A pathway-based approach investigating DNA sequence variants to implicate the inflammatory pathway in the genetic predisposition to Achilles tendinopathy

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A Pathway-Based Approach Investigating DNA Sequence Variants to Implicate the Inflammatory Pathway in the Predisposition to Achilles Tendinopathy

by

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DECLARATION

I, Erica-Mari Nell, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABBREVIATIONS

^{o}C	degrees Celsius
A	adenine
ADAM	a disintegrin and metalloproteinase
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
ANOVA	Analysis of variance
Asp	aspartate
AT	Achilles tendinopathy
AUC	area under the curve
AUS	Australia
Bcl-2	B-cell lymphoma-2
BMI	body mass index
bp	base pairs
C	cytosine
CASP3	gene encoding caspase-3
CASP8	gene encoding caspase-8
CI	confidence interval
COL5A1	gene encoding the α1 chain of type V collagen
COMP	cartilage oligomeric matrix protein
CON	control participants
COX-2	cyclooxygenase-2
COX-2	gene encoding cyclooxygenase-2
DAB2	gene encoding mitogen responsive phosphoprotein
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dw	dry weight
E2F	E2 promoter binding factor
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid

eNOS	endothelial nitric oxide synthase
EP_4	PGE_2 receptor subtype-4
FLIP	FLICE-like inhibitory protein
G	guanine
GAG	glycosaminoglycans
GDF-5	growth differentiation factor-5
GDF5	gene encoding growth differentiation factor-5
His	histidine
HWE	Hardy-Weinberg equilibrium
IGF-I	insulin like growth factor-1
IL-1β	interleukin-1β
IL-1β	gene encoding Interleukin-1β
IL-1R1	interleukin-1 receptor-1
IL-1ra	interleukin-1 receptor antagonist
IL-1RN	gene encoding interleukin-1 receptor antagonist
IL-6	interleukin-6
IL-6	gene encoding interleukin-6
iNOS	inducible nitric oxide synthase
kb	kilobases
LR	likelihood ratio
Mb	megabases
miRNA	micro ribonucleic acid
MMP	matrix metalloproteinase
MMP3	gene encoding matrix metalloproteinase-3
mPGES-1	microsomal prostaglandin E synthase-1
MTJ	musculotendinous junction
NCBI	national centre for biotechnology information
NMDAR	n-methyl-D-aspartate receptor
nNOS	neuronal nitric oxide synthase
NO	nitric oxide

NOS	nitric oxide synthase
NOS2	gene encoding Inducible nitric oxide synthase
NOS3	gene encoding endothelial nitric oxide synthase
NSAIDs	non-steroidal anti-inflammatory drugs
OR	odds ratio
OTJ	osteotendinous junction
p arm	short arm of the chromosome
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDGF-R	platelet derived growth factor receptor
PGs	proteoglycans
PGE_2	prostaglandin E_2
PGH_2	prostaglandin H_2
PTGER4	gene encoding PGE_2 receptor subtype EP_4
PTGS2	gene encoding cyclooxygenase-2, also known as *COX-2*
q arm	long arm of the chromosome
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROC	receiver operating characteristic
SA	South Africa
siRNA	silencing ribonucleic acid
SNP	single nucleotide polymorphism
SP1	stimulatory protein-1
Stat-3	signal transducer and activator of transcription-3
T	thymine
TEN	Achilles tendinopathy participants
TGF-β	transforming growth factor beta
TGFB2	gene encoding transforming growth factor beta-2
TGF-βR1	transforming growth factor beta receptor-1
TGS	total genotype score

TNC	gene encoding tenascin C
TNF-α	tumor necrosis factor alpha
TNFRSF8	tumor necrosis factor receptor superfamily member 8
TIMP	tissue inhibitors of metalloproteinases
UTR	untranslated region
VEGF	vascular endothelial growth factor
VEGF-R	vascular endothelial growth factor receptor
VNTR	variable number tandem repeat
YY1	ying yang 1

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ABSTRACT

INTRODUCTION:

Achilles tendinopathy (AT) is a multifactorial condition for which several extrinsic and intrinsic risk factors, including genetic risk factors, have been identified. Variants within the *IL-1β* and *IL-6* genes were previously collectively associated with AT. The proteins encoded for by these genes are involved in the extracellular matrix (ECM) degradation and apoptosis signalling cascades respectively.

AIMS:

The aims of this dissertation were therefore (i) to follow a pathway-based approach investigating genes encoding proteins involved in the ECM degradation and apoptosis signalling cascade for associations with AT and (ii) to identify a polygenic risk model, comprised of several genetic markers within genes encoding proteins involved in the inflammatory pathway, to predict risk of AT.

METHODS:

A pathway-based case-control genetic association study was conducted. From South Africa (SA), 161 asymptomatic control (SA CON) and 85 clinically diagnosed AT participants (SA TEN), and from Australia (AUS), 205 AUS CON and 82 AUS TEN participants were genotyped for functional or proposed-functional variants: *COX-2* -765G>C*, PTGER4* gene desert A>C and *TGFB2* -876A>C of the ECM degradation signalling cascade and *CASP8* -652 6N del, *CASP8* Asp302His, *NOS3* Glu298Asp and *NOS2* -1026C>A of the apoptosis signalling cascade. Polygenic risk models were constructed incorporating variants within genes from previously published studies and this dissertation, which were associated with AT in SA or AUS and encode proteins functioning within (i) the ECM degradation signalling cascade, (ii) the apoptosis signalling

cascade, and (iii) the inflammatory pathway comprised of variants from both signalling cascades, to assess their effectiveness to predict risk of AT.

