is widely expressed in almost all tissues (Takuwa et al., 2002).

The *PTGS1* gene encodes PTGS1 or cyclooxygenase 1, a constitutively active enzyme, involved in prostaglandin biosynthesis and regulation of angiogenesis in endothelial cells (reviewed in Warner and Mitchell, 2004). PTGS1 is thought to be involved in cell-cell signaling and maintaining tissue homeostasis and PTGS1 protein is regulated by cytokines and growth factors (Warner and Mitchell, 2004). It is possible that it is involved in the pain associated with tendinopathy.

Proteasomes (encoded for by the *PSMB7* gene) are proteolytic enzymes responsible for the degradation of misfolded and denatured proteins in response to different cell stressors as well as mediate other activities such as cell division, metabolism, DNA repair and transcription (reviewed in Maupin-Furlow et al., 2005; Wolf and Hilt, 2004). *RAPGEF1* gene encodes a human guanine nucleotide releasing protein for Ras protein (reviewed in Shivakrupa et al., 2003). It mediates binding events that control the activity and localization of many proteins involved in the transmission of signals from the cell surface to the nucleus. It is expressed, at least at the mRNA level, widely in most tissues, but it is not known whether it is found in tendons. The *NOTCH1* gene encodes a

transmembrane protein, involved in intercellular signaling and also acts as a receptor for membrane bound ligands (Conboy et al., 2003). Incidentally, NOTCH1 signaling is vital for skeletal muscle regeneration and its activity decreases with age (Conboy et al., 2003). The authors suggested that this activity might be a generalized mechanism associated with reduced regenerative capacity of most aged tissues. Because tendinopathy is associated with aging, it is possible that NOTCH1 signalling plays an important role in the tendon regenerative capacity in response to mechanical loading.

In addition to the genetic factors, several non-genetic intrinsic factors, such as amongst others, age, gender, body weight and a history of previous injury; as well as extrinsic factors, such as type of activity and training, have been implicated in the aetiology of Achilles tendon overuse injuries (Jarvinen et al., 2005). Although the subjects in the Achilles tendon injury groups and sub-groups were significantly older than the asymptomatic control subjects when recruited into the trial, there were similarly matched for age when the age of onset of the initial symptoms in the Achilles tendon injury group was used in the analysis. The symptomatic subjects in this study were significantly heavier than the control subjects and also participated for significantly more years in high-impact sports. Any possible interaction of weight and/or physical activity exposure with their genetic make-up was responsible for the development of symptoms of Achilles tendon injuries could not be excluded. This will be discussed further in chapter 7 of this thesis. It should be noted however that because this was a retrospective study, body weight of the subjects at the onset of their initial injury could not be recorded accurately. Anecdotally, many subjects reported increases in their body weight after injury as a result of a decrease or in worst cases a complete cessation in physical activity.

In the current study, a significant number of control subjects either smoked or were ex-smokers compared to the Achilles tendon injury subjects. Two studies

were found in the literature that investigated the possible association between smoking and tendon injury. In one study of wrist tendinopathy, the proportion of smokers in the injury group was significantly higher than would be expected in the general population (Safrin and Graham, 2002). The sample size of the injured subjects was however very small, consisting of only 14 subjects. In the second study, no association was found between smoking and risk of tendon injury (Harris et al., 1999).

It has been suggested that the majority of ruptures occur in the non-dominant or left leg (assuming that most people are right footed) (reviewed in Maffulli et al., 1999). In the current study the vast majority of the subjects were right handed (and assumed to be right footed), 91.9% in the CON and RUP groups. There were slight less right-handed individuals in the ATI (82.0%) and TEN (78.1%) groups. There was however an even distribution in of injuries in either the left or right Achilles tendon in study with just over 30% of the subjects injuring both tendons.

All the control subjects in this study did not have any clinical symptoms of Achilles tendinopathy. The Achilles tendons of only 22 (16.8%) of these subjects were examined using imaging technique (ultrasonography) (refer to Chapter 6). Two of the subjects had some form of abnormality in one of their tendons. This is therefore a limitation of this study, since we did not exclude any of the control subjects with sub-clinical signs of tendinopathy. These sub-clinical signs are known to precede clinical presentation of signs and symptoms of tendinopathy (Khan et al., 1998). Furthermore, the number of signs and symptoms associated with chronic Achilles tendinopathy may be much lower than would be expected because the study was mainly retrospective.

In conclusion, twenty-one candidate genes were identified from a possible 404 genes, which have been mapped to the 9q32-q34.3 locus. Two of the 21 candidate genes, namely *COL5A1* and *TNC*, encode for structural components of tendons and whose expression have previously been shown to be altered during tendon pathology. The association of polymorphisms with these two genes with Achilles tendon overuse injuries was investigated in this thesis. The distribution of ABO blood groups was, however, similar between the control and symptomatic subjects.

CHAPTER THREE - THE *BSTUI* AND *DPNII* RESTRICTION LENGTH POLYMORPHISMS WITHIN THE *COL5A1* GENE AND ACHILLES TENDON INJURY

The data presented in this chapter has been published in the following article:-
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3.1 INTRODUCTION

Although there is a high incidence of chronic tendon injuries during sporting or exercise activities, the aetiologies of these conditions are not yet fully understood (Jarvinen et al., 2005; Jozsa and Kannus, 1997; Kannus and Natri, 1997; Maffulli, 1999). Some studies have suggested that there is, at least in part, a genetic component involved in Achilles tendon (Kannus and Natri, 1997; Maffulli, 1999) and, more recently, rotator cuff tendon injuries (Harvie et al., 2004). None of these studies, have however, identified or proposed specific genes that might be associated with overuse tendon injuries.

As discussed in chapter 2, several investigators have suggested that a gene(s) on the tip of the long arm chromosome 9, closely linked to the *ABO* blood group gene, is associated with Achilles tendon pathology (reviewed in Kannus and Natri, 1997). Recently, Årøen et al (2004) also suggested, based on their findings that individuals who had ruptured an Achilles tendon had an increased risk of rupturing their contralateral tendon, the possible involvement of genetic elements in the aetiology of Achilles tendon pathology. An earlier study found that 41% of individuals with unilateral Achilles tendinopathy developed symptoms of Achilles tendinopathy in the contralateral leg during an 8-year follow-up period (Paavola et al., 2000). Although beyond the scope of this thesis, a recent study has also suggested that there is a genetic component toward tearing the anterior cruciate ligament (Flynn et al., 2005). The above studies all suggest that they may be a genetic predisposition

towards connective tissue disorders associated with mechanical overuse, including tendon injuries.

Tendons have a highly ordered hierarchical structure made up of tightly packed protein bundles consisting predominantly of type I collagen fibres (reviewed in Silver et al., 2003). Trace amounts of other collagens, such as types III and V, form heterotypic fibres with the type I collagen found in tendons (Birk, 2001; reviewed in Silver et al., 2003). The pro-α1(V) chain is found in most of the isoforms of type V collagen and is encoded by the *COL5A1* gene, which has been mapped to the same locus as the *ABO* gene on chromosome 9q34 (Caridi et al., 1992). This gene was therefore identified as one of the ideal candidate genetic markers of Achilles tendon injuries in the previous chapter.

Pro-∝2(V) and pro-∝3(V) chains are also found in some of the type V collagen isoforms. Neither of the genes that encode for these two ∝-chains has however been mapped to human chromosome 9 and was therefore not investigated as candidate genes in this study (reviewed in Myllyharju and Kivirikko, 2001). In addition, several mutations within the *COL5A1* and *COL5A2* genes have been shown to cause more severe connective tissue disorders such as some of the Ehlers-Danlos syndromes (EDS), which have been shown to affect tendons (reviewed in Myllyharju and Kivirikko, 2001; Riley, 2004).

CHAPTER THREE - THE *BST*UI AND *DPN*II RESTRICTION LENGTH POLYMORPHISMS WITHIN THE *COL5A1* GENE AND ACHILLES TENDON INJURY

The *COL5A1* gene contains a *Bst*UI and a *Dpn*II restriction fragment length polymorphisms (RFLPs) within its 3'-untranslated region (UTR) (Greenspan and Pasquinelli, 1994). The influence of these polymorphisms on the expression of the *COL5A1* gene and the ultimate function of type V collagen is currently unknown. The aim of this chapter of the thesis therefore was to determine whether the *Bst*UI and/or *Dpn*II RFLPs within the 3'-UTR of the *COL5A1* gene are associated with Achilles tendon injury.

CHAPTER THREE - THE *BSTUI* AND *DPNII* RESTRICTION LENGTH POLYMORPHISMS WITHIN THE *COL5A1* GENE AND ACHILLES TENDON INJURY

3.2 MATERIALS AND METHODS

3.2.1 Subjects

The recruitment strategy, inclusion and exclusion criteria for the subjects included in the Achilles tendon injury (ATI) and control (CON) groups, as well as the Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP) sub-groups have been described in detail in Chapter 2 (refer to section 2.2.1). One-hundred and sixteen of the 122 Caucasian patients with a current or past clinical history of Achilles tendon injury (ATI) were included in this study. The ATI patients included 76 of the 79 subjects with chronic tendinopathy (TEN) and 40 of the 43 subjects with either a complete (37 of 40) or partial (3 of 40) rupture (RUP). All the subjects were physically active prior to the development of symptoms. One-hundred and twenty-nine of the 131 apparently healthy physically active Caucasian asymptomatic control (CON) subjects without any history of Achilles tendon injury were also included in this study.

3.2.2 Sample collection and total DNA extraction

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 total DNA extraction. Total DNA was extracted from the sample as described by Lahiri and Nurnberger (1991), with some modifications. Briefly, the blood samples were transferred to 15 ml polypropylene tubes, to which two volumes 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 a 10 minute incubation at room temperature, the white blood cells were pelleted by centrifugation at 1 200 x g at room temperature for 10 minutes and washed at least once with one volume of

TKM1 buffer. The washed 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 10 minutes at 55 °C or until the pellets had dissolved. 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 13 000 rpm (15 000 x g) 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 (15 000 x g) for 2 minutes at room temperature. The precipitated DNA was air dried for 30 minutes, resuspended in at least 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) either by incubation for one hour at 65 °C or over-night at room temperature, and stored at 4°C until PCR analysis.

3.2.3 *COL5A1 genotyping*

A 667 bp fragment containing the *Bst*UI and *Dpn*II restriction fragment length polymorphisms (RFLPs) within the 3'-UTR of the *COL5A1* gene was PCR amplified as described by Greenspan and Pasquinelli (1994) (Figure 3.1). The PCR was carried out in a total volume of 60 µl containing at least 100 ng of DNA, 20 pmol of the forward and reverse 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 (Invitrogen Life Technologies, Carlsbad, California, USA), using a PCR Express Thermal Cycler (Hybaid Limited, Middlesex, UK). The amplification was performed with an initial denaturing step at 94°C for 3 min, followed by 35 cycles of denaturing at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1.5 min, and a final extension step at 72°C for 8 min. The PCR products were digested with *Bst*UI to produce 351 bp and 316 bp fragments for the A1 allele or variant (1

*Bst*UI site), 316 bp, 271 bp and 80 bp fragments for the A2 allele or variant (2 *Bst*UI sites) and a 667 bp fragment for the A3 allele or variant (no *Bst*UI site). Since the rare A3 allele did not contain a *Bst*UI restriction site, all the samples with an A3 allele were digested at least in duplicate to confirm the genotype of the subject. The PCR products were also digested with *Dpn*II to produce 418 bp, 194 bp, 40 bp and 15 bp fragments for the B1 allele (3 *Dpn*II sites) and 612 bp, 40 bp and 15 bp fragments for the B2 allele (2 *Dpn*II sites). The resulting fragments were separated, together with 100 bp DNA ladder known size markers (Promega Corporation, Madison, Wisconsin, USA), on 5% non-denaturing polyacrylamide gels and visualized 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 (Figure 3.2).

The *Bst*UI and *Dpn*II RFLPs are probably produced by single nucleotide polymorphisms (SNPs) within the 3'-end of the *COL5A1* gene, which were not identified in the original article in which the A1, A2, A3, B1 and B2 alleles were initially described (Greenspan and Pasquinelli, 1994). The number and sizes of the produced alleles indicate that all three *Bst*UI sites are polymorphic while only the middle *Dpn*II site is polymorphic (Figure 3.1). All the published SNPs within exon 66 of the *COL5A1* gene, which corresponds to the gene's 3'-end, were therefore identified using the NCBI SNP database (www.ncbi.nlm.nih.gov/SNP/) (Figure 3.1). The A1 and A2 alleles of the *Bst*UI RFLP have T and C nucleotides at position 67579, respectively (SNP ID number rs12722). The A3 allele also has a T nucleotide at position 67579. The restriction enzyme *Bst*UI recognised the consensus sequence 5'-CGCG-3' which is destroyed when a T replaces the C at position 67579. There is also a *Bst*UI site at position 67499, which is destroyed to produce the A3 allele. Because the A3 allele is rare, the polymorphic site at position 67499 was not present in the SNP database. Individuals with a A1A1, A1A3 or A3A3 genotype were TT at

nucleotide 67579, while individuals with a A2A2 genotype were CC. Individuals with A1A2 and A3A2 genotype were TC at nucleotide 67579. The B1 and B2 alleles of the *Dpn*II RFLP have T and C nucleotides at position 67395, respectively (SNP ID number rs13946). The *Dpn*II restriction sites at positions 67198 and 67812 are found in both alleles and therefore function as internal digestion sites (i.e. they are not polymorphic). Individuals with a B1B1, B1B2 and B2B2 genotypes are TT, TC and CC at nucleotide 67395, respectively.

3.2.4 Statistical analyses

The required sample size for this study was determined using QUANTO Version 0.5 (http://hydra.usc.edu/gxe) (Gauderman, 2002). Data were analyzed using the STATISTICA version 7 (StatSoft Inc., Tulsa, OK, USA) and GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA, USA) statistical programs. A one-way analysis of variance (ANOVA) was used to determine any significant differences between the characteristics of the ATI and CON groups, as well as the TEN and RUP sub-groups. When the overall F value was significant, a LSD post hoc test was used to identify specific differences. Statistical significance was accepted when $p<0.05$. Where applicable, data are presented as means ± standard deviations (SD) with the number of subjects in parentheses. Pearson's Chi-square analysis was used to analyze differences in the genotype and allele frequencies between the ATI and CON groups. The genotype frequencies of the *Bst*UI RFLP of the COL5A1 gene were analyzed using Monte Carlo simulations (CLUMP version 2.0 program) (Sham and Curtis, 1995). The *Bst*UI (A1A1, A1A2, A2A2, A1A3, A2A3 and A3A3) and DpnII (B1B1, B1B2 and B2B2) RFLP genotypes were converted into their actual nucleotide genotypes (i.e. TT, TC and CC) at positions 67579 and 67395. Lewontin's standardized disequilibrium coefficient (D') was calculated from the actual genotypes using the Linkage Disequilibrium Analyzer 1.0 software (Ding et al., 2003).

```
67166                                   67198
|                                       |
ACCAAGAAAG GCTACCAGAA GACGGTTCTG GAGATCGACA CCCCCAAAGT GGAGCAGGTG 60
                    Forward Primer    DpnII

CCCATCGTGG ACATCATGTT CAATGACTTC GGTGAAGCGT CACAGAAATT TGGATTTGAA 120

                               67312           67326
                               |               |
GTGGGGCCGG CTTGCTTCAT GGGCTAGGAG CCGCCGAGCC CGGGCTCCCG AGAGCAACCT 180
                         stop codon            T (rs12553247)

                                                       67395
                                                       |
CGTGACCTCA GCATGCCATT GCTTCGTGAG TGTCCCGTGC ACGTCCTGAC CCTGGACAGT 240
                                                         T (rs13946)
                                                      DpnII

GAAGGCTTCT CCCTCCCCTC CCACCTGACT TCATCTACGC CTCGGCACCA CGGGGTGTGG 270

                                       67499
                                       |
GACCCCAGCC CGGAGAGAAC AGAGGGAAGG AGCCGCGCCC CCACCTGGAG CTGAATCACA 360
                                       BstUI
                                                            67579
                                                            |
TGACCTAGCT GCACCCCAGC GCCTGGGCCC GCCCCACGCT CTGTCCACAC CCACGCGCCC 420
                                                            T (rs12722)
                                                            BstUI
       67593
       |
CGGGAGCGGG GCCATGCCTC CAGCCCCCCA GCTCGCCCGA CCCATCCTGT TCGTGAATAG 480
       T (rs1134114)

GTCTCAGGGG TTGGGGGAGG GACTGCCAGA TTTGGACACT ATATTTTTTT CTAAATTCAA 540

CTTGAAGATG TGTATTTCCC CTGACCTTCA AAAAATGTTC CAAGGTAAGC CTCGTAAAGG 600

                                                     67812
                                                     |
TCATCCCACC ATCACCAAAG CCTCCGTTTT TAACAACCTC CAACACGATC CATTTAGAGG 660
                                                     DpnII

CCAAATGTCA TTCTGCAGGT GCCTTCCCGA TGGATTAAAG GTGCTTATGT TTTTGTGAGT 720
          Reverse Primer

                 67902
                 |
TTTAAGTAAA TATTTGT                                                737
```

Figure 3.1 The legend is on the next page.

Figure 3.1. Nucleotide sequence of the 3'-end (exon 66) of the *COL5A1* gene. The sequence was obtained from clone RP11-263F14 on chromosome 9 (accession number AL603650). The nucleotide numbers within the clone are indicated. The forward and reverse primer sequences are underlined. The stop codon is also underlined. The *Bst*UI (CG/CG) and *Dpn*II (/GATC) restriction sequence are in bold and double underlined. All three *Bst*UI sites are polymorphic, while only the middle (position 67395) *Dpn*II site is polymorphic. Single nucleotide polymorphisms (SNPs) within exon 66 of the gene were identified using the NCBI SNP database (www.ncbi.nlm.nih.gov/SNP/). The positions of these SNPs, together with there NCBI db SNP ID number, are also shown. The alternative nucleotide is given under the wild type sequence.

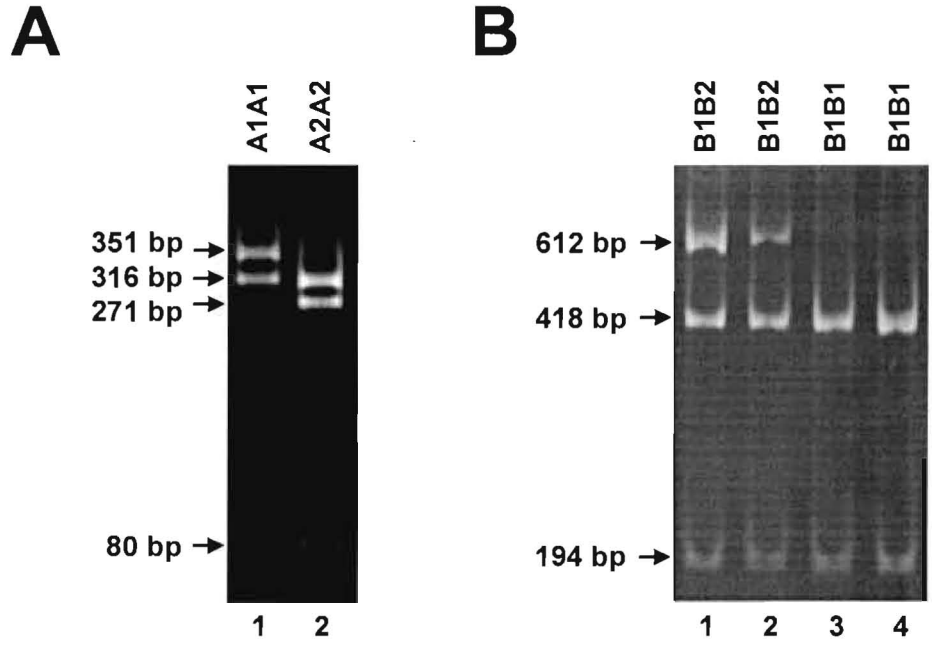

Figure 3.2. Typical 5% non-denaturing polyacrylamide gels showing the common (A) *Bst*UI and (B) *Dpn*II RFLP genotypes. The sizes of the various DNA fragments are indicated on the left size of each gel. Panel A: Lane 1 is an A1A1 genotype, while lane 2 is an A2A2 genotype for the *Bst*UI RFLP. Panel B: Lanes 1 and 2 are a B1B2 genotype, while lanes 3 and 4 are a B1B1 genotype of the *Dpn*II RFLP. The 40 bp and 15 bp products produced during digestion of the PCR fragments with *Dpn*II are not shown in panel B.

3.3 RESULTS

3.3.1 *Subject characteristics*

As shown in Table 3.1, the ATI, TEN, RUP and CON groups were similarly matched for age, height and country of birth. The TEN (71.1%) and CON (61.7%) groups were similarly matched for gender (p=0.224), while the ATI (74.1%, p=0.041) and RUP groups (80.0%, p=0.036) contained significantly more male subjects than the CON group. In addition, the ATI (80.5 ± 14.9 kg, p<0.001), TEN (76.8 ± 13.6 kg, p=0.006) and RUP (87.1 ± 15.1 kg, p<0.001) groups were significantly heavier with a corresponding higher BMI's than the CON (71.1 ± 12.1 kg) group. The RUP sub-group was also significantly heavier with a corresponding higher BMI than the TEN sub-group (p<0.001). The weights of the ATI, groups were recorded at the time of recruitment into the trial on average 8.0 ± 9.2 years, ranging from 0 to 39 years, after the onset of the symptoms. There was however no *Bst*UI or *Dpn*II *COL5A1* RFLP genotype effect on weight (*Bst*UI, p=0.526; *Dpn*II, p=0.735) or BMI (*Bst*UI, p=0.283; *Dpn*II, p=0.915) (data not shown).

3.3.2 *COL5A1 genotype and allele frequencies*

There was a significant difference when the distribution of the three *COL5A1 Bst*UI RFLP alleles or variants (A1, A2 and A3) of the CON group was compared to the ATI group (Pearson's Chi-square=10.2, p=0.006) (Table 3.2 and Figure 3.3A). The frequencies of the A1 and A3 alleles were higher in the ATI group (177 A1, 76.3% and 13 A3, 5.6%) than in the CON group (173 A1, 67.1% and 8 A3, 3.1%), while the frequency of the A2 allele was higher in the CON subjects (77 A2, 29.8%) than in the ATI group (42 ATP, 18.1%) (odds ratio of 1.9; 95% CI 1.3 - 3.0; p=0.003).

Table 3.1. Characteristics of the control (CON) and Achilles tendon injury (ATI) groups, as well as the Achilles tendinopathy (TEN) and Achilles rupture (RUP) sub-groups.

	CON (n=129)	ATI (n=116)	TEN (n=76)	RUP (n=40)
Age (yrs)[1]	41.3 ± 11.0 (122)	40.1 ± 13.8 (112)	39.8 ± 15.2 (72)	40.6 ± 11.2 (40)
Height (cm)	175 ± 9 (121)	176 ± 9 (112)	176 ± 10 (72)	176 ± 8 (40)
Weight (kg)	71.1 ± 12.1 (125)[a,c,e]	80.5 ± 14.9 (112)[a]	76.8 ± 13.6 (72)[c,g]	87.1 ± 15.1 (40)[e,g]
BMI (kg/cm^2)	23.2 ± 2.7 (121)[a,d,e]	25.8 ± 3.9 (112)[a]	24.6 ± 3.2 (72)[d,g]	28.1 ± 4.0 (40)[e,g]
Gender (% males)	61.7 (128)[b,f]	74.1 (116)[b]	71.1 (76)	80.0 (40)[f]
Country of Birth (% South Africa)	71.0 (124)	76.6 (111)	74.7 (71)	80.0 (40)

Values are expressed as mean ± standard deviation or a frequency (%) where applicable. Number of subjects (n) is in parentheses.

[1]The age of the ATP group as well as the TEN and RUP sub-groups are the age of onset of the symptoms of Achilles tendon pathology.

[a]CON vs ATI ($p<0.001$)

[b]CON vs ATI ($p=0.041$)

[c]CON vs TEN ($p=0.006$)

[d]CON vs TEN ($p=0.004$)

[e]CON vs RUP ($p<0.001$)

[f]CON vs RUP ($p=0.036$)

[g]TEN vs RUP ($p<0.001$)

When the ATI group was sub-divided into more homogeneous pathologies, namely, the chronic tendinopathy (TEN) or the rupture (RUP) sub-groups, there was a more significant difference in the frequencies of the distribution of the A1, A2 and A3 alleles when the CON and TEN groups were compared (Pearson's Chi-square=13.3, p=0.001) (Table 3.2 and Figure 3.3A). The frequencies of the A1 and A3 alleles were higher in the TEN group (121 A1, 79.6% and 9 A3, 5.9%) than in the CON group, while the frequency of the A2 allele was higher in the CON group than in the TEN group (22 A2, 14.5%) (odds ratio of 2.5; 95% CI 1.5 - 4.2; p<0.001). Although it should be interpreted with caution because of the small sample size (80 alleles), there was no significant difference in the distribution of these three alleles between the CON and RUP group (56 A1, 70.0%; 20 A2, 25.0% and 4 A3, 5.0 %) (Pearson's Chi-square=1.3, p=0.549) (Table 3.2 and Figure 3.3A).

Table 3.2. Relative frequencies of *Bst*UI RFLP allele of the *COL5A1* gene within the control (CON) and Achilles tendon injury (ATI) groups, as well as the Achilles tendinopathy (TEN) and Achilles rupture (RUP) sub-groups.

Alleles	Nucleotide at pos 67579	CON (n=258)	ATI (n=232)	TEN (n=152)	RUP (n=80)
A1 (%)	T	67.1 (173)	76.3 (177)	79.6 (121)	70.0 (56)
A2 (%)	C	29.8 (77)	18.1 (42)	14.5 (22)	25.0 (20)
A3 (%)	T	3.1 (8)	5.6 (13)	5.9 (9)	5.0 (4)

The values are expressed as percentage with the number of alleles (n) in parentheses. The nucleotide at position 67579 (SNP rs12722) for the three alleles are also shown.

CON vs ATI, Pearson's Chi-square=10.2, p=0.006

CON vs TEN, Pearson's Chi-square=13.3, p=0.001

CON vs RUP, Pearson's Chi-square=1.3, p=0.549

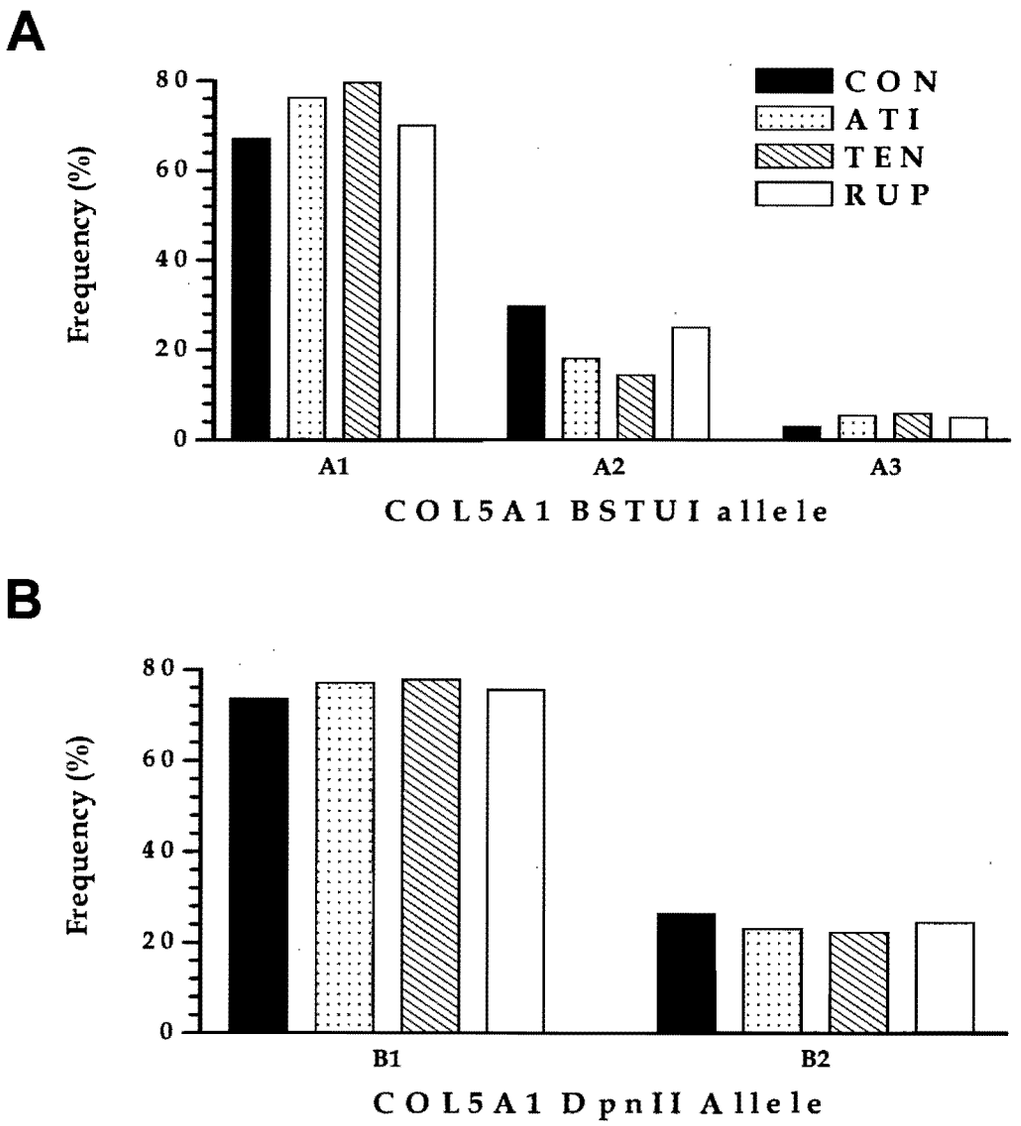

Figure 3.3. Allele frequencies of the (A) *Bst*UI and (B) *Dpn*II restriction fragment length polymorphism within the *COL5A1* gene of the asymptomatic control subjects (CON, solid bars), as well as the symptomatic Achilles tendon injury (ATI, grey bars), chronic Achilles tendinopathy (TEN, hatched bars) and Achilles tendon rupture (RUP, clear bars) patients. (A) p=0.006 CON vs ATI, p=0.001 CON vs TEN, p=0.549 CON vs RUP and (B) p=0.453 CON vs ATI, p=0.424 CON vs TEN, p=0.837 CON vs RUP.

Since the polymorphic nucleotide at position 67579, which produces SNP rs12722, is a T in both the A1 and A3 alleles and a C in the A2 allele, the A1 and A3 alleles were combined and the data re-analysed to determine whether this specific SNP at position 67579 was associated with Achilles tendon over-use injuries (Figure 3.1 and Table 3.2). The frequency of the T allele at position 67579 was significantly higher in the ATI group (190 T, 81.9% and 42 C, 18.1%) than in the CON group (187 T, 72.5% and 77 C, 29.8%) (odds ratio of 1.9; 95% CI 1.2 - 2.9; p=0.004). The frequency of the T allele was also significantly higher in the TEN sub-group (130 T, 85.5% and 22 C, 14.5%) than in the CON group (odds ratio of 2.4; 95% CI 1.4 - 4.1; p<0.001). The frequency of the T allele in the RUP sub-group (60 T, 75.0% and 20 C, 25.0%) was similar to its frequency within the CON group (odds ratio of 1.2; 95% CI 0.7 - 2.2; p=0.571).

Table 3.3. Relative frequencies of *Dpn*II RFLP allele of the *COL5A1* gene within the control (CON) and Achilles tendon injury (ATI) groups, as well as the Achilles tendinopathy (TEN) and Achilles rupture (RUP) sub-groups.

Alleles	Nucleotide at pos 67395	CON (n=258)	ATI (n=222)	TEN (n=144)	RUP (n=78)
B1 (%)	T	73.6 (190)	77.0 (171)	77.8 (112)	75.6 (59)
B2 (%)	C	26.4 (68)	23.0 (51)	22.2 (32)	24.4 (19)

The values are expressed as percentage with the number of alleles (n) in parentheses. The nucleotide at position 67395 (SNP rs13946) for the two alleles are also shown.

CON vs ATI, Pearson's Chi-square=0.6, p=0.453

CON vs TEN, Pearson's Chi-square=0.6, p=0.424

CON vs RUP, Pearson's Chi-square=0.04, p=0.837

Figure 3.3B and table 3.3 shows that there were no significant differences in the distribution of the two *COL5A1 Dpn*II RFLP alleles (B1 and B2) between the CON group and either the entire ATI group (Pearson's Chi-square=0.6, p=0.453) or the TEN (Pearson's Chi-square=0.6, p=0.424) and RUP (Pearson's Chi-square=0.04, p=0.837) sub-groups.

Table 3.4. Relative frequencies of *Bst*UI RFLP genotype of the *COL5A1* gene within the control (CON) and Achilles tendon injury (ATI) groups, as well as the Achilles tendinopathy (TEN) and Achilles rupture (RUP) sub-groups.

Genotype	Genotype at pos 67579	CON (n=129)	ATI (n=116)	TEN (n=76)	RUP (n=40)
A1A1 (%)	TT	62.8 (81)	69.8 (81)	75.0 (57)	60.0 (24)
A1A2 (%)	TC	7.0 (9)	7.8 (9)	6.6 (5)	10.0 (4)
A1A3 (%)	TT	1.6 (2)	5.2 (6)	2.6 (2)	10.0 (4)
A2A2 (%)	CC	26.4 (34)	13.8 (16)	10.5 (8)	20.0 (8)
A2A3 (%)	TC	0 (0)	0.9 (1)	1.3 (1)	0 (0)
A3A3 (%)	TT	2.3 (3)	2.6 (3)	4.0 (3)	0 (0)

The values are expressed as percentage with the number of subjects (n) in parentheses. The genotypes at position 67579 (SNP rs12722) are also shown.
CON vs ATI, Clump's T4 Chi-square=5.9, p=0.117
CON vs TEN, Clump's T4 Chi-square=1.2, p=0.911
CON vs RUP, Clump's T4 Chi-square=2.5, p=0.712

There were no significant differences in the distributions of the genotype frequencies of the *COL5A1 Bst*UI (Table 3.4) and *Dpn*II (Table 3.5) RFLPs between the CON and ATI (Clump T4 Chi-square of the *Bst*UI RFLP =5.9, p=0.117 and Pearson's Chi-

square of the *Dpn*II RFLP =3.7, p=0.160) groups, nor the CON and TEN (Clump T4 Chi-square of the *Bst*UI RFLP =1.2, p=0.911 and Pearson's Chi-square of the *Dpn*II RFLP =2.8, p=0.243) or RUP (Clump T4 Chi-square of the *Bst*UI RFLP =2.5, p=0.712 and Pearson's Chi-square of the *Dpn*II RFLP =1.6, p=0.460) sub-groups.

Table 3.5. Relative frequencies of *Dpn*II RFLP genotype of the *COL5A1* gene within the control (CON) and Achilles tendon injury (ATI) groups, as well as the Achilles tendinopathy (TEN) and Achilles rupture (RUP) sub-groups.

Genotype	Genotype at pos 67395	CON (n=129)	ATI (n=111)	TEN (n=72)	RUP (n=39)
B1B1 (%)	TT	58.1 (75)	58.6 (65)	59.7 (43)	56.4 (22)
B1B2 (%)	TC	31.0 (40)	36.9 (41)	36.1 (26)	38.5 (15)
B2B2 (%)	CC	10.9 (14)	4.5 (5)	4.2 (3)	5.1 (2)

The values are expressed as percentage with the number of subjects (n) in parentheses. The genotypes at position 67395 (SNP rs13946) for the two alleles are also shown.
CON vs ATI, Pearson's Chi-square=3.7, p=0.160
CON vs TEN, Pearson's Chi-square=2.8, p=0.243
CON vs RUP, Pearson's Chi-square=1.6, p=0.460

Since the A1 and A3 alleles both have a T at nucleotide 67579 (refer to figure 3.1), the A1A1, A1A3 and A3A3 genotypes are all a TT genotype at position 67579, while the A1A2 and A2A3 genotypes are also both a TC genotype at this position. Only the A2A2 genotype is a CC genotype at nucleotide 67579. There was a significant difference in the genotype distribution of SNP rs12722 when the CON (86 TT, 66.7%, 9 TC, 7.0% and 34 CC, 26.4%) group was compared to the TEN (62 TT, 81.6%, 6 TC, 7.9% and 8 CC, 10.5%; Chi-square=7.4 and p=0.025) group, but not the ATI (90 TT,

77.6%, 10 TC, 8.6% and 16 CC, 13.8%; Chi-square=6.0 and p=0.051) or RUP (28 TT, 70.0%, 4 TC, 10.0% and 8 CC, 20.0%; Chi-square=0.9 and p=0.635) groups. Individuals with an A2A2 or CC genotype were however under represented in the ATI (odds ratio of 2.1; 95% CI 1.1-4.1; p=0.035) and TEN (odds ratio of 2.9; 95% CI 1.2-6.6; p=0.018) subjects. SNP rs12722 (*Bst*U1 RFLP) and SNP rs13946 (*Dpn*II RFLP) of the *COL5A1* gene were not in linkage disequilibrium (D' for CON=0.476 and D' for ATI=0.383).

The relative frequencies of the *Bst*UI RFLP genotypes of the *COL5A1* gene in the ATI and TEN groups of subjects who had either a single or multiple injuries are shown in table 3.6 while the relative frequencies of those who had a unilateral or bilateral injuries are shown in Table 3.7. There were no significant differences in the *COL5A1* genotype distribution (A1A1, A1A3 and A3A3 versus A2A2, A1A2 and A2A3 genotypes) between Achilles tendon injury (ATI) patients who had single or multiple injuries (Fisher's exact test, p=0.603) as well as between Achilles tendinopathy (TEN) patients who had single or multiple injuries (Fisher's exact test, p=1.00) (Table 3.6). There were also no significant differences in the *COL5A1* genotype distribution (A1A1, A1A3 and A3A3 versus A2A2, A1A2 and A2A3 genotypes) between Achilles tendon injury (ATI) patients who had unilateral and bilateral injuries (Fisher's exact test, p=1.00). There were also no significant differences in the *COL5A1* genotype distribution between Achilles tendinopathy (TEN) patients who had unilateral or bilateral injuries (Fisher's exact test, p=0.757) (Table 3.7).

Table 3.6. The distribution of single and multiple injuries in the Achilles tendon injury (ATI) and Achilles tendinopathy (TEN) subjects.

*Bst*UI RFLP *COL5A1* Genotype	Single Injury ATI (n=80)	Multiple Injuries ATI (n=27)	Single Injury TEN (n=54)	Multiple Injuries TEN (n=13)
A1A1	71.3 (57)	63.0 (17)	72.2 (39)	84.6 (11)
A1A3	3.8 (3)	11.1 (3)	3.7 (2)	0.0 (0)
A3A3	3.8 (3)	0.0 (0)	5.6 (3)	0.0 (0)
A1 Sub-total	**78.8 (63)**	**74.1 (20)**	**81.5 (44)**	**84.6 (11)**
A2A2	15.0 (12)	11.1 (3)	11.1 (6)	7.7 (1)
A1A2	5.0 (4)	14.8 (4)	5.6 (3)	7.7 (1)
A2A3	1.3 (1)	0.0 (0)	1.9 (1)	0.0 (0)
A2 Sub-total	**21.3 (17)**	**25.9 (7)**	**18.5 (10)**	**15.4 (2)**

The values are expressed as percentage with the number of alleles (n) in parentheses. The number of subjects without an A2 allele (A1A1, A1A3 and A3A3 genotype) were added together to produce the A1 sub-total. The number of subjects with an A2 allele (A2A2, A1A2 and A2A3 genotype) were added together to produce the A2 sub-total. There were no significant differences in A1 and A2 sub-total distributions between Achilles tendon injury (ATI) patients who had single or multiple injuries (Fisher's exact test, p=0.603) as well as between Achilles tendinopathy patients who had single or multiple injuries (Fisher's exact test, p=1.00).

