Genetic and Epigenetic Variation within Extracellular Matrix Genes as Risk Factors for Human Tendinopathy

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Dedication

This thesis is dedicated to my parents and sister.

May they be proud of this achievement.

To all professional and recreational athletes of the world: we did not solve the whole genetic mystery that predisposes you to injuries, but this thesis took us several steps forward.

We will get there!

Acknowledgements

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List of Scientific Output from This Thesis

Articles in Peer-Reviewed Journals

***ELN* and *FBN2* Gene Variants as Risk Factors for Two Sports-related Musculoskeletal Injuries**
L. El Khoury, M. Posthumus, M. Collins, W. van der Merwe, C.J. Handley, J. Cook and S.M. Raleigh
International Journal of Sports Medicine (Epub, ahead of print)

Polymorphic Variation Within the *ADAMTS2*, *ADAMTS14*, *ADAMTS5*, *ADAM12* and *TIMP2* Genes and the Risk of Achilles Tendon Pathology: A Genetic Association Study
L. El Khoury, M. Posthumus, M. Collins, C.J. Handley, J. Cook and S.M. Raleigh
Journal of Science and Medicine in Sport, 2013, 16 (6), 493-498

Abstracts in International Journals

The COL5A1 Gene and Risk of Achilles Tendon Pathology in a British Cohort
L. El Khoury, M. Posthumus, M. Collins, W. Ribbans, S.M. Raleigh.
British Journal of Sports Medicine, 2014, 48(Suppl 2):A54-A54.

***TIMP2* and *GDF5* Gene Variants and Achilles Tendon Pathology: Replication Study in a British Case-Control Population**
L. El Khoury, W.J. Ribbans and S.M. Raleigh
British Journal of Sports Medicine, 2014, 48(Suppl 2):A55-A55.

The *ADAMTS2* and *ADAMTS14* Genes and Risk of Achilles Tendon Pathology
L. El Khoury, M. Posthumus, M. Collins, W.J. Ribbans, C.J. Handley, J. Cook and S.M. Raleigh
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Presentations at International Conferences

Jul. 2014 **Poster Presentation*:* Variation within the *MMP3* Gene as a Risk Factor for Achilles Tendon Pathology in a British Population**
L. El Khoury, W.J. Ribbans, and S.M. Raleigh
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Jul. 2013 **Oral Presentation*:* Polymorphic Variation within the *ADAMTS2*, *ADAMTS14*, *ADAMTS5*, *ADAM12* and *TIMP2* Genes and the Risk of Achilles Tendon Pathology: A Genetic Association Study**
L. El Khoury, M. Posthumus, M. Collins, C.J. Handley, J. Cook and S.M. Raleigh
European College of Sports Sciences Congress – Barcelona, Spain

Sept. 2012 **Poster Presentation: The Elastin and Fibrillin2 Genes and the Risk of Achilles Tendon Pathology**
L. El Khoury, M. Posthumus, M. Collins, C.J. Handley, J. Cook and S.M. Raleigh
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Aug. 2012 **Oral Presentation: The *ADAMTS5*, *ADAM12* and *TIMP2* Genes and the Risk of Achilles Tendon Pathology**
L. El Khoury, M. Posthumus, M. Collins, W.J. Ribbans, C.J. Handley, J. Cook and S.M. Raleigh
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Aug. 2011 **Oral Presentation: The *ADAMTS2* and *ADAMTS14* Genes and Risk of Achilles Tendon Pathology**
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3rd International of Sports Science and Sports Medicine Conference – Newcastle, UK

Abbreviations

A Adenine
ACL Anterior cruciate ligament
ADAM A disintegrin and metalloproteinase
ADAM12 A disintegrin and metalloproteinase 12 protein (italics indicate the gene)
ADAMTS A disintegrin-like and metalloproteinase with thrombospondin motifs protein (italics indicate the gene)
ADAMTS2 A disintegrin-like and metalloproteinase with thrombospondin motifs 2 protein (italics indicate the gene)
ADAMTS5 A disintegrin-like and metalloproteinase with thrombospondin motifs 5 protein (italics indicate the gene)
ADAMTS14 A disintegrin-like and metalloproteinase with thrombospondin motifs 14 protein (italics indicate the gene)
ANOVA Analysis of variance
ATP Achilles tendon pathology
AUS Australia / Australian

BDKRB2 Bradykinin receptor B2
BMI Body mass index
bp Base pairs
BPTF Bromodomain PHD finger transcription factor

C Cytosine
CASP8 Caspase 8 protein (italics indicate the gene)
CCA Congenital contractural arachnodactyly
CCD Charge-coupled device
CHD1 Chromodomain helicase DNA binding protein 1
CI Confidence interval
COL1A1 The α1 chain of type I collagen protein (italics indicate the gene)
COL5A1 The α1 chain of type V collagen protein (italics indicate the gene)
COL11A1 The α1 chain of type XI collagen protein (italics indicate the gene)
COL11A2 The α2 chain of type XI collagen protein (italics indicate the gene)
COL12A1 The α1 chain of type XII collagen protein (italics indicate the gene)
COL14A1 The α1 chain of type XIV collagen protein (italics indicate the gene)
COL27A1 The α1 chain of type XXVII collagen protein (italics indicate the gene)
COMP Cartilage oligomeric matrix protein

CON Control

DGCR8 Digeorge syndrome Critical Region Gene 8
DNA Deoxyribonucleic acid
DNMT DNA methyltransferase
dNTP Deoxyribonucleotide triphosphate

ECM Extracellular matrix
ELN Elastin protein protein (italics indicate the gene)
EDS Ehlers-Danlos syndrome

FACIT Fibril Associated Collagen with Interrupted Triple helices
FBN Fibrillin protein protein (italics indicate the gene)
FBN1 Fibrillin 1 fibril protein (italics indicate the gene)
FBN2 Fibrillin 2 fibril protein (italics indicate the gene)

G Guanine
GAG Glycosaminoglycan
GAPDH The gene coding for glyceraldehyde 3-phosphate dehydrogenase
GDF5 Growth/differentiation factor-5 protein protein (italics indicate the gene)
GWAS Genome-Wide Association Studies

HWE Hardy-Weinberg Equilibrium
Hz Hertz
Kb Kilo base (1000 bases)
kN Kilo Newton (1000 Newton)

LD Linkage disequilibrium

IL-1β Interleukin-1β protein (italics indicate the gene)
IL-1RN Gene encoding interleukin-1 receptor antagonist protein (italics indicate the gene)
IL-6 Interleukin-6 protein (italics indicate the gene)
INHAT Inhibitor of histone acetyltransferase
INS Insertional tendinopathy

MEF2A Myocyte enhancer factor 2A
miRNA Micro ribonucleic acid

MMP Matrix Metalloproteinase
MMP2 The gene encoding for the Matrix metalloproteinase-2 protein (italics indicate the gene)
MMP3 The gene encoding for the Matrix metalloproteinase-3 protein (italics indicate the gene)
MMP9 The gene encoding for the Matrix metalloproteinase-9 protein (italics indicate the gene)
MRI Magnetic Resonance Imaging
mRNA Messenger Ribonucleic Acid
MTA Material Transfer Agreement

NCAA National Collegiate Athletic Association
NCBI National Center for Biotechnology Information
ncRNA Non-coding RNA
NON-INS Non-insertional tendinopathy
nt Nucleotide
NuRD nucleosome remodelling and deacetylation complex

OA Osteoarthritis
OR Odds ratio

p Short arm of chromosome
PAGE Polyacrylamide Gel Electrophoresis
PCR Polymerase chain reaction
PGA Power for Genetic Association Analyses package
PGC1A Peroxisome proliferator activated receptor gamma coactivator 1 alpha
PPi Pyrophosphate
Pre-miRNA Premature micro-RNA
Pri-miRNA Primary micro-RNA
PT Patellar tendinopathy
PTP Patellar tendon pathology

q Long arm of chromosome
qPCR Quantitative Polymerase Chain Reaction

REC Research Ethics Committee
RFLP Restriction fragment length polymorphism

RISC RNA Induced Silencing Complex
rpm Revolution per minute
RNOH Royal National Orthopaedic Hospital
RNU6B The gene coding for U6 small nuclear 2
RT-PCR Reverse Transcriptase Polymerase Chain Reaction
RUNX1 Runt-related transcription factor 1
RUP Rupture

SA South Africa / South African
SAM S-adenosylmethionine
SNP Single nucleotide polymorphism

TEN Tendinopathy
TGF-β Transforming growth factor beta protein (italics indicate the gene)
THBS2 Thrombospondin-2 protein (italics indicate the gene)
TIMP Tissue inhibitor of metalloproteinases
TIMP2 Tissue inhibitor of metalloproteinases 2 protein (italics indicate the gene)
TNC Tenacin-C protein protein (italics indicate the gene)

UK United Kingdom
UTR Untranslated region

Abstract

Background and Aims

Regular physical activities have shown to have various health benefits however there is always an accompanying risk of developing musculoskeletal soft tissue injuries. Indeed, damaged tendons account for 30-50% of sports-related injuries where the lifetime risk of Achilles tendon pathology (ATP) approaches 50% in runners, and that of patellar tendon pathology (PTP) is 21% in football players. The exact aetiology and mechanisms of tendon pathologies are still under investigation, however extrinsic and intrinsic risk factors (of which genetic) have been identified. Recent genetic association studies found that gene variants within the *TNC*, *COL5A1*, *MMP3*, *GDF5* and *CASP8* were associated with ATP in a Caucasian Australian and South African population. Furthermore, epigenetic mechanisms such as DNA methylation and microRNA (miRNA) activity have been implicated in a range of diseases but were never investigated for their role in human tendinopathy.

Based on the aforementioned information, this thesis aimed at investigating novel candidate genes that may be associated with ATP and to replicate previously conducted studies in a newly recruited case-control population. Additionally, this thesis aimed at investigating potential differences in DNA methylation profiles and miRNA expression levels between healthy and damaged Achilles and patellar tendons.

One hundred and thirty six UK Caucasian participants with clinically diagnosed ATP and 131 asymptomatic, unrelated, physically active control participants were recruited for this study. Furthermore, the previously recruited 173 clinically diagnosed ATP participants and 238 asymptomatic, unrelated, physically active control participants from Australia and South Africa (AUS+SA) were also included in the studies of this thesis.

Participants within the combined AUS+SA were genotyped for the *ELN* rs2071307, *FBN2* rs331079, *ADAM12* rs3740199, *ADAMTS2* rs1054480, *ADAMTS5* rs226794, *ADAMTS14* rs4747096, and *TIMP2* rs4789932 variants, and the UK participants were genotyped for the *COL5A1* rs71746744, *FBN2* rs331079, *GDF5* rs143833, *MMP3* rs679620, *TIMP2* rs4789932 variants using fluorescent based TaqMan® technology. Furthermore, the UK cohort was genotyped for the *COL5A1* rs12722 variant using polyacrylamide gel electrophoresis.

Moreover, 10 healthy and 10 diseased patellar tendon tissue samples in addition to 4 healthy, and 1 diseased Achilles tendon samples were obtained for the epigenetic studies. The DNA methylation profile within the *TIMP2* and *GDF5* promoter regions were analysed for all samples using pyrosequencing technology. Furthermore, the expression levels of *TIMP2*, miR-21, miR-155, and miR-191 were determined by RT-PCR using TaqMan technology.

Results and Discussion

The genetic association studies conducted showed that the *FBN2* rs331079 GG genotype was over-represented among the tendinopathy (TEN) group and that the *ELN* rs2071307 AA genotype was over-represented in the rupture (RUP) group within the AUS+SA population. Furthermore, the *COL5A1* rs12722 and rs71746744 were associated with RUP (TT genotype over-represented in the RUP group, p=0.004; OR=4.2; 95% CI 1.58-11.97) and ATP (DEL allele over-represented in the CON group, p=0.046; OR=1.61; 95% CI 1.01-2.56) respectively in the male UK cohort. The *GDF5* rs143833, on the other hand, was not associated with ATP (p=0.538) and showed no sign of gender-specific association (female p=0.737; male p=0.319) in the UK population. Furthermore, the CT genotype for the *TIMP2* rs4789932 variant was over-represented (p=0.004; OR=1.77; 95% CI 1.20 - 2.64) in the ATP group of the combined AUS+SA population and the CC genotype was over represented (p=0.016; OR=2.36; 95% CI 1.16 – 5.81) in ATP of the UK male cohort. It was also reported that the *MMP3* rs679620 GG genotype was over represented (p=0.027; OR=2.51; 95% CI

1.11 – 5.64) in the UK RUP group. The *ADAM12* rs3740199 (p=0.633), *ADAMTS2* rs1054480 (p=0.316), *ADAMTS5* rs226794 (p=0.342), and *ADAMTS14* rs4747096 (p=0.849) gene variants were not associated with ATP in the AUS+SA population.

Interestingly, individuals carrying the *ADAMTS14* rs4747096 GG genotype within the AUS+SA population and the *ELN* rs2071307 AA genotype within the AUS population developed their injuries at a significantly (p=0.024; p=0.005, respectively) later stage than other participants. Moreover, UK males diagnosed with tendinosis and carrying the GG genotype at the *MMP3* rs679620 locus developed significantly (p=0.003) thicker tendons than other participants with the AA, and AG genotypes.

The preliminary epigenetic DNA methylation studies showed no differences in the average methylation profiles of the investigated regions within the *TIMP2* (p=0.885) and *GDF5* (p=0.333) genes in the patellar tendon samples. Moreover, no DNA methylation differences (p=0.617) were observed in the investigated region of the *TIMP2* gene in the Achilles tendon samples. Interestingly, the single ATP sample showed a lower *GDF5* average methylation profile than the CON samples. Furthermore, the expression of *TIMP2* was up-regulated, and miR-191 was down-regulated in the ATP tissue sample compared to the CON group. The expression levels of miR-21 and miR-151, however, were not different between the two groups.

Conclusion

This thesis provides evidence that novel genes coding for structural and ECM regulatory enzymes are associated with ATPs in Caucasians. The findings of this thesis should to be replicated in new and larger cohorts from different ethnic backgrounds before being incorporated into multifactorial risk assessment models aiming at reducing the incidence of human tendinopathy.

Chapter 1 Introduction and scope of the thesis

The regular involvement in physical activity is an important component for the maintenance of a healthy lifestyle (Jarvinen et al. 2005). In spite of the numerous health benefits that physical activity might bring to an individual, there is an increased risk of injuries, particularly musculoskeletal soft tissue injuries (Jarvinen et al. 2005). Among these injuries, Achilles tendinopathy and Achilles rupture (hereon referred to as Achilles tendon pathology or ATP) have been described to be serious injuries with an annual prevalence as high as 11% (Jarvinen et al. 2005, Kujala, Sarna & Kaprio 2005, Rees, Wilson & Wolman 2006). ATP represents as much as 18% of tendon injuries acquired during daily physical activity (Collins, Raleigh 2009). The devastating consequence resulting from such an injury stresses the importance of the identification of the risk factors associated with this injury, by conducting scientific research studies, to better understand the aetiology and the mechanisms involved.

Various ATP intrinsic (anatomical variances, gender, and age) and extrinsic (type of physical activity, level of engagement in sports, footwear and equipment) risk factors have been identified (Riley 2004). Researchers started only recently investigating the role of genetic elements as predisposing risk factors for ATP (Mokone et al. 2005). Various musculoskeletal soft tissue injuries have been suggested to have important familiar predispositions (Flynn et al. 2005, Harvie et al. 2004). With this in mind genetic association studies have been initiated to identify gene variants that could associate with ATP. Variants within the *TNC* (Mokone et al. 2005)*, COL5A1* (Mokone et al. 2006)*, MMP3* (Raleigh et al. 2009), and *GDF5* (Posthumus et al. 2010) genes have all been shown to co-segregate with ATP. However, these studies have been limited to Caucasian Australian and South African cohorts only. It is well established that confidence in the results of genetic association studies grows from the repetition of such work in trans-ethnic and trans-geographic cohorts (Greene et al. 2009). Therefore the replication of the aforementioned genetic association studies and the

identification of novel gene variants are crucial to our understanding of the genetic predisposition to ATP.

With this in mind, a first objective of this thesis was to determine whether previously investigated variants within the *COL5A1*, *GDF5*, and *MMP3* genes were associated with ATP in a UK population. Such approach would confirm that these genetic factors are not limited to isolated populations. Indeed the collection of an additional matched population cohort will permit the testing of novel genetic loci that may predispose humans to ATP.

The identification of additional genetic risk factors, will further improve the understanding of the aetiology of ATP. Obtaining a clearer image of the pathways and mechanisms involved in the development of ATP is required for the introduction of appropriate evidence-based preventative measures which may contribute towards the reduction in the number of incidences (Collins, Raleigh 2009).

Based on what is mentioned above, a second objective of this thesis was therefore to identify specific and novel genetic elements which predispose humans to ATP. Genetic association studies (case-control design) were used to test whether sequence variants (single nucleotide polymorphisms) within candidate genes influenced the risk of ATP. Candidate genes were selected, based on their structural and biological function within tendons. Specifically, genes which encode for the basic structural and functional unit of tendons (*FBN2* and *ELN*), and enzymes maintaining the extracellular matrix integrity (*ADAMs*, *ADAMTSs*, and *TIMPs*) were identified as candidate genes.

Furthermore, epigenetic modifications have become of interest in the investigations of human diseases. Mechanisms such as DNA methylation and microRNA activity have been implicated in the alteration and reduction of gene expression. However, those were never investigated for their role in human tendinopathy. Therefore a third objective of this thesis

was to assess whether there were different DNA methylation profiles (within *TIMP2* and *GDF5* genes) and microRNA (miR-21, miR-155, and miR-191) gene expression levels between healthy and diseased Achilles as well as patellar tendons.

In preparation for the exploration of the content of this thesis, chapter 2 will provide a review of the gross anatomy of the tendons, the biomechanics of human tendon, the different tendon pathologies, the epidemiology of tendinopathy, and the identified risk factors for tendinopathies. Chapter 3 will be describing the materials and experimental methods used to conduct the research. All work has been conducted by the author unless otherwise stated. The core experimental chapters will use a case-control candidate gene approach to accomplish the aims and objectives of the thesis.

Chapter 2 Achilles and Patellar Tendon Pathology: A Review

2.1 GROSS ANATOMY OF HUMAN TENDONS

2.1.1 Anatomy of the Achilles Tendon

The Achilles tendon, the strongest and thickest tendon of the body (Benjamin et al. 2007), attaches the gastrocnemius and the soleus muscles to the calcaneus bone (heel) (Maffulli 1999) (Figure 2.1.1). This tendon is suggested to have shaped human evolution since it is critically linked to humans' ability to develop bipedalism and run away from danger (Bramble, Lieberman 2004). The tendon is formed when both gastrocnemius and soleus muscles merge approximately 5 to 6 cm above the calcaneal insertion (Maffulli 1999). In fact, the soleus tendon begins on the posterior surface of the soleus muscle whereas the gastrocnemius tendon originates at the distal margin of the muscle bellies (Cummins, Anson 1946). Both tendons vary in length. The gastrocnemius component ranges from 11-26 cm while that of the soleus ranges from 3-11 cm (Cummins, Anson 1946). The width of the Achilles tendon at its point of insertion into the calcaneus varies from 1.2-3 cm (Schepsis, Jones & Haas 2002, Koch, Tillmann 1995).

The Achilles tendon becomes gradually round in cross section, before inserting into the superior calcaneal tuberosity (Drake et al. 2010). The fibres of the Achilles tendon twist 90 degrees during its descent: the medial fibres rotate posteriorly and the posterior fibres rotate laterally allowing the tendon to stretch and recoil (Schepsis, Jones & Haas 2002). Furthermore, the shape of the Achilles tendon varies from proximal to distal. It is suggested that the distal part of the tendon does not exceed 7 mm in thickness; otherwise this would be regarded as pathologic (Sadro, Dalinka 2000). However, it is considered normal to see a thickness of 2-3 mm at the level of the insertion where the tendon is flattened (Koch, Tillmann 1995).

The calcaneal insertion of the Achilles tendon is highly specialised (Rufai, Ralphs & Benjamin 1996, Rufai, Ralphs & Benjamin 1995). It is composed of layers of hyaline cartilage, and an area of bone that is not covered by periosteum (a membrane covering the surface of all bones). The Achilles tendon inserts on the posterior surface of the calcaneus. Proximal to the point of insertion, between the tendon and the skin, is the subcutaneous (superficial) bursa, a fluid-filled saclike cavity, which reduces friction between the tendon and the surrounding tissues (Maffulli 1999).

The subtendinous (deep) bursa lies between the tendon and the calcaneus (Drake et al. 2010) such that the tendon forms the posterior wall of the bursa which borders the cartilaginous layer of the calcaneal tuberosity, and the synovial lining located proximally would separate the bursa from the proximal fat pad (Karjalainen et al. 2000). Located anterior to the tendon, the fat pad is bordered anteriorly by the flexor hallucis longus muscle and posteriorly by the Achilles tendon. Figure 2.1.1 indicates the anatomical location of the different bursas around the Achilles tendon.

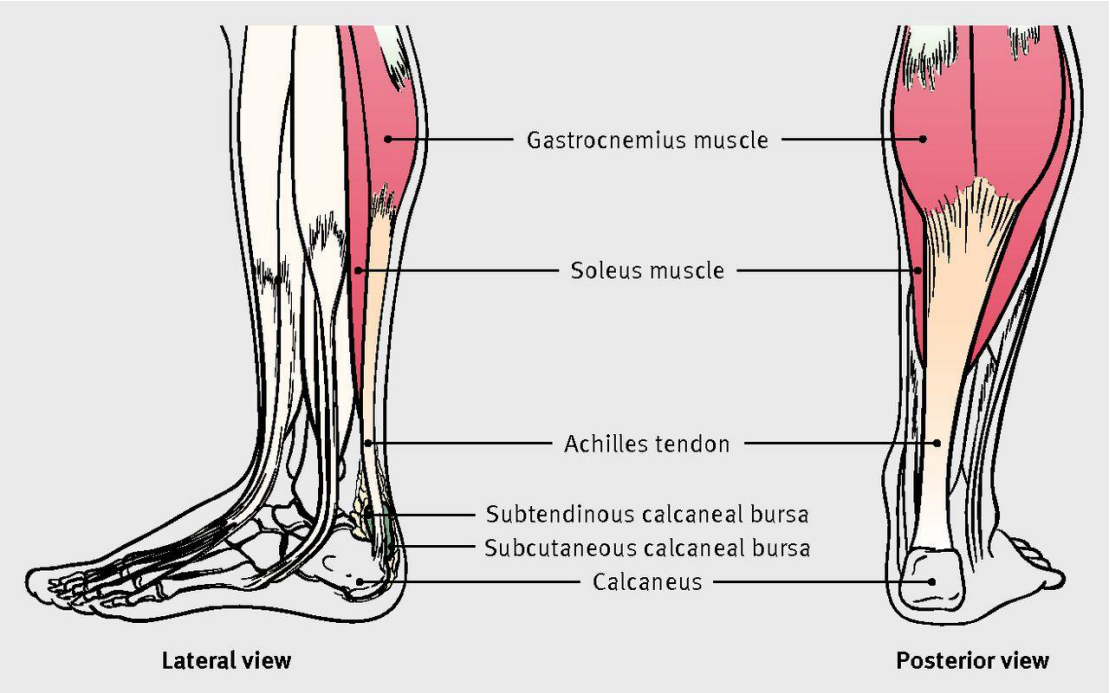


Figure 2.1.1 Anatomy of the Achilles tendon. Obtained from (Asplund, Best 2013) with permission of the publisher.

The plantar aponeurosis (fascia) is a thick connective tissue that supports the arch at the bottom of the foot. It originates from the medial tubercule of the calcaneal and inserts into the phalanges through a network of fibrious tissue (Cheung, Zhang & An 2006). The plantar fascia has a special relation with the Achilles tendon wherein young individuals there is a continuous fibrillar extension from the tendinous insertion of the Achilles into the plantar fascia. However, with age this connection decreases to a point where few, if any, connective fibres remain (Snow et al. 1995). The plantar fascia is known to sustain high tension during weight bearing. Furthermore, a positive correlation has been reported between Achilles tendon loading and plantar fascia tension (Erdemir et al. 2004). This finding indicates that excessive stretching and tightness of the Achilles tendon can represent risk factors for plantar fasciitis.

The Achilles tendon is encased in the paratenon which contains a single layer of cells. The paratenon consists of a highly vascularised fatty tissue responsible for a significant portion of the blood supply to the tendon (Stein et al. 2000). Additionally, blood supply to the tendon comes from two other sources: the musculotendinous junction and the osseous insertion. An area of relative avascularity is located 2-6 cm proximal to the insertion point into the calcaneus (Baxter, Zingas 1995).

2.1.2 Anatomy of the Patellar Tendon

The patellar tendon extends between the tibial tuberosity and the inferior pole of the patella. On the sagital plane, the tendon is 4-5 mm deep, and its width is ~3 cm on the coronal plane (Khan et al. 1998). The patellar tendon is thin and broad proximally, but gradually becomes thick and narrow at the distal end because of the convergence of the fibre bundles as they run down towards the tibial tuberosity (Basso, Johnson & Amis 2001). The patellar tendon is surrounded by the deep and superficial infrapatellar bursas (Snell 2011) which function to reduce friction between the tendon and the surrounding (Maffulli 1999).

2.1.3 Blood Supply

Tendons can receive their blood supply from three sources: the musculotendinous junction, the surrounding connective tissue, and the bone-tendon junction (Drake et al. 2010). The blood flow of the Achilles tendon varies between young and older individuals with higher vascularisation among the former group (Theobald et al. 2005). The midportion of the Achilles tendon has been reported to be poorly vascularised and more prone for damage (Ahmed et al. 1998, Carr, Norris 1989). Nevertheless, the distribution of blood vessels in the tendon is still under investigation. Some studies have reported an even distribution of vessels (Carr, Norris 1989) while others described the middle part to have a lower density of blood vessels compared to the extremities with a variation depending on age, gender and physical activity (Astrom, Westlin 1994).

On the other hand, the patellar tendon receives the blood supplies from the anastomotic ring located within the thin layers of connective tissue surrounding the knee (Snell 2011). The vascularisation of the patellar tendon is primarily provided by the Hoffa's fat pad (Khan et al. 1998). The proximal posterior aspect of the patellar tendon represents the point of entry of the blood supply to the proximal portion of the tendon, which is most often damaged during patellar tendon pathology (Khan et al. 1998).

2.2 THE STRUCTURE OF HUMAN TENDONS

2.2.1 Tenocytes: The Tendon Cells

Tenocytes are the fibroblast cells that make up the connective tissue forming the tendon. These cells are known to have a uniform, spindle shaped nucleus and a thin cytoplasm with a slender and elongated morphology (Chuen et al. 2004). Furthermore, the main role of tenocytes is the production of fibrillar (collagen, versican, aggrican, etc.) and nonfibrillar (enzymes and inflammatory proteins) components of the extracellular matrix (ECM) (Xu, Murrell 2008). Age is a major factor affecting the shape and activity of tenocytes which could provide a mechanistic explanation to the increase risk of tendinopathy in older individuals (Yu et al. 2013).

2.2.2 Hierarchical Micro-Structure of Human Tendons

Type I procollagen molecule represent the base unit of the collagen network. Fibrils, are next in the hierarchy and are formed by the conglomeration of five type I procollagen molecules. The compilation of several fibrils in parallel forms fibres which, in turn, are stacked together to form a fascicle. Each fascicle is wrapped by a sheath containing blood vessels and nerve endings, the endotenon. The fascicles are further encapsulated by the epitenon which is surrounded by the paratenon (figure 2.2.1) (Hoffmann, Gross 2007). One of the many important features of tendons is the crimping alignment of fibres which provides shock-absorbing properties.

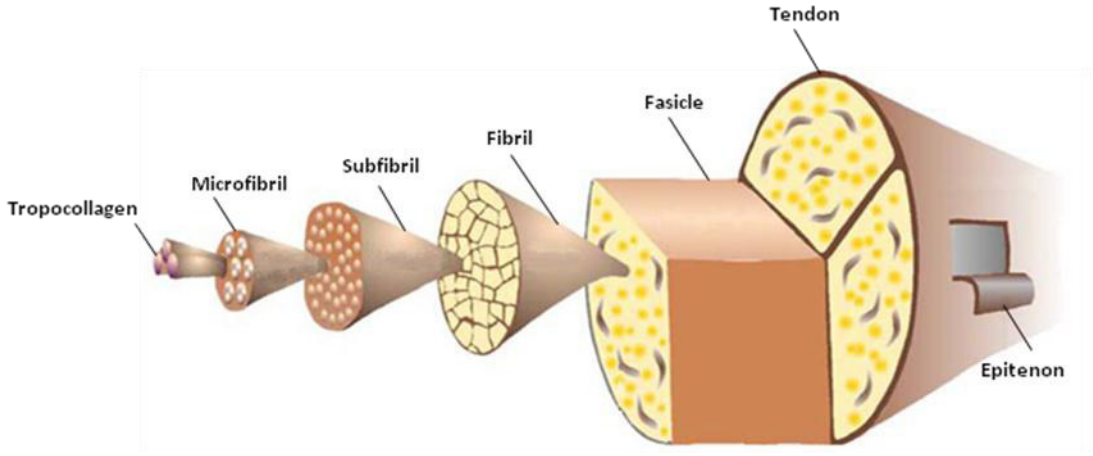


Figure 2.2.1 The hierarchical organisation of a tendon ranging from the smallest subunit (tropocollagen) to a full size tendon. Figure obtained from (Hoffmann, Gross 2007) with permission of the publisher.

2.2.3 Molecular Structure of Tendons

The major molecular components of the ECM within tendons include structural (collagens, glycoproteins, and proteoglycans) and non-structural proteins (metalloproteinases and their inhibitors).

2.2.3.1 Collagen

Two thirds of a tendon consists of water while the rest is made up of structural fibrils where collagen accounts for 70 % of the dry weight (Obrien 1992). Several collagens have been identified and classified into two groups based on their structure and function. The first group consists of fibrillar collagens which form the scaffolding network. The second group consists of non-fibrillar collagens such as the fibril-associated collagens with interrupted triple helices (FACIT) who serve as molecular bridges that are important for the organisation and stability the ECM (Riley 2005). Of the two groups, fibrillar collagen are the most abundant and are further categorised into major (types I, II, III) and minor (type V, VI) collagens (Birk 2001).

Genes coding for collagen proteins play a very important role in determining the function of connective tissues. Mutations within different collagen genes result in serious connective

tissue disorders. For instance, mutations within the gene coding for type I collagen result in osteogenesis imperfecta (Pollitt et al. 2006), and mutations within the type II and XI collagen result in chondrodysplasias (Välkkilä et al. 2001) whereas mutations within the type III and V result in Ehlers-Danlos syndrome (EDS) (Malfait et al. 2005).

Type I collagen, a heterotrimer consisting of two α1 and one α2 chains, is the most abundant structural component of the fibrils (Collins, Raleigh 2009). Type I collagen determines the mechanical and tensile strength of tendons (Hoffmann, Gross 2007). Type III collagen, another important fibrillar collagen of tendon, plays an important role in the healing process and during fibrillogenesis (Banos, Thomas & Kuo 2008). It is believed that type III collagen contributes towards the regulation of the diameter of type I collagen fibrils during healing by limiting lateral growth (Banos, Thomas & Kuo 2008).

When comparing normal to pathological tendons, it can be reported that normal tendons consist mostly of type I collagen, and that pathological tendons contain a high proportion of type III collagen (Eriksen et al. 2002). Interestingly, it was reported that cultures of fibroblast cells from ruptured Achilles tendons produce both type I and type III collagen (Jarvinen et al. 1997). It is important to note that type III collagen is known to be less resistant to tensile forces and therefore its abundance, instead of more elastic structural proteins, may predispose the tendon to damage.

Type V collagen is a minor fibrillar collagen which is co-expressed with type I collagen (Birk 2001). It exists predominantly as a heterotrimer composed of the predominant isoform of type V collagen of two α1 and one α2 chains, however some isoforms homotrimers of three α3 chains exist (Collins, Raleigh 2009). Type V collagen is embedded within the type I collagen molecule as indicated in figure 2.2.2. This interaction allows type V collagen to play a role in the regulation of the diameter of type I collagen fibril as will be discussed later in Chapter 4 (Collins, Posthumus 2011).

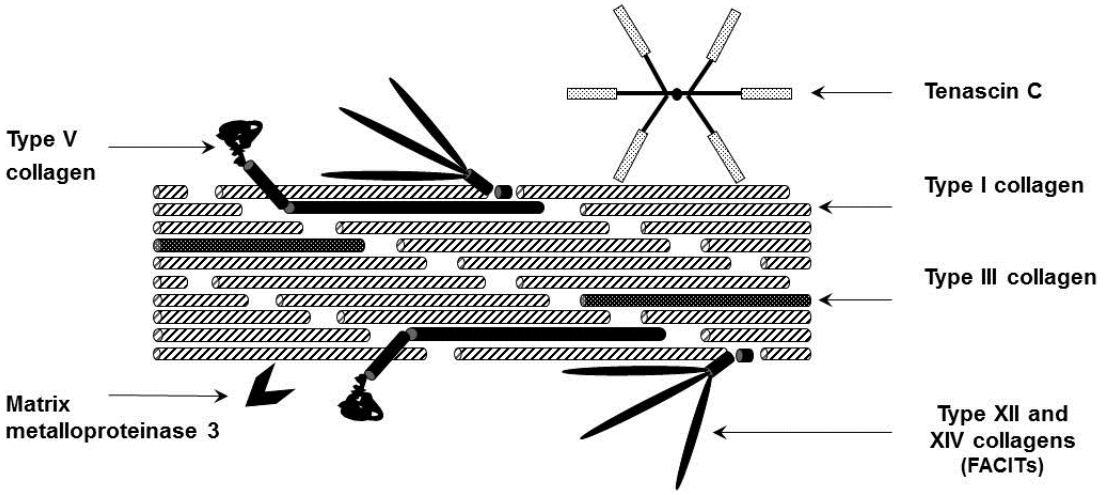


Figure 2.2.2 A schematic diagram of the basic structural unit of a tendon, the collagen fibril. The fibril consist predominantly of type I collagen and of trace amounts of FACITs, type III, and type V collagen. Furthermore, tenascin-C is a glycoprotein expressed during wound healing, and MMP3 is a proteolytic enzyme involved in ECM remodelling. Adapted from Collins and Raleigh (2009).

2.2.3.2 Glycoproteins and Proteoglycans

Glycoproteins are proteins with polypeptide chains covalently linked to oligosaccharide chains. Elastin (discussed in detail in Chapter 4) is considered to be the major glycoprotein in the tendon representing ~2% of the dry weight and has got elastic properties allowing it to stretch and return to its original state (Riley 2005). Furthermore, elastin has got an important load-bearing property, in musculoskeletal tissues, which is used for the storage of mechanical energy (Gosline et al. 2002).

Other glycoproteins like tenascin-C (TNC), fibrillin, laminin and fibronectin execute a variety of functions in the ECM but are predominantly involved in mediating cell-matrix interactions (Riley 2005). Fibrillin (discussed in details in Chapter 4) is a cysteine-rich glycoprotein (Sakai et al. 1991, Sakai, Keene & Engvall 1986) which contributes to the assembly of the microfibril structures. These structures act as architectural frameworks for the deposition of tropoelastin (a precursor of elastin) and the assembly of elastic fibres (Charbonneau et al.

2004). Furthermore, COMP (cartilage oligomeric matrix protein) is another major component of tendon consisting of 5 subunits arranged as branches around a central cylinder (DiCesare et al. 1994). The COMP subunits bind and interact with type I and type III collagens during matrix assembly and healing (Hecht et al. 2005). Moreover, TNC is a mechanosensitive glycoprotein expressed during wound healing and tissue remodelling, especially after the exposure to a high tensile mechanical load (Jarvinen et al. 2003).

Proteoglycans are heavily glycosylated proteins consisting of a core protein with pertruding GAG (glycosaminoglycans) side chains. Proteoglycans are important for the normal functioning of the tendon given their interaction with the fibrillar collagen network (Parkinson et al. 2011). They are known for their resistance to compressive shear forces and their involvement in the regulation of fibrilligenesis, cell migration and differentiation (Rees, Dent & Caterson 2009). Proteoglycans include hyalectan, aggrecan, versican, decorin, biglycan, fibromodulin, and lumican (Riley 2005). The perturbation of the metabolism and proteoglycans has been implicated in tendinopathy (Rees, Dent & Caterson 2009) however this is beyond the scope of this thesis and is expertly discussed elsewhere (Riley 2005, Rees, Dent & Caterson 2009).

2.2.3.3 Matrix Metalloproteinases and their Inhibitors

The essential maintenance of homeostasis in a tendon is guaranteed by the proteolytic activity of matrix metalloproteinase enzymes (Jones, Riley 2005). MMPs (matrix metalloproteinase), ADAMs (a disintegrin and metalloproteinase), and ADAMTSs (a disintegrin-like and metalloproteinase with thrombospondin motifs) degrade collagenous and non-collagenous molecules with the purpose of remodelling the ECM (Jones, Riley 2005). Furthermore, the activity of MMPs, ADAMs and ADAMTSs is regulated by TIMPs (tissue inhibitors of metalloproteinases) which bind to the enzymes and inhibit their attachment to their respective target sites (Jones et al. 2006). Changes in expression of metalloproteinases

and their inhibitors have been implicated in tendinopathy (Jones et al. 2006) and will be discussed in detail in Chapter 5.

2.3 BIOMECHANICS OF HUMAN TENDONS

Tendons act as contractile force transmitters between muscles and bones (Maffulli, Leadbetter & Renstrèom 2005). Vulnerability to injury increases as load is exerted on tendons to accomplish their function. In the case of the Achilles tendon, the gastrocnemius and soleus muscles are the main plantar flexors of the ankle joint and promote locomotion during a walk, a run, or a jump (Schepsis, Jones & Haas 2002). The gastrocnemius which contains a great number of the fast glycolytic type IIX fibres (fast-twitch) acts as an energy provider by contributing to knee flexion and by promoting a strong driving force during sprints and jumps (Ennion et al. 1995). Unlike the fast oxidative type IIA fibres which contain a larger amount of mitochondria and myoglobin, type IIX fibres break down ATP quickly which results in fast burst of power and rapid fatigue (Schiaffino, Reggiani 2011). On the other hand, the soleus contains a high proportion of type I (slow-twitch) fibres (Ennion et al. 1995) which plays a major role in maintaining posture by preventing the body from falling forward when standing immobile (Schepsis, Jones & Haas 2002).

The filamentous proteins present in muscles, actin and myosin, are all involved in the transmission of force from muscles to bones during a tendon's contraction or relaxation mechanisms (Fukashiro et al. 1995b). A force of 2.2 kN has been measured *in vivo* in a resting human Achilles tendon (Fukashiro et al. 1995a, Komi, Fukashiro & Jarvinen 1992). In addition to that, it has been reported that it can withstand a force up to 10 kN (equivalent to 10 times body weight) when running; making it the strongest tendon in a human body (Komi 1990). In a more recent study it has been reported that forces within the Achilles tendon vary between 6 to 8 times the body weight when running (Allenmark 1992); approaching the ultimate strength of the tendon (Schepsis, Jones & Haas 2002).

In the patellar tendon, a force of 0.5 kN is measured during a walk, however this force would increase to reach 8 kN when landing from a jump and 9 kN when sprinting (Zernicke, Garhammer & Jobe 1977, Stanish, Curwin & Mandel 2000). Interestingly, a force as high as 14 kN has been measured in competitive weight lifters (Stanish, Rubinovich & Curwin 1986).

During a normal gait cycle, force increases in the Achilles tendon just before the heel reaches contact with the ground. This force is then suddenly released during early impact. It was previously described that the Achilles tendon has a 90° spiral structure which explains the tendons elasticity when under load (Schepsis, Jones & Haas 2002). Palastanga et al. (1989) explain that upon landing from a jump, the tension is absorbed by the Achilles tendon and the recoil effect is regained as the body remains upright due to the activity in the triceps surae muscle (Palastanga, Field & Soames 1989). Indeed, the Achilles tendon is exposed to different forms of stress such as non-uniform stresses which might cause modifications in an individual muscle's contribution towards mechanical activity (Allenmark 1992). The combination of non-uniform stresses with a poor coordination between agonist and antagonist muscle contractions could possibly predispose to an injury (Maffulli 1998, Allenmark 1992).

To identify the mechanical properties of human tendons, stretching and elongation tests were conducted on tendon specimens. At rest, a tendon has a crimped arrangement, resulting from the crimping of the collagen fibrils and the retraction of the remaining elastic fibres (Obrien 1992). Stressing forces will cause the loss of the crimped arrangement as the fibrils of the structural proteins, mainly collagen, are stretched. As collagen fibres are being pulled apart, they respond in a linear way to the increasing tendon loads (Kirkendall, Garrett 1997). The compliance of the tendon to strains is dependent on intra-tendinous waviness, which plays a direct role in the ability of the gastrocnemius-soleus muscle complex to generate force (Hawkins, Bey 1997). Hence, four different stages that tendons go through before sustaining a rupture have been identified and are illustrated in figure 2.2.1.

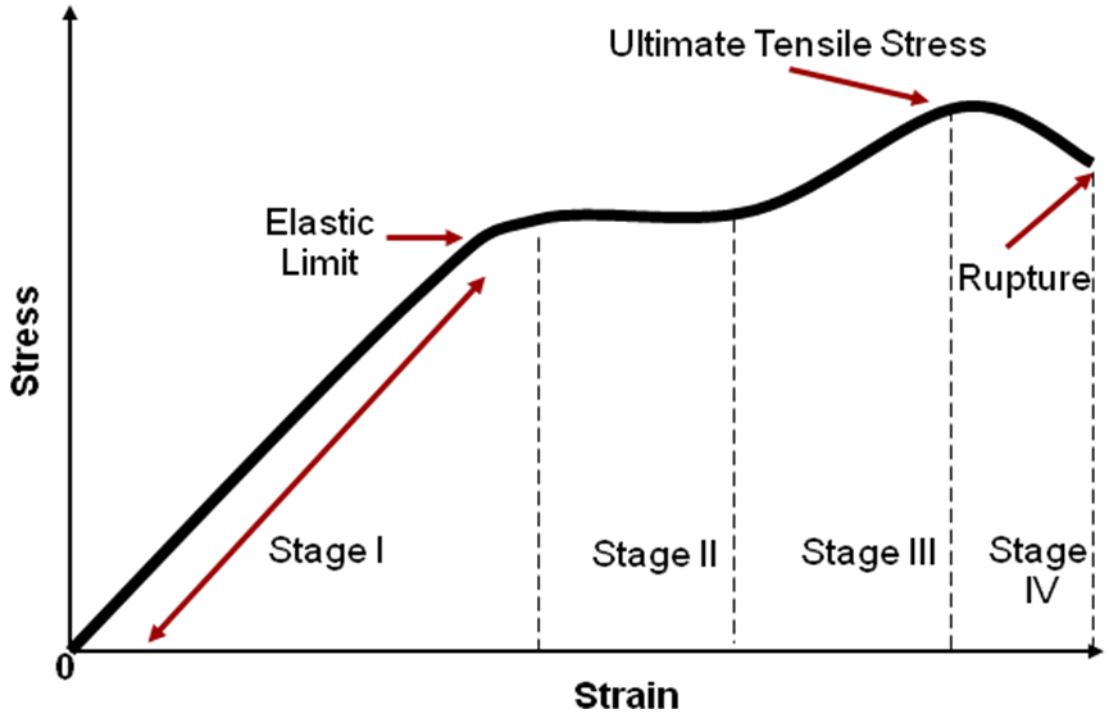


Figure 2.2.1 Illustration demonstrating the different stages that precede tendon rupture. I, toe region; II, linear region; III and IV, failure regions. Adapted from (Maganaris, Narici 2005).

Stage I, also known as the toe stage is when non-damaging forces reduce the crimping of the resting fibres. Also known as the linear stage, stage II is known for the stretching of the fibres beyond their elastic limit. It is at the end of this stage that signs of fibre damage start appearing. Stage II is followed by stage III where additional fibre failure occurs, and stage IV where complete rupture is observed (Vincent 1992, Diamant et al. 1972, Butler et al. 1978, Maganaris & Narici 2005). *In vitro* tests have shown that the Young's modulus, during stage II, ranges between 1 and 2 GPa (Pollock, Shadwick 1994). Furthermore, the ultimate tendon stress (stress at rupture) was reported to be ~100 MPa, and that the tendon strain (strain at rupture) was between 4 and 10% (Butler et al. 1978, Shadwick 1990). On the other hand, *in vivo* studies have measured a tensile stress of 110 MPa which exceeds the *in vitro* measurements (Butler et al. 1978, Shadwick 1990). Such a high tensile stress highlights the possibility of tendon rupture following a single real life movement. Nevertheless, to understand the effect of altered mechanical loading on tendons, cross sectional (Komi,

Fukashiro & Jarvinen 1992) and longitudinal studies (Kubo et al. 2000) were conducted. It was reported that tendons gain stiffness after chronic mechanical loading and become more compliant when loading is decreased (Hansen et al. 2003, Kubo, Kanehisa & Fukunaga 2002). Furthermore, a gender effect has been reported where male tendons are stiffer and more rebound-resilient than female tendons (Kubo, Kanehisa & Fukunaga 2002).

A review by Allenmark (1992) states that the Achilles tendon can be exposed to various forces secondary to subtalar motion because of the tendon' s insertion into the calcaneal. In fact, this can be noticed in pronating individuals. This review paper is therefore suggesting greater chances of developing Achilles tendon pathology among individuals with hyperpronated feet because of the reduced shock absorption attributed to this condition (Allenmark 1992).

James et al. (1978) classify overpronation as a risk factor for non-insertional Achilles tendonitis (James, Bates & Osternig 1978). It is reported that pronation happens during the midstance phase, leading to an internal rotation force on the tibia when the knee extends. Hence, damage to the Achilles tendon takes place at this particular instance when contradictory rotational forces are directed toward the tendon (James, Bates & Osternig 1978, Myerson, McGarvey 1999, Saltzman, Tearse 1998).

2.4 DIFFERENT HUMAN TENDON PATHOLOGIES

2.4.1 Non-Insertional Achilles Tendinopathy

In the late 1990s, non-insertional tendinopathy was classified as a degenerative non-inflammatory condition due to the absence of inflammatory cells and pro-inflammatory prostaglandins, PGE_2 (Alfredson, Thorsen & Lorentzon 1999). However inflammation cannot be excluded as studies in rat models reported an increase of serum TNF-α and other cytokines due to the infiltration of macrophages into the tendon tissue following physical activity (Barbe et al. 2008). Furthermore, higher levels of lymphocytes B and T were detected in patients with Achilles tendinosis when compared to patients who suffered from a sudden Achilles tendon rupture (Schubert et al. 2005). In fact, inflammation and degeneration can co-exist in a tendon (Abate et al. 2009). This has been elucidated by recent genetic association studies which showed the association of gene variants within the interleukin-1β (*IL-1β*), interleukin-6 (*IL-6*), the receptor antagonist of IL-1β, interleukin-1ra (*IL-1ra*), and Caspase-8 (*CASP8*) genes with Achilles tendinopathy and by that implicating the inflammatory pathway in the pathogenesis of human tendinopathy (September et al. 2011, Nell et al. 2012).

Many tendinopathy pathogenesis models have been proposed, however none is considered exclusive since several factors contribute mutually in the progression of the pathology as described in section 2.6. Tendon loading discussed in section 2.3 is received and transduced by tenocytes to initiate either an appropriate or a pathological adaptation. The appropriate response involves the repair and healing of the tendon followed by the adaptation to the experienced load. On the other hand, if the tendon fails to appropriately adapt and the pathological adaptation is initiated, the healing and repair process will be malfunctioning leaving the ECM to degrade which could eventually lead to an injury (Kjaer 2004). The effectiveness of the healing response is dependent on the amount of time allowed for the tendon to recover (Cook, Purdam 2009), and the implementation of a proper post-training stretching program (Judge et al. 2010, Smoljanovic et al. 2009). This model

was further discussed by Cook and Purdam (2009) who describe tendinopathy as a continuum of events, within different areas of the tendon, which proceed back and forth between stages in response to loading and recovery.

When a healthy tendon is subjected to acute tensile loads, an adaptation reaction is initiated which involves increased stiffness and thickening of the tendon. This reaction is characterised by an increase influx of water and proteoglycans into the tendon and does not involve neovascularisation (Cook, Purdam 2009). Tendon properties are expected to revert to normal if the load is reduced and enough time is allowed for recovery. However, if the tendon is not rested and additional load has been exerted, tendon properties will reach the disrepair stage where separation of the collagen fibres and disorganisation within the ECM is reported as a result of the increase in the number of cells and proteins. Once this stage is reached, the reversibility back to the earliest stages of the tendinopathic spectrum is limited. The furthest end of the spectrum is classified as degenerative tendinopathy where the likelihoods of recovering are reduced to the minimum (Cook, Purdam 2009). At this stage the ECM becomes vascularised, full of matrix breakdown products, and acellular as a result of the increased rate of apoptosis. This model supports previous statements suggesting that tendinopathy is the result of an imbalance between adaptation and degradation following an unaccustomed load.

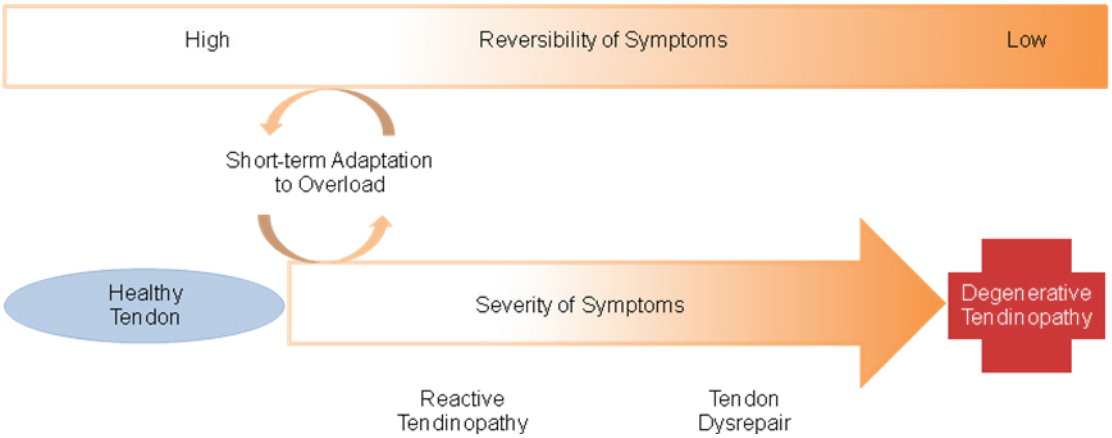


Figure 2.4.1 The tendinopathy continuum shows the four stages a tendon goes through following overload: healthy, reactive tendinopathy, tendon disrepair, and degenerative tendinopathy. The likelihood of the reversibility of symptoms at each stage is indicated in the top heat bar. Adapted from Cook and Purdam (2009).

This section of the thesis will present an overview of the different non-insertional Achilles tendinopathies and how they fit with the aforementioned tendinopathy continuum model.

2.4.1.1 Paratendinopathy

The paratenon is a thin gliding membrane covering the whole of the tendon and acts as an elastic sleeve that facilitates the movement of the tendon (van Dijk et al. 2011, Perry 1997). Paratendinopathy is a swelling of the paratenon where subjects would report localised pain after strenuous activity, and is therefore commonly reported in middle and long distance runners (Schepsis, Jones & Haas 2002). Signs of tenderness and swelling are more frequently found on the medial side of the Achilles tendon because of the increased stress on the medial fibres due to the 90° lateral twist of the Achilles tendon (Schepsis, Jones & Haas 2002) and the presence of the plantaris (van Dijk 2006).

Achilles paratendinopathy is considered to be an overuse injury (Józsa, Kannus 1997). However, circumstances that lead to damaging the paratenon exclusively, without damaging

the tendon body have not been identified (Paavola, Sayana & Maffulli 2007). Nevertheless, several explanations have been proposed such as training mistakes (sharp increase in distances and intensity, and asymmetry), weather, and running surfaces (Kvist 1994). Additionally, hyperpronation (Selvanetti, Cipolla & Puddu 1997) and limited ankle joint mobility were reported in athletes complaining from Achilles paratendinopathy. On a similar note, a mechanical explanation by Kaufman et al. (1999) suggests that the injury results from an increase in hindfoot inversion followed by a decrease in ankle dorsiflexion during knee extension (Kaufman et al. 1999).

2.4.1.2 Non-Insertional Tendinosis

Non-insertional Achilles tendinosis is a condition involving intra-tendinous degeneration and atrophy (Heckman, Gluck & Parekh 2009). This pathology results from a repetitive "whipping action" following excessive hindfoot movement accompanied by foot pronation and a lateral heel strike (Ajis et al. 2007). Such repetitive micro-trauma fits well in the Cook and Purdam (2009) model since they put subjects at risk of tendinosis if they occur at a rate faster than the tenocytes-mediated healing and repair process (Sharma, Maffulli 2006).

Generally, pain is sensed at the beginning and at the end of a training session (Ajis et al. 2007). However, as the pathology progresses, pain may occur during exercise and can interfere with daily life activities (Ajis et al. 2007). A swelling within the tendon is the main symptom of tendinosis and is believed to originate from a combination of biochemical and mechanical causes since high concentrations of the neurotransmitter glutamate have been reported (Alfredson, Thorsen & Lorentzon 1999). Non-insertional tendinosis is a degeneration of collagen accompanied by fibre thinning, followed by scattered vascular development (Józsa, Kannus 1997, Khan, Maffulli 1998, Leadbetter 1992). The reported degenerative changes in tendons are believed to be predominantly a result of the aging process, and a dysregulated cell signalling system combined with chronic mechanical

stresses (Józsa, Kannus 1997). Previous studies have reported that tendon damage can be caused by free radicals resulting from impaired tenocyte apoptosis following ischemia, hyperthermia, and hypoxia (Bestwick, Maffulli 2000).

2.4.2 Insertional Achilles Tendinopathy

Insertional Achilles tendinopathy is a clinically diagnosed condition characterised by pain and swelling in the posterior heel with impaired function of the Achilles tendon. Insertional Achilles tendinopathy is characterised by four elements (insertional tendinosis, Haglund's deformity, intra-tendinous calcification, and retrocalcaneal bursitis) which can occur in combinations or independently. These four elements are described in the sections below.

2.4.2.1 Insertional Tendinosis

Insertional tendinosis is a process most commonly reported in overweight individuals and older or recreational athletes (Schepsis, Jones & Haas 2002). It is frequently associated with Haglund's deformity or retrocalcaneal bursitis. The pathological changes acquired from an insertional tendinosis may include oedema, disruption of collagen fibrils, haemorrhage, and necrosis (Young, Sayana & Maffulli 2007). These may be caused by repetitive tensile load due to strain shielding around the insertion of the tendon (Lyman, Weinhold & Almekinders 2004). Nevertheless, strains within the tendon and around the insertion are not uniform (Vogel et al. 1993). The stress-shielded side of the enthesis may develop cartilage in response to the lack of tensile stress and eventually, this process may induce degeneration within the tendon (Vogel et al. 1993). Such pathology explains why tendinopathy is not strictly related to activity, but also to other factors such as weight and age (Maganaris et al. 2004). Furthermore, changes in the direction of strains can be observed when the joint changes position. At this stage, injury to the cellular and extracellular matrix can result from the internally generated shear forces and heat (Maganaris et al. 2004). Similar to non-insertional tendinosis, hill running, and training errors are well known to exacerbate insertional tendinosis (Heckman, Gluck & Parekh 2009).