RESULTS:

The *PTGER4* gene desert CC genotype was associated with protection from developing AT in AUS (Chapter 3). The *CASP8* -652 del/del and *CASP8* Asp302His GG genotypes were associated with increased risk of AT in AUS and heterozygous genotypes at the *CASP8* -652 6Ndel, *CASP8* Asp302His and *NOS2* -1026A>C loci were associated with protection from developing AT in AUS (Chapter 4). A polygenic risk profile for the ECM degradation (Chapter 3) and apoptosis pathway (Chapter 4) were identified in both SA and AUS and the best predictor of risk for AT was the model incorporating variants from genes encoding proteins in the combined inflammatory pathway (Chapter 5).

CONCLUSION:

This dissertation adds an additional level of evidence for the involvement of the inflammatory pathway in the development of AT. Biological markers were identified within the ECM degradation and apoptosis signalling cascades. Furthermore, the pathway-based approached used in this dissertation has facilitated the identification of a polygenic risk model to identify individuals at an increased risk of developing AT.

PREFACE

…According to Greek mythology, Ἀχιλλεύς or Achilles was the most handsome and mightiest Greek warrior of the civilized world. Achilles' body was mystically protected from harm – a boon of invulnerability bestowed on him as a child when his mother submerged him into the River Styx, suspending him in the water by his heel. Everything that the magical waters touched became invulnerable, but the water did not wet his heel, leaving it unprotected. During the Trojan War, at the height of his power and martial prowess, Achilles was struck by a poisoned arrow on his vulnerable heel and died…

It was this legend that inspired Philip Verheyen in 1693 to describe the tendon located at the heel as the "cord of Achilles" in his textbook *Corporis Humani Anatomia*. This led to this tendon being commonly and medically referred to as the Achilles tendon.

Chapter 1

INTRODUCTION – ACHILLES TENDINOPATHY

INTRODUCTION AND SCOPE OF DISSERTATION

The bone-tendon-muscle complex is remarkably engineered for locomotion to allow a range of functions, from the execution of intricate movements such as writing to the generation of great force needed for jumping. Even though evolution has shaped these structures for their function, extensive and frequent use of the musculoskeletal complex does increase the risk of injury.[1] Injuries to soft tissue (tendons, ligaments and muscle) constitute 43% of referrals to rheumatology practices in the United Kingdom.[2] Tendons are capable of withstanding great forces without sustaining injury, the largest of these forces being produced during tension.[3] However, the fact that tendons have to withstand such high forces makes them vulnerable to overload and injury and thus tendon injuries are common as a result of participation in both physical exercise and repetitive occupational activities.[4] Chronic tendon injuries were reported to account for 30 to 50% of all sporting injuries.[5]

The tendons which are most commonly injured during sporting or physical activities are the rotator cuff, forearm extensor, patellar, Achilles and tibialis posterior tendons.[6] The location of the tendon injury is thought to be sport/activity specific since development of the injury is, at least in part, due to the high level of mechanical stress that these tendons have to endure.[7, 8] The Achilles tendon, which is the focus of this dissertation, is able to withstand tensile forces as high as 9kN while running, which is the equivalent of 12.5 times body weight.[9] Achilles tendon injuries occur mainly in sporting activities which involve running and jumping.[10, 11]

Achilles tendinopathy (AT) is a tendon injury predominantly caused by overuse of the tendon and the biological mechanisms and pathways underlying the development of AT are poorly understood.[5] There is extensive debate in the literature whether tendinopathy is a degenerative or inflammatory condition.[5, 12, 13] It has become apparent that although tendinopathy is primarily a degenerative condition, the inflammatory pathway is involved.[14, 15] The focus of this dissertation was therefore to use a pathway-based approach to investigate mediators of the inflammatory pathway, specifically looking at the extracellular matrix (ECM) degradation and apoptosis signalling cascades, using a case-control genetic association study design.

In preparation for the experimental studies that form part of this dissertation, Chapter 1 provides an overview of the anatomy of the Achilles tendon (Section 1.1), the classification of the types of tendon injuries (Section 1.2.1), tendon injury histopathology (Section 1.2.2) and the risk factors for AT (Section 1.2.3), with an emphasis on the genetic risk factors (Section 1.3).

The focus of the studies within this dissertation was the investigation of the inflammatory pathway in the development of AT and therefore Chapter 2 unravels the intricacies of the current understanding of the extracellular matrix degradation (Section 2.1) and apoptosis (Section 2.2) signalling cascades in the development of AT.

Subsequent experimental chapters employ a pathway-based candidate gene approach to explore genes within the inflammatory pathway leading to ECM degradation (Chapter 3) and apoptosis (Chapter 4). In both these chapters risk models incorporating several loci within the respective pathways were designed and explored for effectiveness to identify risk of AT. Chapter 5 then explores the polygenic risk model incorporating loci from both pathways to determine whether this combined model had a greater discriminating power to predict risk of AT.

The conclusions chapter provides a summary of the novel findings (Section 6.1) and the limitations (Section 6.2) of this dissertation. This chapter also explores the role of genetics in tendinopathy (Section 6.3) and the clinical implications of the findings of this dissertation (Section 6.4). Finally, a hypothesis for the involvement of inflammation in the development of AT is presented (Section 6.5).

1.1. ANATOMY OF THE ACHILLES TENDON

The Achilles tendon is the largest and strongest tendon in the body.[16] Its tensile strength and its ability to withstand high levels of mechanical stress can be attributed to its structure. The detailed anatomy of the Achilles tendon is beyond the scope of this dissertation and has been extensively reviewed elsewhere.[17-19] Only the anatomy relevant to tendon injury will therefore be presented.