Table 3.7. The distribution of unilateral and bilateral injuries in the Achilles tendon injury (ATI) and Achilles tendinopathy (TEN) subjects.

*Bst*UI RFLP *COL5A1* Genotype	Unilateral injuries ATI (n=70)	Bilateral Injuries ATI (n=34)	Unilateral injuries TEN (n=34)	Bilateral Injuries TEN (n=30)
A1A1	65.7 (46)	73.5 (25)	70.6 (24)	76.7 (23)
A1A3	8.6 (6)	0.0 (0)	5.9 (2)	0.0 (0)
A3A3	1.4 (1)	5.9 (2)	2.9 (1)	6.7 (2)
A1 Sub-total	**78.1 (57)**	**79.4 (27)**	**79.4 (27)**	**83.4 (25)**
A2A2	17.1 (12)	8.8 (3)	11.8 (4)	10.0 (3)
A1A2	7.1 (5)	8.8 (3)	8.8 (3)	3.3 (1)
A2A3	0.0 (0)	2.9 (1)	0.0 (0)	3.3 (1)
A2 Sub-total	**21.9 (16)**	**20.6 (7)**	**20.6 (7)**	**16.6 (5)**

The values are expressed as percentage with the number of alleles (n) in parentheses. The number of subjects without an A2 allele (A1A1, A1A3 and A3A3 genotype) were added together to produce the A1 sub-total. The number of subjects with an A2 allele (A2A2, A1A2 and A2A3 genotype) were added together to produce the A2 sub-total. There were no significant differences in A1 and A2 sub-total distributions between Achilles tendon injury (ATI) patients who had unilateral and bilateral injuries (Fisher's exact test, p=1.00). There were also no significant differences in the COL5A1 genotype distribution between Achilles tendinopathy (TEN) patients who had unilateral or bilateral injuries (Fisher's exact test, p=0.757).

3.4 DISCUSSION

The novel and main finding of this study was that the three alleles (or variants) produced by the *Bst*UI restriction fragment length polymorphism (RFLP), or more specifically the T/C single nucleotide polymorphism at position 67579, within the 3'-UTR of the *COL5A1* gene were associated with Achilles tendon injury (p=0.006). Although a genetic predisposition to Achilles tendon (Kannus and Natri, 1997), rotator cuff tendon (Harvie et al., 2004) and anterior cruciate ligament injuries (Flynn et al., 2005) have been suggested, no specific genes have been shown to be associated with these connective tissue pathologies. This is the first study to demonstrate an association between a polymorphism within a gene expressed in tendons, namely *COL5A1*, and symptoms or signs of Achilles tendon pathology in physically active individuals. There was a significant higher frequency of the A2 allele (C nucleotide at position 67579) of this gene in the asymptomatic control subjects (CON 29.8% vs ATI 18.1%). Individuals with the A2 allele were therefore less likely of developing symptoms or signs of Achilles tendon injury (odds ratio = 1.9; 95% CI 1.3-3.0; p=0.003).

The *COL5A1* gene encodes for the pro-α1(V) chain, which is found in most of the isoforms of type V collagen (reviewed in Ristiniemi and Oikarinen, 1989). The major isoform of type V collagen is a heterotrimer consisting of two pro-α1(V) chains and one pro-α2(V) chain. Trace amounts of type V collagen are found in tendons where it forms heterotypic fibres with the major structural collagen, namely type I collagen (reviewed in Silver et al., 2003; Birk, 2001). Although most investigators have speculated, based on the function of type V collagen in the cornea, that the protein plays an important role in regulating fibrillogenesis and modulating fibril growth in tendons, some investigators have suggested that the function of type V collagen in

tendons, and other tissues where its content is low, is actually unknown (reviewed in Riley, 2004; Birk, 2001). Although there is no consensus about the function of type V collagen in tendons, Dressler et al. (2002) have reported an age-dependent increase in the content of the protein, together with a decrease in fibril diameter and the biomechanical properties in the rabbit patellar tendon. In addition, Goncalves-Neto et al. (2002) has shown an increase in types III and V collagen together with a reduction in the content of type I collagen in biopsy samples of degenerative tendons from patients with posterior tibial tendon dysfunction syndrome. Recently, Wenstrup et al. (2004) demonstrated that type V collagen deficient mice could not form collagen fibrils, emphasizing the role of type V collagen in fibrillogenesis. The authors proposed that no other collagen type, including type I, plays such a major role in the control of fibril initiation as type V collagen, even though type V collagen is only a minor fibrillar collagen of tendon and other connective tissue structures.

An additional finding of this study was that the alleles of the *COL5A1 Bst*UI RFLP were strongly associated with chronic Achilles tendinopathy (p=0.001), since individuals with the A2 allele were less likely to present with symptoms or signs of tendinopathy (odds ratio of 2.5; 95% CI 1.5 - 4.2, p=0.0005). This RFLP was however not associated with Achilles tendon ruptures in this study, suggesting that the aetiology of ruptures and tendinopathies may be distinct. These findings must however be interpreted with caution since only 80 alleles were analyzed.

Because of its proximity to the *ABO* gene on chromosome 9 as well as the presence and proposed function of type V collagen in tendons, we propose that the *COL5A1* gene is a better candidate genetic marker than the *ABO* gene for Achilles tendon injury. Although the *COL5A1* gene is an ideal marker for Achilles tendon pathology and more specifically chronic Achilles tendinopathy, the findings of this thesis do not prove that type V collagen is involved in the aetiology of tendon pathology. It is possible, due to the nature of association studies, that another gene closely linked to

the *COL5A1* and *ABO* genes on the tip of the long arm of chromosome 9 encodes for a protein, which is directly involved in the pathogenesis of Achilles tendon injuries. Any possible role of type V collagen in the development of Achilles tendon pathology needs to be investigated. The results of the present study nevertheless suggest that the *COL5A1* gene is a marker of Achilles tendon pathology. As mentioned in chapter 2, a second gene, namely the TNC gene, which is closely linked to the *ABO* gene and encodes for a component of the tendon extracellular matrix, was also identified as an ideal candidate gene. The possible association of polymorphisms within the *TNC* gene and Achilles tendon pathology will be discussed in the next chapter (Chapter 4).

The *Dpn*II RFLP or more specifically the T/C single nucleotide polymorphism at position 67395, within exon 66 of the *COL5A1* was not associated with Achilles tendon injury or Achilles tendinopathy. Although this single nucleotide polymorphism is only 184 bases upstream from the T/C single nucleotide polymorphism at position 67579 responsible for the generation of A2 allele, these polymorphisms were not in linkage disequilibrium. The allele distributions of the *COL5A1 Dpn*II RFLP within the control subjects and the various groups of subjects with symptoms of Achilles tendon pathology were similar to those of previously reported values. In addition there was no significant difference in the allele distribution of the *COL5A1 Bst*UI RFLP when the control subjects where compared to previously reported values ($p=0.206$) (Greenspan and Pasquinelli, 1994).

When the individuals in the ATI and TEN groups were divided into those subjects who experienced unilateral or bilateral injuries, there were no significant differences in the *Bst*UI genotype frequency distributions between the groups. Furthermore, there were no significant *Bst*UI genotype frequency distribution between the subjects who had single or multiple (2 or more) injuries within both the ATI and

TEN groups. Therefore, this polymorphism was not associated the severity of Achilles tendon injury experienced.

In addition to the genetic factors identified in this study, several non-genetic intrinsic factors, such as, amongst others, age, gender, body weight and a history of a previous injury; as well as extrinsic factors, such as type of activity and training, have been implicated in the aetiology of Achilles tendon pathology (reviewed in Jarvinen et al., 2005; Riley, 2005;). Because the symptomatic subjects in this study were significantly heavier than the control subjects and had also participated for a significantly more years in high impact sports (refer to chapter 2), we cannot exclude the possibility that an interaction of body weight and/or physical activity exposure with the *COL5A1* gene was responsible or at least played a part in the development of symptoms and signs of Achilles tendon injury. It should be noted however that because this was a retrospective study we could not record body weight of the subjects accurately at the time of injury. Anecdotally, many subjects reported increases in their body weight after injury as a result of a decrease in physical activity. Increased body weight has been documented as a risk factor for lower extremity injuries in some studies (Murphy et al., 2003), and therefore has also been suggested as an intrinsic risk factor for Achilles tendon injury (Paavola et al., 2002; Riley, 2004). However, to our knowledge no prospective cohort studies have shown that increased body weight is an independent risk factor for Achilles tendon injuries. It can however be noted that there is a reported interaction of obesity with the *COL9A3* gene and lumber disc degeneration (Solovieva et al., 2002). Therefore, the possible interaction of non-genetic factors, such as body weight and exposure to physical activity, with the *COL5A1* gene or any other gene needs to be investigated.

In conclusion, the *Bst*UI RFLP or the T/C single nucleotide polymorphism at position 67579 within the 3'-UTR of the *COL5A1* gene is associated with Achilles tendon injury and more specifically chronic Achilles tendinopathy. This thesis

suggests that individuals with the A2 allele, or a C nucleotide at position 67579, of the *COL5A1* gene are less likely of developing symptoms and signs of chronic Achilles tendinopathy.

CHAPTER FOUR - THE GUANINE-THYMINE (GT) DINUCLEOTIDE REPEAT POLYMORPHISM WITHIN THE TENASCIN-C GENE IS ASSOCIATED WITH ACHILLES TENDON INJURY

The data presented in this chapter has been published in the following article:-

Mokone GG, Gajjar M, September AV, Schwellnus MP, Noakes TD, Collins M (2005) The guanine-thymine dinucleotide repeat polymorphism within the *Tenascin-C* gene is associated with Achilles tendon injuries. American Journal of Sports Medicine. 33 (7): 1016-21.

4.1 INTRODUCTION

As shown in Chapter 3, the *COL5A1* gene is associated with Achilles tendon injuries or more specifically Achilles tendinopathy. Individuals with the A1/A3 alleles were more likely to develop Achilles tendon pathology compared to those with the A2 allele. However, as already discussed, this finding does not necessarily imply that the *COL5A1* gene is directly involved in the aetiology and pathogenesis of Achilles tendon injuries. The *Bst*U1 RFLP of the *COL5A1* gene is only associated with Achilles tendon injuries. Due to the nature of association studies it is possible that another gene, which is closely linked to the *ABO* and *COL5A1* genes may be directly involved in the aetiology of Achilles tendon injuries.

The extracellular matrix glycoprotein, tenascin-C is expressed in a variety of tissues, including tendons, (reviewed in Mackie, 1997) and is encoded by the *tenascin-C* (*TNC*) or *hexabrachion* (*HXB*) gene, which has been mapped to chromosome 9q33 18 Mb upstream from the *ABO* gene (Bennet et al., 1995; Rocchi et al., 1991) (Refer to figure 2.2). Tenascin-C binds other components of the extracellular matrix and cell receptors and plays an important role in regulating cell-matrix interactions (reviewed in Jones and Jones, 2000a). In normal adult tendons, the *TNC* gene is expressed predominantly in regions responsible for transmitting high levels of mechanical force such as the myotendinous and oesteotendinous junctions (Chiquet

and Fambrough, 1984a; Chiquet and Fambrough, 1984b; Jarvinen et al., 1999). The protein is also expressed around the cells and the collagen fibres of the Achilles tendon (Jarvinen et al., 2003). In addition, it has been shown that expression of the *TNC* gene is regulated in a dose-dependent manner by mechanical loading in tendons (Jarvinen et al., 1999; Jarvinen et al., 2003).

Isoforms of the protein, with distinct functions, are produced by alternative splicing of the primary transcript (reviewed in Bosman and Stamenkovic, 2003; Jones and Jones, 2000a). Riley et al (1996) have shown that healthy tendons express a small 200 KDa tenascin-C isoform, while degenerate tendons also express a functionally distinct larger 300 KDa isoform. In investigating this finding, Ireland et al (2001), but not Alfredson et al (2003), have reported an increase in tenascin-C expression in biopsy samples of chronic Achilles tendinopathies using cDNA arrays.

Since (1) several investigators have suggested that either the *ABO* gene or a closely linked gene(s) on the tip of the long arm of chromosome 9 maybe associated with Achilles tendon pathology (Jozsa et al., 1989a; Jozsa et al., 1989b; Kujala et al., 1992), (2) the *TNC* gene has been mapped to chromosome 9q33 which is in close proximity to the *ABO* gene (Bennet et al., 1995; Rocchi et al., 1991), (3) the gene encodes for tenascin-C which is an important structural component of tendons (Jarvinen et al., 1999; Jarvinen et al., 2003) and (4) its expression is regulated by mechanical stimuli (Jarvinen et al., 1999; Jarvinen et al., 2003) and altered during tendon pathology (Ireland et al., 2001; Riley at al., 1996); it is, like the *COL5A1* gene, also an ideal candidate genetic marker of tendon injury. The *TNC* gene contains a guanine-thymine (GT) dinucleotide repeat polymorphism (a tandem repeat consisting of a repeated 2-base pair sequence of varying lengths in different people) within intron 17. The influence of this polymorphism in the expression of the gene or the biological function of tenascin-C is currently unknown. The aim of the study was

therefore to investigate the association of the GT dinucleotide repeat polymorphism within intron 17 of the *TNC* gene with Achilles tendon injuries.

4.2 MATERIALS AND METHODS

4.2.1 Subjects

The recruitment strategy, inclusion and exclusion criteria for the subjects included in the Achilles tendon injury (ATI) and control (CON) groups, as well as the Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP) sub-groups have been described in detail in Chapter 2 (refer to section 2.2.1). One-hundred and fourteen of the 122 Caucasian patients with a current or past clinical diagnosis of Achilles tendon injury (ATI) were included in this study. The ATI patients included 72 of the 79 subjects with chronic tendinopathy (TEN) and 42 of the 43 subjects with either a complete (39 of 42) or partial (3 of 42) rupture (RUP). All the subjects were physically active prior to the development of symptoms. One-hundred and twenty-seven of the 131 apparently healthy physically active Caucasian asymptomatic control (CON) subjects without any history of Achilles tendon injury were also included in this study.

4.2.2 Sample collection and total DNA extraction

Approximately 4.5 ml of venous blood was obtained from each subject by venipuncture of a forearm vein and collected into an EDTA vacutainer tube. Blood samples were stored at 4°C until total DNA extraction could be undertaken as previously described in detail in Chapter 3 (refer to section 3.2.2).

4.2.3 TNC genotyping

The GT dinucleotide repeat polymorphism within the *TNC* gene (Figure 4.1) was PCR amplified as described by Ozelius et al (1992) using the following fluorescently

labeled (56-FAM) forward primer, 5'-ATA GCC CAA AGA GAG GTG CCC-3', and reverse primer, 5'-AGA GCC CTT CTG TCT TCT CC-3', to produce a PCR product of approximately 110 bp. The PCR was carried out in a total volume of 25 µl containing at least 200 ng DNA, 10 pmol of each primer, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP and 0.5 units of DNA Taq polymerase (Invitrogen Life Technologies, Carlsbad, California, USA), using a PCR Express Thermal Cycler (Hybaid Limited, Middlesex, UK). Amplification was performed with an initial denaturing step at 94°C for 3 min, followed by 25 cycles at 94°C for 30 sec, 53°C for 30 sec, 72°C for 40 sec, and a final extension step at 72°C for 7 min. The resultant PCR products were resolved on 4% polyacrylamide gels using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, California, USA). Each individual sample was run with a size standard. The repeat length was calculated by subtracting the forward (21 bp) and reverse (20 bp) primer lengths and the intervening sequences (16 bp from the end of the forward primer to the start of the GT dinucleotide repeat polymorphism and the 25 bp from the end of the repeat polymorphism until the start of the reverse primer) from the resultant PCR product sizes (Figure 4.1). These 82 base pairs are common to all the variants. The number of repeats was determined by dividing the repeat length by two.

4.2.4 Statistical analyses

Data were analysed using the STATISTICA version 7 (StatSoft Inc., Tulsa, OK, USA) statistical programme. A one-way analysis of variance, (ANOVA) was used to determine any significant differences between the characteristics of the Achilles tendon injury (ATI) and control (CON) groups, as well as the Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP) sub-groups. When the overall F value was significant, a LSD post hoc test was used to identify specific differences.

Pearson's chi-square analysis was used to analyse any gender and country of birth differences between the CON and ATI groups and as well as the TEN and RUP sub-groups. The allele frequencies of the *TNC* gene were analysed using the Monte Carlo test (CLUMP version 2.0 programme) (Sham and Curtis, 1995). The odds ratios and the 95% confidence intervals were determined using GraphPad Instat version 3 (GraphPad Software, San Diego, CA, USA) statistical programme. Where applicable, data were presented as means ± standard deviations (SD) or a frequency with the number of subjects in parentheses. Statistical significance was accepted when $p<0.05$.

atagcccaaa gagaggtgcc catgcgcgtg

Forward Primer

gctgcttGTG TGTGTGTGTG TGTGTGTGTG

TGTGTGTGTG Tctcaactgc ctgtgctcca

ggaccaggag aagacagaag ggctct

Reverse Primer

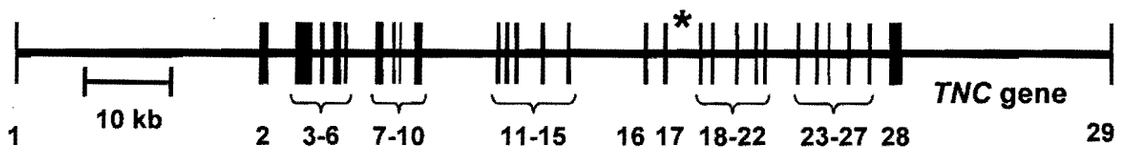

Figure 4.1. (**Top Panel**) The 116 bp PCR product containing 17 GT dinuleotide repeat polymorphism (shown in capital letters) within intron 17 of the *TNC* gene. The underlined sequences denote the forward and reverse primers. The sequence, GenBank accession number Z11654, corresponds to part of intron 17 of the TNC gene. (**Bottom Panel**) A schematic diagramm of the TNC gene showing the relative positions and sizes of the 29 exons (numbered) and the 28 introns. The position of intron 17 is marked with an asterisk.

4.3 RESULTS

4.3.1 Subject characteristics

The ATI, CON, TEN and RUP subjects were similarly matched for age, height, gender and country of birth (Table 4.1). The ATI (80.8 ± 15.3 kg, $p<0.001$), TEN (77.3 ± 14.2 kg, $p=0.003$) and RUP (86.6 ± 15.3 kg, $p<0.001$) groups were significantly heavier with corresponding higher body mass indices (BMIs) than the CON (71.4 ± 11.9 kg) group. In addition, the RUP group was heavier with a corresponding higher BMI than the TEN group ($p<0.001$). The weights of the ATI group were recorded at the time of recruitment into the study on average 7.7 ± 8.7 years, ranging from 0 to 39 yrs, after the onset of symptoms.

4.3.2 TNC genotyping

As shown in table 4.2, 18 different alleles or variants of the GT dinucleotide repeat polymorphism within the *TNC* gene were identified within the ATI (n=14) and CON (n=15) groups. The number of GT repeats within the identified alleles ranged from 3 to 21, with 95.0 % of the alleles containing between 12 and 17 repeats. There was a significant difference in the distribution of the alleles between the ATI and CON groups (Chi-square=51.0 $p=0.001$), with the alleles containing 12 and 14 GT dinucleotide repeats being significantly over represented in the ATI group (Chi-square=21.6 $p<0.001$) (Figure 4.2). The alleles containing 13 and 17 repeats were on the other hand significantly under represented in the subjects diagnosed with Achilles tendon injury (Chi-square=42.4 $p<0.001$) (Figure 4.2). When the ATI group was divided into the TEN and RUP sub-groups, their allele distributions were similar to those of the ATI group (ATI vs TEN, Chi-square=3.1, $p=1.000$ and ATI vs RUP, Chi-square=5.0, $p=0.989$). The allele distribution of these two groups was therefore also significantly different to the CON group (CON vs TEN, Chi-

square=56.0, p<0.001 and CON vs RUP, Chi-square=47.2, p<0.001). In addition, the allele distribution of the TEN and RUP sub-groups were not significantly different (Chi-square=11.0 p=0.855) (Table 4.2).

Table 4.1. Characteristics of the control (CON) and Achilles tendon injury (ATI) groups, as well as the Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP) sub-groups.

	CON (n=127)	**ATI** (n=114)	**TEN** (n=72)	**RUP** (n=42)
Age (yrs)[1]	40.4 ± 10.8 (120)	39.8 ± 13.3 (112)	39.2 ± 14.5 (70)	40.7 ± 11.1 (42)
Height (cm)	175 ± 9 (120)	176 ± 9 (112)	176 ± 9 (70)	176 ± 8 (42)
Weight (kg)	71.4 ± 11.9 (123)[a,b,c]	80.8 ± 15.3 (112)[a,]	77.3 ± 14.2 (70)[b,d]	86.6 ± 15.3 (42)[c,d]
BMI (kg/cm^2)	23.3 ± 2.7 (120)[a,c,e]	26.0 ± 4.0 (112)[a]	24.9 ± 3.5 (70)[d,e]	27.9 ± 4.0 (42)[c,d]
Gender (% males)	63.5 (126)	72.8 (114)	70.8 (72)	76.2 (42)
Country of Birth (% South Africa)	69.7 (122)	76.6 (111)	73.9 (69)	81.0 (42)

Values are expressed as mean ± standard deviation or a frequency where applicable. Number of subjects (n) is in parentheses. [1]The age of the ATI group, as well as the TEN and RUP sub-groups, are the ages of onset of the symptoms.

[a] CON vs ATI, $p < 0.001$, [b] CON vs TEN, $p = 0.003$,

[c] CON vs RUP, $p < 0.001$, [d] TEN vs RUP, $p < 0.001$

[e] CON vs TEN, $p < 0.001$

Table 4.2. The distributions of the *TNC* gene's 18 GT dinucleotide repeat polymorphism alleles within the control (CON) and Achilles tendon injury (ATI) groups, as well as the Achilles tendinopathy (TEN) and Achilles rupture (RUP) sub-groups.

Number of GT Repeats[1]	CON (n=254)	ATI (n=228)	TEN (n=144)	RUP (n=84)
3	0.4 (1)	0.4 (1)	0.0 (0)	1.2 (1)
4	0.4 (1)	0.0 (0)	0.0 (0)	0.0 (0)
5	0.4 (1)	0.4 (1)	0.0 (0)	1.2 (1)
6	0.4 (1)	1.3 (3)	1.4 (2)	1.2 (1)
7	0.0 (0)	0.4 (1)	0.0 (0)	1.2 (1)
8	0.4 (1)	0.0 (0)	0.0 (0)	0.0 (0)
9	0.0 (0)	0.4 (1)	0.7 (1)	0.0 (0)
11	0.0 (0)	1.3 (3)	2.1 (3)	0.0 (0)
12[2]	10.2 (26)	18.9 (43)	17.4 (25)	21.4 (18)
13[3]	24.0 (61)	8.8 (20)	8.3 (12)	9.5 (8)
14[2]	0.8 (2)	9.2 (21)	8.3 (12)	10.7 (9)
15	17.3 (44)	21.5 (49)	24.3 (35)	16.7 (14)
16	22.8 (58)	28.9 (66)	28.5 (41)	29.8 (25)
17[3]	20.1 (51)	7.5 (17)	7.6 (11)	7.1 (6)
18	1.2 (3)	0.4 (1)	0.7 (1)	0.0 (0)
19	0.4 (1)	0.0 (0)	0.0 (0)	0.0 (0)
20	0.4 (1)	0.4 (1)	0.7 (1)	0.0 (0)
21	0.8 (2)	0.0 (0)	0.0 (0)	0.0 (0)

The values are expressed as percentage with the number of alleles (n) in parentheses. [1]The number of GT repeats was calculated from the size of the PCR products and the published sequence, accession no Z11654. [2]Alleles (12 repeats $p=0.010$ and 14 repeats $p<0.001$) are over-represented within the ATI group when compared to the CON group. [3]Alleles (13 repeats $p<0.001$ and 17 repeats $p<0.001$) are significantly lower in the ATI group when compared to the CON group. The distribution of the TEN ($p=1.000$) and RUP ($p=0.989$) sub-groups where similar to the ATI group and to each other ($p=0.855$).

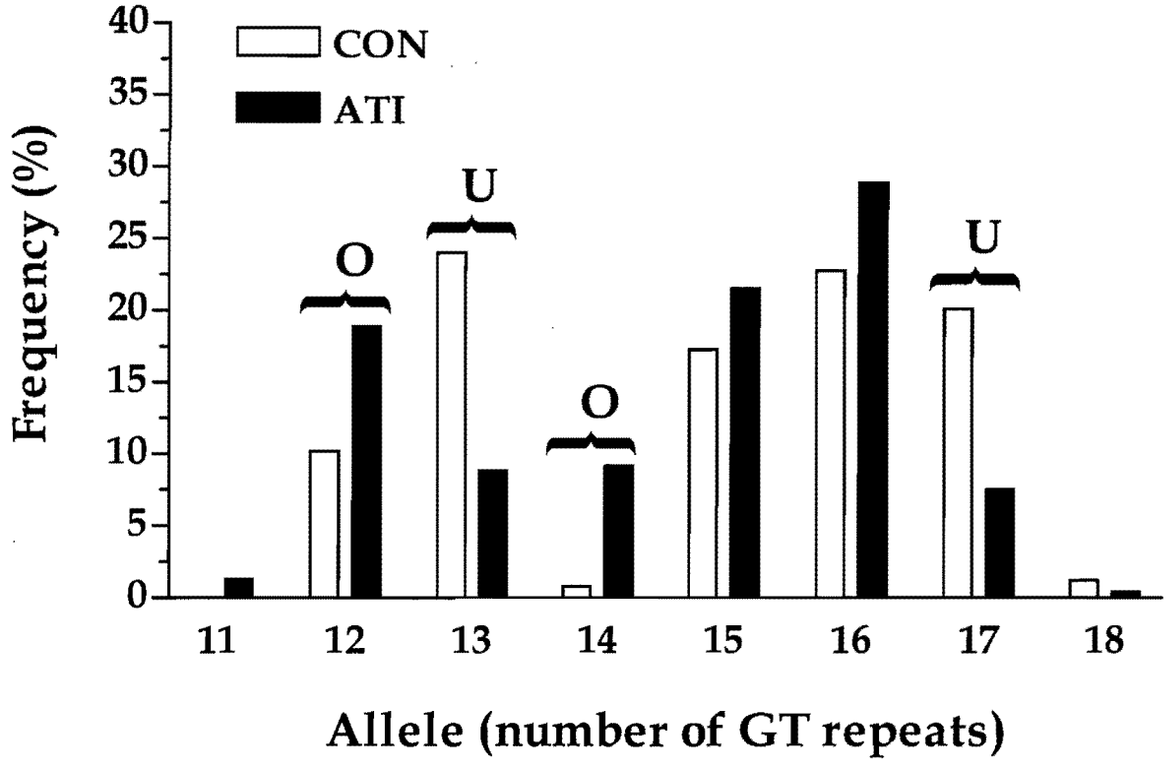

Figure 4.2. Allele frequency of the GT dinucleotide repeats polymorphism within the *TNC* gene of the asymptomatic control (CON, white bars) and symptomatic Achilles injury (ATI, solid bars) groups. The frequencies of the rare alleles containing less than 11 and greater than 18 repeats are not shown. O = over represented alleles and U = under represented alleles.

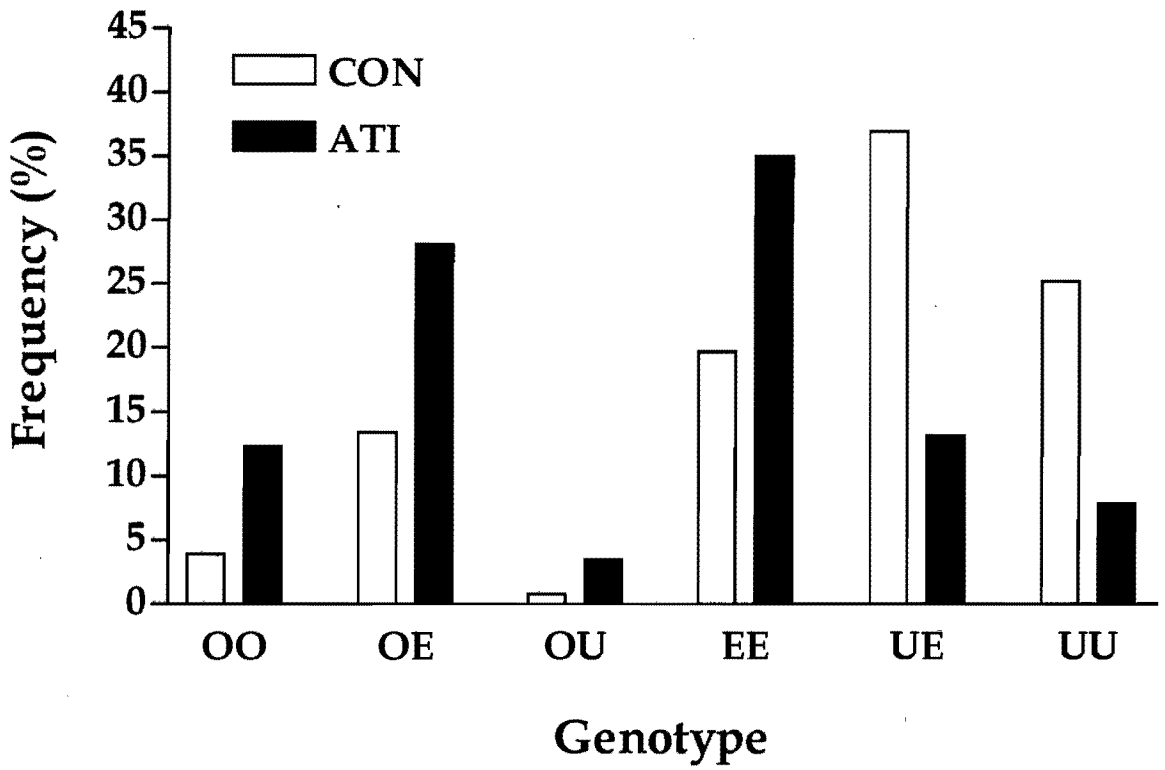

Figure 4.3. Genotype frequency of the GT dinucleotide repeats polymorphism within the *TNC* gene of the asymptomatic control (CON, white bars) and symptomatic Achilles injury (ATI, solid bars) groups. O = over represented alleles containing 12 or 14 GT dinucleotide repeats. U = under represented alleles containing 13 or 17 repeats. E = evenly distributed alleles.

Alleles which were significantly over represented (O) in the ATI group (12 repeats, p=0.010 and 14 repeats, p<0.001), significantly under represented (U) in the ATI group (13 repeats, p<0.001 and 17 repeats, p<0.001) or evenly distributed (E) between groups, were grouped (Figure 4.2). Individuals with a UU (32 CON, 25.2% vs 9 ATI, 7.9%) or UE (47 CON, 37.0% vs 15 ATI, 13.2%) genotype were under represented in the ATI group (odds ratio=6.2, 95% CI 3.5-11.0, p<0.001) (Figure 4.3). Individuals that were either homozygous (OO; 5 CON, 3.9% vs 14 ATI, 12.3%) or heterozygous (OE; 17 CON, 13.4% vs 32 ATI, 28.1% or OU; 1 CON, 0.8% vs 4 ATI, 3.5%) for the O alleles were over represented in the ATI subjects. Interestingly, the frequency of the EE genotype was higher in the ATI group (n=40, 35.1%) than in the CON group (n=25, 19.7%) (Figure 4.3).

Although the sample size is small, the distribution of the *TNC* genotype in the thirty subjects who had bilateral injury was not significantly different to the 73 subjects with unilateral injuries (Table 4.3) (Clump T4 chi-Square=3.3, p=0.488). In addition, there was no significant difference when the over-represented (OO, OE, OU and EE) genotype were added together and compared to the under-represented (UE and UU) genotypes (Fisher's exact test, p=0.416). Individuals with an EE genotype were included with those with the over-represented (OO, OE and OU) genotypes based on the findings presented in figure 4.3.

Similarly, the distribution of the *TNC* genotype in the twenty-six subjects who had multiple injuries was not significantly different from those subjects (n=81) who had only one injury event (Clump T4 chi-square=1.49, p=0.881) (Table 4.4). There was also no significant difference when the over-represented (OO, OE, OU and EE) genotype were added together and compared to the under-represented (UE and UU) genotypes (Fisher's exact test, p=0.582).

Table 4.3. The distribution of unilateral and bilateral injuries in the Achilles tendon injury (ATI) subjects.

TNC Genotype	Unilateral injuries (n=73)	Bilateral Injuries (n=30)
OO	16.4 (12)	6.7 (2)
OE	27.4 (20)	33.3 (10)
OU	4.1 (3)	3.3 (1)
EE	30.1 (22)	43.3 (13)
"O" Sub-total	**78.1 (57)**	**86.6 (26)**
UE	13.7 (10)	6.7 (2)
UU	8.2 (6)	6.7 (2)
"U" Sub-total	**21.9 (16)**	**13.4 (4)**

The values are expressed as percentage with the number of alleles (n) in parentheses. There were no significant differences in the 6 possible genotype distributions between Achilles tendon injury (ATI) patients who had unilateral and bilateral injuries (Clump T4 Chi Square=3.3, p=0.488). The number of subjects with an OO, OE, OU and EE genotypes were added together to produce the "O" sub-total. The number of subjects with an UE and UU genotypes were added together to produce the "U" sub-total. There were no significant differences in "O" and "U" sub-total distributions between ATI patients who had unilateral and bilateral injuries (Fisher's exact test, p=0.461).

Table 4.4. The distribution of single and multiple injuries in the Achilles tendon injury (ATI) subjects.

TNC Genotype	Single Injury (n=81)	Multiple Injuries (n=26)
OO	13.6 (11)	11.5 (3)
OE	28.4 (23)	26.9 (7)
OU	2.5 (2)	7.7 (2)
EE	33.3 (27)	38.5 (10)
"O" Sub-total	**77.8 (63)**	**84.6 (22)**
UE	12.4 (10)	11.5 (3)
UU	9.9 (8)	3.8 (1)
"U" Sub-total	**22.2 (18)**	**15.4 (4)**

The values are expressed as percentage with the number of alleles (n) in parentheses. There were no significant differences in the 6 possible genotype distributions between Achilles tendon injury (ATI) patients who had unilateral and bilateral injuries (Clump T4 chi-square=3.3, p=0.881). The number of subjects with an OO, OE, OU and EE genotypes were added together to produce the "O" sub-total. The number of subjects with an UE and UU genotypes were added together to produce the "U" sub-total. There were no significant differences in "O" and "U" sub-total distributions between ATI patients who had unilateral and bilateral injuries (Fisher's exact test, p=0.582).

CHAPTER FOUR - THE GUANINE-THYMINE (GT) DINUCLEOTIDE REPEAT POLYMORPHISM WITHIN THE *TENASCIN-C* GENE IS ASSOCIATED WITH ACHILLES TENDON INJURY

In an attempt to investigate the combined genotype effects of the *COL5A1* and *TNC* genes on Achilles tendon injuries, the over-represented (TT) and under-represented (CC or TC) genotypes of the *COL5A1* single nucleotide polymorphism (SNP) rs12722 (refer to Table 3.4) were combined with the over-represented (OO, OE and OU) and under-represented (UU and UE) genotypes of the TNC dincluceotide repeat polymorphism (Figure 4.3). Although the E alleles were evenly distributed between the groups (Figure 4.2), the EE genotype was over-represented in the ATI subjects (Figure 4.3). The EE genotype of *TNC* gene was also combined with either the over- or under-represented *COL5A1* genotypes. The distribution of the six groups of combined *COL5A1* and *TNC* genotypes were significantly different between the CON and ATI groups (Clump T4 chi-square=36.3, $p<0.001$), as well as between the CON and the TEN (Clump T4 chi square=25.9, $p<0.001$) and RUP (Clump T4 chi-square=22.4, $p<0.001$) sub-groups (Table 4.5). There were similar combined genotype distributions between the TEN and RUP sub-groups (Clump T4 chi-square=3.5, $p=0.395$).

Individuals with any of the under-represented *TNC* genotypes (UU and UE) combined with any of the three *COL5A1* genotypes (TT, TC or CC) were significantly less likely of developing symptoms of Achilles tendon injury (CON: n=78, 62.9% and ATI: n=24, 23.1%) compared to individuals all the other possible genotype combinations (CON: n=46, 37.1% and ATI: n=80, 77.0%) (Fisher's exact test, $p<0.001$,odds ratio=5.6, 95% CI 3.2-10.1). Although it appears that the *TNC* UU or UE genotype combined with a C allele (CC or TC genotype) of the *COL5A1* gene is the strongest genetic marker associated with developing symptoms of Achilles tendon injuries, the *TNC* UU or UE genotype combined with a TT genotype of the *COL5A1* gene is also a genetic marker but to a lesser extent. Individuals with any of the over-represented *TNC* genotypes (OO, OE and OU) combined with any of the *COL5A1* genotypes, together with the COL5A1 TT genotype combined with the *TNC*

EE genotype, were significantly more likely of developing symptoms of Achilles tendon injury (CON: n=36, 29.0% and ATI: n=71, 68.3%) compared to individuals all the other possible genotype combinations (CON: n=88, 71% and ATI: n=33, 31.8%) (Fisher's exact test, $p<0.001$, odds ratio=5.2, 95% CI 3.0-9.3). The combined *COL5A1* CC and *TNC* EE genotypes were however evenly distributed between the CON (8.1%) and ATI (8.7%) groups.

Table 4.5. The frequency distribution of the combined *COL5A1* SNP rs12722 and *TNC* dinucleotide repeat genotypes within the control (CON) and Achilles tendon injury (ATI) group, as wells as the Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP) sub-groups.

COL5A1 Genotype	*TNC* Genotype	CON (n=124)	ATI (n=104)	TEN (n=67)	RUP (n=37)
TT[1]	[3]OO, OE or OU	14.5 (18)	30.8 (32)	29.9 (20)	32.4 (12)
TT[1]	[4]EE	11.3 (14)	27.9 (29)	29.9 (20)	24.3 (9)
TT[1]	[5]UU or UE	40.3 (50)	19.2 (20)	23.9 (16)	10.8 (4)
CC or TC[2]	[3]OO, OE or OU	3.2 (4)	9.6 (10)	7.5 (5)	13.5 (5)
CC or TC[2]	[4]EE	8.1 (10)	8.7 (9)	7.5 (5)	10.8 (4)
CC or TC[2]	[5]UU or UE	22.6 (28)	3.9 (4)	1.5 (1)	8.1 (3)

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

[1]The SNP rs 12722 TT genotype of the *COL5A1* gene produces the A1A1, A1A3 and A3A3 *Bst*UI RFLPs which are over-represented in the ATI and TEN groups.