2.4.2.2 Haglund's Deformity

Haglund's deformity is a chronic and sometimes painful enlargement of the posterosuperior prominence of the calcaneus (Schepsis, Jones & Haas 2002) which causes pain by the mechanical impingement of the retrocalcaneal bursa (Kang, Thordarson & Charlton 2012). Such chronic condition results from repetitive traction forces (Benjamin, Rufai & Ralphs 2000) which are caused by wearing shoes with rigid and high heels (Kang, Thordarson & Charlton 2012). Indeed, studies report strain shielding of the tendon is predominantly caused by repetitive tensile load (Sayana, Maffulli 2007).

2.4.2.3 Intra-Tendinous Calcification

Intra-tendinous calcification results from the protrusion of a bony spur into the tendon (Johnson, Zalavras & Thordarson 2006). It has been suggested that the bony spur is formed following inflammatory reactions within the tendon (Schepsis, Wagner & Leach 1994); however the exact biochemical interactions have not been described. This pathology results from repetitive mechanical stresses and can result in collagen degradation, fibrosis, and calcific metaplasia (Johnson, Zalavras & Thordarson 2006). Intra-tendinous calcification causes posterior heel pain at the bone-tendon junction and is usually painful after exercise or when wearing closed-heel shoes (Johnson, Zalavras & Thordarson 2006).

2.4.2.4 Retrocalcaneal Bursitis

Retrocalcaneal bursitis is a condition that involves the inflammation of the retrocalcaneal bursa, and is characterized by pain anterior to the Achilles tendon (Heckman, Gluck & Parekh 2009). The prolonged impingement of the retrocalcaneal bursa is thought to be the reason behind the pain in the heel (Schepsis, Jones & Haas 2002). Being situated between the calcaneus and the Achilles tendon, the bursa is squeezed during ankle dorsiflexion

(Maffulli 1999). Retrocalcaneal bursitis is frequently encountered in runners training for uphill running (McGarvey et al. 2002).

2.4.3 Achilles Tendon Rupture

2.4.3.1 Acute Achilles Tendon Rupture

Acute rupture of the Achilles tendon was reported to occur more commonly in middle-aged men during athletic activities (Kannus, Jozsa 1991). Ruptures occur when pushing off with the weight bearing foot while extending the knee (Young, Movin & Maffulli 2007). The rupture can also occur during sudden or violent dorsiflexion of a plantar flexed foot (Heckman, Gluck & Parekh 2009). During tendon load, collagen loses its crimping conformation and responds linearly to the increasing load (Leadbetter 1992). Tendons can take up to 4% strain before showing signs of damage and failure (Leadbetter 1992). Furthermore, tendons are expected to completely rupture if strain levels exceed 8% of the ultimate strain (Leadbetter 1992). The rupture of the Achilles tendon occurs more commonly 2-6 cm proximal to the insertion and may result after the accumulation of various micro-traumatic and degenerative changes (Kannus, Jozsa 1991). Local or systemic corticosteroids (Mahler, Fritschy 1992) and fluoroquinolones (McGarvey, Singh & Trevino 1996) have also been implicated as risk factors for Achilles ruptures. Patients with Achilles tendon ruptures report a sudden snap in their heel with subsequent weakness and discomfort (Schepsis, Jones & Haas 2002). Confirmation of a rupture is determined by the Thomson squeeze test and the palpation of a gap in the tendon (Maffulli 1999).

2.4.3.2 Chronic Achilles Tendon Rupture

The definition of a chronic Achilles tendon rupture varies greatly across the literature but is more commonly known when a patient sustains a rupture that is not diagnosed within 4-6 weeks of the incident (Maffulli, Ajis 2008). Studies have shown that up to 20% of Achilles ruptures are missed during the first examination (Ballas, Tytko & Mannarino 1998). As time passes pain fades away making the diagnosis of chronic ruptures difficult. Following 4 weeks of delay, the tendon sheath may become thickened, would adhere to the retracted tendon ends, and would act as a weakened plantarflexor (Maffulli, Ajis 2008). A detailed examination of a tendon would show the absence of tendinous tissue inside the sheath at the site of the rupture. Furthermore, scar tissue is usually found at the site of the injury bridging both ends of the rupture (Yuan, Wang & Murrell 2003).

2.4.4 Patellar Tendinopathy

Patellar tendinopathy (PT), also known as jumper's knee, is very common in sporting activities involving repetitive jumping such as basketball and volleyball (Khan, Cook & Maffulli 2005). It has been reported that injuries at the level of the patellar tendon are the most common (~26%) knee related pathologies (Kujala, Kvist & Österman 1986, Bollen 2000).

Patellar tendinopathy is considered to be an overuse injury and is characterised by a gradual onset of pain localised at the anterior aspect of the knee. Furthermore, the key feature of patellar tendinopathy is represented by a tenderness of the main tendon body when the knee is fully extended (Blazina et al. 1973). Some studies reported that tendinopathic tendon samples contained yellow-brown disorganised tissue (Reinhart et al. 2000); evidence of mucoid degeneration (Karlsson et al. 1992). However, other studies reported hardness in the tissue indicating a hyaline degeneration (Yu et al. 1995). Similar to Achilles tendinopathy, patellar tendinopathy is characterised by disorganised collagen fibres and the presence of

degenerative necrotic tissue resulting from repetitive micro-traumas (Khan, Cook & Maffulli 2005). The main difference lies in that the damage to the patellar tendon occurs predominantly at the proximal end where as the Achilles tendon is most commonly damaged at mid-substance (Khan, Cook & Maffulli 2005, Jarvinen et al. 2005).

2.4.5 Patellar Tendon Rupture

Patellar tendon ruptures are relatively rare; however, it is not unusual to see them in active individuals around the age of 40 (Siwek, Rao 1981). A patellar tendon rupture, due to an indirect trauma, is believed to be the result of an existing and untreated chronic patellar tendinopathy (Kelly et al. 1984).

Patellar tendon ruptures are usually sustained following a tough eccentric contraction of the knee extensor against the body weight thus placing the knee in a flexed position (Khan, Cook & Maffulli 2005); this is usually seen among weight lifters. Once the tendon is ruptured, pain suddenly shoots, and it becomes impossible for the individual to bare weight if not assisted. Furthermore, it becomes nearly impossible to maintain an extended knee against gravity (Kelly et al. 1984).

It is not uncommon for patellar tendon ruptures to be undiagnosed. In such cases, some knee extension is possible due to the formation of scar tissue which acts as a bridge at the level of the rupture (Takebe, Hirohata 1985). Nevertheless, stair climbing and getting up from sitting position remain hard and painful tasks.

2.5 EPIDEMIOLOGY

There are no accurate records of the global occurrence of Achilles tendon pathology (ATP) or patellar tendon pathology (PTP) but it has been noted that there is an increase in the reported incidences during the 1990s (Fredericson, Misra 2007) as people became more aware of the benefits of exercise. Non-insertional tendinopathies account for 55-65% of ATPs whereas insertional tendinopathies account for 20-25% (Jarvinen et al. 2005). Up to 83% of incidences occur during sports activities (Jarvinen et al. 2005). Leppilahti et al. (1994) estimated the incidence of Achilles tendon rupture in the city of Oulu, Finland, in 1994, to be ~18 per 100,000 individual, and the incidence peak was between the ages 30-39 (Leppilahti, Puranen & Orava 1996b). A more recent study revealed that the average annual mean of Achilles tendon ruptures in Edmonton, Canada was 8.3 ruptures per 100,000 inhabitants (Suchak et al. 2005). Furthermore, a study conducted in Sweden reports that the incidences of Achilles tendon ruptures peak at two intervals. A first and large peak includes young and middle aged individuals. A second smaller peak includes people in their 70s (Möller, Åström & Westlin 1996).

Achilles tendon ruptures occur mainly when taking part in the most popular sport of a country. For instance, higher incidences of Achilles tendon ruptures in Scandinavian countries occur when playing badminton (Fahlstrom, Bjornstig & Lorentzon 1998). In north and central Europe ruptures occur when taking part in football, tennis, track and field, and gymnastics (Józsa, Kannus 1997). Furthermore, incidences in North America are reported in American football, basketball, baseball, and tennis (Józsa, Kannus 1997). On average, 8-20% of patients with Achilles tendon rupture are competitive athletes, 75% are recreational athletes, and 10-12% do not take part in regular physical activities (Leppilahti et al. 1998).

Pathologies of the Achilles tendon are more commonly reported in males, with a male-to-female ratio of 4:1 (Suchak et al. 2005). Although there might be different factors, this figure

reflects the greater involvement of males in sports compared to females. Typically, more men sustain the first Achilles tendon injury during their 30s while women sustain it during their 40s. These individuals tend to occupy white-collar professions, and are occasionally involved in sports (Maffulli 1999).

Achilles tendon ruptures have been described to be unilateral with a slight predominance of injuries in the left foot with a ratio of 1.2:1 (Leppilahti, Orava 1998). Such finding is due to people pushing-off with their left lower limb which predisposes the left Achilles tendon to damage more frequently.

Achilles tendinopathies, as explained earlier, result from repetitive strenuous activities and the subsequent accumulation of micro-traumas. This explains, why running is the main activity for people presenting with ATP. In fact, there is a high incidence rate (11%) of Achilles tendinopathy among middle and long distance runners (Kujala, Sarna & Kaprio 2005).

As mentioned earlier, patellar tendinopathy (PT) is referred to as jumper's knee for its high prevalence (14%) among elite athletes taking part in sport requiring repetitive jumping (Lian, Engebretsen & Bahr 2005). PT was reported to be highly prevalent in volleyball (45%) and basketball (32%) players and represents 15% of soft tissue injuries among military recruits (Lian, Engebretsen & Bahr 2005, Linenger, West 1992). Injuries sustained by athletes require particular attention as they present a career termination risk where the recovery period can be as long as 6 months (Willberg et al. 2011). Similar to ATP, males are reporting more than twice the prevalence of PT than females (Janssen et al. 2014).

A study conducted by Agel et al. (2007) report that PTP among female NCAA volleyball players (season 1988-1989 through 2003-2004) was much lower than previous reports published in the 1980s (Ferretti 1986). It accounted for 3.1% (0.1/1000 athlete) of injuries acquired during matches and 5.4% (0.15/1000 athlete) of injuries during acquired during training (Agel et al. 2007). The authors justify these results by explaining that they only included injuries that resulted in an absence of more than 10 days. Furthermore, PT records among football players in professional European leagues corresponded to 1.5% of all injuries reported between 2001 and 2009 with an incidence of 0.12/1000 hours (Hagglund, Zwerver & Ekstrand 2011). Each season 2.4% of players missed a training session or a match due to PT. Unlike the study on NCAA female volleyball players (Agel et al. 2007), this study included athletes whose injury resulted in absence of less than a week (61% of cases) (Hagglund, Zwerver & Ekstrand 2011).

The prevalence of PTP among Dutch non-elite athletes was reported to be 8.5% (78 of 891 athletes) (Zwerver, Bredeweg & van den Akker-Scheek 2011). The highest numbers of reported injuries were among recreational volleyball (14.4%), handball (13.3%), and basketball (11.8%) players, and the lowest numbers of injuries were among field hockey (5.1%) and football (2.5%) (Zwerver, Bredeweg & van den Akker-Scheek 2011). Furthermore, this study reported a significantly higher prevalence rate in male non-elite athletes (10.2%, 51 of 502) than in female non-elite athletes (6.4%, 25 of 389) and have attributed this to the possibility that females' patellar tendons were exposed to lower forces because they have less quadriceps strength and an inferior jumping capacity (Zwerver, Bredeweg & van den Akker-Scheek 2011).

2.6 FACTORS ASSOCIATED WITH HUMAN TENDON PATHOLOGIES

Numerous factors have been associated with the increased risk of human tendinopathy. Therefore, like most musculoskeletal sports related injuries, tendinopathy is considered a multifactorial condition resulting from a combination of both extrinsic and intrinsic factors (table 2.6.1) as described by Meeuwissie (1994). In his proposed model, Meeuwisse suggests that intrinsic risk factors predispose individuals to the risk of injury. It is important to note that the injury will not take place without the exposure to at least one extrinsic factor, which will determine the susceptibility to the injury. Furthermore, the interaction of intrinsic and extrinsic risk factors does not cause the damage and requires an inciting episode (figure 2.6.1) (Meeuwisse 1994). The inciting event in a tendinopathy is represented in the successive and repetitive micro-traumatic events which occur and place enough strain on the tendon to damage some fibrils and initiate the symptoms. Alternatively, tendon ruptures can result following one macro-traumatic event that exerts enough strain on the tendon and cause a rupture.

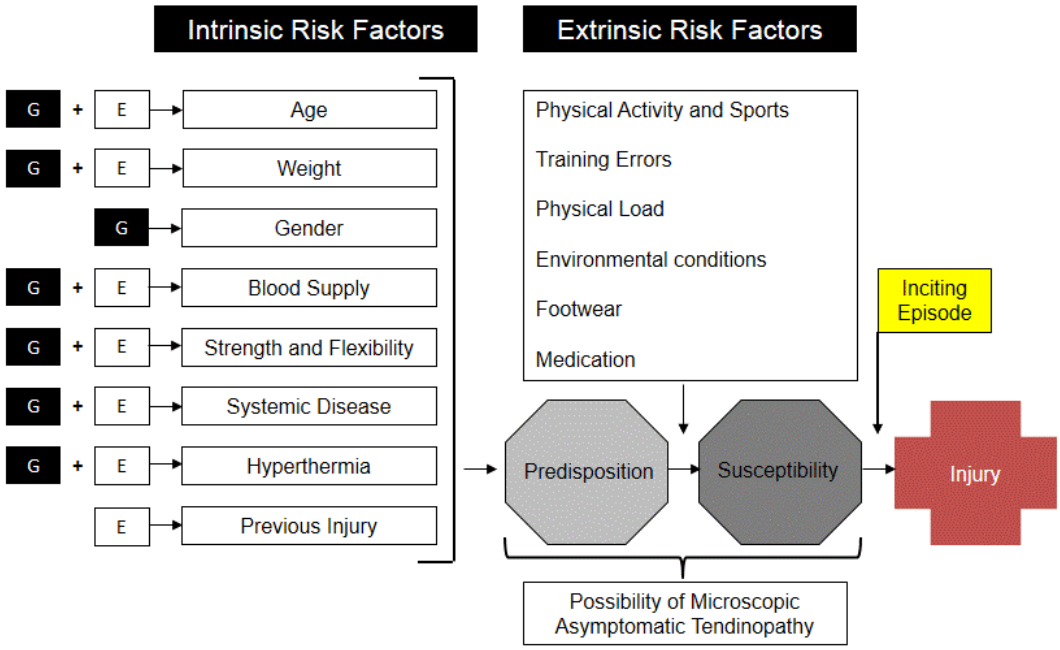


Figure 2.6.1 A schematic diagram illustrating the injury causation model as described by Meeuwisse (1994). The diagram highlights the interaction between the intrinsic, extrinsic factors, and the inciting event in the aetiology of tendinopathy. Many intrinsic factors are multifactorial phenotypes determined by either genetic (G) and environmental (E) factors, or both. This diagram is adapted from Raleigh and Collins (2012) and Ribbans and Collins (2013).

Table 2.6.1 List of major extrinsic and intrinsic tendon pathology risk factors.

Adapted from Riley (2004) and Collins and Raleigh (2009)

Extrinsic factors	**Intrinsic factors**
Daily activity	Age
Type of sport	Gender
Training errors	Anatomical variants
Physical load	Joint laxity
Environmental conditions	Muscle weakness/imbalance
Shoes and equipment	Hyperthermia
Corticosteroids	Systemic disease
Fluoroquinolone Antibiotics	Genetics

Tendon pathologies have been associated with a multitude of disorders, such as inflammatory, autoimmune, genetic, and infectious diseases (Maffulli 1999). However, there is little agreement with regard to its aetiology.

Different disease processes may predispose the tendon to accumulate damage ranging from degeneration to ruptures (Waterston, Maffulli & Ewen 1997). As previously described, the blood flow in the tendon decreases with age (Astrom, Westlin 1994) therefore the area with a decreased vascularity is more prone to sustain damage in comparison with the rest of the tendon (Kuwada 1995). In addition to daily activity, sports place additional stress on tendons leading to the accumulation of micro-traumas which can eventually cause ruptures or alternatively lead to chronic tendinopathy described in Section 2.4 (Maffulli et al. 2007).

2.6.1 Extrinsic Factors Associated With Increased Risk of Tendon Pathologies

2.6.1.1 Daily Activity

It is believed that regular loading of tendons through regular activity and daily exercise increase the tensile strength of tendons (Buchanan, Marsh 2002, Kjaer et al. 2005). This is believed to take place following activity-induced collagen type-I production (Kjaer et al. 2005). On the other hand, reduced activity results in the reduction in collagen synthesis, which gives way to a stimulation of MMP activity (Kjaer et al. 2005). MMPs are known to degrade collagens which would ultimately lead to a decrease in tensile strength resulting in fragile tendons.

2.6.1.2 Training Errors

Among runners, the most common causes of Achilles tendon injuries are training errors, followed by anatomical differences (Fredericson, Misra 2007). Factors such as a sudden increase in training intensity, or the sudden change of inclination are major extrinsic factors predisposing to ATP (Fredericson, Misra 2007). Similar to ATP, PTP could result from

repetitive loading of the tendon such as the repetitive exposure to counter-forces when landing from jumps, and fast progression in intensity (Cook et al. 1997). There is a limited number of studies conducted on training errors and the risk of ATP or PTP. However a 1978 study reports that 60% of running injuries were attributed to training errors (James, Bates & Osternig 1978).

2.6.1.3 Environmental Conditions

Exercising in extreme weather conditions has been suggested as a risk factor for tendinopathy (Riley 2004). However, there is limited evidence to support these claims. A single study conducted on 1500 military recruits reported an elevation in incidence rate of ATP during the winter season (Milgrom et al. 2003). The authors believe that the reduced temperatures increased the viscosity of the lubricant around the tendon which might have lead to increase friction. However, given that training surfaces have been cited as risk factors (Jarvinen et al. 2005), it could be hypothesised that the observed increase in incidence during colder weather could be an interaction between training on moist and muddy ground, and the low temperatures. On the other hand, there is no difference in the number of rugby, football, and American football injuries recorded on natural turf compared to artificial turf (Williams, Hume & Kara 2011). Therefore training surfaces cannot be seen as independent risk factors.

2.6.1.4 Footwear

Sports footwear are seen as protective garments worn during exercise to reduce the impact of collision between the foot and the ground (Robbins, Gouw 1991). In fact, different types of footwear have been reported to show differences in the risks of injuries. There was a 9.9% reduction in tension measured within the Achilles tendon when high-top athletic shoes were worn (Rowson, McNally & Duma 2010). Furthermore, wearing high-top shoes resulted in 7.2% reduction in the angle of ankle dorsiflexion compared to low-top shoes. With that in

mind, Rowson at al (2010) suggested that high-top shoes as well as well-tied laces will act as one body with the foot and will prevent the shoe from moving independently of the foot. Such fixation of the foot in the shoe would help the transmission of forces to the shoe and prevent its accumulation in the foot and the tendons. It should be noted however that footwear orthotics and in-soles can reduce the risk of injury by correcting biomechanical malalignments (Rosenbloom 2011).

2.6.1.5 Corticosteroids

Corticosteroids are a class of chemicals involved in a wide range of physiologic responses, mainly the regulation of inflammation (de Kloet, Oitzl & Joëls 1999). Corticosteroids are administered for a variety of diseases and have been widely implicated in tendinopathies (Mahler, Fritschy 1992). In a study published in 1972, different rabbits' calcaneal tendons were injected with hydrocortisone, and a saline solution. Interestingly, necrosis was reported after 45 minutes in the rabbits injected with hydrocortisone. Furthermore the rabbits injected with hydrocortisone showed a delayed healing response in comparisons with those that received the saline solution (Balasubramaniam, Prathap 1972).

Reviews of corticosteroid treatment of tendinopathy have acknowledged the short term benefits, however they stress on the severity of the long term outcome which include tendon rupture (Coombes, Bisset & Vicenzino 2010). The anti-inflammatory and analgesic properties of corticosteroids tend to mask various symptoms of tendon injury (Rees, Stride & Scott 2013) resulting in athletes maintaining a high level of activity instead of resting.

A meta-analysis published in 1996 has shown that corticosteroid injections do not seem to play a beneficial role in the treatment of Achilles tendinopathy (Shrier, Matheson & Kohl 1996). Other scientists have discussed the presence of corticosteroids in tendinopathic tendons (Unverferth, Olix 1973). Such findings reveal that corticosteroid accumulation at that

particular area compromises the proper functioning and wellbeing of the tendon. Furthermore, orally administered corticosteroids did not show different effects on the potential risks of tendon pathology (Sánchez et al. 2012). Therefore, given the available literature and evidences, corticosteroid administration should probably be avoided.

2.6.1.6 Fluoroquinolones

Fluoroquinolones are a family of antibacterial drugs that act by inhibiting bacterial DNA replication (Blondeau 2004). Fluoroquinolone antibiotics have been implicated in the aetiology of tendinopathies (Childs 2007). There are different ways in which fluoroquinolones may induce tendinopathies. First, they mediate G2/M cell cycle arrest in tendon cells by down-regulation of cyclin B and cyclin-dependent kinase 1 (Tsai et al. 2009). Second, they restrain migration of tenocytes by down-regulation of focal adhesion kinase phosphorylation (Tsai et al. 2009). Third, they boost the enzymatic activity of matrix metalloproteinase-2 which in turn degrades type I collagen (Tsai et al. 2011). Fourth, they decrease the expression of decorin which affects the elastic property of tendons and render them more fragile (Tsai et al. 2011).

In France, between 1985 and 1992, 100 patients being treated with fluoroquinolone antibiotics developed tendon disorders, including 31 ruptures (Royer, Pierfitte & Netter 1994). However, it was hard to implicate only fluoroquinolones since many had also received corticosteroids. Tendinopathy and tendon rupture were reported in 98 patients treated with fluoroquinolones: the principal tendon affected was the Achilles (Khaliq, Zhanel 2003). Therefore fluoroquinolones should not be given to athletes since it represents a hazard of tendon damage when combined with their training load (Lewis, Cook 2014).

2.6.2 Intrinsic Factors Associated With Increased Risk of Human Tendon Pathology

2.6.2.1 Age

Age is often listed as an intrinsic risk factor for tendinopathy. However, these injuries are activity specific since sports induced injuries are reported between the ages of 30-50 (Houshian, Tscherning & Riegels-Nielsen 1998) whereas the non-sports induced injuries occur at a later stage (Suchak et al. 2005). It could be therefore suggested that the mechanisms of injury differ between younger and older individuals. This can be explained by the observed age related changes in tendon properties (Tuite, Renström & O'brien 1997). For instance, there is a reported reduction in density and activity of tenoblasts in addition to a decrease in ECM glycosaminoglycans and increase in collagen content (Dressler, Butler & Boivin 2005). Collagen fibres increase in diameter which affects the structural cross-links rendering the tendon more susceptible to injury (Dressler, Butler & Boivin 2005). Furthermore, as discussed earlier in section 2.1.3 tendons of younger individuals are better vascularised which result in better delivery of oxygen to tendon cells (Theobald et al. 2005). Therefore, it is believed that the reduction in blood flow increases the risk of hypoxia and consequently death of tenocytes (Kannus, Natri 1997).

2.6.2.2 Gender

It has been stated earlier in the epidemiology section that there is a higher male : female ratio of ATP due to the higher involvement of males with sports (Maffulli 1999). Females however were found to have a higher incidence rate of other musculoskeletal injuries such as ACL ruptures (Mountcastle et al. 2007). It was suggested that females are biologically more vulnerable to soft tissue pathology given the hormonal activity accompanying the menstrual cycle (Slauterbeck et al. 2002). Oestrogen for instance, whose receptors have been identified in connective tissue, was reported to negatively affect the production of

collagen thus resulting in a smaller cross-sectional area of tendon which would withstand lower tensile forces (Magnusson et al. 2007).

2.6.2.3 Anatomical Factors

It was once stated that healthy tendons would not rupture (McMaster 1933). Barfred tested this hypothesis and suggested that in cases where straight traction was applied, the forces generated are evenly distributed across the muscle-tendon complex, and would therefore reduce the risk of tendon damage (Barfred 1971). However, in cases where oblique traction is applied, forces are concentrated on the tendon rendering it more prone to damage (Barfred 1971).

Anatomical variances are second to training error as risk factors of tendon injuries (Fredericson, Misra 2007). Tendon damage caused by non-uniform stress between the gastrocnemius and the soleus is due to an uneven force contribution by each muscle (Leadbetter 1992). This results in uneven forces within the tendon which ultimately generate frictional forces between the fibrils (Leadbetter 1992). Furthermore, 2/3 of athletes diagnosed with an ATP showed signs of malalignment e.g. hyperpronation, limited subtalar joint mobility, and limited range of motion within the ankle joint (Kvist 1994). Indeed, Kvist (1994) suggests that heal strike running with excessive pronation intensifies the “whipping action” within the tendon, thus predisposing it to ATP.

In addition to malalignments, leg length discrepancy is also believed to be another potential contributor (Kannus, Natri 1997). In the average person, discrepancies of 25 mm are not considered major risk factors for ATP. However, in elite athletes, a discrepancy greater than 5 mm multiplies the risk of developing a pathology, and therefore the use of shoe insoles is advised (Kannus, Natri 1997). Additionally, both cavus foot and flat foot with excessive pronation, foot abnormalities, and malalignment factors above the ankle, such as genu

varum, can also contribute to increased stress on the Achilles tendon (Schepsis, Jones & Haas 2002).

The intrinsic factors reported for PTP are similar to those described in ATPs. Studies have reported a significant correlation between patellar tendinopathy and leg length inequality (Kujala, Kvist & Österman 1986). Furthermore, the pain reported at the level of the patella insertion correlates with an increased laxity in the knee joint (Kujala, Kvist & Österman 1986).

2.6.2.4 Hyperthermia

Stretching of tendons generates energy which is released when the tendon relaxes (Ker 1981). Such accumulation and release of energy does not damage the tendon. However, the repetition of this physiological behaviour may result in the accumulation of heat from cellular respiration (Maganaris, & Narici 2005). Up to 10 % of the elastic energy stored in tendons may be released as heat (Ker 1981). Wilson and Goodship evaluated the temperatures generated *in vivo* within equine superficial digital flexor tendons during exercise (Wilson, Goodship 1994). The temperature of 45 °C was measured within the core of the tendon after seven minutes of trotting. This is the temperature at which tenocytes can be damaged (Arancia et al. 1989). Therefore, exercise-induced hyperthermia, may contribute to tendon degeneration. Given that good vascularisation and blood supply to tissues should help by cooling the heated area (Hastad, Larsson & Lindholm 1959), it is believed that tendons of older individuals are more at risk of incurring a pathology because the decrease in vascularisation attributed to the aging process decreases the dissipation of heat (Maffulli 1999).

2.6.2.5 Systemic Diseases

Systemic diseases such as diabetes mellitus, alkaptonuria, gout, rheumatoid arthritis, adrenal disorders, thyroid problems, and amyloidosis are believed to contribute to 2% of ATP cases (Abate et al. 2013, Järvinen et al. 2001). As these conditions develop, they lead to a reduction in vascularity in and around tendons, followed by matrix degradation (Abate et al. 2013, Fox et al. 2011). Furthermore, genetic disorder such as Ehlers-Danlos syndrome, Marfan syndrome, and osteogenesis imperfecta which lead to joint hypermobility have also been listed as risk factors of human tendinopathy (Malfait, Wenstrup & De Paepe 2010).

2.6.2.6 Genetic Risk Factors

In 1989, a study investigated the ABO blood group as a genetic marker for tendon pathologies. The investigators found that the O blood group was associated with an increased risk of ATP and other tendon pathologies in a Hungarian population (Kannus, Natri 1997, Jozsa et al. 1989). Nevertheless, several attempts to replicate this study have failed (Leppilahti, Puranen & Orava 1996a, Maffulli et al. 2000).

It was suggested that genes (coding for extracellular matrix proteins) closely located to the ABO locus might represent better candidates for an association with ATP. Knowing that the ABO blood group locus is found on the long arm of chromosome 9, Mokone et al. (2005, 2006) investigated whether a possible association existed between the *TNC* and *COL5A1* genes, located on the same chromosome, and the risk of developing ATP (Mokone et al. 2005, Mokone et al. 2006). In the first genetic association study, a South African (SA) case-control cohort was recruited to investigate the poly-GT micro-satellite variant within the TNC gene. The findings of the study showed that the GT allelic repeats 12 and 14 were over-represented in participants with ATP, while the 13 and 17 repeat alleles were significantly under-represented (Mokone et al. 2005). These results suggest that alleles 12 and 14 could be causative variants whereas alleles 13 and 17 might be protective. Furthermore, the

rs2104772 and rs1330363 gene variants within the TNC genes reported allelic associations (p=0.017; p=0.020 respectively) with ATP (Saunders et al. 2013b). Saunders et al. (2013) further showed an interaction between the *TNC* and *COL27A1* gene. Specifically, the *TNC* rs13321, *TNC* rs2104772 and the *COL27A1* rs946053, which were not independently associated with ATP, formed a CAG haplotype which was significantly (p=0.019) over represented in ATP group.

Furthermore, a second study found that the CC genotype in the *Bst*UI restriction fragment length polymorphism (RFLP) within the 3'- untranslated (UTR) region of the *COL5A1* gene (also known as rs12722) was significantly over-represented in the SA asymptomatic control participants of the study (Mokone et al. 2006). Interestingly, this study was replicated in an Australian (AUS) population group and generated similar findings (September et al. 2009). Therefore it was concluded that carriers of the *COL5A1* rs12722 CC genotype were at reduced risk of ATP in the SA (OR 0.38, 95% CI: 0.18-0.77; p=0.008) (Mokone et al. 2006) and AUS (OR 0.42, 95% CI: 0.20-0.86; P=0.017) (September et al. 2009) populations. Interestingly, the rs12722 variant was associated with additional phenotypes such as ACL ruptures in females (Posthumus et al. 2009), joint range of motion (Brown et al. 2011), and endurance running performance (O'Connell, Posthumus & Collins 2014). Based on that, it has been suggested that the TT allele at the rs12722 variant results in the formation of densely packed thin collagen fibrils which lead to increased risk of injury through the reduction of tensile strength (Collins, Posthumus 2011). This is further discussed in Chapter 4 of this thesis.

A novel gene variant, *COL5A1* rs71746744, was recently associated with ATP in the SA and AUS populations (Abrahams et al. 2013). This variant along with the rs12722 variant were suggested to play a role in the stability of the *COL5A1* mRNA (Laguette et al. 2011). Laguette et al. (2011) report a lower luciferase activity (69.0 ± 22.0%, n=24 vs. 90.6 ± 13.7% n=30, p<0.001) when the C allele at the rs12722 locus is present in comparison to the T

allele. Furthermore, the TCT pseudohaplotypes formed by the *COL11A1* rs3753841, *COL11A1* rs1676486, and *COL11A2* rs1799907 was significantly over-represented in the ATP group (Hay et al. 2013). The three variants were not independently associated with ATP. However, an interaction between *COL11A1*, *COL11A2*, and *COL5A1* was reported where the TCT(AGGG) pseudohaplotype was significantly over-represented in the ATP group (Hay et al. 2013). Additional collagen coding genes have been investigated and showed no association with ATP, but were associated with ACL, such as the *COL1A1* (Posthumus et al. 2009b).

Additional genes encoding proteins involved in biological processes in the Achilles tendon were also examined for possible association with Achilles tendon injuries. For example, the matrix metalloproteinases (MMPs) are enzymes known for their role in maintaining homeostasis within tendons by degrading structural proteins. One of the proteins within this family, MMP3, degrades various collagen types as well as laminin, fibronectin, proteoglycan, decorin and aggrecan (Birkedal-Hansen et al. 1993). In a study aimed at researching a possible association between *MMP3* and ATP, Raleigh et al. (2009) investigated the possible association of three single nucleotide polymorphisms (SNP) within the *MMP3* gene and the risk of developing ATP. Individuals carrying the homozygote genotypes for the rs679620 (GG: OR=2.5, 95% CI 1.2 to 4.90, p=0.010), rs591058 (CC: OR=2.3, 95% CI 1.1 to 4.50, p=0.023), and rs650108 (AA: OR=4.9, 95% CI 1.0 to 24.1, p=0.043) SNPs were found to be at greater risk of developing ATP than those carrying other genotypes (Raleigh et al. 2009). Furthermore, when the G allele of the rs679620 variant within the *MMP3* gene and the T allele within the *COL5A1* rs12722 variant were combined, the risk of ATP was increased therefore indicating an interaction between the two variants (Raleigh et al. 2009).

In addition to *MMP3*, the *GDF5* gene encodes for a protein involved in the maintenance, growth, and repair of cartilage and musculoskeletal soft tissues (Settle Jr et al. 2003). GDF5 proteins are believed to enhance tendon healing (Aspenberg, Forslund 1999) by stimulating collagen synthesis (Wolfman et al. 1997, Mikic 2004). In 2010, Posthumus et al. reported that the TT genotype of the *GDF5* rs143383 variant was significantly over-represented in the ATP group of a Caucasian population. Furthermore, gene variants within the *CASP8*, *IL-6*, *IL-1β*, and *IL-1RN* which are involved in the inflammatory pathway have also been implicated with ATP (September et al. 2011, Nell et al. 2012).

In addition to the aforementioned variants, many gene variants were not associated with ATP such as those found in *COL1A1* (Posthumus et al. 2009a), *COL12A1*, *COL14A1* (September et al. 2008), *TGFβ* (Posthumus et al. 2010), *THBS2*, and *COMP* (Saunders et al. 2013a).

2.7 EPIGENETIC MECHANISMS AS POTENTIAL RISK FACTORS FOR HUMAN TENDINOPATHY

The heritability of phenotypes associated with medical pathologies has been investigated and discussed by different experts (Ehlert, Simon & Moser 2013). Following the completion of the Human Genome Project, it was thought that the obtained genomic data would reveal the origin of different phenotypes. However, gene expression differences in specific tissues between diseased and healthy samples could not be strictly attributed to variations in the genomic sequence. There is a growing number of evidence that heritable changes in gene expression are not influenced by mere genetic factors as described above, but also by numerous epigenetic factors (Jirtle, Skinner 2007).

Epigenetics has been hard to define with consensus given the different regulatory mechanisms involved such as histone modifications, DNA methylation, and the expression

of non-coding RNAs (Berger et al. 2009). Nevertheless, a large number of scientists define epigenetics as heritable changes in gene expression that occur independent of changes in the primary DNA sequence (Russo, Martienssen & Riggs 1996).

The role of epigenetic changes in the development of human tendinopathy was never explored. This subsection will go over different epigenetic mechanisms and their impact on health and physiological phenotypes which could be suspected to contribute to the onset of tendinopathies.

2.7.1 Histone Modifications

Histone modifications deal directly with the accessibility of transcription factors to DNA by changing the chromatin conformation and structure (Zhou, Goren & Bernstein 2010). The nucleosome octamer is composed of eight histones around which the DNA molecule is packed. Histones are subjected to various modifications induced by acetylatio or methylation which would determine a loose or tight chromatin packaging respectively (Zhou, Goren & Bernstein 2010).

Histone acetylation is universally associated with gene activity (Egger et al. 2004). When the acetyl group is added to the amino acid lysine of the histone tail, it reduces the positive charge on the histone which leads to a reduced electrostatic attraction between the positively charged histone and the negatively charged DNA. The end product of such a mechanism is a loose packing of DNA which is more accessible for transcription factors (Grunstein 1997).

Histone methylation can be associated with either gene activity or inactivity. The most commonly discussed methylation marks are the methylation of the fourth lysine on histone H3 (H3K4) which activates gene expression in addition to the methylation of H3K9 and

H3K27 both of which are linked to gene silencing (Zhou, Goren & Bernstein 2010). The methylation of H3K4 facilitates gene expression by recruiting the chromatin remodelling factors CHD1 (Flanagan et al. 2005) and BPTF (Li et al. 2006) which open chromatin, while preventing the binding of repressive complexes like NuRD (Nishioka et al. 2002) and INHAT (Schneider et al. 2004).

On the other hand, the methylation of H3K9 leads, indirectly, to the recruitment of DNA methyltranferase enzyme (DNMT) (Handy, Castro & Loscalzo 2011) which methylates CpG sites on the DNA strand and by that suppressing gene expression as described later in this thesis. Furthermore, DNMT1 will recruit histone deacytelase enzyme which removes the acetyl group from the lysine on the histone tail (Taverna et al. 2007). This leads to a tighter and more compact chromatin where the DNA molecule is not accessed by the transcription factors (Fuks 2005).

Modifications of the histone packaging have been described in various pathologies and therefore suggest an important role in health and disease (Jirtle, Skinner 2007). A common mark of human cancer is the loss of acetylation of lysine 16 and the methylation of lysine 20 in histone H4 (Fraga et al. 2005a). In addition to that, modifications in histones H3 and H4 could be used to predict the recurrence risk of prostate cancer as suggested by Seligson et al. (2005).

Similar to other environmental factors, exercise promotes modifications of histone structure. In a study conducted by Collins et al. (2009) rats' adaptation to new environments following physical exercise were tested. Subjects who exercised coped better with stress than the controls who did not exercise. Upon the investigation of the exercised rats' brain cells, the authors of the paper found an increase in histone acetylation which associates with loosely packed chromatin and thus more accessible DNA (Collins et al. 2009). It can be concluded that gene expression facilitated by histone acetylation helped rats cope with stress.

2.7.2 DNA Methylation

DNA methylation is an epigenetic modification where a methyl group binds to a cytosine base converting it to 5-methylcytosine (Robertson 2005). In particular, a cytosine followed by a guanine forming a CpG site, could potentially attract a methyl group and become methylated (Golbabapour, Abdulla & Hajrezaei 2011). Stretches of DNA with high concentrations of CpG sites are called CpG islands (Bock et al. 2007). CpG islands are assessed according to the criteria discussed by Gardiner-Garden (1987): 1) the island should be greater than 200 bp, 2) the GC contents should be greater than 50%, and 3) the ratio of observed/expected (obs/exp) CpG sites should be greater than 0.6. The obs/exp ratio is calculated as shown in the formula below (Bock et al. 2007, Gardiner-Garden, Frommer 1987):

(Obs/exp) CpG sites = N x [number of CpG sites / (number of C x number of G)]
N = number of nucleotides in the sequence of interest.

The methylation is promoted by DNMT enzymes which are believed to transfer a methyl group from S-adenosylmethionine (SAM) to the cytosine on CpG sites (Golbabapour, Abdulla & Hajrezaei 2011). CpG islands are associated with the promoter region of genes since they can be used to regulate gene activity. In specific, DNA methylation serves to down regulate gene expression by regulating the accessibility of genes to transcription factors (Ehlert, Simon & Moser 2013). It is worth noting that in normal cases CpG islands are not methylated, instead CpG sites outside the promoter region eg. enhancer region of genes, or intragenic sites are more likely to be methylated (Meissner et al. 2008, Maunakea et al. 2010). The down regulation of genes is caused by different factors: 1) cytosine methylation could prevent the binding of transcription factors; or 2) CpG methylation could indirectly interfere with chromatin/histone folding (Weber, Schübeler 2007). DNA methylation

does not act alone on gene regulation but is acting collectively with other epigenetic factors on determining chromatin structure and thus gene accessibility. In some cases DNA methylation could enhance gene expression by silencing the transcription of a silencing element (Suzuki, Bird 2008).

Following fertilisation, zygotes undergo a genome wide demethylation followed by a *de novo* remethylation (Morgan et al. 2005). The *de novo* methylation process is believed to be important for the specialisation of pluripotent cells during growth and development. Nevertheless there are genes that do not undergo demethylation. Instead, their methylation status is passed on to future generations along with the respective phenotypes (Wood, Oakey 2006, Reik, Walter 2001). While the DNA methylation status of some genes is determined primarily in the prenatal and early postnatal stage, and is assumed to be stable with a lifelong effect on gene expression (Farthing et al. 2008), other genes are subjected to changes in methylation levels as discussed in a study on monozygotic twins. This study reports that twins are likely develop different epigenomes as they age and adopt different lifestyles (Fraga et al. 2005b).

The role played by DNA methylation in health and disease has been identified and discussed in different papers (Robertson 2005, Rushton et al. 2014, Javierre et al. 2010). Alterations in DNA methylation statuses have been reported to take place as people age, therefore it is not surprising to associate epigenetic changes with age related conditions such as rheumatic diseases and tendinopathies. A study conducted by Klein et al. (2013) reported an altered methylation status of the promoters of genes associated with the inflammatory cascade in peripheral blood mononuclear cells and fibroblast-like synoviocytes (Klein, Gay 2013). Interestingly, similar results were obtained in epigenome-wide association studies (Nakano et al. 2013, Liu et al. 2013). Likewise, in osteoarthritis (OA) different methylation levels of the promoter regions of genes coding for proteins involved with cartilage structure and maintenance such as COL1A2, COL5A3, MMP13, ADAMTS4 (Barter,

Bui & Young 2012) and GDF5 (Reynard et al. 2011) were reported. These observations are believed to contribute to the differential expression of those genes in OA.

Physical activity and exercise contribute greatly to changes in DNA methylation profiles of different tissue cells. The methylation profile of an individual changes in response to physical activity, in a dose-dependent, gene-specific and tissue-specific manner. Barres et al. (2012) showed that acute exercise alters skeletal muscle cells DNA methylation statuses by increasing methylation of some genes, and reducing the methylation of other genes. Changes to the DNA methylation levels are initiated once an individual exceeds the exercise intensity threshold (Voisin et al. 2014): eg. in response to high intensity exercise skeletal muscle cells release calcium ions which interact with other cellular components to initiate an active demethylation of metabolic genes (Barres et al. 2012). On the other hand, to examine the effects of chronic exercise on DNA methylation levels, Alibegovic et al. (2010) placed young men on bed-rest for 9 days which significantly increased the methylation levels of the *PGC1A* gene, known to act as a regulator of muscle fibre type determination, and as a link between external physiological stimuli and mitochondrial activity. It is worth noting that the methylation levels were coupled with the reduction of *PGC1A* gene expression. These participants were later retrained for a period of 4 weeks. The results showed tendency towards altered methylation however those were not significant (Alibegovic et al. 2010).

A longitudinal genome-wide study by Nitert et al. (2012) compared the skeletal muscles' methylation levels of young sedentary individuals, before and after a 6-month endurance exercise training program. After the completion of the program, the methylation levels of genes coding for proteins associated with endurance phenotypes (RUNX1, MEF2A, and BDKRB2) were decreased. The decrease of *BDKRB2* methylation was coupled with an increase in mRNA levels (Nitert et al. 2012). When attempting to replicate this work in adipose tissue, the same research team found a global increase in methylation levels (Rönn et al. 2013). This increase in methylation levels was coupled with only one third of mRNA

expression changes. These studies revealed an interesting difference in the response of skeletal muscle cells (hypomethylation) and adipose tissue (hypermethylation) to 6 months of endurance exercise.

The literature reviewed above highlights the role played by DNA methylation in altering gene expression in connective tissue pathologies and also highlighted the effect of physical activity and exercise on methylation levels. Therefore it is important to investigate the status of DNA methylation in sports related pathologies such as tendinopathies.

2.7.3 Non-coding RNAs

As the name indicates, non-coding RNAs (ncRNA) are RNAs that do not code for proteins. There are many types of non-coding RNAs grouped into two major classes based on their sizes. Small ncRNAs which include Piwi-interacting RNA, small-interfering RNAs, and micro-RNA are transcripts shorter than 200 nucleotides (nt) (Tang et al. 2014). The second class of ncRNA includes the long non-coding RNAs which range from 200 nt to 100 kb (Gibb et al. 2011). In recent years it became evident that ncRNAs carried on the non-protein coding portion of the genome were functionally active in the determination of physiological and health phenotypes (Mercer, Dinger & Mattick 2009). These molecules have been described to regulate different cell mechanisms such as translation, proliferation, differentiation, and apoptosis by interacting with different acting proteins and are therefore important epigenetic regulators (Wang, Sen 2011).

Although all ncRNAs are expressed in human cells, current research has been focused on micro-RNAs, since they are considered to be a critical component of complex functional pathways (Tang et al. 2014). Therefore this review will focus on them in the section below.

2.7.3.1 Micro-RNA

Micro-RNAs (miRNA) are small (~ 20 nucleotide) non-protein-coding transcription products formed endogenously (Güller, Russell 2010). Details of their biogenesis are described in Chapter 7. They are known to repress gene expression post-transcriptionally by blocking translation or by degrading target mRNAs (Hamilton, Baulcombe 1999, Reinhart et al. 2000). Approximately one third of genes could be regulated by miRNAs (Lewis, Burge & Bartel 2005). In fact, a single miRNA could target several genes (Vella et al. 2004), and a gene could be targeted by several miRNAs (Doench, Sharp 2004).

Micro-RNAs are naturally transcribed by cells to maintain homeostasis through the regulation of gene expression (Tang et al. 2014). The role of miRNA in the onset of chronic diseases has been extensively studied (Mendias, Gumucio & Lynch 2012, Arroyo et al. 2011, Shen, Ambrosone & Zhao 2009, Zufferey, Williams & Spector 2014). The expression levels of tumour miRNA were found to be different than those of normal cell which explains the differences in intra-cellular activities within diseased and healthy cells. These differences can contribute greatly to changes in cellular mechanisms (growth and apoptosis) and therefore the onset of diseases (Tang et al. 2014).

In cancers, both the presence and absence of specific miRNAs play an important role in tumorigenesis. The down regulation of a miRNA can provide a greater opportunity for the expression of an oncogenic gene which is normally silenced by miRNA: eg. deficiency of miR-15a and miR-16-1 expression can lead to overexpression of *Bcl2* (Cimmino et al. 2005), an important anti-apoptotic factor, resulting in decreased apoptosis, thereby promoting tumorigenesis and tumor development (Tie, Fan 2011). On the other hand, up-regulation of a miRNA which targets a tumour suppressor gene can also lead to the initiation and development of tumours: the over-expression of miR-221 or miR-222 suppresses the

expression of the gene coding for Kit protein which controls apoptosis, resulting in tumorigenesis in the thyroid (He et al. 2005).

MicroRNAs have also been described to play a role in musculoskeletal soft tissue pathologies. In human OA, the reported increases in levels of aggrecan were explained by the deficiency of the aggrecanase ADAMTS5 (Bondeson et al. 2008). It was later reported that this deficiency could be resulting from an increase in the levels of miR-140 which decreases ADAMTS5 (Miyaki et al. 2009). Over-expression of other miRNA such as miR-9, miR-98 and miR-146 also disrupts homeostasis in OA by reducing the production of TNFa (Jones et al. 2009). The expression of miRNA also correlates with the degree of the disease as described by Yamasaki et al. (Yamasaki et al. 2009) where the expression of miR-146a decreases when the severity of OA increases. As described above and in Chapter 4, metalloproteinases play an important role in the maintenance of ECM homeostasis. Reductions in miR-27a levels were reported in OA and are believed to disrupt the normal levels of MMP13 (Akhtar et al. 2010). Likewise, structural proteins are subject to miRNA activities where COL2A1 is indirectly up-regulated by the activity of miR-675 (Dudek et al. 2010).

Similar to previous epigenetic mechanisms miRNAs are differently expressed following exercise (Nielsen et al. 2010). Individuals who took part in resistance exercise training displayed different miR-378, miR-29a, miR-26a, and miR-451 expression levels between low and high responders. Such observations suggest a major role played by miRNA activity in determining muscle growth (Davidsen et al. 2011). Furthermore, exercise changes the level of circulating miRNA, as demonstrated by Nielsen et al. (2014). They showed a decrease in blood-circulating miRNA (miR-106a, miR-221, and miR-30b) immediately after an acute bout of exercise. However the levels of other miRNAs (miR-1, miR-133a miR-223 and miR-143) were significantly increased 1 h post-exercise. Based on that, it could be suggested that the sharp drop in the levels of miR-106a, miR-221, and miR-30b was part of the skeletal

muscles' response to acute exercise (Nielsen et al. 2014). It was not however possible to postulate whether the observed increase in miR-1, miR-133a miR-223 and miR-143 circulating levels was indicative of a recovery process, or resulting from damage that skeletal muscles would have incurred as result of the intensity of the activity. It is important to note that circulating miRNAs may enter recipient cells and by that can engage in exogenous interference and regulation of gene expression (Arroyo et al. 2011). Such activity has not been reported in musculoskeletal pathologies; however it is worth investigating whether circulating miRNAs produced following a specific physical activity can penetrate tendon cells and initiate a tendinopathy. No study up to this point has determined whether circulating miRNAs produced post-exercise affect the proper functioning of physiological systems.

After having explored three different epigenetic mechanisms, it is important to appreciate that they do not act independent of one another or of the variations in the genome. Difficulties in identifying genes associated with diseases suggest that phenotypes are determined by the interactions of different epigenetic mechanisms with each other and with genetic variations. For instance, DNA methylation can promote the methylation of H3K9 which leads to a more repressive chromatin state (Fuks et al. 2003). Furthermore, given that miRNAs are coded in the DNA sequence, their expression levels are dependent on the histone and DNA methylation statuses (Saito et al. 2006). With that in mind epigenetic regulations represent a forthcoming avenue of research in human tendinopathy.

2.8 AIMS AND OBJECTIVES OF THE THESIS

As discussed in the previous section, the aetiology and mechanisms of human tendinopathy have not been fully elucidated. It has been established however that human tendinopathies are multifactorial conditions resulting from the interaction of several extrinsic and intrinsic risk factors. As more studies revealing the association of gene variants with human tendinopathies are being published, confidence grows around the existence of genetic predispositions. Therefore it is important to conduct additional studies to identify all variants associated with tendinopathy. Based on that, the primary aim of this thesis was to identify genetic variants associated with human tendinopathy and propose biological mechanisms underlying the genetic risks. The secondary aim was to identify the potential roles played by different epigenetic factors in the development of tendinopathy. The objectives put in place to atcheive the aims of the thesis were:

- To recruit a UK case-control population with the purpose of identifying gene variants as determinant of human tendinopathy.
- To determine whether previously investigated variants within the *COL5A1*, *GDF5*, (Chapter 4) and *MMP3* genes (Chapter 5) were associated with ATP in a UK population.
- To determine if gene variants within structural proteins like *ELN* and *FBN2* were associated with ATP (Chapter 4).
- To determine if gene variants within the *ADAM12*, *ADAMTS2*, *ADAMTS5*, *ADAMTS14*, and *TIMP2* genes were associated with ATP (Chapter 5).
- To determine whether there are different, epigenetic *TIMP2* and *GDF5* DNA methylation profiles between healthy and damaged patellar tendon (Chapter 6) and Achilles tendon samples (Chapter 7).
- To determine whether there are different gene (*TIMP2*) and miRNA (miR-21, miR-155, and miR-191) expression levels between healthy and damaged Achilles tendon samples (Chapter 7)

Chapter 3 Material and Methods

3.1 GENERAL APPROACH TO INVESTIGATING GENE VARIANTS ASSOCIATED WITH COMPLEX DISEASES

It has been stated earlier in this thesis that the main aim was to identify gene variants that predispose humans to tendinopathy. In order to characterise the genetic contributors of a particular medical condition, genetic association studies are conducted. These studies aim at relating genetic information derived from a population to a complex disorder status (Lewis, Knight 2012). Complex disorders result from the interaction of both genetic and environmental factors. In fact, the risk of a complex medical condition is controlled by different gene variants with small to moderate effects (Lewis, Knight 2012).

Variants such as insertion/deletion, copy number variations (CNVs), variable-number tandem repeats (VNTRs) and short tandem repeats (STRs) can be used as genetic markers for association studies. However, the most widely used variants in association studies are the single nucleotide polymorphisms (SNPs) (Lewis, Knight 2012). Genetic associations can be regarded as direct associations, indirect associations, or false-positives. Direct associations involve the association of non-synonymous SNPs which are considered putative causal variants due to their role in altering codons (Cordell, Clayton 2011). Causal variants are not strictly located in the coding region and can be found in the non-coding region where they can play a role in the regulation of gene expression (Tabor, Risch & Myers 2002). There is limited knowledge that can help in predicting which variants play a causal role. With that, direct associations can only reveal some of the genetic causes of complex diseases. Indirect associations do not involve the true disease-causing variants and, instead, involve variants located at a proximity, or in high LD (linkage disequilibrium) with the disease causing variant (Cordell, Clayton 2011). False-positive associations can arise by chance or from systematic type I errors resulting from biases in the frequencies of alleles and genotypes. Additionally, false positive associations can be obtained in under-powered studies with relatively small sample sizes (Lewis, Knight 2012).

There are different approaches to identify gene variants associated with complex diseases. The two most common approaches are the genome wide association studies (GWAS) and the candidate gene studies. Both approaches in addition to the use of genetic association studies in the investigation of ATP will be briefly discussed in the following sections.

3.1.1 Genome Wide Association Studies (GWAS)

GWAS aims at determining which alleles or genotypes appear in a significantly different frequency between a case and a control group. Unlike the candidate gene approach (discussed in section 3.1.2) GWAS tend to be less hypothesis-driven and involves conducting an investigation into a large number of SNPs (Visscher et al. 2012). While the underlying goal of both approaches is similar, data processing and analysis is far more complex in the case of GWAS which would require the usage of special computer operating systems to handle the large volume of data (Foulkes 2009). To account for multiple testing, significance in GWAS would be called at a very small p-value ($p<10^{-7}$) (Grant, Hakonarson 2008). It is important to note that GWAS has gained popularity in recent years following the widespread availability of the “SNP chip” (Visscher et al. 2012).

3.1.2 Candidate Gene Association Studies

A candidate gene study is an investigation into an association for which there is an *a priori* hypothesis about the involvement of the candidate gene into the mechanism of pathology (Tabor, Risch & Myers 2002). There are two candidate gene association approaches: the transmission disequilibrium tests and case-control studies. Transmission disequilibrium tests aim at identifying genetic determinants of complex diseases by measuring the over-transmission of an allele from heterozygous parents to the affected offspring (Zhang et al. 2013). On the other hand, the aim of case-control studies would be to determine whether an allele or genotype of a specific SNP appears at a significantly greater frequency in cases compared to a matched control group (Cordell, Clayton 2011). One of the major challenges faced when designing a candidate gene association study is the selection of SNPs that would be most likely playing a role in the development of the disease. Variants are usually selected on the basis of allele frequency and their potential functional effect on the phenotype (Lewis, Knight 2012). Information on allele frequencies are abundant and easily accessible through bioinformatics databases such as NCBI and Ensembl. However the literature contains limited information describing the biological function of SNPs and therefore rendering it harder to classify associations as direct, indirect, or false positive as described before (Cordell, Clayton 2011, Rebbeck, Spitz & Wu 2004).

The candidate gene association approaches are useful in validating GWAS results and have been widely used for the study of complex diseases (Foulkes 2009). However on many occasions this approach has been criticised for failure to replicate. Tabor et al. (2002) suggested the application of rigorous gene selection and prioritisation principles that could improve the chances of successfully replicating associations. For instance, candidate genes should be selected based on previous associations in linkage or expression studies present in the literature. When designing the study, priority should be given to polymorphisms with greater biological impact (nonsense, missense, insertion/deletions) which are likely to have

an effect on the function or expression of a protein (Tabor, Risch & Myers 2002). Another important aspect to consider when selecting SNPs would be whether the target variants are part of a linkage block. If several SNPs are in complete linkage disequilibrium it becomes possible to infer the genotype of the remaining SNPs based on the genotype of a single SNP (Tabor, Risch & Myers 2002). This can contribute towards the reduction of the tested variants and would in some cases provide greater power to detect associations than simple testing of individual SNPs (Subrahmanyan et al. 2001).