The Achilles tendon originates at the soleus and gastrocnemius muscles of the calf and inserts at the calcaneus (Figure 1.1). Tendons are predominantly composed of ECM with only a few cells.[20] The fibroblasts resident in tendons, known as tenocytes, are the predominant cell type and function mainly to regulate ECM turnover.[21, 22] The metabolic activity of tenocytes is 7.5 fold lower than that of skeletal muscles due to a well developed anaerobic energy generation capacity.[23] However, the low metabolic rate also results in a compromised healing capacity so that the appropriate time needed by tendons for adaptation to training would be longer than that of the well-vascularised muscle tissue.[24]

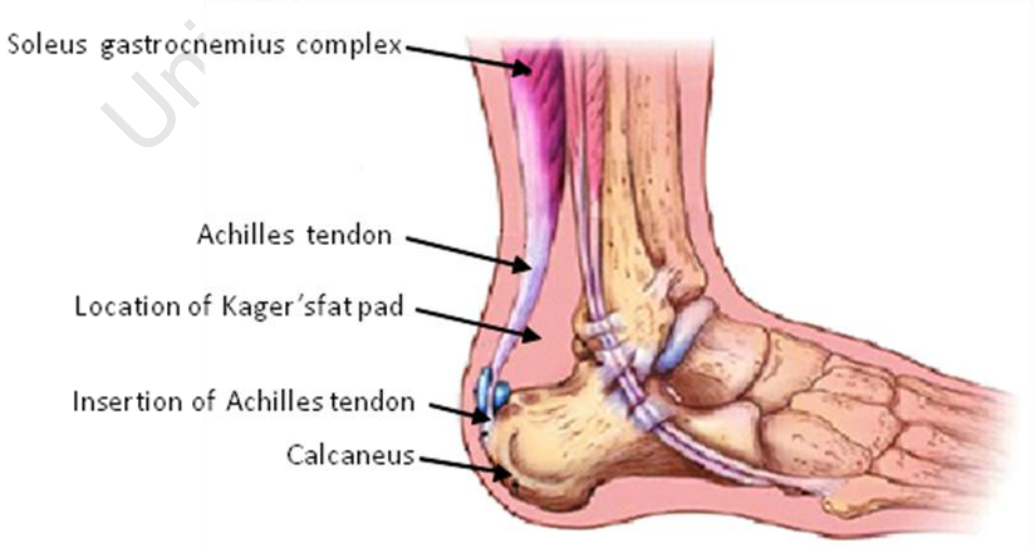

Figure 1.1. Basic anatomy of the heel in the region of the Achilles tendon. Modified from Mazzone *et al*, (2002).[25]

The remainder of the tendon tissue, the ECM, makes up the bulk of the tendon. The dry mass of the ECM, which is 33% of the total mass in water, consists of collagens, glycoproteins, proteoglycans and glycosaminoglycans (GAGs). The main constituent of the tendon ECM is collagen, constituting 60% of the dry weight (dw) of the tendon.[13] Type I collagen is most abundantly expressed in the tendon and most of the collagen fibre bundles are arranged parallel to the long axis of the tendon (Figure 1.2). The parallel organisation is made possible by proteoglycans such as decorin and versican which comprise 0.5% dw and glycoproteins such as tenascin and cartilage oligomeric matrix protein (COMP), which constitute 5% dw.[13] The arrangement of these collagen molecules allows for the role of the tendon to resist tensile forces.[20]

Collagen molecules assemble parallel to each other to form a collagen fibril. A number of collagen fibrils embedded in endotenon form a collagen fibre and collagen fibres are surrounded by an epitenon to form a fascicle. The Achilles tendon is a nonsheathed tendon and therefore the fascicles of the Achilles tendon are encapsulated by a paratenon to form the functional tendon unit (Figure 1.2). The paratenon provides the extrinsic component of the vasculature and nerve supply to the Achilles tendon. In some tendons, such as the extensor tendons of the hand, the paratenon is replaced by a synovial sheath.[17]

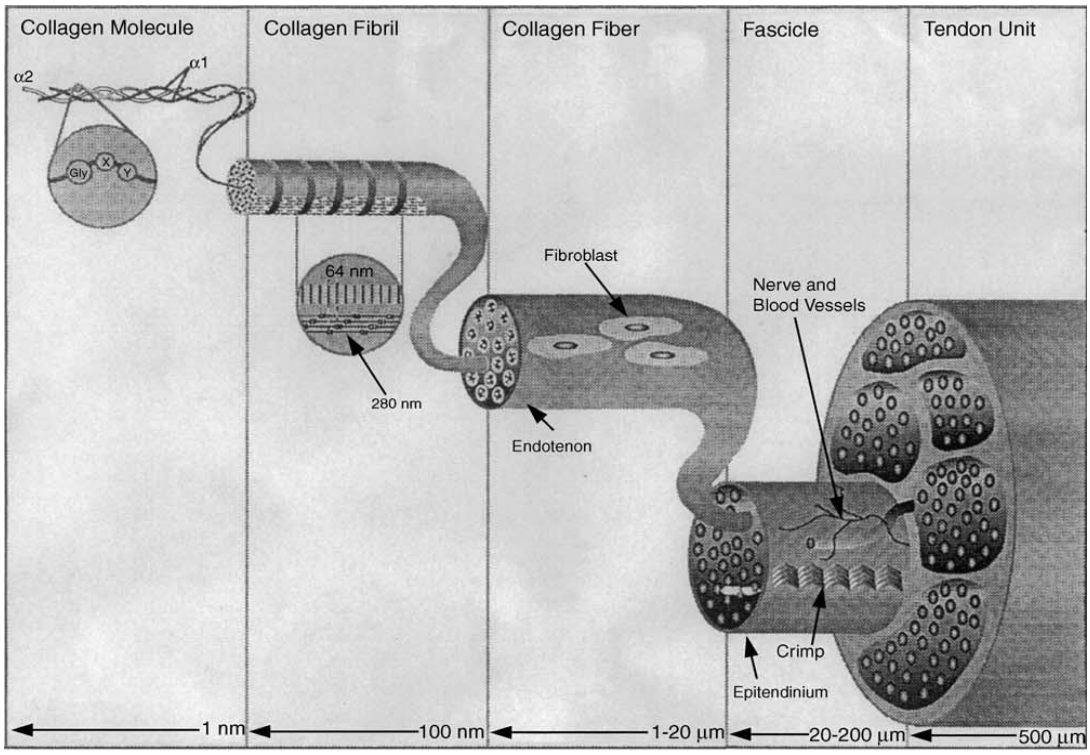

Figure 1.2. Hierarchical organization of the tendon. Modified from Silver *et al.*, (2003) [26] The collagen molecule of type I collagen, the most abundant collagen type in tendons, is comprised of two α1 chains and one α2 chain. Collagen molecules self assemble parallel to form a collagen fibril, which arrange to form a collagen fibre, associated with fibroblasts. The fibres assemble into fascicles which are innervated and vascularised and together form the tendon unit.