[2]The SNP rs 12722 TC genotype of the *COL5A1* gene produces the A1A2 and A2A3 *Bst*UI RFLPs, while the CC genotype produces the A2A2 RFLP. These genotypes are under-represented in the ATI and TEN groups.

[3]The *TNC* OO, OE and OU genotypes are over-represented in the ATI group.

[4]Althought the E alleles were evenly distributed between the groups, the EE genotype was over-represented within the ATI group.

[5]The *TNC* UU and UE genotypes are under-represented in the ATI group.

CON vs ATI, Clump T4 chi-square=36.3, $p<0.001$

CON vs TEN, Clump T4 chi-square=25.9, $p=<0.001$

CON vs RUP, Clump T4 chi-square=22.1, $p<0.001$

TEN vs RUP, Clump T4 chi-square=3.5, $p=0.395$

4.4 DISCUSSION

Several studies have previously suggested there is, at least in part, a genetic component involved in the development Achilles tendon (Kannus and Natri, 1997; Maffulli, 1999; Årøen et al., 2004), rotator cuff tendon (Harvie et al., 2004) and anterior cruciate ligament injuries (Flynn et al., 2005). However, as mentioned (chapter 3, section 3.1) none of these studies have identified specific genes that might be associated with overuse tendon injuries.

The novel finding of this study was that the allele distributions of the GT dinucleotide repeat polymorphism within the *TNC* gene were significantly different between the subjects presenting with signs and symptoms of Achilles tendon injury and the asymptomatic control subjects. The frequencies of the alleles containing 12 and 14 GT repeats were significantly over-represented in the symptomatic subjects, while the frequencies of the alleles containing 13 and 17 GT repeats were significantly under-represented in these subjects. Further analysis demonstrated that individuals who were homozygous or heterozygous for the under represented alleles (containing either 13 or 17 GT repeats) were 6.2 times less likely to develop symptoms of Achilles tendon injury. The heterozygous individuals did not contain an allele with either 12 or 14 repeats. This polymorphism within the *TNC* gene, together with the *Bst*UI RFLP within the *COL5A1* gene (chapter 3), is associated with Achilles tendon over-use injuries.

Further comparison of the allele distribution of the TEN and RUP sub-groups to the CON group yielded similar results obtained for the ATI group. In addition, there were no significant differences in the allele distribution between the TEN and RUP sub-groups. These results suggest that perhaps both tendinopathy and rupture have similar aetiology or the involvement of tenascin-C in both pathologies is via the

same mechanism. This finding is in contrast to the *COL5A1 Bst*UI allele distribution in the Achilles tendon rupture sub-group, which was not different to the CON group (see Chapter 3, sections 3.3 and 3.4). The reasons for this are unclear and warrant further investigation.

When the individuals in the ATI group were divided into those subjects who experienced unilateral or bilateral injuries, there were no significant differences in the *TNC* genotype frequency distributions between the groups. Furthermore, there was no significant *TNC* genotype frequency distribution between the subjects who had single or multiple (2 or more) injuries within the ATI group. Similar to the *Bst*UI RLFP of the *COL5A1* gene, the GT dinucleotide repeat polymorphism of the *TNC* gene was not associated the number of injuries or side of Achilles tendon injury experienced. These findings should however be interpreted with caution and considered preliminary since larger sample sizes need to analysed until a more firm conclusion can be drawn.

Individuals with any of the under-represented *TNC* genotypes (UU and UE) combined with any of the three *COL5A1* genotypes (TT, TC or CC) were less likely of developing symptoms of Achilles tendon injury compared to individuals with all the other possible genotype combinations, suggesting that the GT dinucleotide repeat polymorphism of the *TNC* gene was more strongly associated with Achilles tendon injury. It should be noted however that individuals with both the under-represented *TNC* (UU and UE) and *COL5A1* (CC and TC) genotypes (C allele was over-represented in the asymptomatic control subjects) were the least likely of developing symptoms of Achilles tendon injuries. The over-represented *TNC* genotypes (OO, OE and OU) combined with any of the *COL5A1* genotypes, the over-represented and under-represented, were more likely of developing symptoms of Achilles tendon injury. This also suggests that the *TNC* genotypes are more strongly

associated with Achilles tendon injury. Although polymorphisms in both the *COL5A1* (chapter 3) and *TNC* (this chapter) genes have been associated with Achilles tendon pathology in this study, these results suggest that perhaps the GT dinucleotide repeat polymorphism within intron 17 of the *TNC* is a stronger genetic marker of Achilles tendon injury compared to the *Bst*UI RFLP within the *COL5A1* gene and that the observed association of the *COL5A1* gene with Achilles tendon injuries is merely due to the close proximity of the *COL5A1* gene to the *TNC* gene on the tip of the long arm of chromosome 9. Although, only speculative at this stage, the combined genotype data did not exclude the possibility that both the *COL5A1* and *TNC* genes interact with one another to influence the outcome of Achilles tendon adaptation to chronic repetitive mechanical loading. Further studies with larger sample sizes will have to be done to answering this question.

Tenascin-C is expressed in numerous tissues and, in particular those subject to high tensile stress such as tendons, ligaments and arterial walls (reviewed in Chiquet-Ehrismann and Chiquet, 2003). The expression of this extracellular matrix protein is usually absent or very low in fully differentiated tissues, but is up-regulated during embryogenesis, tissue regeneration, wound healing and certain pathologic conditions (reviewed in Chiquet-Ehrismann and Chiquet, 2003; Jones and Jones, 2000a; Jones and Jones, 2000b). Tenascin-C has a characteristic modular structure consisting of a tenascin assembly (TA) domain, heptad repeats, epidermal growth factor (EGF)-like repeats, fibronectin type III (FN-III) domains and fibrinogen-like globular domain (reviewed in Bosman and Stamenkovic, 2003; Jones and Jones, 2000a). Because of its modular structure, the protein is able to interact with various protein, such as fibronectin, and glycosaminoglycan components of the extracelluar matrix. The glycosaminoglycan components include the family of chondroitin sulphate proteoglycans known as lecticans (aggrecan, versican, brevican and neurocan). Tenascin-C interacts also with numerous cell surface receptors, such as

integrins, cell adhesion molecules (CAM), and annexin II. These diverse interactions are believed to play an important role in regulating cell-matrix interactions.

A recent study have found that a coding single nucleotide polymorphism (SNP) in exon 17 (44413A/T) of *TNC* gene's fibronectin type III-D (Fn-III-D) domain was associated with adult bronchial asthma in Japanese patients (Matsuda et al., 2005). In both Asthma (reviewed in Matsuda et al., 2005) and Achilles tendinopathy (Riley et al., 1996), tenascin-C expression, more especially the larger alternatively spliced form, is increased. Curiously, the GT-dinucleotide repeat polymorphism associated with tendinopathy in the current study is located in intron 17 only 2090 bp drownstream of the functional SNP. This raises a possibility of a functional relationship between intron 17 and exon 17. Further studies need to be conducted to determine whether this SNP in exon 17 is associated with Achilles tendon injury.

Investigators have suggested that apoptosis of tendon cells precedes tendinopathy (reviewed in Murrell, 2002). Abnormal mechanical loading is believed to initiate this programmed cell death in tendons. Since mechanical signals are able to alter the synthesis of tenascin-C (reviewed in Chiquet, 1999; Chiquet and Fambrough, 1984; Chiquet et al., 2003), which in turn is able to regulate cell-matrix interactions, it is tempting to speculate that this protein may play an important role in the proposed apoptotic model of tendinopathy. This theory invites future study.

The association of the *TNC* gene or the *COL5A1* gene (Chapter 3) with symptoms of Achilles tendon injury does not prove that the tenascin-C protein or type V collagen is involved in a cause-effect relationship of Achilles tendon pathology. It is also possible that another gene(s) within this locus, such as any of the other genes identified within chapter 2, may be involved. In addition, it is highly unlikely that a single gene on chromosome 9q32-q34 is exclusively associated with the development

of the symptoms of Achilles tendon injury, since numerous proteins are involved in tendon structure, development and regeneration (reviewed in Birk, 2001; Silver et al., 2003). These alternative possibilities need to be further investigated and will be discussed further in chapter 7.

Besides the involvement of genetic factors, investigators have identified a number of non-genetic intrinsic factors and extrinsic factors that are implicated in the development of Achilles tendon injury. These factors include, amongst others, body weight, type of activity and type of training (reviewed in Riley, 2004). Since the subjects in this study with symptoms of Achilles tendon injury were significantly heavier and had participated for significantly more years in high impact sports than the asymptomatic control subjects, any possible interactions of weight and load with the genetic background in the development of the symptoms of Achilles tendon injury cannot be excluded, and needs to be investigated further (refer to Chapters 2 and 3). Nevertheless, although Achilles tendon injury is typically a chronic condition, the first symptoms usually develop within the first few years of beginning regular, weight-bearing physical activity (Mazzone and Mc Cue, 2002). Since the subjects in the control group had been active for an average of 11.5 years and were currently exercising an average of 5 hours per week of high impact sport, it seems improbable that their apparent resistance to Achilles tendon injury was due purely to a lesser exposure to high impact loading of their Achilles tendons (refer to Chapter 2). The possible gene-gene and gene-environment interactions will be discussed further in chapter 7.

In conclusion, the GT dinucleotide repeat polymorphism within intron 17 of the *TNC* gene is associated with the symptoms of chronic Achilles tendon injury. Alleles containing 12 and 14 GT repeats were over represented in individuals with symptoms of Achilles tendon injury, while the alleles containing 13 and 17 repeats

were under represented. There were no differences in the distribution of these alleles between the RUP and TEN sub-groups.

5.1 INTRODUCTION

Flexibility can be defined as "the ability to move a joint through its complete range of motion" (ROM) (ACSM, 2000). Traditionally, a reduced joint ROM is postulated to increase the risk of muscle-tendon injuries during both recreational and professional sporting activities (Beaulieu, 1981; Renstrom and Kannus, 1991). It is therefore a common practise that many professional and recreational athletes stretch their muscle-tendon units (MTUs), before, during or after exercise or physical activity in order to increase joint ROM (Pope et al., 2000; Smith, 1984). This practise of regular and habitual stretching is based on the belief that stretching will improve musculotendinous flexibility and therefore, minimize the risk of injury (Pope et al., 2000). The relationship between stretching and improved joint ROM is well established (reviewed in Smith, 1994; Nelson and Bandy, 2004). However, the question as to whether an improved joint ROM translates to a decrease in muscle-tendon injury is unclear and is currently being investigated (Andrish et al., 1974; Ekstrand et al., 1984; Laurenson, 1999; Safran et al., 1988; Witvrouv et al., 2001; Worrel and Perrin, 1992; Witvrouw et al., 2004; Pope et al., 2000; van Mechelen et al., 1993; Gleim and McHugh, 1997; Shrier, 1999; Thacker et al., 2004; van Mechelen et al., 2002; Weldon and Hill, 2003; Yeung and Yeung, 2003). In a recent prospective study, it was found that pre-season hip adductor inflexibility was not a risk factor for adductor muscle strains in professional ice hockey players (Tyler et al., 2002). In another study of children (mean age of 12 years) with ligament sprains and fractures, ankle dorsiflesion ROM was found to be significantly lower in the injured compared to an age-matched control group (Tabrizi et al., 2000).

More recently, a prospective study in professional soccer players and athletic population, however, found that pre-season decreased range of motion in the quadriceps and the hamstring muscle groups were associated with increased risk of injury in those muscle groups (Witvrouw et al., 2001). Another two-year

prospective study in an athletic population also demonstrated that lower hamstring and quadriceps flexibility measured before the study was predictive of patellar "tendinitis"after 2 years (Witvrouw et al., 2003). Therefore, it appears that regular stretching results in increase muscle-tendon unit flexibility that can have positive effect and reduce the risk of muscle-tendon unit injuries.

There are no specific studies that have investigated the relationship between general flexibility and Achilles tendon injuries. In a few studies, investigators have studied the relationship between multiple lower extremity injuries, including Achilles tendon injuries, and flexibility. In one prospective study, 303 army male trainees were followed up for 12 weeks (Jones et al., 1993). It was shown that the back and hamstring flexibility (as measured indirectly by the sit-and-reach test) was associated with risk of lower extremity injuries. In this study, 86 (28.4%) subjects experienced lower extremity overuse injuries, which included Achilles tendinopathy, patellofemoral syndrome and stress fractures. Furthermore, individuals with the sit-and-reach test ROM at both extremes (most flexible and least flexible) were at more than two times at greater risk of developing an injury compared to the individuals with an average flexibility. In this study the ROM therefore displayed a U-shaped relationship with injury. It was unclear whether this relationship would be observed with the ankle dorsiflexion-plantarflexion ROM. In another study of military recruits, the control (n=148) and intervention (n=150) groups were enrolled for a 13-week basic military training. The intervention group however included an additional three hamstring stretching sessions conducted in the morning, during lunch and evening. Measurements of hamstring flexibility were recorded before and after military training. After 13 weeks of training, the intervention group showed significantly greater hamstring flexibility compared to the control group. The results of the study also showed that the incidence of lower overuse extremity injuries, which included Achilles tendinopathy, was significantly lower in the intervention group (Hartig and Henderson, 1999) (16.7% injuries, intervension vs 29.1% injuries, control group). The main limitation of the above studies was that

the specific relationship between ankle dorsiflexion ROM and Achilles tendon injury was not studied.

Currently, there are few specific investigative studies (Clement et al., 1984; Kuafman et al., 1999; Wiesler et al., 1996; Pope et al., 1998; Pope et al., 2000; Kvist, 1994) on the role of musculotendinous flexibility in Achilles tendon injury. Data from these studies are mostly in the form of case series and are therefore limited because a cause-effect relationship could not be established. Regular stretching is often prescribed as part of the conservative treatment for chronic Achilles tendon injuries (Clement et al. 1984). In 109 runners, it was found that 41 (38%) displayed insufficiency in strength and flexibility of the gastrocnemius/soleus musclotendinous unit (Clement et al., 1984). It was suggested that musculotendinous flexibility could be one of the aetiological factors in overuse chronic Achilles tendon injury. However, this study included no control group of non-injured runners. In a study on dancers, it was shown that abnormalities in ankle range of motion were a poor predictor of future injuries in modern and ballet dancers and it was instead suggested that "over-stretching can also result in increased risk of injury (Wiesler et al., 1996). It has also been observed that athletes with symptoms of Achilles tendinopathy frequently have decreased ankle range of motion as well as limited subtalar joint mobility (reviewed in Kvist, 1994) and that ankle dorsiflexion measured with straight knee was decreased in individuals with Achilles tendinopathy (Kaufman et al., 1999). In randomised controlled studies of over 1000 army recruits, divided into pre-exercise stretching and pre-exercise non-stretching groups, it was found stretching before performing exercise did not significantly reduce the risk of injury to the Achilles tendon (Pope et al., 1998; Pope et al., 2000). These latter two studies used the same subjects, with the 2000 study expanding the subject numbers to improve the power. More recently, a prospective cohort study suggested that increased ankle dorsiflexion ROM may be associated with Achilles tendinopathy (Mahieu et al., 2006).

In summary, a direct association between reduced or excessive increased ankle ROM and subsequent Achilles tendon injury is still not clear (Jarvinen et al., 2005; Hunter and Spriggs, 2000). Data from most studies do not indicate whether a decreased ankle ROM was the cause or result of Achilles tendon injury because injured individuals were studied without baseline data. More prospective cohort studies will be required to determine this cause-effect relationship.

In addition to the possibility that a decreased joint ROM may be related to Achilles tendon injury, it is also possible that excessive increases in joint ROM may be related to Achilles tendon injury. In particular, the relationship between joint hypermobility, genetic factors and Achilles tendon injury risk need to be explored. Joint hypermobility or laxity is a common unifying feature of the heritable disorders of connective tissue (HDCTs) (Hakim and Grahame, 2003). These include Marfan syndrome, Ehlers Danlos Syndrome (EDS), Osteogenesis imperfecta (OI) and benign joint hypermobility syndrome (BJHS). Joint hypermobility, which can be defined as an asymptomatic increased range of joint or spinal movement, per se is not a disease (Grahame, 1999). Joint hypermobility must therefore be distinguished from BJHS, which is often symptomatic, and presents with chronic joint pain as the cardinal symptom (Grahame, 1999; Hakim and Grahame, 2003).

Mutations in the *COL5A1* gene have been shown to cause types I and II forms of EDS (Loughlin et al., 1995; De Paepe et al., 1997; reviewed in Myllyharju and Kivirikko, 2001; Riley, 2004; Malfait et al., 2005), while mutations in the tenascin X (*TNX*) gene, on the other hand have been shown to be associated with both hypermobility type-Ehlers Danlos Syndrome (HT-EDS) and BJHS (Schalkwilk et al., 2001, Zweers et al., 2005). As discussed in Chapter 4, tenascin X is a member of the same family of matricellular glycoproteins as Tenascin C.

Mutations within the *TNX* gene have also been associated with tenascin X deficiency, since reduced serum levels of Tenascin X are present in 5-10% of patients diagnosed with BJHS or HT-EDS (Zweers et. al., 2003, Zweers et. al.,

2005). In addition, patients with tenascin X haploinsufficiency (which is a mutation in one of the *TNX* alleles resulting in phenotypic alteration, while the other allele remains normal) also show generalized joint hypermobility (Zweers et al., 2005). Tenascin X is produced predominantly by dermal fibroblasts where it has been shown to influence collagen fibre deposition (Chiquet-Ehrismann and Tucker, 2004). Furthermore, it has also been shown that tenascin X is secreted by fibroblasts in the endo-, peri- and epimysium of skeletal muscle (Chiquet-Ehrismann and Tucker, 2004). Since the detailed functions of the family of tenascin proteins are not fully understood, it is possible that tenascin X has other similar functions as Tenascin-C in the tendon extracellular matrix, which has not been described yet.

As described in Chapter 3, the *Bst*UI restriction fragment length polymorphism (RFLP) within the *COL5A1* gene was associated with symptoms of Achilles tendon injuries. Individuals with the A1 and A3 alleles were over-represented in the Achilles tendon injury (ATI) group, while individuals with the A2 allele were under-represented in the ATI group. Furthermore, the GT dinucleotide repeat polymorphism within the *TNC* gene was also associated with symptoms of Achilles tendon injuries (Chapter 4). Individuals with 12 or 14 GT repeats were over-represented (grouped together and termed the O alleles) in the ATI group, while individuals with 13 or 17 GT repeats were under-represented (termed the U alleles) in the ATI group. There were no significant differences in the distribution of the remaining alleles between the two groups (termed the E alleles).

Since mutations within both the *COL5A1* and *TNX* genes have been associated with joint hypermobility pathologies, such as HT-EDS and BJHS, and Tenascin-C belongs to the same family of glycoproteins as Tenascin X, it is possible that the variants of the *COL5A1* and/or *TNC* genes, which are either over- or under-represented within Achilles tendon injury group (Chapters 3 and 4) are associated with alterations in musculotendinous flexibility.

The aim of this present study therefore was to investigate the possible interactions of polymorphisms within the *COL5A1* and/or *TNC* genes and musculotendinous flexibility in symptomatic subjects with Achilles tendon injuries as well as asymptomatic control subjects. A secondary aim was to investigate whether the subjects with Achilles tendon injuries would exhibit either an asymmetrical limb or decreased flexibility compared to non-injured subjects.

5.2 MATERIALS AND METHODS

5.2.1 *Subjects*

The recruitment strategy, inclusion and exclusion criteria for the subjects included in the Achilles tendon injury (ATI) and control (CON) groups, as well as the Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP) sub-groups has been previously described in detail in Chapter 2 (refer to section 2.2.1). The subjects were also informed and gave written informed consent of the sub-study on the measurement of the range of motion to evaluate musculotendinous flexibility. During the same visit to the laboratory when the subjects were provided with information and explanation on the overall study, completed questionnaires and an informed consent form, range of motion (refer to section 5.2.2) was also measured in a those subjects who agreed to participate in the flexibility component of the study. Participation in the flexibility sub-study was based on the subject's willingness to spend extra-time (about 45min) on the trial so that the range of motion could be measured. Therefore, a sub-group of 71 subjects (62% of the ATI group) with a current or past clinical history of Achilles tendon injury (ATI-F) and a sub-group of 37 (29% of the CON group) apparently healthy asymptomatic control (CON-F) subjects were recruited for the flexibility component of the study as well. The ATI-F group consisted of 38 (53% of the TEN group) subjects diagnosed with chronic Achilles tendinopathy (TEN-F) and 33 (79% of the RUP group), all with a complete rupture of the Achilles tendon (RUP-F).

5.2.2 *Subjects grouping according to the BstUI alleles of the COL5A1 gene*

The A1 and A3 alleles of the *Bst*UI restriction fragment length polymorphisms (RFLPs) within the *COL5A1* gene were significantly over-represented in the ATI than in the CON group, while the A2 allele was under-represented in the ATI

subjects (Chapter 3). The CON-F and ATI-F groups were therefore divided into those individuals who were heterozygous or homozygous for the A2 allele, namely the CON-F-A2 and ATI-F-A2 sub-groups and the remaining subjects who did not have an A2 allele, namely the CON-F-A1/A3 and ATI-F-A1/A3 sub-groups.

5.2.3 *Subjects grouping according to the GT repeat alleles of the TNC gene*

As shown in Chapter 4, the 12 and 14 repeats of the GT dinucleotide repeat polymorphism (the O alleles) within the *TNC* gene were significantly over-represented in ATI than in the CON group, while the 13 and 17 repeats (the U alleles) were under-represented in the ATI group. The remaining GT repeats were evenly distributed (the E alleles) between CON and ATI groups. Therefore, the CON-F and ATI-F subjects were also divided into those individuals who were heterozygous or homozygous for the O alleles (OO and OE) (sub-groups CON-F-O and ATI-F-O), individuals homozygous for the evenly distributed alleles (EE) (sub-groups CON-F-E and ATI-F-E) and individuals who had either a UU or UE *TNC* genotype (sub-groups CON-F-U and ATI-F-U).

5.2.4 *Flexibility measurements*

The following flexibility measurements were conducted and recorded in all the subjects: (i) sit and reach test (SR), (ii) passive straight leg raise (SLR), (iii) passive ankle dorsiflexion (ADF), (iv) passive shoulder external (Sho ER) and internal rotation (Sho IR), (v) passive hip external (Hip ER) and internal rotation (Hip IR) and (vi) active elbow extension (Elb Ext) and flexion (Elb Fle). Subjects were requested not to exercise in excess of their normal training habits in the 2 days before being tested in an effort to exclude the effects of delayed onset muscle soreness on flexibility (Cleak and Eston, 1992; reviewed in Marginson et al., 2005). Subjects were not allowed to warm up before taking flexibility measurements and no prior stretching between tests was permitted. The time of the day of the test and instructions given to the subjects was also kept as similar as possible. Except for the sit and reach test, all other measurements were assessed twice and the average score later calculated. The same researcher (GM) tested all the subjects.

In the test-retest pilot study the 17 of the 19 subjects recruited and tested were right handed, while two were left handed. Fifteen of the 17 (88%) right-handed subjects were also right footed. Of the 2 remaining left-handed subjects, 1 was left footed. Therefore, the reported dominant hand in the questionnaire was used to assign the corresponding dominant hand. In comparing the injured versus non-injured limbs, individuals who had experienced bilateral injuries were not included in that particular analysis.

5.2.4.1 The sit and reach test

The standard floor sit-and-reach test was used to assess lower back and hip joint flexibility (ACSM, 2000) as well as an indirect technique to measure hamstring muscle flexibility (Hui et al., 1999). Participants were instructed on how to perform test using the sit-and-reach box in which the zero point is set at 23 cm

(ACSM, 2000). The best of the four test trials were recorded and used for analysis. The furthest distance covered during one of the four trials by the subject was the best sit and reach test score recorded.

5.2.4.2 The straight leg raise test

The straight leg raise was performed with the subject lying supine on a plinth, with the head resting on the plinth, arms placed on the chest, and both knees fully extended and their ankles resting slightly over the end of the plinth (Norkin and White, 2003). An L-shaped ankle splint, constructed from moulded polyurethane and lined with foam cushioning (constructed by Mr. John Rex, an Othortist at Claremont Medical Centre, Cape Town), was attached to the leg that was being tested using three Velcro straps. The ankle joint was held in a neutral position at 90°. One of the straps was fastened across the foot, while the remaining two straps were fastened across the subject's anterior tibia (Hughes, 1996; Cobbing, 1999).

An inclinometer, which was used to measure the range of motion, was secured to the lateral side of the splint using two Velcro straps. The inclinometer was constructed from a 30 cm goniometer. The original swinging arm of the protractor was replaced with a free swinging 2cm wide and 10cm long rectangular Perspex arm to which a small weight was attached. Readings were taken from the inclinometer where the free-swinging Perspex arm bisected the periphery of a standard protractor. Prior to each test the inclinometer was calibrated to a starting point of 90°, with the leg fully extended on the plinth. The subject was instructed to relax the muscles in the leg throughout the testing procedure. The researcher then slowly lifted the subject's experimental leg with one hand placed over the subject's knee and the other over the subject's heel, ensuring that full knee extension was maintained during the test. The researcher stopped raising the leg once the subject indicated a feeling of discomfort. The leg

was then returned to the starting position and the procedure repeated for the second measurement after about 30 seconds of rest.

5.2.4.3 Passive Ankle dorsiflexion test

Ankle dorsiflexion was performed with the subject lying supine on the plinth with the knees fully extended and the ankles relaxed (Clarkson and Gilewich, 1989; Evans, 1994). The start position of ankle was therefore not standardized to a neutral position. For this test, a goniometer was used, where the short arm of the inclinometer was replaced with the original swinging long arm of the instrument. The fulcrum of the goniometer was placed over the lateral malleolus so that the stationary arm was aligned with the lateral midline of the fibula, using the fibular head as a reference point. The swinging arm was aligned with the lateral aspect of the fifth metatarsal.

During this test the ankle of the subject was progressively dorsiflexed while keeping the knee fully extended until discomfort was felt or the ankle could not be moved further into dorsiflexion. The final reading of the moving arm on the goniometer was substracted from the reference reading taken at the start.

5.2.4.4 Passive shoulder internal and external rotation test

Passive internal and external shoulder rotation was performed with the subject lying supine on a plinth (Herrington, 1998; Norkin and White, 2003). The arm that was tested was abducted to 90° and the elbow, which was positioned just off the edge of the plinth, was flexed to 90°. The forearm was therefore upright and perpendicular to the ground with the palm of the hand facing forwards. The fulcrum of the inclinometer was placed over the olecranon between the medial and lateral epicondyles of the humerus. The long stationary arm of the inclinometer was placed perpendicular to the ground, pointing upward and was secured by two Velcro straps to the lower arm, one near the wrist and the other

near the elbow joint. The short free-swinging arm of the inclinometer was hanging facing downwards creating a reference point. To measure passive external rotation, the subject's experimental forearm was rotated backward from the upright to a position where the subject indicated discomfort and this was used as the end point for rotation. Similarly, to measure passive internal rotation, the forearm was gently rotated to a position of maximal forward rotation from vertical position. Throughout the tests, the shoulder of the arm being tested was held firmly against the plinth to limit rotation of the scapula.

5.2.4.5 Passive hip internal and external rotation test

Passive hip internal and external rotation measurements were performed with the subject seated upright on the edge of the plinth (Norkin and White, 2003). The hips were flexed at 90° flexion and kept in 0° abduction/adduction. The lower legs were hanging over the edge of the plinth with their knees at 90° flexion. The fulcrum of the inclinometer was placed over the centre of the anterior surface of the patella. Both the long and short arms of the inclinometer were aligned perpendicular to the floor, facing downwards, and the reference point of the short arm was at an angle of 0°. Passive hip external rotation was recorded as the maximal rotation of the lower leg to a position in which it was rolled outward by moving the foot medially. Passive hip internal rotation was measured as the maximal rotation of the lower leg by moving the foot laterally. The subject was asked to keep their hands behind their back on the plinth to stabilize themselves and their pelvis was not allowed to rotate up off the plinth. The subject was instructed by the researcher to remain upright and relaxed during all passive movements of the hip.

5.2.4.6 Active elbow flexion and extension test

Elbow flexion/extension was performed with the subject kneeling on both legs on a mat, with the upper arm placed horizontally on the plinth and the elbow flexed 90° (Norkin and White, 2003). The elbow joint was placed just off the corner and edge of the plinth, to allow for any possible elbow extension beyond 90°. The fulcrum of the inclinometer was placed over the medial epicondyle of the humerus. The long stationary arm of the inclinometer was aligned with medial surface of the ulna, using the ulna styloid process as a reference point. Elbow flexion was recorded as the maximal movement of the forearm toward the humerus, and extension as the maximal movement of the fulcrum away from the humerus to a fully extended position.

5.2.5 *Test-retest reliability of the flexibility tests*

Before actively testing all the subjects for flexibility measurements, the tester performed a test-retest repeatability study on asymptomatic subjects after written informed consent had been obtained. The reliability study was performed for two main reasons; (i) to establish if the inexperienced examiner was consistent with flexibility measurements and (ii) to establish the consistency of the modified goniometer used. Each of the subjects was tested on two different days so that the second visit was separated from the first visit by between 2 to 6 days (mean ± SD: 2.7± 1.0 days). All subjects performed the range of flexibility measures as described above. Most of the nineteen (7 males and 12 females) asymptomatic physically active Caucasian volunteers were recruited from the Sports Science Institute of South Africa. One subject was excluded from the study on account of age, (a 52 year old female) which was outside the narrow age range. A second subject (a 30 year old female) was also excluded for only completing the first set of tests. The subjects were included in this test-retest study if they participated in some form of regular physical activity and free of musculoskeletal injury and had never been treated for Achilles tendon injury.

The subject's mean age, weight and height were 25.5 ± 3.3 yrs (range 21-34), 68.7 ± 11.5 kg (range 51-87) and 173.1 ± 10.6 cm (range 153-186), respectively. The test-retest reliabilities (Rs) for the 17 range of motion (ROM) measurements ranged from 0.736 to 0.995 (Table 5.1). Six (35%) of the seventeen measurements showed a moderate (Rs between 0.80 and 0.89) and eight (47%) of the measurements showed high (Rs > 0.90) consistency between test 1 and test 2. Only three measurements (non-dominant elbow extension, dominant and non-dominant elbow flexion, ICC < 0.8) were less reliable, but acceptable. In addition, except for the dominant hip internal rotation (p=0.024) and dominant ankle dorsiflexion (p=0.023), all other measurements were repeatable, when the test 1 and test 2 were analysed using the paired t-test.

Table 5.1 Intra-observer reliability of the lower and upper limb flexibility (°) and sit and reach (cm) measurements of the subject's (n=17).

	Test 1	Test 2	ICC (R)	P value[a]
D-Sho ER	95.3 ± 10.0	93.6 ± 12.3	0.863	0.366
D-Sho IR	59.0 ± 9.4	59.1 ± 7.4	0.918	0.901
ND-Sho ER	88.6 ± 10.1	89.7 ± 12.0	0.893	0.509
ND-Sho IR	73.4 ± 7.8	72.4 ± 7.1	0.890	0.396
D-Elb Ext	92.1 ± 6.5	92.3 ± 5.9	0.840	0.880
D-Elb Fle	54.5 ± 5.7	54.1 ± 5.6	0.738[b]	0.786
ND-Elb Ext	94.5 ± 5.4	92.7 ± 5.6	0.799[b]	0.100
ND-Elb Fle	55.1 ± 4.7	57.0 ± 6.0	0.736[b]	0.128
D-Hip ER	69.2 ± 11.9	68.9 ± 12.3	0.976	0.686
D-Hip IR	55.6 ± 7.8	58.9 ± 9.6	0.865	0.024
ND-Hip ER	68.9 ± 12.3	69.6 ± 13.0	0.939	0.673
ND-Hip IR	56.3 ± 8.5	57.7 ± 8.7	0.901	0.274
D-SLR	97.2 ± 21.1	94.4 ± 19.1	0.970	0.097
ND-SLR	93.7 ± 19.8	93.0 ± 18.8	0.971	0.651
D-ADF	63.4 ± 7.7	60.3 ± 7.7	0.848	0.023
ND-ADF	67.4 ± 11.9	68.7 ± 12.8	0.933	0.394
SR (cm)	28.2 ± 9.4	28.3 ± 9.4	0.995	0.715

Values are expressed as mean ± standard deviation.

D, Dominant; **ND**, Non-Dominant; **Sho ER**, Shoulder External Rotation, **Sho IR**, Shoulder Internal Rotation; **Elb Ext**, Elbow Extension, **Elb Fle**, Elbow Flexion, **Hip ER**, Hip External Rotation; **Hip IR**, Hip Internal Rotation; **SLR**, Straight Leg Raise; **ADF**, Ankle Dorsiflexion, **SR**, Sit and Reach. [a]The P values of the repeated measures ANOVA and the paired t-test are identical. [b]The ICC values which were less than 0.8.

5.2.6 *Statistical analysis*

Data were analyzed using Statistica for Windows (Version 7, Statsoft Inc., Tulsa, OK, USA). A one-way analysis of variance (ANOVA) was used to determine any significant differences between the characteristics of the CON, TEN and RUP groups. When the overall F value was significant, a Tukey's HSD post hoc test was used to identify specific differences. Unless otherwise specified, statistical significance was accepted when $p<0.05$. Where applicable, data are presented as means ± standard deviations (SD) with the number of subjects in parentheses. Pearson's Chi-square or Fisher's exact test analyses were used to analyze differences in the percentage of males between the CON, TEN and RUP groups. Differences in the right versus left, dominant versus non-dominant or right versus dominant or left versus non-dominant sides in the CON group were tested using a two-tailed dependent t-test (Stefanyshyn and Engsberg, 1994; Ekstrand et al., 1982) with a Bonferroni adjustment as four multiple t-tests of interest were conducted (Vincent, 1999). Therefore, the significance level was adjusted to 0.0125 (calculated from the alpha level of 0.05 divided by 4 comparisons). For the differences between the injured and non-injured lower limb in the TEN-F and RUP-F groups a paired t-test was used without a Bonferroni adjustment as only 1 comparison was made within each group (alpha level was set at $p<0.05$). When both legs were injured, the data were not included in the comparison of injured versus non-injured. An intra-class reliability (R) or intra-class correlation coefficient (ICC) was calculated using values obtained from a repeated measures analysis of variance (refer to Appendix 5) for test-retest reliability between tests 1 and 2 measurements. In addition, paired t-test was performed to determine the consistency between tests 1 and 2 measurements. Flexibility measurements were compared between the CON-F, TEN-F and RUP-F groups using a one-way analysis of variance (ANOVA).

5.3 RESULTS

5.3.1 Subject characteristics of the CON-F, TEN-F and RUP-F groups

The CON-F, TEN-F and RUP-F groups were similarly matched for height and gender (Table 5.2). The CON-F group was however significantly younger than the TEN-F and RUP-F groups. The CON-F group was also significantly lighter, with a corresponding lower BMI, than the RUP-F group. There were no significant differences in the weight and BMI of the CON-F and TEN-F groups. The range of motion within the TEN-F and RUP-F groups were tested 9.8 ± 10.3 (range 0-33) and 8.1 ± 9.2 (range 0-40) years after the age of onset for the Achilles tendon injury, respectively.

Table 5.2 Characteristics of the control (CON-F), Achilles tendinopathy (TEN-F) and Achilles Rupture (RUP-F) groups.

	CON-F	TEN-F	RUP-F
Age (yrs)	33.3 ± 11.0 (37)[a,b]	47.3 ± 12.7 (38)[a]	48.7 ± 11.5 (33)[b]
Height (cm)	173 ± 10 (35)	176 ± 9 (37)	175 ± 9 (33)
Weight (kg)	69.5 ± 13.0 (37)[b]	76.2 ± 14.8 (37)	85.1 ± 15.3 (33)[b]
BMI (kg/m^2)	22.8 ± 2.7 (35)[b]	24.7 ± 3.6 (36)	27.9 ± 4.2 (33)[b]
Gender (% males)	62.2 (37)	63.2 (38)	69.7 (33)

Values are expressed as mean ± standard deviation or a frequency (%) where applicable. Number of subjects (n) is in parentheses. **BMI**, Body Mass Index.

[a]CON vs TEN ($p<0.001$), [b]CON vs RUP ($p<0.001$).

5.3.2 *Achilles tendon injury and flexibility of the CON, TEN and RUP groups*

When co-varied for age and body weight at the time of measurement there were significant group differences in only the active dominant elbow flexion (D-Elb Fle, p=0.049) and the non-dominant active elbow extension (ND-Elb Ext, p=0.038) (Table 5.3). No significant differences were observed between the CON-F, TEN-F and RUP-F groups in the remaining upper (Table 5.3) and all lower (Table 5.4) limb flexibility measurements.

Table 5.3 Upper limb flexibility (°) measurements of the control (CON-F), Achilles tendinopathy (TEN-F) and Achilles rupture (RUP-F) groups.

	CON-F	TEN-F	RUP-F
D-Sho ER	95.0 ± 11.8 (36)	86.3 ± 11.2 (27)	88.3 ± 14.8 (11)
D-Sho IR	70.9 ± 12.7 (36)	71.3 ± 9.8 (27)	82.1 ± 14.6 (11)
ND-ShoER	85.7 ± 11.8 (36)	81.0 ± 12.6 (27)	84.7 ± 10.4 (11)
ND-ShoIR	80.4 ± 14.4 (36)	78.9 ± 13.1 (27)	84.8 ± 11.9 (11)
D-Elb Fle[a]	54.5 ± 8.1 (26)	56.3 ± 13.8 (16)	57.9 ± 5.2 (9)
D-Elb Ext	96.2 ± 11.2 (26)	87.5 ± 17.4 (16)	92.8 ± 11.6 (9)
ND-Elb Fle	55.9 ± 8.9 (26)	52.8 ± 12.8 (16)	59.3 ± 7.1 (9)
ND-Elb Ext[a]	95.6 ± 9.9 (26)	91.3 ± 11.6 (16)	94.6 ± 12.6 (9)

Values are expressed as mean ± standard deviation, with the number of subjects (n) in parentheses.

D, Dominant; **ND**, Non-dominant; **Sho ER**, Shoulder External Rotation; **Sho IR**, Shoulder Internal Rotation; **Elb Fle**, Elbow Flexion; **Elb Ext**, Elbow Extension

[a]$P<0.05$ (after covarying for age and weight at the time of measurement)

Table 5.4 Lower limb flexibility (°) and sit and reach (cm) measurements of the control (CON-F), Achilles tendinopathy (TEN-F) and Achilles rupture (RUP-F) groups.

	CON-F	TEN-F	RUP-F
D-Hip ER	70.2 ± 12.8 (31)	68.1 ± 12.0 (19)	59.8 ± 8.6 (11)
D-Hip IR	59.3 ± 9.7 (32)	59.4 ± 8.5 (19)	65.6 ± 5.1 (11)
ND-Hip ER	72.7 ± 13.6 (31)	71.1 ± 13.5 (18)	64.5 ± 8.6 (11)
ND-Hip IR	63.2 ± 11.2 (32)	61.0 ± 9.5 (18)	67.8 ± 8.5 (11)
D-SLR	88.0 ± 20.1 (37)	81.6 ± 15.0 (38)	86.0 ± 18.7 (28)
ND-SLR	88.6 ± 19.3 (37)	80.8 ± 14.2 (38)	85.9 ± 17.7 (28)
D-ADF	58.1 ± 7.9 (37)	56.8 ± 9.6 (37)	53.5 ± 7.9 (29)
ND-ADF	60.0 ± 9.3 (37)	59.8 ± 10.3 (37)	58.2 ± 8.8 (27)
SR (cm)	27.9 ± 10.8 (35)	21.3 ± 11.6 (38)	21.9 ± 12.1 (33)

Values are expressed as mean ± standard deviation, with the number of subjects (n) in parentheses.