3.1.3 Genetic Association Studies in Relation to Achilles Tendon Pathology

Case-control candidate gene studies have been successfully employed in identifying risk variants for musculoskeletal injuries like Achilles tendon pathology, and ACL ruptures (Collins, Raleigh 2009, Raleigh, Collins 2012). These studies relied on the accurate selection of a well defined clinical phenotype i.e. injuries to the Achilles tendons. In a successful genetic association study, confounding factors such as population stratification (differences in allelic frequencies due to differences in ancestry) should be taken into consideration when designing the study (Lewis 2002). Population stratification can be eliminated by matching cases and controls according to ancestry and geographical origins (Wacholder, Rothman & Caporaso 2002). In the case of multifactorial conditions such as tendon injuries, intrinsic and extrinsic factors discussed in Chapter 2 have to be considered and controlled for when recruiting participants for both the case and control groups. The severity of the injury should also be taken into consideration when defining the inclusion and exclusion criteria (section 3.2.2). Furthermore, the selection of appropriately matched controls (biometrics and physical activities) is as important as the selection of the cases.

3.2 PARTICIPANTS

3.2.1 Ethics Approval

Approval for the work outlined in this thesis was obtained from the relevant research ethics committees (RECs) (Appendix I). Those involved included: The University of Northampton Research Ethics Committee, The University of Northampton School of Health Research Ethics Committee, The University of Cape Town Research Ethics Committee, Monash University Research Ethics Committee and La Trobe University Research Ethics Committee. Research on the Achilles tendon tissue samples obtained by the Royal National Orthopaedic Hospital (RNOH) had been approved by the National Research Ethics Service Committee within the National Health Services. Storage and use of these materials at the University of Northampton was subject to a material transfer agreement (MTA) that was set up between the RNOH and The University of Northampton. All procedures were compliant with the Human Tissue Act 2004.

3.2.2 British Cohort for Genetic Association Studies

One hundred and thirty six British Caucasian participants with clinically diagnosed Achilles tendon pathology (ATP) were recruited for this thesis and subsequent studies from The County Clinic in Northampton, UK.

Of the 136 ATP cases, 109 (80.1%) participants were diagnosed with an Achilles tendinopathy as previously described by Mokone et al. (2005): 1) pain for at least 6 months, 2) morning stiffness and pain, 3) swelling of the tendon, 4) tenderness to palpation, 5) nodular thickening in the tendon, 6) movement of the painful area with plantar-dorsiflexion. The width and the exact pathology of damaged tendons (non-insertional tendinopathy, insertional tendinopathy, or tendon rupture) were determined by Prof Ribbans using MRI images. The imaging was conducted by experienced staff at The Three Shires Hospital on a Toshiba Advantage 1.5T Atlas Machine (Excelart Vantage Powered

by Atlas; Toshiba Medical Systems, Tokyo, Japan) using the standard sequences used for imaging of the Achilles tendon: sagittal and axial T1 weighted sequences, sagittal and axial T2 weighted fat suppressed sequences and a coronal T2 fast spin echo sequence, slice thickness 3 mm with a field of view of 20 cm. There were 80 (58.8%) cases of non-insertional tendinopathy, and 49 (36%) cases of insertional tendinopathy in addition to 36 (26.3%) cases diagnosed with midpoint Achilles tendon rupture. Nevertheless, several participants displayed a combination of pathologies. Furthermore, 3 (2.2%) participants, initially recruited for the asymptomatic control group, developed an ATP after recruitment and were therefore re-categorised as cases. Unfortunately, it was not possible to obtain details of their diagnosis since they were not recruited through The County Clinic. Figure 3.2.1 provides a clearer image of the breakdown of pathologies in this newly recruited Caucasian British population (UK cohort).

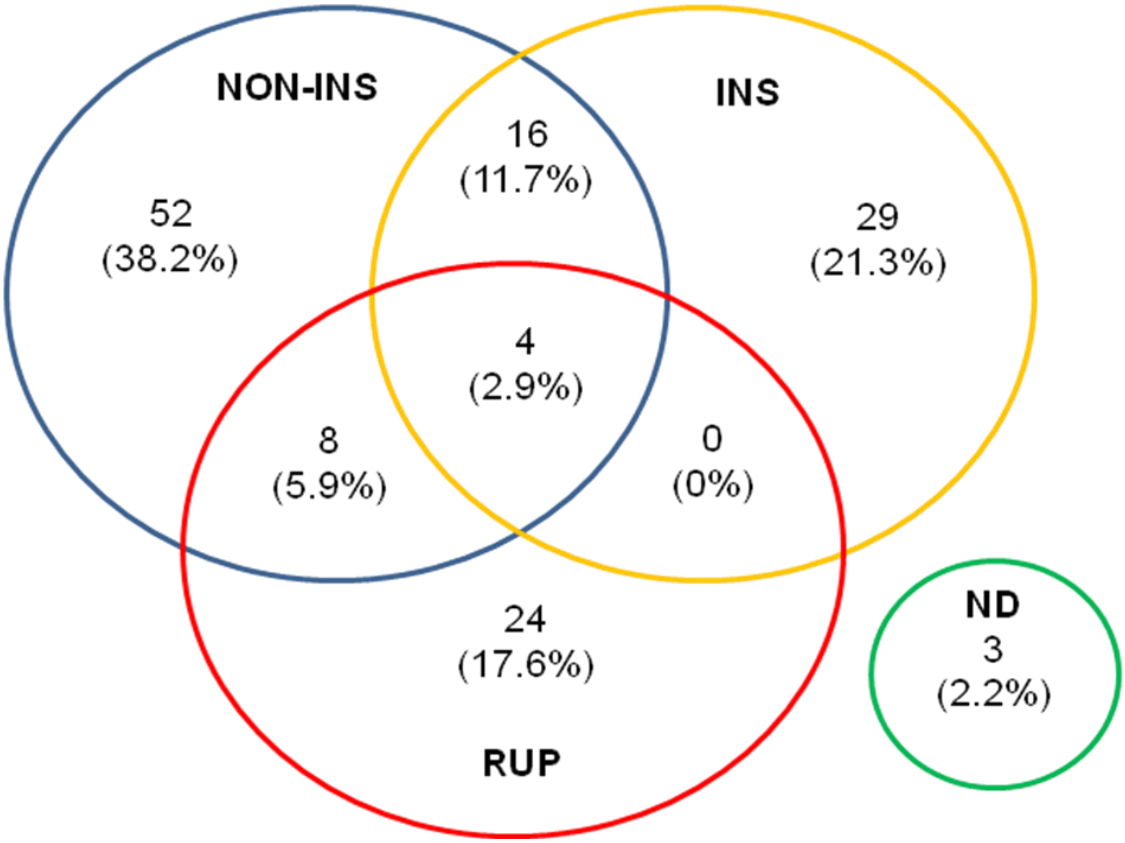


Figure 3.2.1 Venn diagram showing the distribution of the UK ATP cases among the different diagnostic groups. NON-INS, Achilles non-insertional tendinopathy; INS, Achilles insertional tendinopathy; RUP, Achilles tendon rupture; ND, not diagnosed. Values are reported as the count followed by the frequency in parenthesis.

In addition to the 136 ATP cases, 131 asymptomatic, healthy, unrelated, and physically active British Caucasians were recruited from sports clubs within the county of Northamptonshire to form the control group (CON). The criteria for inclusion into the CON group required potential participants to be older than 25 years of age since sports related injuries are commonly reported after that age (Renstrom, Woo 2008). Furthermore, control participants had to be physically active i.e. high intensity exercise for a period of 2 hours per week or more (Schneider et al. 2006, Lee, Paffenbarger 2000).

Table 3.2.1 shows a list of the most common sports played by both the UK CON and the ATP groups. In summary, 58.5% (48 of 82) and 57.7% (47 of 82) of males within the control group were dedicated at some point to playing rugby and football respectively. In contrast, 46.4% (39 of 84) and 38.1% (32 of 84) of males within the ATP group were committed to football and running respectively, at some point in their life. Furthermore, running was the most popular sport among females with 63.3% (31 of 49) and 46.1% (24 of 52) of females within the CON and ATP group respectively.

Table 3.2.1 Table showing the most commonly played sports by UK males and females in the CON and ATP groups

	Males				Females			
	CON (n=82)		ATP (n=84)		CON (n=49)		ATP (n=52)	
Rugby	48	58.5%	27	32.1%	3	6.1%	1	1.9%
Football	47	57.3%	39	46.4%	2	4.1%	0	0.0%
Running	19	23.2%	32	38.1%	31	63.3%	24	46.2%
Cricket	15	18.3%	15	17.9%	0	0.0%	2	3.8%
Squash	13	15.9%	27	32.1%	5	10.2%	7	13.5%
Cycling	12	14.6%	27	32.1%	9	18.4%	11	21.2%
Tennis	8	9.8%	28	33.3%	6	12.2%	11	21.2%
Swimming	8	9.8%	25	29.8%	16	32.7%	16	30.8%
Hockey	6	7.3%	2	2.4%	11	22.4%	14	26.9%
Badminton	5	6.1%	11	13.1%	5	10.2%	9	17.3%
Netball	0	0.0%	0	0.0%	5	10.2%	17	32.7%

Prior to participation in the study, all participants gave informed written consent (Appendix II). Furthermore, all participants were asked to fill a questionnaire detailing their involvement in physical activities as well as their medical and injury history (Appendix III).

3.2.2.1 DNA Extraction from Saliva.

The Oragene DNA Genotek OG-500 tubes (DNA Genotek, Inc, Ottawa, Ontario, Canada) were used to collect saliva samples from all participants in the UK cohort. Participants were asked to provide 2 ml of saliva, and were also instructed to refrain from drinking, eating and smoking for a period of 30 minutes ahead of the sample collection procedure. The OG-500

tubes contain a preserving solution that will keep saliva stable at room temperature for a period longer than a year (Birnboim 2004). Therefore, the saliva samples were stored on the laboratory bench surface until the time of DNA extraction.

DNA was extracted using the manufacturer's protocol (Appendix IV) with slight modification. The OG-500 tubes containing saliva samples were incubated in a water bath set at 50 °C for one hour. After incubation, 500 µl of saliva was transferred to 1.5 ml minicentrifuge tubes and mixed with 20 µl of PT-L2P solution. The PT-L2P solution contains a detergent to lyse the epithelial cells present in the saliva. Additionally, the PT-L2P solution is known to isolate and precipitate impurities and inhibitors (Nunes et al. 2012). To maximise the effectiveness of the PT-L2P solution, the minicentrifuge tubes were incubated on ice for 10 minutes and then centrifuged at room temperature for 10 minutes at 15,000 x *g*. The supernatant containing the DNA was transferred to new tubes while the tubes containing the pellet and impurities were discarded. To precipitate the DNA, 500 µl of 95-100% (v/v) ethanol was added to each of the new minicentrifuge tubes. The tubes were inverted 10 times and were allowed to stand for 10 minutes at room temperature. Once the 10 minutes have elapsed, the tubes were centrifuged for 3 minutes at 15,000 x *g*. The pellet of DNA was then visible at the bottom of the tube. At this stage, the supernatant was discarded, and an ethanol wash was conducted using 250 µl of 70% (v/v) ethanol. The tubes were left to stand for 1 minute, then ethanol was completely removed, and the tubes were left open to air dry. Once the tubes had dried, 100 µl of pH 8 TE buffer was added to each tube. DNA was left on the laboratory bench surface to re-suspend at room temperature over-night. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to measure the DNA concentration and purity. Once the DNA concentration was measured, working stocks were prepared in 96 wells storage plates (Costar, Corning, NY, USA) and refrigerated at 4 °C whereas, samples for long term storage were archived at -20 °C.

3.2.3 Australian and South African Cohorts

In addition to the samples obtained from the newly recruited British cohort, samples from previously recruited populations (Mokone et al. 2005, September et al. 2009) were also included in this thesis as part of collaborative studies. The inclusion of these samples in this study was approved by the Research Ethics Committees of the Faculty of Health Sciences at the University of Cape Town, La Trobe University, Monash University, and the University of Northampton.

The Australian cohort (AUS) was composed of 149 asymptomatic healthy controls and 60 clinically diagnosed ATP cases. For the South African cohort, 98 asymptomatic healthy controls and 115 clinically diagnosed ATP cases were included. Both cohorts were recruited using the same clinical criteria used for the UK cohort (section 3.1.1) as documented by Mokone et al. (2005) and September et al. (2009).

For the AUS group, staff at the Musculoskeletal Research Centre at La Trobe University extracted DNA from whole blood using a Flexigene DNA Kit (Qiagen, Valencia, CA, USA) following the manufacturer protocol. As for the SA group, DNA was extracted from whole blood, by Dr G. Mokone at the Sport Science Institute of South Africa, according to the Lahiri and Nurnberg (1991) protocol and modified by Mokone et al. (2005 and 2006).

3.2.4 British Achilles Tendon Samples for Gene Expression and Epigenetic Studies

Achilles tendon samples were obtained from the Royal National Orthopaedic Hospital (RNOH). One damaged tendon sample was obtained from a male individual undergoing an Achilles tendon repair surgery whereas, 4 healthy Achilles tendon samples (3 males and 1 female) were obtained from participants undergoing surgery in their lower limbs for medical conditions not involving tendon pathologies. The tissue samples collected were cut into sections of 4x4x4 mm.

To stabilise and preserve the DNA, RNA, and miRNA in the tissue, the samples were stored in minicentrifuge tubes containing Allprotect Tissue Reagent ® (Qiagen, Hilden, Germany) and were kept at 4 °C until DNA or RNA extraction day.

3.2.4.1 DNA Extraction

To extract DNA from the Achilles tendon samples, tissues were mashed and lysed using the TissueLyser LT (Qiagen, Hilden, Germany) with slight modifications to the manufacturer's guidelines (Appendix IV). Tissue sections, not exceeding 25 mg in weight, were transferred into precooled (in 70% (v/v) ethanol at -80 °C, overnight) 2 ml microcentrifuge tubes containing 1 stainless steel bead (5 mm in diameter). The tubes containing the samples were further incubated in -80 °C cooled 70% (v/v) ethanol for 15 minutes. Once the 15 minutes incubation was over, the tubes were placed in the tissue lyser for 2 minutes at room temperature. Next, 180 µl of ATL buffer from the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was added to each tube. The tubes were allowed to lyse in the tissue lyser for 40 seconds at a speed of 30 Hz/s. A second 5 mm stainless bead was then added to each tube, and the lysis procedure was allowed to go on for a period of 5 minutes at a speed of 50 Hz/s. To achieve a complete lysis, 20 µl of proteinase K was then added to each tube and incubated for 3 hours at 56 °C in an Eppendorf Thermomixer comfort (Eppendorf, Hamburg, Germany) at a speed of 1400 rpm. Once the 3 hours had elapsed, 200 µl of AL buffer was

added to the samples and left to incubate for 10 minutes at 70 °C. Next, 200 µl ethanol (95-100%) was added to each tube. Upon the addition of ethanol, DNA precipitation was observed. The mixtures were then transferred to QIAamp Mini spin columns and were centrifuged for 1 minute at 6,000 x *g*. At this point the mixture went through a series of column washing where 500 µl of AW1 and AW2 buffers were added and centrifuged for 1 minute at 6,000 x *g* and for 3 minutes at 20,000 x *g* respectively. The columns were then transferred into 1.5 ml microcentrifuge tubes. To elute the washed and isolated DNA, 200 µl of AE buffer was added to each column and allowed to spin for 1 minute at 6,000 x *g*. To confirm the success of the extraction and to quantify the DNA, the concentration of the eluted solution was measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

3.2.4.2 RNA and miRNA Extraction

RNA and miRNA were extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) and the TissueLyser LT (Qiagen, Hilden, Germany) with a slight modification to the manufacturer's protocol (Appendix IV). Tissue sections, not exceeding 30 mg in weight, were transferred into precooled (in 70% (v/v) ethanol at -80 °C, overnight) 2 ml microcentrifuge tubes containing 1 stainless steel bead (5 mm in diameter). The tubes containing the samples were further incubated in -80 °C cooled 70% (v/v) ethanol for 15 minutes. Once the 15 minutes incubation was over, the tubes were placed in the tissue lyser for 2 minutes at room temperature. Next, 700 µl of QIAzol Lysis Reagent was added to each tube. The tubes were allowed to shake in the tissue lyser for 40 seconds at a speed of 30 Hz/s. A second 5 mm stainless bead was added to each tube, and the lysis procedure was allowed to go longer for a period of 5 minutes at a speed of 50 Hz/s. Upon completion of the mashing procedure, 140 µl of chloroform was added to the tubes containing the homogenates, followed by vigorous shaking for 15 seconds. The tubes were then centrifuged for 15 minutes at 12,000 x *g* at 4 °C. Following this step, the samples were separated into 3

phases: the upper aqueous phase containing the RNA; a middle white interphase; and a lower organic phase. The aqueous solutions (~350 µl each) were transferred to new microcentrifuge tubes and were mixed using the pipette with 525 µl 95-100% ethanol. The mixtures were next transferred to RNeasy Mini spin columns and were centrifuged for 15 seconds at 9,000 x *g* at room temperature. Next the spin columns went through a series of washing steps where 700 µl of RWT buffer and 500 µl of RPE buffer was added and centrifuged at 9,000 x *g* for 15 seconds and 2 minutes respectively. To elute the RNA and miRNA preserved in the columns, 30 µl of RNase-free water was added to each column and centrifuged for 1 minute at 9,000 x *g*. To confirm the success of the extraction and to quantify the RNA, the concentration of the eluted solution was measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

3.2.5 Australian Patellar Tendon Samples for DNA Methylation

In addition to the Achilles tendon samples used for DNA methylation analysis, additional DNA samples from patellar tendons were obtained from La Trobe University, Melbourne, Australia. Ten healthy patellar tendons were obtained from male participants undergoing ACL reconstruction surgeries using a patellar tendon graft, and 10 abnormal samples were obtained from male individuals undergoing surgery for patellar tendinopathy as previously described by Parkinson et al. (2010). DNA was isolated by staff members at La Trobe University, using the PureLink® Genomic DNA Kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's recommendations.

3.3 GENOTYPING

3.3.1 SNP Genotyping

SNP genotyping was conducted using custom made TaqMan® SNP genotyping assays (Applied Biosystems, Foster City, CA, USA). A 6 µl PCR mixture was prepared according to the volumes in table 3.3.1.

Table 3.3.1 List of reagents included for each TaqMan® SNP genotyping PCR reaction

Reagents	Volume (µl)
TaqMan® Universal PCR Master Mix, No AmpErase® UNG	3
40x SNP genotyping assay	0.15
Nuclease-free water	2.25
Template DNA (10 ng/µl)	0.6
Total volume	6

Volumes modified from Applied Biosystems' recommendations

All qPCR reactions were prepared in 96 well plates. The 96 well plates contained 88 different DNA samples in addition to 4 no-template negative control reactions and 4 sample repeats acting as positive controls. Before the start of the reactions the 96 well plates were covered using transparent MicroAmp® Optical Adhesive Films (Applied Biosystems, Foster City, CA, USA).

All SNP genotyping reactions were conducted on an Applied Biosystems StepOnePlus platform (Applied Biosystems, Foster City, CA, USA) according to the manufacturers protocols described in table 3.3.2.

Table 3.3.2 qPCR protocol for TaqMan® SNP genotyping

Stage/step	Pre PCR read	Thermal Cycling			Post PCR read
	Holding stage	Holding stage	Cycling (40 cycles)		Holding stage
			Denature	Anneal & elongate	
Temperature	60 °C	95 °C	92 °C	60 °C	60 °C
Time (mm:ss)	00:30	10:00	00:15	01:00	00:30

Upon completion of the reaction, the data was analysed using the StepOne software v2.1 (Applied Biosystems, Foster City, CA, USA). All genotypes were automatically called by the software on allelic discrimination plots.

3.3.2 *COL5A1* rs12722 Polyacrylamide Gel Electrophoresis Genotyping

The *Bst*UI RFLP SNP rs12722 is located on the 3'-UTR of the *COL5A1* gene. A 667 bp fragment containing this gene variant was amplified with a slight modification from the description of Greenspan and Pasquinelli (1994) and Mokone et al. (2006). The PCR reaction was conducted in a volume of 60 µl. The PCR mix contained 20 pmol of forward (5'-GAA GAC GTT TCT GGA GGA TC-3') and reverse (5'-GGA GGC ACC TGC AGA ATG AC-3') primers, a buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 0.5 mM dNTP solution, and 2.5 units of Taq polymerase (New England Biolabs, Ipswich, Massachusetts, USA). The amplification was performed on a XP Thermal Cycler Block (Bioer Technology Co, Middlesex, UK) according to the steps in table 3.3.3. The PCR products were run on 2% agarose gel to confirm the amplification.

Table 3.3.3 PCR conditions for the amplification of the 667 bp *Bst*UI RFLP within the 3' UTR of the *COL5A1* gene

	Thermal Cycling					
		Cycling (35 cycles)				
Stage/step	Initial Denaturation	Denature	Anneal	Extend	Final Extension	Final Incubation
Temperature	94 °C	94 °C	59 °C	72 °C	72 °C	4 °C
Time (mm:ss)	03:00	01:00	01:00	01:30	08:00	∞

Upon confirmation of amplification, the 667 bp PCR products were digested overnight at 60 °C using *Bst*UI (5 units) enzyme and NEB2 buffer (New England Biolabs, Ipswich, Massachusetts, USA) in a 25 µl reaction described in table 3.3.4.

Table 3.3.4 list of reagents needed for *Bst*UI digestion of the *COL5A1* 667 bp PCR product

Reagents	Volume (µl)
*Bst*UI Enzyme (5 units)	0.5
NEB 2 Buffer	2.5
Molecular Grade Water	2
DNA (PCR product)	20
Total volume	25

The resulting digestion products and a 100 bp ladder (MBI Fermentas, UK) were stained using SYBR® Gold nucleic acid gel stain (Invitrogen Molecular Probes™, Oregon, USA) were separated on 6% non-denaturing polyacrylamide gels and visualised under UV light using Uvitec photodocumentation system (Uvitec Limited, Cambridge, UK). The genotypes were determined according to the size of fragments. The observation of 351 bp and 316 bp fragments is indicative of the presence of a T allele, where as the observation of 316 bp, 271 bp, and 80 bp fragments is indicative of the presence of a C allele.

3.4 GENE EXPRESSION

A two-step (reverse transcription and real-time PCR) gene expression experiment was conducted using TaqMan® Gene Expression Assay according to the manufacturer's protocols (Applied Biosystems, Foster City, CA, USA).

3.4.1 Reverse Transcription

The preparation of cDNA from previously extracted RNA (section 3.3.4.2) was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a 20 µl volume reaction as recommended by the manufacturer and described in table 3.4.1.

Table 3.4.1 Volumes of reagents included in the reverse transcriptase reaction

Reagents	Volume (µl)
10x RT Buffer	2
25x dNTP Mix (100 mM)	0.8
10x RT Random Primers	2
MultiScribe™ Reverse Trancriptase	1
Nuclease-free Water	4.2
RNA Template (10 ng/µl)	10
Total volume	20

The PCR reaction was conducted in a Techne TC-512 thermocycler (Bibby Scientific Ltd, Staffordshire, UK) according to the conditions recommended by Applied Biosystems and described in table 3.4.2.

Table 3.4.2 Reverse transcriptase conditions for the transcription of cDNA from RNA using the High Capacity cDNA Transcription Kit

Stage/step	Step 1	Step 2	Step 3	Step 4
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 min	120 min	5 min	∞

The generated cDNA was stored at -20 °C until the day of the real-time PCR quantification.

3.4.2 Real-time PCR

The gene expression analysis was conducted on the previously produced cDNA (section 3.4.1) using TaqMan® Gene Expression Assay (Applied Biosystems, Foster City, CA, USA).

The 20 µl RT-PCR reaction was prepared as described in table 3.4.3 with a slight modification to the manufacturer's recommendations (Appendix IV).

Table 3.4.3 Volumes of reagents included in the Real-time PCR reaction

Reagents	Volume (µl)
TaqMan® Universal PCR Master Mix, No AmpErase® UNG	10
20x TaqMan® Gene Expression Assay	1
RNase-free Water	7
cDNA Template (10 ng/µl)	2
Total volume	20

Gene expressions were conducted in triplicates for the gene of interest along with *GAPDH* gene as a housekeeping gene. The real-time PCR was conducted on an ABI StepOnePlus

(Applied Biosystems, Foster City, CA, USA) according to the manufacturer's cycling conditions recommendations described in table 3.4.4.

Table 3.4.4 Real-time PCR cycling conditions for TaqMan® Gene Expression Assay.

	Thermal Cycling		
		Cycling (40 cycles)	
Stage/step	Holding stage	Denature	Anneal& elongate
Temperature	95 °C	95 °C	60 °C
Time (mm:ss)	10:00	00:15	01:00

The real-time PCR amplification plots were visualised on the StepOne Software v2.0 (Applied Biosystems, Foster City, CA, USA).

3.5 miRNA EXPRESSION

Using a similar method to the gene expression work, miRNA expression was conducted using a two step (reverse transcription and real-time PCR) reaction. However, miRNA expression was performed using TaqMan® miRNA assay (Applied Biosystems, Foster City, CA, USA).

3.5.1 Reverse Transcription

The preparation of cDNA from previously extracted miRNA (section 3.2.4.2) was performed using the TaqMan® miRNA Assay (Applied Biosystems, Foster City, CA, USA) containing MuLV reverse transcriptase in a 15 µl volume reaction as recommended by the manufacturer and described in table 3.5.1.

Table 3.5.1 Volumes of reagents included in the reverse transcriptase reaction

Reagents	Volume (µl)
10x RT Buffer	1.5
25x dNTP Mix (100 mM)	0.15
5x RT Primers	3
MultiScribe™ Reverse Trancriptase (50 U/µl)	1
RNase Inhibitor (20 U/µl)	0.19
Nuclease-free Water	4.16
RNA Template (1 ng/µl)	5
Total volume	15

The PCR reaction was conducted in a Techne TC-512 thermocycler (Bibby Scientific Ltd, Staffordshire, UK) according to the conditions recommended by Applied Biosystems and described in table 3.5.2.

Table 3.5.2 Reverse transcriptase conditions for the transcription of cDNA from miRNA using the TaqMan® miRNA Assay

Stage/step	Step 1	Step 2	Step 3	Step 4
Temperature	16 °C	42 °C	85 °C	4 °C
Time	30 min	30 min	5 min	∞

The generated cDNA was stored at -20 °C until the day of the real-time PCR experiment.

3.5.2 Real-Time PCR

The miRNA expression analysis was conducted on the previously generated cDNA (section 3.5.1) using TaqMan® miRNA Assay (Applied Biosystems, Foster City, CA, USA).

The 20 µl RT-PCR reaction was prepared as described in table 3.5.3 according to the manufacturer's recommendations.

Table 3.5.3 Volumes of reagents included in the real-time PCR reaction

Reagents	Volume (µl)
TaqMan® Universal PCR Master Mix, No AmpErase® UNG	10
20x TaqMan® miRNA Assay	1
RNase-free Water	7.67
cDNA Template (1 ng/µl)	1.33
Total volume	20

Micro-RNA expressions was conducted in triplicates for the miRNA of interest along with *RNU6B* gene as a housekeeping gene which is a widely used endogenous reference in miRNA quantification studies (Matera, Terns & Terns 2007). The real-time PCR was conducted on an ABI StepOnePlus (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's cycling conditions recommendations described in table 3.5.4.

Table 3.5.4 Real-time PCR cycling conditions for TaqMan® Gene Expression Assay.

	Thermal Cycling		
		Cycling (40 cycles)	
Stage/step	Holding stage	Denature	Anneal& elongate
Temperature	95 °C	95 °C	60 °C
Time (mm:ss)	10:00	00:15	01:00

The real-time PCR amplification plots were visualised on the StepOne Software v2.0 (Applied Biosystems, Foster City, CA, USA).

3.6 DNA METHYLATION AND PYROSEQUENCING

Pyrosequencing consists of a series of cascade reactions where DNA is sequenced by synthesis (Ahmadian et al. 2000). This technique relies on the release of pyrophosphate (PPi) following the incorporation of each nucleotide and the formation of a base pair. With the help of ATP sulfurylase, the released PPi is converted to ATP which provides energy for luciferase to oxidise luciferin and generate light (Ronaghi 2001). The generated light is picked up by the CCD camera which indicates the incorporation of a new known nucleotide by showing a peak on the pyrogram (Huse et al. 2007) as shown in figure 3.6.1. The sequence of the template DNA can be determined because the added nucleotide is known. Pyrosequecing is a technology used to measure the level of DNA methylation within the promoter region of the gene of interest. Specifically, this procedure aims at quantifying the amount of methylated cytosine that were not converted to uracil during bisulfite conversion (Tost, Gut 2007).

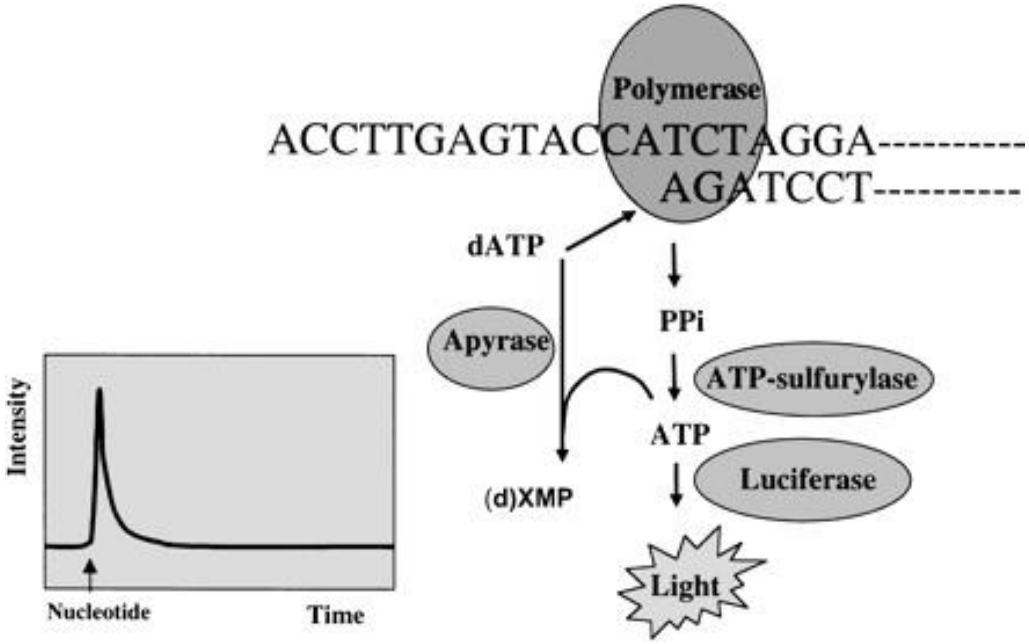


Figure 3.6.1 Schematic representation of the pyrosequencing cascade reaction. The nucleotides are added to the sample according to a defined order. When the added nucleotide forms a base-pair, the PPi is released and is converted to ATP by ATP-sulfurylase. The energy from ATP is used by luciferase to oxidise luciferin and generate light. The light signal is picked up by the CCD camera and a peak is displayed on the pyrogram. Excess nucleotides are degraded by apyrase. Figure obtained from (Ronaghi 2001) with permission of the publisher.

This experiment is divided into three steps: the bisulphite conversion, the PCR amplification of the target region within the promoter of the gene, and the pyrosequencing.

3.6.1 Bisulphite Conversion

Bisulphite conversion is a step during which all the unmethylated cytosines within the promoter region of genes are converted to uracil, leaving the methylated cytosine unchanged.

Bisulphite conversion was performed using the EpiTect® fast DNA bisulphite Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations described in table 3.6.1. All reactions were performed in 200 µl PCR tubes.

Table 3.6.1 Volumes of reagents included in a bisulfite conversion reaction.

Reagents	Volume (µl)
DNA Protect Buffer	35
Bisulfite Solution	85
Nuclease-free Water	18
DNA (~50 ng)	2
Total volume	140

A successful bisulfite conversion requires the maintenance of a low pH. The DNA Protect buffer behaves as a pH indicator as it changes colour from blue to green indicating that the acidity within the solution is adequate. Next, all reaction tubes were introduced into the thermal cycler Techne TC-512 (Bibby Scientific Ltd, Staffordshire, UK) for the conversion to proceed based on the cycling stages described in table 3.6.2.

Table 3.6.2 Bisulfite conversion thermal cycler conditions

Stage/step	Denaturation	Incubation	Denaturation	Incubation	Hold
Temperature	95	60	95	60	20
Time	5 min	10 min	5 min	10 min	∞

Upon completion of the bisulfite conversion, the converted DNA underwent a series of clean up steps where 310 µl of BL buffer and 250 µl ethanol (95-100%) were allowed to mix with the reaction by briefly vortexing. Next, the entire content of the reaction was transferred to a MinElute® DNA spin column. The tube was centrifuged to separate the converted DNA from the reaction leftovers. Following that, 500 µl of BW and BD were passed, separately, through the column (1 min; ~9.5 x *g*). Also, 250 µl ethanol (95-100%) were added to the spin columns for a final wash step. To evaporate any remaining liquid in the spin columns, they were placed on a heating block for 5 minutes at 60 °C. Finally, the bisulfite converted DNA was eluted in 15 µl EB buffer and was quantified using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

3.6.2 PCR Amplification of Target Region

The amplification of the target of interest was performed using the PyroMark® PCR Kit (Qiagen, Hilden, Germany) in addition to pre-designed primers from the selection of PyroMark® CpG Assays (Qiagen, Hilden, Germany). PCR was performed on a Techne TC-512 thermocycler (Bibby Scientific Ltd, Staffordshire, UK), according to the manufacturer recommendations and described in table 3.6.3.

Table 3.6.3 PCR conditions for the amplification of gene promoter sites using PyroMark® CpG Assays primers

	Thermal Cycling					
		Cycling (45 cycles)				
Stage/step	Initial Denaturation	Denature	Anneal	Extend	Final Extension	Final Incubation
Temperature	95 °C	94 °C	56 °C	72 °C	72 °C	4 °C
Time (mm:ss)	15:00	00:30	00:30	00:30	10:00	∞

Amplification of the target region was confirmed by running a 1% agarose gel stained with SYBR® Safe DNA gel stain dye (Invitrogen, Carlsbad, CA, USA) along with a 100 bp ladder (MBI Fermentas, UK) as demonstrated in Chapter 6.

3.6.3 Pyrosequencing

Pyrosequencing is the final stage of the DNA methylation experiment. At this stage, the PCR product has to be treated and prepared to be pyrosequenced.

The first step of this stage involved immobilising the PCR product. This was performed by mixing the biotinylated PCR product with Streptavidin-coated Sepharose high-performance 35 µm beads (GE Healthcare, Buckinghamshire, UK) in the presence of PyroMark® binding buffer according to the manufacturer's recommendations described in table 3.6.4.

Table 3.6.4 List of reagents and their respective volumes required for the immobilisation of the PCR product

Reagents	Volume (µl)
Streptavidin-coated Sepharose high-performance beads	2
PyroMark® binding buffer	40
Nuclease-free Water	31
PCR Product (0.5 - 1 µg/µl)	7
Total volume	80

The immobilised PCR product was captured using the PyroMark® Q24 vacuum workstation as described in table 3.6.5

Table 3.6.5 Steps required for capturing the immobilised PCR product using PyroMark® Q24 vacuum workstation

Stage/step	70% (v/v) ethanol washing	Denaturation in PyroMark® Denaturation solution	Neutralisation in PyroMark® Wash Buffer
Volume	50 ml	40 ml	50 ml
Time	5 sec	5 sec	10 sec

Following the multiple washing steps, the captured PCR product was released on a 24 well PyroMark® Q24 plate containing 2.5 µl sequencing primer (3 µM) diluted in 22.5 µl PyroMark® annealing buffer. For the sequencing primer to anneal onto the PCR product, the plate was incubated on a pre-heated 80 °C hot plate for 2 minutes. The Q24 plate was allowed to cool for 10 minutes and was loaded into the PyroMark® Q24 pyrosequencer. The PyroMark® Q24 software was used to generate the dispensation order and the appropriate amounts of enzyme, substrate, and dNTPs to load into the PyroMark® cartridge (Qiagen, Hilden, Germany) and into the pyrosequencer.

Upon the completion of the run, the PyroMark® Q24 software generated different pyrograms showing the percent methylation values for the various sequenced samples.

3.7 STATISTICS

3.7.1 Power Calculations

The power calculation for this work was conducted on the power calculation software Quanto v1.2 (http://hydra.usc.edu/gxe) and the Power for Genetic Association Analyses (PGA) package (http://dceg.cancer.gov/bb/tools/pga) using a recessive model and a disease population prevalence of 10%. To detect an allelic OR of 2.0 at a power of 80% and a significance level of 5%, a 60% risk allele frequency was assumed.

3.7.2 Data Analysis

Data were analysed using SPSS Version 20 (SPSS Science Inc, Chicago, Ill, USA) statistical program and graphs were designed using Microsoft Office Excel 2007 and Graphpad InStat Version 5 (Graphpad Software, San Diego, California, USA). Allelic and genotypic group distribution differences were evaluated using Chi-squared (χ^2) analysis or Fisher's exact test if the expected count in a cell was less than 5% (Chapter 4 and 5). Independent t-tests were conducted to compare differences between means of continuous data between groups i.e. differences in mean biological data (age, height, weight, BMI, etc) or DNA methylation percentages between cases and controls (Chapter 4, 5, and 6). Furthermore, one way analysis of variance (ANOVA) was conducted to assess mean differences between groups when investigating interactions between genotypes and age or tendon width (Chapter 4 and 5). Moreover, tests of linear regression were conducted to look for interactions between two continuous data: tendon width vs biological data (age, height, weight, and BMI) (Chapter 5). A one-sample t-test was conducted to compare the mean Ct of the control group to the Ct value of the single subject in the case group (Chapter 7).

Adjustments for multiple testing were not conducted as it has been previously described (Posthumus et al. 2011) that no appropriate method exists. Furthermore, the Bonferroni adjustment was considered too conservative and inappropriate for the studies conducted in this thesis since prior evidence that the gene of interest is associated with a trait exist (Perneger 1998). Hardy-Weinberg equilibrium was determined using Michael H. Court's (2005–2008) online calculator (www.tufts.edu/~mcourt01/Documents/Court%20lab%20%20HW%20calculator.xls).

Significance for all test was called at $p<0.05$.

Chapter 4 Variants Within Genes Encoding Structural and Extra-Cellular Matrix Proteins as Risk Factors for Achilles Tendon Pathology

Some of the findings in this chapter have been published in the International Journal of Sports Medicine

L. El Khoury, M. Posthumus, M. Collins, W. van der Merwe, C.J. Handley, J. Cook and S.M. Raleigh 2014, " *ELN* and *FBN2* Gene Variants as Risk Factors for Two Sports-related Musculoskeletal Injuries ", *International Journal of Sports Medicine ,* no. Epub, ahead of print.

4.1 INTRODUCTION

Achilles tendon pathologies (ATPs) such as Achilles tendinopathy and Achilles tendon ruptures have been identified as debilitating conditions resulting from either acute or repetitive overuse loading mechanisms (Kvist 1994). Intrinsic (including genetic) and extrinsic factors have been identified and have contributed towards a better understanding of the underlying aetiology of ATP (Collins, Raleigh 2009). As previously discussed in this thesis, genetic studies have been actively revealing the association of several gene variants (Collins, Raleigh 2009, Raleigh, Collins 2012) and have also discussed the potential biochemical roles played by the respective proteins in the predisposition to ATP (Foster et al. 2012).

Collagen is the most abundant structural protein in the extracellular matrix of tendons (Obrien 1992). In particular, type I collagen fibrils represent the majority of the hierarchical formation of tendons (Obrien 1992). They are also known to play a role in determining the strength of tendons (Hoffmann, Gross 2007). Gene variants within the *COL1A1* have been associated with the reduction of the risk of ACL rupture (Posthumus et al. 2009, Ficek et al. 2013). Furthermore, type V collagen is present on a smaller scale in tendons where, along with type I collagen, it regulates the size and shape of fibrils (Imamura, Scott & Greenspan 2000, Wenstrup et al. 2011). It has been reported that an increase in the amount of type V collagen reduces the diameter of type I collagen fibrils in chick corneal stroma (Birk et al. 1990). Furthermore, a reduction in the deposition of type V collagen results in the formation of large type I collagen fibrils (Birk 2001, Birk et al. 1990) as depicted in figure 4.1.1. It is worth noting that this experimental model was not tested in humans. As mentioned in Chapter 2, variants within the *COL5A1* gene were associated with ATP in two Caucasian populations (Mokone et al. 2006, September et al. 2009, Abrahams et al. 2013). In specific, the CC genotype of the rs12722 variant was associated with a reduction in the risk of ATP unlike the II genotype of the rs71746744 variant which associates with an increased risk.

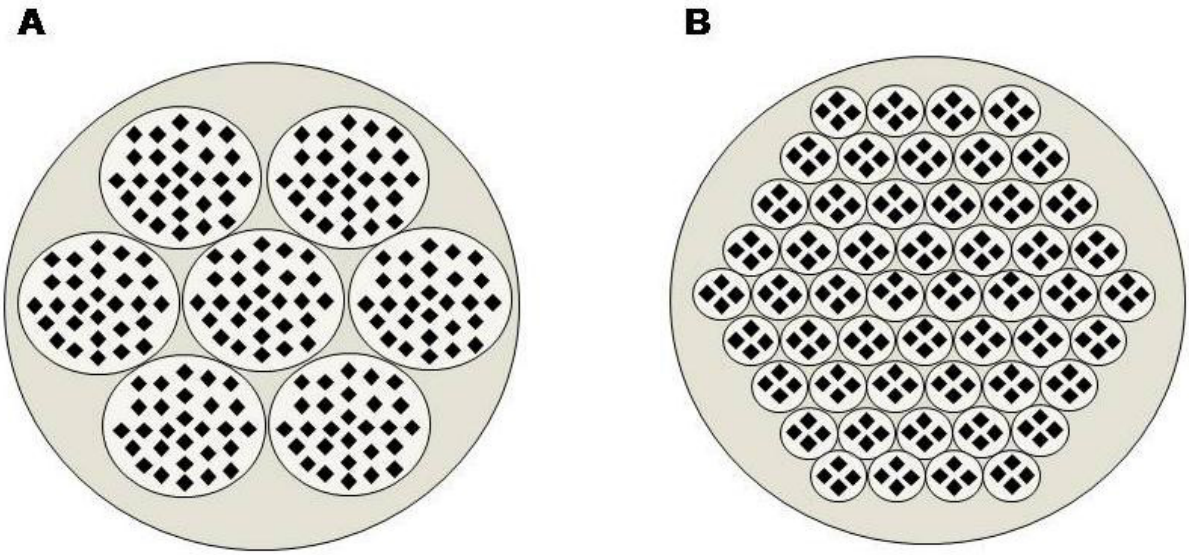


Figure 4.1.1 A cross-sectional view of collagen. The fibrils are represented by the circles in the middle, and the sub-fibrils are represented by the black dots. A) A healthy tendon with large and less densely packed fibrils. B) An injured tendon with small and densely packed fibrils. Adapted from (Ribbans, Collins 2013, Birk et al. 1990).

In addition to collagen which accounts for 65% of the dry mass of a tendon, elastin and fibrillin are structural proteins which provide tissue with stretching and recoiling abilities (Rosenbloom, Abrams & Mecham 1993). The changes in the structural composition and protein density of the extracellular matrix (ECM) in tendons are likely to be reflected in the biomechanical properties of these connective tissues (Aoyama et al. 1994).

Elastin (ELN) is an insoluble polymer composed of several tropoelastin molecules covalently bound to each other by cross-links. Individual ELN molecules are aligned on a scaffolding of microfibrils composed of fibrillin (Aoyama et al. 1994) where they are stabilised by Desmosine: intermolecular crosslinks known to stabilise microfibril alignments (Rosenbloom, Abrams & Mecham 1993). The widely accepted model is depicted in figure 4.1.2.

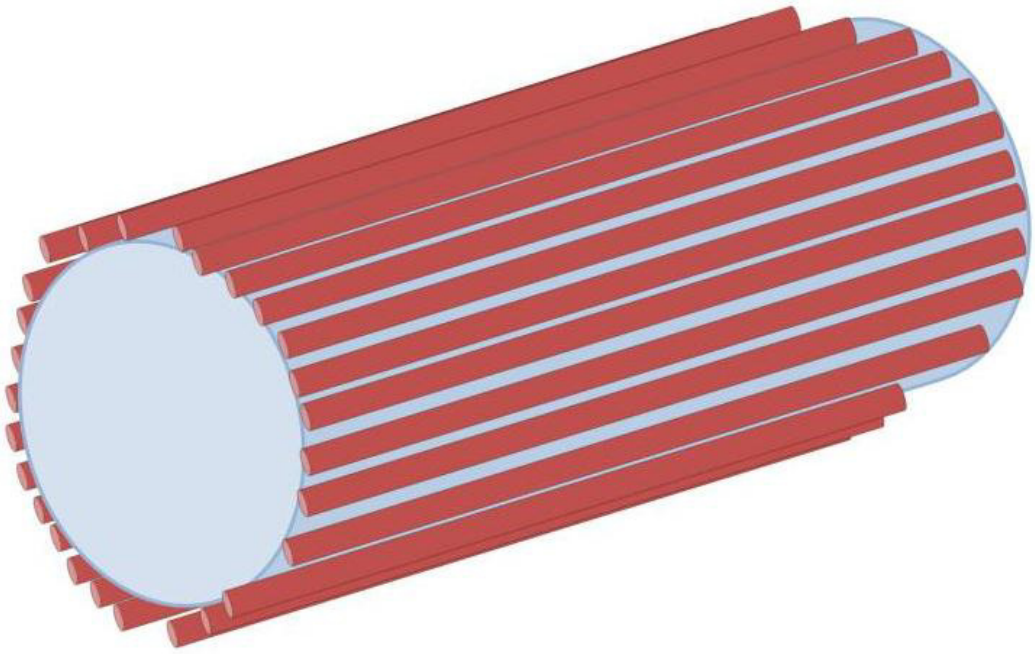


Figure 4.1.2 A schematic diagram of the assembly of fibrillin and elastin to form the elastic fibre. The large blue structure in the middle represents elastin. The red filaments surrounding elastin are the fibrillin microfibrils. Adapted from (Kielty 2006).

ELN is composed predominantly of hydrophobic amino acids such as glycine, and proline (Debelle, Tamburro 1999). The individual polypeptide chains are linked with the rubberlike network through highly mobile lysine-derived crosslinks (Debelle, Tamburro 1999). Although its nature is hydrophobic, ELN is highly hydrated by solvent water which swells the polymer *in vivo*, enhancing the entropy of the molecule (Tamburro 1981). It is suggested that ELN can last for the entire life span of an individual because of its stability and its very slow turnover (Debelle, Tamburro 1999).

ELN proteins provide tendons with the needed elasticity, allowing them to stretch and return to their original state (Kielty 2006). These proteins have an important load-bearing role in musculoskeletal tissues and are used in places where the storage of mechanical energy is required (Gosline et al. 2002).

The gene variant rs2071307 within the *ELN* gene was found to be associated with aortic stenosis, (Ellis et al. 2012) and aortic aneurysm (Saracini et al. 2012). The *ELN* rs2071307 (G/A) variant located on exon 20 is a non-synonymous SNP that substitutes the hydrophobic amino acid glycine with a hydrophilic serine (http://www.ncbi.nlm.nih.gov/projects/SNP/). Such substitution may disrupt the integrity of the microfibrils rendering them more prone to damage (He et al. 2012). Therefore, the investigation of the role of this gene variant in the predisposition to ATP is empirical.

Fibrillins are large, cysteine-rich glycoproteins present in the extracellular matrix of tendons (Sakai, Keene & Engvall 1986). The assembly of fibrillins contributes towards the assembly of microfibril structures. These structures act as architectural frameworks for the deposition of tropoelastin (a precursor of elastin) and the assembly of elastic fibres (Charbonneau et al. 2004). There are two highly homologous fibrillin proteins involved in providing strength and flexibility to tendons: Fibrillin-1 (FBN1) and fibrillin-2 (FBN2). FBN2 is found preferentially in elastic tissues, such as tendons and ligaments, in addition to the tunica media layer of the aorta, and along the bronchial tree (Zhang, Hu & Ramirez 1995) where it plays a role in directing the assembly of elastic fibres (Rongish et al. 1998).

Mutations within the *FBN2* gene, located in chromosome 5q23, have been shown to cause congenital contractural arachnodactyly (CCA) also known as Beals Syndrome (Gupta et al. 2002). This suggests that gene variants within the *FBN2* gene are suitable candidates to include in a genetic association study focused on the disruption of the ECM. The rs331079 variant within intron 7 of *FBN2* has been associated with the disruption of the ECM in intracranial aneurysms (Ruigrok et al. 2006) and would therefore be a suitable candidate gene to investigate in ATP.

Tendon growth, differentiation, and maintenance are regulated by the TGF-β superfamily which includes growth differentiation factors (GDFs) proteins. A particular member of this

family, GDF5, is involved in the maintenance and repair of musculoskeletal structures (Settle Jr et al. 2003). Interestingly, the specific role of GDF5 in tendons is unknown. However, Wolfman et al. suggested that GDF5 plays a role in the formation of new collagen fibrils (Wolfman et al. 1997). This was confirmed when GDF5-deficient mice had a reduced concentration of collagen in their tendons (Mikic et al. 2001). Furthermore, mutations within the *GDF5* gene underlie several genetic disorders such as Du Pan Syndrome (Szczaluba et al. 2005), brachydactyly type C (Schwabe et al. 2004), Grebe and Hunter-Thompson dysplasia (Basit et al. 2008). Interestingly, the *GDF5* rs143383 promoter gene variant was associated with osteoarthritis (Southam et al. 2007), congenital hip dysplasia (Rouault et al. 2010), lumbar disk degeneration (Williams et al. 2011), as well as with ATP in a combined Australian and South African Caucasian population (Posthumus et al. 2010).

As described in this section, gene variants within the *COL5A1*, *ELN*, *FBN2*, and *GDF5* genes have been associated with different soft tissue pathologies. Accordingly, the aim of this study was to determine whether the *FBN2* rs331079 and *ELN* rs2071307 variants were associated with ATP in Caucasian cohorts. Furthermore, this study aimed at replicating, in the UK cohort, the investigation previously conducted on the *COL5A1* rs12722, *COL5A1* rs71746744 and *GDF5* rs143883 variants in both AUS and SA cohorts.

4.2 MATERIAL AND METHODS

4.2.1 Participants

As described in Chapter 3, 136 (52 females and 84 males) participants diagnosed with Achilles tendon pathology (ATP group) and 131 (49 females and 82 males) asymptomatic healthy controls (CON group), were recruited for this case-control genetic association study. All participants gave written informed consent (Appendix II) and completed a medical questionnaire (Appendix III). Participants within the CON group were involved in high intensity physical activities and sports for a minimum period of 2 hours per week (Schneider et al. 2006, Lee, Paffenbarger 2000). The pathologies (non-insertional tendinopathy, insertional tendinopathy, or tendon rupture) of the participants within the ATP group were identified using MRI scans and ultra-sound imaging.

4.2.2 DNA Extraction

Approximately 2 ml of saliva was collected from each participant into OG-500 tubes (DNA Genotek, Inc, Ottawa, Ontario, Canada). The OG-500 tubes containing the saliva were stored at room temperature until the day of total DNA extraction. DNA was extracted using the manufacturer's protocol as described in Chapter 3.

4.2.3 TaqMan® Genotyping

SNP genotyping was performed using fluorescence-based custom-made TaqMan® SNP Genotyping Assays for the *FBN2 rs331079* (figure 4.2.1), *ELN rs2071307* (figure 4.2.2) and *GDF5* rs143883 (figure 4.2.3) variants. Similarly to SNP genotyping, the DNA samples were genotyped for the insertion/deletion (AGGG) *COL5A1* rs71746744 variant using a custom designed TaqMan® Assay (figure 4.2.4). PCR reactions were performed in a total volume of 6 µl. Each reaction included primers and probes, and PCR mastermix containing AmpliTaq DNA Polymerase Gold (Applied Biosystems, Foster City, CA, USA). Furthermore, Rox was used as a passive reference, and each run contained repeat samples acting as positive controls in addition to no-template negative controls. qPCR reactions were conducted on an Applied Biosystems StepOnePlus platform (Applied Biosystems, Foster City, CA, USA) and genotypes were automatically called using the StepOne software v2.1 (Applied Biosystems, Foster City, CA, USA) as shown in figure 4.2.5.