Kager's fat pad is a mass of adipose tissue anterior to the Achilles tendon, superior to the calcaneus (Figure 1.1).[27] The fat pad lies within the paratenon of the Achilles tendon, so that the adipose tissue lies adjacent to the Achilles tendon.[27] The function of the fat pad has received little attention in the role it may play in injury. The fat pad is of interest in this dissertation since adipose tissue is a known source of inflammatory cytokines [28] and it is therefore tempting to speculate that Kager's fat pad may initiate or exacerbate inflammatory signalling cascades within tenocytes of the adjacent Achilles tendon.

Three regions of the Achilles tendon can be defined based on the subtle differences in biochemical composition (i) the musculotendinous junction (MTJ), (ii) the midsubstance of the tendon and (iii) the osteotendinous junction (OTJ) (Figure 1.3).

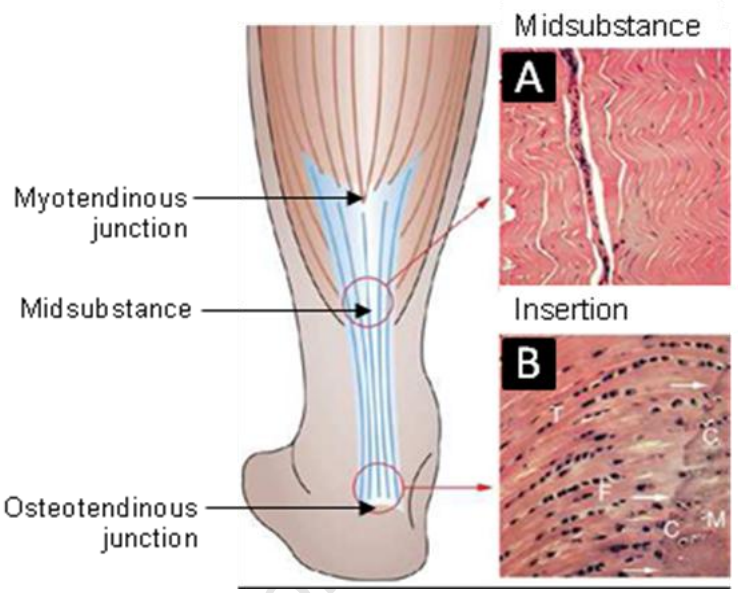

Figure 1.3. The midsubstance and insertional regions of the Achilles tendon. A) The midsubstance of the tendon showing the 'crimped' arrangement of fibres. B) The osteotendinous junction showing the transition from tendon (T) to fibrocartilage (F) to calcified fibrocartilage (C) to mineralized bone (M). Collagen is stained pink and cells stained purple with Haematoxylin and Eosin (H&E) staining. Modified from Riley, (2008).[13]

The detailed structure of the MTJ and OTJ are specialized and beyond the scope of this review. However, the structure of MTJ and OTJ has been detailed in [13, 20]. The midsubstance of the Achilles tendon is the region which is most commonly injured [29] and will therefore be described. The midsubstance is predominantly made up of type I collagen fibres, arranged parallel to the long axis of the tendon. Other components of the midsubstance ECM include glycoproteins, the proteoglycans versican and decorin and minor collagens, such as type V collagen.[13] Longitudinal

sections of the tendon viewed at rest reveal that fibre bundles have a wavelike or ‘crimped’ structure (Figure 1.3 A).[30] The stretching out of the crimp may act as a buffer against fibre damage.[30]

The blood supply to the Achilles tendon is derived mainly by the posterior tibial artery entering from three regions, namely the MTJ, the OTJ and the paratenon.[31] The Achilles tendon has poor vascularity throughout its length contributing to decreased healing capacity of the tendon tissue and contributing to vulnerability to injury.[32] The midsubstance of the Achilles tendon is the region which is most commonly injured, perhaps due to reduced vascularity in this region in comparison to the rest of the tendon.[33] In most cases, the injury is localised to the medial side of the Achilles tendon, reflecting increased torsion in the medial aspect of the tendon with hyperpronation of the foot.[29]

1.2. **ACHILLES TENDON INJURIES**

As mentioned earlier, Achilles tendon injuries occur mainly in sporting activities requiring high levels of tendon loading, such as running and jumping.[10, 11] Nevertheless the incidence of Achilles tendon injuries in non-athletes is high, 25 to 30%.[34] Achilles tendon injuries accounted for 6 to 18% of all sporting injuries during the running boom of the 1980s.[35] The prevalence of Achilles tendon injuries may have declined since then, due to awareness and prevention amongst runners, however the incidence of lower leg running injuries is still noted to be as high as 9 to 32%.[36-38] and it was reported that one in every two male runners will develop an Achilles overuse injury before the age of 45,[39] making Achilles tendon injuries predominantly a running injury.