D, dominant; **ND**, non-dominant; **I**, injured; **NI**, non-injured; **ER**, External Rotation; **IR**, Internal Rotation; **SLR**, straight leg raise; **ADF**, ankle dorsiflexion; **SR**, Sit and Reach.

5.3.3 *Lower and upper limb dominance and flexibility in the control group*

Most of the control subjects were right handed (33 of 37) and none of the subjects indicated that they were ambidextrous. None of the ROM measurements, therefore, showed any differences between the right limb versus the dominant limb or the left limb versus the non-dominant limb (data not shown). Therefore, only the dominant and non-dominant ROM measurements are reported.

Table 5.5 The comparison of the dominant and non-dominant flexibility (°) measurements of the control subjects in the upper and lower limbs.

	Dominant	Non-dominant	P value
Sho-ER	95.0 ± 11.8 (36)	85.7 ± 11.8 (36)	<0.001
Sho-IR	70.9 ± 12.7 (36)	80.4 ± 14.4 (36)	<0.001
Elb-Ext	96.2 ± 11.2 (26)	95.6 ± 9.9 (26)	0.685
Elb-Fle	54.5 ± 8.1 (26)	55.9 ± 8.9 (26)	0.205
Hip-ER	70.2 ± 12.8 (31)	72.7 ± 13.6 (31)	0.100
Hip-IR	59.3 ± 9.7 (32)	63.2 ± 11.2 (32)	0.007
SLR	88.0 ± 20.1 (37)	88.6 ± 19.3 (37)	0.390
ADF	58.1 ± 7.9 (37)	60.0 ± 9.3 (37)	**0.048**

Values are expressed as mean ± standard deviation, with the number of subjects (n) in parentheses.

Sho ER, Shoulder external rotatio; **Sho IR**, Shoulder internal rotation; **Elb Ext**, Elbow extension; **Elb Fle**, Elbow flexion; **Hip ER**, Hip external rotation; **Hip IR**, Hip internal rotaion; **SLR** Straight leg raise; **ADF**, Ankle dorsiflexion.

As shown in table 5.5, there were no significant differences between the dominant versus non-dominant limb measurements of the active elbow flexion and extension, straight leg raise as well as the passive hip external rotation ROM. The dominant shoulder external rotation (95.0 ± 11.6°) was significantly larger ($p<0.001$) than the non-dominant shoulder external rotation (85.7 ± 11.7°). The dominant shoulder internal rotation (70.9 ± 12.7°) was significantly lower ($p<0.001$) than the non-dominant shoulder internal rotation (80.4 ± 14.4°). The dominant hip external rotation (70.2 ± 12.8°) was not significantly lower ($p=0.100$) than the non-dominant hip external rotation (72.7 ± 13.6°). The dominant hip internal rotation (59.3 ± 9.7°) was significantly lower ($p=0.007$) than the non-dominant hip internal rotation (63.2 ± 11.2°). Finally, the dominant ankle dorsiflexion (58.1 ± 7.9°) was significantly lower than the non-dominant ankle dorsiflexion (60.0 ± 9.3°) ($p=0.048$).

5.3.2 *Lower limb flexibility and Achilles tendon injury*

Table 5.6 shows a summary of the results of injured and non-injured ROM measured in the hip and lower leg for the TEN-F and RUP-F groups. In the TEN-F group none of the ROM measurements (Hip ER, Hip IR, SLR and ADF) showed significant differences between the injured versus non-injured limbs. However, the ROM of the injured hip external rotation (65.4 ± 9.4°) was significantly higher ($p=0.021$) than the non-injured hip internal rotation (58.9 ± 7.0°) in the RUP-F group. In addition, the ROM of the injured hip internal rotation (69.1 ± 6.7°) was significantly higher ($p=0.045$) than the non-injured hip internal rotation (64.4 ± 6.5°) in the RUP-F group. The ROM of the SLR and ADF were however not significantly different between the injured and non-injured limbs in the RUP-F group.

Table 5.6 The differences in injured and non-injured limb flexibility (°) of the Achilles tendinopathy (TEN-F) and Achilles tendon rupture (RUP-F) subjects.

	TEN-F		RUP-F	
	Injured	**Non-injured**	**Injured**	**Non-injured**
Hip-ER	66.5 ± 11.2 (11)	66.6 ± 10.6 (10)	65.4 ± 9.4 (11)[a]	58.9 ± 7.0 (11)[a]
Hip-IR	59.4 ± 6.2 (11)	60.1 ± 8.5 (10)	69.1 ± 6.7 (11)[b]	64.4 ± 6.5 (11)[b]
SLR	76.8 ± 13.6 (19)	78.6 ± 14.8 (19)	87.7 ± 16.7 (26)	87.5 ± 15.7 (26)
ADF	55.9 ± 9.2 (19)	55.9 ± 9.8 (19)	54.7 ± 10.3 (24)	57.1 ± 7.4 (26)

Values are expressed as mean ± standard deviation, with the number of subjects (n) in parentheses.

Hip ER, Hip external rotation; **Hip IR**, Hip internal rotation; **SLR**, Straight leg raise; **ADF**, Ankle dorsiflexion

[a]RUP-F (Injured) vs RUP-F (Non-injured), p=0.021

[b]RUP-F (Injured) vs RUP-F (Non-injured), p=0.045.

5.5.3 The COL5A1 allelic grouping and flexibility in the CON-F and ATI-F groups

Tables 5.7 and 5.8 show the characteristics of the CON-F and ATI-F subjects when divided into their *COL5A1* gene A2 and A1/A3 allelic groupings, respectively. The control subjects with an A2 allele (CON-F-A2) and those with either an A1 or A3 allele (CON-F-A1/A3) were similarly matched for age, height, weight and BMI and gender (Table 5.7). Similarly, the Achilles tendon injury subjects with an A2 allele (ATI-F-A2), were matched for age, height, weight and gender when compared to the Achilles tendon injury subjects with either an A1 or A3 (ATI-F-A1/A3) (Table 5.8). The ATI-F-A2 sub-group however had a significantly higher BMI than the ATI-F-A1/A3 sub-group. The two control *COL5A1* allelic sub-groups were however both younger and lighter with a corresponding lower BMIs than the two Achilles tendon injury COL5A1 allelic sub-groups (data not shown).

Table 5.7 Characteristics of the control subjects with an A2 (CON-F-A2) and either an A1 or A3 (CON-F-A1/A3) alleles of the *COL5A1* gene.

	CON-F-A1/A3	CON-F-A2	P value
Age (yrs)	35.7 ± 11.2 (23)	29.4 ± 9.6 (14)	0.095
Height (cm)	173 ± 10 (22)	173 ± 10 (13)	0.920
Weight (kg)	69.8 ± 14.6 (23)	69.1 ± 10.3 (14)	0.874
BMI (kg/m^2)	23.0 ± 3.0 (22)	22.4 ± 2.3 (13)	0.577
Gender (% males)	60.9 (23)	64.3 (14)	1.00

Values are expressed as mean ± standard deviation, with the number of subjects (n) in parentheses.

BMI, Body Mass Index

Table 5.8 Characteristics of the Achilles tendon injury subjects with an A2 (ATI-F-A2) and either an A1 or A3 (ATI-F-A1/A3) alleles of the *COL5A1* gene.

	ATI-F-A1/A3	ATI-F-A2	P value
Age (yrs)	47.1 ± 12.7 (49)	51.1 ± 10.6 (17)	0.257
Height (cm)	175± 10 (48)	175 ± 7 (17)	0.837
Weight (kg)	78.4 ± 14.4 (48)	85.2 ± 17.1 (17)	0.113
BMI (kg/m^2)	25.4 ± 3.7 (48)	27.9 ± 4.8 (17)	0.040
Gender (% males)	65.3 (49)	70.6 (17)	0.773

Values are expressed as mean ± standard deviation, with the number of subjects (n) in parentheses. Significant differences are highlighted in bold.
BMI, Body Mass Index.

There were no differences found in the lower limb flexibility measurements between the CON-F-A2 and CON-F-A1/A3 groups (Table 5.9). Except for the non-dominant ankle dorsiflexion (ND-ADF), which was significantly larger in the ATI-F subjects with either an A1 or an A3 allele than in the subjects with an A2, there were no other differences found in the lower limb flexibility measurements between the ATI-F-A2 and ATI-F-A1/A3 sub-groups (Table 5.10).

Table 5.9 Lower limb flexibility (°) and sit and reach (cm) measurements of the control subjects with an A2 (CON-F-A2) and either an A1 or A3 (CON-F-A1/A3) alleles of the *COL5A1* gene.

	CON-F-A1/A3	CON-F-A2	P value
D-SLR	86.9 ± 22.9 (23)	89.8 ± 14.9 (14)	0.681
ND-SLR	87.4 ± 21.8 (23)	90.5 ± 15.1 (14)	0.655
D-ADF	57.0 ± 8.6 (23)	59.9 ± 6.7 (14)	0.298
ND-ADF	59.5 ± 9.0 (23)	60.8 ± 10.0 (14)	0.685
SR (cm)	26.3 ± 12.0 (21)	30.3 ± 8.5 (14)	0.290

Values are expressed as mean ± standard deviation, with the number of subjects (n) in parentheses.

D, dominant; **ND**, non-dominant; **SLR**, Straight leg raise; **ADF**, Ankle dorsiflexion; **SR**, sit and reach.

Table 5.10 Lower limb flexibility (°) and sit and reach (cm) measurements of the Achilles tendon injury subjects with an A2 (ATI-F-A2) and either an A1 or A3 (ATI-F-A1/A3) alleles of the *COL5A1* gene.

	ATI-F-A1/A3	ATI-F-A2	P value
D-SLR	86.3 ± 15.2 (46)	78.1 ± 18.5 (15)	0.088
ND-SLR	85.4 ± 14.2 (46)	79.4 ± 17.9 (15)	0.191
I-SLR	86.9 ± 15.2 (29)	77.8 ± 14.5 (11)	0.094
NI-SLR	86.7 ± 14.6 (29)	80.5 ± 15.8 (11)	0.247
D-ADF	56.7 ± 8.9 (45)	53.7 ± 9.1 (16)	0.256
ND-ADF	60.7 ± 9.3 (45)	55.0 ± 8.9 (15)	0.046
I-ADF	55.9 ± 8.9 (29)	53.4 ± 10.7 (10)	0.477
NI-ADF	57.7 ± 8.2 (29)	56.2 ± 9.0 (11)	0.607
SR (cm)	22.9 ± 10.6 (49)	18.9 ± 13.9 (17)	0.227

Values are expressed as mean ± standard deviation, with the number of subjects (n) in parentheses.

D, Dominant; **ND**, Non-dominant; **I**, Injured; **NI**, Non-Injured **SLR**, Straight Leg Raise; **ADF**, Ankle Dorsiflexion, **SR**, Sit-and-Reach.

5.5.4 The TNC allelic grouping and flexibility in the CON-F and ATI-F groups

The control subjects with the genotypes UU and UE (CON-F-UU/UE), EE (CON-F-EE) and OO and OE (CON-F-OO/OE) were similarly matched for gender, height, weight and BMI (Table 5.11). The CON-F-EE sub-group was significantly younger than the CON-F-OO/OE sub-group (p=0.019), but not the CON-F-UU/UE sub-group. There was also no significant difference in age between the CON-F-OO/OE and CON-F-UU/UE sub-groups. The Achilles tendon injury subjects with genotypes UU and UE (ATI-F-UU/UE), EE (ATI-F-EE) and OO and OE (ATI-F-OO/OE) were similarly matched for age, height, weight and BMI and

gender (Table 5.12). The three *TNC* genotype CON-F sub-groups were however both younger and lighter with a corresponding lower BMIs than the three TNC genotype ATI-F sub-groups (data not shown).

Table 5.11 Characteristics of the control subjects with UU or UE (CON-F-UU/UE), EE (CON-F-EE) and OO or OE (CON-F-OO/OE) genotypes of the *TNC* gene.

	CON-F-UU/UE	CON-F-EE	CON-F-OO/OE	P value
Age (yrs)	31.8 ± 10.2 (13)	27.4 ± 4.6 (9)[a]	39.9 ± 11.3 (11)[a]	0.019
Height (cm)	171 ± 9 (12)	174 ± 11 (9)	176 ± 10 (11)	0.380
Weight (kg)	67.7 ± 13.2 (13)	69.3 ± 9.4 (9)	73.8 ± 15.4 (11)	0.517
BMI (kg/m^2)	22.5 ± 3.0 (12)	22.9 ± 2.4 (9)	23.5 ± 2.8 (11)	0.695
Gender (% males)	61.5 (13)	66.7 (9)	72.7 (11)	NS

Values are expressed as mean ± standard deviation, with the number of subjects (n) in parentheses.

BMI, Body Mass Index.

[a]CON-F-EE vs CON-F-OO/OE, P=0.024

NS, not significant, where CON-F-UU/UE vs CON-EE, p=1.00; CON-F-EE vs CON-F-OO/EE, p=1.00, CON-F-UU/UE vs CON-F-OO/OE, p=0.679

Table 5.12 Characteristics of the Achilles tendon injury subjects with UU or UE (CON-F-UU/UE), EE (CON-F-EE) and OO or OE (CON-F-OO/OE) genotypes of the *TNC* gene.

	ATI-F-UU/UE	ATI-F-EE	ATI-F-OO/OE	P value
Age (yrs)	46.1 ± 12.6 (15)	48.6 ± 10.0 (24)	47.5 ± 13.0 (28)	0.815
Height (cm)	174 ± 11 (14)	176 ± 8 (24)	173 ± 9 (28)	0.505
Weight (kg)	75.2 ± 12.3 (14)	80.3 ± 15.3 (24)	82.1 ± 17.8 (28)	0.413
BMI (kg/m^2)	24.8 ± 3.2 (14)	26.0 ± 4.7 (24)	27.1 ± 4.3 (27)	0.267
Gender (% males)	46.7 (15)	75.0 (24)	64.3 (28)	NS

Values are expressed as mean ± standard deviation, with the number of subjects (n) in parentheses.

BMI, Body Mass Index

NS, not significant, where ATI-F-UU/UE vs ATI-EE, p=0.0953; ATI-F-EE vs ATI-F-OO/EE, p=0.549, ATI-F-UU/UE vs ATI-F-OO/OE, p=0.338

There were no significant differences in the lower limb flexibility measurements between the CON-F-UU/UE, CON-F-EE and CON-F-OO/OE sub-groups (Table 5.13). The dominant ankle dorsiflexion (D-ADF) of the ATI-F-UU/UE was significantly greater than in the ATI-F-OO/OE sub-group (p=0.046), but not in the ATI-F-EE sub-group (Table 5.14). The dominant ankle dorsiflexion (D-ADF) of the ATI-F-OO/OE sub-group was similar to the ATI-F-EE sub-group. All the other flexibility and the sit and reach measurements were similar between the *TNC* genotype ATI-F sub-groups.

Table 5.13 Lower limb flexibility (°) and sit and reach (cm) measurements of the control subjects with UU or UE (CON-F-UU/UE), EE (CON-F-EE) and OO or OE (CON-F-OO/OE) genotypes of the *TNC* gene.

	CON-F-UU/UE	CON-F-EE	CON-F-OO/OE	P value
D-SLR	86.5 ± 17.2 (13)	97.2 ± 28.1 (9)	82.2 ± 17.3 (11)	0.271
ND-SLR	86.7 ± 17.4 (13)	97.6 ± 25.4 (9)	83.5 ± 17.7 (11)	0.277
D-ADF	56.2 ± 9.4 (13)	62.1 ± 4.8 (9)	57.7 ± 6.0 (11)	0.186
ND-ADF	59.5 ± 11.8 (13)	61.2 ± 6.0 (9)	60.4 ± 6.8 (11)	0.906
SR (cm)	27.3 ± 10.1 (12)	30.2 ± 11.5 (9)	26.5 ± 12.5 (10)	0.752

Values are expressed as mean ± standard deviation, with the number of subjects (n) in parentheses.

D, Dominant; **ND**, Non-dominant; **I**, Injured; **NI**, Non-Injured **SLR**, Straight Leg Raise; **ADF**, Ankle Dorsiflexion, **SR**, Sit-and-Reach.

Table 5.14 Lower limb flexibility (°) and sit and reach (cm) measurements of the Achilles tendon injury subjects with UU or UE (ATI-F-UU/UE), EE (ATI-F-EE) and OO or OE (ATI-F-OO/OE) genotypes of the *TNC* gene.

	ATI-F-UU/UE	ATI-F-EE	ATI-F-OO/OE	P value
D-SLR	86.8 ± 13.9 (14)	80.8 ± 16.9 (23)	83.1 ± 18.7 (25)	0.593
ND-SLR	86.9 ± 12.9 (14)	81.7 ± 18.2 (23)	81.9 ± 16.2 (25)	0.589
I-SLR	90.4 ± 11.3 (9)	81.3 ± 15.0 (15)	80.4 ± 19.1 (19)	0.301
NI-SLR	92.0 ± 13.1 (9)	82.2 ± 16.1 (15)	80.9 ± 16.8 (19)	0.218
D-ADF	60.5 ± 7.7 (14)	54.1 ± 6.8 (22)	53.5 ± 10.7 (26)	0.047
ND-ADF	60.2 ± 9.9 (13)	59.3 ± 9.3 (22)	58.5 ± 10.6 (25)	0.878
I-ADF	57.4 ± 7.7 (8)	53.5 ± 7.7 (15)	54.9 ± 12.1 (18)	0.670
NI-ADF	57.5 ± 6.1 (9)	56.5 ± 8.0 (15)	55.7 ± 10.2 (19)	0.877
SR (cm)	26.3 ± 10.8 (15)	22.7 ± 11.2 (24)	18.6 ± 12.4 (28)	0.118

Values are expressed as mean ± standard deviation, with the number of subjects (n) in parentheses.

D, Dominant; **ND**, Non-dominant; **I**, Injured; **NI**, Non-Injured **SLR**, Straight Leg Raise; **ADF**, Ankle Dorsiflexion, **SR**, Sit-and-Reach.

5.4 DISCUSSION

The study investigated whether the *Bst*UI RFLP within the *COL5A1* gene and/or the GT dinucleotide repeat polymorphism within the *TNC* gene were associated with the ROM in symptomatic patients with Achilles tendon injuries and asymptomatic controls. The study investigated whether there was a genotype effect on muscle-tendon flexibility as represented by the measurement of ROM. Because few studies (Clement et al., 1984) have investigated the specific role of flexibility on Achilles tendon injuries, a further secondary aim was to test whether flexibility was associated with the risk of developing Achilles tendon injuries.

The main finding of the study was that the different polymorphisms of both *COL5A1* and *TNC* genes were not associated with measurements of ROM of all joints tested in the study. The results suggest that both *COL5A1* and *TNC* genes do not influence the ROM measured and therefore have no effect on the corresponding muscle-tendon flexibility in the joints measured.

Most treatment programs for chronic Achilles tendinopathy include stretching because of the notion that "inflexibility is a major cause of the injury" (El Hawary et. al., 1997). However, there is conflicting evidence with regard to the role of flexibility in tendon injury (reviewed in Shrier, 1999; Witvrouv et. al., 2003; Weldon and Hill, 2003; Yeung and Yeung, 2001). It has been shown that both decreased and increased flexibility could be related to an increased risk of tendon injury (Jones et. al., 1993). It has also been suggested that the relationship between flexibility and injury risk is U-shaped, with the number of injuries increasing at both extreme ranges of motion (Taimela et. al., 1990; Jones et. al., 1993).

In support of this, there is a group of heritable disorders of connective tissue (HDCTs) with a unifying characteristic of joint hypermobility. As well as having joint ROM in the extreme, these conditions are all characterized by increased musculo-skeletal fragility. These conditions in various forms of expressions or severity may represent the one of the spectrum in U-shape relationship between injury risk and joint ROM. Two of these HDCTs, namely Ehlers Danlos syndrome (EDS) and Benign Joint Hypermobility syndromes (BJHS) have been associated with mutations in the *COL5A1* and *TN-X* genes. Because (i) TNC belongs to the same family of proteins as TNX and possibly share some functional roles in the extracellualar matrix, (ii) both *COL5A1* and *TNC* genes have been associated with Achilles tendinopathy (chapter 3 and 4) and (iii) both *COL5A1* and *TNX* genes have been associated with pathologies associated with hyperflexible joints, it is probable that these genes and their protein products play a role in the quality of connective tissue including those of tendons.

However, the passive properties of the muscle-tendon unit have recently been shown to be similar between female patients with BJHS and an age and gender matched control group, and the authors interpreted these findings to suggest that connective tissue of the muscle-tendon unit is unaffected in BJHS (Magnusson et al., 2001). This notion argues against the belief that the increased musculoskeletal flexibility associated with BJHS is a result of altered mechanical properties of the connective tissue (Bilkey et al., 1981). Subjects with BJHS tolerated stretch loading as shown by their greater peak moment compared to the controls. However, the structures and mechanisms that allow BJHS subjects to tolerate more stretch loading are not known (Magnusson et al., 2001).

Although it makes intuitive sense that a more flexible and less stiff tendon should be at lower risk for tendon injury, it is also understood that Achilles tendon injury is a manifestation of many factors (Riley, 2004; Jarvinen et al., 2005). These factors include amongst others, the magnitude, rate and type of mechanical load and other environmental factors such training surfaces as well as other

physiological (such as age, gender, ankle dorsiflexion and hamstring ROM), psychological and other random factors (Gleim and Mc Hugh, 1997; Riley, 2004). It is therefore very difficult to isolate and quantify the contribution of all these factors and control their effects in order to study the effects of a single factor such as flexibility.

The second finding of this study was that there were no significant differences between joint range of motion measurements between the control (CON-F), Achilles tendinopathy (TEN-F) and Achilles tendon rupture (RUP-F) groups. These results suggest that flexibility is perhaps not associated with Achilles tendon injury. Although the CON-F, TEN-F and RUP-F groups were matched for gender and height, the symptomatic groups were consistently heavier and older. Because there were generally no differences in the ROM measurements of most joints (except for the elbow measurements, Table 5.3) between the TEN-F and RUP-F groups, these two sub-groups were combined for the genotype studies to help increase the sample size. Interestingly, both the dominant elbow flexion (D-Elb Fle) and non-dominant elbow flexion (ND-Elb Fle) produced the lowest repeatability scores (ICC of 0.738 and 0.745 respectively). Furthermore, it is highly unlikely that elbow ROM has any effect on Achilles tendon function.

Although, there is unequivocal evidence that stretching improves the ROM around most joints and therefore improves muscle-tendon joint flexibility (Witvrouw et al., 2004), scientific evidence relating decreased ROM to increased risk of muscle-tendon injury is at best controversial (Yeung and Yeung, 2001; Gleim and McHugh, 1997; Witvrouw et al., 2004; Thacker et al., 2004; Weldon and Hill, 2003; Shrier, 1999). To illustrate the importance of muscle-tendon flexibility, guidelines from the America College of Sports Medicine and the World Health Organization include flexibility as one of the tenets of health and wellness (ACSM, 2000). Furthermore, stretching to increase flexibility is routinely prescribed as part of training programs for both recreational and professional athletes (Witvrouw et al., 2004). It is therefore not surprising that

flexibility has become part of most athlete's fitness training and testing programs. In a recent review, it was concluded that due to improper study design and small sample sizes, only one study so far has fulfilled the two criteria in trying to understand risk factors, which included the role of muscle-tendon flexibility, associated with lower extremity injuries (Bahr and Holme., 2003).

The lower limb ROM measurements were of interest in this study, in particular the ankle dorsiflexion ROM, as a decreased ankle dorsiflexion ROM has been associated with an increased risk of Achilles tendon injury (Clement et. al., 1984). These investigators retrospectively examined runners with Achilles tendinopathy and found that they had insufficient strength and flexibility in the triceps surae muscle complex (Clement et al., 1984). However, the study could not distinguish cause and effect and did not have an age- and physical activity matched control. Our study was also a retrospective study, but it included a control group, which due to an age-at-onset matching attempt for the larger study, could not be precisely matched for present age-at-testing of subjects. Nonetheless, no differences were found in the flexibility measurements between the CON-F, TEN-F and RUP-F groups, in particular, the ankle dorsiflexion ROM, even after controlling for differences in age and body weight between groups.

Because of the nature of their design, case-control studies are unable to distinguish between risk factors and consequence of injury (Bahr and Holme, 2003). Therefore, lack of baseline flexibility data before Achilles tendon injury occurrence would make it difficult to determine if reduced or increased flexibility was as a result of the injury or a risk factor for the injury. However, such information points towards conducting a prospective cohort study or randomised controlled trial. At least one study (Hartig and Henderson, 1999), has shown reduction of muscle-tendon injuries as a result of increased flexibility.

Compared to other movements around joints, the range of ankle dorsiflexion is largely fixed, probably due to bony factors, rather than connective tissue stretch

(Bennell et. al., 2001). For instance, a six-week static stretching failed to elicit a change in active ankle dorsiflexion range of motion (Youdas et. al., 2003). However, other studies have found an increased ankle dorsiflexion ROM after immediate intense stretching session or after 10 weeks of static stretching exercises and weights (Swank et al., 2003; Wiktorsson-Moller et al., 1983).

Although shown to be reliable and valid, goniometric measurements are static, therefore it is difficult to extrapolate the values to dynamic, continous and functional range of motion that occurs during human body movement. These measurements are also performed in a non-weight bearing position, however body movement often occur with some weight bearing activity. This makes it difficult to interpret these measurements in the context of range of motion during activity. Hunter and Spriggs (2000) have suggested there are differences in the functional correlates of passive and active ROM around joint. Therefore, it could mean that measurement of active ROM would result in possibly different results compared to the current results, as all of our measurements were passive ROM, except for the elbow flexion and extension ROM and sit-and-reach test.

Because flexibility is highly specific to joints (ACSM, 2000), upper body flexibility measurements were recorded in this study in order to correlate some lower body versus upper body range of motion. No specific relationships or trend could be observed between lower and upper body limb ROM, perhaps supporting the notion that one joint ROM such as the sit-and reach test, does not represent the individual's general flexibility (Boyle et al., 2004; ACSM, 2000).

The role of limb dominance in flexibility was investigated in the control, but not in symptomatic subjects, as they were uninjured. The dominant shoulder external rotation was significantly greater than the non-dominant shoulder external rotation. Interestingly in the opposite movement, the dominant shoulder internal rotation was significantly lower than the non-dominant shoulder internal rotation. When the two movements in each limb were added to

give total shoulder rotation, there were no significant differences in the total shoulder ROM (data not presented). The dominant hip internal rotation and ankle dorsiflexion were lower than in the non-dominant sides, although the differences (3.9° Hip IR and 1.9° Ankle dorsiflexion) were probably not of clinical importance. Whether these limb ROM differences may predispose individuals to an injury is not known, as there was no baseline data before injury for the symptomatic subjects to compare with CON subjects. Nonetheless, it has been suggested that asymmetrical limb flexibility could predispose a joint or soft tissue to injury (Ash and Werlinger, 1997).

In the TEN-F group, there were no significant differences between the injured and non-injured hip external rotation (Hip ER), hip internal rotation (Hip IR), straight leg raise (SLR) and ankle dorsiflexion (ADF). The result of comparisons of injured versus non-injured limbs in the TEN-F group are interesting especially for the ankle dorsiflexion as no differences were found in passive ROM. The result could suggest that either the measurement of passive ADF is unable to detect the differences in changes in connective tissue of the muscle-tendon or that passive ADF is not associated with tendinopathy. It is possible that active ADF range of motion will yield different results.

However, both the Hip ER and Hip IR, of the injured limb had a significantly greater ROM than that of the non-injured limb in the RUP-F group. It is not clear why both the Hip ER and Hip IR are larger in the injured limb compared the non-injured sides. However, it is possible that during the period leading up to Achilles tendon rupture and during recovery, the ipsilateral hip to the injured Achilles tendon probably has to adapt in some way to increase both its internal and external ROM. However, it is difficult to make that conclusion as no baseline data before injury were obtained in these subjects. The SLR and ADF range of motions were similar for the injured and non-injured limb in the RUP-F group. These latter results suggest that either the Achilles tendon passive properties are unaffected by rupture and surgery, or alternatively surgery is effective in the

healing of Achilles tendon rupture to restore the passive property of the Achilles tendon. Over ninety percent (30 of 33) of the subjects in the RUP-F group had undergone surgical operation of their Achilles tendons. None of the subjects in TEN-F group were surgically repaired.

An additional finding was that the measurements of flexibility were found to have in the majority a moderate to high reliability as determined by the intra-class coefficient (ICC) (Vincent, 1999; Thomas and Nelson, 1996). The reliability and validity of hand held goniometers have been shown to be fairly good (Gajdosik and Bohannon, 1987). The results show that data obtained from flexibility measurements were reliable. Hartig and Henderson, 1999 found a test-re-test reliability coefficient of 0.98 (n=20) for passive hamstring ROM, when the same researcher performed the tests, which is similar to some of our results. Significant statistical difference ($p<0.001$) of 4° was found between the experimental (n=148, 41.7±8.3°) and the control (n=150, 45.9 ± 6.5°) groups at the beginning of the intervention. This difference was significant ($p<0.001$) again after 13 weeks (experimental, 34.7° vs control, 42.9°) of the training intervention. In this case, the lower values indicate a more flexible hamstring muscle-tendon unit. Murphy et al. (2003) attributed the lack of consistent findings in most injury studies to improper study design and small sample sizes. This study serves to demonstrate the value of larger sample sizes in determining small to moderate differences in ROM measurements.

In the current study, the same researcher conducted the testing as it is well documented that intra-observer error is less than inter-observer error in goniometric measurements (Low, 1976). It has been shown that taking more than one measurement recording further enhances measurement reliability (Low, 1976). Therefore, in this study, an average of two recordings were measured for each joint range of motion, except for the sit and reach test, where the best and furthest of 4 measurements was recorded. Furthermore, all subjects were not allowed warm-up before the measurements in order to standardize the testing

procedure as it has been shown that warm-up may improve range of motion around joints (Low, 1976).

Furthermore, it has been shown that certain joint range of motion measurements were associated with less variation compared with others (Low, 1976). In our study, certain joint measurements were more reproducible than others. It has also been suggested that it was easier to measure certain joints more accurately than others (Low, 1976). Other factors affecting the measurements could be the difficulty associated with aligning the goniometer arms with bony marks, as well as the development of tissue tension from day to day which might influence the result of test-retest measurements.

One of the limitations of the present study is that plantar-flexion ROM was not assessed. The reason for not including this measurement was a poor reproducibility of this measurement during the pilot testing. It could be that the total available ROM, e.g. the sum of ankle plantar- and flexion, at any joint is more important than a single isolated unidirectional movement in joint function, although that remains speculation. It was also impossible to control for age of individual's flexibility testing as matching was conducted for the larger studies (chapters 3 and 4).

It was also not possible to test range of motion measurements in each subject, as only individuals who were prepared to give an extra hour of testing enrolled for the range of motion measurement testing. This may have introduced a selection bias in subject recruitment for this component of the study. Those subjects who were likely to benefit from the flexibility information feedback were more likely to participate. Thus the majority of the symptomatic subjects volunteered for this component of the study compared to the control subjects. Furthermore, some subjects were visited at their clubs for a collection of blood sample for DNA extraction and therefore their flexibility could not be tested.

The study's sample size was small (Murphy et al., 2003) and the data collection was largely retrospective. There was also large inter-subject variability in the value obtained for most range of motion in the CON-F and ATI-F groups. However, Hartig and Henderson (1999) study showed that the intervention group had reduced number of injuries as a result of the stretching intervention, suggesting that perhaps a 7°degree change in ROM in the hamstring may be clinically important. In our study, some ROM measurement differences between groups were much higher than 7°, but due to the small sample sizes and in most cases large variations between individuals (SDs), the study probably lacked enough power to detect differences.

The present study was unable to show a relationship between the range of motion measurements and polymorphisms with both the *COL5A1* and *TNC* genes. The range of motion of the CON-F-A1/A3 and CON-F-A2 groups measured was similar as well as those of ATI-F-A1/A3 versus ATI-F-A2 group. The results were similar for the TNC genotypes. This suggested a lack of genotype interaction between these studied polymorphisms of the *COL5A1* and *TNC* genes with the ROM or flexibility in the development of Achilles tendon injuries. There were also no significant differences between the CON-F, TEN-F and RUP-F groups in the range of motion measured, without regard for the genotype, suggesting that the range of motion in the joint measured is not associated with Achilles tendon injuries. However, the results need to be interpreted cautiously as the sample sizes were small. (Murphy et al., 2003; Bahr and Holme, 2003).

Further research is needed in larger sample size randomised controlled trials or prospective cohort studies to define the precise role of flexibility or ROM on Achilles tendon injury. Furthermore, the exact role of genetic factors in tendon connective tissues need to be further investigated. Although, goniometric

measurement of ROM has been found to be reliable and valid, the role of passive versus active in muscle-tendon function needs to be investigated.

6.1 INTRODUCTION

In chapters 3 and 4, it was shown that polymorphisms within both the *COL5A1* and *TNC* genes, located on chromosome 9q32-q34, are associated with Achilles tendinopathy. Further support for the products of these genes being involved in Achilles tendon injury could be gained by investigating any associations of these polymorphisms with Achilles tendon morphology using non-invasive imaging techniques.

Several imaging methods such as soft tissue radiography, computerized tomography (CT), ultrasonography (US) and magnetic resonance imaging (MRI) have been used to assist clinicians in the diagnosis of Achilles tendon injuries (Astrom et al., 1996). MRI and US are currently considered superior to radiography and CT and are therefore the methods of choice in imaging the Achilles tendon (Astrom et al., 1996). Although MRI has been shown to provide marginally better images, it is more expensive and less available than US. In addition, US device are also portable and dynamic. US can also be used to show real time pictures and offers assessment of versatile functions such as blood flow measurements. It is therefore often the preferred method to assess tendon morphology (Astrom et al., 1996). Assessment of the paratenon, however, using both MRI and US techniques has been shown to be less reliable than the measurements within the tendon substance itself (Astrom et al., 1996).

The shape, sagittal diameter, contour, intratendinous structure and signal intensity of the Achilles tendon can be determined using either MRI or US (Neuhold et al., 1992;Nehrer et al., 1997; Astrom et al., 1996; Gibbon et al., 2000; Schweitzer and Karasick, 2000). The normal Achilles tendon is oval in shape, approximately 6 mm in diameter, with a well-defined contour, and a sagittal scan shows the antero-posterior (AP) margins to be parallel to one another. The intratendinous structure appears homogenous and the signal intensity

hypointense (i.e. appears black) using MRI while the intratendinous structure appears honeycomb and whiter using US.

In contrast, a pathological Achilles tendon is rounder in shape, greater than 6 mm in diameter (generally 10-18 mm thick), with an ill-defined contour. Sagittal scans show that the AP margins are no longer parallel to one another and the intratendinous structure is seen with increased signal intensity (i.e. appears whiter) using MRI while it appears hypoechoic (i.e. black) using US. The increased signal intensity by MRI and hypoechoic intratendinous scans by US are believed to indicate tendon degeneration. These changes in the signal intensity have been shown to correlate well with the histopathology of Achilles tendinopathy (Astrom et al., 1996; Maffulli et al., 2000; Jozsa and Kannus, 1991).

As shown in Chapter 3 the alpha 1 type V collagen (*COL5A1*) gene, which encodes for a minor structural component of tendons, is associated with chronic Achilles tendinopathy. The A2 variant (A1A2, A2A2 and A2A3 genotypes) of this gene was under-represented in individuals with symptoms of chronic Achilles tendinopathy compared to asymptomatic control subjects. Individuals who are homozygous (A1A1 or A3A3) or heterozygous (A1A3) for the A1 or A3 variants were however over-represented in individuals with symptoms of Achilles tendinopathy compared to asymptomatic control subjects. Type V collagen forms hetrotypic fibers with type I collagen, which are the major structural units of tendons, where it is believed to regulate the formation and growth of the fibres (Birk, 2001). Wenstrup et al. (2004) have recently shown that initiation of fibril assembly in col5a1-deficient mice is completely dependent on type V collagen.

It was also shown (Chapter 4), that the GT dinucleotide repeat polymorphism within the *TNC* gene was associated with chronic Achilles tendinopathy. Individuals with the 12 and 14 GT repeats (O alleles) were over-represented,

whereas the 13 and 17 GT repeats (U alleles) were under-represented in the Achilles tendinopathy group compared to the control subjects. The *TNC* gene encodes for a protein which is also an important component of tendons and whose expression is regulated by mechanical stress (Jarvinen et al., 1999; Jarvinen et al., 2003).

Since both type V collagen and tenascin C are structural components of tendon and expression of both their genes are altered during tendon pathology (Dressler et al., 2002; Ireland et al., 2001; Riley et al., 1996), it is possible that individuals who are at a greater genetic risk for developing symptoms of Achilles tendinopathy would display greater tendon morphological abnormalities. These tendon morphological abnormalities may be assessed using imaging techniques such soft tissue diagnostic ultrasonography.

Therefore, the aim of this chapter of the thesis was to investigate the association between Achilles tendon morphology and the *COL5A1* and/or *TNC* genotype, using soft tissue diagnostic ultrasonography (US).

6.2 MATERIALS AND METHODS

6.2.1 Subjects

The recruitment strategy, inclusion and exclusion criteria for the subjects included in the Achilles tendon injury (ATI) and control (CON) groups, as well as the Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP) sub-groups has been previously described in detail in Chapter 2 (refer to section 2.2.1). During the same visit to the laboratory when the subjects were provided with information and explanation on the overall study, they were also informed and gave written informed consent of the US component of the study on the measurement of the morphological characteristics of the Achilles tendon using ultrasonography (US). Similar to the flexibility study (Chapter 5), participation in the US component of the study was based on the subject's known *COL5A1* and *TNC* genotype and availability at the time of the ultrasonography. A sub-group of 36 male subjects (45.6% of the Achilles tendinopathy (TEN) group) with a current or past clinical history of Achilles tendinopathy (TEN-M) and a sub-group of 22 (16.8% of the CON group) apparently healthy asymptomatic male control (CON-M) subjects were recruited for the US component of the study. Only male subjects were included in this study as it was anticipated that few subjects would be enrolled and therefore a homogeneous sub-group was required. Subjects who had an Achilles tendon rupture were not included in the study as most of them were no longer experiencing symptoms associated with injury. For the purpose of this thesis it was assumed that the dominant leg of each subject was the same as their self-reported dominant hand on the completed questionnaire.

6.2.2 *Subjects grouping according to the A1, A2 and A3 alleles of the COL5A1 gene*

The A1 and A3 alleles of the *Bst*UI restriction fragment length polymorphism (RFLP) within the *COL5A1* gene were significantly over-represented in the ATI than in the CON group, while the A2 allele was under-represented in the ATI subjects (Chapter 3). The CON-M and TEN-M groups were therefore divided into those individuals who were heterozygous (A1A2 or A3A2) or homozygous (A2A2) for the A2 allele, namely the CON-M-A2 and TEN-M-A2 sub-groups, respectively. The remaining subjects who did not have an A2 allele (i.e. those with an A1A1, A1A3 or A3A3 genotype) were divided into the CON-M-A1/A3 and TEN-M-A1/A3 sub-groups.