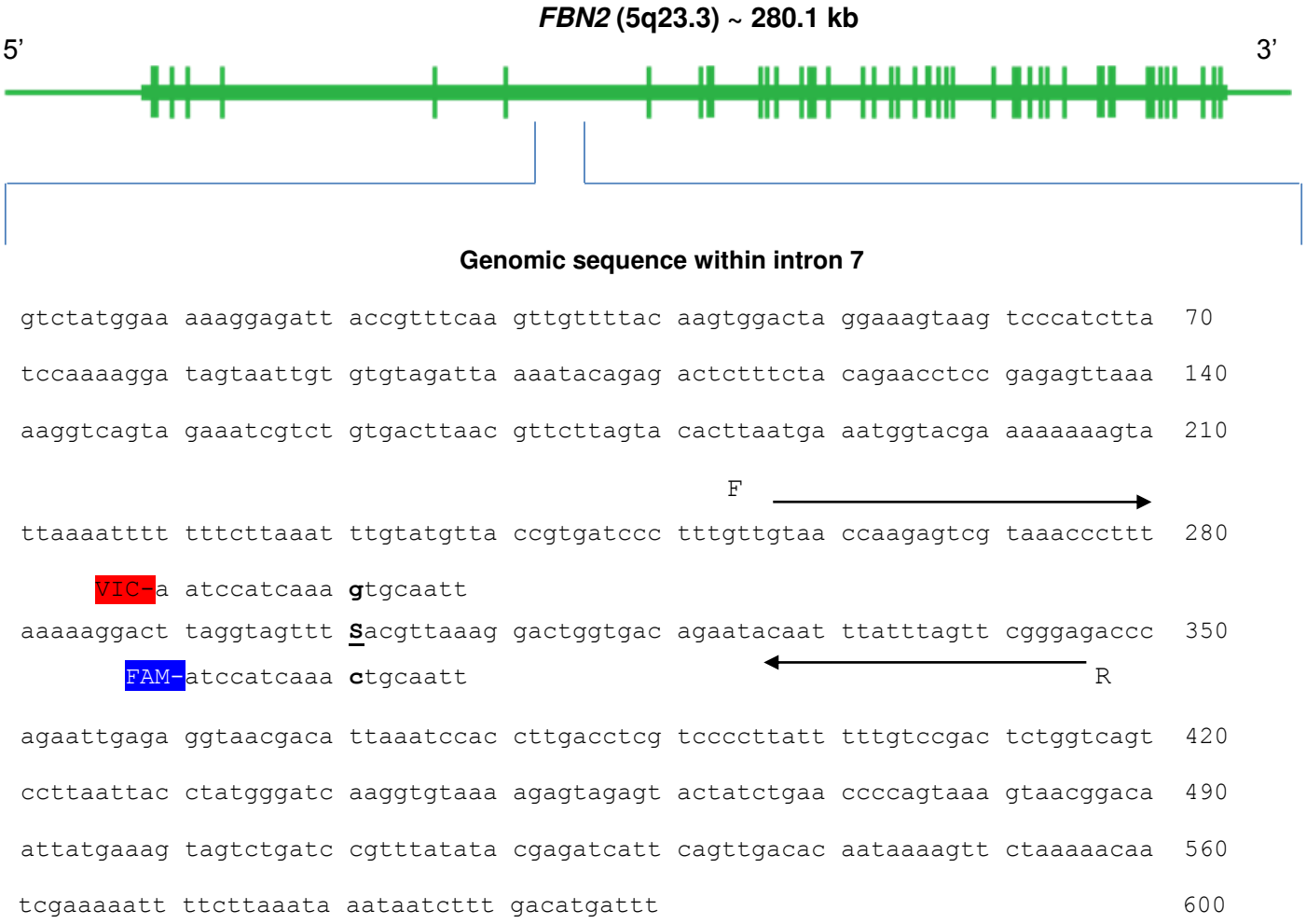


Figure 4.2.1 a schematic representation of exons (vertical lines), and introns (thick horizontal line) within the *FBN2* gene as well as the 5' and 3' UTR (thin horizontal line). The genomic sequence consists of the 300 bp residing on both ends of the rs331079 variant. The letter "S" at position 301 is the IUPAC code for a G/C SNP. The forward and reverse primers are indicated with the arrows preceded by the letters "F" and "R" respectively. The sequences of the VIC and FAM probes are highlighted in red and blue respectively. Adapted from the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/projects/SNP/

***ELN* (7q11.23) ~ 42.1 kb**

Genomic sequence within intron 19, exon 20, and intron 20

```
cggggtgtaa aaaaaatttc cgaagtacca cctccacgac ccctgggtcc gtagggtcaa aagacagaaa  70

atacctgttc cggacccccc ttaaatgtag gagaaagggt taggtagtcg tagggagtct cgggcgggtc  140

ggagagagtg actcgaagaa aagatgaacc gagggaaggg agacgtcCCC GGTCCGGGCC TCAGCCTCAA  210

                                                      F
CCTCCGTAAG GATGAATGCC CCAACCTCGA CCCCCGAAAG GGCCGAAACC ACAGCCTCAG CCTCCATAGG  280

       VIC-AGGTGTCCCT GGTGTCG
GACCTCAGCG TCCACAGGGA RCACAGCCTC CACAAGGGCC TCAGCCTCCA CAGGGCCCTC AACCGTAAAG  350
       FAM-AGGTGTCCCT AGTGTCG                                  R

GGcactcgga atcagtgtgg acccctgtac ccaactcttc cctacccccg aagaacagac gagccgagac  420

gtccccgtca cccctgacat ctagcccgaa cttacacgag tccctcctca acccccctctt cttccctcca  490

gcataggtac ggaatgtccc gtcttctcga aatttgtgcc gagcctcctc tgggtccgtg ccgaagactc  560

ccagagaaag aaagagcaaa ggaacatcgg cttcgagtcc                                    600
```

Figure 4.2.2 a schematic representation of exons (vertical lines), and introns (thick horizontal line) within the *ELN* gene as well as the 5' and 3' UTR (thin horizontal line). The genomic sequence consists of the 300 bp residing on both ends of the rs2071307 variant. The sequences in small letters represent the intronic region, and the sequences in capital letters are the exonic regions. The letter "R" at position 301 is the IUPAC code for a G/A SNP. The forward and reverse primers are indicated with the arrows preceded by the letters "F" and "R" respectively. The sequences of the VIC and FAM probes are highlighted in red and blue respectively. Adapted from the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/projects/SNP/

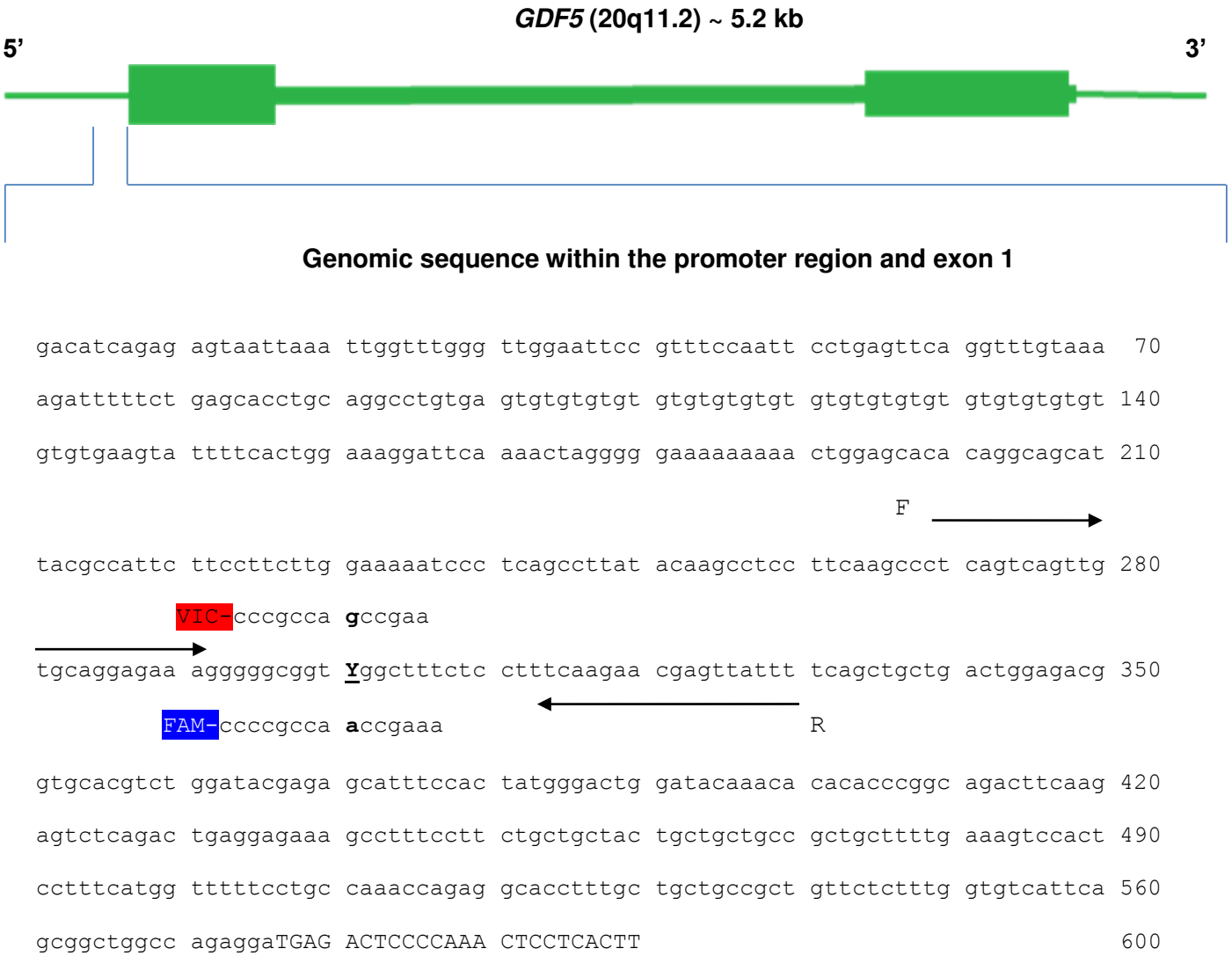
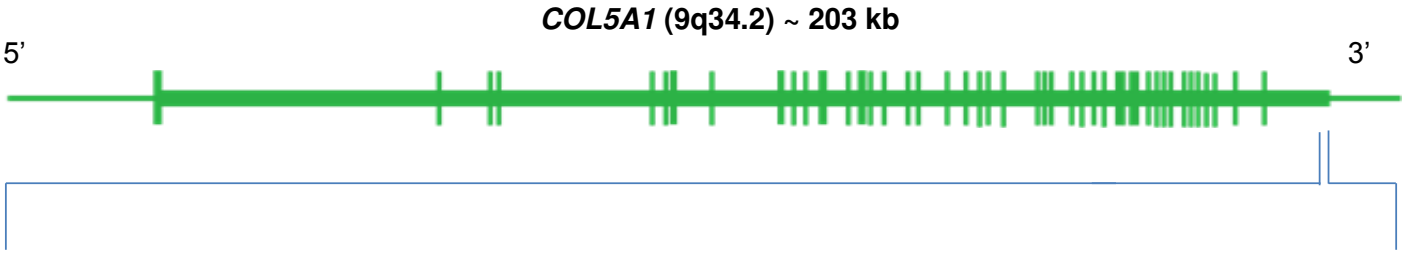


Figure 4.2.3 a schematic representation of exons (boxes), and introns (thick horizontal line) within the *GDF5* gene as well as the 5' and 3' UTR (thin horizontal line). The genomic sequence consists of the 300 bp residing on both ends of the rs143833 variant. The sequences in small letters represent the intronic region, and the sequences in capital letters are the exonic regions. The letter "Y" at position 301 is the IUPAC code for a C/T SNP. The forward and reverse primers are indicated with the arrows preceded by the letters "F" and "R" respectively. The sequences of the VIC and FAM probes are highlighted in red and blue respectively. Adapted from the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/projects/SNP/



Genomic sequence within the 3' UTR

```
tgagttttaa gtaaatattt gtattgtatt gttataaatg ttaagtgtgc ctggctttca atcatgcacg   70

gaaacccagt ctcagtccca cggacagaat gggcgaggca tggattctgg gttgcagtac cgttctgatt  140

agaaatagga agtctcccca cccccgccct ggccaagaac gtgcaataaa ttggaagttt gccccggggc  210

                                          F ---------->

agcaagaatt tatgctgcca ttgaaaagca ggtaccagtg ccccttttca gacagttttt gattcgctct  280

          VIC-aaaatta tccctccctt t

agacttttt ttttttaat agggaaaaaa tttgataatt ttctttttc tacatgcact taagactaaa  350

          FAM-aaaatta tcccttttt aaac                                    <---- R

acacaggttt ggattaattt tatttgcttc ctttttccgc ttttcttccc gcagagcctg atgggagaat  420

<---------- R

gtccagggca gggaaaccac atttttgta ggtgataact caatgaaaat tggtgcttat tttttacact  490

tctctcttgt ggctctcttg tggtgctatc tatctgtttt aaggtctcct tgaaggcgca ctggggaccc  560

tggccatgcc tcgttctccc tgctttcttt atcctgttat                                  600
```

Figure 4.2.4 a schematic representation of exons (vertical lines), and introns (thick horizontal line) within the *COL5A1* gene as well as the 5' and 3' UTR (thin horizontal line). The genomic sequence consists of the 300 bp residing on both ends of the rs71746744 variant. The (aggg) STR polymorphism is located at position 301 and is typed in bold characters. The forward and reverse primers are indicated with the arrows preceded by the letters "F" and "R" respectively. The sequences of the VIC and FAM probes are highlighted in red and blue respectively. Adapted from the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/projects/SNP/

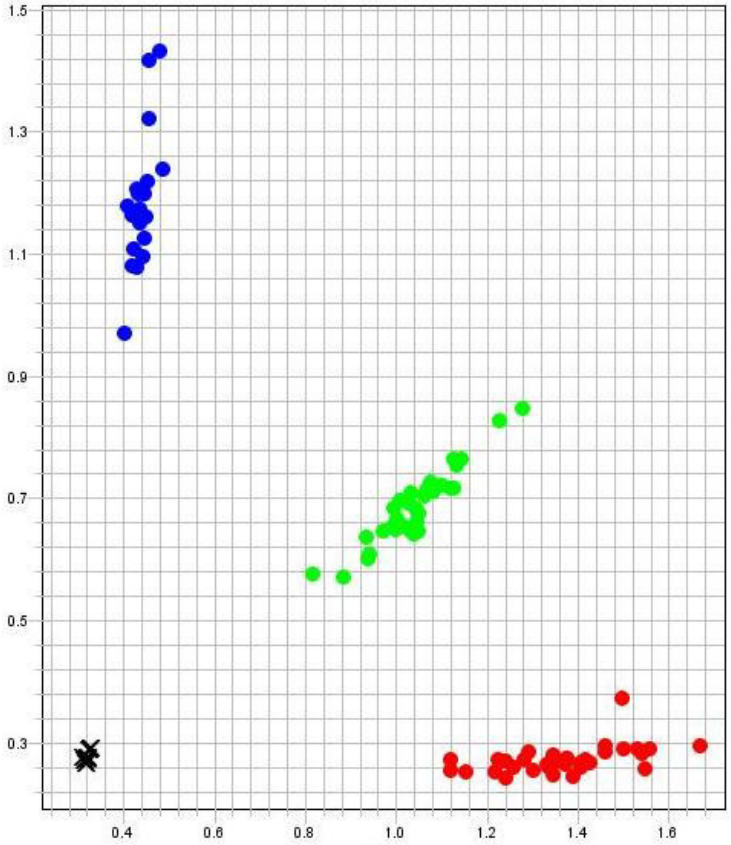


Figure 4.2.5 A typical allelic discrimination plot on the StepOne software v2.1 (Applied Biosystems, Foster City, CA, USA) showing automatic genotypic calls for the *ELN* rs2071307 variant. Each dot corresponds to an individual's genotype. The blue dots represent the homozygous AA genotypes, the green dots represent the heterozygous GA genotypes, and the red dots represent the GG genotype. The "X" signs at the bottom left represent the negative no template controls.

4.2.4 PAGE Genotyping

All participants were genotyped for the *COL5A1* rs12722 variant using the polyacrylamide gel electrophoresis (PAGE) method described in Chapter 3. The 667 bp fragment containing the *Bst*UI RFLP rs12722 SNP within the 3'-UTR of the *COL5A1* gene (figure 4.2.6) was amplified in a 60 µl PCR reaction based on the Greenspan and Pasquinelli (1994) protocol and modified by Mokone et al. (2006).

As discussed in Chapter 3, the 667 bp PCR product was digested using 5 units of *Bst*UI enzyme (New England Biolabs, Ipswich, Massachusetts, USA) in a 25 µl reaction containing NEB2 buffer (New England Biolabs, Ipswich, Massachusetts, USA) and incubated at 60 °C overnight. The digestion products were separated alongside a 100 bp DNA ladder on 6% PAGE gels stained with SYBR® Gold nucleic acid gel stain (Invitrogen Molecular Probes™, Oregon, USA).

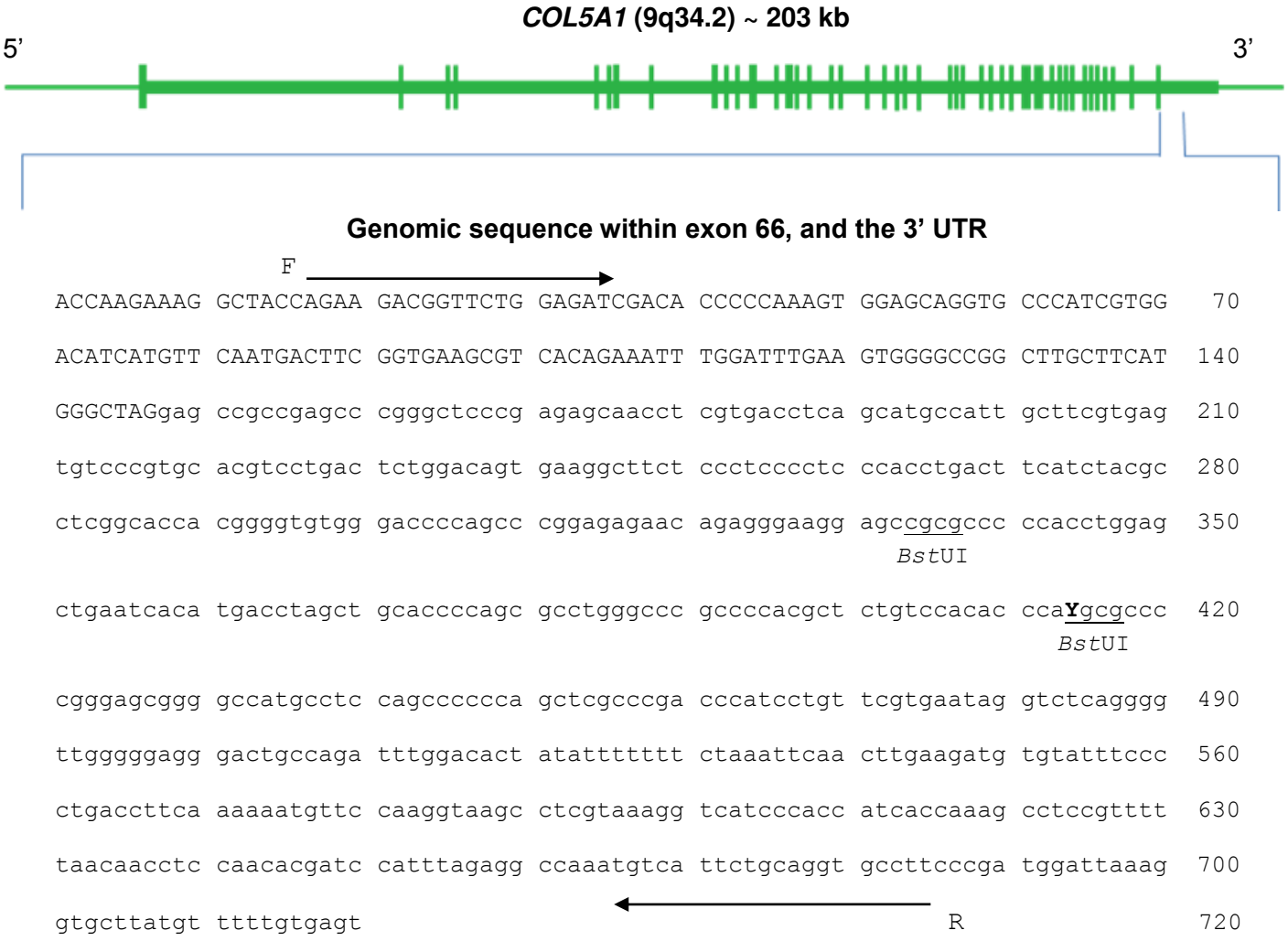


Figure 4.2.6 A schematic representation of exons (vertical lines), and introns (thick horizontal line) within the *COL5A1* gene as well as the 5' and 3' UTR (thin horizontal line). The genomic sequence consists of the 412 bp residing upstream and 306 pb residing downstream of the rs12722 variant. The sequences in small letters represent the intronic region, and the sequences in capital letters are the exonic regions. The letter "Y" at position 413 is the IUPAC code for a C/T SNP. The forward and reverse primers are indicated with the arrows preceded by the letters "F" and "R" respectively. Adapted from the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/projects/SNP/

.

The C and T alleles were identified based on the sizes of fragments as indicated in figure 4.2.7. DNA sequences carrying the T allele for the rs12722 variant would produce two fragments of varying sizes: 351 bp and 316 bp. On the other hand, DNA sequences carrying the C allele produce three fragments: 316 bp, 271 bp, and 80 bp.

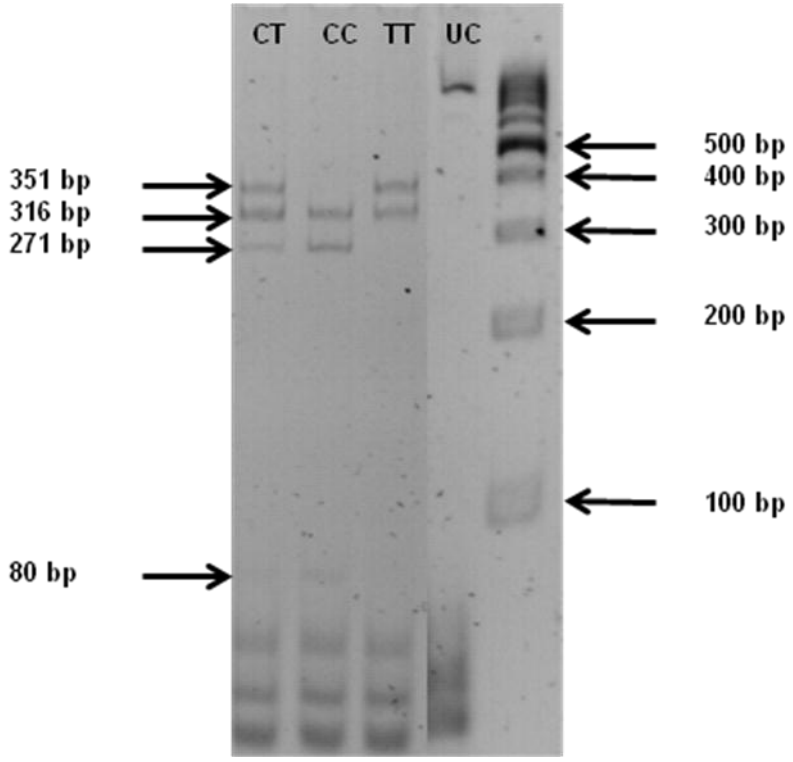


Figure 4.2.7 A typical 6% non-denaturing polyacrylamide gel showing the genotypes of the *COL5A1* *Bst*UI RFLP. The digestion of the 667 bp PCR product with *Bst*UI produced 351 bp and 316 bp fragments for the T allele, and 316 bp, 271 bp and 80 bp fragments for the C allele. The 80 bp fragments are very faint but are indicated by the arrow. The right lane contains the 100 bp molecular weight marker with the appropriate fragment sizes given in base pairs (bp). The second lane from the right contains the uncut (UC) 667 bp PCR product

4.2.5 RNA Secondary Structure

The secondary structures of exon 20 within the *ELN* gene were generated using the SFold online statistical algorithms (http://sfold.wadsworth.org) (Ding, Lawrence 2003, Ding, Chan & Lawrence 2005). SFold generates the RNA secondary structures using statistical algorithms from the Boltsman ensemble of secondary structures. All structures were folded at 37 °C and 1 M NaCl in the absence of divalent ions.

4.2.6 Statistical Analysis

The power calculations for this study were conducted using Quanto v1.2 (http://hydra.usc.edu/gxe) and the Power for Genetic Association Analyses (PGA) package (http://dceg.cancer.gov/bb/tools/pga). The initial calculations were done using a recessive model and a disease population prevalence of 10%.

Data analysis was conducted using SPSS Version 20 (SPSS Science Inc, Chicago, Ill, USA) statistical program. Chi-squared (χ^2) analysis or Fisher's exact test were used to analyse differences in the genotype and allele frequencies, as well as other categorical data between the groups. Furthermore, a one-way analysis of variance was used to determine significant differences between the characteristics of the ATP and CON groups within all three cohorts. Deviation of groups from the Hardy-Weinberg equilibrium was tested for using Michael H. Court's (2005–2008) online calculator (www.tufts.edu). The significance of all statistical testing was accepted at $p<0.05$.

4.3 RESULTS

4.3.1 Power Calculations

The conducted power calculation reported that a case-control population of 136 matched individuals was adequate to detect an allelic OR of 2.0 at a power of 80% and a significance level of 5% when the allele frequency is estimated at 60% (figure 4.3.1) (Appendix V).

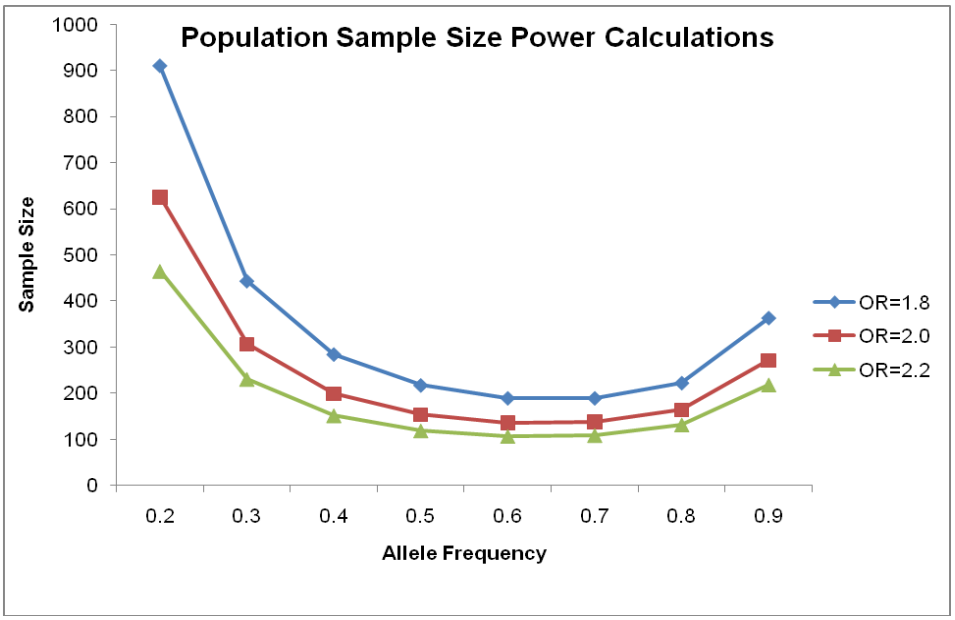


Figure 4.3.1 Graphical representation of the power calculations to detect OR= 1.8, 2, and 2.2. The minimum sample size was determined at a 10% prevalence of ATP with a power of 80%.

4.3.2 Participants' Characteristics

In this study, the UK ATP and CON groups were matched for all characteristics except for age; the ATP group was significantly older (p=0.033) at the time of injury than the CON group at the time of recruitment. When stratified between genders, the female ATP and CON groups were matched for age, however, the male ATP group was significantly older (p=0.015) than the CON group. Furthermore, the male CON group was significantly (p=0.006) taller than the ATP group, and the female ATP had a significantly higher BMI (p=0.026) than then CON group (table 4.3.1).

The CON and ATP groups within the combined AUS and SA cohort were similarly matched for age and height. However, the ATP group was significantly heavier and larger (weight, $p<0.001$; BMI, $p<0.001$) than the CON group. There was a significantly ($p<0.001$) greater number of males in the ATP group than in the CON group. When split into gender, the CON and ATP groups were similarly matched for all characteristics except for weight in males ($p=0.001$) and BMI in females ($p=0.016$) (table 4.3.2).

Interestingly, participants in the AUS TEN group carrying the *ELN* rs2071307 AA (53.1 ± 11.6, n=10) genotype were significantly ($p=0.005$) older at the time of their initial Achilles tendon injury when compared to those with a GG (37.2 ± 12.6, n=16) or a GA (37.8 ± 13.6, n=32) genotype (figure 4.3.2 B). There were, however, no significant differences in the average ages of the three genotype groups in the CON AUS group (GG: 40.7 ± 11.8, n=48; GA: 37.4 ± 12.2, n=68; AA: 40.1 ± 12.1, n=24; $p=0.323$) (figure 4.3.2 A). No age, height, weight or BMI interaction with genotype was detected in either the SA or the UK cohorts.

Table 4.3.1 General characteristics of the UK Achilles tendon pathology group (ATP) and the respective asymptomatic control (CON) group

				Female			Males		
	CON (n=131)	**ATP (n=136)**	**P-value**	**CON (n=49)**	**ATP (n=52)**	**P-value**	**CON (n=82)**	**ATP (n=84)**	**P-value**
Age (Years)[a]	41.6 ± 11.6 (124)	45.1 ± 14.1 (135)	**0.033**	44.2 ± 11.4 (48)	45.2 ± 14.2 (51)	0.716	40.2 ± 11.5 (77)	45.1 ± 14.1 (84)	**0.015**
Gender (% male)	62.6 (82)	61.8 (84)	0.887						
Height (cm)	174.9 ± 10.4 (123)	172.6 ± 9.4 (133)	0.062	165.5 ± 7.1 (48)	164.1 ± 6.5 (51)	0.314	180.9 ± 7.2 (76)	177.9 ± 6.7 (82)	**0.006**
Weight (kg)[b]	80.1 ± 19.6 (124)	78.1 ± 15.0 (99)	0.388	63.7 ± 10.9 (48)	67.4 ± 11.1 (40)	0.122	90.7 ± 16.4 (77)	85.4 ± 12.9 (59)	0.052
BMI (kg/m^2)	25.9 ± 4.5 (123)	26.3 ± 4.1 (98)	0.665	23.2 ± 3.5 (48)	25.2 ± 4.7 (40)	0.026	27.6 ± 4.4 (76)	26.9 ± 3.4 (59)	0.532

Values are expressed as mean ± SD or a frequency (%). The total number of participants (n).

The maximum number (n) of participants in each category is also indicated.

[a] age of the ATP group is at the age of initial injury, while the age of the CON group is at the age of recruitment.

[b] weight of the ATP group is at the time of initial injury, while the weight of the CON groups is at the time of recruitment

cm, centimetres; kg, kilograms; m, metres

Table 4.3.2 General characteristics of the combined AUS+SA Achilles tendon pathology group (ATP)

and the respective asymptomatic control (CON) group

				Female			Males		
	CON (n=238)	**ATP (n=174)**	**P-value**	**CON (n=121)**	**ATP (n=50)**	**P-value**	**CON (n=124)**	**ATP (n=122)**	**P-value**
Age (Years)[a]	38.2 ± 11.2 (230)	40.2 ± 13.6 (166)	0.112	37.7 ± 11.1 (119)	38.5 ± 12.8 (48)	0.694	38.2 ± 11.3 (117)	40.9 ± 13.8 (119)	0.095
Gender (% male)	50.6 (124)	70.9 (122)	<0.001						
Height (cm)	173.1 ± 9.5 (235)	175.0 ± 9.1 (161)	0.054	166.9 ± 7.6 (120)	165.0 ± 6.2 (46)	0.135	179.5 ± 6.7 (121)	179.2 ± 6.8 (116)	0.693
Weight (kg)	72.7 ± 13.2 (238)	80.6 ± 14.8 (166)	<0.001[b]	65.4 ± 10.7 (121)	68.8 ± 12.1 (47)	0.073	80.3 ± 11.3 (123)	85.6 ± 13.5 (120)	0.001
BMI (kg/m^2)	24.2 ± 3.6 (235)	26.2 ± 4.0 (161)	<0.001[b]	23.5 ± 3.9 (120)	25.3 ± 4.6 (46)	0.016	24.9 ± 3.2 (121)	26.6 ± 3.8 (116)	0.532

Values are expressed as mean ± SD or a frequency (%). The total number of participants (n).

The maximum number (n) of participants in each category is also indicated.

[a] age of the ATP group is at the age of initial injury, while the age of the CON group is at the age of recruitment.

[b] co-varied for age of recruitment.

cm, centimetres; kg, kilograms; m, metres

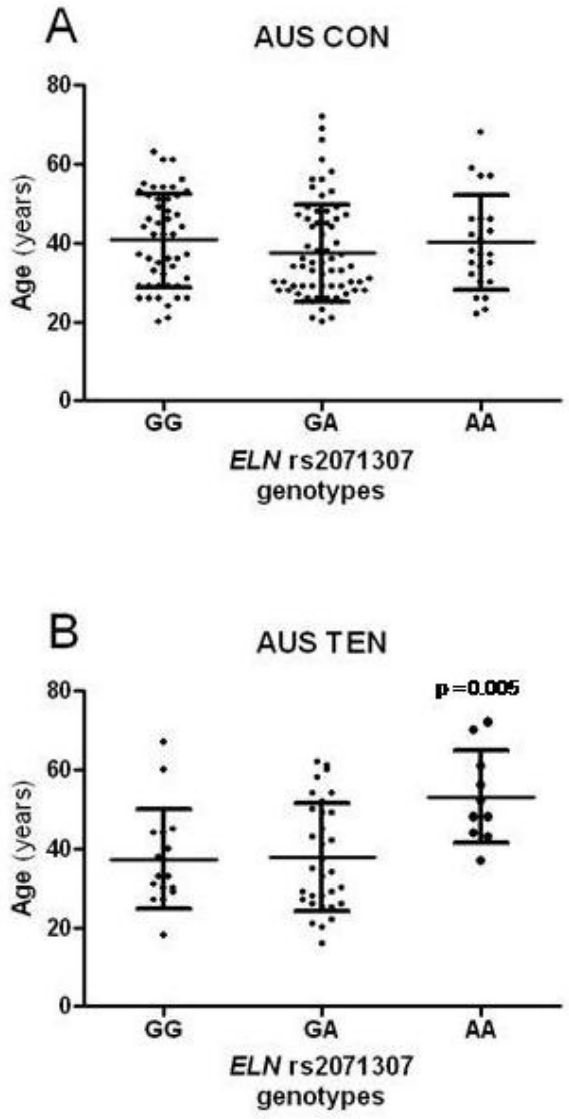


Figure 4.3.2 Column scatter graph showing the mean and standard deviation of the age of recruitment of the CON group and the age of injury of the TEN group based on genotype at the *ELN* rs2071307 variant in the AUS cohort. A) There is no significant difference (p=0.323) in the age of recruitment of the CON group. B) Participants carrying the AA genotype were significantly older (p=0.005) than participants with GG and GA genotypes when they first developed the injury.

4.3.3 Genotype and Allele Frequencies

No significant genotype distribution frequency was detected in the UK cohort between the CON and ATP for all investigated variants: *FBN2* rs331079 (p=0.507), *COL5A1* rs12722 (p=0.658), *COL5A1* rs71746744 (p=0.319) and *GDF5* rs143383 (p=0.538). However, a significant allelic distribution difference of the *FBN2* rs331079 variant was reported between the CON and the RUP group (p=0.033). The C allele was significantly (p=0.033; OR=2.09; 95% CI 1.05-4.16) over-represented in the RUP group. Furthermore, when the data was split according to gender, a significant genotypic (p=0.004) and allelic (p<0.001) distribution difference of the *FBN2* rs331079 variants was detected in females between the CON and the RUP groups. There was a significant over-representation of the CC genotype (p=0.004; OR=7.70; 95% CI 1.95-30.39) and the C allele (p<0.001; OR=6.76; 95% CI 2.02-22.68) in the RUP group (table 4.3.3).

In addition to that, the genotype (p=0.009) and allele (p=0.031) distribution frequencies of the *COL5A1* rs12722 variant were significantly different between the male CON and RUP groups. The TT genotype was significantly (p=0.004; OR=4.2; 95% CI 1.58-11.97) over-represented in the RUP group, and the C allele was significantly (p=0.031; OR=2.07; 95% CI 1.06-4.05) over-represented in the CON group. Likewise, within the male population, the results show an allelic association where the *COL5A1* rs71746744 DEL allele was significantly over-represented (p=0.046; OR=1.61; 95% CI 1.01-2.56) in the CON group when compared to the ATP group.

The genotype frequency distributions of *FBN2* rs331079 and *ELN* rs2071307 within the SA and AUS CON and ATP groups were not significantly different and therefore the two cohorts were looked at collectively. The genotype frequencies for the independent AUS and SA cohorts can be found in appendix VI. There was no significant genotype distribution difference of the *ELN* rs2071307 variant between the CON and ATP groups in the combined

AUS+SA cohort. However, there was a significant genotypic (p=0.015) and allelic (p=0.026) distribution difference between the CON and the RUP group (table 4.3.4). In fact, both the AA genotype (p=0.005; OR=2.89; 95% CI 1.38-6.06) and the A allele (p=0.026; OR=1.73; 95% CI 1.06-2.82) were significantly over-represented in the RUP group.

On the other hand, there was a significant genotypic (p=0.044) and allelic (p=0.011) distribution difference for the *FBN2* rs331079 variant between the CON and the ATP group in the AUS+SA cohort (table 4.3.4). Both the GG genotype (p=0.019; OR=1.88; 95% CI 1.11-3.18) and the G allele (p=0.011; OR=1.86; 1.14-3.04) were over-represented in the ATP group. Furthermore, when the analysis was conducted between the CON and the TEN groups, both the genotypic (p=0.035) and allelic (p=0.017) distributions were significantly different. The GG genotype (p=0.035; OR=1.83; 95% CI 1.04 – 3.25) and the G (p=0.017; OR=1.90; 95% CI 1.11 – 3.27) allele were significantly over-represented in the TEN group.

4.3.4 *ELN* RNA Secondary Structure

To identify any structural differences between the G and A allele, the secondary structures of the *ELN* exon 20 were generated using SFold as illustrated in figure 4.3.3. There was a clear structural difference observed at position 114 (the location of the SNP). In the presence of the G allele, the nucleotides at positions 53, 54, 113, and 114 were not aligned with the neighbouring sequences. When the A allele was present however, the nucleotides at positions 53, 54, 113, and 114 were in line with the neighbouring sequences resulting in a different structure. Furthermore, the substitution of the G allele by the A allele resulted in a reduction of the free energy of the molecule: G allele, $\Delta G°$=-70.6 kcal/mol; A allele, $\Delta G°$=-71.6 kcal/mol.

Table 4.3.3 Genotype and allele frequency distribution of the *FBN2* rs331079 the *COL5A1* rs12722, and the *COL5A1* rs74746744 variants within the UK control (CON) and Achilles tendon pathology (ATP), as well as the pathological sub-groups Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP). The genotype and allele frequency distributions are also shown when the dataset was categorised into gender.

					Female				**Male**			
	CON	**ATP**	**TEN**	**RUP**	**CON**	**ATP**	**TEN**	**RUP**	**CON**	**ATP**	**TEN**	**RUP**
***FBN2* rs331079**	n=129	n=131	n=94	n=37	n=49	n=50	n=38	n=12	n=80	n=81	n=56	n=25
GG	78.3 (101)	74.8 (98)	78.7 (74)	64.9 (24)	89.8 (44)	82.0 (41)	86.8 (33)	66.7 (8)	71.2 (57)	70.4 (57)	73.2 (41)	64.0 (16)
GC	21.7 (28)	22.9 (30)	20.2 (19)	29.7 (11)	10.2 (5)	16.0 (8)	13.2 (5)	25.0 (6)	28.8 (23)	27.2 (22)	25.0 (14)	32.0 (8)
CC	0.0 (0)	2.3 (3)	1.1 (1)	5.4 (2)	0.0 (0)	2.0 (1)	0.0 (0)	8.3 (1)	0.0 (0)	2.5 (2)	1.8 (1)	4.0 (1)
P Value	0.507[a]		0.939[b]	0.098[c]	0.271[a]		0.669[b]	**0.004[c]**	0.902[a]		0.802[b]	0.493[c]
HWE	0.167	0.698	0.857	0.625	0.707	0.432	0.432	0.929	0.133	0.943	0.876	1.000
C allele	10.9 (28)	13.7 (36)	11.2 (21)	20.3 (15)	5.1 (5)	10.0 (10)	6.6 (5)	26.7 (8)	14.4 (23)	16.0 (26)	14.3 (16)	20.0 (10)
P Value	0.316[a]		0.171[b]	**0.033[c]**	0.193[a]		0.678[b]	**<0.001[c]**	0.676[a]		0.983[b]	0.340[c]
***COL5A1* rs12722**	n=125	n=127	n=93	n=34	n=47	n=48	n=38	n=10	n=78	n=79	n=55	n=24
CC	21.6 (27)	22.8 (29)	21.5 (20)	26.5 (9)	14.9 (7)	25.0 (12)	21.1 (8)	40.0 (4)	25.6 (20)	21.5 (17)	21.8 (12)	20.8 (5)
CT	51.2 (64)	45.7 (58)	50.5 (47)	32.4 (11)	44.7 (21)	52.1 (25)	55.3 (21)	40.0 (4)	55.1 (43)	41.8 (33)	47.3 (26)	29.2 (7)
TT	27.2 (34)	31.5 (40)	28.0 (26)	41.2 (14)	40.4 (19)	22.9 (11)	23.7 (9)	20.0 (2)	19.2 (15)	36.7 (29)	30.9 (17)	50.0 (12)
P Value	0.658[a]		0.992[b]	0.134[c]	0.151[a]		0.257[b]	0.365[c]	**0.050[a]**		0.299[b]	**0.009[c]**
HWE	0.761	0.369	0.885	**0.048**	0.762	0.770	0.513	0.598	0.344	0.198	0.729	0.076
C allele	47.2 (118)	45.7 (116)	46.8 (87)	42.6 (29)	37.2 (35)	51.0 (49)	48.7 (37)	60.0 (12)	53.2 (83)	42.4 (67)	45.5 (50)	35.4 (17)
P Value	0.730[a]		0.929[b]	0.504[c]	0.055[a]		0.133[b]	0.060[c]	0.055[a]		0.213[b]	**0.031[c]**
***COL5A1* rs71746744**	n=126	n=125	n=90	n=35	n=46	n=48	n=36	n=12	n=80	n=77	n=54	n=23
D/D	7.9 (10)	8.0 (10)	8.9 (8)	5.7 (2)	0.0 (0)	8.3 (4)	8.3 (3)	8.3 (1)	12.5 (10)	7.8 (6)	9.3 (5)	4.3 (1)
D/I	52.4 (66)	43.2 (54)	44.4 (40)	40.0 (14)	45.7 (21)	41.7 (20)	50.0 (18)	16.7 (2)	56.2 (45)	44.2 (34)	40.7 (22)	52.2 (12)
I/I	39.7 (50)	48.8 (61)	46.7 (42)	54.3 (19)	54.3 (25)	50.0 (24)	41.7 (15)	75.0 (9)	31.2 (25)	48.1 (37)	50.0 (27)	43.5 (10)
P Value	0.319[a]		0.514[b]	0.303[c]	0.135[a]		0.256[b]	0.205[c]	0.091[a]		0.516[b]	0.636[c]
HWE	0.064	0.683	0.726	0.781	**0.045**	0.953	0.453	0.166	0.138	0.636	0.866	0.266
DEL allele	34.1 (86)	29.6 (74)	31.1 (56)	25.7 (18)	22.8 (21)	29.2 (28)	33.3 (24)	16.7 (4)	40.6 (65)	29.9 (46)	29.6 (32)	30.4 (14)
P Value	0.276[a]		0.511[b]	0.183[c]	0.322[a]		0.134[b]	0.513[c]	**0.046[a]**		0.066[b]	0.210[c]

The values are expressed as a frequency with the number of participants (n) in parenthesis.

[a] CON vs ATP; [b] CON vs TEN; [c] CON vs RUP

Table 4.3.4 Genotype and allele frequency distribution of the *FBN2* rs331079 and the *ELN* rs2071307 variants within the combined AUS+SA control (CON) and Achilles tendon pathology (ATP), as well as the pathological sub-groups Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP). The genotype and allele frequency distributions are also shown when the dataset was categorised into gender.

					Female				Male			
	CON	**ATP**	**TEN**	**RUP**	**CON**	**ATP**	**TEN**	**RUP**	**CON**	**ATP**	**TEN**	**RUP**
***FBN2* rs331079**	n=238	n=174	n=135	n=39	n=116	n=50	n=40	n=10	n=120	n=121	n=94	n=27
GG	76.9 (183)	86.2 (150)	85.9 (116)	87.2 (34)	76.7 (89)	86.0 (43)	85.0 (34)	90.0 (9)	77.5 (93)	86.0 (104)	86.2 (81)	85.2 (23)
GC	21.0 (50)	13.2 (23)	14.1 (19)	10.3 (4)	20.7 (24)	14.0 (7)	15.0 (6)	10.0 (1)	20.8 (25)	12.2 (16)	13.8 (13)	11.1 (3)
CC	2.1 (5)	0.6 (1)	0.0 (0)	2.6 (1)	2.6 (3)	0.0 (0)	0.0 (0)	0.0 (0)	1.7 (2)	0.8 (1)	0.0 (0)	3.7 (1)
P Value	0.044[a]		**0.035[b]**	0.225[c]	0.174[a]		0.269[b]	0.455[c]	0.214[a]		0.106[b]	0.445[c]
HWE	0.473	0.907	0.379	0.083	0.382	0.595	0.608	0.868	0.831	0.662	0.471	0.078
C allele	12.6 (60)	7.2 (25)	7.0 (19)	7.7 (6)	12.9 (30)	7.0 (7)	7.5 (6)	5.0 (1)	12.1 (29)	7.4 (18)	6.9 (13)	9.3 (5)
P Value	0.011[a]		**0.017[b]**	0.214[c]	0.115[a]		0.189[b]	0.483[c]	0.086[a]		0.074[b]	0.558[c]
***ELN* rs2071307**	n=238	n=171	n=133	n=38	n=117	n=47	n=38	n=9	n=119	n=121	n=94	n=27
GG	36.1 (86)	34.5 (59)	36.1 (48)	28.9 (11)	38.5 (45)	34.0 (16)	36.8 (14)	22.2 (2)	33.6 (40)	34.7 (42)	36.2 (34)	29.6 (8)
GA	47.1 (112)	46.2 (79)	49.6 (66)	34.2 (13)	45.3 (53)	40.4 (19)	44.7 (17)	22.2 (2)	48.7 (58)	48.8 (59)	52.1 (49)	37.0 (10)
AA	16.8 (40)	19.3 (33)	14.3 (19)	36.8 (14)	16.2 (19)	25.5 (12)	18.4 (7)	55.6 (5)	17.6 (21)	16.5 (20)	11.7 (11)	33.3 (9)
P Value	0.804[a]		0.795[b]	**0.015[c]**	0.389[a]		0.949[b]	**0.032[c]**	0.985[a]		0.483[b]	0.184[c]
HWE	0.730	0.479	0.628	0.055	0.611	0.203	0.649	0.134	0.997	0.925	0.291	0.179
A allele	40.3 (192)	42.4 (145)	39.1 (104)	53.9 (41)	38.9 (91)	45.7 (43)	40.8 (31)	66.7 (12)	42.0 (100)	40.9 (99)	37.8 (71)	51.9 (28)
P Value	0.555[a]		0.741[b]	**0.026[c]**	0.253[a]		0.768[b]	**0.021[c]**	0.805[a]		0.374[b]	0.188[c]

The values are expressed as a frequency with the number of participants (n) in parenthesis.

[a] CON vs ATP; [b] CON vs TEN; [c] CON vs RUP

Table 4.3.5 Genotype and allele frequency distribution of the *GDF5* rs143383 variant within the UK control (CON) and Achilles tendon pathology (ATP), as well as the pathological sub-groups Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP). The genotype and allele frequency distributions are also shown when the dataset was categorised into gender.

					Female				**Male**			
	CON	**ATP**	**TEN**	**RUP**	**CON**	**ATP**	**TEN**	**RUP**	**CON**	**ATP**	**TEN**	**RUP**
***GDF5* rs143383**	n=131	n=119	n=84	n=35	n=48	n=47	n=35	n=12	n=83	n=72	n=49	n=23
TT	37.4 (49)	37.8 (45)	36.1 (30)	42.9 (15)	49.6 (19)	31.9 (15)	25.7 (9)	50.0 (6)	31.6 (30)	41.7 (30)	42.9 (21)	39.1 (9)
CT	50.4 (66)	45.4 (54)	45.8 (38)	45.7 (16)	43.8 (21)	48.9 (23)	58.6 (17)	50.0 (6)	54.2 (45)	43.1 (31)	42.9 (21)	43.5 (10)
CC	12.2 (16)	16.8 (20)	19.0 (16)	11.4 (4)	16.7 (8)	19.1 (9)	25.7 (9)	0.0 (0)	9.6 (8)	15.3 (11)	14.3 (7)	17.4 (4)
P Value	0.538[a]		0.381[b]	0.839[c]	0.737[a]		0.356[b]	0.512[c]	0.319[a]		0.419[b]	0.497[c]
HWE	0.385	0.581	0.525	0.932	0.596	0.972	0.866	0.248	0.130	0.527	0.641	0.675
C allele	37.4 (98)	39.5 (94)	41.7 (70)	34.3 (24)	38.5 (37)	43.6 (41)	50.0 (35)	25.0 (6)	36.7 (61)	36.8 (53)	35.7 (35)	39.1 (18)
P Value	0.631[a]		0.377[b]	0.631[c]	0.477[a]		0.141[b]	0.216[c]	0.991[a]		0.866[b]	0.767[c]

The values are expressed as a frequency with the number of participants (n) in parenthesis.

[a] CON vs ATP; [b] CON vs TEN; [c] CON vs RUP

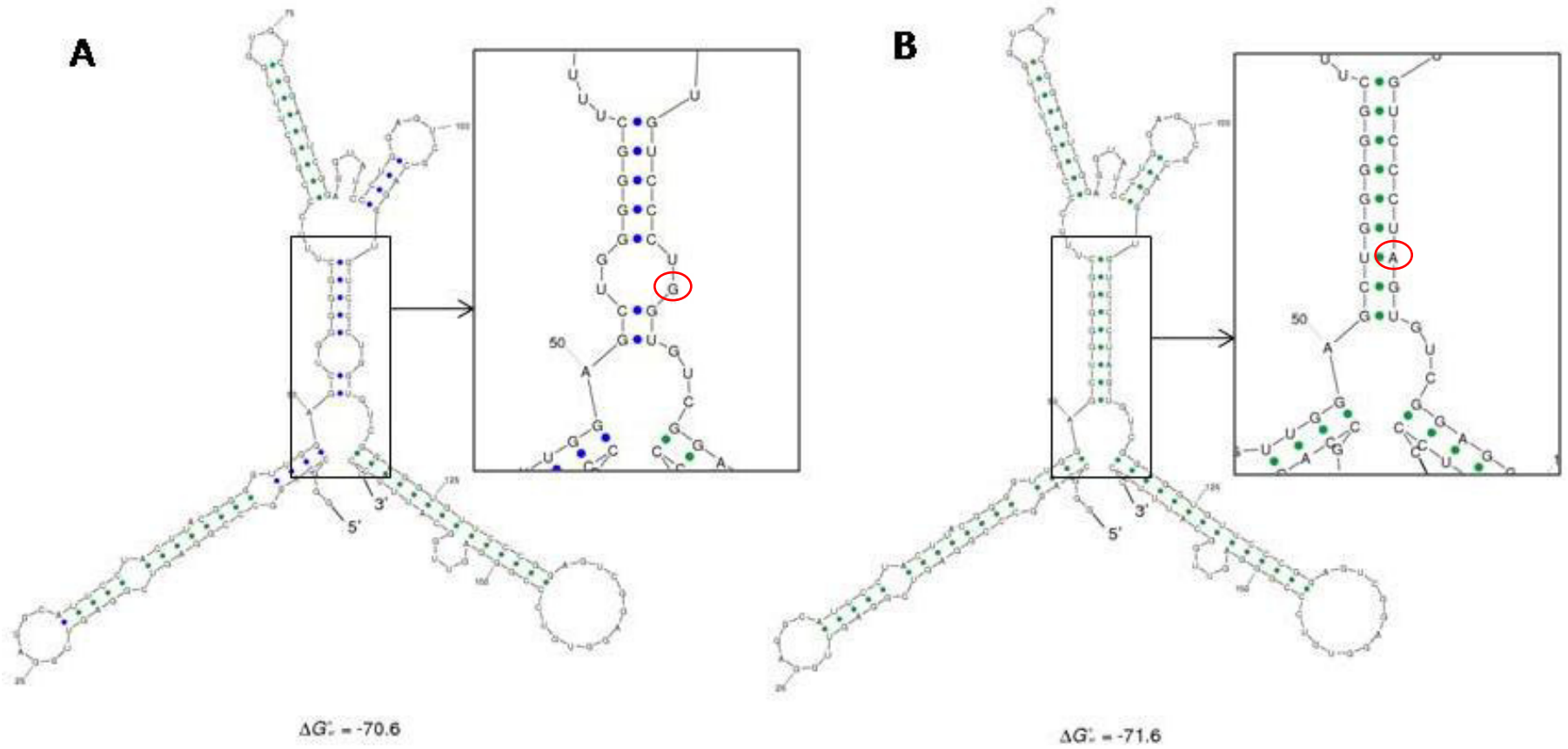


Figure 4.3.3 Comparison of the secondary structure of the RNA transcribed from exon 20 of the *ELN* gene. A) Secondary RNA structure when the G allele is present at the rs2071307 variant. B) Secondary structure when the A allele is present at the rs2071307 variant. The structural differences are magnified in the box, and the alleles are highlighted by a red circle. The secondary structures were generated using the Sfold online RNA folding tool (available at http://sfold.wadsworth.org). The algorithm generates RNA secondary structures using a statistical sample from the Boltzmann ensemble of secondary structures. All structures were folded at 37 °C and 1M NaCl in the absence of divalent ions.

4.4 DISCUSSION

This study found various associations between the investigated gene variants and the various Achilles tendon pathologies. The data report a genotypic and allelic association of the *FBN2* rs331079 variant with Achilles tendinopathy in the combined Australian and South African population where the GG genotype and G allele were over-represented in the TEN group. Interestingly in the UK cohort, and unlike in the AUS+SA cohort, the *FBN2* rs331079 variant was associated with Achilles tendon rupture. This variant was previously shown to associate with the disruption of the extracellular matrix in intra-cranial aneurysms in a Dutch population (Ruigrok et al. 2006). However, in the Dutch study it was the C allele that was found to be the risk factor as opposed to the G allele. *FBN2* mRNA levels have been shown to be elevated in rat Achilles tendons undergoing repair with the expression of *FBN2* reported to be increased for ten days post injury (Jelinsky et al. 2011). Similarly, an increase in the expression of *FBN2* has been found in other pathologies such as mitral valve prolapse (Radermecker et al. 2003).

ELN and FBN-2 are known to form a network of microfibrils that maintains the tendon's architecture (Rosenbloom, Abrams & Mecham 1993). An increase in FBN-2 levels might be expected to increase the density of the tendon and lead to an increase in tendon stiffness and rigidity possibly affecting the compliance of the tendon in response to muscle movement (Cook, McDonagh 1996). On the other hand, a decrease in FBN-2 levels could result in weaker tendons caused by structural deficiencies in the microfibril network (Robinson, Godfrey 2000). Any impairment of the function of FBN-2 is believed to be a major determinant of microfibrillopathy (Robinson, Godfrey 2000) which is speculated to precede a tendinopathy. Furthermore, the increase in *FBN2* expression levels observed during tendon repair (Jelinsky et al. 2011) is consistent with an important role for FBN-2 in maintaining the tendon's architectural integrity.

Mutations such as the G3532T and G3590A substitutions have been found within the *FBN2* gene and lead to the development of connective tissue disorders such as congenital contractural arachnodactyly (Gupta et al. 2002). The rs331079 variant that was investigated in this study resides within an intronic region of the *FBN2* gene (www.snpper.chip.org). Although intronic variants do not determine the primary sequence of a protein molecule (Berget, Moore & Sharp 1977), they may have other, hitherto, undiscovered roles that are necessary for appropriate expression of protein molecules. However, at present it is unknown why this variant predisposes individuals to ATP. The rs331079 variant is known to be part of a linkage block in Caucasians and is in high linkage disequilibrium (D'=1) with the *FBN2* rs331081, rs331082, and rs331085 variants (www.ensembl.com). All three of these additional variants are also located within intron 7 of the *FBN2* gene (www.snpper.chip.org). The linkage disequilibrium between the investigated rs331079 variant and rs331081, rs331082, and rs331085 means that it is conceivable that one of these linked variants may also have a role in predisposing to ATP.