1.2.1. **Types of Achilles tendon injuries**

There is a spectrum of injuries that can affect the tendon and surrounding structures.[40] These include achillodinia, peritendinitis, tendinosis, tendinitis and rupture.[41]

The use of the term tendonitis to describe chronic painful tendon conditions has fallen out of favour, since it implies that the underlying mechanism of pathogenesis is inflammatory and most chronically painful tendons is primarily degenerative and not inflammatory.[42, 43] Tendinosis, on the other hand, refers to degeneration of the tendon in the absence of inflammation.[44, 45] The underlying aetiology and mechanisms of chronically painful tendons are however are not fully characterised. The term “tendinopathy” was therefore chosen to describe chronic tendon pain in this dissertation, because it makes no assumptions about the underlying pathology as the and several distinct pathological processes seem to exist in the tendon at the same time.[46]

The most common injuries affecting the midsubstance of the Achilles tendon are (i) spontaneous rupture of the tendon and (ii) chronic Achilles tendinopathy.[47] Spontaneous rupture is an acute onset injury occurring in the absence of previous symptoms and presenting as a partial or complete tear of the fibres making up the tendon; therefore resulting from a macrotraumatic event.[40] Chronic AT on the other hand results from an accumulation of microscopic damage to the tendon fibres, most commonly due to repetitive strain or chronic overuse. This microscopic damage occurs either directly by microtrauma to the fibrils or indirectly through mechanical loading mediated degradative activity by tenocytes and inflammatory cells. AT is characterised by pain, swelling, tenderness and stiffness of the affected tendon.[43, 48]

1.2.2. **Histopathology and biochemistry of tendon injuries**

The histopathology of chronic tendon injuries is vast and complicated and has been extensively reviewed.[43, 44] For the purpose of this dissertation a summary of the literature is briefly presented to assist in the understanding of the histological changes observed in tendinopathy. Histopathological examination of tendon pathology reveals a primarily degenerative condition of the tendon ECM.[43] Characteristic degenerative changes include collagen fibril disorganisation and microtears, decreased fibre diameter, tenocyte dedifferentiation, hypo- and acellularity and increased GAG content.[43, 49, 50] In contrast, other regions of the tendinopathic tendon show signs of repair: fibrosis and proliferation of fibroblasts.[44, 51-53] These histological changes are characteristic to both tendinopathy and ruptured tendons,[43, 54] with a greater degree of degeneration being observed in ruptured tendons.[49] It may seem counterintuitive that, despite being more degenerative, rupture often occurs in the absence of symptoms. However this may be explained by the fact that tendinopathic tendons are

characterised by neurovascular proliferation,[43] while ruptured tendons display much less neovascularisation.[55] Since abnormal vascularity has been associated with tendon pain,[56] the difference in symptoms may be accounted for by the difference in vascularisation and associated neural innervation observed in these two pathologies. The abundance of GAGs, neurovascular ingrowth and tenocyte morphology which accompany the collagen breakdown substantiates the notion that several distinct pathological processes seem to exist in the tendon at the same time.[46]

Several disease models have been proposed for the degenerative process observed in tendinopathy, including the mechanical overload theory,[6] failed healing response [57] and the neuropeptide synthesis theory.[58] While these hypotheses all focus on specific aspects of tendinopathy development, one can hypothesise that there may be varying combinations of all these biological processes occurring simultaneously in the tendon which results in pathology.

It was recently proposed that the progression of tendon pathology follows a series of defined steps along a continuum, in response to overload [59] and this model is worth elaborating on in light of the focus of this dissertation. The tenocytes are the components of the tendon which are able to respond to mechanical stimuli and initiate the adaptation response [60] therefore in response to overload, the first observation is a compensatory proliferation of tenocytes and not collagen fibre disruption.[61] This cell proliferation is presumably to increase the rate at which ECM homeostasis is restored.[62] The first matrix macromolecules to be secreted by tenocytes as a short term, quick adaptive response to loading, are proteoglycans (PGs) such as aggrecan and versican, and GAGs such as hyaluronan, which are able to draw water. This leads to an altered composition of the tendon ground substance and increased thickness of the tendon (presenting as swelling), resulting in reduced stress and increased stiffness.[63] At this stage,

tendinopathy is thought to be reversible and given sufficient rest or reduction in load, may revert to normal.[59] If loading continues, the altered balance of PGs may disrupt the ordered collagen structure of the ECM (causing the ECM degeneration characteristic of tendinopathy) and finally this disruption allows for vascular ingrowth.[61] It is further hypothesised that at the end stages in the progression of the disease focal tenocyte apoptosis occurs, resulting in areas of hypocellularity accompanied by the ECM degeneration which is commonly described in the literature investigating end stage disease.[43, 53, 62, 64] The apoptosis of tenocytes at end stage tendinopathy is not compensated for by concomitant cell proliferation to maintain cell numbers (as with normal healing) and perhaps this is due to loss of tension between tenocytes and the ECM secondary to altered cell-matrix interaction.[65] It is at this point that the scale is tipped from reactive tendinopathy, which may be reversed, to irreversible tendon disrepair.[59] It should be noted that, according to this continuum model, the whole process is a non-inflammatory cell response.[59] However it is possible that microtrauma to the collagen fibrils due to mechanical loading causes infiltration of inflammatory cells to initially aid tenocytes in the reparative process.[66-69] Furthermore the continuum model suggests that tenocyte apoptosis (without concomitant proliferation to maintain cell numbers) only occurs at the end stages in the progression of the pathological process, however it is possible that apoptosis of other cell types occur throughout the early stages of the disease, such as the inflammatory cells and fibroblasts which may be involved in the repair process.[70]

New molecular techniques have gone a long way to providing a comprehensive molecular picture of tendinopathy. Although many questions still remain, it is now possible to propose that it is the tenocytes, which are continually responding to external stimuli such as mechanical loading by remodelling the ECM to adapt to load.[60] However, this cell-matrix response, which is required for maintaining the homeostasis of ECM

components, could possibly fail at several points resulting in accumulation or breakdown of ECM components and cell transformation.[64] The proposed algorithm for the onset of overuse tendinopathy involves altered cell-matrix interactions in response to repetitive loading (Figure 1.4). The tenocytes are unable to maintain the ECM effectively in conditions of repetitive loading with insufficient time to attain ECM homeostasis before the next bout of loading, leading to degeneration of the matrix and transient weakness, which makes it vulnerable to pathology if loading continues.