6.2.3 *Subjects grouping according to the GT repeat alleles of the TNC gene*

As shown in Chapter 4, the 12 and 14 repeats of the GT dinucleotide repeat polymorphism (the O alleles) within the *TNC* gene were significantly over-represented in TEN than in the CON group, while the 13 and 17 repeats (the U alleles) were under-represented in the TEN group. The remaining GT repeats were evenly distributed (the E alleles) between CON and ATI groups. Therefore, the CON-M and TEN-M subjects were also divided into those individuals who were heterozygous or homozygous for the O alleles (i.e. an OO, OE or OU genotype) to produce the CON-M-O and TEN-M-O sub-groups, respectively. The remaining individuals within the CON-M and TEN-M groups who had either a UU, UE or EE *TNC* genotype were divided into the CON-M-U and TEN-M-U sub-groups, respectively.

6.2.4 Testing Procedure

Three experienced musculoskeletal radiologists, who were blinded to the subject group, conducted the soft tissue diagnostic ultrasound investigation. The majority of the subjects were examined by the same radiologist (RdV, n=46), and in a few cases a different radiologist was involved (CG, n=6 and BM, n=6). The method of analysing the Achilles tendon using soft tissue diagnostic US has previously been described (Gibbon et al., 2000). A Toshiba Nemio scanner (Nemio 20, Toshiba Diagnostic Ultrasound Systems, Tochigi-Ken, Japan) and a 12-MHz linear-array transducer were used. During each examination, the subject was in a prone position with the feet overhanging the examination couch. The ankles were in their naturally relaxed position with the Achilles tendon fairly taut. For each subject, both Achilles tendons were examined from the musculotendinous junction to the calcaneal insertion in both the transverse and longitudinal axes. The sonographic probe was placed parallel to the Achilles tendon for transverse scans to avoid anisotrophy artifacts (Fornage, 1987).

Tendon degeneration was defined as an area of decreased echogenecity, heterogeneous echogenecity, or both within a tendon, with or without associated thickening (Gibbon et al., 2000). Paratendinitis was diagnosed if the paratenon was abnormally thickened or contained fluid (Gibbon et al., 2000). Bursitis was defined by an area of increased fluid distention, adjacent oedema, or both in the region of the retrocalcaneal or superficial precalcaneal bursa (Gibbon et al., 2000). Colour Doppler was used to detect an increased blood flow in both the tendon substance and the paratenon. Additionally, tendon sagittal and transverse diameters in the antero-posterior positions were measured (mm) at the thickest point of Achilles tendon (Astrom et al., 1996).

Tendon shape, sagittal diameter, and echogenecity were classified as either normal (score of 0) or abnormal (score of 1) (Table 6.1). Blood flow was assessed in both the tendon substance (maximum score of 1 for each leg) and the paratenon (maximum score of 1 for each leg), therefore the maximum score for blood flow was 2. For the tendon dimensions measured, only the sagittal diameter was used and the mean diameter ± 2SD of the CON-M group was used to define the normal range (score of 0) (Schimdt et al., 2004). The normal mean sagittal diameter was 5.1 mm ± 2SD, therefore any sagittal diameter less than 3.9 mm or greater than 6.3 mm was considered abnormal and assigned a score of 1. A pathology score (ranging from 0 to 10) was calculated as the sum of the dominant and non-dominant scores of the (i) tendon shape (maximum score of 1 for each leg), (ii) sagittal diameter (maximum score of 1 for each leg), (iii) tendon and paratendon blood flow (maximum score of 2 for each leg) and (iv) echogenecity (maximum score of 1 for each leg). Therefore, the worst pathology score (10 points) for an individual would be as follows: abnormal tendon shape (2 points), increased sagittal diameter (2 points), increased blood flow (4 points), decreased echogenecity (2 points). Other characteristics such as the sizes of the retrocalcaneal bursa, Kager's fat pad, structure of calcaneal bone and the myotendinous junction (MTJ) were also recorded (Appendix 6.1) to exclude other conditions, such as inflammation of the retrocalcaneal bursa (bursitis), formation of bone spurs and tricep surae muscle injuries associated with the MTJ, as the cause of the subject's pain and discomfort.

Both Achilles tendons were examined and investigated in all the subjects. Morphological characteristics of the dominant (D) and non-dominant (ND) leg were compared. Within the pathology group, morphological characteristics of injured (I) versus non-injured (NI) leg were also compared.

Table 6.1. Ultrasonography morphological pathology score determination in the dominant and non-dominant Achilles tendons.

Variable	Achilles Tendon Pathology			
Dominant Hand	Left ☐	Right ☐	Both ☐	
Injured Achilles Tendon	Left ☐	Right ☐	Both ☐	
Tendon Shape	Normal (0) Left ☐ Right ☐		Fusiform (1) Left ☐ Right ☐	
AP sagittal diameter (mm)	Left _____		Right _____	
Blood Flow	Normal (0) Left ☐ Right ☐		Increased vascularity of the tendon (1) Left ☐ Right ☐ Increased vascularity of the paratenon (1) Left ☐ Right ☐	
Echogenecity	Normal (0) Left ☐ Right ☐		Hypoechogenic (1)	Left ☐ Right ☐
Pathology Score[a]	(min 0 and max 10)			

AP, anteroposterior

[a]**Pathology score** is the sum scores of both the left and right Achilles tendon measurements of the (i) tendon shape, (ii) sagittal diameter, (iii) blood flow and (iv) echogenecity. The Pathology score has a minimum value of zero and a maximum value of 10 for each individual.

6.2.3 *Statistical analysis*

Data were analyzed using Statistica for Windows (Version 7 Statsoft, Tulsa, OK, USA) software. The differences between group subject's characteristics were performed using a one-way analysis of variance. Differences between the frequency counts of the injured (I) versus non-injured (NI) sides were tested using a Fisher's Exact test, while the differences in the sagittal diameters were analysed using the paired t-test. Differences between all subjects in the control (CON-M) and tendinopathy (TEN-M) groups were analysed using the Fisher's Exact test to compare the frequencies of abnormal morphological changes. Ultrasonography scores were compared between the CON-M-A1/A3, CON-M-A2, TEN-M-A1/A3, TEN-M-A2 or the CON-M-U, CON-M-O, TEN-M-U and TEN-M-O groups using the Fisher's Exact test. When the contingency table was larger than 2x2, the results were collapsed into a 2x2 table and the Fisher's exact test performed using Graphpad Instat (version 3) software. The differences in the pathology scores between groups were analyzed using the non-parametric Kruskal-Wallis ANOVA and the median test. Values were reported as percentages of individual with abnormal morphological changes for a particular variable. Where appropriate, group values were reported as mean and standard deviation (SD). Significance for all analyses was set at $p<0.05$.

6.3 RESULTS

6.3.1 Subject characteristics of the CON-M and TEN-M groups

The characteristics of all the subjects included in CON-M and TEN-M groups are shown in Table 6.2. The CON-M and TEN-M groups were similarly matched for body height and weight. However, the TEN-M group was older ($p<0.001$) and had a higher BMI ($p=0.035$) than the CON-M group (Table 6.2). The sonographic measurements of individuals in the TEN-M group were recorded on average 7.7 ± 7.7 years, ranging from 0 to 30 years, after the age of onset of the symptoms for Achilles tendinopathy.

Because age and BMI were significantly different between the CON-M and TEN-M groups, a correlation analysis of age, weight and BMI with the pathology scores of the TEN-M only group was conducted. There was a significant positive correlation between weight and pathology score ($r=0.39$, $p=0.019$). However, the correlations between age and pathology score ($r=0.32$, $p=0.059$) as well as BMI and pathology score ($r=0.30$, $p= 0.112$) were not significant.

Table 6.2. Characteristics of all the subjects included in the control (CON-M) and Achilles tendinopathy (TEN-M) groups.

	CON-M (n=22)	TEN-M (n=36)	P value
Age[1] (yrs)	39.2 ± 12.1 (22)	51.9 ± 10.7 (36)	< 0.001
Height (cm)	179 ± 7 (22)	180 ± 6 (35)	0.520
Weight (kg)	78.1 ± 10.0 (22)	84.0 ± 12.6 (35)	0.071
BMI (kg/m^2)	24.4 ± 2.4 (22)	26.2 ± 3.5 (30)	0.035

Values are expressed as mean ± standard deviation with the number of subjects (n) in parentheses. [1]Age=the age at which the ultrasound recordings were performed.

6.3.2 Achilles tendinopathy and morphological characteristics of the CON-M and TEN-M groups

Five (22.7%) of the 22 asymptomatic control subjects (CON-M) had only one ultrasonographic sign of Achilles tendon pathology. Three of the five subjects had increased blood flow in only one of their Achilles tendons, while the fourth subject had increased bilateral sagittal diameters (left, 6.7 mm and right, 6.6 mm). The fifth subject had a slight decreased echogenecity of the left (non-dominant) Achilles tendon. All the other measured features within these five subjects were normal (refer to Table 6.7). The tendon shape, sagittal and transverse diameters, blood flow as well as the echogenecity of the remaining 17 subjects were normal in both tendons. The retrocalcaneal bursa, Kager's fat pad, calcaneal bone and the myotendinous junction were normal in all the subjects within the CON-M group.

Thirty four (94.5%) of the 36 TEN-M subjects diagnosed with clinical symptoms of Achilles tendinopathy had ultrasonographic signs of tendinopathy. Based on their clinical symptoms, 18 (50%) of the 36 subjects were diagnosed with bilateral Achilles tendinopathy. However, 25 (69.4%) of the TEN-M subjects were shown ultrasonographically to have bilateral tendinopathy. The sagittal diameter was abnormal in both tendons in 19 (52.7%) of these subjects. Abnormal tendon shape, decreased echogenecity and increased blood flow were observed in both Achilles tendons of 21 (58.3%), 21 (58.3%) and 9 (25%) subjects, respectively. In addition to the abnormal findings in the tendon, 8 of the subjects also had abnormalities in either the retrocalcaneal bursa, Kager's fat pad, calcaneal bone and/or the myotendinous junction. One subject had bilateral calcaneal bone spurs, bilateral retrocalcaneal bursitis and a unilateral abnormal Kager's fat pad. A second subject had a unilateral abnormal MTJ, unilateral retrocalcaneal bursitis and a bilateral swollen Kager's fat pad. A third subject had unilateral abnormal Kager's fat pads, while an additional 5 and 3 subjects subjects had unilateral and bilateral swollen Kager's fat pads, respectively.

The two subjects whose diagnoses were not confirmed by US were clinically diagnosed with bilateral Achilles tendinopathy. Both subjects reported gradual progressive pain, swelling and stiffness in the Achilles tendon area during the time of the injury. Once recruited into the study, the clinical diagnosis was confirmed (MS) in one of the subjects. During the examination there was tenderness to palpation, palpable nodular thickening and movement of the painful area in the Achilles tendon with plantar-dorsiflexion (positive shift test). The second subject saw a doctor who initially diagnosed him as having a partial rupture of the right Achilles tendon 10 years ago and was treated by a physiotherapist.

There were no significant differences in the number of musculotendinous junction, Kager's fat pad, retrocalcaneal bursa and the calcaneous abnormalities within the dominant and non-dominant Achilles tendons of the CON-M group when compared to the TEN-M group (data not shown) (refer to Appendix 6).

Table 6.3. Ultrasonography measurements of the Achilles tendon diameters of the subjects in the control (CON-M) and Achilles tendinopathy (TEN-M) groups.

	CON-M (n=22)	TEN-M (n=36)	P value
D-AP Sagittal Diameter (mm)	5.1 ± 0.6 (22)	7.8 ± 2.4 (36)	<0.001
ND-AP Sagittal Diameter (mm)	5.1 ± 0.6 (22)	7.8 ± 2.5 (36)	<0.001
D-AP Transverse Diameter (mm)	5.1 ± 0.5 (22)	7.6 ± 2.8 (36)	<0.001
ND-AP Transverse Diameter (mm)	5.0 ± 0.5 (22)	7.2 ± 2.5 (36)	<0.001

Values are expressed as mean ± standard deviation with the number of subjects (n) in parentheses. **D**, Dominant; **ND**, Non-dominant; **AP**, anteroposterior.

The mean anteroposterior sagittal diameter of the CON-M group's dominant (5.1 ± 0.6 mm) and non-dominant (5.1 ± 0.6 mm) Achilles tendon were both significantly smaller (dominant and non-dominant comparisons were both $p<0.001$) than that of the TEN-M group (dominant 7.8 ± 2.5 mm and non-dominant 7.8 ± 2.5 mm). The transverse diameters of the dominant and the non-dominant Achilles tendons were also significantly different when the average measurements of the CON-M and TEN-M groups were compared (Table 6.3). The mean sagittal diameter ± 2 standard deviation of the CON-M group was used to define the normal size range (score=0) (Schidmt et al., 2004). Tendon sagittal diameters of less than 3.9 mm or greater than 6.3 mm were therefore classified as abnormal and obtained a score of 1. As expected the relative sonographic score for anteroposterior sagittal diameter of the dominant and non-dominant Achilles tendons were significantly different when the CON-M group (dominant 4.6 % and non-dominant 4.6%) was compared to the TEN-M group (dominant 65.7 %, $p<0.001$ and non-dominant 68.6 %, $p<0.001$) (Table 6.4). Since the mean transverse diameters were similar to the mean sagittal diameters, only the sagittal diameter was included in the subsequent analyses of ultrasonographic scores.

The average ultrasonographic scores of all the other measurements in the CON-M and TEN-M subjects are shown in Table 6.4. There were significant differences in the tendon shapes when the dominant (0.0% CON-M vs 72.2% TEN-M, $p<0.001$) and non-dominant (0.0% CON-M vs 69.4% TEN-M, $p<0.001$) CON-M group was compared to the TEN-M group. There were also significant differences in the blood flow when the dominant (9.1% CON-M vs 41.7% TEN-M, $p=0.009$) and non-dominant (4.6% CON-M vs 36.1% TEN-M, $p=0.010$), as well as in the echogenicity of the dominant (0.0% CON-M vs 66.7% TEN-M, $p<0.001$) and non-dominant (4.6% CON-M vs 69.4% TEN-M, $p<0.001$) CON-M and TEN-M groups were compared.

Table 6.4. Percentage of subjects in the control (CON-M) and Achilles tendinopathy (TEN-M) groups with abnormal findings in the dominant and non-dominant legs.

	CON-M (n=22)	TEN-M (n=36)	P value
D-Tendon Shape	0.0 (0)	72.2 (26)	<0.001
ND-Tendon Shape	0.0 (0)	69.4 (25)	<0.001
D-AP Sagittal Diameter	4.6 (1)	65.7 (23)	<0.001
ND-AP Sagittal Diameter	4.6 (1)	68.6 (24)	<0.001
D-Blood flow	9.1 (2)	41.7 (15)	0.009
ND-Blood flow	4.6 (1)	36.1 (13)	0.010
D-Echogenicity	0.0 (0)	66.7 (24)	<0.001
ND-Echogenecity	4.6 (1)	69.4 (25)	<0.001
Pathology Score[a]	0.27 ± 0.55 (0-2)	5.16 ± 2.9 (0-9)	<0.001
Pathology Score[b]	0--0--0	3--5.5--8.0	<0.001

Values are expressed as percentages and the number of subjects with abnormal morphology in parentheses.

[a]Values are expressed as means ± SD (range).

[b]Values are expressed as first quartile--median--third quartile.

D, Dominant; ND, Non-dominant; AP, anteroposterior.

6.3.3 *The sonographic morphological characteristics of the injured and non-injured Achilles tendons.*

For those subjects who sustained a unilateral Achilles tendinopathy (n=18), the mean anteroposterior sagittal diameter of the injured tendon (8.3 ± 2.7 mm) was significantly larger than the non-injured tendon (6.2 ± 1.9 mm) (p=0.007). Except for the percentage abnormal score for the sagittal diameter (p=0.032), the tendon shape, blood flow and echogenicity were not significantly different between the injured and non-injured tendon (Table 6.5).

Table 6.5. The comparison of percentage of abnormal morphological characteristics in the injured and non-injured leg of the Achilles tendinopathy (TEN-M) subjects who sustained a unilateral injury, n=18[a]

	CON-M (n=22)[c]	Injured limb (n=18)	Non-Injured limb (n=18)	P value[b]
Tendon Shape	0.0 (0/22)	77.7 (14/18)	50.0 (9/18)	0.164
AP Sagittal Diameter	4.6 (1/22)	76.5 (14/17)	41.7 (7/17)	0.032
Blood Flow	9.1 (2/22)	44.4 (8/18)	27.8 (5/18)	0.489
Echogenicity	4.6 (1/22)	72.2 (13/18)	44.4 (8/18)	0.176

Values are expressed as percentages with the number of subjects (n) in parentheses. [a]Subjects who sustained a bilateral injury were not included in this analysis. **AP,** anteroposterior.

[b] Non-injured versus injured only

[c]Column added to illustrate the differences between CON-M and non-injured subjects

6.3.4 *The COL5A1 allelic effects on the sonographic morphological characteristics of the CON-M and TEN-M groups.*

Tables 6.6 and 6.7 show the characteristics of the CON-M and TEN-M subjects when divided into their COL5A1 gene A2 and A1/A3 allelic groupings, respectively. The control subjects with an A2 allele (CON-M-A2; 2 A1A2, 5 A2A2, 0 A2A3) and those with either an A1 or A3 allele (CON-M-A1/A3; 13 A1A1, 1 A1A3, 1 A3A3) were similarly matched for age, height, weight and BMI (Table 6.6). Similarly, the Achilles tendon injury subjects with an A2 allele (ATI-M-A2; 4 A1A2, 4 A2A2, 0 A2A3) were matched for age, height, weight and BMI when compared to the Achilles tendon injury subjects with either an A1 or A3 (TEN-M-A1/A3; 21 A1A1, 1 A1A3, 2 A3A3) (Table 6.7). The two control COL5A1 allelic sub-groups were however both younger and lighter with a corresponding lower BMIs than the two Achilles tendinopathy COL5A1 allelic sub-groups (data not shown).

There were no differences found in the percentages of abnormal sonographic morphological characteristics between the CON-M-A2 and CON-M-A1/A3 groups (Table 6.8). Similarly, there were no differences found in the sonographic morphological characteristics between the TEN-M-A2 and TEN-M-A1/A3 sub-groups (Table 6.9). However, the percentages of abnormal sonographic characteristics were higher in the two TEN-M groups compared to the two COM-M groups. Although very few, some abnormal changes were found in asymptomatic subjects (Table 6.8). However, all of the five subjects with a sub-clinical abnormalities detected via US were in the CON-M-A1/A3 group only and therefore did not have the A2 allele.

Table 6.6. Characteristics of the control (CON-M) subjects with A2 and A1/A3 alleles.

	A1/A3 (n=15)	A2 (n=7)	P value
Age (yrs)	40.7 ± 10.9 (15)	36.1 ± 14.9 (7)	0.428
Height (cm)	179 ± 9 (15)	180 ± 4 (7)	0.642
Weight (kg)	78.2 ± 11.3 (15)	78.0 ± 7.3 (7)	0.972
BMI (kg/m^2)	24.5 ± 2.5 (15)	24.0 ± 2.2 (7)	0.693

Values are expressed as mean ± standard deviation. Number of subjects (n) is in parentheses.

Table 6.7. Characteristics of the Achilles tendinopathy (TEN-M) subjects with A2 and A1/A3 alleles.

	TEN-M-A1/A3	TEN-M-A2	P value
Age (yrs)	53.3 ± 11.7 (23)	51.8 ± 7.7 (6)	0.781
Height (cm)	181± 6 (23)	180 ± 9 (7)	0.839
Weight (kg)	84.0 ± 11.5 (23)	87.1 ± 10.9 (7)	0.534
BMI (kg/m^2)	25.7 ± 3.1 (23)	26.7 ± 2.5 (6)	0.477

Values are expressed as mean ± standard deviation. Number of subjects (n) is in parentheses.

Table 6.8. Percentages of abnormal ultrasonography recordings of the control (CON-M) subjects with A1/A3 and A2 alleles.

	CON-M-A1/A3 (n=15)	CON-M-A2 (n=7)	P value
D-Tendon Shape	0 (0/15)	0 (0/7)	NS
ND-Tendon Shape	0 (0/15)	0 (0/7)	NS
D-Ap Sagittal Diameter	6.7 (1/15)	0.0 (0/7)	1.00
ND-AP Sagittal Diameter	6.7 (1/15)	0.0 (0/7)	1.00
D-Blood Flow	13.3 (2/15)	0.0 (0/7)	1.00
ND-Blood Flow	6.7 (1/15)	0.0 (0/7)	1.00
D-Echogenicity	0.0 (0/15)	0.0 (0/7)	NS
ND-Echogenicity	6.7 (1/15)	0.0 (0/7)	1.00
Pathology Score[a]	0.4 ± 0.6 (0–2)	0.0 ± 0.0 (0-0)	0.091
Pathology Score[b]	0—0--1	0—0--0	0.082
ND-Sag Diameter (mm)	5.0 ± 0.6	5.2 ± 0.5	0.608
D-Sag Diameter (mm)	5.2 ± 0.6	5.0 ± 0.6	0.461

Values are expressed as percentages with number of subjects (n) in parentheses.

D, Dominant; **ND**, Non-dominant

[a]Values are expressed as means ± SD (range).

[b]Values are expressed as first quartile--median--third quartile.

Table 6.9. The percentages of abnormal ultrasonography recordings of the Achilles tendinopathy (TEN-M) subjects with A1/A3 and A2 alleles.

	TEN-M-A1/A3 (n=23)	TEN-M-A2 (n=7)	P value
D-Tendon Shape	73.9 (17/23)	85.7 (6/7)	1.00
ND-Tendon Shape	69.6 (16/23)	71.4 (5/7)	1.00
D-AP Sagittal Diameter	73.9 (17/23)	71.4 (5/7)	1.00
ND-AP Sagittal Diameter	69.6 (16/23)	71.4 (5/7)	1.00
D-Blood Flow	43.5 (10/23)	28.6 (2/5)	0.669
ND-Blood Flow	30.4 (7/23)	57.2 (4/7)	0.372
D-Echogenicity	69.6 (16/23)	71.4 (5/7)	1.00
ND-Echogenicity	69.6 (16/23)	71.4 (5/7)	1.00
Pathology Score[a]	5.4 ± 2.9 (0-9)	5.6 ± 2.6 (3-9)	0.941
Pathology Score[b]	3--6—8	3--4—8	0.666
ND-Sag Diameter (mm)	7.2 ± 2.0	7.5 ± 2.3	0.064
D-Sag Diameter (mm)	8.1 ± 2.3	7.8 ± 2.8	0.134

Values are expressed as percentages with number of subjects (n) in parentheses.

D, Dominant; ND, Non-dominant

[a]Values are expressed as means ± SD (range).

[b]Values are expressed as first quartile--median--third quartile.

6.3.5 *The TNC allelic effects on the sonographic morphological characteristics of the CON-M and TEN-M groups*

The control subjects with the genotypes UU, UE and EE (CON-M-U; 6 UU, 5 UE, 4 EE) and OO and OE (CON-M-O; 1 OO and 4 OE) were similarly matched for age, height, weight and BMI (Table 6.10). Similarly, the Achilles tendinopathy subjects with genotypes UU, UE and EE (TEN-M-U; 1 UU, 3 UE, 16 EE) and OO and OE (TEN-M-O; 1 OO and 9 OE) were similarly matched for age, height, weight and BMI (Table 6.11). The two *TNC* genotypes of CON-M sub-groups were however both younger and lighter with a corresponding lower BMIs than the two TNC genotypes of the TEN-M sub-groups (data not shown). However, this differences. However, these differences do not affect the results as the comparisons were made between either the two CON-M groups or the two TEN-M, which were matched for age, weight and BMI.

The percentages of abnormal sonographic morphological characteristics for the tendon shape, diameter, blood flow, echogenecity and pathology score were all higher in the two TEN groups compared with the two CON-M groups. There were no significant differences in the percentages of sonographic morphological characteristics between the two CON-M groups or between the two TEN-M groups (Table 6.12 and 6.13). However, percentages of individuals with increased blood flow and/or sagittal diameter in the TEN-M-U group were significantly higher than in the TEN-M-O group in the non-dominant Achilles tendons (Table 6.13).

Table 6.10. Characteristics of the control (CON-M) subjects with UU, UE, EE, OO or OE genotypes.

	CON-M-U (n=15)	CON-M-O (n=5)	P value
Age (yrs)	39.7 ± 13.3 (15)	39.6 ± 11.1 (5)	0.984
Height (cm)	179 ± 6 (15)	181 ± 12 (5)	0.554
Weight (kg)	75.7 ± 8.0 (15)	84.8 ± 14.8 (5)	0.092
BMI (kg/m^2)	23.8 ± 2.4 (15)	25.7 ± 1.7 (5)	0.112

Values are expressed as mean ± standard deviation. Number of subjects (n) is in parentheses.

Table 6.11. Characteristics of the Achilles tendinopathy (TEN-M) subjects with UU, UE, EE, OO or OE genotypes.

	TEN-M-U (n=20)	TEN-M-O (n=11)	P value
Age (yrs)	50.8 ± 8.8 (20)	53.5 ± 12.1 (10)	0.481
Height (cm)	180 ± 7 (20)	179 ± 4 (11)	0.675
Weight (kg)	84.5 ± 13.0 (20)	85.6 ± 12.4 (11)	0.809
BMI (kg/m^2)	26.3 ± 3.4 (18)	26.1 ± 4.0 (11)	0.676

Values are expressed as mean ± standard deviation. Number of subjects (n) is in parentheses.

Table 6.12. Ultrasonography measurements of the control (CON-M) subjects with UU, UE, EE, OO or OE genotypes.

	CON-M-U (n=15)	CON-M-O (n=5)	P value
D-Tendon Shape	0.0 (0/15)	0.0 (0/5)	1.00
ND-Tendon Shape	0.0 (0/15)	0.0 (0/5)	1.00
D-AP Sagittal Diameter	0.0 (0/15)	20.0 (1/5)	0.250
ND-AP Sagittal Diameter	0.0 (0/15)	20.0 (1/5)	0.250
D-Blood Flow	13.3 (2/15)	0.0 (0/5)	1.00
ND-Blood Flow	6.67 (1/15)	0.0 (0/5)	1.00
D-Echogenicity	0.0 (0/15)	0.0 (0/5)	1.00
ND-Echogenicity	0.0 (0/15)	20.0 (1/5)	0.250
Pathology Score[a]	0.2 ± 0.4 (0-1)	0.6 ± 0.9 (0-2)	0.299
Pathology Score[b]	0--0--0	0—0--0.5	0.371
ND-Sag Diameter (mm)	5.1 ± 0.4	5.0 ± 0.9	0.713
D-Sag Diameter (mm)	5.2 ± 0.6	5.4 ± 0.8	0.497

Values are expressed as percentages with number of subjects (n) in parentheses.

D, Dominant; **ND**, Non-dominant

[a]Values are expressed as means ± SD (range).

[b]Values are expressed as first quartile--median--third quartile.

Table 6.13. Ultrasonography measurements of the Achilles tendinopathy (TEN-M) subjects with UU, UE, EE, OO or OE genotypes.

	TEN-M-U (n=15)	TEN-M-O (n=11)	P value
D-Tendon Shape	75.0 (15/20)	72.7 (8/11)	1.00
ND-Tendon Shape	70.0 (14/20)	54.6 (6/11)	0.452
D-AP Sagittal Diameter	65.0 (13/20)	63.6 (7/11)	1.00
ND-AP Sagittal Diameter	80.0 (16/20)	36.4 (4/11)	0.023
D-Blood Flow	35.0 (7/20)	36.4 (4/11)	1.00
ND-Blood Flow	55.0 (11/20)	9.1 (1/11)	0.020
D-Echogenicity	65.0 (13/20)	72.7 (8/11)	1.00
ND-Echogenicity	70.0 (14/20)	54.6 (6/11)	0.452
Pathology Score[a]	5.6 ± 3.0 (1-9)	4.2 ± 2.7 (0-8)	0.220
Pathology Score[b]	3.0—5.5—8.5	2.0—5.0—6.0	0.465
ND-Sag Diameter (mm)	7.6 ±2.1	7.2 ±3.3	0.671
D-Sag Diameter (mm)	7.6 ± 2.2	7.4 ± 1.8	0.869

Values are expressed as percentages with number of subjects (n) in parentheses.

D, Dominant; **ND**, Non-dominant

[a]Values are expressed as means ± SD (range).

[b]Values are expressed as first quartile--median--third quartile.

6.4 DISCUSSION

The aim of this chapter of the thesis was to investigate whether the morphology of the Achilles tendon displayed greater abnormality in individuals of the A1/A3 variant of the *COL5A1* gene or 12 and 14 GT repeats (OO and OE) within the *TNC* gene, when compared to individuals with the A2 variant of the *COL5A1* gene or the 13 and 17 GT repeats (UU, UE and EE) within the *TNC* gene, respectively, irrespective of the underlying pathology. The rationale was that because subjects with the A2 allele of the COL5A1 gene or the 13 and 17 GT repeats within the *TNC* gene would be at lower risk for developing Achilles tendon injury, and should therefore display less morphological changes associated with tendon abnormality.

There were two main findings of this chapter of the thesis. Firstly, as expected, the TEN-M group clearly had significantly more abnormal features within their Achilles tendon substance than the CON-M group. The abnormal features included a fusiform shape, a larger sagittal or transverse tendon diameter, increased blood flow in the tendon but rarely in the paratenon and a non-homogenous and/or hypoechogenic areas. However, individuals in the TEN-M group were older and had a higher BMI compared to the individuals in the CON-M group. The age difference was due the fact that subjects were matched for age-at-onset of Achilles tendinopathy for the studies described in Chapters 3 and 4. Therefore the age-at-sonography measurements did not always coincide with age-at-onset of injury as the US study was largely retrospective. However, the differences in the age, weight and BMI were eliminated during the genotype analysis by comparing either the two sub-groups of the CON-M (e.g. CON-M-A2 versus CON-M-A1/A3) or the TEN-M (eg. TEN-M-A2 versus TEN-M-A1/A3) groups as genetic differences sought were theoretically independent of the injury to the Achilles tendon.

The use of non-invasive techniques such as ultrasonography adds another dimension to the understanding of the tendon pathology. It provides an opportunity to measure live tissue and its response to mechanical loading such as blood flow rather than biopsy tendon samples obtained from cadavers that are usually used in histopathological studies of Achilles tendinopathy. However, such non-invasive techniques need to be reliable and valid so as to reflect the extent of pathology in the tendon that is true. Currently, the validity of sonography as measure of pathology in the tendon is complicated by a lack of a universally acceptable quantitative pathology score.

The second finding was that polymorphisms within both *COL5A1* and *TNC* genes were not associated with the Achilles tendon morphological characteristics found in individuals with tendinopathy. However, for the *TNC* gene, the percentages of individuals with increased and therefore abnormal blood flow and sagittal diameter of the TEN-M-U group were significantly higher in the TEN-M-O group in the non-dominant Achilles tendons. The reason for these differences is not clear. For the COL5A1 gene, it is interesting to note that although the sample size is small and that these are preliminary results, abnormal changes in the control group only occurred in the CON-M-A1/A3 group, with none occurring in the CON-M-A2 group. There are currently no other studies with which to compare these findings, as there have been no studies on the association of gene polymorphisms and Achilles tendinopathy.

In a study describing the standard reference values for musculoskeletal ultrasonography of 102 healthy but non-sporting individuals (mean age 38 yrs; range=20-60; 54 women), there were no statistical differences in US morphology between the dominant and non-dominant sides (Schmidt et al., 2004). There were

also no US morphological differences between subjects of age <40 years and age >40 years and between subjects engaging in low and high physical activity at work (Schmidt et al., 2004). Although the study sample size in our study was relatively smaller, the CON-M group (n=22, mean age=40.5 yrs, range=24-69; mean weight=78kg, range=64-110) had a similar mean age, weight distribution compared to the Schimdt et al., 2004 male group (n=48), with mean age of 39 yrs (range, 20-60), mean weight of 80kg (range=62-103).

In our study, the Achilles tendon mean sagittal diameter was 5.1mm, 95%CI 3.9-6.3 (range=4.2-6.7mm), which was similar to the 5.3mm and 5.2mm reported for Spanish men (Civiera et al., 1988) and Chinese subjects (Ying et al., 2003), respectively. However, the CON-M mean sagittal diameter was larger compared to that of 4.6mm obtained by Schmidt et al. (2004), but it was smaller than the 6.2mm (range=4-9) reported by Mathieson et al., (1988).

In the Schimdt et al (2004) study, all diameter measurements were carried out at the same defined location (2cm proximal to the calcaneus). In a sample of healthy individuals, it is reasonable to assume that the tendon diameter should not vary greatly along its length. However, in symptomatic tendons, the location of hypoechogenic regions or focal degenerations for instance could occur anywhere along the length of the Achilles tendon. These might be the reason for discrepancies in the reported results in the mean sagittal diameter. For example, we chose to take dimension measurements at a position of greatest diameter, which might not necessarily relate to the position of increased blood flow. To report increased blood flow, the greatest area of abnormality along the tendon independent of the tendon thickness was chosen. Therefore for every parameter, measurements were recorded at location of greatest abnormality for that parameter.

An additional finding in our study was that a larger percentage of the injured Achilles tendons displayed features of abnormality compared to the uninjured Achilles tendon within TEN-M group. Furthermore, the frequency of abnormal US morphological features found in the uninjured legs of the tendinopathy subjects (50% altered tendon shape, 41.7% increased sagittal diameter, 27.8% increased blood flow and 44.4% hypoechoic) were higher than would be expected in normal tendons. The corresponding mean values in the normal tendons were 0% altered tendon shape, 4.6% increased sagittal diameter, 9.1% increased blood flow and 4.6% hypoechoic tendon substance (Tables 6.4 and 6.5). These results suggest that a unilateral Achilles tendinopathy is a good predictor of sub-clinical pathology in the other leg of the same individual.

There were some limitations to this study. Firstly, there were few individuals with the A2 allele in the Achilles tendinopathy group as a whole (Chapter 3), and this therefore meant it was difficult to obtain large enough sample size (about 10-15 subjects as desired) in both CON-M-A2 and TEN-M-A2 groups. The same argument applies equally to the O allele, which was overrepresented in the Achilles tendinopathy (TEN) group (chapter 4). Secondly, although we used information on the dominant leg, this was based on the known dominant hand obtained via the questionnaire. However, the correlation between dominant leg and hand is high and therefore this method would not have significantly altered the results. Thirdly, we only obtained access to the ultrasound equipment and the radiologist 3 years into the study. However, only 12 (33.3%) of the 36 injured subjects included in this component of the study were examined via sonography an average of 1.5 years after the initial date of testing (ie blood samples, questionnaire and others). Fourthly, subjects enrolled into the study an average of 7.7 years after first date of injury. Although the tendons of the TEN-M group clearly exhibited more abnormal features than those of the CON-M group, we may not have determined the true

representation of their abnormality. Fifthly, there is no universally acceptable Achilles tendon pathology score determined from US measurements that could be used to compare results of different studies.

Finally, in case-control genetic association studies, the asymptomatic subjects must be matched to cases on an age-at-diagnosis or age-at-onset, which may not necessarily coincide. In addition, age-at-onset (a self reported measure) can be difficult to accurately establish especially with chronic Achilles tendinopathy, for example, compared to an acute complete Achilles tendon rupture. For this present chapter, the age at which measurements were made was considered more important, but as shown the Achilles tendinopathy (TEN-M) group was significantly older than the control group (CON-M).

In conclusion, this pilot investigation did not show that polymorphisms within the *COL5A1* and *TNC* genes were associated with the morphology of the Achilles tendon. However, the investigation showed that there was significant pathology in the TEN-M group compared with the CON-M group. Therefore, the results of this chapter of the thesis support the findings of other studies that have shown sonography as a valid technique that could be used to confirm the clinical diagnosis of Achilles tendinopathy. Another interesting observation of this investigation was the increased number of abnormal US morphological features in the uninjured tendons of subjects who had sustained a unilateral injury (TEN-M) when compared to the asymptomatic subjects (CON-M). In this study, the ultrasonographic features such as the tendon shape, diameter, blood flow and echogenecity were identified as important in diagnosing Achilles tendinopathy, while features such as the musculotendinous junction, calcaneus, bursars, Kager's fat pad may be less important. However, a reliable quantitative ultrasound Achilles tendinopathy score, which provides information on extent of abnormality, is currently unavailable.

Finally, it is recommended that a larger prospective study need to be conducted to investigate the changes in tendon morphology and the association with genetic factors in Achilles tendinopathy. Also, because US is widely used in the diagnosis of Achilles tendinopathy, it is important that a reliable and valid ultrasonography pathology score be developed that can be used universally in other research studies.

The aetiology of Achilles tendon injuries is believed to be multifactorial. A number of extrinsic and intrinsic risk factors for Achilles tendon injuries have been proposed. It has also been suggested for several years that genetic factors, at least in part, play a role in the development of tendon injuries (Kannus and Jozsa, 1997). More recently, investigators have suggested that there is a possible genetic component to rotator cuff tendon and anterior cruciate ligament injuries (Flynn et al., 2005; Harvie et al., 2004). To date, none of the studies have proposed or shown that specific genes are associated with these injuries. However, since previous studies have suggested that the ABO blood group is associated with Achilles tendon injuries, it has been proposed that specific genes in the proximity of the *ABO* gene on chromosome 9q34 might be associated with tendon injury (Kannus and Natri, 1997; Kujala et al., 1992; Maffulli et al., 2002).

The purpose of this thesis therefore was to explore the possible intrinsic risk factors for Achilles tendon injuries. More specifically the aim was to identify specific genes on chromosome 9 in close proximity to the *ABO* gene that might be associated with Achilles tendon injuries and to investigate whether these genes were associated with Achilles tendon injuries in physically active South African Caucasian subjects. The specific objectives and results were:

1. To identify any candidate gene(s) that are located on the tip of the long arm of chromosome 9 (more specifically the 9q32-q34 locus) closely linked to the *ABO* gene which could theoretically be associated with Achilles tendon overuse injuries. In addition, the association of ABO blood group system with Achilles tendon rupture or chronic tendinopathy in a South African Caucasian population was investigated.

Twenty-one candidate genes were identified from a possible 404 genes and pseudogenes, which have been mapped to the 9q32-q34.3 locus. Two of the 21 candidate genes, namely *COL5A1* and *TNC*, encode for structural components of tendons. Another two genes, the *COL27A1* and *LAMC3*, encode for components of the extracellular matrix, namely the alpha chain of type XXVII collagen and gamma chain of laminin 12, respectively (Pace et al., 2003; Koch et al., 1999). Neither type XXVII Collagen nor laminin 12 have been shown to be structural components of tendons. It must however be pointed out that due to the limited amount of work done on the recently identified type XXVII collagen, it is theoretically possible that the protein plays a role in tendons. Although none of the other 17 proposed candidate genes are known to encode structural components of Achilles tendons, it was theoretically possible based on the proposed functions of the proteins that they could be involved in the aetiology of Achilles tendon pathology. Further studies need to be done to determine whether any of these other genes are associated with tendon injuries. In addition, it is possible that there are other genes located on the tip of the long arm of chromosome 9, which have not been accurately mapped or whose function is currently poorly understood, that might be associated with these injuries. However, both the identified *COL5A1* and *TNC* gene expression had previously been shown to be altered during tendon pathology. Thus, the associations of polymorphisms within these two genes with Achilles tendon injuries were chosen to be investigated in this thesis.