Furthermore, the data shows an association of the *ELN* rs2071307 variants with Achilles tendon rupture in the UK cohort. This variant was also found to be associated with the disruption of the extracellular matrix of other medical conditions such as intracranial aneurysms (Yang et al. 2013), (Saracini et al. 2012) and abdominal aortic aneurysms (Saracini et al. 2012). ELN is known to show little turnover and shows remarkable durability following enormous stretch and recoil cycles. Therefore it could be suggested that the non-synonymous polymorphism rs2071307, known to cause a change of amino acid from hydrophobic glycine to hydrophilic serine (www.snpper.chip.org), could alter the architecture of elastic fibres rendering them more prone to failure (He et al. 2012). He et al. (2012) report an alteration of the mechanical properties of ELN following the glycine to serine substitution. Specifically, they suggest that such amino acid substitution could reduce the entropy of the protein structure upon extension.

Furthermore, as indicated in figure 4.3.3 the substitution of the G allele with the A allele resulted in the generation of a different secondary RNA structure with alternative molecular properties. The difference in free energy change (ΔG°) suggests that there is a greater force driving the spontaneous forward reaction of the A allele RNA than that of the G allele RNA (Alberts et al. 2002). These thermodynamic properties could explain an over-expression of ELN mRNA as observed in post-injury Achilles tendons of mice (Guerquin et al. 2013).

It is important to note however that the A allele is present in Caucasian populations in a frequency as high as 40%. The elevated abundance of this allele could be suggestive of a positive role it plays in improving the performance of elastin fibres (He et al. 2012). This could explain the reported age genotype interaction where individuals in the AUS population carrying the AA genotype at the *ELN* rs2071307 locus developed an Achilles tendinopathy at a significantly later age than those with the GG and GA genotype.

The results obtained from the investigation of the role of two genetic variants within the 3'UTR of the *COL5A1* gene partly agree with previous findings (Mokone et al. 2006, September et al. 2009, Abrahams et al. 2013). Unlike previous studies where the TT genotype within the *COL5A1* rs12722 variant was associated with ATP in the SA cohort (Mokone et al. 2006), or Achilles tendonipathy in both AUS and SA (September et al. 2009), the TT genotype was associated with Achilles tendon rupture, strictly, in the UK male participants. Furthermore, the data suggest that individuals carrying the T allele are twice at risk of sustaining a rupture than those carrying the C allele. In addition to that, the results of the UK cohort found a significant association of the insertion (I) allele with an increased risk of ATP among male participants. This variant however, was previously reported to be associated with Achilles tendinopathy in the combined AUS and SA cohort (Abrahams et al. 2013). The findings of functional analysis studies suggest that the presence of the T allele at the rs12722 locus and the insertion allele at the rs71746744 locus significantly increase the stability of the transcribed mRNA (Laguette et al. 2011). It has not been confirmed however

whether the T and I alleles play a role in increasing the risk of injury by increasing the synthesis of COL5A1 (Laguette et al. 2011) which could lead to the production of smaller and densely packed collagen fibrils (Ribbans, Collins 2013) as demonstrated in figure 4.1.1. Interestingly, a recent study showed no association of the *COL5A1* rs12722 variant with the volume of the patellar tendon (Foster et al. 2014). One might think that these findings contradict the suggestion put forward by Collins and Posthumus (2011) about fibril arrangement and density, but no findings have described a relationship between tendon properties and risk of damage (Foster et al. 2014). Furthermore, the *COL5A1* rs12722 T allele was also associated with other musculoskeletal soft tissue phenotypes such as: increased sit and reach range of motion (Brown et al. 2011), and increased risk of ACL rupture among females (Posthumus et al. 2009).

The *GDF5* rs143383 variant was not found to be associated with ATP in the UK cohort. This finding agrees with a previously published study where the rs143383 variant was not associated with ATP in a Caucasian South African population (Posthumus et al. 2010). The rs143383 variant is functional and exerts a reduction in the expression of *GDF5* in cartilage when the T allele is carried, relative to the C allele (Egli et al. 2009). The reduction in expression of *GDF5* is believed to increase the risk of osteoarthritis (Reynard et al. 2011). In fact, females carrying the CC genotype are 28% less likely of developing osteoarthritis when compared to females with the TT genotype (Vaes et al. 2009). Posthumus et al. (2010) report an increased risk of acquiring ATP when carrying the TT genotype. In fact, the observed reduction in the expression of *GDF5* caused by the T allele at the rs143383 locus, reported by Egli et al. (2009), and the reduction in the concentration of collagen fibrils in tendons of GDF5-deficient mice, reported by Mikic et al. (2001), could explain the elevated risk of developing an ATP in carriers of the TT genotype. However, the results obtained from the UK population did not match those from the AUS+SA population. It is important to note that the transcriptional effect of the rs143383 variant is influenced by a neighbouring C/T SNP, rs143384, whose T allele also contributes towards a reduced expression of *GDF5*

(Dodd, Syddall & Loughlin 2013). Therefore it is important to conduct genetic association studies on the rs143384 variant and assess whether genotype combinations between both variants (rs143383 and rs143384) could contribute towards an increase in the risks of developing ATP.

Finally, it is recognised that data from genetic association studies should be replicated in different cohorts. This study was conducted on Caucasian cohorts and it is therefore imperative to replicate it in cohorts of other ethnicities. A limitation of the present study is the relatively small sample size. However, the relatively elevated OR reported suggest a large effect. Furthermore, since the number of cases in the RUP group is very small (n=38) and indicates a reduced study power for the association, it is important to treat all findings related to Achilles tendon rupture with care.

Chapter 5 Polymorphic Variations within Metalloproteinases and Metalloproteinase Inhibitors and the Risk of Achilles Tendon Pathology

Some of the findings in this chapter have been published in the Journal of Science and Medicine in Sports

El Khoury, L., Posthumus, M., Collins, M., Handley, C.J., Cook, J. & Raleigh, S.M. 2013, "Polymorphic variation within the *ADAMTS2*, *ADAMTS14*, *ADAMTS5*, *ADAM12* and *TIMP2* genes and the risk of Achilles tendon pathology: A genetic association study", *Journal of science and medicine in sport,* vol. 16, no. 6, pp. 493-498.

5.1 INTRODUCTION

Achilles tendon pathology (ATP), consisting of chronic Achilles tendinopathy and acute Achilles tendon ruptures, typically occurs as a result of acute or repetitive mechanical loading during occupational and sporting activities (Kvist 1994, Rees, Dent & Caterson 2009). Although the exact underlying aetiology of ATP remains to be defined, a number of intrinsic (including genetic) and extrinsic risk factors have been identified (Riley 2004). Previous studies have identified variants within the *TNC* (Mokone et al. 2005)*, COL5A1* (Mokone et al. 2006, September et al. 2009)*, MMP3* (Raleigh et al. 2009), *GDF5* (Posthumus et al. 2010) and *CASP8* (Nell et al. 2012) genes which independently associate with risk of ATP. Since ATP is a multifactorial condition, its development is likely to have a complex genetic component and additional candidate genes should be investigated (Collins, Raleigh 2009).

All genes, which have been shown to associate with Achilles tendinopathy encode for proteins that are either structural and/or regulatory in function (Collins, Raleigh 2009). Therefore additional genes encoding for extracellular matrix (ECM) specific proteinases and their inhibitors are suitable candidates for further investigation. In addition to the MMP (matrix metalloproteinase) family of proteins, the ADAM (a disintegrin and metalloproteinase), ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs), and the TIMP (tissue inhibitor metalloproteinase) family of proteins are all involved in the regulation of tendon and other connective tissues (Jones, Riley 2005).

Metalloproteinases (MMPs, ADAMs, and ADAMTSs) are enzymes with a catalytic mechanism involving a metal, predominantly zinc. Metalloproteinases are structurally and evolutionarily related. Therefore they exhibit common domain designs that enable them to degrade various components of the ECM (figure 5.1.1).

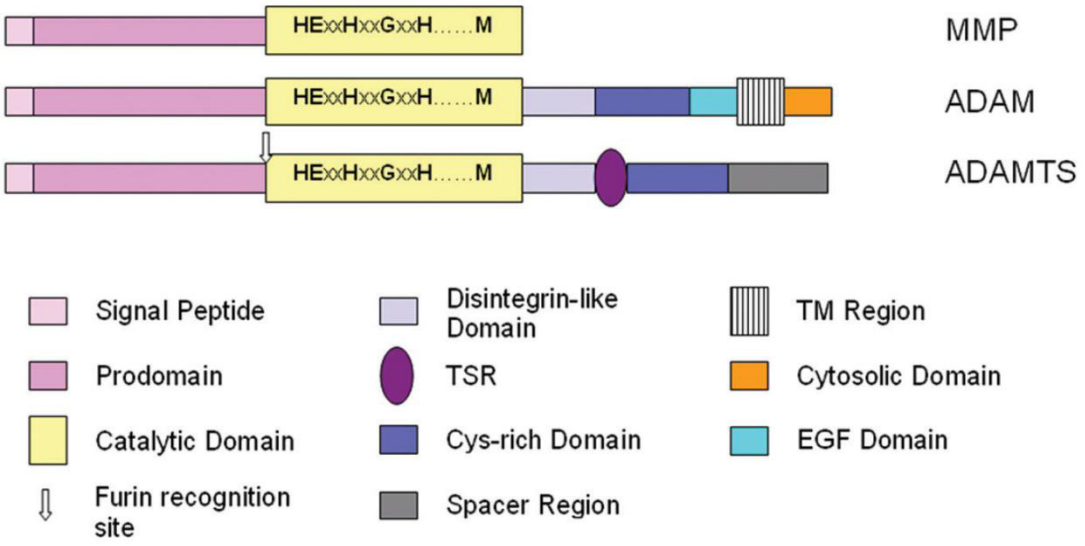


Figure 5.1.1 A schematic representation of the domain organisation of MMP, ADAM, and ADAMTS. Figure obtained from (Jones, Riley 2005) with permission of the publisher.

The MMP family made of 23 different matrix proteins, plays a major role in the proteolytic processing of the ECM (Flannery 2006). In specific, MMP3 is known to degrade various structural components such as: types II, IV, IX, X collagens, as well as fibronectin, decorin and aggrecan (Somerville, Oblander & Apte 2003, Birkedal-Hansen et al. 1993). Interestingly, a decrease in the expression of *MMP3* has been reported in damaged Achilles tendons (Ireland et al. 2001). Furthermore, the *MMP3* rs679620 was previously associated with ATP in a Caucasian South African cohort (Raleigh et al. 2009).

The ADAM family of trans-membrane proteins belong to the super-family of zinc proteases (Seals, Courtneidge 2003). There are 19 different ADAM genes, which are involved in various functions such as: cell-cell interaction, fertilization, and muscle development (Iba et al. 2000, Evans 2001, Eto et al. 2000). The mRNA expression of *ADAM12* was shown to be significantly elevated within painful (Achilles tendinopathy) compared to normal tendons (Jones et al. 2006). Interestingly, the *ADAM12* rs3740199 variant has recently been shown to co-segregate with osteoarthritis (OA) in a Caucasian cohort (Kerna et al. 2009).

The ADAMTS proteinases are a group of secreted enzymes known to influence development, angiogenesis, and coagulation, as well as maintaining homeostasis in the ECM (Kevorkian et al. 2004). *ADAMTS* genes can be divided into four categories according to their function: hyalectanases, procollagen N-propeptidases, von Willebrand factor cleaving protease, and other ADAMTS which as yet have an unclassified role (Jones, Riley 2005). Procollagen N-propeptidases plays an important regulatory role by cleaving the N-terminal propeptides of types I, type II, and type III procollagens to yield mature collagen molecules (Fernandes et al. 2001). A major tendon procollagen N-propeptidase, ADAMTS2, is reported to be highly expressed in pathologic compared with healthy tendons (Jones et al. 2006). Also, mutations within the *ADAMTS2* gene have shown to be associated with other soft tissue pathologies such as Ehlers-Danlos syndrome VIIC (Colige et al. 1999). Furthermore, ADAMTS14 is a homologue of ADAMTS2 (Colige et al. 2002) and a major type I procollagen N-propeptidase in tendons (Jones, Riley 2005, Jones et al. 2006). The *ADAMTS14* rs4747096 variant, a putative deleterious non-synonymous SNP has been associated with osteoarthritis (Rodriguez-Lopez et al. 2009). The *ADAMTS2* rs1054480, also a non-synonymous SNP, is predicted to be deleterious (Ng, Henikoff 2003).

Hyalectanases are known to cleave hyalectan as well as aggrecan, versican, fibromudilin, decorin, and COMP (cartilage oligomatrix protein) in order to maintain homeostasis within the ECM (Jones, Riley 2005). Previous studies reported a decrease in the expression level of ADAMTS5, a hyalectanase, in Achilles tendinopanthy samples compared to healthy controls obtained from cadavers (Jones et al. 2006). ADAMTS5 has been implicated in aggrecan degradation in OA (Malfait et al. 2002). Although genetic variations in the *ADAMTS5* gene have not been associated with osteoarthritis, a putative deleterious non-synonymous variant, *ADAMTS5* rs226794, showed a tendency to be over-represented within a European Caucasian cohort with osteoarthritis (Rodriguez-Lopez et al. 2008).

The *TIMP* (tissue inhibitor metalloproteinase) genes encode proteins that inhibit the action of MMPs, ADAMs and ADAMTSs (Seals, Courtneidge 2003, Nagase, Visse & Murphy 2006). Among the TIMP proteins, TIMP2 is responsible for inhibiting ADAMTS1 (Kevorkian et al. 2004) as well as the collagenolytic activity of type IV procollagenase (Stetler-Stevenson, Krutzsch & Liotta 1989). In mice, TIMP2 is required for the successful activation of pro-MMP2 (Wang, Juttermann & Soloway 2000). Elevated expression of *TIMP2* RNA was found in ruptured Achilles tendon samples compared to healthy controls (Karousou et al. 2008). In contrast an earlier study reported lower levels of *TIMP2* RNA in ruptured Achilles tendon relative to corresponding healthy tissue (Jones et al. 2006). In addition to transcript levels, serum TIMP2 protein has been documented to remain high even as long as three years post Achilles tendon injury (Pasternak et al. 2010). The *TIMP2* rs4789932 variant is a promoter SNP that has been previously investigated for its role in the development of different cancers. Although it was not significantly associated with breast cancer, there was a tendency towards association (Peterson et al. 2009).

According to the previous description of the role played by metalloproteases and their inhibitors in the Achilles tendon, the aim of this study was to determine whether the *ADAMTS2* rs1054480, *ADAMTS5* rs226794, *ADAMTS14 rs4747096*, *ADAM12* rs3740199, and *TIMP2* rs4789932 variants were associated with ATP in Caucasians. Furthermore, this study aimed at determining whether there was any association between *MMP3 rs679620* and ATP in the British cohort.

5.2 MATERIALS AND METHODS

5.2.1 Participants

One hundred and seventy-three (59 Australian (AUS) and 114 South African (SA)) self-reported Caucasian participants diagnosed with Achilles tendon pathology (ATP) and 248 (145 AUS and 96 SA) asymptomatic Caucasian controls (CON) were recruited for this case-control genetic association study as previously described (Mokone et al. 2006, September et al. 2009). Furthermore, a British cohort (UK) made of 121 ATP cases and 131 physically active asymptomatic controls individuals were recruited as described in Chapter 3. The diagnosis of all participants was performed using specific clinical criteria based on the descriptions of (Kader et al. 2002, Paavola et al. 2002, Schepsis, Jones & Haas 2002) and adapted by (Mokone et al. 2006, September et al. 2009) as described in Chapter 3.

5.2.2 DNA Collection and Genotyping

For the Australian cohort, DNA was extracted from whole blood using the Flexigene DNA Extraction Kit (Qiagen P/L, Valencia, California, USA) as per the manufacturer's recommendations. DNA for the South African cohort was extracted from whole blood using the procedure described by Lahiri and Nurnberg (1991) and modified by Mokone et al. (2005, 2006). Furthermore, DNA from the UK cohort was extracted from saliva collected in OG-500 (DNA Genotek, Inc., Ottawa, Canada) tubes and extracted as per the manufacturer's recommendations described in Chapter 3.

All subjects were genotyped for the *TIMP2* rs4789932 (figure 5.2.1) gene variant. The AUS and SA groups were genotyped for the *ADAMTS2* rs1054480 (figure 5.2.2), *ADAMTS14* rs4747096 (figure 5.2.3) and *ADAM12* rs3740199 (figure 5.2.4) and *ADAMTS5* rs226794 (figure 5.2.5) variants while the *MMP3* rs679620 (figure 5.2.6) was used to genotype the UK group. These gene variants were carefully selected on the basis that they should meet at

least one of the selection criteria: (1) they are known to be located within an exon with a deleterious or non-synonymous attribute; or (2) are located in either an intron or an exon and have been found to be associated with a medical condition, preferably a musculoskeletal soft tissue pathology. All gene variants were genotyped using custom-designed TaqMan® Assays (Applied Biosystems, Foster City, CA, USA). Each PCR reaction contained probes and primers in a PCR mastermix containing AmpliTaq DNA Polymerase Gold (Applied Biosystems, Foster City, CA, USA) in a reaction volume of 6 µL. PCR was performed on an Applied Biosystems StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Genotypes were automatically called using Applied Biosystems StepOnePlus™ real-time PCR software Version 2.1 (Applied Biosystems, Foster City, CA, USA). Rox was used as a passive reference and each PCR run included both positive (known genotype) and negative (water) controls for quality control.

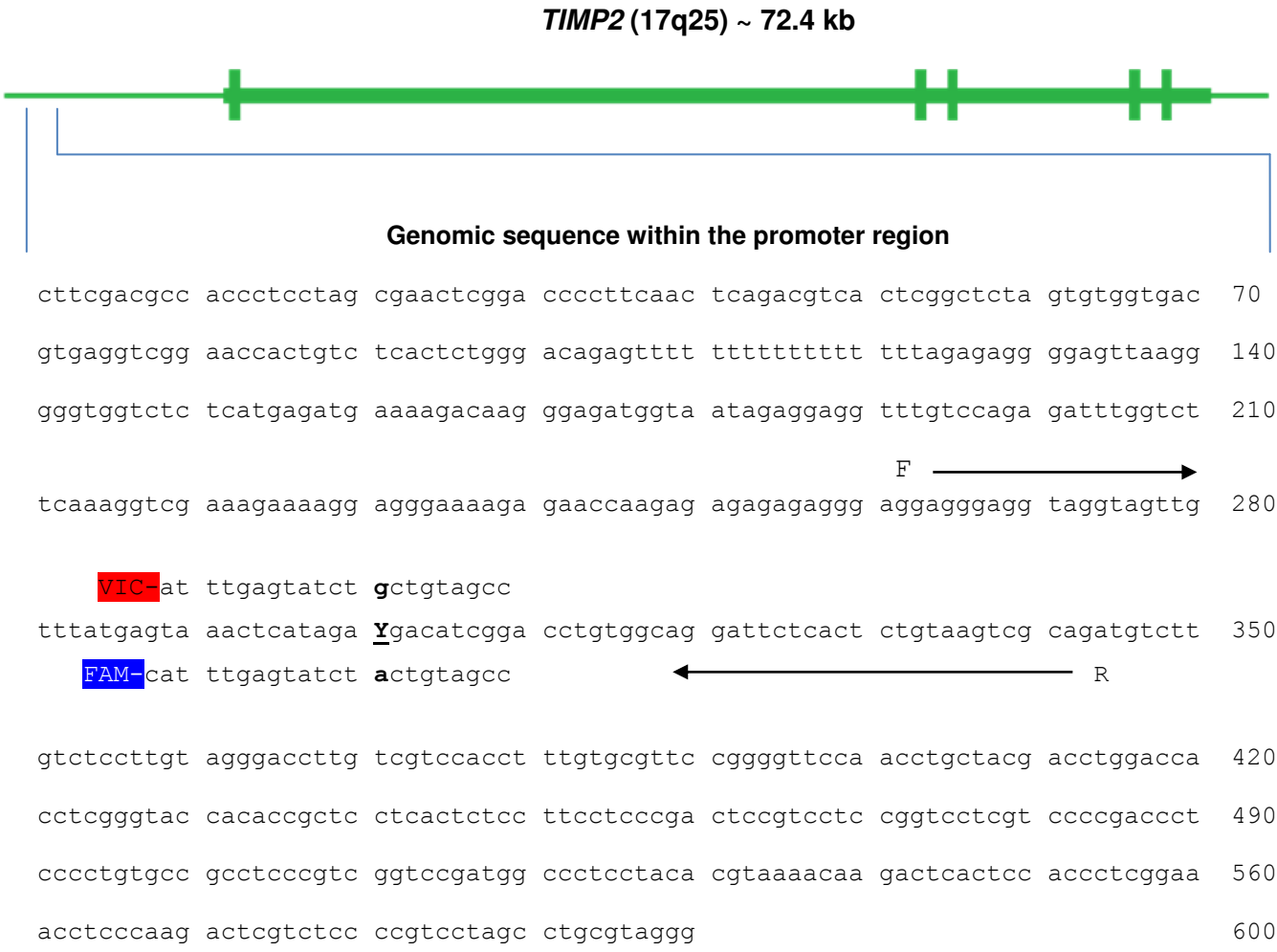
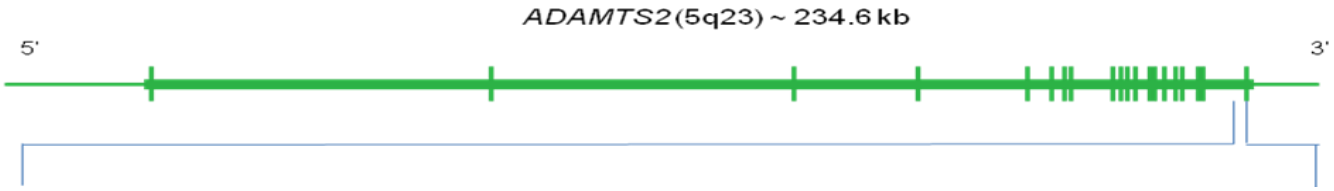


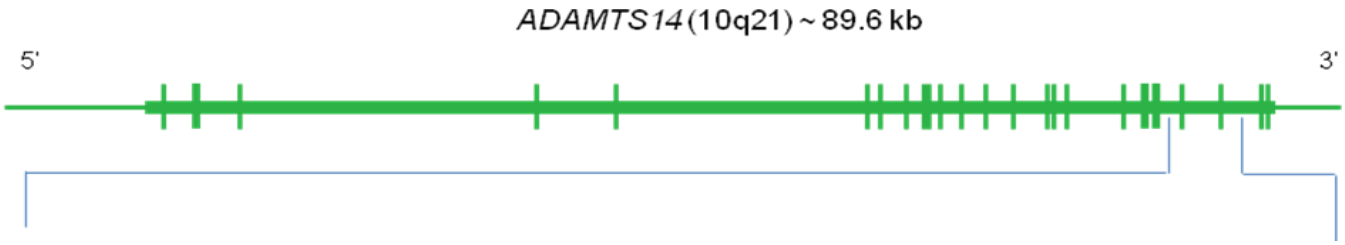
Figure 5.2.1 a schematic representation of exons (vertical lines), and introns (thick horizontal line) within the *TIMP2* gene as well as the 5' and 3' UTR (thin horizontal line). The genomic sequence consists of the 300 bp residing on both ends of the rs4789932 variant. The letter "Y" at position 301 is the IUPAC code for a C/T SNP. The forward and reverse primers are indicated with the arrows preceded by the letters "F" and "R" respectively. The sequences of the VIC and FAM probes are highlighted in red and blue respectively. Adapted from the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/projects/SNP/ .



Genomic sequence within intron 21 and exon 22

```
acccttcgtg tgcacgattg gatcaatggt gtacgagtac ggatagaaag aatgcagtaa ggggagaaa  70

caaaagagtt ttggaaaacc ttagtcctta aaactgaagt agaggtactg tgtcctaaac atagggtac  140

cttatagaga tattcgttct ttttttacga tccctacgat agaaaggtaa aataATCTTG AAGGCTCGT  210

                                F ------------------------>

AGAGAAAGAA GGCGTAGAGT AGTTACTCGA GAACCTAAGA AACCAAAGAC CAGAAAAGTA CCCCGAGCC  280

        VIC-TTAGGTTGG GTGGCTGG
AGCAGCTCCC TAATCCAACC YACCGACCTG AAGTAGAAGG TCCGGTACCT AAAACATCCC AGTAGATGC  350
       FAM-ATTAGGTTGG ATGGCTGG <------------------------ R

CGTAACCAAA GACCCACTAG GAGACACCGT AACCACGACC TCCGTAACTC TCCCTGGAGG CCCCCCACG  420

AACCACTACC GGCGTGGAGG TACCGATGTC ACCCGTGACC CTCCCATCCG TACTTGTGCA TTACAGCAA  490

CACGAAGGGT CCGCCACCGA GATAGGACGG GAGGTGCAAC CACTCCAACA ACATGTCCAA GTCCTGAAC  560

GTCGTGTCGA ACAACATCGG ACCCTACCTC GTTATCGCC                                   600
```

Figure 5.2.2 a schematic representation of exons (vertical lines), and introns (thick horizontal line) within the *ADAMTS2* gene as well as the 5' and 3' UTR (thin horizontal line). The genomic sequence consists of the 300 bp residing on both ends of the rs1054480 variant. The sequences in small letters represent the intronic regions, and the sequences in capital letters are the exonic regions. The letter "Y" at position 301 is the IUPAC code for a C/T SNP. The forward and reverse primers are indicated with the arrows preceded by the letters "F" and "R" respectively. The sequences of the VIC and FAM probes are highlighted in red and blue respectively. Adapted from the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/projects/SNP/ .

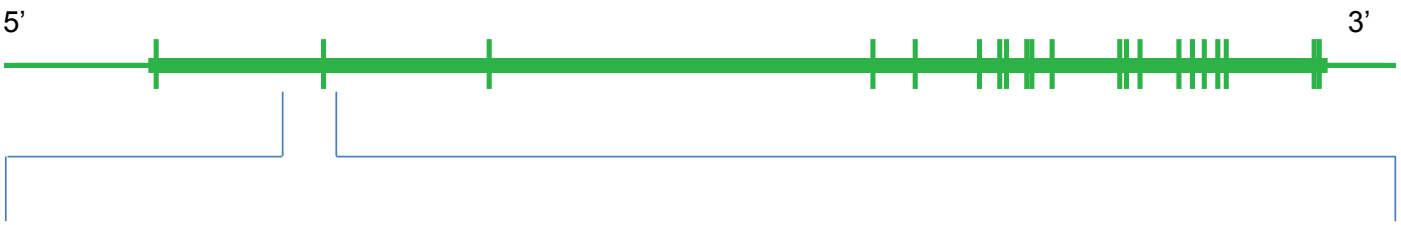


Genomic sequence within intron 19, exon 20, intron 20, exon 21, and intron 21

```
ccacggcagT GCTCTGCCAC CTGTGGAGAG GGCATCCAGC AGCGGCAGGT GGTGTGCAGG CCAACGCCA  70

ACAGCCTCGG GCATTGCGAG GGGGATAGGC CAGACACTGT CCAGGTCTGC AGCCTGCCCG CTGTGGAGg  140

tgagccagag gggatgggga ggccaggtcc agtcccttgg gccaaagtcc caaatactag attgcttct  210

                                                    F
tcctgctcac agGAAATCAC CAGAACTCCA CGGTGAGGGC CGATGTCTGG GAACTTGGGA GCCAGAGGG  280

           VIC-TTAGAC TTGGGGATGT A
GCAGTGGGTG CCACAATCTG RACCCCTACA TCCCATTAAC AAGATATCAT CAAgtaagta agtctatgg  350
           FAM-TTAGAC CTGGGGATG

accctaccct gctccccaga cctttcagtg gccctggtgt tgcagcatgg gccactcagt gagcaaacc  420
                          R
cagcttgtag ctgtctgtgt gaccttcacc tccctgagcc tcagatacct cagttatgaa tggggagga  490

ggctctactg gtccatgagc cgttacctgc aattccaaaa tgtttatttc taagtttggt caaacttac  560

ctggcagcaa aatctgacct gaactaatga gatcatttat                                  600
```

Figure 5.2.3 a schematic representation of exons (vertical lines), and introns (thick horizontal line) within the *ADAMTS14* gene as well as the 5' and 3' UTR (thin horizontal line). The genomic sequence consists of the 300 bp residing on both ends of the rs4747096 variant. The sequences in small letters represent the intronic regions, and the sequences in capital letters are the exonic regions. The letter "R" at position 301 is the IUPAC code for a G/A SNP. The forward and reverse primers are indicated with the arrows preceded by the letters "F" and "R" respectively. The sequences of the VIC and FAM probes are highlighted in red and blue respectively. Adapted from the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/projects/SNP/ .

***ADAM12* (10q26) ~ 376.2 kb**



Genomic sequence within intron 1, exon 2, and intron 2

```
atctgggact gtaacccctg gtttttgttc aaaaactaat tttcagtaga tgcaaataat ttggatccc  70

tcatcagcac tgtcacatca aaattggttt gcatattttt ggcttaagaa taaagcttcc ttctctaag  140

gatagagtta gcattaaagt gagtagagac aacccttgaa tgagagaata ccaaagcagc aatgtgatt  210

                                                          F ---------->
taaaagcttc ggcagtctca aagctgagaa aaggagaGGA ACTCACCTTG GAGTCGAAGC CTTCACTGG  280

F ---------->
          VIC-GTGAGG GTTGTCTCCG T
GATCCAGAGG TCCCCACTCC SAACAGAGGC ACTGACAACT TCATCAGCTC TTCCTTGGTT CATAAgctc  350
          FAM-GTGAGG CTTGTCTCCG T <------------------------------------- R

acccctgaat caaaaggaaa acagccctat atttcactct agtcaggaag agtttggcat agaagctta  420

ttacataaat gacacagtga aaatatttgg aagttaaata agcccaggga aacactagaa ccattaagt  490

catgaaaagt gttaaaactg ctgaattctc tgtcaatcta accatgaaaa ttttgtaact aaagaaaac  560

aaaagttaat taatttgttc cttcaagacc tgtaacatgt
```

Figure 5.2.4 a schematic representation of exons (vertical lines), and introns (thick horizontal line) within the *ADAM12* gene as well as the 5' and 3' UTR (thin horizontal line). The genomic sequence consists of the 300 bp residing on both ends of the rs3740199 variant. The sequences in small letters represent the intronic regions, and the sequences in capital letters are the exonic regions. The letter "S" at position 301 is the IUPAC code for a G/C SNP. The forward and reverse primers are indicated with the arrows preceded by the letters "F" and "R" respectively. The sequences of the VIC and FAM probes are highlighted in red and blue respectively. Adapted from the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/projects/SNP/ .

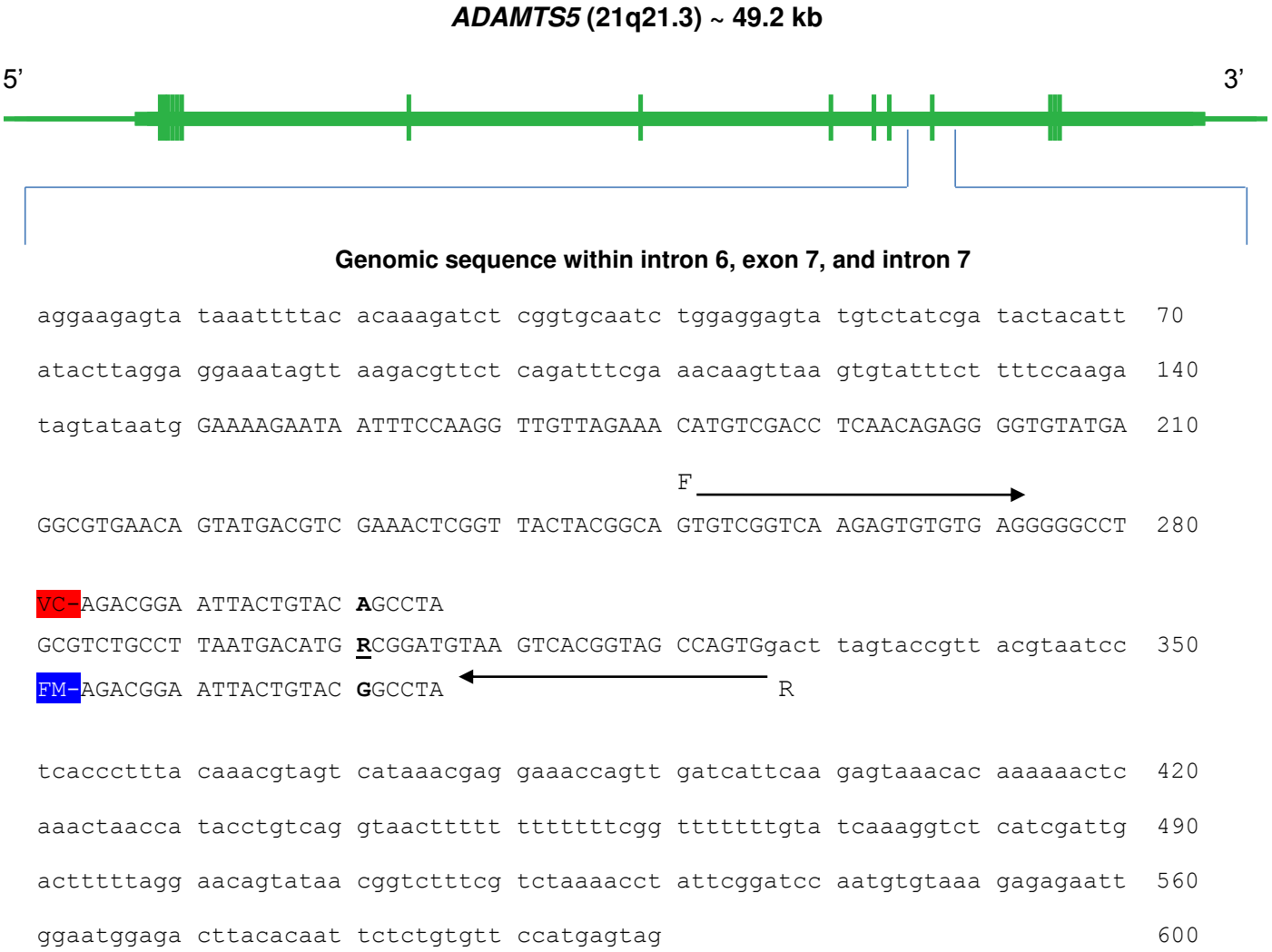


Figure 5.2.5 a schematic representation of exons (vertical lines), and introns (thick horizontal line) within the *ADAMTS5* gene as well as the 5' and 3' UTR (thin horizontal line). The genomic sequence consists of the 300 bp residing on both ends of the rs226794 variant. The sequences in small letters represent the intronic regions, and the sequences in capital letters are the exonic regions. The letter "R" at position 301 is the IUPAC code for a G/A SNP. The forward and reverse primers are indicated with the arrows preceded by the letters "F" and "R" respectively. The sequences of the VIC and FAM probes are highlighted in red and blue respectively. Adapted from the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/projects/SNP/ .

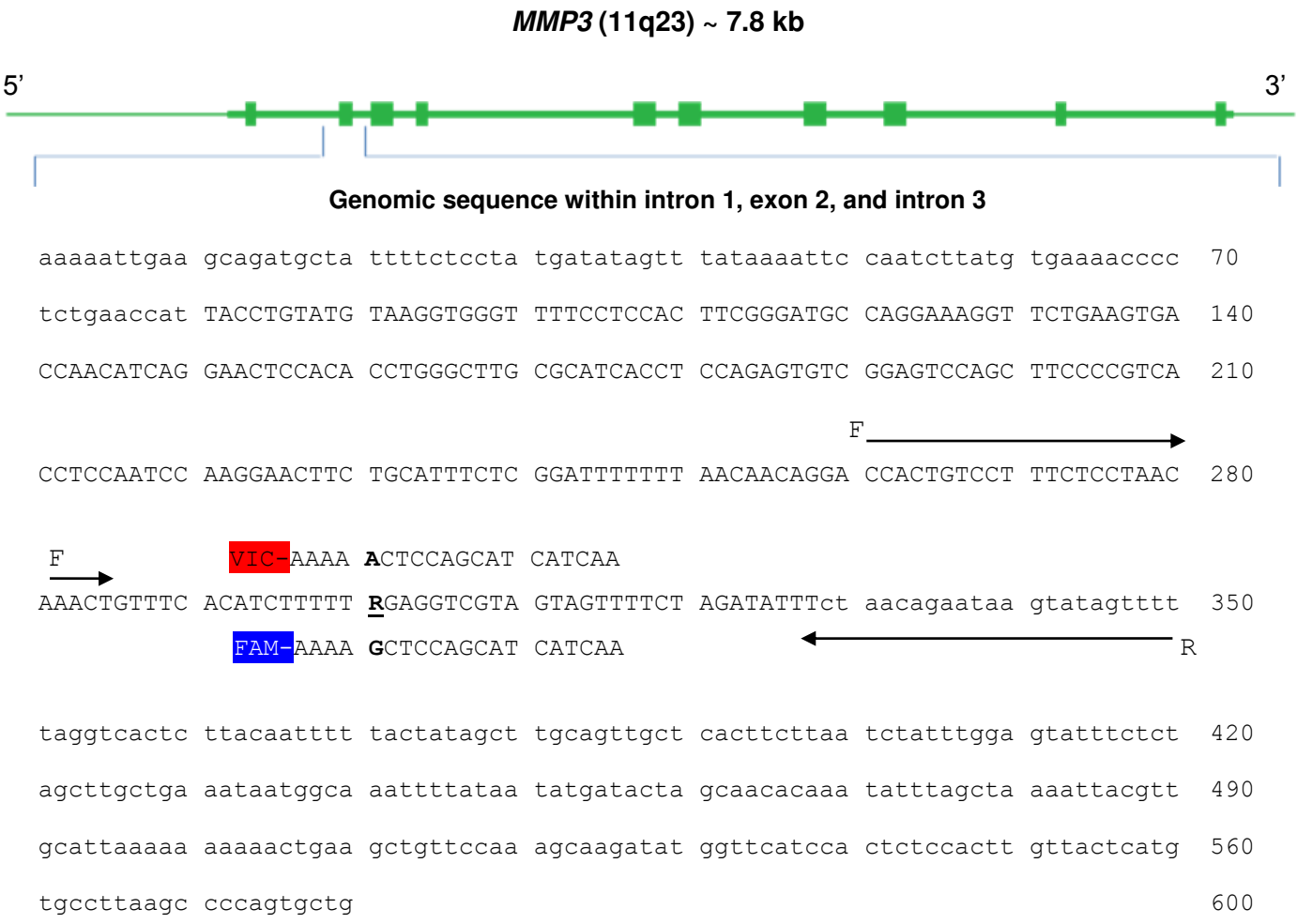


Figure 5.2.6 a schematic representation of exons (vertical lines), and introns (thick horizontal line) within the *MMP3* gene as well as the 5' and 3' UTR (thin horizontal line). The genomic sequence consists of the 300 bp residing on both ends of the rs679620 variant. The sequences in small letters represent the intronic regions, and the sequences in capital letters are the exonic regions. The letter "R" at position 301 is the IUPAC code for a G/A SNP. The forward and reverse primers are indicated with the arrows preceded by the letters "F" and "R" respectively. The sequences of the VIC and FAM probes are highlighted in red and blue respectively. Adapted from the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/projects/SNP/ .

5.2.3 Statistical Analyses

Data were analysed using SPSS Version 20 (SPSS Science Inc, Chicago, Ill, USA) and Graphpad InStat Version 5 (Graphpad Software, San Diego, California, USA) statistical programs. Sample sizes for this study were based on those calculated in Chapter 3 as well as those previously published by Mokone et al. (2005, 2006). A one-way analysis of variance was used to determine significant differences between the characteristics of the ATP and CON groups within all three cohorts. A chi-squared (χ^2) analysis or Fisher's exact test was used to analyse differences in the genotype and allele frequencies, as well as other categorical data between the groups. The significance was accepted when $p<0.05$. Hardy-Weinberg equilibrium was established using Michael H. Court's (2005–2008) online calculator (www.tufts.edu).

5.3 RESULTS

5.3.1 Participant Characteristics

The SA CON, AUS CON, SA ATP and AUS ATP groups were similarly matched for age and country of birth (table 5.3.1). There was however significantly more females in the AUS CON group ($p<0.001$) when compared to the other three groups. When co-varied for sex, the four groups were similarly matched for height. The AUS ATP and SA ATP groups were recruited on average 8.8 ± 10.0 (58) and 8.0 ± 9.0 (107) years, respectively, after their initial injury. Even when co-varied for sex and age of recruitment both the AUS and SA TEN groups were significantly heavier ($p<0.001$) with larger BMIs ($p<0.016$) than the AUS and SA CON groups (table 5.3.1). Except for the AUS CON group, which had a significantly higher BMI than the SA CON group ($p=0.006$), the TEN and CON groups were matched for BMI and weight.

Furthermore, the UK CON and ATP groups were similarly matched for gender, weight, and BMI. However, participants within the ATP group were significantly smaller in height ($p=0.049$) at the time of recruitment and older ($p=0.013$) at the time of injury when compared to the mean height and age of the CON group at the time of recruitment (table 5.3.2).

Interestingly, participants in the (AUS+SA) ATP group with a *ADAMTS14* GG genotype (55.0 ± 7.2, n=6) were significantly ($p=0.024$) older at their initial Achilles tendon injury in comparison with those with either an AA (39.6 ± 14.1 years, n=114) or AG (39.4 ± 11.7 years, n=37) genotype (figure 5.3.1 B). There were no significant differences in the average ages of the three genotype groups in the CON groups (AA: 37.4 ± 11.1 years, n=163; AG: 39.2 ± 11.5 years, n=51; GG: 41.5 ± 11.7 years, n=6; $p=0.437$) (figure 5.3.1 A).

Table 5.3.1 General characteristics of the Australian Achilles tendon pathology (AUS ATP) and the South African Achilles tendon pathology (SA ATP) groups, as well as their respective control (AUS CON and SA CON) groups

	AUS CON (n=152)	AUS ATP (n=59)	SA CON (n=96)	SA ATP (n=114)	P-Value
Age (years) [a]	38.5 ± 11.9 (149)	40.3 ± 14.1 (58)	37.1 ± 10.0 (91)	40.2 ± 12.3 (107)	0.266
Gender (% male) [b]	39.7 (151) [c,d]	67.8 (59) [c]	66.3 (95) [d]	73.0 (111)	<0.001 [b]
Height (cm)	171.7 ± 9.3 (150)	173.8 ± 9.5 (57)	175.4 ± 9.7 (93)	175.9 ± 8.7 (103)	0.345 [c]
Weight (kg)	73.3 ± 13.9 (151)	80.4 ± 15.0 (59)	72.0 ± 12.3 (95)	80.8 ± 14.9 (106)	<0.001 [d]
BMI (kg/m^2)	24.8 ± 4.0 (150)	26.6 ± 4.1 (57)	23.3 ± 2.8 (93)	26.0 ± 3.9 (103)	<0.001 [d]
Country of Birth (% Australian)	84.4 (138)	75.9 (58)	n.a.	n.a.	0.163
Country of Birth (% South African)	n.a.	n.a.	75.8 (95)	75.0 (108)	1.000

Values are expressed as mean ± SD or a frequency (%). The total number of participants (n) with non-missing data is in parentheses. The maximum number (n) of participants in each category is also indicated.

[a] age of the ATP groups is the age of initial injury, while of age of the CON groups is the age of recruitment; [b] AUS ATP vs SA CON vs SA ATP, P=0.559; [c] co-varied for sex; [d] co-varied for sex and age of recruitment. Both the ATP groups were significantly heavier (p<0.001) and had larger BMIs (p<0.016) than the CON groups. The AUS CON group also had a significantly higher BMI than the SA CON group (p=0.006). cm, centimetres; kg, kilograms; m, metres; n.a., non-applicable

Table 5.3.2 General biological characteristics of the British Achilles tendon pathology (ATP) group and the asymptomatic control (CON) group, as well as the gender sub-groupings

				Female			Males		
	CON (n=131)	**ATP (n=121)**	**P-value**	**CON (n=47)**	**ATP (n=47)**	**P-value**	**CON (n=81)**	**ATP (n=74)**	**P-Value**
Age (Years)[a]	41.4 ± 11.3 (123)	45.6 ± 14.5 (117)	**0.013**	43.4 ± 10.7 (46)	44.9 ± 14.4 (44)	0.559	40.2 ± 11.5 (77)	45.9 ± 14.7 (73)	**0.008**
Gender (% male)	67.8 (81)	61.1 (74)	0.730						
Height (cm)	175.0 ± 10.4 (122)	172.4 ± 9.6 (118)	**0.049**	165.3 ± 7.2 (46)	163.9 ± 6.5 (45)	0.340	180.9 ± 7.1 (76)	177.9 ± 6.9 (72)	**0.011**
Weight (kg)	80.3 ± 19.5 (123)	77.9 ± 14.8 (84)	0.315	63.7 ± 11.1 (46)	66.4 ± 9.1 (34)	0.260	90.3 ± 16.5 (77)	85.8 ± 12.6 (50)	0.090
BMI (kg/m^2)	25.9 ± 4.5 (122)	26.2 ± 3.7 (84)	0.665	23.3 ± 3.5 (46)	24.8 ± 4.1 (34)	0.076	27.5 ± 4.3 (76)	27.1 ± 3.2 (50)	0.532

Values are expressed as mean ± SD or a frequency (%). The total number of participants (n) with non-missing data is in parentheses. The maximum number (n) of participants in each category is also indicated.

[a] age of the ATP group is the age at the initial injury, while of age of the CON groups is the age of recruitment.

[b] weight of the ATP group is at the time of the initial injury, while the weight of the CON is at the age of recruitment

cm, centimetres; kg, kilograms; m, meters

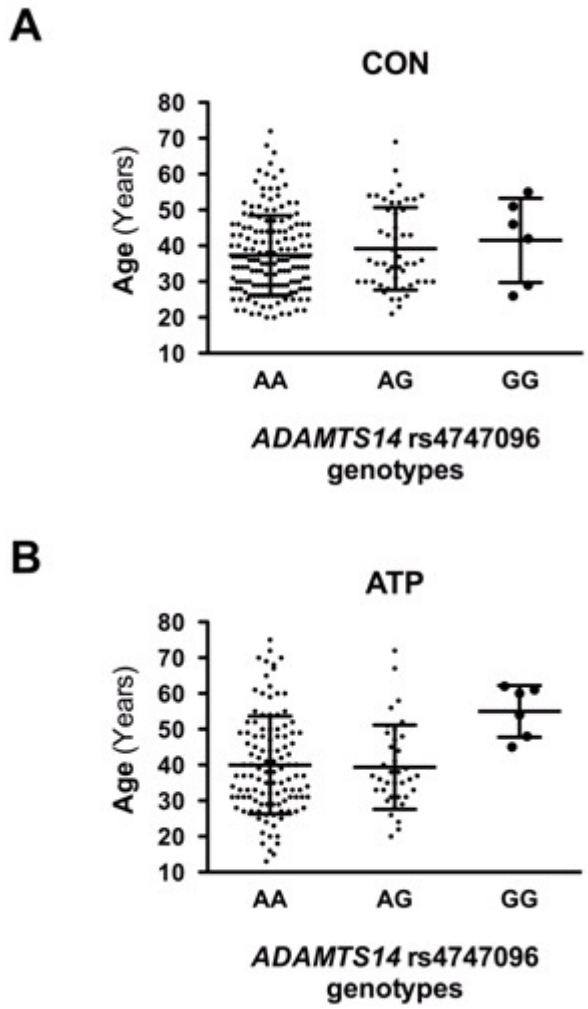


Figure 5.3.1 Column scatter graph showing the standard deviation of genotypes of the *ADAMTS14* rs4747096 SNP in the combined AUS+SA control (CON) and Achilles tendon pathology (ATP). Obtained from El Khoury et al. (2013) with permission of the publisher.

Also, there was a relationship between the *MMP3* rs679620 variant and Achilles tendon width among males within the UK cohort showing signs of tendinosis with severely enlarged tendons (>10 mm). Specifically, males with the GG genotype had significantly (p=0.003) thicker tendons at the mid-portion (15.3 ± 1.6, n=4) when compared to men with either the GA (11.7 ± 0.8, n=7) or AA (12.6 ± 1.7, n=6) genotypes (figure 5.3.2 and figure 5.3.3). Tendon thickness was not significantly correlated with age (r=-0.044; p=0.867), height (r=0.332; p=0.209), weight (r=0.057; p=0.868), and BMI (r=-0.019; p=0.956), and was therefore not co-varied for these variables.

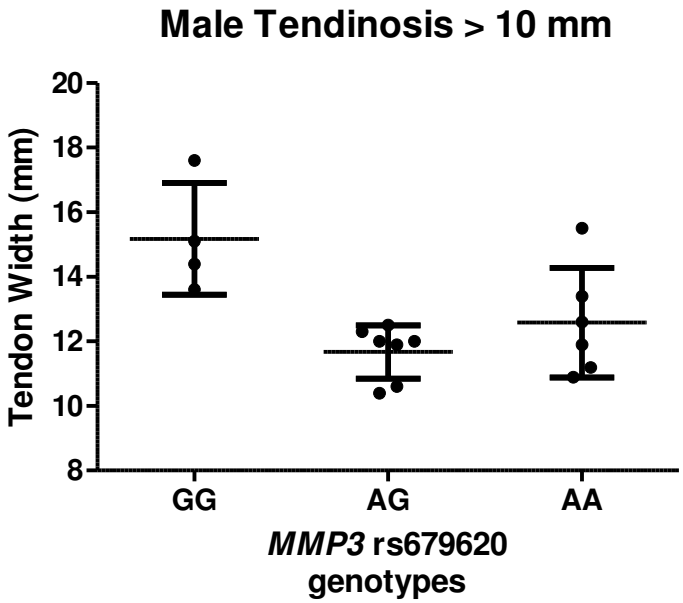


Figure 5.3.2 Column scatter graph showing the mean and standard deviation of tendon widths for genotypes of *MMP3* rs679620 in males suffering from tendinosis and presenting with thick tendons (> 10 mm).

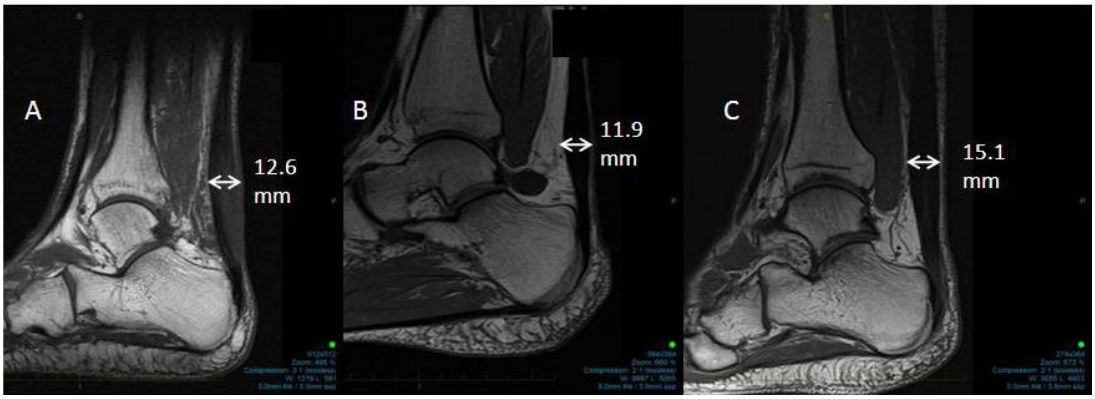


Figure 5.3.3 MRI of the lower leg showing the measurements of enlarged Achilles tendons (>10 mm) with A) the AA genotype B) the AG genotype and C) the GG genotype. The double sided arrows indicate the widest point in the tendon and the measurements are reported in millimetres (mm). Courtesy of Prof William Ribbans, The County Clinic, Northampton, UK

5.3.2 Genotype and Allele Frequencies

The genotype frequency distributions of *ADAMTS2* rs1054480, *ADAMTS5* rs226794, *ADAMTS14* rs4747096, *ADAM12* rs3740199 and *TIMP2* rs4789932 within the AUS and SA CON and ATP groups were not significantly different and therefore the two cohorts were looked at collectively. The genotype frequencies for the independent AUS and SA cohorts can be found in appendix VI.

There were no significant genotype frequency differences for the *ADAMTS2* rs1054480 (p=0.316) *ADAMTS5* rs226794 (p=0.323), *ADAMTS14* rs4747096 (p=0.849), and *ADAM12* rs3740199 (p=0.633) gene variants between the (AUS+SA) CON and ATP groups (table 5.3.3). However, the *TIMP2* rs4789932 genotype frequency was significantly different (p=0.016) between the CON (86 CC, 36%; 100 CT, 42%, 50 TT, 21%) and ATP (46 CC, 27%; 98 CT, 57%, 29 TT, 17%) groups. When combined, the CC genotype was significantly over-represented within the CON group (p=0.042; OR=1.58; 95% CI 1.03 - 2.43) and the CT genotype was significantly over-represented within the ATP group (p=0.004; OR=1.77; 95% CI 1.20 - 2.64) (table 5.3.3). There were no significant differences in allele frequency for any of the investigated variants (table 5.3.3).

Table 5.3.3 The genotype and allele frequency distribution of four selected candidate variants within the combined AUS+SA control (CON) and Achilles tendon pathology (ATP) groups, as well as, the chronic Achilles tendinopathy (TEN) and Achilles rupture (RUP) sub-groups.

	CON	ATP	TEN	RUP
***ADAM12* rs3740199**	n=238	n=172	n=133	n=39
GG	30.7 (73)	29.7 (51)	30.8 (41)	25.6 (10)
GC	47.5 (113)	51.7 (89)	50.4 (67)	56.4 (22)
CC	21.9 (52)	18.6 (32)	18.8 (25)	18.0 (7)
P-Value	0.633		0.767[a]	0.585[b]
C allele	45.6 (217)	44.4 (153)	43.9 (117)	46.1 (36)
P-Value	0.816		0.674[a]	0.926[b]
***ADAMTS2* rs1054480**	n=214	n=160	n=125	n=35
GG	50.5 (108)	53.1 (85)	52.8 (66)	54.3 (19)
GA	38.8 (83)	40.6 (65)	40.8 (51)	40.0 (14)
AA	10.8 (23)	6.3 (10)	6.4(8)	5.7 (2)
P-Value	0.316		0.407[a]	0.652[b]
A allele	30 (129)	26.5 (85)	26.8 (67)	25.7 (18)
P-Value	0.284		0.355[a]	0.452[b]
***ADAMTS5* rs226794**	n=236	n=173	n=134	n=39
GG	79.2 (187)	80.9 (140)	79.9 (107)	84.6 (33)
GA	20.3 (48)	17.3 (30)	17.9 (24)	15.4 (6)
AA	0.4 (1)	1.7 (3)	2.2 (3)	0.0 (0)
P-Value	0.342		0.247[a]	0.437[b, c]
A allele	10.6 (50)	10.4 (36)	11.2 (30)	7.7 (6)
P-Value	0.752		0.800[a]	0.433[b]
***ADAMTS14* rs4747096**	n=228	n=165	n=127	n=38
AA	74.1 (169)	73.3 (121)	73.2 (93)	73.7 (28)
AG	23.3 (53)	23.0 (38)	22.1 (28)	26.3 (10)
GG	2.6 (6)	3.6 (6)	4.7 (6)	0.0 (0)
P-Value	0.849		0.572[a]	0.956[b, c]
G allele	14 (65)	15 (50)	15 (40)	13 (10)
P-Value	0.724		0.591[a]	0.799[b]

Values are expressed as a frequency with the number of participants (n) in parentheses.