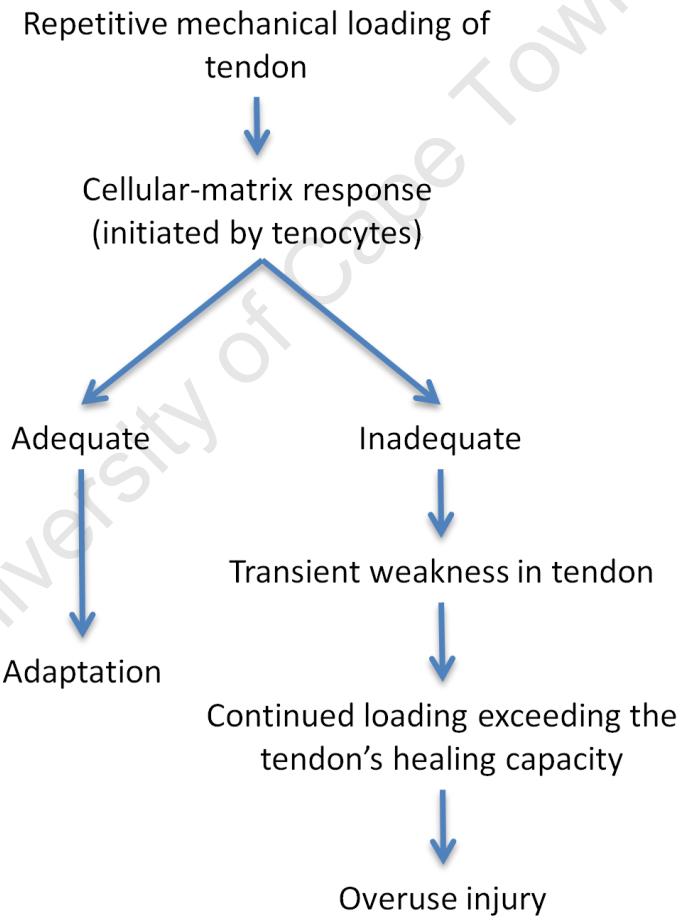

Figure 1.4. A proposed algorithm of the aetiopathogenesis of tendinopathy as modified from Archambault *et al*., (1995).[71]

There is an increasing body of evidence to suggest that tendinopathy is characterised by an increase in degradative enzymes, such as the matrix metalloproteinases, as well as an induction of unregulated excessive apoptosis.[72-80] Investigators have suggested that it is the tenocytes mechanobiological response to excessive loading that initiates the degenerative cascade which leads to tendinopathy and this includes the production of inflammatory cytokines and prostaglandins which are thought to be the mediators of tendinopathy.[76, 81-84] While this is a feasible algorithm for the development of tendinopathy, the exact mechanisms of the altered cell-matrix interactions are a work in progress.

1.2.3. **Intrinsic and extrinsic risk factors**

AT is considered to be a complex, multifactorial condition caused by the interaction of many extrinsic and intrinsic risk factors.[22, 85] A detailed description of the risk factors for AT are beyond the scope of this dissertation and have been widely reviewed.[5, 22, 48, 86, 87] It is worth noting though that most of the risk factors that have been identified for AT have a level of evidence of $\geq$ III,[88] highlighting that there are a lack of well-designed randomised controlled trials and prospective studies to identify risk factors. The only risk factor with level I evidence (meeting the above criteria) is a previous injury.[89] Some of the proposed risk factors for development of tendon injuries are outlined in Table 1.1.

Table 1.1. The intrinsic and extrinsic risk factors for development of tendinopathy.

Intrinsic Risk factors	Extrinsic Risk Factors
Age	Occupation
Gender	Physical activity
Adiposity	Training errors
Previous injury	Cold weather
Tendon vascularity	Training surface
Lower limb malalignment	Footwear
Reduced flexibility	Smoking
Systemic disease	Medication use e.g. corticosteroids,
GENETIC FACTORS [90]	fluoroquinolone

In the development of complex, multifactorial conditions, it is proposed that the intrinsic risk factors, which include genetic factors (Table 1.1. Intrinsic Risk Factors), predispose an individual to injury. When exposed to a combination of extrinsic risk factors (Table 1.1. Extrinsic Risk Factors), the predisposed individual becomes susceptible to injury. It should be noted that intrinsic and extrinsic risk factors do not necessarily result in injury, but rather, it is only at the occurrence of a subsequent inciting event that injury occurs (Figure 1.5).[90, 91]

This proposed injury model was designed for the developed for acute injuries, however it is also applicable to chronic overuse injuries, such as AT, as long as the definition of the inciting event is clarified for such chronic injuries. For acute injuries, as stated above, the inciting event defines the event that the tendon becomes acutely injured (such as complete or partial rupture of the tendon), while for chronic overuse injuries, we hypothesise that the inciting event would be defined as the event when the individual becomes aware of the injury (in the form of discomfort or pain).

Figure 1.5. The proposed mechanism for development of complex multifactorial conditions, as adapted from Meeuwisse, (1994).[91] Intrinsic risk factors, which include genetic factors, predispose an individual to injury and exposure to extrinsic risk factors such as training regime, environmental conditions, medication use etc. will increase the individual's susceptibility to injury. For acute injuries, a specific inciting event, such as excessive strain, is required for the development of injury. In chronic injuries, the inciting event would be defined as the action when the individual becomes aware of symptoms.