In addition, the distribution of ABO blood groups was compared between the asymptomatic and symptomatic subjects. It was found that the ABO blood group O distribution and the A/O ratio were similar between the control and symptomatic subjects. Blood group O and the A/O ratio have previously been shown to be over-represented in some, but not all populations, with Achilles tendon injuries (Aroen et al., 2004; Jozsa et al., 1989; Kujala et al., 1992; Leppilahti et al., 1996; Maffulli et al., 2000; Mahrlein et al., 1995).

2. To determine whether the *Bst*UI RFLP within the 3'-UTR of the *COL5A1* gene, which encodes a tendon protein, is associated with the symptoms of Achilles tendon injury (ATI).

It was found that the frequency of the A2 allele (or C allele) of the *COL5A1* gene was significantly under-represented in the ATI group when compared to the CON group. The frequency of the A1 and A3 alleles (or T allele) of the *COL5A1* gene on the other hand, was over-represented when the ATI group was compared to the CON group. Therefore, individuals containing the A2 allele of the *COL5A1* gene are less likely of developing Achilles tendon injury (P=0.004, odds ratio of 1.9; 95%CI 1.3-3.0). This association was even stronger when the CON group was compared to a sub-group of ATI subjects diagnosed with chronic Achilles tendinopathy (TEN) (P=0.0005, odds ratio of 2.6; 95%CI 1.5-4.5). Although it should be interpreted with caution because of the small sample size, the *Bst*UI RFLP within the *COL5A1* gene was not associated with a sub-group of subjects diagnosed with Achilles tendon ruptures (RUP).

The *COL5A1* gene codes for the pro-α1(V) chain, which is found in most of the isoforms of type V collagen (Caridi et al., 1992). The major isoform of type V collagen is a heterotrimer consisting of two pro-α1(V) chains and one pro-α2(V) chain (Caridi et al., 1992). Trace amounts of type V collagen are found in tendons where it forms heterotypic fibres with the major structural type I collagen (reviewed in Birk, 2001). Type V collagen is believed to play an important role in regulating fibrillogenesis and modulating fibril growth in tendons (Birk et al., 1990). An age-dependent increase in the content of the protein, together with a decrease in fibril diameter and the biomechanical properties in the rabbit patellar tendon has been reported (Dressler et al., 2002; Goncalves-Neto et al., 2002). In addition, some investigators have shown an increase in types III and V collagen together with a

reduction in the content of type I collagen in biopsy samples of degenerative tendons from patients with posterior tibial tendon dysfunction syndrome (Goncalves-Neto et al., 2002). Recently, it was demonstrated that type V collagen deficient mice could not form collagen fibrils, emphasizing the role of type V collagen in fibrillogenesis (Wenstrup et al., 2004).

3. To investigate whether the GT dinucleotide repeat polymorphism within intron 17 of the *TNC* gene, which also encodes a tendon protein, is also associated with Achilles tendon injury.

Altogether, this thesis established 18 different alleles of the GT dinucleotide repeat polymorphism within the *TNC* gene, ranging from 3 to 21 GT repeats. The frequencies of the alleles containing the 12 and 14 repeats were significantly over-represented in the ATI group, while the frequencies of the alleles containing the 13 and 17 repeats were significantly under-represented in the ATI group. The rest of the GT repeats were evenly distributed when the CON and ATI groups were compared. Further analysis showed that individuals containing alleles with the 13 and 17 repeats and did not contain the 12 and 14 repeats have a lower risk of developing Achilles tendon injuries ($P<0.001$, odds ratio of 6.2, 95%CI 3.5-11.0). When the ATI group was divided into the TEN and RUP sub-groups, their allele distribution were similar to each other and to those of the ATI group. Therefore, unlike the polymorphism within the *COL5A1* gene which appeared to be specifically associated with Achilles tendinopathies, the GT dinucleotide repeat polymorphism within the *TNC* gene was associated with both Achilles tendinopathy and ruptures.

The *tenascin-C* (*TNC*), or *hexabrachion* (*HXB*) gene as it was originally called, encodes for the extracellular matrix glycoprotein, tenascin-C, and is located 18 Mb upstream from the *ABO* gene on locus 9q33 (Rocchi et al., 1991). Tenascin-C is expressed in a variety of tissues, including tendons, where it binds to other components of the

extracellular matrix and cell receptors and plays an important role in regulating cell-matrix interactions (Mackie, 1997; Jones and Jones, 2000a). In normal adult tendons, the *TNC* gene is expressed predominantly in regions responsible for transmitting high levels of mechanical force such as the myotendinous and osteotendinous junctions (Chiquet and Fambrough, 1984a, Chiquet and Fambrough, 1984b; Jarvinen et al., 1999). The protein is also expressed around the cells and the collagen fibres of the Achilles tendon. In addition, it has been shown that expression of the *TNC* gene is regulated in a dose-dependent manner by mechanical loading in tendons (Jarvinen et al., 1999; Jarvinen et al., 2003).

Isoforms of the protein, with distinct functions, are produced by alternative splicing of the primary transcript. It has been shown that healthy tendons express a small 200 KDa tenascin-C isoform, while degenerate tendons also express a functionally distinct larger 300 KDa isoform (Riley et al., 1996). One study, but not a second, has so far reported an increase in tenascin-C expression in biopsy samples of chronic Achilles tendinopathy human subjects using cDNA arrays (Ireland et al., 2001).

Although there has been research on the expression of various genes coding for proteins found within the tendon in normal versus tendinopathic tendons, this is to our knowledge, the first study to identify specific genes shown to be associated with Achilles tendon injuries. However, because this thesis was a case-control association design, causality cannot be established. It is possible that other genes on chromosome 9 closely linked to both the *COL5A1* and *TNC* genes might be directly involved in aetiology and pathogenesis of tendon injuries. As previously mentioned, although the *COL27A1* gene was not analysed in this thesis because there is to date no evidence that the gene is expressed in tendons, its expression in tendons has not been excluded. It is theoretically possible that due to the presence of type XXVII collagen in cartilage, eye, ear, lung and colon, that the protein is expressed in tendon.

In addition, the results of this thesis need to be replicated in an independent Caucasian population. Whether these polymorphisms are also associated with Achilles tendinopathy in other ethnic groups also needs to be investigated. Other alternative methods to confirm these associations would involve studies of siblings and family members of the affected subjects (linkage studies).

If the *TNC* and/or *COL5A1* genes are directly involved in the aetiology of Achilles tendinopathy, the functional polymorphisms within these genes need to be identified. Recently, Matsuda et al. (2005) have identified a single nucleotide polymorphism (SNP) located in exon 17 of *TNC* gene, which interestingly is adjacent to the GT repeat dinucleotide polymorphism within intron 17 of this gene, which creates an amino acid substitution (Leu1677Ile) believed to change structural stability of the fibronectin III domain contained in the tenascin C protein. The association of this SNP with Achilles tendon injury needs to be investigated.

It is known that other proteins, besides type V collagen and tenascin C, are involved in the structure and/or function of tendons and therefore the possible association of polymorphisms (eg. single nucleotide polymorphisms (SNPs), restriction fragment length polymorphisms (RFLPs), microsatellites) in the multiple genes located on other chromosomes, which encode for these proteins (see Table 7.1), with Achilles tendinopathy also need to be investigated. It is unlikely that a single gene is associated with this complex condition and that Achilles tendinopathies are rather polygenic in nature.

Table 7.1. The genes encoding for tendon extracellular matrix (ECM) proteins.

Collagen	Structure/Type	Gene(s) and Location (s)
I	Fibril-forming	*COL1A1* (17q21.3-q22.1); *COL1A2* (7q22.1)
II	Fibril-forming	*COL2A1* (12q13.11-q13.2)[a]
III	Fibril-forming	*COL3A1* (2q31)
IV	Forms meshwork	*COL4A1* & *COL4A2* (13q34); *COL4A3* & *COL4A4* (2q36-q37); *COL4A5* & *COL4A6* (Xq22.3)
V	Fibril-forming	*COL5A1* (9q34.2-q34.3); *COL5A2* (2q31); *COL5A3* (19q13.2)
VI	Forms beaded filaments	*COL6A1* & *COL6A2* (21q22.3); *COL6A3* (20q13.3)
IX	FACIT[b]	*COL9A1* (6q13); *COL9A2* (1p33-32.2); *COL9A3* (20q13.3)
X	Forms meshwork	*COL10A1* (6q21-q22.3)
XI	Fibril-forming	*COL11A1* (1p21); *COL11A2* (6p21.3); *COL2A1* (12q13.11-q13.2)[a]
XII	FACIT[b]	*COL12A1* (6q12-q13)
XIV	FACIT[b]	*COL14A1* (8q23)

[a]*COL2A1* = *COL11A3*

[b]**FACIT**, Fibril-associated collagens with interrupted triple helix

Table 7.1 (Continued) The genes encoding for tendon extracellular matrix (ECM) proteins.

	Protein	Structure/Type	Gene (s) and Locations
Proteoglycan	Decorin	SLRP[c]	*DCN* (12q21.33)
	Biglycan	SLRP[c]	*BGN* (Xq28)
	Fibromodullin	SRLP[c]	*FMOD* (7q22.1)
	Lumican	SLRP[c]	*LUM* (12q21.3-q22)
	Aggrecan	Hyalectan	*AGC1* (15q26.1)
	Versican	Hyalectan	*CSPG2* (5q14.3)
Glycoprotein	Elastin	Branched network	*ELN* (7q11.23)
	Fibrillin	Linear arrays	*FBN1* (15q21.1); *FBN2* (5q23.-q31)
	Tenascin-C	Branched molecule	*TNC* (9q33)
	COMP[d]	Branched molecule	*COMP* (19p13.1)
	Fibronectin	Modular protein	*FN1* (2q34)
	Laminin[e]	Modular protein	
	Thrombospondin	Modular protein	*THBS1* (15q15); *THBS2* (6q27); *THBS3* (1q21); THBS4 (5q13)
	Link protein	Globular protein	*CTR1* (5q13-q14)
	Tenomodulin	Transmembrane protein	*TNMD* (Xq21.33-q23)

[c]**SLRP**, small leucine-rich repeat proteoglycan; [d]**COMP**, cartilage oligomeric matrix protein; [e]Multiple genes, coding for multiple proteins.

(Adapted from Riley, 2005b).

For instance, both type XII and XIV collagens respond to mechanical loading and are involved in fibrillogenesis, processes that both *COL5A1* and *TNC* are involved in as well (Fluck et al., 2000). In a rat model of rotator cuff tendinopathy, it has been shown that type XII collagen is up-regulated during tendon healing (Thomopoulos et al., 2002). Therefore it would be interesting investigate the association of genes coding for some of the other collagen and non-collagenous proteins found in tendons.

Further studies also need to investigate the possible role of the interaction of the *COL5A1*, *TNC* and any other genes (gene-gene interactions) that might be identified in the further studies in tendon pathology. As already mentioned in thesis, it is generally well-established that the aetiology of Achilles tendon injuries is multi-factorial. Therefore, future studies also need to investigate the extent of the interaction, if any, of the different intrinsic and extrinsic risk factors with genetic risk factors, the gene-environment interactions, in tendon pathology. The interaction of other intrinsic factors such as age and body weight, as well as extrinsic factors such as the type and amount of physical activity and training, with either the *COL5A1* and/or *TNC* genes could not be excluded in this thesis. In support of this, an interaction of increased body weight and the *COL9A3* gene has been reported in lumbar disc degeneration (Solovieva et al., 2002). Therefore, the possible interaction of non-genetic factors, such as body weight and physical activity with the *COL5A1*, *TNC* and/or any other genes needs to be investigated.

As previously mentioned, investigators have shown that there is a genetic component to rotator cuff tendon and anterior cruciate ligament injuries. Further research also needs to be conducted to establish whether the TNC and COL5A1 genes are also associated with other tendon as well as ligament injuries.

4. To investigate whether there was any *COL5A1* or *TNC* genotype effect on the muscle-tendon unit range of motion (ROM).

The thesis established that both *COL5A1* and *TNC* genotypes did not influence the individual's muscle-tendon unit ROM in the joints examined. Comparison of individuals who had sustained a chronic Achilles tendinopathy and control subjects, irrespective of their genotype also showed similar measurements of ROM. This is in contrast to a recent finding that showed that an increased ankle dorsiflexion was a risk factor for the development of Achilles tendinopathy. However, our study had limitations as the measurements of ROM were conducted in individuals who had already been injured (either chronic Achilles tendinopathy or rupture), therefore we did not have baseline ROM data on all our ATI subjects. We also studied injuries from various sports. It may be ideal to study a cohort of individual beginners of a single sport such as running, which is known to be highly associated with Achilles tendinopathy and obtain baseline and periodic measurements on the cohort.

Since mutations in *COL5A1* and *TNX* genes have been associated with both hypermobility type-Ehlers Danlos Syndrome (HT-EDS) and benign joint hypermobilty syndrome (BJHS) and tenascin C belongs to the same family as tenascin X, therefore it will be interesting to investigate further the function of tenascin X in tendons.

5. To investigate whether there was any *COL5A1* or *TNC* genotype effect on the morphological changes associated with Achilles tendon injury by using grey scale and colour Doppler ultrasonography.

There was no genotype effect of both the *COL5A1* and *TNC* genes on the morphological changes of the Achilles tendon as determined via grey scale and colour Doppler ultrasonography. In this thesis, 34 of the 36 sub-group of

individuals who had been clinically diagnosed with Achilles tendinopathy were confirmed via ultrasonographic examination to have Achilles tendinopathy as they satisfied the majority of the four conditions associated with tendinopathy. The current thesis suggests that four main characteristics, which were found to be informative, include increased tendon diameter, increased tendon flow, decreased echogenecity and an alteration in tendon shape. These four characteristics support previous histopathological findings which indicates that in chronic tendinopathy a tendon becomes thicker hence the increased diameter and change in shape. The tendon also has increased ground substance, which is hydrophilic and therefore attracts water into the extracellular matrix. This increased water content causes tendon swelling and the decrease in tendon echogenecity with ultrasonography. It is proposed that a number of intratendinous changes need to be present, not just one, to predict future development of symptoms of tendon injury.

Although the sample size was too small, and the results are therefore only preliminary, it was observed that none of the 7 control (CON-M) subjects with the A2 allele of the *COL5A1* gene had tendon abnormalities. Six of the 15 (40%) subjects with the A1A1 allele genotype had at least one tendon abnormality as determined by a pathology score of 1 or more (range of scores= 1-4).

Because of (i) the sample size for the ultrasonography component of the thesis and (ii) there was an interesting result concerning control subjects containing the A2 allele of the *COL5A1* gene, further research is warranted to increase the sample size and perhaps replicate the results in a prospective cohort study. The majority of studies in the literature investigating possible risk factors for tendon injuries have been retrospective and therefore, future research need to design and conduct prospective cohort studies to identify risk factors for Achilles tendon injuries. In one prospective cohort study, asymptomatic soccer players with preseason US

abnormality had increased risk for developing tendinopathy during the season (Fredberg and Bolvig, 2002).

In conclusion, polymorphisms within the *COL5A1* and *TNC* genes, which are both located on the long arm of chromosome 9 in close proximity with the ABO gene have been shown in this thesis to be associated with Achilles tendon injury in a physically active South African Caucasian population. This thesis has shown that although many extrinsic and intrinsic factors have been implicated in Achilles tendon injury, the level of evidence for the majority (21 of 29 (72%)) of these factors have a level of evidence of weak. So far the level of evidence for genetic factors as independent risk factors for Achilles tendon injuries has been weak (Chapter 1, Table 1.8). However, this thesis has strengthened the level of evidence for genetic factors from weak to limited (Table 7.2). There was no *COL5A1* or *TNC* genotype effects on the muscle tendon unit range of motion or flexibility, at least in the measured joints, as well as in the Achilles tendon morphology as measured via ultrasonography.

Table 7.2 Intrinsic risk factors associated with acute and overuse Achilles tendon injuries

Intrinsic risk factor	Level of evidence	Key references
1. Age	Weak	Leppilahti et al., 1996
2. Gender	Limited	Jozsa et al., 1989
3. Body mass and size	Weak	Kannus and Natri, 1997
4. Previous injury	Strong	Fredberg and Bolvig, 2002
5. Tendon blood supply	Weak	Knobloch et al., 2006
6. Tendon temperature	Weak	Wilson and Goodship, 1994
7. Biomechanical factors:		
• Flexibility	Limited	Mahieu et al., 2006
• Leg length inequality	Weak	Leppilahti et al., 1998
• Leg dominance	Weak	Levi, 1997
• Foot morphology	Weak	Kvist, 1994; Leppilahti et al., 1998
• Muscle strength	Limited	Mahieu et al., 2005; Kvist, 1994
• Associated Plantaris	Weak	Daseler and Anson, 1943
8. Genetic factors		
• ABO blood group	Weak	Jozsa et al., 1989
• HLA	Weak	Olivieri et al., 1987
• Ethnic group	Weak	Davis et al., 1999
• COL5A1 gene	Limited	Mokone et al., 2006
• TNC gene	Limited	Mokone et al., 2005
9. Systemic diseases	Weak	Jozsa et al., 1989
• Hyperlipidemia	Limited	Ozgurtas et al., 2003

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Appendix 1.1 Levels of evidence for primary research question

		Prognostic studies
Strong evidence	Level I	•High quality prospective study (all patients were enrolled at the same point in their disease with ≥ 80% follow-up of enrolled patients •Systematic review of level-I Studies
Limited evidence	Level II	•Retrospective study •Untreated controls from a randomized controlled trial •Lesser quality prospective study (e.g., patients enrolled at different time points in their disease or ≤ 80% follow-up •Systematic review of level-II Studies
	Level III	•Case-control study
Weak evidence	Level IV	•Case Series
	Level V	•Expert opinion

Notes:

Adapted from the level-of-evidence rating system used by JBJS-A (Reprinted from : Instructions to Authors, The Journal of Bone and Joint Surgery (American), December 2005.)

Detailed explanations of the studies are provided by JBJS-A.

APPENDIX 2.1

RECRUITMENT LETTER

Clinician's Name
Clinician's Address

Date

Dear

RE: STUDY ON THE GENETIC BASIS OF CHRONIC TENDON PATHOLOGY

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 recently approached me about a research study on the genetic basis of chronic tendon pathology they are currently undertaking. The researchers have asked me to inform you about this study and to invite you to participate in their research.

If you would like to participate in this study, you will be required to donate five millilitres of venous blood or a mouthwash sample containing some of the loose cells which lined the inside of your cheeks. After receiving written consent, the blood or mouthwash sample will be used for the extraction and analysis of genetic material (DNA). You will also be required to complete personal particulars, sporting participation, medical history, stretching and warm up questionnaires. All the information collected during the study will be treated with the strictest confidentiality and will only be used for scientific research purposes. Your name and personal particulars will not be released under any circumstances and all the data obtained will be analysed anonymously. There will also be other additional tests that we will ask you to do if you agree to participate in this study.

If you would like to participate in the study and/or obtain any additional information, please contact one of the following:-

Mr. George Mokone, BSc (Hons)
(021) 650-4569
gmokone@sports.uct.ac.za

Dr. Malcolm Collins, PhD
(021) 650-4574
mcollins@sports.uct.ac.za

Yours faithfully

Clinician's Name and Qualifications

APPENDIX 2.2

ETHICS APPROVAL LETTER

UNIVERSITY OF CAPE TOWN

Research Ethics Committee
Faculty of Medicine
Anzio Road, Observatory, 7925
Queries : Martha Jacobs
Tel : (021) 406-6492 Fax: (021) 406-6390
E-mail : Martha@medicine.uct.ac.za

17 April 2000

REC REF: 321/99

Mr G Mokone
BERU

Dear Mr Mokone

GENETIC BASIS OF ACHILLES TENDON RUPTURES AND CHRONIC ACHILLES TENDINOPATHY

Thank you for your application submitted to the Research Ethics Committee on 14 December 1999.

I have pleasure in informing you that the Research Ethics Committee has **formally approved** the above study on 24 January 2000.

You may proceed with the trial once the financial agreement/contract and protocol have been processed through the department of Research Development and duly signed by the authorised University of Cape Town signatories.

Included is a list of Research Ethics Committee Members who have formally approved your protocol.

Please quote the above Reference number in all correspondence.

Yours sincerely,

PROFESSOR DM DENT
ACTING-CHAIRPERSON

Queries: Martha Jacobs
Research Ethics Committee
Room 212 Werner and Beit
UCT Medical School
Anzio Road, Observatory, 7925
Tel: (021) 406-6492 Fax: (021) 406-6390

APPENDIX 2.3

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 tendinopathy. 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 2.4

GENETIC BASIS OF EXERCISE-INDUCED TENDON INJURY QUESTIONNAIRE

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 ☐		
Nationality		Dominant Handedness	Left ☐ Right ☐
Country of Birth			
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			
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			
Years in competitive sport			
Professional or amateur			
Hours of training per week (last 3 months)			
Hours of training per week (3-12 months)			
Hours of training per week (12-24 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			
Years in competitive sport			
Professional or amateur			
Hours of training per week (last 3 months)			
Hours of training per week (3-12 months)			
Hours of training per week (12-24 months)			

C. MEDICAL DETAILS (GENERAL TENDON INJURY)

How many times have you had tendon injuries?	Event	Date	Acute or Chronic Injury	Sudden[1] or Gradual[2] Onset
[1]Sudden onset is within a few seconds or minutes	1			
[2]Gradual onset is over days or weeks	2			
	3			
	4			
	5			

Please complete a separate form, Part C only, for each Tendon Injury you have had

Event Number (1,2,3,4,or 5)	
Which tendon did you injure?	Rotator cuff tendon ف • Supraspinatus ف • Infraspinatus ف • teres minor ف Patellar tendon ف Wrist extensor tendons ف Achilles tendon ف
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 ☐ none of the above ☐ not sure
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 ☐ none of the above ☐ not sure
How were you injured? (e.g. sport, walking)	
Which of the following symptoms were present before the injury	Pain (less than 1 week) ☐ Stiffness ☐ Pain (1-4 weeks) ☐ Swelling ☐ Pain (> 4 weeks) ☐ None ☐

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. ف Doctor _____ ف Physiotherapist _____ ف Biokineticist _____ ف Podiatrist _____ ف Other _____

To what extent was your Tendon ruptured?	complete ☐	partial ☐	none ☐
If applicable, what diagnostic imaging was performed?	ultrasound ☐	MRI ☐	Other _____
If applicable, who did the imaging?	Clinician _____	Phone _____	
Specifics			
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 ☐
General Information			

Have you ever used oral corticosteroids (cortisone tablets)?	yes ☐	no ☐	If Yes, How long Ago ☐ 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 the injured 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
Do you suffer from any Connective Tissue Disorders?	yes ☐	no ☐	If Yes, please state the condition _____
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 ☐		
If you had a tendon rupture. How was it treated?	Surgically ☐	Non-surgically ☐		
If you have or had chronic tendon pain, what seems to alleviate the pain?				

D. STRETCHING AND WARM UP					
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?					

List of Common Connective Tissue Disorders

1. heumatoid Arthiritis
2. Ankylosing Spondylitis
3. Systemic Lupus Erythematosus
4. Vasulatis
5. Polyarteritis Nodosa
6. Temporal Arteritis
7. Wegener's Granulomatosis
8. Crohn's Disease
9. Discoid Lupus Erythromatosus
10. Relapsing Polychrondritis
11. Systemic Sclerosis
12. Eosinophilic Fascitis
13. Polymyositis & Dermatomyositis
14. Polymyalgia Rheumatica
15. Sjogren's Syndrome
16. Behcet's Syndrome

APPENDIX 2.5

CLINICAL DIAGNOSIS OF ACHILLES TENDINOPATHY

SUBJECT NUMBER/CODE: _____

Clinical criteria [1; 2]	Present
Gradual progressive pain over the posterior lower leg - Achilles tendon area (> 6 weeks)	
Early morning pain	
Early morning stiffness	
History of swelling over the Achilles tendon area	
Tenderness to palpation over the Achilles tendon	
Palpable nodular thickening over the affected Achilles	
Positive "shift" test (movement of the nodular area with plantar-/dorsi-flexion)	

Date: _____ / _____ / 2003

Investigator: Prof M Schwellnus

Signature: _____

References:

1. Schepsis AA, Jones H, Haas AL. Achilles tendon disorders in athletes. *Am.J Sports Med* 2002;**30**:287-305.

2. Kader D, Saxena A, Movin T, Maffulli N. Achilles tendinopathy: some aspects of basic science and clinical management. *Br.J Sports Med* 2002;**36**:239-49.

APPENDIX 2.6

CLINICAL DIAGNOSIS OF ACHILLES TENDON RUPTURES

SUBJECT NUMBER/CODE: _____

Clinical criteria [1]	Present
Acute sever pain over the posterior lower leg - Achilles tendon area	
Audible snap at the time of injury	
Unable to weight bear on affected side	
Positive "Thompson" squeeze test	
Palpable defect over the Achilles tendon	

Date: _____ / _____ / 2003

Investigator: Prof M Schwellnus

Signature: _____

References:

1. Schepsis AA, Jones H, Haas AL. Achilles tendon disorders in athletes. *Am.J Sports Med* 2002;**30**:287-305.

APPENDIX 2.7

LIST OF ALL THE GENES MAPPED TO CHROMOSOME 9q32 - 9q34.3

No	Cytogenetic Location	Start (Mb)	Gene Name	Short Description
1	9q32	105	FLJ13385	Hypothetical protein FLJ13385
2	9q32	105	ZNF462	Zinc finger protein 462
3	9q32	105	LOC389782	LOC389782
4	9q31.2	105	RAD23B	RAD23 homolog B (S. cerevisiae)
5	9q31	106	KLF4	Kruppel-like factor 4 (gut)
6	9q32	106	LOC392380	Similar to peptidylprolyl isomerase A (cyclophilin A)
7	9q32	106	LOC392381	Similar to large subunit ribosomal protein L36a
8	9q32	106	LOC392382	Similar to ribosomal protein L31
9	9q32	107	LOC347292	Similar to ribosomal protein L36; 60S ribosomal protein L36
10	9q31	107	ACTL7B	Actin-like 7B
11	9q31	107	ACTL7A	Actin-like 7A
12	9q31	107	IKBKAP	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein
13	9q32	107	FLJ20457	hypothetical protein FLJ20457
14	9q31.2	107	CTNNAL1	Catenin (cadherin-associated protein), alpha-like 1
15	9q31	107	C9orf5	chromosome 9 open reading frame 5
16	9q31	107	C9orf4	chromosome 9 open reading frame 4
17	9q31	107	EPB41L4B	erythrocyte membrane protein band 4.1 like 4B
18	9q32	107	FLJ21596	hypothetical protein FLJ21596
19	9q31	108	PTPN3	protein tyrosine phosphatase, non-receptor type 3
20	9q32	108	LOC402375	similar to DNA-binding protein B
21	9q32	108	LOC389783	LOC389783
22	9q31-q33	108	AKAP2	A kinase (PRKA) anchor protein 2
23	9q31-q33	108	PALM2	paralemmin 2
24	9q32	108	LOC401546	similar to RIKEN cDNA D630039A03 gene
25	9q31	108	TXN	thioredoxin
26	9q32	108	LOC255220	similar to RIKEN cDNA 4930429J24
27	9q31.3-q32	109	MUSK	muscle, skeletal, receptor tyrosine kinase
28	9q32	109	EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2
29	9q32	109	OR2K2	olfactory receptor, family 2, subfamily K, member 2
30	3	109	OR2AR1P	olfactory receptor, family 2, subfamily AR, member 1 pseudogene
31	9q32	110	KIAA0368	KIAA0368
32	9q32	110	ZNF483	zinc finger protein 483
33	9q32	110	LTB4DH	leukotriene B4 12-hydroxydehydrogenase
34	9q32	110	LOC401547	similar to KIAA0563 gene product
35	9q32	110	GNG10	guanine nucleotide binding protein (G protein), gamma 10
36	9q32	110	C9orf84	chromosome 9 open reading frame 84
37	9q31	110	UGCG	UDP-glucose ceramide glucosyltransferase
38	9q31.3-q33.1	110	SUSD1	sushi domain containing 1
39	9q33.1	110	ROD1	ROD1 regulator of differentiation 1 (S. pombe)
40	9q33.1	110	EPF5	EPF5 pseudogene
41	9q33.1	111	LOC392384	similar to 60S ribosomal protein L32

No	Cytogenetic Location	Start (Mb)	Gene Name	Short Description
42	9q33.1	111	MGC10940	hypothetical protein MGC10940
43	9q33.1	111	LOC389784	LOC389784
44	9q33.1	111	KIAA1958	KIAA1958
45	9q32	111	C9orf80	chromosome 9 open reading frame 80
46	9q33.1	111	LOC389785	similar to RIKEN cDNA 4732481H14
47	9q33.1	111	LOC401548	similar to RIKEN cDNA 4732481H14
48	9q32	111	TSCOT	thymic stromal co-transporter
49	9q33.1	111	LOC169834	hypothetical protein LOC169834
50	9q32	111	ZFP37	zinc finger protein 37 homolog (mouse)
51	9q33.1	111	LOC401549	hypothetical gene supported by BX647840
52	9q33.1	111	LOC286333	hypothetical protein LOC286333
53	9q31-q32	111	SLC31A2	solute carrier family 31 (copper transporters), member 2
54	9q33.1	111	KIAA0674	KIAA0674 protein
55	9q31-q32	111	SLC31A1	solute carrier family 31 (copper transporters), member 1
56	9q32	111	CDC26	cell division cycle 26
57	9q31-q33	111	PRPF4	PRP4 pre-mRNA processing factor 4 homolog (yeast)
58	9q33.1	111	MGC4734	hypothetical protein MGC4734
59	9q33.1	111	WDR31	WD repeat domain 31
60	9q33.1	111	BSPRY	B-box and SPRY domain containing
61	9q33.1	112	MGC12904	hypothetical protein MGC12904
62	9q34	112	ALAD	aminolevulinate, delta-, dehydratase
63	9q33	112	POLE3	polymerase (DNA directed), epsilon 3 (p17 subunit)
64	9q33.1	112	C9orf43	chromosome 9 open reading frame 43
65	9q32	112	RGS3	regulator of G-protein signalling 3
66	9q33.1	112	FLJ31713	hypothetical protein FLJ31713
67	9q33.1	112	LOC392385	similar to programmed cell death 2 isoform 1; zinc finger protein Rp-8; programmed cell death 2/Rp8 homolog
68	9q33.1	112	KIAA1952	KIAA1952 protein
69	9q32-q33	112	AMBP	alpha-1-microglobulin/bikunin precursor
70	9q33.1	112	KIF12	kinesin family member 12
71	9q33.1	112	COL27A1	Collagen, type XXVII, alpha 1
72	9q31-q32	112	ORM1	orosomucoid 1
73	9q32	112	ORM2	orosomucoid 2
74	9q32	112	AKNA	AT-hook transcription factor AKNA
75	9q33.1	113	CIP98	CASK-interacting protein CIP98
76	9q33.1	113	ATP6V1G1	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G isoform 1
77	9q33.1	113	DKFZp547P234	DKFZp547P234 protein
78	9q33.1	113	LOC389786	LOC389786
79	9q32	113	TNFSF15	tumor necrosis factor (ligand) superfamily, member 15
80	9q33.1	113	LOC402376	similar to KIAA0536 protein
81	9q33	113	TNFSF8	tumor necrosis factor (ligand) superfamily, member 8
82	9q33	113	TNC	Tenascin C (hexabrachion)
83	9q32	113	38322	deleted in esophageal cancer 1
84	9q31.3	114	C9orf27	chromosome 9 open reading frame 27
85	9q33.2	114	PAPPA	pregnancy-associated plasma protein A
86	9q33.2	115	ASTN2	astrotactin 2
87	9q33.2	115	TRIM32	tripartite motif-containing 32

No	Cytogenetic Location	Start (Mb)	Gene Name	Short Description
88	9q32-q33	116	TLR4	toll-like receptor 4
89	9q33.2	116	LOC340477	hypothetical LOC340477
90	9q33.2	116	LOC389787	similar to Translationally controlled tumor protein (TCTP) (p23) (Histamine-releasing factor) (HRF)
91	9q33.3	117	LOC347165	similar to beta-tubulin 4Q
92	9q32-q33	117	DBC1	deleted in bladder cancer 1
93	9q33.3	119	CDK5RAP2	CDK5 regulatory subunit associated protein 2
94	9q32-q33.3	119	EGFL5	EGF-like-domain, multiple 5
95	9q34.11	119	LOC392387	similar to Ahcy protein
96	9q34	119	FBXW2	F-box and WD-40 domain protein 2
97	9q34.11	119	LOC402377	similar to beta-1,3-N-acetylglucosaminyltransferase 5
98	9q34.11	119	PSMD5	proteasome (prosome, macropain) 26S subunit, non-ATPase, 5
99	9q34.11	119	PHF19	PHD finger protein 19
100	9q33-q34	119	TRAF1	TNF receptor-associated factor 1
101	9q33-q34	119	C5	complement component 5
102	9q33-q34	119	CEP1	centrosomal protein 1
103	9q32-q34.11	119	RAB14	RAB14, member RAS oncogene family
104	9q34.11	119	LOC392388	similar to ATPase, H+ transporting, lysosomal 56/58kD, V1 subunit B, isoform 2
105	9q33	119	GSN	gelsolin (amyloidosis, Finnish type)
106	9q34.11	119	MOST2	MOST2 protein
107	9q34.1	119	STOM	stomatin
108	9q34.11	120	a1/3GTP	alpha-1,3-galactosyltransferase pseudogene
109	9q33.1-q33.3	120	DAB2IP	DAB2 interacting protein
110	9q34.11	120	LOC158135	similar to H23L24.3.p
111	9q34.11	120	LOC401550	hypothetical gene supported by BC039180
112	9q33.2-q34.11	120	NDUFA8	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8, 19kDa
113	9q34.11	120	FLJ46909	FLJ46909 protein
114	9q34.11	120	LHX6	LIM homeobox 6
115	9q34.11	120	RBM18	RNA binding motif protein 18
116	9q34.11	120	MRRF	mitochondrial ribosome recycling factor
117	9q32-q33.3	121	PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
118	9q34.11	121	OR1J5	olfactory receptor, family 1, subfamily J, member 5
119	9q34.11	121	LOC347168	similar to Olfactory receptor 1J1
120	9q34.11	121	OR1J4	olfactory receptor, family 1, subfamily J, member 4
121	9q34.11	121	LOC138883	similar to Olfactory receptor 1N3
122	9q34.11	121	LOC138882	similar to Olfactory receptor 1N2
123	9q34.11	121	LOC138881	similar to Olfactory receptor 1L8
124	9q34.11	121	LOC392389	similar to G protein-coupled receptor homolog clone H8
125	9q34.11	121	LOC158131	similar to Olfactory receptor 1Q1 (Olfactory receptor TPCR106) (Olfactory receptor 9-A) (OR9-A) (OST226)
126	9q34.11	121	LOC347169	similar to Olfactory receptor 1B1 (Olfactory receptor 9-B) (OR9-B)
127	9q34.11	121	LOC158130	similar to tousled-like kinase 1; serine threonine protein kinase
128	9q34.11	121	LOC254973	similar to Olfactory receptor 1L4 (Olfactory receptor 9-E) (OR9-E) (OST046)

No	Cytogenetic Location	Start (Mb)	Gene Name	Short Description
129	9q34.11	121	LOC392390	similar to Olfactory receptor 1L6
130	9q34.11	121	LOC389789	LOC389789
131	9q34.11	121	LOC392391	similar to Olfactory receptor 5C1 (Olfactory receptor 9-F) (OR9-F)
132	9q34.11	121	LOC392392	similar to Olfactory receptor 1K1
133	9q12-q13	121	PDCL	phosducin-like
134	9q34.11	121	LOC402378	similar to Keratin, type I cytoskeletal 18 (Cytokeratin 18) (K18) (CK 18)
135	9q34	121	MNAB	membrane-associated nucleic acid binding protein
136	9q34.11	121	ZNF482	zinc finger protein 482
137	9q34.11	121	ZNF481	zinc finger protein 481
138	9q34.11	121	RABGAP1	RAB GTPase activating protein 1
139	9q33	121	GPR21	G protein-coupled receptor 21
140	9q34.11	121	C9orf45	chromosome 9 open reading frame 45
141	9q33.3	121	STRBP	spermatid perinuclear RNA binding protein
142	9q34.11	122	FLJ38464	hypothetical protein FLJ38464
143	9q34.11	122	KIAA1608	KIAA1608
144	9q33-q34.1	122	LHX2	LIM homeobox 2
145	9q33.3-q34.11	122	NEK6	NIMA (never in mitosis gene a)-related kinase 6
146	9q34.11-q34.12	122	PSMB7	proteasome (prosome, macropain) subunit, beta type, 7
147	9q34.11	123	GPR144	G protein-coupled receptor 144
148	9q33	123	NR5A1	nuclear receptor subfamily 5, group A, member 1
149	9q33-q34.1	123	NR6A1	nuclear receptor subfamily 6, group A, member 1
150	9q34.11	123	LOC169611	hypothetical protein LOC169611
151	9q34.11	123	LOC401551	similar to hypothetical protein FLJ25955
152	9q34.1	123	RPL35	ribosomal protein L35
153	9q34.11	123	ARPC5L	actin related protein 2/3 complex, subunit 5-like
154	9q34.11	123	GOLGA1	golgi autoantigen, golgin subfamily a, 1
155	9q34.11	123	FLJ36664	hypothetical protein FLJ36664
156	9q34.11	123	PPP6C	protein phosphatase 6, catalytic subunit
157	9q32-q34.1	123	NDUFB3P2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa pseudogene 2
158	9q34.11	123	RAB9P40	Rab9 effector p40
159	9q33-q34.1	123	HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)
160	9q34.11	123	DKFZP434C212	DKFZP434C212 protein
161	9q34.12	124	MAPKAP1	mitogen-activated protein kinase associated protein 1
162	9q34.12	124	LOC51145	erythrocyte transmembrane protein
163	9q33-q34	124	PBX3	pre-B-cell leukemia transcription factor 3
164	9q34.12	124	C9orf28	chromosome 9 open reading frame 28
165	9q34	125	LMX1B	LIM homeobox transcription factor 1, beta
166	9p24.1-q22.33	125	ZNF297B	zinc finger protein 297B
167	9q34	125	ANGPTL2	angiopoietin-like 2
168	9q34.13	125	DKFZp761J1523	hypothetical protein DKFZp761J1523
169	9q34.13	126	SLC2A8	solute carrier family 2, (facilitated glucose transporter) member 8
170	9q34	126	ZNF79	zinc finger protein 79 (pT7)
171	9q34	126	RPL12	ribosomal protein L12
172	9q34.13	126	LRSAM1	leucine rich repeat and sterile alpha motif containing 1