[a] CON vs TEN; [b] CON vs RUP; [c] Major homozygote vs heterozygote and minor homozygote

Table 5.3.4 The genotype and allele frequency distribution of the *TIMP2* rs4789932 candidate variant within the combined AUS+SA control (CON) and Achilles tendon pathology (ATP) groups, as well as, the chronic Achilles tendinopathy (TEN) and Achilles rupture (RUP) sub-groups

	CON	ATP	TEN	RUP
***TIMP2* rs4789932**	n=236	n=173	n=134	n=39
CC	36.4 (86)	26.6 (46)	26.9 (36)	25.6 (10)
CT	42.4 (100)	56.7 (98)	56.7 (76)	56.4 (22)
TT	21.2 (50)	16.8 (29)	16.4 (22)	18.0 (7)
P-Value	0.016		0.029[a]	0.249[b]
T allele	42.4 (200)	45.1 (156)	44.8 (120)	46.2 (36)
P-Value	0.439		0.525[a]	0.532[b]

Values are expressed as a frequency with the number of participants (n) in parentheses.

[a] CON vs TEN; [b] CON vs RUP

Interestingly, when investigated in the UK cohort, the *TIMP2* rs4789932 gene variant was also associated with ATP, however strictly among males. The genotype frequencies were significantly different (p=0.043) between the CON (CC, 20.5%; CT, 60.2%; TT, 19.3%) and ATP (CC, 37.8%; CT, 43.2%; TT, 18.9%) groups. Furthermore, the CT genotype was over represented among the CON group (p=0.033; OR=1.99; 95% CI 1.05 – 3.76) whereas the CC genotype was over represented in the ATP group (p=0.016; OR=2.36; 95% CI 1.16 – 5.81). No significant genotypic distribution difference was found for the *TIMP2* rs4789932 variant between the CON and ATP groups within female participants (p=0.700), or in the combined male + female group (p=0.279).

The *MMP3* rs679620 gene variant was not associated with ATP in either the female group (p=0.253), the male group (p=0.735), or the combined female + male UK cohort (p=0.349). Nevertheless, there was a significant (p=0.029) genotype distribution difference between the UK CON (AA, 26.7%; AG, 54.2%; GG, 19.1%) and RUP (AA, 31.4%; AG, 31.4%; GG, 37.1%). Specifically, the GG genotype was significantly over represented among the RUP group (p=0.027; OR=2.51; 95% CI 1.11 – 5.64), whereas the AG genotype was significantly over represented among the CON group (p=0.019; OR=2.6; 95% CI 1.17 – 5.70). Interestingly, the UK RUP group was not in HWE (p=0.029). The remaining investigated groups were all in HWE.

Table 5.3.5 The genotype and allele frequency distribution of *MMP3* rs679620 and *TIMP2* rs4789932 variants within the British control (CON) and Achilles tendon pathology (ATP) groups, as well as the chronic Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP) sub-groups.

					Female				Male			
	CON	**ATP**	**TEN**	**RUP**	**CON**	**ATP**	**TEN**	**RUP**	**CON**	**ATP**	**TEN**	**RUP**
***MMP3* rs679620**	n=131	n=121	n=86	n=35	n=48	n=47	n=35	n=12	n=83	n=74	n=51	n=23
AA	26.7 (35)	29.8 (36)	29.1 (25)	31.4 (11)	25.0 (12)	25.5 (12)	25.7 (9)	25.0 (3)	27.7 (23)	32.4 (24)	31.4 (16)	34.8 (8)
AG	54.2 (71)	45.5 (55)	51.2 (44)	31.4 (11)	60.4 (29)	46.8 (22)	51.4 (18)	33.3 (4)	50.6 (42)	44.6 (33)	51.0 (26)	30.4 (7)
GG	19.1 (25)	24.8 (30)	19.8 (17)	37.1 (13)	14.6 (7)	27.7 (13)	22.9 (8)	41.7 (5)	21.7 (18)	23.0 (17)	17.6 (9)	34.8 (8)
P-Value	0.349[a]		0.902[b]	**0.029[c]**	0.253[a]		0.589[b]	0.113[c]	0.735[a]		0.818[b]	0.207[c]
HWE	0.301	0.329	0.765	**0.029**	0.125	0.663	0.862	0.276	0.886	0.389	0.780	0.061
G allele	46.2 (121)	47.5 (115)	45.3 (78)	52.9 (37)	44.8 (43)	51.1 (48)	48.6 (34)	58.3 (14)	47.0 (78)	45.3 (67)	43.1 (44)	50.0 (23)
P-Value	0.764[a]		0.864[b]	0.320[c]	0.387[a]		0.503[b]	0.235[c]	0.760[a]		0.539[b]	0.717[c]
***TIMP2* rs4789932**	n=131	n=121	n=86	n=35	n=48	n=47	n=35	n=12	n=83	n=74	n=51	n=23
CC	26.7 (35)	34.7 (42)	36.0 (31)	31.4 (11)	37.5 (18)	29.8 (14)	31.4 (11)	25.0 (3)	20.5 (17)	37.8 (28)	39.2 (20)	34.8 (8)
CT	54.2 (71)	44.6 (54)	46.5 (40)	40.0 (14)	43.8 (21)	46.8 (22)	45.7 (16)	50.0 (6)	60.2 (50)	43.2 (32)	47.1 (24)	34.8 (8)
TT	19.1 (25)	20.7 (25)	17.4 (15)	28.6 (10)	18.8 (9)	23.4 (11)	22.9 (8)	25.0 (3)	19.3 (16)	18.9 (14)	13.7 (7)	30.4 (7)
P-Value	0.279[a]		0.339[b]	0.288[c]	0.700[a]		0.820[b]	0.763[c]	**0.043[a]**		0.062[b]	0.094[c]
HWE	0.301	0.325	0.735	0.238	0.519	0.681	0.640	1.000	0.062	0.375	0.962	0.146
T allele	46.2 (121)	43.0 (104)	40.7 (70)	48.6 (34)	40.6 (39)	46.8 (44)	45.7 (32)	50.0 (12)	49.4 (82)	40.5 (60)	37.3 (38)	47.8 (22)
P-Value	0.469[a]		0.260[b]	0.722[c]	0.390[a]		0.513[b]	0.406[c]	0.116[a]		0.052[b]	0.850[c]

Values are expressed as a frequency with the number of participants (n) in parentheses
[a] CON vs ATP; [b] CON vs TEN; [c] CON vs RUP

5.4 DISCUSSION

This study reports for the first time that the *TIMP2* rs4789932 variant was significantly associated with the risk of ATP. The CC genotype of this variant was significantly over-represented within asymptomatic controls, whereas the CT genotype was over-represented in the ATP group when the South African and the Australian population were collectively analysed. In contrast, the CT genotype was over represented in the CON group and the CC genotype in the ATP group among the male UK participants. These findings might appear initially to be perplexing as they are contradictory to each other. However, although the direction of the genetic association found in the UK male cohort is opposite to the association in the AUS+SA cohort it is still true to say that the *TIMP2* rs4789932 variant does in some way modify the risk of ATP. It has recently been noted that such reverse effects in genetic association studies may reveal additional information about a susceptibility locus that was previously unknown. For example minor alterations in allele frequency between populations, differences in interacting loci gene-gene and gene-environment interactions may all contribute to a reverse effect, even in populations of the same ethnicity but separated geographically (Greene et al. 2009).

TIMP2 mRNA and enzyme levels have previously been shown to be elevated in ruptured Achilles tendon samples compared to healthy controls (Karousou et al. 2008). In addition to transcript levels, serum TIMP2 protein has been documented to remain high even as long as three years post Achilles tendon rupture (Pasternak et al. 2010). Within degenerate human Achilles tendons, *TIMP2* mRNA expression has been shown to be reduced (Jones et al. 2006). Similarly, a decrease in *TIMP2* mRNA levels has also been reported in torn rotator cuff tendons compared with healthy cadaveric controls (Lo et al. 2004). The discrepancy in mRNA expression levels in the studies described above might be attributed to the fact that in the Jones et al. (2006) study control specimens were from cadavers while in the Karousou et al. (2008) study control samples were harvested from living individuals undergoing surgical

repair of an Achilles tendon rupture (Karousou et al. 2008). It is known that TIMP2 is an inhibitor of MMP (Karousou et al. 2008) and disruption to the TIMP/MMP balance has been suggested as a plausible determinant of tendon injury (Pasternak et al. 2010). An increase in TIMP2 levels would be expected to limit the activity of MMPs which, in turn, would repress the degradation of the ECM. Such activity might result in tendon stiffness and rigidity. On the other hand, a decrease in TIMP2 levels could result in higher levels of MMPs with a commensurate increase in the degradation of collagen and other structural proteins in the tendon (Pasternak et al. 2010).

At present a precise mechanism of how the rs4789932 variant within the *TIMP2* gene increases the risk of ATP is unknown. However, as the rs4789932 locus resides within the promoter region of the *TIMP2* gene (Peterson et al. 2009), one may consider that this variant alters the binding affinity of transcription factors and would therefore influence the expression of the gene. The rs4789932 variant is located -2803 bp from the starting point of exon 1 on the *TIMP2* gene (www.snpper.chip.org). TIMP2 expression is under complex control with regulatory *cis*-acting elements, including an AP-1, two AP-2s, five SP-1s and three PEA 3 sites located within a region spanning approximately -2600 bp upstream of the gene (Hammani et al. 1996). PEA-3 is known to a member of the Ets transcription factors, capable of activating transcription (Bojović, Hassell 2001). Therefore it can be speculated that a possible interaction between the CT genotype of *TIMP2* rs4789932 and PEA-3 could alter the transcription of the gene. Subsequent exposure of the Achilles tendon to an excessive or prolonged loading force might exacerbate the genetic predisposition by causing localised musculoskeletal tissue maladaptations resulting in ATP. Although this hypothesis is unproven it is consistent with the literature in which gene-environment interactions are thought to play a major role in the development of musculoskeletal soft tissue injuries (Collins, Raleigh 2009, September et al. 2006).

However, further work is required to determine if the rs4789932 variant itself, or a neighbouring variant, is responsible for the risk reported in this study. The rs4789932 variant forms part of a linkage block in Caucasians and is in high linkage disequilibrium with rs9894526 (D'= 1) and rs4789855 (D'= 0.94) (www.ensembl.com). Both these C/T SNPs are located in the promoter region and are 2811 bp and 6767 bp away from the rs4789932 SNP respectively. The *TIMP2* rs9894526 variant has been investigated for its role in the development of periodontitis (Letra et al. 2012b) and nonsyndromic oral cleft (Letra et al. 2012a) but was not found to be associated with either condition. The rs4789855 variant, on the other hand, has not previously been investigated for a role in the development of a disease. Hence it is possible that any of the linked loci might underlie the risk of tendinopathy.

Another finding of this study was the significant association (p=0.029) between the *MMP3* rs679620 variant and Achilles tendon rupture, where the GG genotype was over represented in the RUP group when compared to the CON group in the UK population. In an earlier study the GG genotype at the rs679620 locus was found to be a risk factor for Achilles tendinopathy in Caucasian South Africans (Raleigh et al. 2009). However, in that study no association was found in individuals who had a Achilles tendon rupture. In addition to that, the genotypes for the *MMP3* rs679620 within the UK RUP group were not in HWE (p=0.029) whereas those within the UK CON group did not violate the HWE assumption (p=0.301). Participants in the UK cohort were carefully selected to exclude any possibility of biases caused by assortative mating, or sampling from different ethnic groups. Based on that, it is fair to say that the results of the UK cohort suggest a true association between *MMP3* rs679620 and Achilles tendon rupture (Schaid, Jacobsen 1999). Moreover, it is important to note that the data revealed a preliminary association between the *MMP3* rs679620 variant and the width of Achilles tendons in UK males presenting with signs of tendinosis who had severely enlarged tendons (> 10 mm). Specifically, subjects with the GG genotype appeared to have a thicker Achilles tendon when compared to subjects with either a GA, or AA

genotype. However, it would be prudent to treat this result as a preliminary finding due to the small number of affected males in the dataset with a severely thick Achilles tendon. The G allele of the rs679620 locus was previously believed to increase the levels of *MMP3* expression (Foster et al. 2012). Over-expression of *MMP3* might contribute towards tendon degeneration since MMP3 plays a role in the degradation of ECM substrates such as proteoglycan, decorin, and laminin (Birkedal-Hansen et al. 1993). In healthy tendons, Type III collagen is present in small amounts (Ippolito et al. 1980). However, in response to tendon damage, the production of type III collagen is induced (Maffulli et al. 2000) which could explain the difference in tendon thickness for male participants carrying the GG genotype for the rs679620 locus. As type III collagen is less resistant to tensile force, degenerate tendons with greater amounts of type III collagen are at greater risk of rupture (Maffulli et al. 2000). This could explain the association of the GG genotype of the *MMP3* rs679620 variant with Achilles tendon rupture. Furthermore, there were no significant effects of the *MMP3* rs679620 variant on tendon width in females ($p=0.408$) or when both genders were combined ($p=0.276$).

Furthermore, there was no significant association between the *ADAM12* rs3740199, *ADAMTS2* rs1054480, *ADAMTS5* rs226794 and *ADAMTS14* rs4747096 variants and ATP in the AUS+SA cohort. Indeed, association was not detected when either the SA or AUS cohorts were analysed as separate datasets or when they were combined. Although no relationship was found between the above variants and the risk of ATP; altered mRNA levels of *ADAM12* were detected in a recent study on tendinopathy using array technology (Jelinsky et al. 2008). Altered levels of ADAM12, ADAMTS2 and ADAMTS5 have been detected in tendinopathic tissue compared to controls (Riley 2008). The mechanism that underlies these transcriptional changes is still not fully understood and requires clarification. As previously mentioned, the investigated variants *ADAM12* rs3740199 (Kerna et al. 2009), *ADAMTS5* rs226794 (Rodriguez-Lopez et al. 2008), and *ADAMTS14* rs4747096 (Rodriguez-

Lopez et al. 2009), have all been shown to play a role in osteoarthritis. Therefore, it cannot be excluded that other variants within these genes are associated with ATP.

In summary, this study identified that the rs4789932 SNP within the *TIMP2* gene is a risk factor for ATP and that *MMP3* rs679620 is a risk factor for Achilles tendon rupture. It is also necessary to repeat these studies in cohorts of different countries and ethnicities since it was shown in this study that geographic locations could contribute towards genetic differences in populations. Furthermore, the *MMP3* rs679620 GG genotype might be related to increased tendon width in individuals presenting with tendinosis with severely enlarged tendons. Also the *ADAMTS14* rs4747096 GG genotype might be playing a role in delaying the onset of ATP. In terms of limitations, this study would have greater power if conducted on a larger sample size. This is particularly true for the preliminary work reported with respect to the *MMP3* rs679620 variant and tendon thickness where only 17 males were included. Finally, although this study has shown that the *TIMP2* rs4789932 and the *MMP3* rs679620 are risk variants, the pathological phenotype is only likely to manifest as a result of a complex interaction between other genetic variants and environmental factors.

Chapter 6 DNA Methylation and Human Patellar Tendinopathy

6.1 INTRODUCTION

Patellar tendinopathy (PT), also known as jumper's knee, is a degenerative condition characterised by activity-dependant anterior pain to the knee (Lian, Engebretsen & Bahr 2005). Injuries at the level of the patellar tendon have been reported to be one of the most common knee related injuries (~26%) (Kujala, Kvist & Österman 1986, Bollen 2000). Cases of PT are highly reported among elite volleyball and basketball players, with a prevalence of 45% and 32% respectively (Lian, Engebretsen & Bahr 2005). Degeneration and disorganisation have been reported within tendinopathic patellar tendon tissue (Karlsson et al. 1992, Yu et al. 1995). The degree of disorganisation of the collagen fibres and the presence of necrotic tissue observed in PT was similarly observed in Achilles tendinopathy (Khan, Cook & Maffulli 2005).

Gene variants within the promoter regions of the *TIMP2* and the *GDF5* genes have been investigated in musculoskeletal soft tissue pathologies. The *TIMP2* rs4789932 C/T variant was associated with Achilles tendon pathology, as reported in Chapter 5 and published by (El Khoury et al. 2013) and other soft tissue pathologies such as intracranial aneurisms (Ruigrok et al. 2006). The TIMP2 protein is known to inhibit the activity of metalloproteinases (Nagase, Visse & Murphy 2006); however, the functionality of the rs4789932 variant has not been described yet. Interestingly, there is a discrepancy in describing the level of expression of the *TIMP2* gene in healthy versus diseased Achilles tendons; Jones et al. (2006) reported a decrease in *TIMP2* expression where as Karousou el al (2010) reported an increase in *TIMP2* expression in diseased compared to healthy tendons. Furthermore, a decrease in *TIMP2* mRNA level was detected in ruptured rotator cuff tendons (Lo et al. 2004).

Conversely, the *GDF5* rs143383 C/T variant was found to be associated with a range of musculoskeletal conditions including, Achilles tendon pathology (Posthumus et al. 2010), congenital hip dysplasia (Rouault et al. 2010), hip fractures (Vaes et al. 2009), lumbar disc

degeneration (Williams et al. 2011) and osteoarthritis (Chapman et al. 2008, Valdes et al. 2011). The rs143383 variant was found to be functional where it affects the expression of the *GDF5* gene. The T allele was found to cause a significant decrease in gene expression when compared with the C allele in cartilage and joint tissue (Reynard et al. 2011).

The role played by epigenetic factors in musculoskeletal conditions is a relatively new avenue of research. Different DNA methylation patterns have been revealed between fibroblast-like synoviocytes obtained from rheumatoid arthritis cases and healthy control (Nakano et al. 2013). On the other hand, the role of DNA methylation as a predisposing factor for human tendinopathy was first presented in detail at the 4th meeting for Clinical Sports Medicine in 2010 by S. Raleigh. A review by Collins and Raleigh (2009) highlighted the importance of investigating the potential association of epigenetic mechanisms with the development of musculoskeletal pathologies, but as yet research in this area has not been forthcoming.

DNA methylation is an epigenetic modification where a methyl group binds to a cytosine base converting it to 5-methylcytosine (Robertson 2005). In particular, a cytosine followed by a guanine forming a CpG site, could potentially attract a methyl group and become methylated (Golbabapour, Abdulla & Hajrezaei 2011). Stretches of DNA with high concentrations of CpG sites are called CpG islands (Bock et al. 2007). CpG islands are assessed according to the criteria discussed by Gardiner-Garden and Frommer (1987): 1) the island should be greater than 200 bp, 2) the GC contents should be greater than 50%, and 3) the ratio of observed/expected (obs/exp) CpG sites should be greater than 0.6. The obs/exp ratio is calculated as shown in the formula below (Bock et al. 2007, Gardiner-Garden, Frommer 1987):

(Obs/exp) CpG sites = N x [number of CpG sites / (number of C x number of G)]

N = number of nucleotides in the sequence of interest.

The methylation is promoted by DNA methyltransferase (DNMT) enzymes which are believed to transfer a methyl group from S-adenosylmethionine (SAM) to the cytosine on CpG sites (Golbabapour, Abdulla & Hajrezaei 2011). CpG islands are associated with the promoter region of genes since they can be used to regulate gene activity. In specific, DNA methylation serves to down regulate gene expression (Reynard et al. 2011). The down regulation of genes is caused by different factors: 1) cytosine methylation could prevent the binding of transcription factors; or 2) CpG methylation could indirectly interfere with chromatin/histone interaction (Weber, Schübeler 2007).

In view of the above, the aim of this study was to investigate the role of DNA methylation as an epigenetic factor contributing to the risk of patellar tendinopathy. Primarily, the study explored the DNA methylation profiles within selected regions of the *TIMP2* and *GDF5* promoter and assessed any significant difference in the methylation pattern of these two genes between healthy and PT tissue samples.

6.2 MATERIAL AND METHODS

6.2.1 Participants

Tendon tissue samples were obtained from 10 male participants with healthy patellar tendons (CON) undergoing ACL reconstruction surgeries using a patellar tendon graft. In addition to that, 10 tendinopathic (PT) samples were obtained from male individuals undergoing surgical debridement for recalcitrant overuse patellar tendinopathy as previously described (Parkinson et al. 2010). All obtained samples were harvested from the patellar enthesis and were 15 mm in length.

All participants gave written informed consent. In addition to the approval obtained, for the use of DNA samples by the University of Northampton's School of Health Research Ethics Committee approval was also obtained by the La Trobe University Human Ethics Committee.

6.2.2 DNA Collection and Bisulfite Conversion

The patella tissue samples were homogenised and the DNA was isolated by staff at La Trobe University using the PureLink® Genomic DNA Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations.

The isolated DNA in addition to an unmethylated human DNA control from the EpiTect® PCR Control DNA Set (Qiagen, Hilden, Germany) were bisulfite converted at the University of Northampton using the EpiTect® fast DNA bisulphite Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation and as described in Chapter 3. The quantity of DNA that was bisulfite converted for each sample was ~50 ng.

6.2.3 Loci Selection and Assay Design

As indicated in the introduction of this chapter, single nucleotide polymorphisms (SNPs) within the promoter region of the *TIMP2* (El Khoury et al. 2013) and *GDF5* genes (Posthumus et al. 2010) have been associated with musculoskeletal soft tissue pathologies. Furthermore, CpG islands within the promoter regions of the two stated genes were reported to show a variation in methylation profiles when comparing the cases to the control groups (Reynard et al. 2011, Pulukuri et al. 2007).

The CpG assays used in this study were selected from the pre-designed PyroMark® CpG Assay selection offered by Qiagen (Qiagen, Hilden, Germany). Assay details were obtained using the online GeneGlobe Web Portal from Qiagen (http://www.qiagen.com/geneglobe/). There was only a single assay designed for the CpG islands within the *TIMP2* promoter region (table 6.2.1). On the other hand, the assay chosen for the CpG islands within the promoter region of *GDF5* was selected to cover a wider area of the promoter region and to include a greater number of CpG sites (table 6.2.1).

Table 6.2.1 Pyromark® CpG assays specifications as provided by the GeneGlobe Web Portal

	TIMP2
Gene location	Chr 17q25 (76921897 – 76922051)
GeneGlobe cat. no.	PM00177765
Amplicon length	154
Location of the sequence to analyse	Chromosome 17 76 921 927 – 76 921 960
Number of CpG sites	4
Sequence to analyse	GC**C**GTTCCCTGT**C**GCCACCTCCC**C**GTTTCTGTGGG**C**GGGT
Bisulfite converted sequence	GTYGTTTTTTGTYGTTATTTTTTYGTTTTTGTGGGYGGGT
Dispensation order	TGTCGTTGATCGTAGTTCGCTTCTGTGTCG

	GDF5
Gene location	Chr 20q11.2 (34043229 – 34043468)
GeneGlobe cat. no.	PM00196861
Amplicon length	239
Location of the sequence to analyse	Chromosome 20 34 043 345 – 34 043 373
Number of CpG sites	6
Sequence to analyse	GGGAG**C**GAGGGG**C**GGGGGGG**C**GGCAGAG**C**G**C**GG**C**GC
Bisulfite converted sequence	GGGAGYGAGGGGYGGGGGGGYGGTAGAGYGYGGYGT
Dispensation order	TGAGTCGTATGGTCGGTCGTCAGAGTCGTCGTCG

The C bases in bold characters in the sequence to analyse represent the CpG sites.

The letter Y in the bisulfite converted sequence represents the cytosine of the CpG site.

The PCR primer sequences are proprietary and are therefore not provided by Qiagen.

6.2.4 Template Preparation for Pyrosequencing

All 21 (20 samples and 1 human hypomethylated control) bisulfite converted DNA samples in addition to 2 additional controls (1 hypermethylated and 1 hypomethylated bisulfite converted by the manufacturer) from the EpiTect PCR Control DNA Set (Qiagen, Hilden, Germany) were amplified using the pre-designed primer sets of the PyroMark® CpG Assays (Qiagen, Hilden, Germany). The target sequences were amplified using the PyroMark® PCR Kit in a Techne TC-512 thermocycler (Bibby Scientific Ltd, Staffordshire, UK) as described in Chapter 3. To confirm the success of the PCR amplification of the target region, PCR products were run on a 1x agarose gel alongside a GeneRuler^{TM} 100 bp DNA ladder (MBI Fermentas, UK) (figure 6.3.1 and figure 6.3.2).

6.2.5 Pyrosequencing Reactions

As described in Chapter 3, the biotinylated PCR products were immobilised to Streptavidin-coated Sepharose high-performance beads (GE Healthcare, Buckinghamshire, UK) according to the PyroMark® Q24 manufacturer's recommendations (Qiagen, Hilden, Germany). The immobilised PCR products were captured using the PyroMark® Q24 vacuum (Qiagen, Hilden, Germany). The captured DNA was later released into a 24 well PyroMark® Q24 plate containing 1x sequencing primer diluted in 25 µl PyroMark® Annealing Buffer. Before placing the Q24 plate into the pyrosequencer, the annealing of the sequencing primer was conducted on a preheated hot-plate at 80 °C for 2 min followed by room temperature incubation for 10 min.

6.2.6 Data Analysis

Upon completion of the pyrosequencing reactions, pyrogrammes were visualised and percentage methylation of the CpG islands was automatically called by the PyroMark® Q24 v2.0.6 software as shown in figures 6.3.3 and 6.3.4. Statistical data analysis was performed using SPSS Version 20 program (SPSS Science Inc, Chicago, Ill, USA). A t-test analysis was performed to compare the mean percentage methylation of each CpG site between the CON and the PT samples. Significant difference in methylation was called when $p<0.05$.

6.3 RESULTS

In this study, the CON and PT groups were similarly matched for age ($p=0.449$) and gender. It was not possible however to obtain additional characteristic data such as the height, weight, and BMI of participants.

The PCR reaction conducted to amplify the target regions using the PyroMark® Assay primer sets was successful as shown in figures 6.3.1 and 6.3.2.

As mentioned in the previous section, the selected *TIMP2* assay spanned over 4 CpG sites. There was no significant difference ($p=0.885$) in the average methylation percentages across the 4 sites between the CON and the PT groups. Furthermore, there was no significant difference in methylation for any of the 4 CpG sites when analysed independently between the CON and the PT groups as shown in table 6.3.1 and figure 6.3.5 A.

The *GDF5* assay extended over 6 CpG sites. The data analysis show no significant difference for the mean percentages of methylation across the 6 sites between the CON and the PT groups ($p=0.333$). There was no significant difference in the methylation of any of the 6 CpG sites within the *GDF5* promoter region when analysed independently between CON and PT as shown in table 6.3.1 and figure 6.3.5 B.

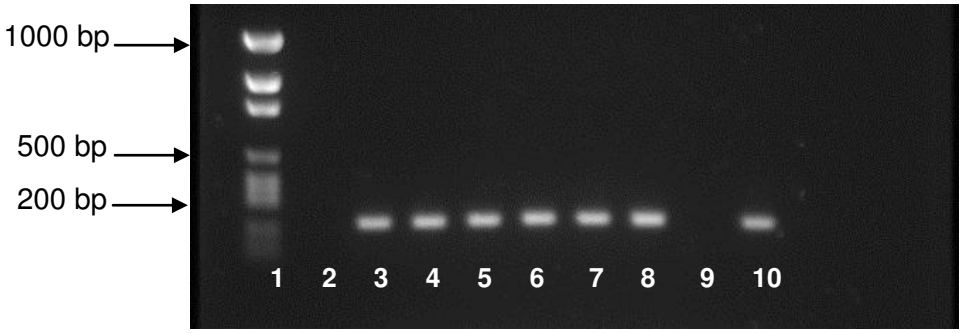


Figure 6.3.1 A 1% agarose gel electrophoresis used to confirm the amplification of the PCR reaction using the PyroMark® CpG Assay PCR primers for the *TIMP2* gene. Well 1, 100 bp ladder; well 2, blank; well 3, CON sample 1; well 4, CON sample 2; well 5, PT sample 1; well 6, PT sample 2; well 7, hypermethylated control; well 8, hypomethylated control; well 9, no template (negative control); well 10, bisulfite converted hypomethylated control.

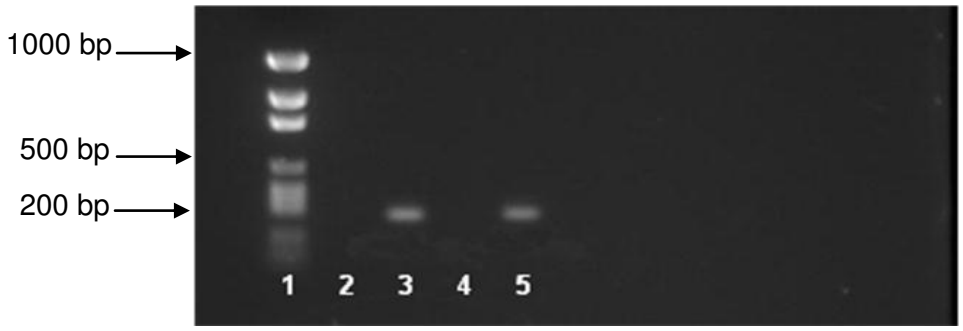


Figure 6.3.2 A 1% agarose gel electrophoresis used to confirm the amplification of the PCR reaction using the PyroMark® CpG Assay PCR primers for the *GDF5* gene. Well 1, 100 bp ladder; well 2, blank; well 3, PT sample; well 4, no template (negative control); well 5, PT sample.

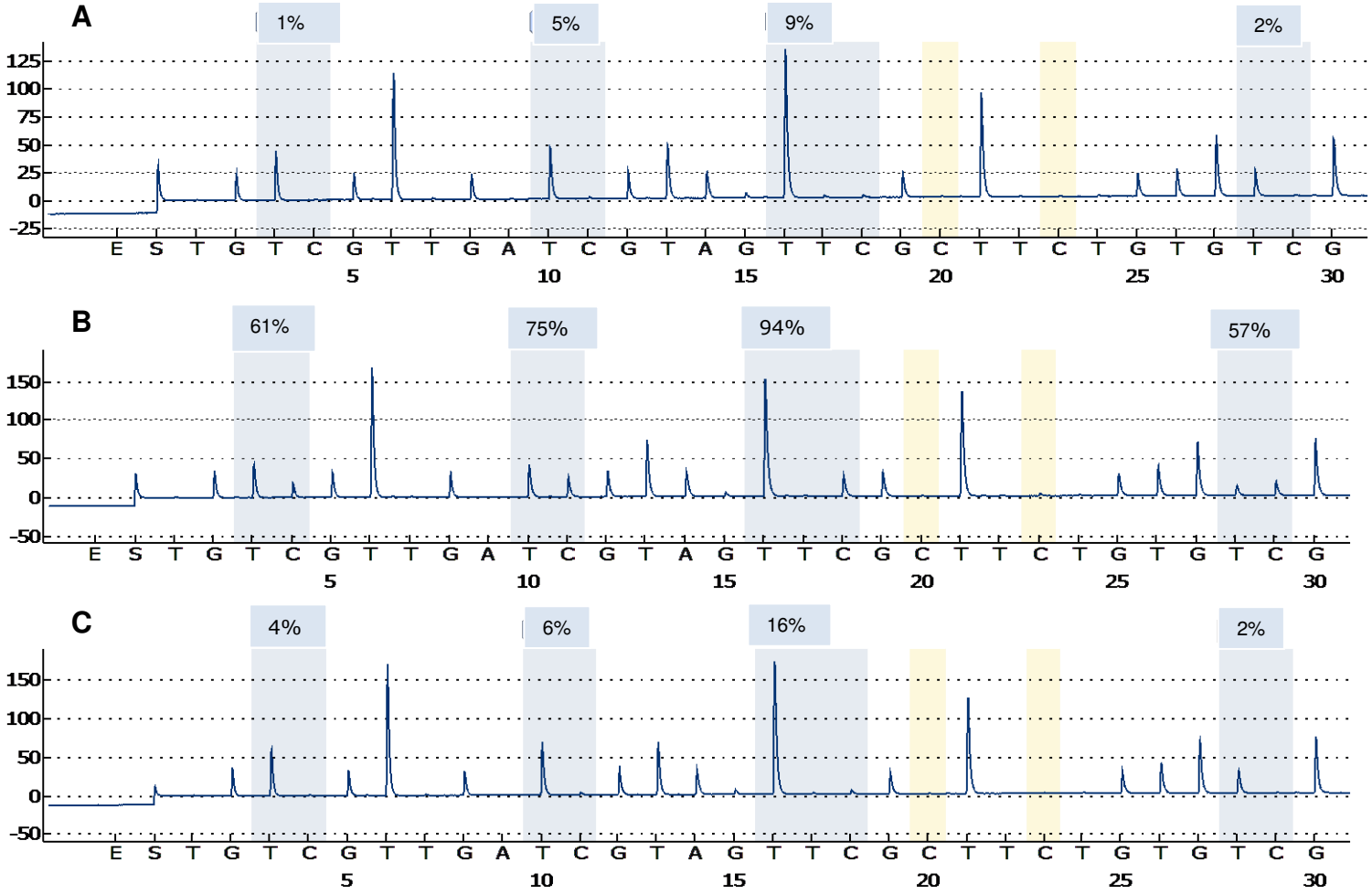


Figure 6.3.3 Typical pyrogram of the *TIMP2* assay showing the CpG sites in the shaded area, and the methylation percentage indicated above the CpG sites' peaks. A) PT sample, B) Hypermethylated control, C) hypomethylated control.

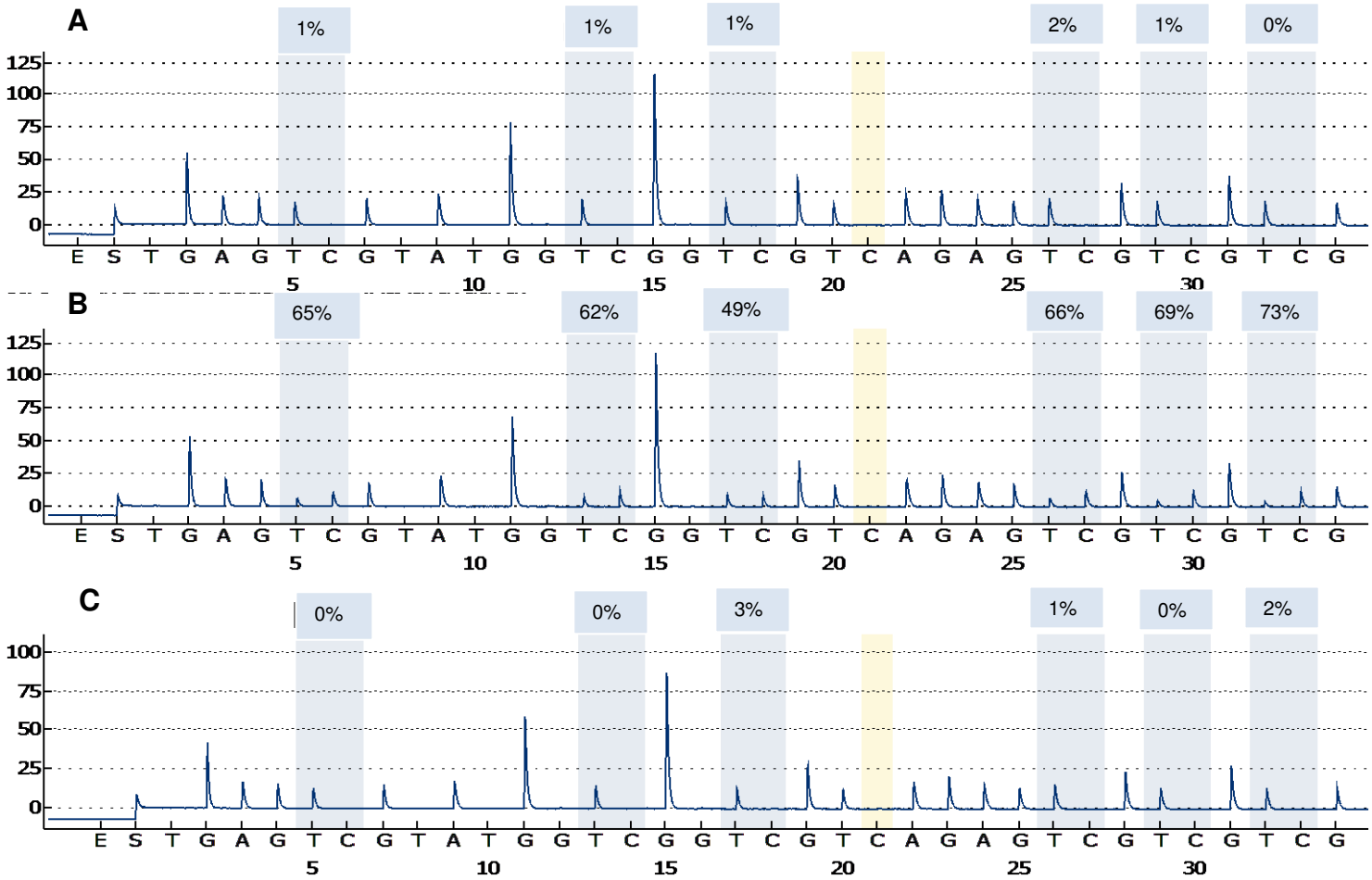


Figure 6.3.4 Typical pyrogram of the *GDF5* assay showing the CpG sites in the shaded area, and the methylation percentage indicated above the CpG sites' peaks. A) PT sample, B) Hypermethylated control, C) Hypomethylated control.

Table 6.3.1 Comparison of the mean methylation percentages of CpG islands between the asymptomatic controls (CON) and the patellar tendinopathy (PT) groups.

	CON	PT	P-Value
TIMP2 **CpG 1**	2.4 ± 1.2 (10)	2.5 ± 1.6 (10)	0.878
TIMP2 **CpG 2**	5.3 ± 2.1 (10)	5.3 ± 1.6 (10)	1.000
TIMP2 **CpG 3**	8.0 ± 3.2 (10)	8.4 ± 2.2 (10)	0.747
TIMP2 **CpG 4**	2.3 ± 1.8 (10)	2.2 ± 1.2 (9)	0.915
TIMP2 **Avg**	4.5 ± 1.7 (10)	4.6 ± 1.4 (10)	0.885
GDF5 **CpG 1**	4.1 ± 6.4 (10)	1.0 ± 1.1 (10)	0.149
GDF5 **CpG 2**	3.2 ± 6.3 (10)	1.9 ± 1.9 (10)	0.540
GDF5 **CpG 3**	3.5 ± 5.8 (10)	1.6 ± 1.2 (10)	0.326
GDF5 **CpG 4**	3.9 ± 5.8 (10)	2.4 ± 1.6 (10)	0.443
GDF5 **CpG 5**	3.3 ± 6.3 (9)	1.9 ± 1.3 (10)	0.490
GDF5 **CpG 6**	4.1 ± 7.2 (9)	1.5 ± 2.3 (10)	0.293
GDF5 **Avg**	3.6 ± 6.2 (10)	1.7 ± 0.4 (10)	0.333

Values are expressed as the mean, followed by the standard deviation and the number of participants (n) in parenthesis.

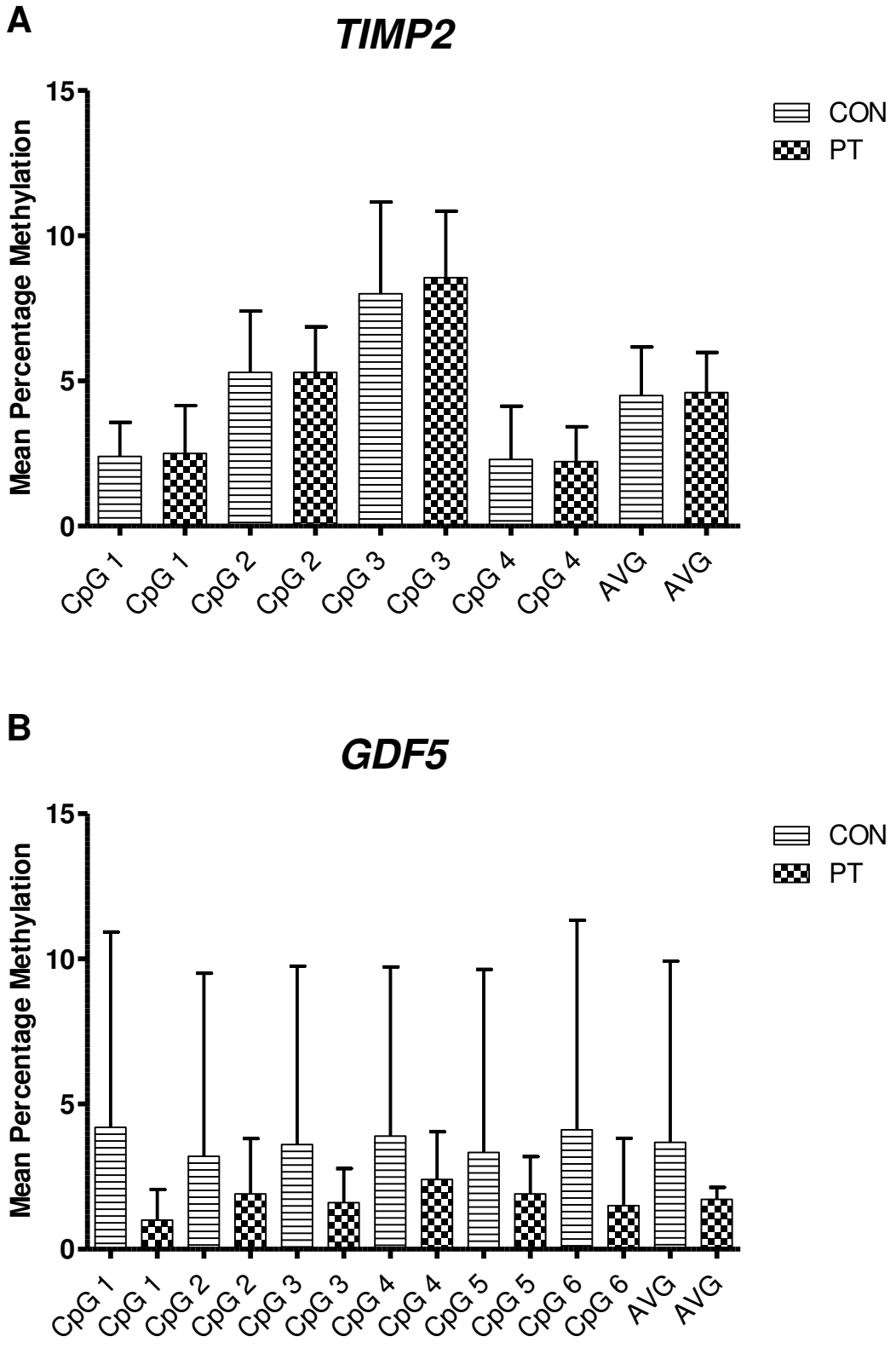


Figure 6.3.5 Bar chart showing the mean and SD of methylation percentages for the CpG sites investigated in addition to the average (AVG) methylation in the A) *TIMP2* and B) *GDF5* genes.

6.4 DISCUSSION

The initial aim of this study was to investigate whether different *TIMP2* and *GDF5* methylation profiles could be observed between healthy and damaged patellar tendons.

Pulukuri at al (2007) identified a CpG island on the promoter region of the *TIMP2* gene extending from the transcription initiation site upstream for approximately 900 bp. The methylation of the CpG sites located between positions -295 and -145 bp was found to reduce the expression of *TIMP2* in prostate cancer samples (Pulukuri et al. 2007). Likewise, the overlapping region ranging from -350 to -240 bp was also found to be hypermethylated in cervical cancer (Ivanova et al. 2004). As observed, the CpG island within the first 1 kb region upstream of the *TIMP2* gene was hyper-methylated in medical conditions such as cancer; however, this chapter study showed that the region investigated within the same CpG island ranging from position -488 to -455 bp did not show a different methylation pattern between the CON and PT tissue samples. In fact, all analysed samples showed very low methylation suggesting a limited role played by DNA methylation at the 4 investigated CpG sites in moderating the expression of the *TIMP2* gene in human patellar tendinopathy.

The DNA methylation pattern within the promoter region of the *GDF5* gene was not significantly different between the CON and the PT group. In particular, the studied region between position -805 and -777 showed hypomethylation. Interestingly, this methylation pattern is similar to the one observed between -1081 and -755 bp in osteoarthritis synovial join tissue (Reynard et al. 2011). However, the CpG islands located at position -1344 to -1208 and in the proximal promoter between -453 and +301 were reported to show hypermethylation in osteoarthritis (Reynard et al. 2011). Interestingly, the rs143383 C/T variant, which was associated with a range of multi-factorial conditions discussed in the introduction of this chapter, is located at position -275 bp and forms a CpG site which was also found to be hypermethylated in osteoarthritis (Reynard et al. 2011). It is important to

note that a reduction in the expression of *GDF5* increases the risk of OA. In that respect, carrying a T allele or a methylated C allele increase the risk of OA by decreasing the expression of *GDF5*. Such statement could be applicable in human tendinopathy but remains to be tested and proven.

This study has revealed a common hypomethylation pattern between OA and PT samples. This similarity justifies conducting additional work on the PT samples with focus on the regions showing hypermethylation of the *GDF5* promoter in OA. Furthermore, additional DNA methylation studies within the *TIMP2* promoter could be conducted on the CpG sites proximal to the transcription starting site showing hypermethylation in prostate (Pulukuri et al. 2007) and cervical cancer (Ivanova et al. 2004).

Chapter 7 DNA Methylation and miRNA Expression Profiles in Achilles Tendon Pathology: A Case Study

7.1 INTRODUCTION

Recent studies have been associating epigenetic mechanisms and susceptibility to disease in humans (Jirtle, Skinner 2007). As previously described, ATP results from intra-tendinous degeneration following the disruption of homeostasis within the extra-cellular matrix (ECM) (Józsa, Kannus 1997). A normal tendon is made up of straight parallel packed collagen fibres with tenocytes, blood vessels and nerve endings located between the bundles (Jones et al. 2006). In contrast, a degenerate tendon contains irregular alignment of collagen fibres, an alteration in the amount of non-collagenous matrix, and a variation in vascularisation (Movin et al. 1997). The intra-tendinous disruption of the ECM could be attributed in part to the activity of metalloproteinases (MMPs, ADAMs, and ADAMTSs) and the metalloproteinase inhibitors (TIMPs) (Jones et al. 2006). Gene variants within the *MMP3* (Raleigh et al. 2009) and *TIMP2* (El Khoury et al. 2013) genes have been associated with the risk of ATP. Furthermore, variations in gene expression have been reported where *MMP3* is down-regulated in pathologic tendons when compared to controls (Ireland et al. 2001). Variations in the expression of *TIMP2* have been reported; however there have been contradicting reports as to the direction of the variation. Jones et al. (2006) reported a down regulation of *TIMP2* in pathologic tendons where as Karousou et al. (2008) reported an up-regulation.

Metalloproteinases regulate homeostasis within the ECM by degrading and preventing abnormal build-ups of structural proteins in the tendon (Jones et al. 2006). A reduction in the production of metalloproteinases or their inhibition by TIMPs could result in the accumulation of collagenous and non-collagenous proteins (Jones et al. 2006) which could result in stiffer and more rigid tendons.

Epigenetic mechanisms and their role as risk factors in human tendinopathy were discussed (Collins, Raleigh 2009) but never investigated prior to this thesis. The previous chapter

investigated the role of DNA methylation profiles as epigenetic factors predisposing to patellar tendinopathy. A second epigenetic mechanism reported to contribute towards human pathologies is micro-RNA (miRNA) activity.

Micro-RNAs are small (~20 nucleotide) non-protein-coding transcription products formed endogenously (Güller, Russell 2010). They are known to repress gene expression post-transcriptionally by blocking translation or by degrading target mRNA (Hamilton, Baulcombe 1999, Reinhart et al. 2000). Approximately one third of genes could be regulated by miRNAs (Lewis, Burge & Bartel 2005). In fact, a single miRNA could target several genes (Vella et al. 2004), and a gene could be targeted by several miRNAs (Doench, Sharp 2004).

The biogenesis of miRNA initiates in the nucleus when RNA polymerase II transcribes miRNA genes into primary miRNAs (pri-miRNA) which are several kb in length (Bartel 2004). The Drosha / DGCR8 (Digeorge syndrome Critical Region Gene 8) complex then cleaves the pri-miRNA into a hairpin-like ~70 nt premature miRNA (pre-miRNA) (Lee et al. 2003). The pre-miRNA is then exported to the cytoplasm for the final maturation steps where the Dicer enzyme will remove the hairpin by cleaving the pre-miRNA into a ~20 nt double stranded molecule (Lee et al. 2003). The resulting RNA complex associates with the miRNA-induced silencing complex (RISC) where one strand is degraded while the remaining one turns into mature miRNA (Schwarz et al. 2002). An overview of the biogenesis is illustrated in figure 7.1.1.

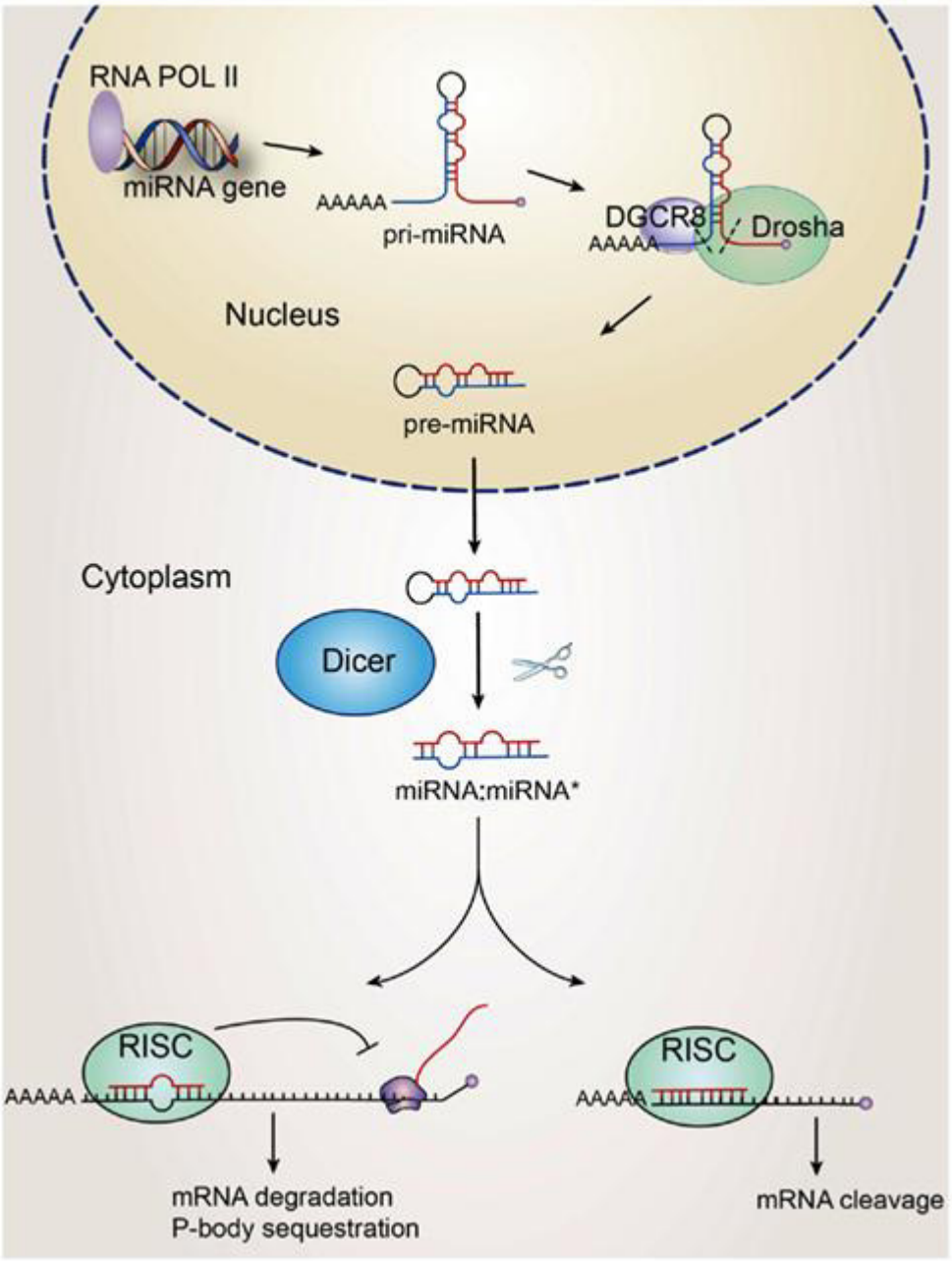


Figure 7.1.1 Biosynthesis of miRNA. The miRNAs are transcribed from the genome using RNA Polymerase II which produces the pri-miRNAs. The pri-miRNAs are converted into pre-miRNAs with the help of the Drosha/DGCR8 complex. The pre-miRNAs are then exported to the cytoplasm where they are cleaved by the Dicer to produce mature miRNAs that will bind to the RISC complex to become active. The image is reproduced from (Lu, Barca 2012) with the permission of the publisher.

Micro-RNAs are expressed in normal tissue to moderate gene expression. There are several potential ways in which miRNAs could lose their normal function and therefore contribute to the onset of pathologies: 1) miRNA could acquire a mutation that would result in the loss of gain of a function, 2) a target site could acquire a mutation and the miRNA could no longer be able to bind (Mills, Cowin 2013). Various miRNA have been associated with human diseases by repressing the expression of genes needed for the normal functioning of a tissue (Alvarez-Garcia, Miska 2005). For instance, miR-21 (miRNA-21) was found to be up-regulated in cardiac hypertrophy (van Rooij et al. 2006), skin cancer (Zhu et al. 2008), and psoriasis (Bostjancic, Glavac 2008). It is believed that miR-21 predisposes to pathologies by inhibiting the production of type I collagen (Li et al. 2011). Furthermore, the activity of miR-155 was associated with cardiovascular conditions where it targets the expression of angiotensin I receptor gene and thus interrupts the rennin angiotensin system (Elton, Sansom & Martin 2010). In addition to cardiovascular conditions, miR-155 was found to be up-regulated in synovial fluid collected from rheumatoid arthritis patients where it is believed to target the expression *MMP3* (Stanczyk et al. 2008). On the other hand, miR-191 was predicted to play a role in the tumorigenesis of breast cancer by targeting the expression of *TIMP2* (Shen, Ambrosone & Zhao 2009). The three miRNA discussed are believed to target the synthesis of proteins playing crucial roles in the maintenance of homeostasis in the ECM. Furthermore, these proteins are expressed by genes previously associated with musculoskeletal soft tissue pathologies (Raleigh et al. 2009, El Khoury et al. 2013, Posthumus et al. 2009).

With the above in mind, the objectives of this study were: 1) to explore the DNA methylation patterns within the promoter regions of the *TIMP2* and *GDF5* genes in ATP, 2) to investigate whether the expression of miR-21, miR-155, and miR-191 varies in ATP, and 3) to identify the variation in gene expression of *TIMP2* in ATP.

7.2 MATERIALS AND METHODS

7.2.1 Participants

Achilles tendon tissue samples were obtained from the Royal National Orthopaedic Hospital under the approval of the National Research Ethics Service Committee within the National Health Services and in accordance with the Human Tissue Authority guidelines as described in Chapter 3. One damaged tendon tissue sample (ATP) was obtained from a male individual undergoing a repair surgery for a degenerative Achilles tendon and 4 healthy (3 males and 1 female) Achilles tendon samples (CON) were obtained from individuals undergoing surgery in their lower limb for medical conditions not involving the Achilles tendon. The collected samples were cut into sections of 4x4x4 mm and were submerged and stored in microcentrifuge tubes containing Allprotect Tissue Reagent ® (Qiagen, Hilden, Germany) to preserve DNA, RNA and miRNA as described in Chapter 3.