In other words, in overuse injuries, the tendon does display a certain degree of pathology before presenting with symptoms, due to, for example, insufficient time between loading sessions to allow for proper healing and adaptation of the tendon. This is in accordance with the aptly named iceberg theory, which proposes that pain is the "tip of the iceberg" in the accumulation of tendon abnormalities (Figure 1.6).[92] The iceberg theory also explains recurrent tendinopathy in individuals who resume training once pain has subsided just below the detection threshold, but the underlying tendon pathology still exists.

There are at least four signalling cascades, namely the matrix degradation, fibrogenesis, tenocyte apoptosis and tenocyte proliferation cascades, which are activated in tenocytes in response to loading.[64] These inflammatory pathways form part of the normal healing strategy of the tendon. It is only when the tendon is not able to cope with the demands for matrix reorganisation, due to, for example, continued repetitive loading, that homeostasis cannot be maintained and tendinopathy results.[64]

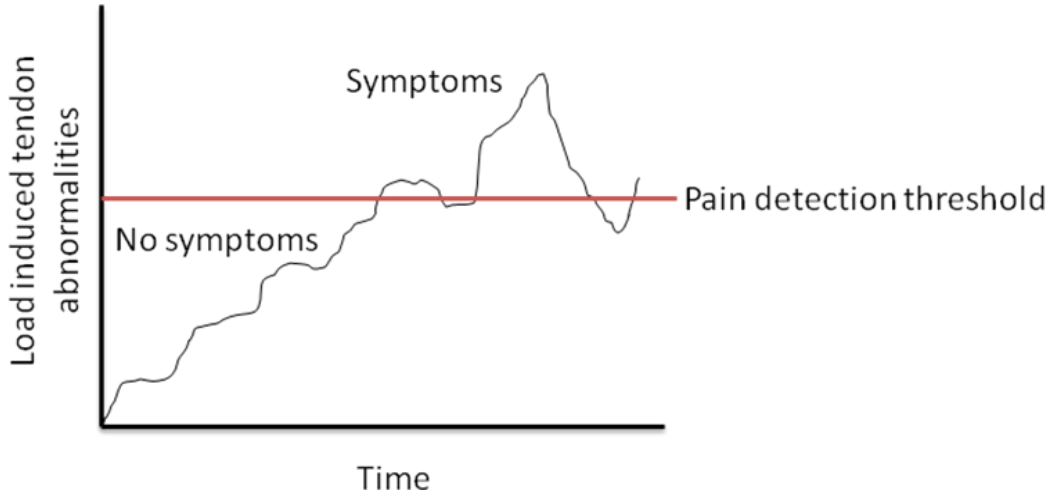

Figure 1.6. A schematic of iceberg theory, illustrating that a certain degree of tendon pathology exists in the tendon before the onset of symptoms. Adapted from Fredberg and Stengaard-Pedersen, (2008).[92]

van Mechelen *et al.*, (1992) [93] proposed that a reduction in the incidence of injuries, such as AT, will only be possible once the aetiology and mechanisms underlying the injury are understood so that appropriate evidence-based preventative measures can be introduced. It was suggested that one of the strongest intrinsic factors to contribute to risk of injury may be the genetic component and may explain why some individuals develop AT, while others do not, despite being the same age and gender and exposed to the same levels of physical activity.[94] The genetic component is therefore an important risk factor and has until recently been under investigated. For this reason, the studies presented in this dissertation, will make use of genetic association studies as a tool to identify the biological pathways leading to AT.[95, 96]

1.3. GENETIC RISK FACTORS FOR ACHILLES TENDINOPATHY

Several studies have shown that intrinsic genetic factors that could predispose individuals to developing a multifactorial disease can successfully be identified by genetic association studies.[97] If these associations are confirmed in large independent populations, the associated genes highlight a particular biological pathway that may be involved in the aetiology of the complex condition, such as AT.[98] Once the pathways and mechanisms are elucidated, these genetic risk factors and other intrinsic risk factors can be incorporated into designing a model which can be used to determine risk and prognosis in individuals and assist in improving the effective clinical management of the patient. Identifying the biological mechanisms underlying the condition can also potentially lead to the development of therapeutic interventions both in treating and preventing injury.[99]

1.3.1. The ABO blood group

The first study to implicate a genetic component in the development of Achilles tendon injuries was a study by Jozsa *et al.*, (1989) [100] in which blood type O was associated with increased risk of Achilles tendon rupture. Several subsequent publications investigated the association in different populations, some in agreement [101, 102] and others not finding an association.[103-105] The ABO blood group is determined by a single gene on chromosome 9q and as such, other candidate genes on chromosome 9q were investigated since the causal variant may be inherited together with a variant in a gene in the vicinity of the ABO blood group gene. Hence, genes encoding tendon proteins on chromosome 9q were identified for investigation.[87] This was the same strategy as was employed with nail patellar syndrome, a rare autosomal dominant disorder in which an association to the ABO blood group was found before the disease causing gene was mapped to the same locus as the ABO blood group gene.[106]

To date, published studies have identified sequence variants, or polymorphisms, within

- the *TNC* gene, encoding tenascin C
- the *COL5A1* gene, encoding the pro-α1(V) chain of type V collagen
- the *MMP3* gene, encoding matrix metalloproteinase-3 (MMP-3)
- the *GDF5* gene, encoding growth differentiation factor-5 (GDF-5) and
- the *IL-1β,IL-1RN* and *IL-6* genes, encoding interleukin-1β (IL-1β), nterleukin-1 receptor antagonist (IL-1ra) and interleukin 6 (IL-6), respectively,

to be associated with AT (Table 1.2).[107-113]

Table 1.2. Polymorphisms which are associated with Achilles tendinopathy in Caucasian individuals.