No	Cytogenetic Location	Start (Mb)	Gene Name	Short Description
173	9q34.13	126	C9orf88	chromosome 9 open reading frame 88
174	9q34.1	126	STXBP1	syntaxin binding protein 1
175	9q34.13	126	LOC286207	hypothetical protein LOC286207
176	9q34.13	126	LOC138428	hypothetical protein LOC138428
177	9q34.13	126	TTC16	tetratricopeptide repeat domain 16
178	9q34.11	126	TOR2A	torsin family 2, member A
179	9q34.13	126	SH2D3C	SH2 domain containing 3C
180	9q34.1	126	CDK9	cyclin-dependent kinase 9 (CDC2-related kinase)
181	9cen-q34	126	FPGS	folylpolyglutamate synthase
182	9q33-q34.1	126	ENG	endoglin (Osler-Rendu-Weber syndrome 1)
183	9q34.1	126	AK1	adenylate kinase 1
184	9q34.13	126	ST6GALNAC6	CMP-NeuAC:(beta)-N-acetylgalactosaminide (alpha)2,6-sialyltransferase member VI
185	9q34	126	SIAT7D	sialyltransferase 7D ((alpha-N-acetylneuraminyl-2,3-beta-galactosyl-1,3)-N-acetyl galactosaminide alpha-2,6-sialyltransferase)
186	9q34.13	126	PIP5KL1	phosphatidylinositol-4-phosphate 5-kinase-like 1
187	9q34.13	126	FLJ10232	hypothetical protein FLJ10232
188	9q34.13	126	LOC389790	similar to phosphatidylinositol phosphate kinase-like protein
189	9q34.13	126	DPM2	dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit
190	9q34.13	126	C9orf90	chromosome 9 open reading frame 90
191	9q34.13	126	MCSC	mitochondrial Ca2+-dependent solute carrier
192	9q34.13	126	LOC401552	hypothetical gene supported by AK124723; AL833509
193	9q34.13	126	PTGES2	prostaglandin E synthase 2
194	9q34.13	126	LOC389791	LOC389791
195	9q34	126	LCN2	lipocalin 2 (oncogene 24p3)
196	9q34.1	126	C9orf16	chromosome 9 open reading frame 16
197	9q34.1	126	CIZ1	CDKN1A interacting zinc finger protein 1
198	9q34	126	DNM1	dynamin 1
199	9q34.13	126	GOLGA2	golgi autoantigen, golgin subfamily a, 2
200	9q34.13	126	LOC375757	hypothetical protein LOC375757
201	9q34.13	126	FLJ21673	hypothetical protein FLJ21673
202	9q34.13	126	TRUB2	TruB pseudouridine (psi) synthase homolog 2 (E. coli)
203	9q34.13	126	COQ4	coenzyme Q4 homolog (yeast)
204	9q34.13	126	SLC27A4	solute carrier family 27 (fatty acid transporter), member 4
205	9q34.13	126	TMSL4	thymosin-like 4
206	9q34.13	127	C9orf74	chromosome 9 open reading frame 74
207	9q34.13	127	CEECAM1	cerebral endothelial cell adhesion molecule 1
208	9q34.13	127	LOC392394	similar to hypothetical protein
209	9q34.11	127	ODF2	outer dense fiber of sperm tails 2
210	9q34.13	127	GLE1L	GLE1 RNA export mediator-like (yeast)
211	9q33-q34	127	SPTAN1	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)
212	9q34.13	127	WDR34	WD repeat domain 34
213	9q34	127	SET	SET translocation (myeloid leukemia-associated)
214	9q34.13	127	pknbeta	protein kinase PKNbeta
215	9q34.13	127	ZDHHC12	zinc finger, DHHC domain containing 12
216	9q34.13	127	C9orf60	chromosome 9 open reading frame 60

No	Cytogenetic Location	Start (Mb)	Gene Name	Short Description
217	9q34.13	127	TBC1D13	TBC1 domain family, member 13
218	9q34.1	127	ENDOG	endonuclease G
219	9q34.13	127	HSPC109	hypothetical protein HSPC109
220	9q34.13	127	CCBL1	cysteine conjugate-beta lyase; cytoplasmic (glutamine transaminase K, kyneurenine aminotransferase)
221	9q34.13	127	LRRC8	leucine rich repeat containing 8
222	9q34.13	127	PHYHD1	phytanoyl-CoA dioxygenase domain containing 1
223	9q34.13	127	TMEM15	transmembrane protein 15
224	9q34	127	SH3GLB2	SH3-domain GRB2-like endophilin B2
225	9q34.13	127	C9orf54	chromosome 9 open reading frame 54
226	9q34.1	127	DOLPP1	dolichyl pyrophosphate phosphatase 1
227	9q34.1	127	CRAT	carnitine acetyltransferase
228	9q34	127	PPP2R4	protein phosphatase 2A, regulatory subunit B' (PR 53)
229	9q34.13	127	LOC389792	similar to RIKEN cDNA 2610524G09
230	9q34.13	127	LOC389793	LOC389793
231	9q34.2	128	LOC389794	LOC389794
232	9q34.2	128	LOC401553	hypothetical gene supported by BC019073; BC036842; BC044229
233	9q34.2	128	LOC375759	hypothetical protein LOC375759
234	9q34.2	128	AD-003	AD-003 protein
235	9q34.13	128	ASB6	ankyrin repeat and SOCS box-containing 6
236	9q34.1	128	PRRX2	paired related homeobox 2
237	9q34.3	128	PTGES	prostaglandin E synthase
238	9q34	128	TOR1B	torsin family 1, member B (torsin B)
239	9q34.2	128	C9orf78	chromosome 9 open reading frame 78
240	9q34	128	DYT1	dystonia 1, torsion (autosomal dominant; torsin A)
241	9q34.2	128	USP20	ubiquitin specific protease 20
242	9q34	128	FNBP1	formin binding protein 1
243	9q34.2	128	GPR107	G protein-coupled receptor 107
244	9q34.2	128	LOC401554	hypothetical gene supported by AK128673
245	9q34	128	FREQ	frequenin homolog (Drosophila)
246	9q34.2	128	LOC392395	similar to hemicentin
247	9q34.2	128	DKFZp434P0216	hemicentin-2
248	9q34.2	129	LOC389796	similar to CDNA sequence BC034076
249	9q34.2	129	LOC389797	similar to cDNA sequence BC034076
250	9q34.1	129	ASS	argininosuccinate synthetase
251	9q34.2	129	FUBP3	far upstream element (FUSE) binding protein 3
252	9q34.2	129	LOC402379	similar to hypothetical protein
253	9q33-q34	129	PRDM12	PR domain containing 12
254	9q34	129	RRP4	homolog of Yeast RRP4 (ribosomal RNA processing 4), 3'-5'-exoribonuclease
255	9q34.1	129	ABL1	v-abl Abelson murine leukemia viral oncogene homolog 1
256	9q34.2	129	P518	P518 precursor protein
257	9q34.2	129	FIBCD1	fibrinogen C domain containing 1
258	9q31-q34	129	LAMC3	laminin, gamma 3
259	9q34.13-q34.3	129	C9orf58	chromosome 9 open reading frame 58
260	9q34.1	129	NUP214	nucleoporin 214kDa
261	9q34	129	C9orf59	chromosome 9 open reading frame 59

No	Cytogenetic Location	Start (Mb)	Gene Name	Short Description
262	9q34.2-q34.3	129	C9orf67	chromosome 9 open reading frame 67
263	9q34.1	130	POMT1	protein-O-mannosyltransferase 1
264	9q34.3	130	UCK1	uridine-cytidine kinase 1
265	9q34.3	130	GRF2	guanine nucleotide-releasing factor 2 (specific for crk proto-oncogene)
266	9q34.1-q34.3	130	CRSP8	cofactor required for Sp1 transcriptional activation, subunit 8, 34kDa
267	9q34	130	NTNG2	netrin G2
268	9q34.3	130	KIAA0625	senataxin
269	9q34.3	131	TTF1	transcription termination factor, RNA polymerase I
270	9q34.3	131	LOC389799	LOC389799
271	9q34	131	BARHL1	BarH-like 1 (Drosophila)
272	9q34.3	131	DDX31	DEAD (Asp-Glu-Ala-Asp) box polypeptide 31
273	9q34.3	131	GTF3C4	general transcription factor IIIC, polypeptide 4, 90kDa
274	9q34.3	131	FLJ32704	hypothetical protein FLJ32704
275	9q34	131	C9orf9	chromosome 9 open reading frame 9
276	9q34	131	TSC1	tuberous sclerosis 1
277	9q34.13	131	GFI1B	growth factor independent 1B (potential regulator of CDKN1A, translocated in CML)
278	9q34	131	LOC158078	eukaryotic translation elongation factor 1 alpha 1 pseudogene
279	9q34.3	131	LOC389801	LOC389801
280	9q34	131	GTF3C5	general transcription factor IIIC, polypeptide 5, 63kDa
281	9q34.3	131	CEL	carboxyl ester lipase (bile salt-stimulated lipase)
282	9q34.3	131	CELP	carboxyl ester lipase pseudogene
283	9q34.3	131	RALGDS	ral guanine nucleotide dissociation stimulator
284	9q34.13-q34.3	131	FS	Forssman glycolipid synthetase
285	9q34	131	OBP2B	odorant binding protein 2B
286	9q34.3	131	LOC286310	similar to Von Ebners gland protein precursor (VEG protein) (Tear prealbumin) (TP) (Tear lipocalin) (Lipocalin 1)
287	9q34.1-q34.2	131	ABO	ABO blood group (transferase A, alpha 1-3-N-acetylgalactosaminyltransferase; transferase B, alpha 1-3-galactosyltransferase)
288	9q34.3	131	LOC392396	similar to Von Ebners gland protein precursor (VEG protein) (Tear prealbumin) (TP) (Tear lipocalin) (Lipocalin 1)
289	9q34.2	131	SURF6	surfeit 6
290	9q34.2	131	SURF5	surfeit 5
291	9q34	131	RPL7A	ribosomal protein L7a
292	9q34.2	131	SURF1	surfeit 1
293	9q34.2	131	SURF2	surfeit 2
294	9q34.2	132	SURF4	surfeit 4
295	9q34.3	132	MGC43306	hypothetical protein MGC43306
296	9q34	132	ADAMTS13	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 13
297	9q34	132	C9orf7	chromosome 9 open reading frame 7
298	9q34	132	SLC2A6	solute carrier family 2 (facilitated glucose transporter), member 6
299	9q34	132	DBH	dopamine beta-hydroxylase (dopamine beta-monooxygenase)
300	9q33-q34	132	SARDH	sarcosine dehydrogenase
301	9q34.1	132	VAV2	vav 2 oncogene
302	9q34.3	132	LOC389803	similar to bA74P14.2 (novel protein)

No	Cytogenetic Location	Start (Mb)	Gene Name	Short Description
303	9q34	132	FLJ35348	FLJ35348
304	9q34	132	BRD3	bromodomain containing 3
305	9q34	132	ARF4P	ADP-ribosylation factor 4 pseudogene
306	9q34.3	133	LOC158226	hypothetical LOC158226
307	9q34.3	133	RXRA	retinoid X receptor, alpha
308	9q34.3	133	LOC157943	hypothetical LOC157943
309	9q34.2-q34.3	133	COL5A1	collagen, type V, alpha 1
310	9q34.3	133	LOC389804	LOC389804
311	9q34.3	133	LOC392397	similar to hypothetical protein
312	9q34	133	FCN2	ficolin (collagen/fibrinogen domain containing lectin) 2 (hucolin)
313	9q34	133	FCN1	ficolin (collagen/fibrinogen domain containing) 1
314	9q34.3	133	OLFM1	olfactomedin 1
315	9q34.3	134	LOC401557	hypothetical gene supported by AK127261
316	9q34.3	134	LOC402380	similar to ligand-independent activating molecule for estrogen receptor
317	9q34.3	134	C9orf62	chromosome 9 open reading frame 62
318	9q34.3	134	KIAA0649	KIAA0649
319	9q34.3	134	MGC29761	hypothetical protein MGC29761
320	9q34	134	MRPS2	mitochondrial ribosomal protein S2
321	9q34	134	LCN1	lipocalin 1 (tear prealbumin)
322	9q34	134	OBP2A	odorant binding protein 2A
323	9q34	134	PAEP	progestagen-associated endometrial protein (placental protein 14, pregnancy-associated endometrial alpha-2-globulin, alpha uterine protein)
324	9q34.3	134	LOC401558	hypothetical gene supported by AK094119
325	9q34.3	134	LOC138159	beta-lactoglobulin pseudogene
326	9q34.3	134	GLTDC1	galactosyltransferase family 6 domain containing 1
327	9q34.3	134	LOC392399	similar to Putative MUP-like lipocalin precursor
328	9q34.3	134	LOC402381	similar to LOC286220 protein
329	9q34.3	134	KCNT1	potassium channel, subfamily T, member 1
330	9q34.3	134	CAMSAP1	calmodulin regulated spectrin-associated protein 1
331	9q34.3	134	UBADC1	ubiquitin associated domain containing 1
332	9q34.3	134	LOC401559	hypothetical gene supported by BC029166
333	9q34.3	134	BTBD14A	BTB (POZ) domain containing 14A
334	9q34.3	134	LOC402382	similar to F4N2.10
335	9q34.3	134	LOC389806	LOC389806
336	9q34.3	134	LOC90120	hypothetical gene supported by AK023162
337	9q34.3	135	LHX3	LIM homeobox 3
338	9q34.3	135	QSCN6L1	quiescin Q6-like 1
339	9q34.3	135	GPSM1	G-protein signalling modulator 1 (AGS3-like, C. elegans)
340	9q34.3	135	LOC389810	LOC389810
341	9q34.3	135	LOC401560	similar to DNL zinc finger (3D41)
342	9q34.3	135	CARD9	caspase recruitment domain family, member 9
343	9q34.3	135	SNAPC4	small nuclear RNA activating complex, polypeptide 4, 190kDa
344	9q34.3	135	SDCCAG3	serologically defined colon cancer antigen 3
345	9q34.3	135	PMPCA	peptidase (mitochondrial processing) alpha
346	9q34.3	135	INPP5E	inositol polyphosphate-5-phosphatase, 72 kDa

No	Cytogenetic Location	Start (Mb)	Gene Name	Short Description
347	9q34.3	135	KIAA0310	KIAA0310
348	9q34.3	135	FLJ36779	hypothetical protein FLJ36779
349	9q34.3	135	NOTCH1	Notch homolog 1, translocation-associated (Drosophila)
350	9q34.3	135	LOC401561	hypothetical gene supported by AY129027
351	9q34.3	135	LOC389811	similar to HSPC324
352	9q34.3	135	EGFL7	EGF-like-domain, multiple 7
353	9q34.3	135	AGPAT2	1-acylglycerol-3-phosphate O-acyltransferase 2 (lysophosphatidic acid acyltransferase, beta)
354	9q34.3	135	MGC20262	hypothetical protein MGC20262
355	9q34.3	135	MGC16037	hypothetical protein MGC16037
356	9q34.3	135	LCN6	lipocalin 6
357	9q34.3	135	LCN8	lipocalin 8
358	9q34.3	135	UNQ2541	MSFL2541
359	9q34.3	135	LOC392401	similar to vacuolar H(+)-ATPase subunit; V-ATPase subunit; M16
360	9q34.3	135	MGC14141	hypothetical protein MGC14141
361	9q34.3	135	KIAA1984	KIAA1984
362	9q34.3	135	C9orf86	chromosome 9 open reading frame 86
363	9q34.3	135	LOC389813	similar to CG15216-PA
364	9q34.3	135	PHP14	phosphohistidine phosphatase
365	9q34.3	135	AEGP	apical early endosomal glycoprotein precursor
366	9q34.3	135	EDF1	endothelial differentiation-related factor 1
367	9q34	135	TRAF2	TNF receptor-associated factor 2
368	9q34.3	135	FBXW5	F-box and WD-40 domain protein 5
369	9q34.3	135	C8G	complement component 8, gamma polypeptide
370	9q34.3	135	MGC48935	hypothetical protein MGC48935
371	9q34.3	135	LOC203235	hypothetical LOC203235
372	9q34.2-q34.3	135	PTGDS	prostaglandin D2 synthase 21kDa (brain)
373	9q34.3	135	LOC401562	hypothetical gene supported by AK127160
374	9q34.3	135	LOC286257	hypothetical protein LOC286257
375	9q34.3	135	CLIC3	chloride intracellular channel 3
376	9q34	135	ABCA2	ATP-binding cassette, sub-family A (ABC1), member 2
377	9q34.3	135	FUT7	fucosyltransferase 7 (alpha (1,3) fucosyltransferase)
378	9q34.3	135	LOC401563	hypothetical gene supported by AK093587; AK124899
379	9q34.3	135	LOC389814	similar to LPAL6438
380	9q34.3	135	NPDC1	neural proliferation, differentiation and control, 1
381	9q34	135	ENTPD2	ectonucleoside triphosphate diphosphohydrolase 2
382	9q34.3	135	LOC89958	hypothetical protein LOC89958
383	9q34.3	135	UAP1L1	UDP-N-acteylglucosamine pyrophosphorylase 1-like 1
384	9q34	135	MAN1B1	mannosidase, alpha, class 1B, member 1
385	9q34.3	135	DPP7	dipeptidylpeptidase 7
386	9q34.3	135	LOC389815	LOC389815
387	9q34.3	135	GRIN1	glutamate receptor, ionotropic, N-methyl D-aspartate 1
388	9q34.3	135	LOC389816	similar to CDNA sequence BC004853
389	9q34.3	135	LOC389817	LOC389817
390	9q34.3	135	ANAPC2	anaphase promoting complex subunit 2
391	9q34.3	135	SSNA1	Sjogren's syndrome nuclear autoantigen 1
392	9q34.3	135	C9orf75	chromosome 9 open reading frame 75

No	Cytogenetic Location	Start (Mb)	Gene Name	Short Description
393	9q34.3	135	MGC14327	hypothetical protein MGC14327
394	9q34.3	136	NELF	nasal embryonic luteinizing hormone-releasing hormone factor
395	9q34.3	136	FLJ43070	FLJ43070 protein
396	9q34.3	136	MRPL41	mitochondrial ribosomal protein L41
397	9q34.3	136	LOC92715	hypothetical protein BC017335
398	9q34.3	136	ZMYND19	zinc finger, MYND domain containing 19
399	9q34.3	136	ARRDC1	arrestin domain containing 1
400	9q34.3	136	C9orf37	chromosome 9 open reading frame 37
401	9q34.3	136	LOC392402	similar to protein phosphatase 2A inhibitor-2 I-2PP2A
402	9q34.3	136	Eu-HMTase1	euchromatic histone methyltransferase 1
403	9q34	136	CACNA1B	calcium channel, voltage-dependent, L type, alpha 1B subunit
404	9q34.3	136	LOC286222	similar to beta-tubulin 4Q

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The *COL5A1* gene and Achilles tendon pathology

G. G. Mokone[1], M. P. Schwellnus[1], T. D. Noakes[1], M. Collins[2]

[1]UCT/MRC Research Unit for Exercise Science and Sports Medicine, Department of Human Biology, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa, [2]Medical Research Council of South Africa, Cape Town, South Africa
Corresponding author: Dr Malcolm Collins, UCT/MRC Research Unit for Exercise Science and Sports Medicine, UCT, PO Box 115, Newlands 7725, South Africa. Tel.: +27 21 650 4574, Fax: +27 21 686 7530, E-mail: mcollins@sports.uct.ac.za

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Purpose: **There is an increase in the incidence of Achilles tendon injuries as a result of the participation in physical activity. It has been suggested that some individuals have a genetic predisposition to Achilles tendon pathology (ATP). The aim of this study was to determine whether the α 1 type V collagen (*COL5A1*) gene, which encodes for a tendon protein, is associated with the symptoms of ATP.** ***Methods:*** **One-hundred and eleven Caucasian subjects diagnosed with ATP and 129 Caucasian control (CON) subjects were genotyped for the *Bst*UI and *Dpn*II restriction fragment length polymorphisms (RFLPs) within the *COL5A1* gene.** ***Results:*** **There was a significant difference in the allele frequencies of the *COL5A1 Bst*UI RFLP between the ATP and CON subjects ($P = 0.006$). The frequency of the A2 allele was significantly higher in the CON group (29.8%) than in the ATP group (18.0%) (odds ratio of 1.9; 95% confidence interval (CI) 1.3–3.0; $P = 0.004$). This allele had a stronger protective role when only the 72 patients diagnosed with chronic Achilles tendinopathy were analyzed (odds ratio of 2.6; 95% CI 1.5–4.5).** ***Conclusions:*** **The *COL5A1 Bst*UI RFLP is associated with ATP and more specifically, chronic Achilles tendinopathy. Individuals with an A2 allele of this gene are less likely of developing symptoms of chronic Achilles tendinopathy.**

There is a reported increase in the incidence of injuries of the Achilles tendon in those who participate in competitive and recreational physical activity (Jozsa et al., 1989b). Although the causes of Achilles tendon injuries are poorly understood, both intrinsic and extrinsic factors have been implicated in its etiology. The relationship of these factors to Achilles tendon pathology (ATP) has recently been reviewed (Paavola et al., 2002; Riley, 2004). Specific intrinsic factors that have been identified include age, gender, body weight, vascular perfusion, nutrition, anatomical variants, joint laxity, muscular weakness or imbalance, the use of certain drugs, systemic disease and a history of a previous injury. Extrinsic factors include the type of activity, occupation, training, physical load, shoes and environmental conditions.

It has been proposed that, in addition to these non-genetic factors, certain genetic elements might, in part, be associated with an individual's susceptibility to Achilles tendon injuries. More specifically, several investigators have suggested that a gene(s) on the tip of chromosome 9q, closely linked to the *ABO* blood group gene, is associated with ATP (reviewed in Kannus & Natri, 1997). Jozsa et al. (1989a), Jozsa et al. (1989b), Kujala et al., (1992) and Kannus and Natri (1997) have shown that blood group O, and by implication the *ABO* gene, is associated with tendon injuries in a group of Hungarian or Finnish patients. But other studies have not shown an association between the ABO blood group with tendon pathology (Leppilahti et al., 1996; Maffulli et al., 2000). Recently, Årøen et al. also suggested, based on their findings that individuals who had ruptured an Achilles tendon had an increased risk of rupturing their contralateral tendon, the possible involvement of genetic elements in the etiology of ATP (Årøen et al., 2004).

Tendons have a highly ordered hierarchical structure made up of tightly packed protein bundles consisting predominantly of type I collagen fibers (reviewed in Silver et al., 2003). Trace amounts of other collagens, such as types III and V, form heterotypic fibers with the type I collagen found in tendons (reviewed in Birk, 2001; Silver et al., 2003). Increases in type V collagen content have been reported with age in the rabbit patellar tendon and in biopsy samples of degenerative tendons (Dressler et al., 2002; Goncalves-Neto et al., 2002).

The pro-α1(V) chain is found in most of the isoforms of type V collagen and is encoded by the α 1 type V collagen (*COL5A1*) gene, which has been mapped to the same locus as the *ABO* gene on chromosome 9q34 (Caridi et al., 1992). The gene was therefore identified as an ideal candidate genetic marker of ATP. Pro-α2(V) and pro-α3(V) chains are

also found in some of the type V collagen isoforms. Neither of the genes that encode these two α-chains have, however, been mapped to human chromosome 9 and were therefore not investigated as candidate genes in this study (reviewed in Myllyharju & Kivirikko, 2001). In addition, several mutations within the *COL5A1* and *COL5A2* genes have been shown to cause more severe connective tissue disorders such as some of the Ehlers–Danlos syndromes (EDS), which have been shown to affect tendons (reviewed in Myllyharju & Kivirikko, 2001; Riley, 2004).

The *COL5A1* gene contains a *Bst*UI and a *Dpn*II restriction fragment length polymorphisms (RFLPs) within its 3′-untranslated region (UTR) (Greenspan & Pasquinelli, 1994). To our knowledge, the influence of these polymorphisms on the expression of the *COL5A1* gene and the ultimate function of type V collagen is unknown. The aim of the study therefore was to determine whether the *Bst*UI and/or *Dpn*II RFLPs within the 3′-UTR of the *COL5A1* gene are associated with ATP.

Material and methods

Subjects

One-hundred and eleven Caucasian patients with a current or past clinical history of ATP were recruited from the Medical Practice at the Sports Science Institute of South Africa and other Clinical Practices within the Cape Town area in South Africa. The ATP patients included 72 with chronic tendinopathies (TEN) and 39 with either a complete (36 of 39) or partial (three of 39) rupture (RUP). They were all physically active prior to the development of symptoms. The clinical practices invited their eligible patients to participate in the study, by sending them a letter informing them about the study. Subjects were requested to contact the investigators if they were interested in volunteering for the study. An experienced clinician initially made the diagnosis of chronic Achilles tendinopathy (69 of 72) using clinical criteria. The diagnostic criteria for every subject were reviewed and confirmed by one of the investigators (M. S.). 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) (Kader et al., 2002; Paavola et al., 2002; Schepsis et al., 2002). In addition to these clinical diagnostic criteria, soft-tissue ultrasound examination was performed in a sub-group (22 of 72) of subjects to confirm the diagnosis of the affected Achilles tendon.

The diagnosis of Achilles tendon rupture was made clinically using standard validated criteria (Leppilahti & Orava, 1998; Schepsis et al., 2002; Maffulli et al., 2003) and confirmed in all cases by examination at the time of surgery (34 of 39) and/or by ultrasound imaging (five of 39), magnetic resonance imaging (MRI) (two of 39) or computerized tomography (CT) scan (one of 39).

One-hundred and twenty-nine apparently healthy physically active Caucasian control (CON) subjects without any history of ATP were also recruited for this study from various recreational sporting clubs. The subjects were matched for age and gender. To avoid any possible effects of population stratification, the ATP and CON groups were also similarly matched for their country of birth.

Approval for this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences within the University of Cape Town. Once recruited, both the ATP and CON subjects were required to complete an informed consent form and personal particulars, physical activity and medical history questionnaires prior to participation. Subjects 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 prior to the onset of symptoms were excluded from the study. This was necessary because of the known association between fluoroquinolone antibiotic (van der Linden et al., 2001) or possibly corticosteroids use, and an increased risk of Achilles tendon rupture (Leppilahti & Orava, 1998). Furthermore, ATP and CON subjects who had been diagnosed with any connective tissue disorders or any other systemic diseases believed to be associated with ATP, such as, but not limited to, EDS, benign hypermobility joint syndrome, rheumatoid arthritis, systemic lupus erythematosus, hyperparathyroidism, renal insufficiency, diabetes mellitus and familial hypercholesterolemia were also excluded from the study (Leppilahti & Orava, 1998).

Sample collection, total DNA extraction and blood grouping analysis

Approximately 4.5 mL of venous blood was collected from each subject into ethylenediaminetetraacetic acid (EDTA) vacutainer tubes by venipuncture of a forearm vein and stored at 4 °C until total DNA extraction. Total DNA was extracted from the sample as described by Lahiri and Nurnberger (1991) with some modifications. Briefly, the blood samples were transferred to 15 mL polypropylene tubes, to which two volumes 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 (Sigma, St. Louis, MO, USA) was added to lyse the red blood cells. After a 10 min incubation at room temperature, the white blood cells were pelleted by centrifugation at 1200 × *g* at room temperature for 10 min and washed at least once with one volume of TKM1 buffer. The washed 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% sodium dodecyl sulfate by incubation for at least 10 min at 55 °C or until the pellets had dissolved. One hundred and fifty microlitres 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 s. The samples were transferred to 1.5 mL microfuge tubes and the protein precipitated by centrifugation at 13 000 r.p.m. (15 000 × g) for 5 min at room temperature. Five hundred microlitres 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 r.p.m. (15 000 × g) for 2 min at room temperature. The precipitated DNA was air dried for 30 min, resuspended in at least 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) either by incubation for 1 h at 65 °C or over-night at room temperature, and stored at 4 °C until polymerase chain reaction (PCR) analysis.

COL5A1 genotyping

A 667 bp fragment containing the *Bst*UI and *Dpn*II RFLPs within the 3′-UTR of the *COL5A1* gene was PCR amplified as described by Greenspan and Pasquinelli (1994). The PCR was carried out in a total volume of 60 μL containing at least 100 ng DNA, 20 pmol of the forward and reverse primers, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dTTP, dCTP and dGTP) and 2.5 U of DNA *Taq* polymerase (Invitrogen Life Technologies, Carlsbad, California, USA), using a PCR Express Thermal Cycler (Hybaid Limited, Middlesex, UK). The amplification was performed with an initial denaturing step at 94 °C for 3 min, followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 53 °C for 1 min, extension at 72 °C for 1.5 min, and a final extension step at 72 °C for 8 min. The PCR products were digested with *Bst*UI to produce 351 and 316 bp fragments for the A1 allele, 316, 271 and 80 bp fragments for the A2 allele and a 667 bp fragment for the A3 allele. The PCR products were also digested with *Dpn*II to produce 418, 194, 40 and 15 bp fragments for the B1 allele and 612, 40 and 15 bp fragments for the B2 allele. The resulting fragments were separated, together with 100 bp DNA ladder known size markers (Promega Corporation, Madison, Wisconsin, USA), on 5% non-denaturing polyacrylamide gels and visualized 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 (Fig. 1).

Statistical analyses

The required sample size for this study was determined using QUANTO Version 0.5 (http://hydra.usc.edu/gxe) (Gauderman, 2002). Data were analyzed using the STATISTICA version 6.1 (StatSoft Inc., Tulsa, Oklahoma, USA) and GraphPad InStat version 2.05a (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 ATP and CON groups, as well as the TEN and RUP sub-groups.

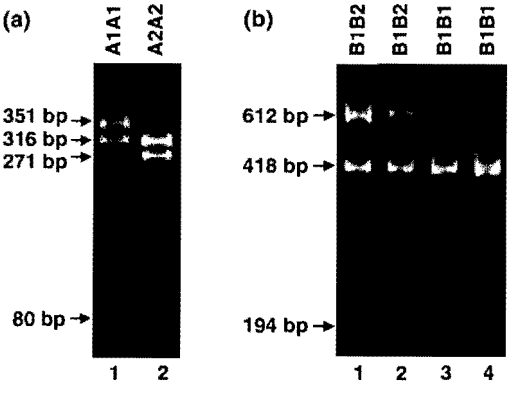

Fig. 1. Typical 5% non-denaturing polyacrylamide gels showing the common (a) *Bst*UI and (b) *Dpn*II restriction fragment length polymorphism (RFLP) genotypes. The sizes of the various DNA fragments are indicated on the left size of each gel. The 40 and 15 bp products produced during digestion of the polymerase chain reaction fragments with *Dpn*II are not shown in panel (b). (Panel a) Lane 1 is an A1A1 genotype, while lane 2 is an A2A2 genotype for the *Bst*UI RFLP. (Panel b) Lanes 1 and 2 are a B1B2 genotype, while lanes 3 and 4 are a B1B1 genotype of the *Dpn*II RFLP.

When the overall F value was significant, a least significant difference post-hoc test was used to identify specific differences. Statistical significance was accepted when $P<0.05$. Where applicable, data are presented as means ± standard deviations (SD) with the number of subjects in parentheses. Pearson's chi-square analysis was used to analyze differences in the genotype and allele frequencies between the ATP and CON groups. The genotype frequencies of the *Bst*UI RFLP of the *COL5A1* gene were analyzed using Monte Carlo simulations (CLUMP version 2.0 program) (Sham & Curtis, 1995).

Results

Subject characteristics

As shown in Table 1, the ATP, TEN, RUP and CON groups were similarly matched for age, height and country of birth. The ATP, TEN and CON groups were similarly matched for gender, while the RUP group (79.5%) contained significantly more male subjects than the CON group (61.7%, $P=0.040$). In addition, the ATP, TEN and RUP groups were significantly heavier with corresponding higher body mass indexes than the CON group.

In the TEN group, the additional documented clinical criteria to confirm the diagnosis were tenderness to palpation (59 of 72), early morning stiffness (39 of 72), a history of swelling (24 of 72), early morning pain (15 of 72), palpable thickening (14 of 72) and a positive "shift" test (nine of 72). In 22 of the 72 subjects, the diagnosis was confirmed by soft-tissue ultrasound examination of the affected Achilles tendon. There were 29 of the 72 subjects with confirmed bilateral Achilles tendinopathy.

In the RUP group, 38 of 39 subjects experienced acute severe pain in the posterior lower leg as the main presenting symptom. The diagnosis was confirmed in all these subjects by either direct examination at the time of surgery (34 of 39) or by imaging (eight of 39) (soft-tissue ultrasound, MRI or CT). Four of the 39 subjects had confirmed bilateral ruptures of the Achilles tendon, and 16 of 39 subjects had a history of tendinopathy prior to rupture.

Range of motion and flexibility were assessed in 36 of the 129 CON and 65 of the 111 ATP subjects (data not shown). None of the subjects tested were hypermobile. In addition, none of the subjects included in this study had symptoms or signs (skin hyperextensibility, bruising, recurrent joint effusions, subluxations or dislocations, ocular manifestations or cardiovascular manifestations) of EDS or benign hypermobility joint syndrome when their medical examinations were reviewed by the investigating clinician (M. S.).

The activity resulting in injury in the majority of the ATP subjects was running (47 of 111, 42.3%) or playing squash (17 of 111, 15.3%), while the remain-

Table 1. Characteristics of the control (CON) and Achilles tendon pathology (ATP) groups, as well as the Achilles tendinopathy (TEN) and Achilles rupture (RUP) sub-groups

	CON	ATP	TEN	RUP
Age (years)*	40.3 ± 11.0 (122)	40.1 ± 14.0 (108)	39.7 ± 15.3 (69)	40.8 ± 11.3 (39)
Height (cm)	175 ± 9 (121)	176 ± 9 (108)	176 ± 10 (69)	176 ± 8 (39)
Weight (kg)	71.1 ± 12.1 (125)[a,c,e]	80.8 ± 15.1 (108)[a]	77.1 ± 13.7 (69)[e,h]	87.3 ± 15.3 (39)[c,h]
Body mass index (kg/cm^2)	23.2 ± 2.7 (121)[b,d,f]	25.9 ± 3.9 (108)[b]	24.7 ± 3.3 (69)[f,i]	28.1 ± 4.1 (39)[d,i]
Gender (% males)	61.7 (128)[g]	73.0 (111)	69.4 (72)	79.5 (39)[g]
Country of birth (% South Africa)	71.0 (124)	75.7 (107)	73.5 (68)	79.5 (39)

Values are expressed as mean ± standard deviation or a frequency (%) where applicable. Number of subjects (n) is in parentheses.
[a,b]CON vs ATP ($P<0.001$); [c,d]CON vs RUP ($P<0.001$); [e]CON vs TEN ($P=0.006$); [f]CON vs TEN ($P=0.004$); [g]CON vs RUP ($P=0.040$); [h,i]TEN vs RUP ($P<0.001$).
*The age of the ATP group as well as the TEN and RUP sub-groups are the age of onset of the symptoms of Achilles tendon pathology.

Table 2. Participation in physical activity and training of the control (CON) and Achilles tendon pathology (ATP) groups, as well as the Achilles tendinopathy (TEN) and Achilles rupture (RUP) sub-groups

	CON	ATP	TEN	RUP
Running (years)	7.9 ± 8.1 (129)	8.6 ± 10.6 (111)	9.4 ± 10.4 (72)	7.0 ± 10.9 (39)
Running in the last 2 years (h/week)	3.3 ± 2.9 (126)[a,b]	2.1 ± 2.6 (98)[a]	2.6 ± 2.8 (63)	1.2 ± 1.9 (35)[b]
High-impact sports (years)	11.5 ± 8.5 (129)[c,d,e]	20.2 ± 13.7 (111)[c]	18.5 ± 14.1 (72)[d]	23.5 ± 12.4 (39)[e]
High-impact sports in the last 2 years (h/week)	5.0 ± 4.7 (129)	4.1 ± 5.1 (111)	4.5 ± 5.2 (72)	3.2 ± 5.0 (39)

Values are expressed as mean ± standard deviation. Number of subjects (n) is in parentheses.
[a]CON vs ATP ($P=0.002$); [b,e]CON vs RUP ($P<0.001$); [c]CON vs ATP ($P<0.001$); [d]CON vs TEN ($P<0.001$).

ing injuries occurred as a result of participating in a variety of sports and activities.

As shown in Table 2, all the subject groups and sub-groups were matched for the number of years participated in running. Of the CON, ATP, TEN and RUP subjects, 74%, 56%, 61% and 46%, respectively, participated in running. Over the last 2 years, both the ATP and RUP groups, but not the TEN group, trained significantly less than the CON group. The three symptomatic Achilles pathology groups, however, participated for significantly more years in high-impact sports than the CON group. There were no significant differences in the hours of training in high-impact sports over the last 2 years between the groups. There were also no significant differences in years of participation and the level of training over the last 2 years when only the squash players were analyzed (data not shown).

COL5A1 genotype and allele frequencies

There was a significant difference when the three *COL5A1 Bst*UI RFLP alleles (A1, A2 and A3) of the CON subjects were compared with the ATP group (Pearson's $\chi^2 = 10.3$, $P = 0.006$) [Fig. 2(a)]. The frequencies of the A1 and A3 alleles were higher in the ATP group (169 A1, 76.1% and 13 A3, 5.9%) than in the CON group (173 A1, 67.1% and eight A3, 3.1%), while the frequency of the A2 allele was higher in the CON subjects (77 A2, 29.8%) than in the ATP group (40 ATP, 18.0%) (odds ratio of 1.9; 95% confidence interval (CI) 1.3–3.0; $P = 0.004$).

When the ATP group was sub-divided into more homogeneous pathologies, namely, chronic TEN or RUP sub-groups, there was a stronger significant difference in the frequencies of the distribution of the A1, A2 and A3 alleles when the CON and TEN groups were compared (Pearson's $\chi^2 = 14.0$, $P = 0.0009$) [Fig. 2(a)]. The frequencies of the A1 and A3 alleles were higher in the TEN group (115 A1, 79.9% and nine A3, 6.2%) than in the CON group. The frequency of the A2 allele was higher in the CON group than in the TEN group (20 A2, 13.9%) (odds ratio of 2.6; 95% CI 1.5–4.5; $P = 0.0005$). Although it should be interpreted with caution because of the small sample size (78 alleles), there was no significant difference in the distribution of these three alleles between the CON and RUP group (54 A1, 69.2%; 20 A2, 25.7% and four A3, 5.1%) (Pearson's $\chi^2 = 1.1$, $P = 0.578$) [Fig. 2(a)].

Figure 2(b) shows that there were no significant differences in the distribution of the two *COL5A1 Dpn*II RFLP alleles (B1 and B2) between the CON group and either the entire ATP group (Pearson's $\chi^2 = 0.6$, $P = 0.453$) or the TEN (Pearson's $\chi^2 = 0.6$, $P = 0.424$) and RUP (Pearson's $\chi^2 = 0.04$, $P = 0.837$) sub-groups.