7.2.2 Extraction of DNA, RNA and miRNA

Tissue samples were mashed, lysed, and homogenised using the TissueLyser LT (Qiagen, Hilden, Germany) as described in Chapter 3. DNA was extracted from sections not exceeding 25 mg in weight using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. RNA and miRNA were extracted together from tissue sections not exceeding 30 mg in weight using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. All extracted genetic material was quantified using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and were stored at -20 °C until the day of analysis.

7.2.3 Bisulfite Conversion, DNA Methylation, and Pyrosequencing

All 5 samples (1 ATP and 4 CON) were bisulfite converted using the method described in Chapter 3. Following that, PCR amplification using the PyroMark® CpG assays for the regions within *TIMP2* and *GDF5* genes used in Chapter 6 was performed. Upon completion of the PCR reaction all samples were prepared and run through the PyroMark® Q24 to assess the DNA methylation patterns.

7.2.4 Gene Expression

A two step gene expression experiment was conducted using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) as described in Chapter 3. A *TIMP2* pre-designed TaqMan® Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) was selected for this study. The expression of *TIMP2* was normalised against the *GAPDH* housekeeping gene. Furthermore, miRNA expression was assessed using predesigned TaqMan® miRNA Assays (Applied Biosystems, Foster City, CA, USA) for mir-21, mir-155, and mir-191. All miRNA expression experiments were normalised against the *RNU6B* gene which is a widely used endogenous reference in miRNA quantification studies (Matera, Terns & Terns 2007). The data was visualised on the StepOne Software v2.1 (Applied Biosystems, Foster City, CA, USA). A comparative Ct analysis was performed and fold-changes in expression were calculated according to the formula: $2^{(-\Delta\Delta Ct)}$ (Livark, Schmittgen 2001). In both, gene and miRNA expression, experiments the CON sample with the highest mean Ct value was used as the calibrator.

7.2.5 Data Analysis

Statistical data analysis was performed for in this chapter as it is a case study with only one ATP participant.

7.3 RESULTS

In this study, the mean age of the CON group (45.75 ± 16.5) was lower than that of the single ATP case (63 years old). It was not possible however to obtain additional characteristic data such as the height, weight, and BMI of the participants.

As mentioned in Chapter 6 the PyroMark® CpG assays used for the *TIMP2* and *GDF5* genes spanned over 4 and 6 CpG sites respectively. In correspondence with the study conducted on patellar tendons, this study on Achilles tendons showed no significant difference in methylation for any of the 4 CpG sites within *TIMP2* or the 6 CpG sites within *GDF5* when analysed independently between the CON group and the single ATP case as shown in table 7.3.1 and figure 7.3.1. Furthermore, there was no difference in average DNA methylation percentages for *TIMP2* between the CON group and the ATP individual. However, there was an observed difference in average methylation of the *GDF5* CpG sites between the CON group and the ATP participant (table 7.3.1 and figure 7.3.1 B).

The gene expression analysis showed interesting results where *TIMP2* within the ATP sample was up-regulated 42.31 fold relative to the mean of the CON group (figure 7.3.2 A). Furthermore, the miRNA expression of all three selected miRNA showed a down-regulation in the ATP sample relative to the mean miRNA expression within the CON group with miR-191 displaying the greatest reduction in expression (3.42 fold) followed by miR-155 (2.31 fold) and miR-21 (1.15 fold) (figure 7.3.2 B, C and D).

Table 7.3.1 Comparison of the mean methylation percentages of CpG islands between the asymptomatic controls (CON) group and the single Achilles tendon pathology (ATP) sample.

	CON	ATP
TIMP2 **CpG 1**	1.5 ± 1.3 (4)	1.0 (1)
TIMP2 **CpG 2**	3.0 ± 1.4 (4)	3.0 (1)
TIMP2 **CpG 3**	7.5 ± 4.0 (4)	7.0 (1)
TIMP2 **CpG 4**	1.2 ± 1.9 (4)	1.0 (1)
TIMP2 **Avg**	3.3 ± 1.1 (4)	3.0 (1)
GDF5 **CpG 1**	1.0 ± 1.4 (4)	0.0 (1)
GDF5 **CpG 2**	0.5 ± 1.0 (4)	1.0 (1)
GDF5 **CpG 3**	2.7 ± 2.5 (4)	0.0 (1)
GDF5 **CpG 4**	3.5 ± 4.1 (4)	0.0 (1)
GDF5 **CpG 5**	0.2 ± 0.5 (4)	0.0 (1)
GDF5 **CpG 6**	2.5 ± 3.8 (4)	1.0 (1)
GDF5 **Avg**	1.7 ± 0.9 (4)	0.3 (1)

Values are expressed as the mean, followed by the standard deviation and the number of participants (n) in parenthesis.

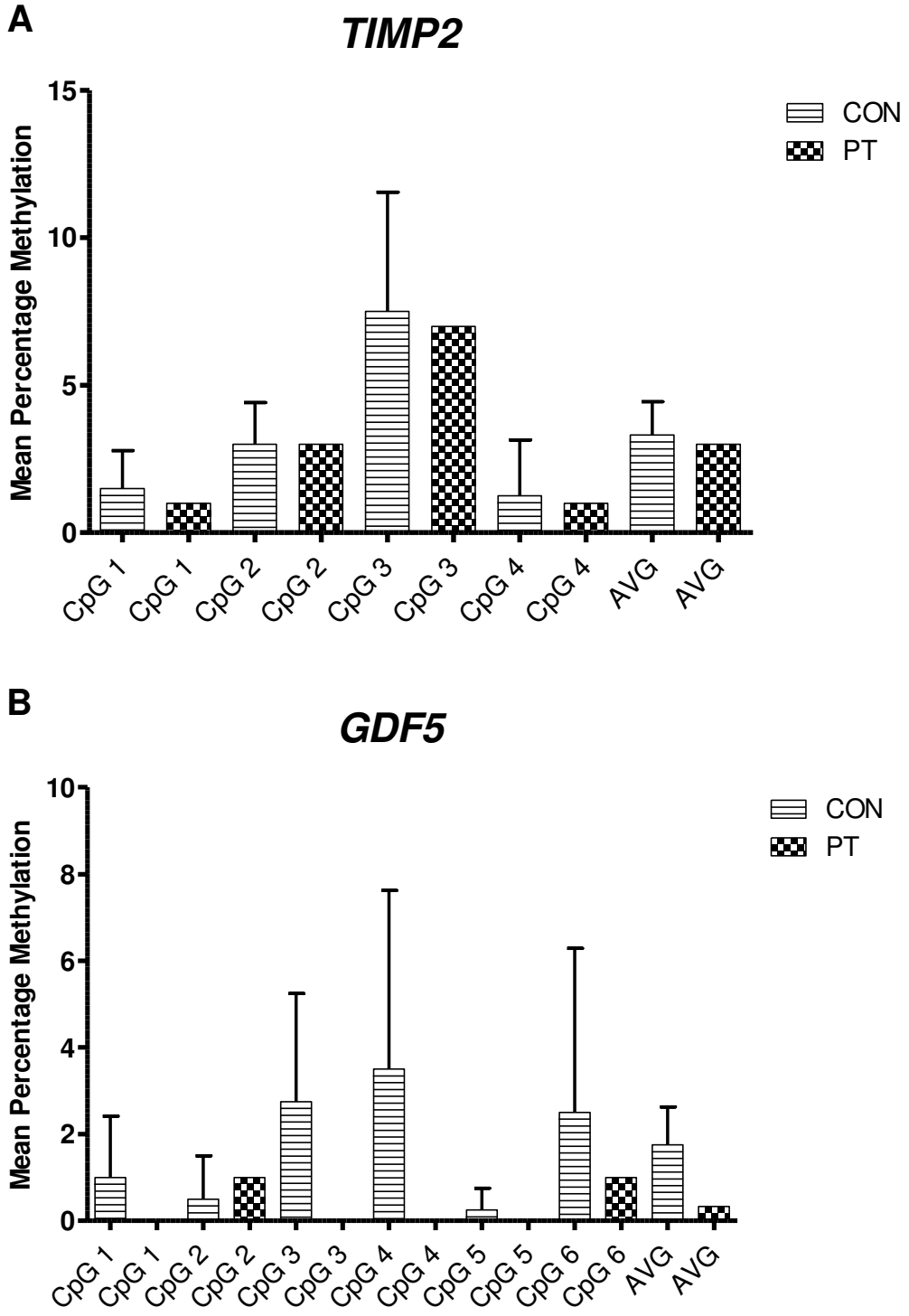


Figure 7.3.1 Bar chart showing the mean and SD of methylation percentages for the CpG sites investigated in addition to the average (AVG) methylation in the A) *TIMP2* and B) *GDF5* genes.

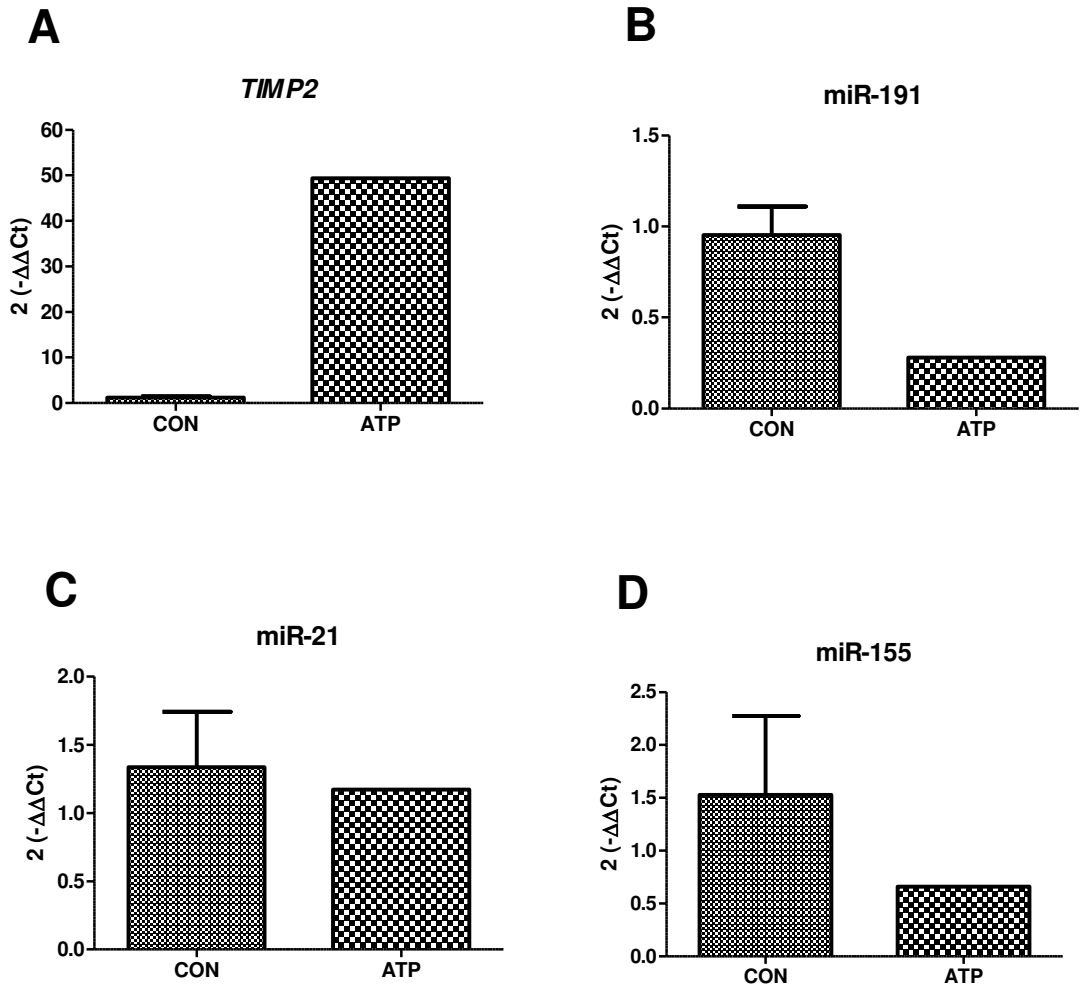


Figure 7.3.2 Bar chart showing the change in expression folds between the CON group and the ATP sample. The relative quantity of mRNA or miRNA is calculated using the $2^{(-\Delta\Delta Ct)}$ formula. The data is displayed as the mean and standard deviation of the relative quantity of the CON group and the single ATP sample. A) A 42.31 fold up-regulation in gene expression of *TIMP2* in the ATP sample compared to the CON group. B) A 3.42 fold down regulation in the miRNA expression of miR-191 in the ATP sample compared to the CON group. C) A 1.15 fold down regulation in the miRNA expression of miR-21 in the ATP sample compared to the CON group. D) A 2.31 fold down regulation in the miRNA expression of miR-155 in the ATP sample compared to the CON group.

7.4 DISCUSSION

This study has, for the first time, combined gene expression and epigenetic investigations on Achilles tendon tissue samples. This combined investigation has helped draw an image of the factors and mechanisms involved in the expression of the *TIMP2* gene in the Achilles tendon. As observed in figure 7.3.1-A the results of this study show an up-regulation of *TIMP2* expression in the ATP sample when compared to the CON group. It is important to note however that this study was conducted with a single ATP sample which is not representative of the ATP population. Nevertheless, these findings correlate with Karousou et al. (2008) who reported an up-regulation of *TIMP2* in damaged Achilles tendons. The DNA methylation experiment showed hypomethylation patterns of the investigated region (-488 to -455 bp) within the *TIMP2* promoter. Although these results suggest the absence of methylation in the investigated region, DNA methylation as a whole cannot be excluded as a mechanism affecting gene expression without, at least, investigating the methylation pattern of the whole CpG island residing within the first kb of the promoter region. This is important because the CpG sites residing within the regions -295 to -145 bp and -350 to -240 bp were found to be hypermethylated in prostate (Pulukuri et al. 2007) and cervical cancer (Ivanova et al. 2004) respectively.

Another finding of this study was the difference in mean DNA methylation percentage for the *GDF5* gene between the ATP sample and the CON group. The exact role played by GDF5 in tendons is not fully explained (Eliasson, Fahlgren & Aspenberg 2008); however it is believed to be involved in the maintenance and repair of musculoskeletal soft tissues (Eliasson, Fahlgren & Aspenberg 2008, Mikic 2004). It has been proposed that a GDF5 deficiency could increase the risk of osteoarthritis (Reynard et al. 2011). Although a difference in DNA methylation levels was observed between the ATP and CON group, any deficiency of GDF5 cannot be attributed to the methylation of the 6 investigated CpG sites as those were very low for all participants.

In addition to DNA methylation, the second epigenetic mechanism investigated in this study was the activity of miRNAs. The results have shown a 3.7 fold down-regulation of the miR-191 in the ATP sample when compared to the mean expression level of the CON group. Since *TIMP2* is a target for the binding of miR-191 (Shen, Ambrosone & Zhao 2009), it can be speculated that the sharp decrease in the expression of miR-191 observed in the ATP case would facilitate the opportunity for *TIMP2* to be expressed and could therefore explain the observed over-expression. Furthermore, serum levels of TIMP2 have been documented to remain elevated as high as three years post Achilles tendon injury (Pasternak et al. 2010). Assuming that the tested ATP sample is not an outlier, then the Pasternak et al. (2010) observation could be explained by the up-regulation of the gene.

As described in Chapter 5, TIMP2 plays a role in inhibiting the activation of MMP2 (Wang, Juttermann & Soloway 2000) and also suppresses the activity of ADAMTS1 (Kevorkian et al. 2004). With this in mind, it could be postulated that the over-expression of *TIMP2* would compromise the TIMP/MMP balance within the ECM which could ultimately result in over-accumulation of structural proteins leading to stiffer and more rigid tendons. On the other hand, it could be argued that the up-regulation of *TIMP2* was a protective response to regulate the activity of metalloproteinases which were over-degrading structural proteins in the ECM (Stanczyk et al. 2008, Aoki et al. 2007).

This study showed minimal variation in the expression of miR-21 between the ATP sample and the CON group. Although miR-21 targets the expression of *COL1A1* (Li et al. 2011), and was found to be up-regulated in dermal (Bostjancic, Glavac 2008) and cardiac conditions (van Rooij et al. 2006), its expression did not vary in the ATP sample.

A tendinopathy is believed to occur following the failure of fibroblasts in regenerating from mechanical loadings which induced damage within the ECM (Khan, Cook 2003). In such a situation, TGF-β is released to induce tendon development (Pryce et al. 2009). Interestingly,

miRNA levels can be regulated by exercise, and reports have shown an up-regulation of miR-21 when mice were treated with TGF-β after exercise (Mendias, Gumucio & Lynch 2012). Moreover, Aoi et al. (2010) report an increase in miR-21 extracted from the gastrocnemius of mice after 4 weeks of treadmill exercise. Given the small number of ATP cases, and the lack of information related to the physical activity of the participant in this thesis study, it is not possible to ascertain whether this person is physically active and would therefore be stimulating the activity of TGF-β and miR-21.

In addition to miR-21 and miR-191, this study shows a 2.31 down-regulation in the expression of miR-155 between the single ATP sample and the CON group. An up-regulation of miR-155 has been reported in rheumatoid arthritis where it suppresses the production of MMP3 and MMP1 (Stanczyk et al. 2008). The activity of miR-155 has been discussed in various inflammatory situations especially that its expression is up-regulated in murine macrophages after exposure to TNF-α (O'Connell et al. 2007). This mechanism has not been confirmed in humans and would therefore require additional investigation. It could be hypothesised that miR-155 plays a protective role by reducing the expression of MMPs following inflammation, and thereby limiting tissue damage (Stanczyk et al. 2008). It is unclear how the tissue would balance between the expression of TIMPs and miR-155 to control the activity of MMPs. Nevertheless, the results of this chapter study show a 42.31 up-regulation of *TIMP2* and a much smaller change in the expression of miR-155 in the ATP sample. It could be suggested that TIMPs are the primary agents released to control the activity of MMPs followed by miRNAs. Moreover, the elucidation of the functional role of miR-155 in tendinopathy requires further studies.

The introduction of this chapter discussed potential ways in which miRNAs can contribute to disease. However, it is not clear yet whether a variation in miRNA expression between the tendinopathy and control samples is causative of the disease, or on the other hand could be a normal response to the disease. Answering this question will help a lot in designing

interventions to deal with tendinopathy. If mutations are acquired, it would be ideal to adopt a gene therapy approach to rectify sequence changes. Additionally, miRNAs are being used as blood markers for several diseases such as asthma (Garbacki et al. 2011) and coronary artery disease (Hoekstra et al. 2010). The spectrum of release of miRNA by tenocytes into the blood stream is still unknown. However, once a clearer image of tendinopathy is established, miRNA blood levels could be indicative of an ongoing damage.

Chapter 8 Summary and Perspectives

The number of musculoskeletal soft tissue pathologies such as tendinopathies in humans is considered to be increasing in a population who is becoming more aware of the need to incorporate sport and physical activity in their daily life to reduce the risks of acquiring chronic medical conditions such as cardiovascular diseases and cancer (Jarvinen et al. 2005). Achilles tendon pathology (ATP) and patellar tendon pathology (PTP) are two conditions highly reported in sports involving the lower limbs such as running, football, basketball, and volleyball (Jarvinen et al. 2005, Bollen 2000). The clinical, imaging, and histological diagnostic criteria for both pathologies have been elucidated (Khan, Cook & Maffulli 2005). However, the underlying biochemical aetiologies remain unclear. The literature has classified human tendinopathy as a multi-factorial condition to which intrinsic and extrinsic factors have been described (Riley 2004). Chapter 2 displayed evidence that show indeed the role of genetic variations as a major intrinsic factor. Genetic variations within genes coding for structural proteins in addition to enzymes involved in the maintenance of homeostasis within the extra-cellular matrix (ECM) have been associated with ATP (Raleigh, Collins 2012). The number of associated gene variants in addition to the different biological pathways they are involved in emphasises the polygenic nature of the pathology and understates the role of a single gene. Indeed, it seems more likely that many polymorphisms with small or moderate effect contribute collectively to the onset of human tendinopathy. The primary aim of this thesis was to identify novel genetic variations associated with ATP and to propose biological mechanisms underlying the genetic risks. Furthermore, the secondary aim was to identify the potential roles played by different epigenetic factors in ATP and PTP.

In Chapter 4 and 5, polymorphic variations within genes coding for ECM components and regulatory enzymes have been identified as suitable candidate genetic variants based on: 1) their location within genes, 2) their minor allele frequency, 3) the polymorphic change that

can be implicated in a modification of a crucial biological function, and 4) any previous association with musculoskeletal soft tissue pathologies, or medical conditions involving the disruption of the ECM. The most suitable study design was a case-control genetic association study where differences in the distribution of genotypes between the two groups would be assessed.

In Chapter 4 of this thesis, gene variants within the *COL5A1* gene which were previously associated with ATP in an Australian and South African population (Mokone et al. 2006, Abrahams et al. 2013) were investigated for a potential association with ATP in a UK population. Specifically, the functional variant rs12722 would, in addition to the rs71746744 variant, alter the secondary structure of the 3' UTR of the *COL5A1* gene and subsequently, affect the mRNA's stability (Laguette et al. 2011). However, the results of the studies conducted in Chapter 4 showed no association of either the rs12722 or the rs71746744 variants with ATP in the UK cohort. Nevertheless, when looking at male participants, the *COL5A1* rs12722 variant was associated with Achilles tendon rupture, and the *COL5A1* rs71746744 was associated with ATP. These results are different to what was previously described by Mokone et al. (2006) and Abrahams et al. (2013) who found both variants to be associated with Achilles tendinopathy in the combined AUS+SA cohort. It is not clear however, why the *COL5A1* variants showed different associations.

Additional and novel genes coding for structural proteins were investigated in Chapter 4. The gene variants *FBN2* rs331079 and the *ELN* rs2071307 which have previously been associated with medical conditions involving the disruption of the ECM (Ruigrok et al. 2006, Yang et al. 2013), have been investigated in all three cohorts except for the *ELN* rs2071307 variant which was only investigated in the AUS and SA population. Within the combined AUS+SA population, the *FBN2* rs331079 was found to be associated with Achilles tendinopathy, and the *ELN* rs2071307 variant was associated with Achilles tendon rupture. The mechanism by which the *FBN2* rs331079 variant predisposes to the injury is not clear

and requires further investigation. It was possible however to suggest a possible role played by the *ELN* rs2071307 in the predisposition to injury. The G/A substitution at the rs2071307 locus that results in the alteration of the secondary structure of the mRNA coded for by exon 20 could lead to a variation of the characteristics of the elastic fibres rendering them more prone to injury.

In addition to structural proteins, Chapter 4 investigated the potential association of the gene coding for GDF5, a protein involved in maintenance and repair or tendons, with ATP in the UK population. The *GDF5* rs143383 variant was previously associated with ATP in a combined AUS+SA cohort (Posthumus et al. 2010). However, these findings did not replicate in the UK population. Lack of replication in this study could have resulted from differences in heterogeneity between studies which lead to different conclusions about the role of a particular variant in the risk of disease. For instance, Lewis and Knight (2012) discuss that different ages of diagnosis could contribute towards a difference in heterogeneity. In fact, the mean age of onset of ATP was significantly different (p=0.002) between the UK (45.1 ± 14.1, n=135) and AUS+SA (40.2 ± 13.6 n=166) cohorts. Furthermore, a small change in allele frequency (<0.1) can reduce the power drastically, and consequently result in a failure to replicate (Greene et al. 2009).

In Chapter 5 an investigation into metalloproteases enzymes (MMPs, ADAMs, and ADAMTSs) and their inhibitors, the TIMPs, was undertaken. The work detailed in this chapter showed that the CT genotype for the *TIMP2* rs4789932 variant was associated with an increased risk of ATP in the combined AUS+SA cohort. Interestingly, in the UK population the CC genotype of the rs4789932 variant was associated with ATP only in the male participants. As discussed in Chapter 5, these findings might appear perplexing as it infers a reverse effect. However it has been suggested that reverse effects in genetic association studies may reveal further information about a susceptibility locus that was previously unknown such as an interaction with one or more functional variants through

epistasis (Greene et al. 2009). Furthermore, It is not clear whether this promoter variant is functional and therefore the mechanism by which it predisposes to the injury is yet to be described. Furthermore, the non-synonymous *MMP3* rs679620 variant which was previously associated with Achilles tendinopathy in the South African population (Raleigh et al. 2009), was associated with Achilles tendon rupture among the UK population. Although the association is not with the identical pathology, it is however the same GG genotype that associates with the risk. It is not clear why the same genotype is associated with the risk of tendinopathy in one cohort, and the risk of rupture in another. However it could be possible that another gene variant involved in a protective mechanism is showing a reverse effect between the two populations; such as protecting the SA population from rupture but not the UK. Furthermore, the A/G substitution at the rs679620 locus results in substituting the polar basic amino acid lysine with the polar acidic amino acid glutamic acid (http://www.ncbi.nlm.nih.gov/projects/SNP). Interestingly, this substitution does not lead to changes in Achilles tendon's mechanical properties (Kubo, Yata & Tsunoda 2013) and therefore the functional significance of the amino acid change remains unknown. Therefore, it is wise to consider *MMP3* rs679620 to be a risk predisposing variant. Non-synonymous gene variants such as the *ADAMTS2* rs1054480, *ADAMTS5* rs226794, *ADAMTS14* rs4747096, and the *ADAM12* rs3740199 were not associated with ATP although they were independently associated with other soft tissue pathologies.

The fact that the variants named above (*FBN2* rs331079, *ELN* rs2071307, *COL5A1* rs12722, *COL5A1* rs71746744, *MMP3* rs679620, and *TIMP2* rs4789932) have been associated with ATP opens the door to their possible incorporation into a clinical risk model. For example, Nell et al. (2012) have designed a risk assessment model for Achilles tendinopathy where they have included gene variants involved in with the inflammatory response. They report that the genetic markers used were collectively able to discriminate between CON and TEN individuals. Furthermore, they added that the effectiveness of this model increases as additional biomarkers are added to it (Nell et al. 2012). However, it becomes challenging to

allocate the correct genotype risk score in a situation where two different genotypes for the same variant show reverse effects such as in the *TIMP2* rs4789932 example, where the CC genotype was associated with increasing the risk of ATP in the UK cohort, and the CT genotype was associated with increasing the risk of ATP in the AUS+SA cohort. Furthermore, these models assume the risk causing genotype to be homozygous. Since the heterozygous CT genotype for the rs4789932 variant was associated with ATP, the suitability of such model must be challenged. Moreover, these models assume an equal effect size of the associated gene variants. Such an assumption may not be valid as some variations can result in greater gene expressions than other variants and could possibly lead to detrimental structural effects which cannot be equated with minor effects of small contributors to the pathology. For instance, a recently designed ATP total genotype score genetic risk assessment model looking at 6 gene variants successfully assessed control participants as not being at risk with 90% accuracy, but showed an accuracy of 37% when assessing the ATP participants as at risk (Saunders 2013). Therefore it is best to include the associated gene variants in the risk models once the effect sizes have been assessed. The total genotype score approach has also been used to assess other polygenic phenotypes such as muscle strength and power where the results showed an elevated similarity between profiles (Hughes et al. 2011). Genetic tests may be beneficial in predicting the risk of injury to put in place personalised preventive measures. However, genetic risk assessments are time dependent and are likely to miss some predictions at the time when the tests are conducted (Williams, Wackerhage 2009).

Moreover, Chapter 5 reports for the first time a relationship between genetics and clinicopathological characteristics of ATP. Specifically, male individuals suffering from tendinosis and carrying the GG genotype for the *MMP3* rs679620 variant, developed thicker Achilles tendons in comparison to those carrying the GA and AA genotypes. As described in the discussion of Chapter 5, there was no correlation between tendon width and biometric characteristics (age, height, weight and BMI), and were therefore not co-varied for. Although

the numbers included in the analysis were small and the results should be treated as preliminary data, these findings provide valuable information on the potential use of genetics in predicting the grade of pathology.

Both chapters 4 and 5 reported a novel age vs genotype interaction where it was found that the AA genotype for the *ELN* rs2071307 variant and the GG genotype for the *ADAMTS14* rs4747096 variant might play a protective role by delaying the onset of injury. This is the first time genotypes have been reported to associate with a delay in the onset of a musculoskeletal soft tissue injury. These findings need to be replicated and confirmed by other studies before these genotypes could be used as genetic predictors of the time of injury. However, the prediction of the risk of injury is also dependent on other gene variants. The reason that the *ADAMTS14* rs4747096 GG genotype and the *ELN* rs2071307 AA genotype are protective until a certain point is not clear. Nonetheless, it is worth noting that both genotypes are the minor genotypes for their respective variants. Evolutionary speaking, it would be thought that a protective genotype should be preserved by natural selection and therefore would be expected to be the most common. However, it could also be suggested that the benefits of these genotypes have only recently been showing, and therefore they are steadily increasing in the population (Bamshad, Wooding 2003). This cannot be confirmed without conducting a population wide evolutionary genetic study. Interestingly, epigenetic studies have shown that global DNA methylation increases with age (Christensen et al. 2009). With this in mind, it could be suggested that the elevation in DNA methylation has altered the level of gene expression which resulted in a biochemical change within the ECM, and therefore contributed towards the onset of the pathology.

In Chapter 6 and 7 preliminary studies were conducted on epigenetic factors (DNA methylation) contributors to the risk of ATP and PTP. DNA methylation profiles of selected CpG region were investigated, within the promoter region of the *TIMP2* (33 bp) and *GDF5* (28 bp) genes in both PTP and ATP using pyrosequencing technology. The two genes were

selected on the basis of the previous association of promoter variants with ATP (Posthumus et al. 2010, El Khoury et al. 2013). The results of the investigation conducted in Chapter 6 showed that the investigated CpG sites in both genes were hypo-methylated with no significant methylation difference between the PTP cases and control samples. This is rather interesting because the CpG sites located 324 bp downstream of the *GDF5* investigated region and 100 bp downstream of the *TIMP2* investigated CpG sites within the promoter regions were hyper-methylated in osteoarthritis (*GDF5*) (Reynard et al. 2011) and in a range of cancers (*TIMP2*) (Ivanova et al. 2004, Pulukuri et al. 2007). Similarly, the results in Chapter 7 reported no difference in DNA methylation between a single ATP case and four controls for the investigated CpG sites within the *TIMP2* gene. Interestingly, there was an average DNA methylation difference between the single ATP case (0.3, n=1) and four controls (1.7 ± 0.9, n=4) for the *GDF5* gene. It is important not to assume that the decrease in *GDF5* methylation in the single ATP sample that we observed could result in an increased gene expression of *GDF5*. In fact, from previous research it seems that musculoskeletal pathologies are more likely to occur when the expression of *GDF5* is reduced (Reynard et al. 2011). Therefore the findings in Chapter 7 should be treated with caution due to the fact that there was only one ATP sample.

In addition to DNA methylation, the experiments outlined in Chapter 7 looked at miRNAs (an additional epigenetic factor). MicroRNAs are small non-coding RNAs that can repress gene expression by blocking translation or by degrading target mRNA (Hamilton, Baulcombe 1999, Reinhart et al. 2000). The purpose of this study was to explore the potential differences in miRNA expression between ATP cases and controls. The miRNAs were selected on the basis of their target genes which are known to be involved in maintaining the integrity of tendons (Rutnam, Wight & Yang 2013). The data showed that miR-191, was 3.4 fold down regulated in the single ATP sample relatively to the control samples. Interestingly, the expression of *TIMP2* in that same sample was up-regulated (43 fold) when compared to the control group. This observation is consistent with a previous study by Karousou et al.

(2008). The observed inverse expression between *TIMP2* and miR-191 can be expected since *TIMP2* mRNA is negatively regulated by MiR-191 (Shen, Ambrosone & Zhao 2009). However the magnitude of up-regulation of *TIMP2* does not match the magnitude of down-regulation of miR-191. This could indicate the involvement of additional factors, along with miR-191, in the regulation of *TIMP2*. Furthermore, the expression of miR-21 and miR-155, which target *COL1A1* (Li et al. 2011) and *MMP3* (Stanczyk et al. 2008) respectively, was not altered in the ATP sample relatively to the control samples. This could indicate a limited role played by these two miRNAs in regulating the expression of *COL1A1* and *MMP3* in ATP. Although these data were conducted on small numbers of clinically resected tissue it must be noted that they are the first experiments that address a possible role for miRNA in human tendinopathy.

The work outlined in this thesis has a number of strengths and some limitations. Firstly, a major strength of this study lies in the in the fact that genotyping was conducted on three geographically distinct populations and hence our association data are not isolated to a single cohort. Secondly, studies conducted on the combined cohorts had good statistical power. Furthermore, successful attempts were made to match the height, weight, BMI and gender distribution of participants within the UK case and control groups however, there were differences in the mean age of participants. On the other hand, the AUS+SA cohort was matched for age and height but not for weight and BMI. There were differences in the amount of information collected from the three cohorts: for instance, the weight and BMI at the time of injury could not be obtained for the AUS or the SA cohorts. Another limitation of this thesis was the modest sample size of participants in the genetic association studies, as well as the epigenetic studies. Specifically, this thesis did not primarily aim at investigating gender specific genetic risk factors, therefore some of the gender specific associations were under powered due to the reduced numbers. Furthermore, there were four control Achilles tendon tissue samples and a single ATP sample to conduct DNA methylation, gene expression, and miRNA expression studies. The medical and familial history of the tissue

donors could not be obtained therefore it was not possible to assess the type or level of exposure to physical activity.

8.1 Future Directions

The work conducted in this thesis has opened a number of interesting lines for future research. Firstly, it has shed the light on the identification of additional biological pathways involved in the regulation and maintenance of the extracellular matrix within tendons. The identification of these pathways will contribute towards the expansion of the list of target genes which should consequently be tested for association with ATP. The gene variants identified as having an association with ATP, namely *FBN2* rs331079, *ELN* rs2071307, *COL5A1* rs12722, *COL5A1* rs71746744, *MMP3* rs679620, and *TIMP2* rs4789932 are likely to interact with other genetic and environmental factors that might also predispose to the pathology. Understanding the nature of such interactions is important in building risk assessment models. Furthermore, the current studies were conducted on a population size which provided a sufficient power of 80%. Obtaining a larger sample size will guarantee a greater confidence in the results and reduces the likelihood of an association due to error. A larger population size would allow population stratification without reducing the power to a level beneath 80%. Moreover, the replication of these studies in populations of different ethnicities is empirical as those have got different allelic and genotypic distribution frequencies which could reveal different associations with different effect sizes.

The functionality of the *TIMP2* rs4789932 and the *FBN2* rs2071307 has not been described. Therefore to understand the potential role played those variants in ATP, cloning experiments where DNA fragments holding the variant of interest are inserted into reporter plasmids, as performed by Laguette et al. (2011), could be conducted. Furthermore, conducting haplotype and pseudo-haplotype studies would add valuable information to our understanding of the linkage blocks associated with elevated risk since they have been successful in the mapping

of complex-disease genes (Niu et al. 2002). In fact, variants associated with disease could occur independently but share a common background haplotype. This would suggest that the associated variants have a common origin and raises the possibility of an undiscovered strongly predisposing variant (Croucher et al. 2003).

This thesis has for the first time investigated epigenetic factors in human tendinopathy. Differences in DNA methylation profiles between cases and controls have only been investigated in selected CpG sites. Future studies should widen the investigation to include additional genes, and assess the methylation profile of the whole promoter region.

Other epigenetic factors such as miRNAs have been investigated in relation to human tendinopathy for the first time in this thesis. However, this study was limited since there was only one ATP case with scarce information about the mode of injury, the age at the onset of symptoms, and the grade of damage in the tendon. Therefore, it would be highly advisable to repeat this study on a larger number of samples, and in tissue from patients from different ethnic populations. Furthermore, Chapter 7 discusses potential ways in which miRNAs can contribute to the pathology. However, it is not clear yet whether variation in miRNA expression between the tendinopathy and control samples is causative of the pathology, or on the other hand could be a normal response to the onset of the pathology. Answering this question will improve our understanding of the biochemical interactions within tendons before, during, and after the injury. Interestingly, if mutations in miRNA coding sequences are acquired, then it would be possible, for future studies, of thinking about designing gene therapy interventions. Moreover, miRNAs represent potentially good blood markers for several diseases such as asthma (Garbacki et al. 2011) and coronary artery disease (Hoekstra et al. 2010). Moreover, it has been described that exercise regulates miRNA expression tendon fibroblasts (Mendias, Gumucio & Lynch 2012) but the amount of miRNA released from tendons into the blood stream has not been established. Therefore, investigating exercise induced pathogenesis through miRNA alterations represents a good

field for future research. Once a better understanding of the expression and release of miRNA from tendon cells into the blood has been obtained, it would be possible to discuss the possibility of using serum miRNA levels as a screening tool for the risk of tendinopathy along with genetic risk assessment models.

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APPENDIX I

Ethical Approvals

UNIVERSITY OF CAPE TOWN

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

02 October 2008

REC REF: 289/2004

A/Prof M Collins
Human Biology

Dear A/Prof Collins

PROJECT TITLE: THE GENETIC BASIS OF ACHILLES TENDON PATHOLOGY IN AN AUSTRALIAN POPULATION

Thank you for your letter to the Research Ethics Committee dated 29th September 2008.

It is a pleasure to inform you that the Ethics Committee has **approved** the amendment with reference to the above-mentioned study.

Please can we have, if not yet submitted an updated report for 289/2004.

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

Please quote the REC. REF in all your correspondence.

Yours sincerely

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

lemjedi

THE UNIVERSITY OF NORTHAMPTON
School of Health

Thornby 2
Park Campus
Boughton Green Road
Northampton NN2 7AL
Tel: 01604 892816
Fax: 01604 791954

Dr Stuart Raleigh
Division of Sport, Exercise and Life Sciences
Brampton Building
Park Campus

Dear Stuart

Re: The Genetic Basis of Tendinopathy

Thank you for your application and associated paperwork for ethics approval for the above research project in collaboration with Professor Collins at the University of Cape Town.

I can now confirm that the ethics panel of the School of Health met on 1st October 2008 and has approved an extension to your original project involving genotyping Australian DNA samples.

May I take this opportunity to wish you well with your research.

Best wishes

Ali Ewing
Principal Lecturer

cc. Professor Carol Phillips
Associate Dean

THE UNIVERSITY OF NORTHAMPTON

The Graduate School
The University of Northampton
Boughton Green Road
Northampton
NN2 7AL

Mr Louis El Khoury
66 NewLife
Crispin Street
Northampton
NN1 2JH

8 November 2012

Dear Louis

I can confirm that the University of Northampton Research Ethics Committee considered your research proposal entitled 'Novel Genetic Factors that Predispose Humans to Achilles Tendon Pathologies' at its meeting on 16 June 2011.

The proposal was considered to be thorough, well thought out and informative. Full approval was given for you to carry out the proposed research.

If I can be of any further assistance, please contact me.

Yours sincerely

David Watson
Research Student Manager

Royal National Orthopaedic Hospital NHS
NHS Trust

R&D Office
% UCL-IOMS
RNOH, Brockley Hill
Stanmore HA7 4LP
Tel: 020 8909 5529
Fax: 020 8909 5273
E-mail: iva.hauptmannova@moh.nhs.uk

NHS R&D Management Approval Letter for Research

To: Professor Allen Goodship (cc Prof. A. Flanagan)

From: Iva Hauptmannova (R&D Manager)

Date: 10/01/10

Project Title: The Stanmore Musculoskeletal Tissue Bank

(REC Ref. 08/H0304.78 R&D Ref.09.016)

Sponsor: RNOH

I am writing on behalf of the Royal National Orthopaedic Hospital NHS Trust Stanmore, to inform you that the above named project has been approved by the Trust and may now proceed.

To maintain this approval, the following conditions must be met:

1. All staff involved in the running of this study must adhere to Trust and Research Governance Framework requirements.

2. As Chief/Principal Investigator you are required to formally advise the R&D Office of **ANY** changes to the project including:
   - Any changes to the status of the project, e.g. abandoned, completed etc
   - Any changes to the protocol – however minor.
   - Any changes to the funding arrangements.

3. The Chief/Principal Investigator is also required to:
   - Notify the R&D, in a timely fashion, any Serious Adverse Events relating to the Research and the appropriate urgent safety measures taken in line with HTA requirements.
   - Ensure that the R&D Office has copies of all annual and final progress reports.
   - Ensure that annual progress report forms are submitted to REC, which has given a favourable opinion.

- Ensure all researchers involved in the project hold the necessary expertise required and have Honorary Contracts should they need to.
- Ensure adequate and accurate reporting and monitoring of said project.
- Co-operate with all internal Trust monitoring and auditing procedures.

4. Because it is a statutory requirement to submit annual reports, this approval will automatically lapse if no annual report on this study is received at the R&D office, 14 months from the date of this letter. If you need help on how to prepare your annual report, please contact the R&D Office at the address on this letter.

Yours sincerely,

Iva Hauptmannova
R&D Manager

RESEARCH SERVICES

MEMORANDUM

To:	Dr Tom Samiric, School of Human Biosciences, Faculty of Health Sciences
From:	Secretary, La Trobe University Human Ethics Committee
Subject:	Review of Human Ethics Committee Application No. 12-086
Title:	Epigenetic investigation of normal and abnormal human patellar tendons
Date:	31 October 2012

Thank you for your recent correspondence in relation to the research project referred to above. The project has been assessed as complying with the *National Statement on Ethical Conduct in Human Research*. I am pleased to advise that your project has been granted ethics approval and you may commence the study.

The project has been approved from the date of this letter until 31 December 2015.

Please note that your application has been reviewed by a sub-committee of the University Human Ethics Committee (UHEC) to facilitate a decision about the study before the next Committee. meeting. This decision will require ratification by the full UHEC at its next meeting and the UHEC reserves the right to alter conditions of approval or withdraw approval. You will be notified if the approval status of your project changes. The UHEC is a fully constituted Ethics Committee in accordance with the National Statement on Ethical Conduct in Research Involving Humans- March 2007 under Section 5.1.29.

The following standard conditions apply to your project:

- **Limit of Approval.** Approval is limited strictly to the research proposal as submitted in your application while taking into account any additional conditions advised by the UHEC.

- **Variation to Project.** Any subsequent variations or modifications you wish to make to your project must be formally notified to the UHEC for approval in advance of these modifications being introduced into the project. This can be done using the appropriate form: *Ethics - Application for Modification to Project* which is available on the Research Services website at *http://www.latrobe.edu.au/research-services/ethics/HEC_human.htm*. If the UHEC considers that the proposed changes are significant, you may be required to submit a new application form for approval of the revised project.

- **Adverse Events.** If any unforeseen or adverse events occur, including adverse effects on participants, during the course of the project which may affect the ethical acceptability of the project, the Chief Investigator must immediately notify the UHEC Secretary on telephone (03) 9479 1443. Any complaints about the project received by the researchers must also be referred immediately to the UHEC Secretary.

- **Withdrawal of Project.** If you decide to discontinue your research before its planned

completion, you must advise the UHEC and clarify the circumstances.

- **Annual Progress Reports.** If your project continues for more than 12 months, you are required to submit an *Ethics - Progress/Final Report Form* annually, **on or just prior to 12 February**. The form is available on the Research Services website (see above address). Failure to submit a Progress Report will mean approval for this project will lapse. An audit may be conducted by the UHEC at any time.

- **Final Report.** A Final Report (see above address) is required within six months of the completion of the project or by **30 June 2016.**

If you have any queries on the information above or require further clarification please contact me through Research Services on telephone (03) 9479-1443, or e-mail at: **humanethics@latrobe.edu.au**.

On behalf of the University Human Ethics Committee, best wishes with your research!

Ms Barbara Doherty
Administrative Officer (Research Ethics)
University Human Ethics Committee
Research Compliance Unit / Research Services
La Trobe University Bundoora, Victoria 3086
P: (03) 9479 – 1443 / F: (03) 9479 - 1464
http://www.latrobe.edu.au/research-services/ethics/HEC_human.htm

APPENDIX II

Information Sheet and Consent Form

PARTICIPANT INFORMATION

Background
Thank you for showing an interest in this new study that is going to be conducted at The University of Northampton. The study is being carried out by Dr Stuart Raleigh who is a Reader and a researcher in Molecular Biology, Professor William Ribbans who is a consultant orthopaedic surgeon and Mr Louis El Khoury who is a researcher and PhD student. All three are based at the University of Northampton's School of Health and Professor Ribbans is also based at the County Clinic, Northampton. The contact details of the team are found towards the end of this leaflet. Please feel free to contact them at any time if you require any additional information.

What is the study about?
We are trying to establish whether certain individuals carry particular versions of genes (known as alleles) that may increase their risk of Achilles tendon problems. We are particularly interested to learn more about the types of Achilles tendon problems called Achilles tendinopathy (swollen, painful tendon) and Achilles tendon rupture. The study is about Achilles tendon problems in humans – we never use animals – and we would greatly value you as a volunteer.

Why are we interested?
We are interested because many people with relatively active lifestyles as well as professional athletes experience Achilles tendon problems during or after sports activities. This can sometimes lead to long term disability. In some cases the injury needs surgery and some individuals are reluctant, or have difficulty returning to a sport, or physical activity, they once enjoyed. This research might eventually lead to a way of reducing the number of people who get tendon problems in the future or to better treatment for those already affected.

What's involved if I participate?
We would like individuals to donate a small sample of their spit (saliva) for DNA analysis. We only want about 2 ml of your saliva (the same as one teaspoonful!) which can be taken from simply spitting into a tube. The procedure would take about 3 minutes to perform. You don't even have to attend a clinic. We can send you the tube to spit into. We would also like you to answer some background questions and details about your health and any exercise that you do. If you would like to take part, please complete, sign, and return the consent from in the paid post envelope and we will send you a questionnaire and a saliva collection tube. The total time taken to collect the saliva sample and complete the questionnaire is about 10-15 minutes.

What will happen to my saliva sample?
Your saliva sample will ONLY be used for Achilles tendon studies. Once we receive your sample, it will be labelled with a unique number only and stored in a secure freezer at the University of Northampton's Park campus. We will isolate DNA from your saliva sample. We will then compare the genes between various groups of individuals to establish whether samples from people who have had Achilles tendon problems contain different versions compared to people who don't have tendon problems. We would like to investigate many genes from your sample and in order for us to do this we would like to retain your sample indefinitely. There may be occasions when we might want to send a small amount of your sample to scientists

that we do joint studies with. This would only be done if they could help us with our study. However, if this does happen the sample they receive will only have the number on it and it would not be possible for you to be identified.

Will my sample be used for studies investigating any other disease?
No. The genes that we are interested in analysing are only relevant to musculoskeletal soft tissue injuries (tendon injuries). It will not be possible for us to investigate other illnesses such as: cancer and cardiovascular diseases.

Will the information I provide be kept private?
Yes. We will not send any of your private contact details to any other party. Your personal contact details will only be seen by the three members of the study team. Indeed your personal contact details will be stored securely in a locked filing cabinet at the university and on a password protected computer. The data will not be on any networked computer drives. To make things even more secure your saliva sample and the DNA that we isolate from it will only be labelled with a number. It would not be possible for any person, other than the study team, to identify you from the number that your sample will be allocated.

How long will you be keeping my personal information?
We would like to retain you personal information for an indefinite amount of time. Nevertheless, if you want to end your participation in this project at any time we will immediately destroy your personal information as well as your DNA sample. However any data obtained from the analysis of your sample up until that point will remain part of the research and will be included in any published work.

Why do you need information about my previous medical history?
We need to ask these questions because certain ailments that you may have had in the past might be important to Achilles tendon studies. If we don't have the data we could make a false conclusion in our study.

Why do you need to know about my lifestyle?
This is because it may be lifestyle factors that are most important in causing people to have Achilles problems. Providing this information will help us to establish whether this is true or not.

Why do you need to know about my ethnicity?
This is because certain genetic factors might be different in people of different ethnicity. Having details about a person's ethnicity can help us build a more accurate assessment of the role of genes in causing any Achilles tendon problems. Without details on ethnicity the data we generate might be flawed.

Why do you need my name and contact details?
This is for two main reasons. Firstly we may want to invite you to be part of some future study and might send you a letter. Of course, it would be entirely up to you whether or not you replied to any future invites. You can always send us a request not contact you for any future studies. Secondly, very rarely, samples that are provided for a study can be accidently damaged during the laboratory procedure. If this does happen we would like to think that we could contact you again for a replacement sample. If we were not able to contact you then the valuable sample that you kindly donated would have been lost from the investigation and this might lead to less accurate scientific results.

What happens if I want to be involved but later change my mind?
You simply contact us and let us know. We will then immediately destroy any sample and information that you have donated to us.

Will I be able to obtain information about my own DNA sample?
The study that we are conducting is aimed at understanding how genetic factors affect the Achilles tendon by studying a group of people. It will be difficult to predict any risk on an individual basis. This is why this study is not designed to test individual genetic status. For this reason we do not plan to give back individual results to participants. If you require addition information about this, please contact us on the numbers or e-mails provided on the last page.

How can I obtain information about the progress of the research?
After roughly 18 months, information about the preliminary results of the study will be posted on the University of Northampton School of Health website. For more information please visit:
(www.northampton.ac.uk/info/20038/school-of-health)

What will happen to the data obtained from this study?
We would like to publish this in scientific journals. The publications would never reveal any participant identity or contact details.

What benefit do I get from being involved in this study?
Many scientific studies rely on sample donations from the general population. By taking part in this study you will help to further our understanding of why some people, particularly active sportspeople, develop Achilles tendon problems. Eventually the findings may help people to be treated for the condition. Furthermore, it might be possible that in the future we could identify those most at risk of developing the problem. If this is the case we could educate people about the suitability of sports that they do and advise them on their risk. The finding may also be used to enhance our understanding of how human tendons work under stressed conditions.

We thank you for reading the above information. If you feel that you would like to be involved then please complete and return the consent form on the next page. Please remember that if you have any questions then please contact the study team who will be very pleased to talk you through any aspect of this study.

Contact Details

Louis El Khoury
MX – 05
University of Northampton
Boughton Green Road
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Tel: 01604 892512
louis.elkhoury@northampton.ac.uk

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Tel: 01604 795414
wjribbans@uk-consultants.co.uk

CONSENT FORM

If you would like to participate in this study then please complete the consent form below. If you have any further questions relating to the study, or if there is something that you wish to discuss with us then please contact us at the addresses provided above.

Please tick the box that corresponds to your choice.

	Yes	No
I have read the study information sheet and understand what is involved.	☐	☐
I understand that my sample will be analysed anonymously and my contact details kept confidential. My contact details will only be used by the researcher if my sample is accidently damaged. It would be up to me whether I provided another sample.	☐	☐
I understand that I can withdraw my participation at any time and that my sample and information would be destroyed upon my request.	☐	☐
I am willing to participate in this project.	☐	☐
I would be interested in receiving information about future studies. My agreement to this would mean my contact details would be retained securely and for this purpose alone.	☐	☐

Signed: Date:

Name (Print)
Address

Phone Number E-mail:

We appreciate your time and your willingness to participate in this study.
Participant number (to be entered by the research team)

APPENDIX III

Questionnaires

Achilles Tendon Datasheet

We greatly appreciate your interest in participating in this project. Please complete as much of this questionnaire as possible keeping in mind the following points:

1. This questionnaire is printed on both sides of every sheet. Please pay attention at answering the questions on the back of each sheet.
2. Throughout the questionnaire you might come across scientific terms followed by small numbers such as: ACL[5]. These terms are explained in the glossary on the last page of this questionnaire.
3. Please feel free to skip any question you are having difficulty with.
4. Please mail us back the questionnaire and a team member will contact you to go over any question you are having difficulty with.
5. If you have any concern about the aspect of this project please feel free to contact us on the numbers or e-mails below..

Louis El Khoury
Tel: 01604 892512
louis.elkhoury@northampton.ac.uk

Dr Stuart Raleigh
Tel: 01604 892306
stuart.raleigh@northampton.ac.uk

Prof William Ribbans
Tel: 01604 795414
wjribbans@uk-consultants.co.uk

ACHILLES TENDON STUDY – Control

We would greatly appreciate you providing us with your personal details. However if you prefer not to fill in this section, please proceed to the next page.

SECTION 1: PERSONAL DETAILS			
Study Number To be completed by research team so leave blank			
Surname		First Name	
Address		Contact Telephone Number	
e-mail		Occupation	

Date of birth Age	D D / M M / Y e a r	Years and months		
Height	Cms:	Ft and Ins:	Gender	male female
Weight (in underwear)	Kgs:	Stone & Lbs:	BMI To be completed by the research team so leave blank.	
Ethnic group	Black/African Mixed Ancestry	White Chinese	Asian Other	
Nationality			Dominant Hand	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 different sports or physical activities, please complete section 2 on Sporting and Recreational Details.

SECTION 2: SPORTING AND RECREATIONAL DETAILS			
Type of sport/ physical activity 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			
Have you been involved in the sport at a professional level? If so, please state how many years.			
Level of Participation: e.g. Leisure/Club/Regional/International			
Average hours of training per week - in the last 12 months			
Type of sport/ physical activity 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			
Have you been involved in the sport at a professional level? If so, please state how many years.			
Level of Participation: e.g. Leisure/Club/Regional/International			
Average hours of training per week - in the last 12 months			

If you Run/Jog, please specify the distance crossed every week: .....

SECTION 3: GENERAL MEDICAL DETAILS

Do you suffer from any Connective Tissue and Rheumatological Diseases and Disorders[1]?

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

Has any member of your family (Blood relatives) suffered from any Achilles tendon injury/problem?	Yes ☐ No ☐ Unknown ☐	If Yes, please specify the family member (eg Mother, Son) and type of injury _____ Rupture ☐ 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 ☐ Unknown ☐
Have you been diagnosed with any of the following diseases?	☐ Diabetes mellitus ☐ Adrenal disorders ☐ Thyroid disorders ☐ Amyloidosis ☐ Renal disease ☐ Other endocrine and metabolic disease (Specify) _____ _____ _____	

Drug and Allergy History	If yes, how long ago (or how many times, where applicable) did you use the medication?	
Have you ever used oral corticosteroids[2] (cortisone tablets)?	Yes ☐ No ☐ Unknown ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months
Have you ever been given an injection with corticosteroids?	Yes ☐ No ☐ Unknown ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months
Have you ever been given an injection of corticosteroids in or around a tendon?	Yes ☐ No ☐ Unknown ☐	☐ Once ☐ Twice ☐ 3 times ☐ >3 times
Have you ever used anabolic steroids[3]?	Yes ☐ No ☐ Unknown ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months
Have you ever used fluoroquinolone antibiotics? (see list below)	Yes ☐ No ☐ Unknown ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months

If yes, please select from the list below:

☐ CIPROFLOXACIN	☐ MOXIFLOXACIN	☐ NORFLOXACIN
☐ CIPROXIN	☐ AVELOX	☐ UTINOR
☐ LEVOFLOXACIN	☐ NALIDIXIC ACID	☐ OFLOXACIN
☐ TAVANIC	☐ URIBEN	☐ TARVID

What medication, if any, are you currently using? (please list)	
Do you suffer from any allergy? (please list)	Yes ☐ No ☐ Unknown ☐

SECTION 4: HISTORY OF LIGAMENT AND TENDON INJURIES/PROBLEMS				
Have you ever had a ligament[4] injury/problem in the past?	Yes ☐ No ☐ Unknown ☐			
If yes, please specify which ligaments? (You may tick more than one block, please select either L (left) or R (right)) The Abreviated medical terms are explained in the glossary on the last page.		L R		L R
	Knee (ACL)[5]	☐ ☐	Knee (PCL)[7]	☐ ☐
	Knee (MCL)[6]	☐ ☐	Knee (LCL)[8]	☐ ☐
	Ankle lateral ligaments	☐ ☐	Ankle medial ligaments	☐ ☐
	Spinal ligaments	☐ ☐	Finger ligaments	☐ ☐
	Shoulder ligaments	☐ ☐	Wrist ligaments	☐ ☐
	Elbow ligaments	☐ ☐	Other ligaments	☐ ☐
To your knowledge, have any other members of your family suffered from any ligament injury/problem?	Yes ☐ No ☐ Unknown ☐	If Yes, please specify the family member ☐ Mother ☐ Father ☐ Sibling ☐ Son / daughter ☐ Other family member …….. and condition: Please choose ligament injury from the list above ……		
Have you ever injured a tendon[9] in the past?	Yes ☐ No ☐ Unknown ☐			
If yes, please specify which tendon? (You may tick more than one block, please select either L (left) or R (right))	Foot and ankle:		L R	
		Achilles tendon	☐ ☐	
		Tibialis posterior	☐ ☐	
		Plantar fascia	☐ ☐	
	Knee:	Patellar tendon	☐ ☐	
	Elbow and wrist:	Wrist extensor tendons	☐ ☐	
	Shoulder:	Subscapularis	☐ ☐	
		Supraspinatus	☐ ☐	
		Infraspinatus	☐ ☐	
		Teres minor	☐ ☐	
	Other:……			

To your knowledge, have any other members of your family suffered from any tendon injury/problem?	Yes ☐ No ☐ Unknown ☐	If Yes, please specify the family member ☐ Mother ☐ Father ☐ Sibling ☐ Son / daughter ☐ Other family member:…… Condition: Please choose tendon injury from the list above …….….
Have you ever suffered from any of the following injuries?	☐ Acute shoulder dislocation ☐ Chronic shoulder instability ☐ Chronic ankle instability _____ _____	

Thank you for taking time to complete this questionnaire. Your participation is greatly appreciated. Please go on to sign the consent document that you have been sent.

Please return your **saliva sample**, **completed questionnaire** and **consent form** to us as described in the "participant information" sheet.

If you are unsure about any aspect of this project or require further information please contact the study team using the contact numbers, or emails, found on the front page of this questionnaire.

Glossary

1. **Rheumatologic disease**: A type of disease involving inflammation of muscles, joints, and other tissues
2. **Corticosteroid**: a group of steroid hormones used to treat inflammation
3. **Anabolic steroid**: a synthetic steroid hormone that resembles testosterone in promoting the growth of muscle. Such hormones are used medicinally to treat some forms of weight loss and, by some athletes and others to enhance physical performance
4. **Ligament**: A short band of tough, flexible, fibrous connective tissue that connects two bones or cartilages or holds together a joint
5. **ACL**: Anterior cruciate ligament
6. **MCL**: medial collateral ligament
7. **PCL**: Posterior cruciate ligament
8. **LCL**: Lateral collateral ligament
9. **Tendon**: A flexible but inelastic cord of strong fibrous collagen tissue attaching a muscle to a bone

Achilles Tendon Datasheet

We greatly appreciate your interest in participating in this project. Please complete as much of this questionnaire as possible keeping in mind the following points:

6. This questionnaire is printed on both sides of every sheet. Please pay attention at answering the questions on the back of each sheet.
7. Throughout the questionnaire you might come across scientific terms followed by small numbers such as: ACL[5]. These terms are explained in the glossary on the last page of this questionnaire.
8. Please feel free to skip any question you are having difficulty with.
9. Please mail us back the questionnaire and a team member will contact you to go over any question you are having difficulty with.
10. If you have any concern about the aspect of this project please feel free to contact us on the numbers or e-mails below.

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ACHILLES TENDON DATASHEET – Case

We would greatly appreciate you providing us with your personal details. However if you prefer not to fill in this section, please proceed to the next page.

SECTION 1: PERSONAL DETAILS			
Study Number To be completed by research team so leave blank			
Surname		First Name	
Address		Contact Telephone Number	
e-mail		Occupation	

Date of birth Age	DD/MM/Year Years and months			
Height	Cms:	Ft and Ins:	Gender	male female
Weight (currently and at first injury)	Kgs:	Stone & Lbs:	BMI To be completed by the research team so leave blank.	
Ethnic group	Black/African White Asian Mixed Ancestry Chinese Other			
Nationality			Dominant Hand	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 different sports, please complete section 2 on Sporting and Recreational Details.

SECTION 2: SPORTING AND RECREATIONAL 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			
Have you been involved in the sport at a professional level? If so, please state how many years.			
Level of Participation: e.g. Leisure/Club/Regional/International			
Average hours of training per week - in the last 12 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			
Have you been involved in the sport at a professional level? If so, please state how many years.			
Level of Participation: e.g. Leisure/Club/Regional/International			
Average hours of training per week - in the last 12 months			

If you Run/Jog, please specify the distance crossed every week: .....

SECTION 3: GENERAL MEDICAL DETAILS

Do you suffer from any Connective Tissue and Rheumatological Diseases and Disorders[1]?

- ☐ Ankylosing Spondylitis
- ☐ Aspartylglycosaminuria (AGU)
- ☐ Behcet's Syndrome
- ☐ Crohn's Disease
- ☐ Discoid Lupus Erythematosus
- ☐ Ehlers-Danlos syndrome (EDS)
- ☐ Eosinophilic Fascitis
- ☐ Giant Cell (Temporal) Arthritis
- ☐ Gout
- ☐ Hypertensive Vasculitis
- ☐ Lipid Storage Diseases
- ☐ Marfan Syndrome
- ☐ Menkes Kinky Hair Syndrome
- ☐ Mucopolysaccharidoses
- ☐ Myopathies and Dystrophies
- ☐ Ochronosis (Homocystinuria)
- ☐ Osteoarthritis
- ☐ Osteogenesis imperfecta (OI)
- ☐ Polyarteritis Nodosa
- ☐ Polymyalgia Rheumatica
- ☐ Polymyositis & Dermatomyositis
- ☐ Pseudogout
- ☐ Reactive Arthritis
- ☐ Reiter's Syndrome
- ☐ Relapsing Polychrondritis
- ☐ Rheumatoid Arthrits
- ☐ Scleroderma
- ☐ Sjogren's Syndrome
- ☐ Systemic Lupus Erythematosus (SLE)
- ☐ Systemic Sclerosis
- ☐ Wegener's Granulomatosis
- ☐ No, I don't suffer of any of these diseases

Have any other members of your family (Blood relatives) suffered from any Achilles tendon injury/problem?	Yes ☐ No ☐ Unknown ☐	If Yes, please specify the family member (eg Mother, Son) and type of injury _____ Rupture ☐ 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 ☐ Unknown ☐
Have you been diagnosed with any of the following diseases?	☐ Diabetes mellitus ☐ Adrenal disorders ☐ Thyroid disorders ☐ Amyloidosis ☐ Renal disease ☐ Other endocrine and metabolic disease (Specify) _____ _____ _____	

Drug and Allergy History	If yes, how long ago (or how many times, where applicable) did you use the medication?	
Have you ever used oral corticosteroids[2] (cortisone tablets)?	Yes ☐ No ☐ Unknown ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months
Have you ever been given an injection with corticosteroids?	Yes ☐ No ☐ Unknown ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months
Have you ever been given an injection of corticosteroids in or around a tendon?	Yes ☐ No ☐ Unknown ☐	☐ Once ☐ Twice ☐ 3 times ☐ >3 times
Have you ever used anabolic steroids[3]?	Yes ☐ No ☐ Unknown ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months
Have you ever used fluoroquinolone antibiotics? (see list below)	Yes ☐ No ☐ Unknown ☐	☐ 3 months ☐ 6 months ☐ 12 months ☐ 24 or more months

If yes, please select from the list below:

☐ CIPROFLOXACIN	☐ MOXIFLOXACIN	☐ NORFLOXACIN
☐ CIPROXIN	☐ AVELOX	☐ UTINOR
☐ LEVOFLOXACIN	☐ NALIDIXIC ACID	☐ OFLOXACIN
☐ TAVANIC	☐ URIBEN	☐ TARVID

What medication, if any, are you currently using? (please list)	
Do you suffer from any allergy? (please list)	Yes ☐ No ☐ Unknown ☐

SECTION 4: HISTORY OF LIGAMENT AND TENDON INJURIES/ PROBLEMS				
Have you ever had a ligament[4] injury/problem in the past?	Yes ☐ No ☐ Unknown ☐			
If yes, please specify which ligaments? (You may tick more than one block, please select either L (left) or R (right)) The abbreviated medical terms are explained in the glossary on the last page.		L R		L R
	Knee (ACL)[5]	☐ ☐	Knee (PCL)[7]	☐ ☐
	Knee (MCL)[6]	☐ ☐	Knee (LCL)[8]	☐ ☐
	Ankle lateral ligaments	☐ ☐	Ankle medial ligaments	☐ ☐
	Spinal ligaments	☐ ☐	Finger ligaments	☐ ☐
	Shoulder ligaments	☐ ☐	Wrist ligaments	☐ ☐
	Elbow ligaments	☐ ☐	Other ligaments	☐ ☐
To your knowledge, have any other members of your family suffered from any ligament injury/problem?	Yes ☐ No ☐ Unknown ☐	If Yes, please specify the family member ☐ Mother ☐ Father ☐ Sibling ☐ Son / daughter ☐ Other family member …….. and condition: Please choose ligament injury from the list above ……		
Have you ever had a tendon[9] injury/problem in the past?	Yes ☐ No ☐ Unknown ☐			
If yes, please specify which tendon? (You may tick more than one block, please select either L (left) or R (right))	Foot and ankle:	L R		
		Achilles tendon ☐ ☐		
		Tibialis posterior ☐ ☐		
		Plantar fascia ☐ ☐		
	Knee:	Patellar tendon ☐ ☐		
	Elbow and wrist:	Wrist extensor tendons ☐ ☐		
	Shoulder:	Subscapularis ☐ ☐		
		Supraspinatus ☐ ☐		
		Infraspinatus ☐ ☐		
		Teres minor ☐ ☐		
	Other:……			

To your knowledge, have any other members of your family suffered from any tendon injury/problem?	Yes ☐ No ☐ Unknown ☐	If Yes, please specify the family member ☐ Mother ☐ Father ☐ Sibling ☐ Son / daughter ☐ Other family member:…… Condition: Please choose tendon injury from the list above ……
Have you ever suffered from any of the following injuries?	☐ Acute shoulder dislocation ☐ Chronic shoulder instability ☐ Chronic ankle instability _____ _____	

SECTION 5: DETAILS OF ACHILLES TENDON INJURY							
How many times have you had tendon injuries/problems? *Sudden onset is within a few seconds or minutes ^Gradual onset is over days or weeks	Event	Date	Right or Left	Acute or Chronic Injury	Sudden* or Gradual^ Onset	Effect of injury in first month following injury	Grade of injury currently
	1.					☐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	☐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
	2.					☐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	☐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
	3.					☐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	☐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

	4.					☐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	☐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
	5.					☐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	☐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

Thank you for taking time to complete this questionnaire. Your participation is greatly appreciated.

Please can you return your **saliva sample**, and the **completed questionnaire** to us as described in the 'participant information' sheet.

If you are unsure about any aspect of this project or require further information please contact the study team using the contact numbers, or emails, found on the front page of this questionnaire.

Glossary

10. **Rheumatologic disease**: A type of disease involving inflammation of muscles, joints, and other tissues
11. **Corticosteroid**: a group of steroid hormones used to treat inflammation
12. **Anabolic steroid**: a synthetic steroid hormone that resembles testosterone in promoting the growth of muscle. Such hormones are used medicinally to treat some forms of weight loss and, by some athletes and others to enhance physical performance
13. **Ligament**: A short band of tough, flexible, fibrous connective tissue that connects two bones or cartilages or holds together a joint
14. **ACL**: Anterior cruciate ligament
15. **MCL**: medial collateral ligament
16. **PCL**: Posterior Cruciate Ligament
17. **LCL**: Lateral collateral ligament
18. **Tendon**: A flexible but inelastic cord of strong fibrous collagen tissue attaching a muscle to a bone

SECTIONS 7, 8, 9 AND 10 TO BE FILLED OUT BY CLINICIAN

SECTION 7: ASSESSMENT OF ACHILLES TENDON INJURY BY CLINICIAN		
Event Number (1,2,3,4,or 5)		
FINDING	YES	NO
Symptoms > 6/12		
Early morning pain over the Achilles area		
Early morning stiffness over the Achilles area		
History of swelling over the Achilles area		
Tenderness to palpation over the Achilles area		
Palpable nodular thickening over the Achilles tendon		
Positive Shift test: **Movement of painful/swollen area with DF/PF of ankle**		
Other comments:		

SECTION 8: DESCRIPTION OF ACHILLES TENDON INJURY Please complete a separate form for each Tendon Injury			**Event Number:**	
			Right	Left
Rupture:				
	Full			
	Partial			
	Insertional avulsion			
Tendinopathy:				
	Non-insertional:			
		Peritendonitis		
		Tendinosis		
		Peritendonitis + Tendinosis		
		Other		
		Unknown		
	Insertional:			
		Haglund's		
		Lateral Calcaneal Ridge		
		Intra-tendinous calcification		
		Achilles Bursitis - superficial		
		Achilles Bursitis -deep		
		Tendinosis		

SECTION 9: IMAGING OF ACHILLES TENDON INJURY Please complete a separate form for each Tendon Injury			**Event Number:**
Type	Date	Location	Details

SECTION 10: DETAILS O F TREATMENT Please complete a separate form for each Tendon Injury				**Event Number:**
Date	Type	Location	Duration	Outcome

APPENDIX IV

Experimental Protocols

prepIT®·L2P

Laboratory protocol for manual purification of DNA from 0.5 mL of sample

For the purification of genomic DNA from the Oragene® and ORAcollect® families of collection kits.

Visit our website at www.dnagenotek.com for any additional languages and protocols.

The following step-by-step protocol describes how to purify DNA from a 500 µL aliquot of sample.

Reagents included

- prepIT®•L2P (catalog #: PT-L2P)

Equipment and reagents

- Microcentrifuge capable of running at 15,000 × *g*
- 1.5 mL microtubes (e.g., Axygen #MCT-150-C)
- Air or water incubator at 50°C
- Ethanol (95% to 100%) at room temperature
- Ethanol (70%) at room temperature
- DNA storage buffer: TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or similar solution

Procedure

Purification steps	Notes
1. Mix the sample in the DNA Genotek kit by inversion and gentle shaking for a few seconds.	• This is to ensure that viscous samples are properly mixed.
2. Incubate the sample at 50°C in a water incubator for a minimum of 1 hour or in an air incubator for a minimum of 2 hours. **Note:** The use of an air incubator may be preferable since the sample tubes may float in a water bath. If a water bath must be used, ensure the sample-containing portion of the tube remains immersed in water.	• This heat-treatment step is essential to ensure that DNA is adequately released and that nucleases are permanently inactivated. • This incubation step may be performed at any time after sample is collected and before it is purified. • The entire sample must be incubated in the original collection tube before aliquoting to ensure sample homogeneity. • The sample may be incubated at 50°C overnight if it is more convenient. • A longer time is required in an air incubator because temperature equilibration is slower than in a water incubator.
3. Transfer 500 µL of the mixed sample to a 1.5 mL microcentrifuge tube.	• The remainder of the sample can be stored at room temperature or frozen (-15°C to -20°C).
4. For 500 µL of sample, add 20 µL (1/25th volume) of PT-L2P to the microcentrifuge tube and mix by vortexing for a few seconds.	• The sample will become turbid as impurities and inhibitors are precipitated.

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Purification steps	Notes
5. Incubate on ice for 10 minutes.	• Room temperature incubation can be substituted but will be slightly less effective in removing impurities.
6. Centrifuge at room temperature for 5 minutes at 15,000 × *g*.	• A longer period of centrifugation (up to 15 minutes) may be beneficial in reducing the turbidity (high A_{320}) of the final DNA solution.
7. Carefully transfer the clear supernatant with a pipette tip into a fresh microcentrifuge tube. **Discard the pellet containing impurities.**	• The pellet contains turbid impurities. If accidentally disturbed, the tube should be re-centrifuged.
8. To 500 µL of supernatant, add 600 µL of room temperature 95% to 100% ethanol. Mix gently by inversion 10 times.	• During mixing with ethanol, the DNA will be precipitated. This may appear as a clot of DNA fibers or as a fine precipitate, depending upon the amount of DNA in the sample. • Even if no clot is seen, DNA will be recovered by carefully following the next steps.
9. Allow the sample to stand at room temperature for 10 minutes to allow the DNA to fully precipitate.	• Incubation at -20°C is not recommended because impurities may co-precipitate with the DNA.
10. Place the tube in the microcentrifuge in a known orientation. Centrifuge at room temperature for 2 minutes at 15,000 × *g*.	• For example, place each tube in the microcentrifuge with the hinge portion of the cap pointing away from the centre of the rotor. After centrifugation, the position of the pellet can be located (even if too tiny to be easily visible), it will be at the tip of the tube below the hinge.
11. Carefully remove the supernatant with a pipette tip and discard it. Take care to avoid disturbing the DNA pellet.	• This pellet contains DNA. Loss of the pellet will result in loss of the DNA. • Rotating the tube such that the pellet is on the upper wall will allow you to safely move a pipette tip along the lower wall and remove all of the supernatant. • The supernatant may contain impurities and should be removed as completely as possible. • Excessive drying of the pellet can make the DNA more difficult to dissolve.
12. Ethanol wash: Carefully add 250 µL of 70% ethanol. Let stand at room temperature for 1 minute. **Completely remove the ethanol without disturbing the pellet.**	• **It is important to remove all ethanol from the sample. Carryover of ethanol may impact the performance of the assay.** • Take care not to disturb the DNA pellet. • The DNA pellet may be small. • Should the pellet detach, centrifuge the sample for 5 minutes at 15,000 x *g*. • After removing the 70% ethanol the tube can be pulse-spun to allow removal of residual ethanol.

Purification steps	Notes
13. Add 100 µL of TE solution (see Page 1) to dissolve the DNA pellet. Vortex for at least 5 seconds.	• If a higher concentration of DNA is desired, 50 µL of TE should be used. • **Note:** large amounts of high molecular weight DNA can be slow to hydrate (dissolve) completely. • Incomplete hydration of the DNA is a cause of inaccuracy in estimating DNA concentration and of failure of downstream applications such as PCR.
14. To ensure complete rehydration of the DNA (pellet and smear) incubate at room temperature overnight followed by vortexing or at 50°C for 1 hour with occasional vortexing.	• Incomplete rehydration of the DNA is a cause of inaccuracy in estimating DNA concentration and potential failure of downstream applications such as PCR.
15. Options for storage of the fully rehydrated DNA: a) **Recommended** in TE, in aliquots at -20°C for long-term storage, or b) In TE at 4°C for up to 2 months.	• Freezing of purified DNA in TE will cause DNA to precipitate. When thawing a sample of frozen purified DNA, pay careful attention to rehydration, as discussed in step 14.

Quantification of DNA

By fluorescence method

Assays that use fluorescent dyes are more specific than absorbance at 260 nm for quantifying the amount of double-stranded DNA (dsDNA) in a DNA sample. We recommend using fluorescent dyes such as PicoGreen® or SYBR® Green I to quantify dsDNA since there is less interference by contaminating RNA. An inexpensive protocol using SYBR Green I is described in PD-PR-075, *DNA quantification using SYBR Green I Dye and a micro-plate reader*[1]. Alternatively, commercially available kits such as Invitrogen's Quant-iT™ PicoGreen dsDNA Assay Kit (Cat. No. Q-33130) can be used. For either protocol, we recommend that the purified DNA be diluted 1:50 with TE solution and that 5 µL be used in the quantification assay.

By absorbance method

If you choose to quantify DNA by absorbance, we recommend that you first treat the purified sample with RNase to digest contaminating RNA and then remove the RNA fragments by ethanol precipitation of the DNA. A detailed protocol is described in PD-PR-040, *RNA removal by double-RNase digestion*[2]. Please note that DNA from an oral sample typically contains appreciably more RNA than found in blood samples. Ensure that alcohol-precipitated DNA is fully dissolved before reading the absorbance.

Conversion factor: An absorbance of 1.0 at 260 nm corresponds to a concentration of 50 ng/µL (50 µg/mL) for pure dsDNA.

Ensure that absorbance values are within the linear range of the spectrophotometer. Re-dilute and re-measure samples that fall outside of the linear range. See your instrument documentation for more information.

Protocol: Purification of RNA or Multiple Analytes from Animal and Human Tissues

This protocol provides guidelines on disrupting animal and human tissues for purification of RNA or for simultaneous purification of DNA and RNA or DNA, RNA, and protein. If using a QIAGEN sample purification kit (see Tables 1, 2, and 6 on pages 7, 8, and 10), refer to the supplied handbook, which contains a complete protocol for sample disruption and purification.

Important points before starting

- Before beginning the procedure, read "Important Notes" (page 13).
- Ensure that you are familiar with operating the TissueLyser LT by referring to the *TissueLyser LT User Manual*.
- If using a QIAGEN sample purification kit, read the supplied handbook carefully before starting.
- After storage in RNA*later* RNA Stabilization Reagent or Allprotect Tissue Reagent, tissues become slightly hard. If disrupting in Buffer RLT, we recommend increasing the volume of this buffer according to the protocols in the *RNeasy Mini Handbook*.
- When disrupting tough or very tough samples, we recommend using one or two 7 mm stainless steel beads, respectively, instead of one 5 mm stainless steel bead to guarantee optimal disruption.

Procedure

1. **Place 2 ml microcentrifuge tubes containing 1 stainless steel bead (5 mm mean diameter) on dry ice for at least 15 min. Keep the insert of the TissueLyser LT Adapter at room temperature (15–25°C).**
2. **Transfer up to 30 mg fresh or frozen tissue to the precooled tubes and incubate for another 15 min on dry ice.**

   If handling tissue samples stabilized with RNA*later* RNA Stabilization Reagent or Allprotect Tissue Reagent, cooling on dry ice is not necessary.
3. **Place the tubes into the insert of the TissueLyser LT Adapter, and incubate at room temperature for 2 min to avoid freezing of lysis buffer in step 4.**

   Do not incubate for longer than 2 min, otherwise the tissue will thaw, resulting in potential RNA degradation.

4. **Immediately add the appropriate volume of lysis buffer (e.g., Buffer RLT, Buffer RLT Plus, or QIAzol Lysis Reagent) to each tube.**

   Note: If using Buffer RLT Plus, we recommend adding Reagent DX to prevent excessive foaming. For details, see "Disruption and homogenization in Buffer RLT Plus" (page 16).

5. **Place the insert with sample tubes into the base of the TissueLyser LT Adapter, which is attached to the TissueLyser LT. Place the lid of the TissueLyser LT Adapter over the insert, and screw the knob until the lid is securely fastened.**

6. **Operate the TissueLyser LT for 2–5 min at 50 Hz.**

   The duration of disruption and homogenization depends on the tissue being processed and can be extended until no tissue debris is visible.

   If processing fiber-rich tissues, complete disruption and homogenization may sometimes not be possible. However, small amounts of debris have no effect on subsequent RNA purification with QIAGEN kits and are usually digested in the proteinase K step.

7. **Proceed with RNA, DNA/RNA, or DNA/RNA/protein purification.**

   Do not reuse the stainless steel beads.

Protocol: Purification of DNA from Animal and Human Tissues

This protocol provides guidelines on disrupting animal and human tissues for subsequent DNA purification.

Important points before starting

- Before beginning the procedure, read "Important Notes" (page 13).
- Ensure that you are familiar with operating the TissueLyser LT by referring to the *TissueLyser LT User Manual*.
- If using a QIAGEN kit for DNA purification, read the supplied handbook and appropriate supplementary protocol carefully before starting.
- When disrupting tough or very tough samples, we recommend using one or two 7 mm stainless steel beads, respectively, instead of one 5 mm stainless steel bead to guarantee optimal disruption.

Procedure

1. **Place 2 ml microcentrifuge tubes containing 1 stainless steel bead (5 mm mean diameter) on dry ice for at least 15 min. Keep the insert of the TissueLyser LT Adapter at room temperature (15–25°C).**
2. **Transfer up to 25 mg fresh or frozen tissue to the precooled tubes and incubate for another 15 min on dry ice.**
3. **Place the tubes into the insert of the TissueLyser LT Adapter, and incubate at room temperature for 2 min to avoid freezing of lysis buffer in step 4.**

   Do not incubate for longer than 2 min, otherwise the tissue will thaw, resulting in potential DNA degradation.
4. **Immediately add the appropriate volume of lysis buffer (e.g., Buffer ATL) to each tube.**
5. **Place the insert with sample tubes into the base of the TissueLyser LT Adapter, which is attached to the TissueLyser LT. Place the lid of the TissueLyser LT Adapter over the insert, and screw the knob until the lid is securely fastened.**
6. **Operate the TissueLyser LT for 40 s at 30 Hz.**

   Note: Depending on the type of tissue, exceeding this homogenization time and intensity may lead to significant fragmentation of genomic DNA. However, for tough samples, it may be necessary to exceed this homogenization time and/or intensity to improve disruption efficiency.

   If working with fibrous tissues, cutting the tissue into smaller pieces before starting disruption will improve disruption efficiency.

Protocol: DNA Purification from Tissues (QIAamp DNA Mini Kit)

This protocol is for purification of total (genomic, mitochondrial, and viral) DNA from tissues using the QIAamp DNA Mini Kit.

Important points before starting

- All centrifugation steps are carried out at room temperature (15–25°C).
- Use carrier DNA if the sample contains <10,000 genome equivalents (see page 17).
- Avoid repeated freezing and thawing of stored samples, since this leads to reduced DNA size.
- Transcriptionally active tissues, such as liver and kidney, contain high levels of RNA which will copurify with genomic DNA. RNA may inhibit some downstream enzymatic reactions, but will not inhibit PCR. If RNA-free genomic DNA is required, include the RNase A digest, as described in step 5a of the protocol.

Things to do before starting

- Equilibrate the sample to room temperature (15–25°C).
- Heat 2 water baths or heating blocks: one to 56°C for use in step 3, and one to 70°C for use in step 5.
- Equilibrate Buffer AE or distilled water to room temperature for elution in step 11.
- Ensure that Buffers AW1 and AW2 have been prepared according to the instructions on page 16.
- If a precipitate has formed in Buffer ATL or Buffer AL, dissolve by incubating at 56°C.

Procedure

1. **Excise the tissue sample or remove it from storage. Determine the amount of tissue. Do not use more than 25 mg (10 mg spleen).**

   Weighing tissue is the most accurate way to determine the amount.

   If DNA is prepared from spleen tissue, no more than 10 mg should be used.

   The yield of DNA will depend on both the amount and the type of tissue processed.

   1 mg of tissue will yield approximately 0.2–1.2 µg of DNA.

2. **Cut up (step 2a), grind (step 2b), or mechanically disrupt (step 2c) the tissue sample.**

   The QIAamp procedure requires no mechanical disruption of the tissue sample, but lysis time will be reduced if the sample is ground in liquid nitrogen (step 2b) or mechanically homogenized (step 2c) in advance.

2a. **Cut up to 25 mg of tissue (up to 10 mg spleen) into small pieces. Place in a 1.5 ml microcentrifuge tube, and add 180 µl of Buffer ATL. Proceed with step 3.**

   It is important to cut the tissue into small pieces to decrease lysis time.

   2 ml microcentrifuge tubes may be better suited for lysis.

2b. **Place up to 25 mg of tissue (10 mg spleen) in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into 1.5 ml microcentrifuge tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw, and add 180 µl of Buffer ATL. Proceed with step 3.**

2c. **Add up to 25 mg of tissue (10 mg spleen) to a 1.5 ml microcentrifuge tube containing no more than 80 µl PBS. Homogenize the sample using the TissueRuptor or equivalent rotor–stator homogenizer. Add 100 µl Buffer ATL, and proceed with step 3.**

   Some tissues require undiluted Buffer ATL for complete lysis. In this case, grinding in liquid nitrogen is recommended. Samples cannot be homogenized directly in Buffer ATL, which contains detergent.

3. **Add 20 µl proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.**

   Note: Proteinase K must be used. QIAGEN Protease has reduced activity in the presence of Buffer ATL.

   Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h. Lysis overnight is possible and does not influence the preparation. To ensure efficient lysis, a shaking water bath or a rocking platform should be used. If not available, vortexing 2–3 times per hour during incubation is recommended.

4. **Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.**

5. **If RNA-free genomic DNA is required, follow step 5a. Otherwise, follow step 5b.**

   Transcriptionally active tissues, such as liver and kidney, contain high levels of RNA which will copurify with genomic DNA. RNA may inhibit some downstream enzymatic reactions, but will not inhibit PCR.

Tissues

5a. **First add 4 µl RNase A (100 mg/ml), mix by pulse-vortexing for 15 s, and incubate for 2 min at room temperature (15–25°C). Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid before adding 200 µl Buffer AL to the sample. Mix again by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.**

It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of Buffer AL. In most cases it will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

5b. **Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.**

It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

6. **Add 200 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.**

It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the QIAamp Mini spin column. This precipitate does not interfere with the QIAamp procedure or with any subsequent application.

Do not use alcohols other than ethanol since this may result in reduced yields.

7. **Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.***

Close each spin column to avoid aerosol formation during centrifugation.

It is essential to apply all of the precipitate to the QIAamp Mini spin column.

Centrifugation is performed at 6000 x *g* (8000 rpm) to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through.

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 6 for safety information.

8. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.***

9. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.**

10. **Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

This step helps to eliminate the chance of possible Buffer AW2 carryover.

11. **Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.**

12. **Repeat step 11.**

A 5 min incubation of the QIAamp Mini spin column loaded with Buffer AE or water, before centrifugation, generally increases DNA yield.

A third elution step with a further 200 µl Buffer AE will increase yields by up to 15%.

Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation.

Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield (see Table 5, page 25). Eluting with 4 x 100 µl instead of 2 x 200 µl does not increase elution efficiency.

For long-term storage of DNA, eluting in Buffer AE and placing at –20°C is recommended, since DNA stored in water is subject to acid hydrolysis.

Yields of DNA will depend both on the amount and the type of tissue processed. 25 mg of tissue will yield approximately 10–30 µg of DNA in 400 µl of water (25–75 ng/µl), with an A_{260}/A_{280} ratio of 1.7–1.9.

For more information about elution and how to determine DNA yield, length, and purity, refer to pages 24–25 and Appendix A, page 50.

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 6 for safety information.

Tissues

Protocol: Purification of Total RNA, Including Small RNAs, from Animal Tissues

Important points before starting

- If using the miRNeasy Mini Kit for the first time, read "Important Notes" (page 11).
- It is important not to overload the RNeasy Mini spin column, as overloading will significantly reduce RNA yield and quality. Read "Determining the amount of starting material" (page 11).
- If working with RNA for the first time, read Appendix E (page 39).
- For optimal results, stabilize harvested tissues immediately in RNA*later* RNA Stabilization Reagent or Allprotect Stabilization Reagent. Tissues can be stored in the reagent for up to 1 day at 37°C, 7 days at 15–25°C, or 4 weeks at 2–8°C, or archived at –20°C or –80°C.
- Fresh, frozen, or RNA*later* or AllProtect stabilized tissues can be used. Tissues can be stored for several months at –70°C. Do not allow tissues to thaw during weighing or handling prior to disruption in QIAzol Lysis Reagent. Homogenized tissue lysates (in QIAzol Lysis Reagent, step 3) can also be stored at –70°C for several months. To process frozen homogenized lysates, incubate at 37°C in a water bath until completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity. Continue with step 4.
- Generally, DNase digestion is not required since the combination of QIAzol and RNeasy technologies efficiently removes most of the DNA without DNase treatment. In addition, miScript Primer Assays and most other assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA. In these cases, small residual amounts of DNA can be removed by on-column DNase digestion (see Appendix B, page 34) or by DNase digestion after RNA purification (please contact QIAGEN Technical Service for a protocol).
- Buffer RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- QIAzol Lysis Reagent and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 5 for safety information.
- Except for phase separation (step 7), all protocol and centrifugation steps should be performed at room temperature. During the procedure, work quickly.

Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required amounts of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- If performing optional on-column DNase digestion, prepare DNase I stock solution as described in Appendix B (page 34).

Procedure

1. **Excise the tissue sample from the animal or remove it from storage. Determine the amount of tissue. Do not use more than 50 mg flash-frozen tissue, 25 mg liver, thymus, spleen, or RNA*later* stabilized tissue, or 100 mg adipose tissue.**

   Unless you are working with RNA*later* or AllProtect stabilized tissue, do not allow the tissue to thaw before placing in QIAzol Lysis Reagent.

2. **If the entire piece of tissue can be used for RNA purification, place it directly into 700 µl QIAzol Lysis Reagent in a suitably sized vessel for disruption and homogenization.**

   If only a portion of the tissue is to be used, determine the weight of the piece to be used and place it into 700 µl QIAzol Lysis Reagent in a suitably sized vessel for disruption and homogenization.

   RNA in tissues is not protected after harvesting until the sample is stabilized in RNA*later* or AllProtect Reagent, flash-frozen, or disrupted and homogenized in step 3. Frozen animal tissue should not be allowed to thaw during handling.

   Note: Use a suitably sized vessel with sufficient headspace to accommodate foaming, which may occur during homogenization.

3. **Homogenize immediately using the TissueLyser II, TissueLyser LT, TissueRuptor, or another method until the sample is uniformly homogeneous (usually 20–40 s).**

   See pages 15–17 for a more detailed description of disruption and homogenization methods.

   Note: Homogenization with the TissueRuptor or the TissueLyser II/TissueLyser LT (see Appendix C, page 37, or the appropriate *TissueLyser Handbook*) generally results in higher total RNA yields than with other homogenization methods.

   Foaming may occur during homogenization, especially of brain tissue. If this occurs, let the homogenate stand at room temperature for 2–3 min until the foam subsides before continuing with the protocol.

   Note: Homogenized tissue lysates can be stored at –70°C for several months.

4. **Place the tube containing the homogenate on the benchtop at room temperature (15–25°C) for 5 min.**

   This step promotes dissociation of nucleoprotein complexes.

Purification of Total RNA from Animal Tissues

5. **Add 140 µl chloroform to the tube containing the homogenate and cap it securely. Shake the tube vigorously for 15 s.**

   Thorough mixing is important for subsequent phase separation.

6. **Place the tube containing the homogenate on the benchtop at room temperature for 2–3 min.**

7. **Centrifuge for 15 min at 12,000 x *g* at 4°C. After centrifugation, heat the centrifuge up to room temperature (15–25°C) if the same centrifuge will be used for the next centrifugation steps.**

   After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. For tissues with an especially high fat content, an additional, clear phase may be visible below the red, organic phase. The volume of the aqueous phase should be approximately 350 µl.

   Note: If you want to purify a separate miRNA-enriched fraction, follow the steps in Appendix A (page 31) after performing this step.

8. **Transfer the upper aqueous phase to a new collection tube (supplied). Add 1.5 volumes (usually 525 µl) of 100% ethanol and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay with step 9.**

   A precipitate may form after addition of ethanol, but this will not affect the RNeasy procedure.

9. **Pipet up to 700 µl of the sample, including any precipitate that may have formed, into an RNeasy Mini spin column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at ≥8000 x *g* (≥10,000 rpm) for 15 s at room temperature (15–25°C). Discard the flow-through.***

   Reuse the collection tube in step 10.

10. **Repeat step 9 using the remainder of the sample. Discard the flow-through.***

    Reuse the collection tube in step 11.

    Optional: If performing optional on-column DNase digestion (see "Important points before starting"), follow the steps in Appendix B (page 34) after performing this step.

11. **Add 700 µl Buffer RWT to the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.†**

    Skip this step if performing the optional on-column DNase digestion (page 34).

    Reuse the collection tube in step 12.

* Flow-through contains QIAzol Lysis Reagent and is therefore not compatible with bleach. See page 5 for safety information.

† Flow-through contains Buffer RWT and is therefore not compatible with bleach. See page 5 for safety information.

12. **Pipet 500 µl Buffer RPE into the RNeasy Mini spin column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.**

    Reuse the collection tube in step 13.

13. **Add another 500 µl Buffer RPE to the RNeasy Mini spin column. Close the lid gently and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to dry the RNeasy Mini spin column membrane.**

    The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

    Note: Following centrifugation, remove the RNeasy Mini spin column from the collection tube carefully so the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

14. **Optional: Place the RNeasy Mini spin column into a new 2 ml collection tube (not supplied), and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 1 min.**

    Perform this step to eliminate any possible carryover of Buffer RPE or if residual flow-through remains on the outside of the RNeasy Mini spin column after step 13.

15. **Transfer the RNeasy Mini spin column to a new 1.5 ml collection tube (supplied). Pipet 30–50 µl RNase-free water directly onto the RNeasy Mini spin column membrane. Close the lid gently and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA.**

16. **If the expected RNA yield is >30 µg, repeat step 15 with a second volume of 30–50 µl RNase-free water. Elute into the same collection tube.**

    To obtain a higher total RNA concentration, this second elution step may be performed by using the first eluate (from step 15). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

Purification of Total RNA from Animal Tissues

Prepare the PCR reaction mix

For duplex reactions using VIC dye-labeled, primer-limited assays, see Appendix C, "Duplex Reactions" on page 49.

1. For each sample (to be run in quadruplicate), pipet the following into a nuclease-free 1.5-mL microcentrifuge tube:

PCR reaction mix component	Volume per 20-µL reaction (µL)	
	Single reaction	Four replicates#
20X TaqMan® Gene Expression Assay	1.0	5.0
2X TaqMan® Gene Expression Master Mix‡	10.0	50.0
cDNA template (1 to 100 ng)§	4.0	20.0
RNase-free water	5.0	25.0

‡ (Optional) Use TaqMan® Fast Advanced Master Mix or TaqMan® Universal Master Mix. If you add AmpErase® UNG (uracil-N-glycosylase), the final concentration must be 0.01 U/µL. Reduce the volume of water in the PCR reaction mix to compensate for additional volume from the UNG.

§ Applied Biosystems recommends that no more than 20% of the PCR be composed of the reverse transcription reaction.

Replicate volumes include 20% excess to compensate for volume loss from pipetting.

2. Cap the tube and invert it several times to mix the reaction components.
3. Centrifuge the tube briefly.

Load the plate

1. Transfer 20 µL of PCR reaction mix into each well of a 48-, 96-, or 384-well reaction plate.

   See Table 12 on page 42 for a list of compatible reaction plates and accessories.
2. Seal the plate with the appropriate cover.
3. Centrifuge the plate briefly.
4. Load the plate into the instrument.

APPENDIX V

Power Calculations

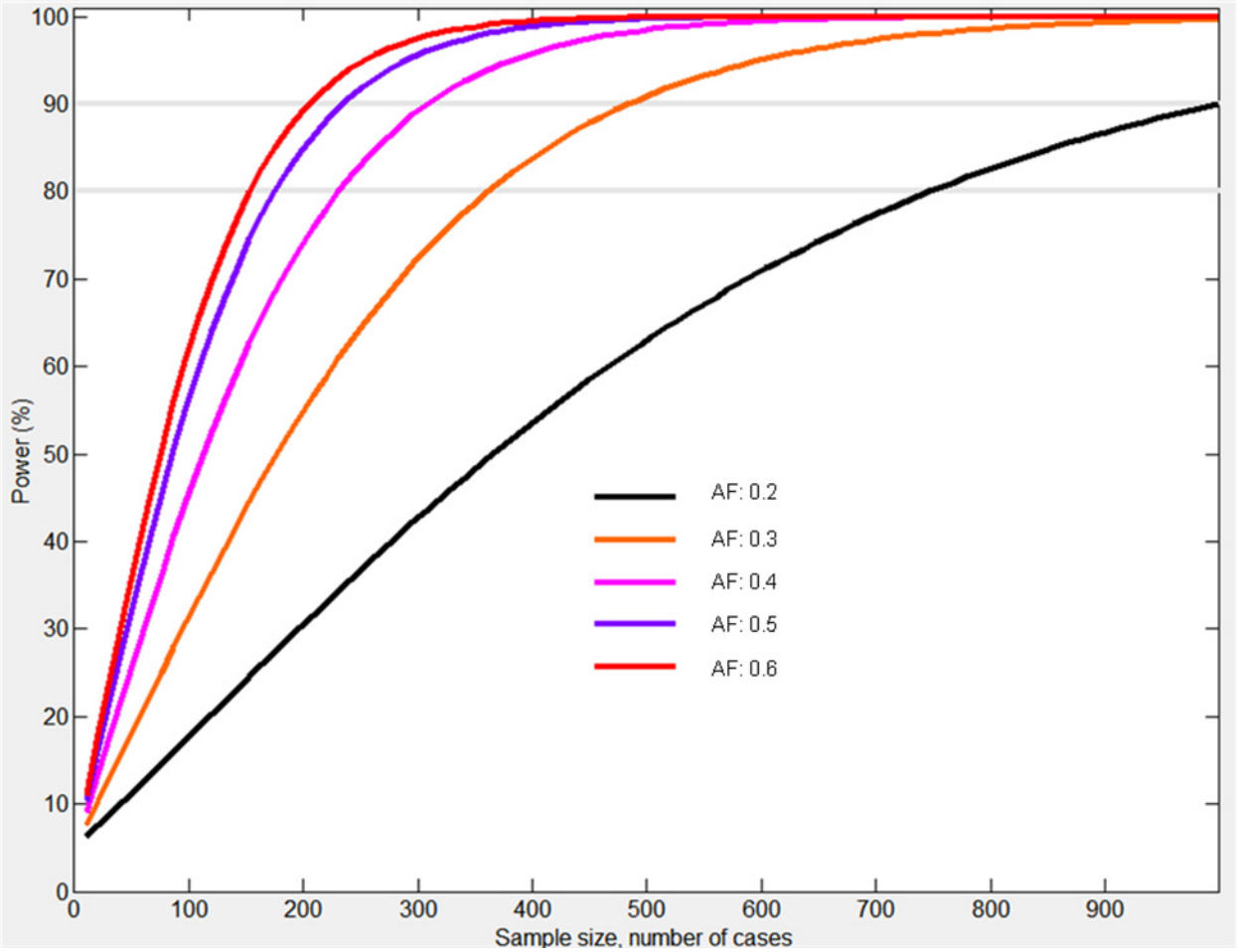

Figure A-V 1 Graphical representation of the power calculations conducted on the PGA package to detect OR=1.8. The minimum sample size was determined at a 10% prevalence of ATP with a power of 80%. Each coloured line corresponds to a different allele frequency (AF).

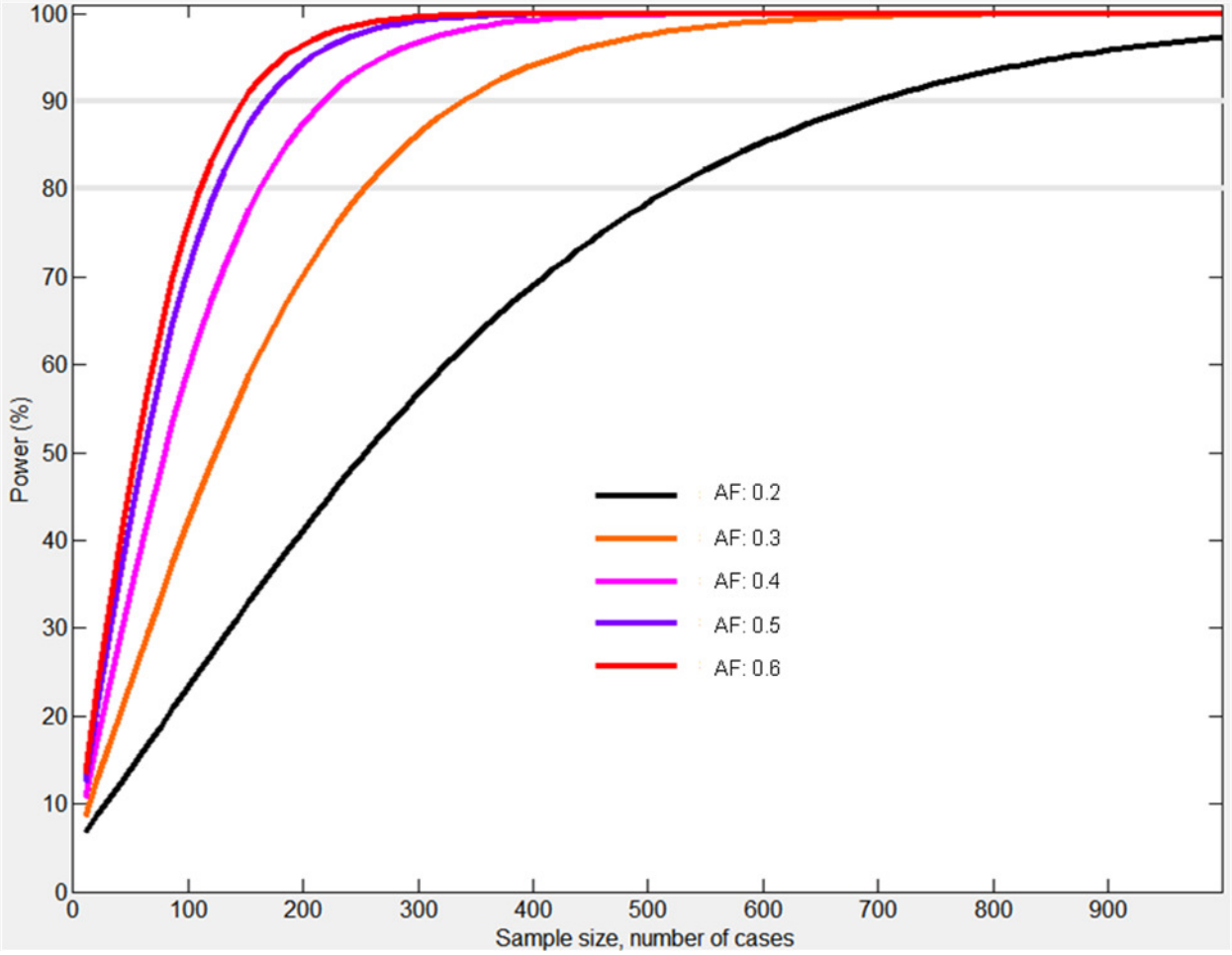

Figure A-V 2 Graphical representation of the power calculations conducted on the PGA package to detect OR=2.0. The minimum sample size was determined at a 10% prevalence of ATP with a power of 80%. Each coloured line corresponds to a different allele frequency (AF).

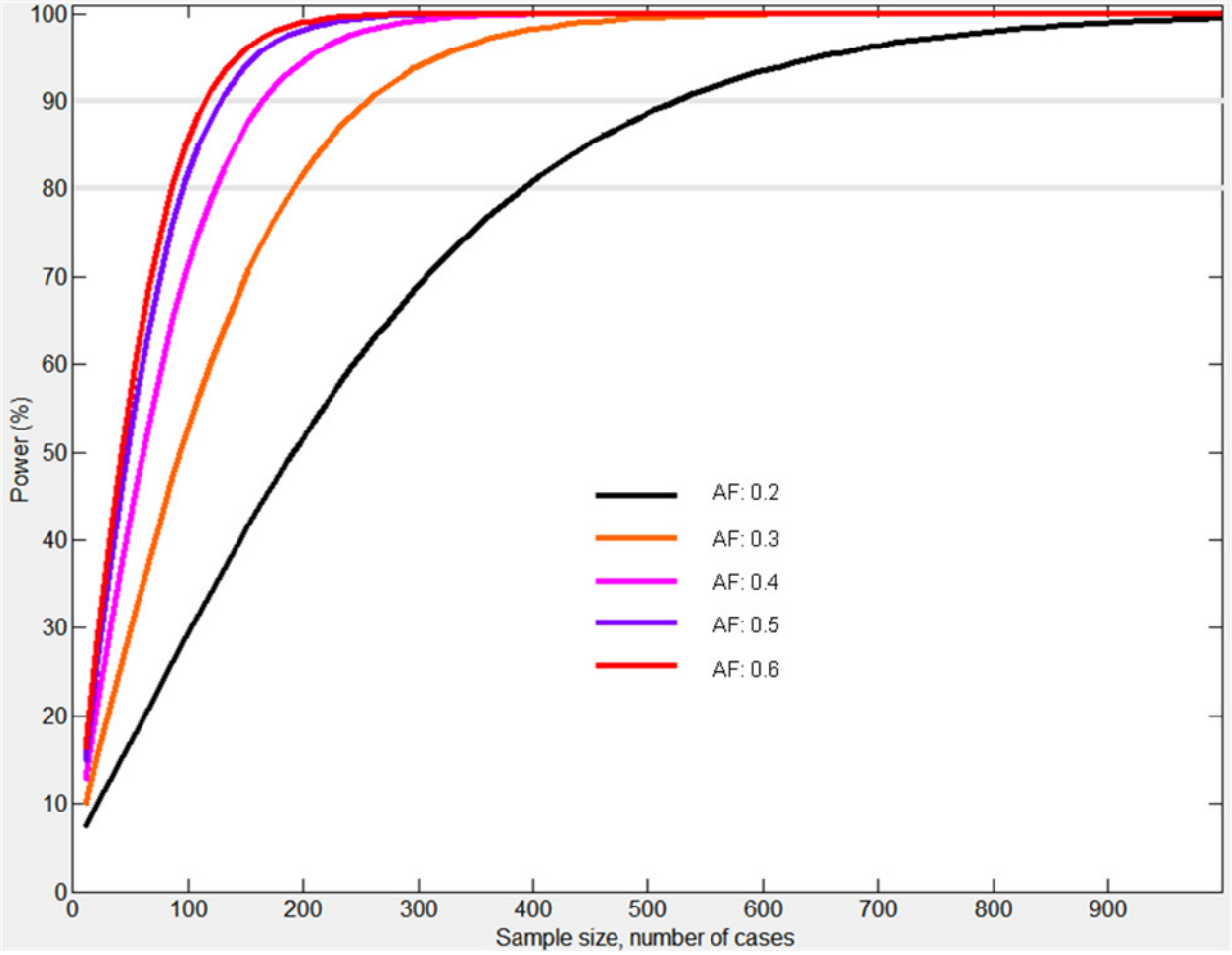

Figure A-V 3 Graphical representation of the power calculations conducted on the PGA package to detect OR=2.2. The minimum sample size was determined at a 10% prevalence of ATP with a power of 80%. Each coloured line corresponds to a different allele frequency (AF).

APPENDIX VI

Supplementary Genotyping Tables

Table A-VI 1 The genotype and allele frequency distribution of the *ELN* rs2071307 and *FBN2* rs331079 gene variants within the Australian (AUS) and South African (SA) control (CON) and Achilles tendinopathy (TEN) groups.

***ELN* rs2071307**	**AUS CON (n= 143)**	**AUS TEN (n= 59)**	**SA CON (n= 95)**	**SA TEN (n= 74)**
GG	35.0 (50)	28.8 (17)	37.9 (36)	41.9 (31)
GA	47.6 (68)	54.2 (32)	46.3 (44)	45.9 (34)
AA	17.5 (25)	16.9 (10)	15.8 (15)	12.2 (9)
P-Value	0.650		0.758	
A allele	41.3 (118)	44.1 (52)	38.9 (74)	35.1 (52)
P-Value	0.603		0.472	
HWE	0.820	0.441	0.799	0.945
***FBN2* rs331079**	AUS CON (n= 142)	AUS TEN (n= 60)	SA CON (n= 96)	SA TEN (n= 75)
GG	74.6 (106)	85.0 (51)	80.2 (77)	86.7 (65)
GC	21.8 (31)	15.0 (9)	19.8 (19)	13.3 (10)
CC	3.5 (5)	0.0 (0)	0.0 (0)	0.0 (0)
C allele	14.4 (41)	7.5 (9)	9.9 (19)	6.7 (10)
P-Value	0.053		0.287	
HWE	0.166	0.529	0.281	0.536
GG	74.6 (106)	85.0 (51)	80.2 (77)	86.7 (65)
GC + CC	25.4 (36)	15.0 (9)	19.8 (19)	13.3 (10)
P-Value	0.106		0.264	

Table A-VI 2 The genotype and allele frequency distribution of the *ADAMTS14* rs4747096, *ADAMTS2* rs1054480, *ADAM12* 3740199, and *ADAMTS5* 226794 gene variants within the Australian (AUS) and South African (SA) control (CON) and Achilles tendon pathology (ATP) groups

	AUS CON	**AUS ATP**	**SA CON**	**SA ATP**
***ADAMTS14* rs4747096**	n=133	n=55	n=94	n=110
AA	76.7 (102)	67.3 (37)	70.2 (66)	76.4 (84)
AG	20.3 (27)	23.6 (13)	27.7 (26)	22.7 (25)
GG	3.0 (4)	9.1 (5)	2.1 (2)	0.9 (1)
P-Value	0.159		0.531	
HWE	0.198	0.034	0.762	0.561
G allele	13.2 (35)	20.9 (23)	15.9 (30)	12.3 (27)
P-Value	0.058		0.285	
***ADAMTS2* rs1054480**	n=137	n=56	n=75	n=104
GG	49.6 (68)	55.4 (31)	52.0 (39)	52.9 (55)
GA	40.1 (55)	37.5 (21)	36.0 (27)	41.3 (43)
AA	10.2 (14)	7.1 (4)	12.0 (9)	5.8 (6)
P-Value	0.695		0.310	
HWE	0.563	0.864	0.216	0.521
A allele	30.3 (83)	25.9 (29)	30.0 (45)	26.4 (55)
P-Value	0.387		0.459	
***ADAM12* rs3740199**	n=140	n=58	n=96	n=114
GG	26.4 (37)	29.3 (17)	37.5 (36)	29.8 (34)
GC	50.0 (70)	53.4 (31)	42.7 (41)	50.9 (58)
CC	23.6 (33)	17.2 (10)	19.8 (19)	19.3 (22)
P-Value	0.614		0.425	
HWE	0.992	0.518	0.247	0.757
C allele	48.6 (136)	43.9 (51)	41.1 (79)	44.7 (102)
P-Value	0.448		0.459	
***ADAMTS5* rs226794**	n=138	n=59	n=96	n=114
GG	76.8 (106)	74.6 (44)	84.4 (81)	84.2 (96)
GA	23.2 (32)	23.7 (14)	14.6 (14)	14.0 (16)
AA	0.0 (0)	1.7 (1)	1.0 (1)	1.8 (2)
P-Value	0.306		0.906	
HWE	0.123	0.925	0.656	0.189
A allele	11.6 (32)	13.6 (16)	8.3 (16)	8.8 (20)
P-Value	0.585		0.873	

Table A-VI 3 The genotype and allele frequency distribution of the *TIMP2* rs4789932 gene variant within the Australian (AUS) and South African (SA) control (CON) and Achilles tendon pathology (ATP) groups

	AUS CON	AUS ATP	SA CON	SA ATP
***TIMP2* rs4789932**	n=140	n=59	n=96	n=114
CC	32.9 (46)	22.0 (13)	41.7 (40)	28.9 (33)
CT	45.0 (63)	59.3 (35)	38.5 (37)	55.3 (63)
TT	22.1 (31)	18.6 (11)	19.8 (19)	15.8 (18)
P-Value	0.165		0.051	
HWE	0.289	0.149	0.062	0.183
T allele	44.6 (125)	48.3 (57)	39.1 (75)	43.4 (99)
P-Value	0.503		0.366	