There were no significant differences in the genotype frequencies of the *COL5A1 Bst*UI (Table 3) and *Dpn*II (Table 4) RFLPs between the CON and ATP (Pearson's χ^2 of the *Bst*UI RFLP = 5.2, $P = 0.169$

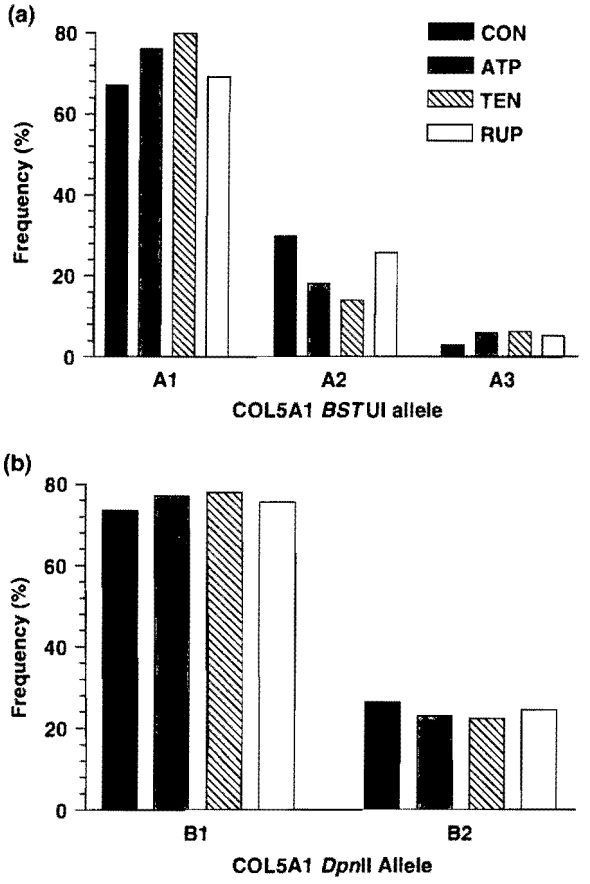

Fig. 2. Allele frequencies of the (a) *Bst*UI and (b) *Dpn*II restriction fragment length polymorphism (RFLP) within the α 1 type V collagen (*COL5A1*) gene of the asymptomatic control subjects (CON, solid bars), as well as the symptomatic Achilles tendon pathology (ATP, gray bars), chronic Achilles tendinopathy (TEN, hatched bars) and Achilles tendon rupture (RUP, clear bars) patients. (a) $P = 0.006$, CON vs ATP; $P = 0.0009$, CON vs TEN; $P = 0.578$, CON vs RUP and (b) $P = 0.453$, CON vs ATP; $P = 0.424$, CON vs TEN; $P = 0.837$, CON vs RUP.

and Pearson's χ^2 of the *Dpn*II RFLP = 3.7, $P = 0.160$) groups, nor the CON and TEN (Pearson's χ^2 of the *Bst*UI RFLP = 7.7, $P = 0.057$ and Pearson's χ^2 of the *Dpn*II RFLP = 2.8, $P = 0.243$) or RUP (Pearson's χ^2 of the *Bst*UI RFLP = 6.6, $P = 0.089$ and Pearson's χ^2 of the *Dpn*II RFLP = 1.6, $P = 0.460$) sub-groups. Individuals with an A2A2 genotype were under-represented in the ATP (odds ratio of 2.1; 95% CI 1.1–4.1; $P = 0.035$) and TEN (odds ratio of 2.9; 95% CI 1.2–6.6; $P = 0.018$) subjects.

Discussion

The main finding of this study was that the three alleles produced by the *Bst*UI RFLP within the 3′-UTR of the *COL5A1* gene were associated with ATP

Table 3. Relative frequencies of *Bst*UI restriction fragment length polymorphism genotype of the *COL5A1* gene within the control (CON) and Achilles tendon pathology (ATP) groups, as well as the Achilles tendinopathy (TEN) and Achilles rupture (RUP) sub-groups

Genotype	CON ($n = 129$)	ATP ($n = 111$)	TEN ($n = 72$)	RUP ($n = 39$)
A1A1 (%)	62.8 (81)	70.3 (78)	76.4 (55)	59.0 (23)
A1A2 (%)	7.0 (9)	6.3 (7)	4.2 (3)	10.3 (4)
A1A3 (%)	1.6 (2)	5.4 (6)	2.8 (2)	10.3 (4)
A2A2 (%)	26.4 (34)	14.4 (16)	11.1 (8)	20.5 (8)
A2A3 (%)	0 (0)	0.9 (1)	1.4 (1)	0 (0)
A3A3 (%)	2.3 (3)	2.7 (3)	4.2 (3)	0 (0)

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

Table 4. Relative frequencies of *Dpn*II restriction fragment length polymorphism genotype of the *COL5A1* gene within the control (CON) and Achilles tendon pathology (ATP) groups, as well as the Achilles tendinopathy (TEN) and Achilles rupture (RUP) sub-groups

Genotype	COn ($n = 129$)	ATP ($n = 111$)	TEN ($n = 72$)	RUP ($n = 39$)
B1B1 (%)	58.1 (75)	58.6 (65)	59.7 (43)	56.4 (22)
B1B2 (%)	31.0 (40)	36.9 (41)	36.1 (26)	38.5 (15)
B2B2 (%)	10.9 (14)	4.5 (5)	4.2 (3)	5.1 (2)

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

($P = 0.006$). There was a significant higher frequency of the A2 allele of this gene in the asymptomatic control subjects (CON 29.8% vs ATP 18.0%). Individuals with the A2 allele were therefore less likely of developing symptoms of tendon pathology (odds ratio = 1.9; 95% CI 1.3–3.0; $P = 0.004$).

It has previously been suggested that a gene(s) on the tip of the long arm of chromosome 9, closely linked to the ABO blood group gene, is associated with ATP (reviewed in Kannus & Natri, 1997). Since the *ABO* gene on chromosome 9q34 encodes for distinct transferases, some investigators have speculated that these enzymes, not only determine the structure of the glycoprotein antigens on red blood cells, but also the structure of some proteins making up the ground substance of tendons (Jozsa et al., 1989a; Bennett et al., 1995). It is however more likely that genes, such as *COL5A1*, which have also been mapped to chromosome 9q34 (Caridi et al., 1992), and known to encode for proteins involved in tendon structure, development and regeneration, are better candidate genes for ATP than the *ABO* gene.

The *COL5A1* gene encodes for the pro-α1(V) chain which is found in most of the isoforms of type V collagen (reviewed in Ristiniemi & Oikarinen, 1989). The major isoform of type V collagen is a heterotrimer consisting of two pro-α1(V) chains and one pro-α2(V) chain. Trace amounts of type V collagen are found in tendons where it forms heterotypic

fibers with the major structural collagen, namely type I collagen (reviewed in Birk, 2001; Silver et al., 2003). Although most investigators have speculated, based on the function of type V collagen in the cornea, that the protein plays an important role in regulating fibrillogenesis and modulating fibril growth in tendons, some investigators have suggested that the function of type V collagen in tendons, and other tissues where its content is low, is actually unknown (reviewed in Birk, 2001; Riley, 2004). Although there is no consensus about the function of type V collagen in tendons, Dressler et al. (2002) have reported an age-dependent increase in the content of the protein, together with a decrease in fibril diameter and the biomechanical properties in the rabbit patellar tendon. In addition, Goncalves-Neto et al. (2002) have shown an increase in types III and V collagen together with a reduction in the content of type I collagen in biopsy samples of degenerative tendons from patients with posterior tibial tendon dysfunction syndrome. Because of (i) its proximity to the *ABO* gene on chromosome 9 and (ii) the presence and proposed function of type V collagen in tendons, we propose that the *COL5A1* gene is a better candidate genetic marker than the *ABO* gene for ATP.

The allele distributions of the *COL5A1 Dpn*II RFLP within the control subjects and the various groups of subjects with symptoms of ATP were similar to those of previously reported values. In addition there was no significant difference in the allele distribution of the *COL5A1 Bst*UI RFLP when the control subjects were compared with previously reported values ($P = 0.206$) (Greenspan & Pasquinelli, 1994).

An additional finding of this study was that the alleles of the *COL5A1 Bst*UI RFLP were strongly associated with chronic Achilles tendinopathy ($P = 0.0009$), since individuals with the A2 allele were less likely to present with symptoms of tendinopathy (odds ratio of 2.6; 95% CI 1.5–4.5, $P = 0.0005$). This RFLP was however not associated with Achilles tendon ruptures in this study, suggesting that the etiology of ruptures and tendinopathies are distinct. These findings must however be interpreted with caution since only 78 alleles were analyzed.

Although the *COL5A1* gene is an ideal marker for ATP and more specifically chronic Achilles tendinopathy, the findings of this study do not prove that type V collagen is involved in the etiology of tendon pathology. It is possible that another gene closely linked to the *COL5A1* and *ABO* genes on the tip of the long arm of chromosome 9 encodes for a protein, which is directly involved in the pathogenesis of Achilles tendon injuries. One such gene, the tenascin-C (*TNC*) or hexabrachion (*HXB*) gene is expressed in tendons (Chiquet & Fambrough, 1984; Jarvinen et al., 1999). Since tenascin-C is able to bind to various components of the extracellular matrix and to cell receptors, it is believed to play an important role in regulating cell–matrix interactions (reviewed in Jones & Jones, 2000). In normal adult tendons, tenascin-C is localized predominantly in regions responsible for transmitting high levels of mechanical force such as the myotendinous and osteotendinous junctions (Chiquet & Fambrough, 1984; Jarvinen et al., 1999). The protein is also localized around the cells and the collagen fibers (Jarvinen et al., 2003). In addition, Jarvinen et al. (1999, 2003) have shown that expression of the *TNC* gene is regulated in a dose-dependent manner by mechanical loading in tendons. Mokone et al. (in press) have recently shown that the GT dinucleotide repeat polymorphism within intron 17 of the *TNC* gene is also associated with ATP. The possible role(s) of type V collagen and/or tenascin-C in the development of ATP needs to be investigated. Our results nevertheless suggest that both the *COL5A1* and *TNC* genes are markers of ATP.

Finally, it is highly unlikely that a single gene or genes in the vicinity of chromosome 9q34 are exclusively associated with the development of the symptoms of ATP. It is perhaps more probable that this condition is polygenic in nature and that other genes which encode for important structural components of tendons are also associated with ATP.

In addition to the genetic factors identified in this study and others that may be identified in the future, several non-genetic intrinsic factors, such as, amongst others, age, gender, body weight and a history of a previous injury; as well as extrinsic factors, such as type of activity and training, have been implicated in the etiology of ATP (reviewed in Riley, 2004). Because the symptomatic subjects in this study were significantly heavier than the control subjects and had also participated for a significantly more years in high-impact sports, we cannot exclude the possibility that an interaction of weight and/or physical activity exposure with the *COL5A1* gene was responsible for the development of symptoms of ATP. It should be noted however that because this was a retrospective study we could not record body weight of the subjects accurately at the time of injury. Anecdotally, many subjects reported increases in their body weight after injury as a result of a decrease in physical activity. Increased body weight has been documented as a risk factor for lower extremity injuries in some studies (Murphy et al., 2003), and therefore has also been suggested as an intrinsic risk factor for ATP (Paavola et al., 2002; Riley, 2004). However, to our knowledge no studies have shown that increased body weight is a specific risk factor for Achilles tendon injuries. It can however be noted that

there is a reported interaction of obesity with the *COL9A3* gene and lumber disc degeneration (Solovieva et al., 2002). Therefore, the possible interaction of non-genetic factors, such as body weight and exposure to physical activity, with the *COL5A1* gene or any other gene needs to be investigated.

In conclusion, the *Bst*UI RFLP within the *COL5A1* gene is associated with ATP and more specifically chronic Achilles tendinopathy and that the A2 allele of this gene appears to protect individuals from developing symptoms.

Perspectives

Although a genetic predisposition to ATP has been suggested, no specific genes have been shown to be associated with this pathology. This is the first study to demonstrate an association between a polymorphism within a gene expressed in tendons, namely *COL5A1*, and symptoms of ATP in physically active individuals. The novel finding of this study is that the *Bst*UI RFLP within the 3'-UTR of the *COL5A1* gene is associated with ATP and more specifically chronic Achilles tendinopathy. The association of the *COL5A1* gene with Achilles tendon injuries however does not prove that type V collagen, an important component of tendons, is directly involved in the development of this pathology. Perhaps other closely linked genes are involved. Besides the involvement of genetic factors, various non-genetic intrinsic and extrinsic factors have been implicated in the etiology of ATP. The interaction of these factors with an individual's genetic background in the development of ATP needs to be investigated. In conclusion, this study suggests that individuals with the A2 allele of the *COL5A1* gene are less likely of developing symptoms of chronic Achilles tendinopathy.

Key words: genetics, polymorphism, type V collagen, tendinopathy, rupture.

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The Guanine-Thymine Dinucleotide Repeat Polymorphism Within the Tenascin-C Gene Is Associated With Achilles Tendon Injuries

Gaonyadiwe G. Mokone,* BSc(Med)(Hons), Mamta Gajjar,[†‡] BSc, Alison V. September,[‡] PhD, Martin P. Schwellnus,* MBChB, MD, Jacquie Greenberg,[‡] PhD, Timothy D. Noakes,* MBChB, MD, DSc(Med), and Malcolm Collins,[§||] PhD

*From the *UCT/MRC Research Unit for Exercise Science and Sports Medicine, Department of Human Biology, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa, the [‡]Division of Human Genetics, Department of Clinical Laboratory Sciences, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa, and the [||]Medical Research Council of South Africa, Tygerberg, South Africa*

Background: Although there is a high incidence of tendon injury as a result of participation in physical activity, the mechanisms responsible for such injuries are poorly understood. Investigators have suggested that some people may have a genetic predisposition to develop tendon injuries; in particular, genes on the tip of the long arm of chromosome 9 might, at least in part, be associated with this condition. The tenascin-C gene, which has been mapped to chromosome 9q32-q34, encodes for a structural component of tendons.

Hypothesis: The tenascin-C gene is associated with Achilles tendon injury.

Study Design: Case control study; Level of evidence, 3.

Methods: A total of 114 physically active white subjects with symptoms of Achilles tendon injury and 127 asymptomatic, physically active white control subjects were genotyped for the guanine-thymine dinucleotide repeat polymorphism within the tenascin-C gene.

Results: A significant difference in the allele frequencies of this polymorphism existed between the 2 groups of subjects (χ^2 = 51.0, P = .001). The frequencies of the alleles containing 12 repeats (symptomatic group, 18.9% vs control group, 10.2%) and 14 repeats (symptomatic group, 9.2% vs control group, 0.8%) were significantly higher in the symptomatic group, while the frequencies of the alleles containing 13 repeats (symptomatic group, 8.8% vs control group, 24.0%) and 17 repeats (symptomatic group, 7.5% vs control group, 20.1%) were significantly lower in this same group. Subjects who were homozygous or heterozygous for the underrepresented alleles (13 and 17 repeats) but who did not possess an overrepresented allele (12 and 14 repeats) may have a lower risk of developing Achilles tendon injuries (odds ratio, 6.2; 95% confidence interval, 3.5-11.0; P < .001).

Conclusions: The guanine-thymine dinucleotide repeat polymorphism within the tenascin-C gene is associated with Achilles tendon injury. Alleles containing 12 and 14 guanine-thymine repeats were overrepresented in subjects with tendon injuries, while the alleles containing 13 and 17 repeats were underrepresented.

Clinical Relevance: Persons who have variants of the tenascin-C gene with 12 and 14 guanine-thymine repeats appear to have a 6-fold risk of developing Achilles tendon injuries.

Keywords: genetics; tendinopathy; rupture; risk factor

Many Achilles tendon injuries, ranging from chronic tendinopathy to rupture, are associated with participation in either recreational or competitive sports.[17] To date, research on Achilles tendon injury has focused primarily on its management rather than on causal mechanisms. Both intrinsic and extrinsic factors have nevertheless been implicated in the causes of this condition.[21,29] Intrinsic factors believed to be associated with the condition include previous injury, increased age, reduced blood supply to the tendon, poor nutrition, lower limb malalignment, reduced

[§]Address correspondence to Malcolm Collins, PhD, UCT/MRC Research Unit for Exercise Science and Sports Medicine, University of Cape Town, PO Box 115, Newlands 7725, South Africa (e-mail: mcollins@sports.uct.ac.za).

[†]Mamta Gajjar died in October 2002.

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flexibility, male gender, systemic disease, and corticosteroids and quinoline antibiotics. Extrinsic factors include occupation, physical activity, training errors, cold weather, running surface, and shoes and other equipment.

It has also been suggested that certain genetic elements are associated with a person's susceptibility to Achilles tendon injury. This idea has recently been highlighted by the finding of a significantly higher involvement of bilateral Achilles tendon ruptures in subjects with a previous unilateral rupture.[2] In addition, some investigators have shown that persons with blood group O are more susceptible to tendon injuries.[16,17,19,20] These findings suggest that either the *ABO* gene or a closely linked gene(s) may be associated with tendon injury.

The extracellular matrix glycoprotein tenascin-C is expressed in a variety of tissues, including tendons,[24] and is encoded by the tenascin-C (*TNC*) or hexabrachion (*HXB*) gene, which is closely linked to the *ABO* gene on chromosome 9q32-q34.[3,31] Tenascin-C binds other components of the extracellular matrix and cell receptors, and it plays an important role in regulating cell-matrix interactions.[14] In normal adult tendons, it is expressed predominately in regions responsible for transmitting high levels of mechanical force, such as the myotendinous and osteotendinous junctions.[7,8,12] The protein is also expressed around the cells and collagen fibers of the Achilles tendon.[13] In addition, Järvinen et al[12,13] have shown that expression of the *TNC* gene is regulated in a dose-dependent manner by mechanical loading in tendons.

Isoforms of the protein, with distinct functions, are produced by alternative splicing of the primary transcript.[5,14] Riley et al[30] have shown that healthy tendons express a small, 200-kd tenascin-C isoform, while degenerate tendons also express a functionally distinct, larger 300-kd isoform. In investigating this finding, Ireland et al,[11] but not Alfredson et al,[1] have reported an increase in tenascin-C expression in biopsy samples of chronic Achilles tendinopathies using cDNA arrays.

Tenascin-C is an ideal candidate genetic marker of tendon injury for the following reasons: (1) Several investigators have suggested that either the *ABO* gene or a closely linked gene(s) on the tip of the long arm of chromosome 9 may be associated with Achilles tendon injuries[16,17,20]; (2) the *TNC* gene has been mapped to chromosome 9q32-q34, which is in close proximity to the *ABO* gene[3,31]; (3) the gene encodes for tenascin-C, which is an important structural component of tendons[12,13]; and (4) tenascin-C expression is regulated by mechanical stimuli[12,13] and altered during tendon injury.[11,30] The *TNC* gene contains a guanine-thymine (GT) dinucleotide repeat polymorphism (a tandem repeat consisting of a repeated 2–base pair sequence of varying lengths in different people) within intron 17 (an intervening DNA sequence within a gene which does not encode for a protein). The influence of this polymorphism in the expression of the gene or the biological function of tenascin-C is, to our knowledge, unknown. The aim of this study was therefore to investigate the association of the GT dinucleotide repeat polymorphism within intron 17 of the *TNC* gene with Achilles tendon injuries.

MATERIALS AND METHODS

Subjects

The Medical Practice at the Sports Science Institute of South Africa and other clinical practices within the greater Cape Town area of South Africa invited patients with a current or past Achilles tendon injury (ATI) to participate in the study. Eligible patients were sent a letter informing them about the study and requesting those interested in volunteering to contact one of the investigators (G.G.M.). As a result, 114 physically active white patients with symptomatic Achilles tendon injuries (72 chronic Achilles tendinopathies and 42 acute Achilles tendon ruptures) were recruited for this study.

The diagnostic criteria for every subject were reviewed by one of the investigators (M.P.S.). The clinical diagnostic criterion for the subjects with chronic Achilles tendinopathy was gradual, progressive pain in the posterior lower limb, localized to the Achilles tendon by manual examination, for longer than 6 months. In addition, one of the following criteria had to be fulfilled for inclusion in the study: (1) early-morning pain in the Achilles tendon area, (2) early-morning stiffness in the Achilles tendon area, (3) history of swelling in the Achilles tendon area, (4) tenderness to palpation in the Achilles tendon, (5) palpable nodular thickening in the affected Achilles tendon, or (6) movement of the painful area in the Achilles tendon with plantar-dorsiflexion (positive shift test).[18,28,32] The diagnosis was confirmed by soft tissue ultrasound examination of the affected Achilles tendon in 22 subjects with tendinopathy.

For the subjects with Achilles tendon rupture, the diagnosis was made clinically using standard criteria.[23,25,32] In all cases of rupture, the diagnosis was confirmed by either examination at the time of surgery, ultrasound imaging, MRI, or CT scan.

In addition, 127 apparently healthy physically active white subjects without any history of symptomatic Achilles tendon injuries were recruited as a control (CON) group for this study. The ATI and CON groups were similarly matched for age, gender, and, to avoid any possible effects of population stratification, country of birth.

Approval of this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences within the University of Cape Town (Reference Number 170/2002). Once recruited, the subjects were required to sign an informed consent form, as well as to provide personal information and to complete physical activity and medical history questionnaires before participation.

Sample Collection, Total DNA Extraction, and Blood Grouping Analysis

Approximately 4.5 mL of venous blood was obtained from each subject by venipuncture of a forearm vein and collected into an ethylenediaminetetraacetic acid (EDTA) vacutainer tube. Blood samples were stored at 4°C until total DNA extraction, as described by Lahiri and Nurnberger,[22] could be undertaken.

TABLE 1
Characteristics of the Control (CON) and Achilles Tendon Injury (ATI) Groups[a]

	CON (n = 127)	ATI (n = 114)
Age, y	40.4 ± 10.8 (120)	39.8 ± 13.3[b] (112)
Height, cm	175 ± 9 (120)	176 ± 9 (112)
Weight, kg	71.4 ± 11.9 (123)	80.8 ± 15.3 (112)[c]
Body mass index, kg/cm^2	23.3 ± 2.7 (120)	26.0 ± 4.0 (112)[c]
Gender, % males	63.5 (126)	72.8 (114)
Country of birth, % South Africa	69.7 (122)	76.6 (111)

[a]Values are expressed as means ± standard deviations or percentages where applicable. Numbers of subjects are in parentheses.
[b]Age at onset of symptoms.
[c]$P < .001$.

TABLE 2
Participation in Physical Activity and Training of the Control (CON) and Achilles Tendon Injury (ATI) Groups[a]

	CON (n = 127)	ATI (n = 114)
Running, y	7.9 ± 8.1 (127)	8.5 ± 10.6 (114)
Running in the past 2 years, h/wk	3.3 ± 2.9 (123)	2.0 ± 2.6 (102)[b]
High-impact sports, y	11.5 ± 8.6 (127)	19.6 ± 13.0 (114)[b]
High-impact sports in the past 2 years, h/wk	5.1 ± 4.9 (127)	3.9 ± 5.0 (114)

[a]Values are expressed as means ± standard deviations. Numbers of subjects are in parentheses.
[b]$P < .001$.

TNC Genotyping

The GT dinucleotide repeat polymorphism within the *TNC* gene was polymerase chain reaction (PCR)–amplified as described by Ozelius et al.[27] The resultant PCR products were resolved with 4% polyacrylamide gels using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, Calif).

Statistical Analyses

Data were analyzed using the STATISTICA version 6.1 program (StatSoft Inc, Tulsa, Okla). A 1-way analysis of variance was used to determine any significant differences between the characteristics of the ATI and CON groups. A Pearson χ^2 test was used to analyze any differences in gender and country of birth between the experimental groups. The allele frequencies of the *TNC* gene were analyzed using the Monte Carlo test (CLUMP program, version 2.0).[33] The odds ratios and 95% confidence intervals were determined using GraphPad InStat version 2.05a (GraphPad Software, San Diego, Calif). Where applicable, data were presented as means ± standard deviations or as frequencies with numbers of subjects in parentheses. Statistical significance was accepted when $P < .05$.

RESULTS

Subject Characteristics

The ATI and CON subjects were similarly matched for age, height, gender, and country of birth (Table 1). The ATI group, however, was significantly heavier, with a corresponding higher body mass index than that of the CON group.

In the ATI group, 72 subjects were diagnosed with chronic Achilles tendinopathy and 42 with either a partial (n = 3) or complete (n = 39) rupture of the Achilles tendon. Seventeen patients had a history of tendinopathy before the rupture. In addition, 41 subjects diagnosed with Achilles tendon rupture experienced acute pain in the posterior lower leg. The diagnosis in the group with acute ruptures was confirmed at the time of surgery (37 patients), with soft tissue diagnostic ultrasound (5 patients), MRI (2 patients), or CT scan (1 patient). Diagnosis was confirmed by surgery as well as by one of the imaging techniques in 3 patients. In the subjects diagnosed with tendinopathy, 70 patients experienced gradual progressive pain, 59 were tender to palpation, while 16, 39, and 23 patients reported early-morning pain, early-morning stiffness, and a history of swelling, respectively. Palpable thickening was documented in 13 patients, and a positive shift test was documented in 8 patients. Twenty-two of the tendinopathy diagnoses were confirmed by ultrasound. Thirty subjects in the ATI group had bilateral Achilles tendon injuries (tendinopathy or rupture).

The majority of subjects injured the Achilles tendon while running (n = 49, 43.0%) or playing squash (n = 19, 16.7%). Because 65% of the subjects participated in distance running, the ATI and CON groups were matched for the number of years they participated in running activities ($P = .651$) (Table 2). During the past 2 years, the ATI subjects trained for running significantly less than did the CON group ($P < .001$). The ATI group, however, participated for significantly more years in high-impact sports than did the CON group ($P < .001$). There was no significant difference in the hours of training in high-impact sports during the past 2 years between the 2 groups ($P = .07$).

TNC Genotyping

As shown in Table 3, 18 different alleles (alternative forms of a specific gene) of the GT dinucleotide repeat polymorphism within the *TNC* gene were identified within the 2 groups (ATI, 14 alleles; CON, 15 alleles). The number of GT repeats within the identified alleles ranged from 3 to 21, with 95.0% of the alleles containing between 12 and 17 repeats. There was a significant difference in the distribu-

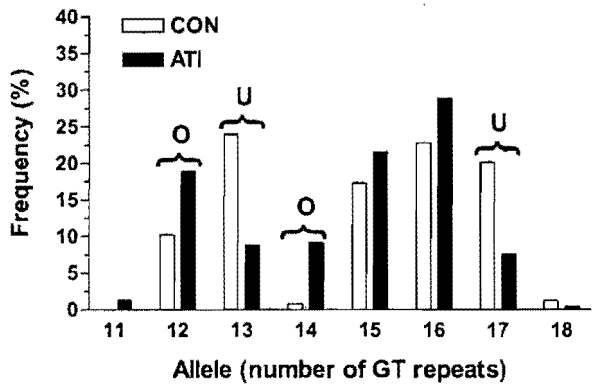

Figure 1. Allele frequencies of the guanine-thymine (GT) dinucleotide repeat polymorphism within the tenasin-C gene of the asymptomatic control (CON) and symptomatic Achilles injury (ATI) groups. The frequencies of the rare alleles containing less than 11 and greater than 18 repeats are not shown. O, overrepresented alleles; U, underrepresented alleles.

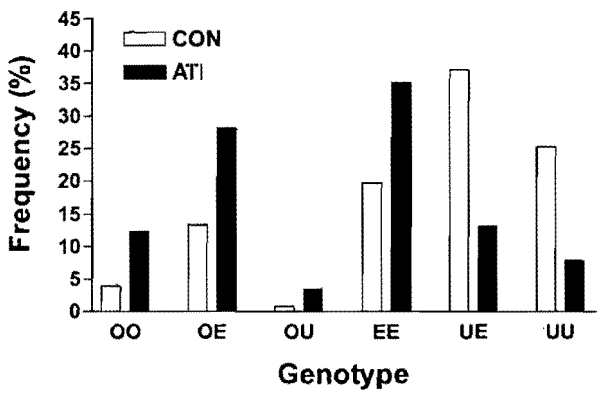

Figure 2. Genotype frequencies of the guanine-thymine (GT) dinucleotide repeat polymorphism within the tenasin-C gene of the asymptomatic control (CON) and symptomatic Achilles injury (ATI) groups. O, overrepresented alleles containing 12 or 14 GT dinucleotide repeats; U, underrepresented alleles containing 13 or 17 repeats; E, evenly distributed alleles.

tion of the alleles between the ATI and CON groups (χ^2 = 51.0, P = .001), with the alleles containing 12 and 14 GT dinucleotide repeats being significantly overrepresented in the ATI group (χ^2 = 21.6, P < .001) (Table 3 and Figure 1). The alleles containing 13 and 17 repeats were, on the other hand, significantly underrepresented in the ATI subjects (χ^2 = 42.4, P < .001) (Table 3 and Figure 1). Alleles were grouped according to those significantly overrepresented, containing 12 or 14 GT dinucleotide repeats (O), those significantly underrepresented, containing 13 or 17 repeats (U), or alleles evenly distributed (E), and were then paired by genotype (genetic constitution) (Figure 2). Subjects with a genotype of UU (32 CON, 25.2% vs 9 ATI, 7.9%) or UE (47 CON, 37.0% vs 15 ATI, 13.2%) were significantly underrepresented in the ATI group (odds ratio, 6.2; 95% confidence interval, 3.5-11.0; P < .001). With respect to the O alleles, subjects who were either homozygous (containing 2 identical alleles; OO) or heterozygous (containing 2 different alleles; OE or OU) were overrepresented in the ATI subjects (OO = 5 CON, 3.9% vs 14 ATI, 12.3%; OE = 17 CON, 13.4% vs 32 ATI, 28.1%; OU = 1 CON, 0.8% vs 4 ATI, 3.5%). Interestingly, the frequency of the EE genotype was higher in the ATI group (n = 40, 35.1%) than in the CON group (n = 25, 19.7%) (Figure 2).

TABLE 3
The Distributions of the *TNC* Gene's 18 Guanine-Thymine (GT) Dinucleotide Repeat Polymorphism Alleles Within the Control (CON) and Achilles Tendon Injury (ATI) Groups[a]

Number of GT Repeats[b]	CON (n = 254)	ATI (n = 228)
3	0.4 (1)	0.4 (1)
4	0.4 (1)	0.0 (0)
5	0.4 (1)	0.4 (1)
6	0.4 (1)	1.3 (3)
7	0.0 (0)	0.4 (1)
8	0.4 (1)	0.0 (0)
9	0.0 (0)	0.4 (1)
11	0.0 (0)	1.3 (3)
12[c]	10.2 (26)	18.9 (43)
13[d]	24.0 (61)	8.8 (20)
14[c]	0.8 (2)	9.2 (21)
15	17.3 (44)	21.5 (49)
16	22.8 (58)	28.9 (66)
17[d]	20.1 (51)	7.5 (17)
18	1.2 (3)	0.4 (1)
19	0.4 (1)	0.0 (0)
20	0.4 (1)	0.4 (1)
21	0.8 (2)	0.0 (0)

[a]Values are expressed as percentages (numbers of alleles).
[b]The number of GT repeats was calculated from the size of the polymerase chain reaction products and the published sequence, accession no. Z11654.
[c]Alleles significantly overrepresented within the ATI group (12 repeats, P = .010 and 14 repeats, P < .001).
[d]Alleles significantly underrepresented in the ATI group (13 repeats, P < .001 and 17 repeats, P < .001).

DISCUSSION

The novel finding of this study was that the allele distributions of the GT dinucleotide repeat polymorphism within the *TNC* gene were significantly different between the subjects presenting with symptoms of Achilles tendon injuries and the asymptomatic subjects. The frequency of the alleles containing 12 and 14 GT repeats was significantly higher in the symptomatic subjects, whereas the frequency of the alleles containing 13 and 17 GT repeats was significantly higher in the asymptomatic control subjects. Further analysis demonstrated that those subjects who were homozygous or heterozygous for the underrepresented alleles (containing either 13 or 17 GT repeats) were 6.2 times less likely to develop injuries of the Achilles tendon. The heterozygous subjects did not contain an allele

with either 12 or 14 repeats. Interestingly, Årøen et al[2] have recently suggested, based on their findings, that there was an increased risk for a rupture of the contralateral Achilles tendon in patients who had previously ruptured the other side, that a genetic predisposition for Achilles tendon ruptures is likely.

Tenascin-C is expressed in numerous tissues and, in particular, areas subject to high tensile stress such as tendons, ligaments, and arterial walls.[10] The expression of this extracellular matrix protein is usually absent or very low in fully differentiated tissues but is upregulated during embryogenesis, tissue regeneration, wound healing, and certain pathologic conditions.[10,14,15] Tenascin-C has a characteristic modular structure consisting of a tenascin assembly (TA) domain, heptad repeats, epidermal growth factor (EGF)–like repeats, fibronectin type III (FN-III) domains, and fibrinogen-like globular domain.[5,14] Because of its modular structure, the protein is able to interact with various other proteins such as fibronectin and with glycosaminoglycan components of the extracellular matrix. The glycosaminoglycan components include the family of chondroitin sulphate proteoglycans known as lecticans (aggrecan, versican, brevican, and neurocan). Tenascin-C also interacts with numerous cell surface receptors, such as integrins, cell adhesion molecules (CAM), and annexin II. These diverse interactions are believed to play an important role in regulating cell-matrix interactions.

Investigators have suggested that apoptosis of tendon cells precedes tendinopathy.[26] Abnormal mechanical loading is believed to initiate this programmed cell death in tendons. Because mechanical signals are able to alter the synthesis of tenascin-C,[6,7,9] which in turn is able to regulate cell-matrix interactions, it is tempting to speculate that this protein may play an important role in the proposed apoptotic model of tendinopathy. This theory invites future study.

The association of the *TNC* gene with symptoms of Achilles tendon injury does not prove that the tenascin-C protein is involved in a cause-effect relationship. It is also possible that another gene(s) within this locus may be involved. In addition, it is highly unlikely that a single gene on chromosome 9q32-q34 is exclusively associated with the development of Achilles tendon injuries, since numerous proteins are involved in tendon structure, development, and regeneration.[4,34] These alternative possibilities need to be further investigated.

Besides the involvement of genetic factors, investigators have identified a number of nongenetic intrinsic and extrinsic factors that are implicated in the development of Achilles tendon injuries. These factors include body weight, type of activity, and type of training.[29] Because the subjects in this study with symptoms of Achilles tendon injury were significantly heavier and had participated for significantly more years in high-impact sports than the asymptomatic control subjects, any possible interactions of weight and load with genetic background in the development of Achilles tendon injury cannot be excluded and needs to be further investigated. Nevertheless, although Achilles tendon injuries are typically chronic, the first symptoms usually develop within the first few years of beginning regular weightbearing physical activity. Because the subjects in the control group had been active for an average of 11.5 years and were currently participating in high-impact sports for an average of 5 hours per week, it seems improbable that their apparent resistance to Achilles tendon injuries was due purely to a lesser exposure to high-impact loading of their Achilles tendons.

In conclusion, the GT dinucleotide repeat polymorphism within intron 17 of the *TNC* gene is associated with the symptoms of chronic Achilles tendon injury. Alleles containing 12 and 14 GT repeats were overrepresented in subjects with Achilles tendon injuries, while the alleles containing 13 and 17 repeats were underrepresented.

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Appendix 5.1 Calculation of Intra-Class Coefficient (ICC) or R

Example: Using the dominant passive straight leg raise (D-SLR)

Step 1. Perform a **repeated measures analysis of variance** and obtain the following results;

Effect	SS	df	MS	F	P
Subjects	12686.4	16	792.9	_	_
D-SLR (trials)	65.0	1	65.0	3.0982	0.097480
D-SLR X Subjects (residuals)	335.5	16	21.0	_	_

Key: **SS**, sum of squares; **df**, degrees of freedom; **MS**, mean square; **F**, calculated F value; **P**, significant level.

Step 2. Calculate **MS error** using the following equation:

MS error = (SS trials + SS residuals) / (df trials + df residuals)

= (65.0 + 335.5) / (1 + 16)

= 400.5/17

= 23.55882353

Step 3. Calculation of **R (or ICC)**

R = (MS subjects – MS error) / MS subjects

= (792.9 – 23.55882353) / 792.9

= 769.3411765 / 792.9

= 0.970287774

= **0.970**

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Appendix 5.1 continued

Raw data from an excel spreadsheet showing the calculations for ICC (R)

Variable	SStrials	SSresidu	DFtrials	DFresidu	MS error	MSsubj	R	F trials	p trials
D-SLR	65	335.5	1	16	23.55882	792.9	0.970288	3.0982	0.09748
ND-SLR	4.6	346.3	1	16	20.64118	723.8	0.971482	0.2123	0.65114
D-Sho ER	26.5	489.8	1	16	30.37059	220.9	0.862514	0.865	0.366236
D-Sho IR	0.2	182.7	1	16	10.75882	131.3	0.918059	0.0161	0.900614
ND-Sho ER	11.2	391.7	1	16	23.7	222.4	0.893435	0.457	0.508756
ND-Sho IR	8.5	179	1	16	11.02941	99.9	0.889595	0.76	0.39629
D-Hip ER	1.2	117.1	1	16	6.958824	286.8	0.975736	0.1697	0.685808
D-Hip IR	89	230.8	1	16	18.81176	138.9	0.864566	6.1684	0.024464
ND-Hip ER	3.6	307.4	1	16	18.29412	300.2	0.93906	0.1852	0.672674
ND-Hip IR	16.9	211.1	1	16	13.41176	134.9	0.90058	1.2843	0.273801
D-ADF	78	195.9	1	16	16.11176	106	0.848002	6.372	0.022542
ND-ADF	14.2	296.3	1	16	18.26471	272.7	0.933023	0.7688	0.393567
D-ElbExt	0.3	180.7	1	16	10.64706	66.6	0.840134	0.023	0.880249
D-Elb Fle	1.1	221.4	1	16	13.08824	50	0.738235	0.077	0.785633
ND-Elb Ext	28.3	148	1	16	10.37059	51.5	0.798629	3.056	0.0996
ND-Elb Fle	29.2	181.4	1	16	12.38824	47	0.736421	2.574	0.128221
SR	0.12	13.63	1	16	0.808824	176.02	0.995405	0.1381	0.715072

Legend: **D**, Dominant; **ND**, Non-Dominant; **Sho ER**, Shoulder External Rotation, **Sho IR**, Shoulder Internal Rotation; **Elb Ext**, Elbow Extension, **Elb Fle,** Elbow Flexion, **Hip ER**, Hip External Rotation; **Hip IR**, Hip Internal Rotation; **SLR**, Straight Leg Raise; **ADF**, Ankle Dorsiflexion; **SR**, Sit and Reach

Appendix 6.1 Radiological data sheet used in the Achilles tendon injury study

Subject Name & CODE			**Age:**	**Date:**
Variable	**Right Leg**		**Left Leg**	
1.Musculotendinous Junction	Normal 0 Abnormal 1		Normal 0 Abnormal 1	
2.Shape	Normal 0 Fusiform 1		Normal 0 Fusiform 1	
3.Ap sagittal diameter (cm)				
4.Ap transverse diameter (cm)				
5.Colour Doppler	Normal 0	↑vasc(tendon) 1 ↑vasc(paraten) 1	Normal 0	↑vasc(tendon) 1 ↑vasc(paraten) 1
6.Kager's Fat Pad	Normal 0	↑Echogenecity 1	Normal 0	↑Echogenecity 1
7.Retrocalcaneal Bursar	Retrocalcaneal: Normal 0 Abnormal 1		Retrocalcaneal: Normal 0 Abnormal 1	
8.Echogenecity	Normal 0	Hypoechogenic 1	Normal 0	Hypoechogenic 1
9.Calcaneous	Normal 0	Abnormal 1	Normal 0	Abnormal 1
10.Miscellaneous	Normal 0	Abnormal 1	Normal 0	Abnormal 1