Gene	Polymorphism	Association	Population (and gender if applicable)	OR (95% CI)	Reference
TNC	GT repeat in intron 17	13 and 17 repeats ↓ risk of AT 12 and 14 repeats ↑ risk of AT	SA	not available	[107]
COL5A1	rs12722 (T/C)	CC genotype ↓ risk of AT	SA AUS	2.6 (1.5 – 4.5) 2.4 (1.2 – 5.0)	[108] [109]
MMP3	rs591058 (T/C)	CC genotype ↑ risk of AT	SA	2.3 (1.1 – 4.5)	[110]
	rs679620 (A/G)	GG genotype and G allele ↑ risk of AT		2.5 (1.2 – 4.9)	
	rs650108 (G/A)	AA genotype ↑ risk of AT		4.9 (1.0 – 24.1)	
GDF5	rs143383 (T/C)	TT genotype ↑ risk of AT	AUS (AUS+SA)	2.2 (1.2 – 4.2) 1.8 (1.2 – 2.7)	[111]
IL-1β	rs16944 (T/C)	TT genotype ↓ risk of AT	SA males	3.4 (1.2 – 9.6)	[113]
	rs1143627 (T/C)	C allele ↓ risk of AT	SA males	1.7 (1.0 – 2.7)	
IL-6	rs1800795 (G/C)	GG genotype ↑ risk of AT G allele ↑ risk of AT	AUS	2.1 (1.2 – 3.6) 1.5 (1.0 – 2.2)	[112]

The accession numbers of single nucleotide polymorphisms (SNPs) were identified from databases hosted by the national centre for biotechnology information (NBCI) (http://www.ncbi.nlm.nih. gov/). Major and minor alleles of SNPs are reported in parenthesis, respectively. Up arrow (↑), increased; down arrow (↓), decreased; AT, Achilles tendinopathy; AUS, Australia; *COL5A1*, gene encoding type V collagen; *GDF5*, gene encoding growth differentiation factor 5; *IL-1β*, gene encoding interleukin-1β; *IL-6*, gene encoding interleukin-6; *MMP3*, gene encoding matrix metalloproteinases; OR (95% CI), odds ratio (95% confidence interval); SA, South Africa; *TNC*, gene encoding tenascin C.

1.3.2. **Genes on chromosome 9: *TNC* and *COL5A1***

Polymorphisms within two genes on chromosome 9 are associated with Achilles tendon injuries.

i) A GT dinucleotide repeat polymorphism within the *TNC* gene was shown to be associated with both tendinopathy and rupture in a South African population.[107] The *TNC* gene encodes a glycoprotein tenascin C, which regulates cell-matrix interactions and is upregulated by mechanical loading.[114] Alleles of the gene containing 12 and 14 repeats were overrepresented in the Achilles tendon injury group thus increasing risk of developing AT, while the alleles containing 13 and 17 repeats were underrepresented, conferring protection from developing AT (Table 1.2).[107]

ii) The single nucleotide polymorphism (SNP) rs12722, within the 3' untranslated region (UTR) of the *COL5A1* gene was associated with tendinopathy, but not ruptures, in South Africans [108] and Australians.[109] The *COL5A1* gene encodes the pro-α1(V) chain of type V collagen, the rate limiting component of the assembly of type V collagen trimer and a structural component of the tendon.[115] In the initial study, carried out in South African individuals, the frequency of the CC genotype of the *COL5A1 Bst*UI RFLP (rs12722) was significantly higher in the control group compared to the AT group.[108] The second study investigated rs12722, as well as other SNPs within the 3'UTR of the *COL5A1* gene in South African and Australian individuals. The CC genotype of rs12722 was again found to be associated with decreased risk of developing tendinopathy, in both South African and Australian individuals (Table 1.2). Furthermore, a significant over-representation of the TC haplotype (consisting of SNPs rs12722 and rs3196378) was found in the South African tendinopathy group compared to controls.[109] These studies collectively implicate the 3'UTR of

the *COL5A1* gene as a potential predisposing genetic region for the development of AT.

1.3.3. *MMP3* gene

Using the candidate gene approach, a third locus, within the *MMP3* gene on chromosome 11q22.2 was shown to be associated with increased risk of developing AT in South Africans.[110] The *MMP3* gene encodes MMP-3, which is involved in catalysing the degradation of type II, IV, V, IX and X collagen, laminin, fibronectin, decorin and aggrecan.[116] The three SNPs investigated (rs679620, rs591058 and rs650108) were found to be associated with increased risk of developing tendinopathy (Table 1.2). Furthermore a significant interaction was found between this locus and the *COL5A1* rs12722 SNP.[110]

1.3.4. *GDF5* gene

A SNP within the *GDF5* gene located on chromosome 20q11.22, was associated with risk of developing AT.[111] The *GDF5* gene encodes a cytokine, GDF-5, which regulates the maintenance and healing of the tendon.[117] The TT genotype of the variant rs143383 located within the 5'UTR of the *GDF5* gene, was overrepresented in individuals with AT in Australian individuals, independently and when combined with South African individuals (Table 1.2).[111]

1.3.5. *IL-1β*, *IL-1RN* and *IL-6* genes

The most recent study evaluated variants within the *IL-1β*, *IL-1RN* and *IL-6* genes.[118] These genes encode the cytokines IL-1β, IL-1ra and IL-6 respectively and are involved in the ECM degradation and apoptosis inflammatory processes. All four loci investigated in this study (*IL-1β* -31T>C,

IL-1β -511C>T, *IL-1RN* VNTR and *IL-6* -172G>C) together with the *COL5A1* *Bst*UI RFLP (Section 1.3.2) were collectively significantly associated with risk of AT.[118] This study suggested that the collectively the variants within genes of the inflammatory pathway may contribute to risk of AT and thus the inflammatory pathway warrants further investigation.

Chapter 2

THE INFLAMMATORY PATHWAY IN ACHILLES TENDINOPATHY

THE INFLAMMATORY PATHWAY IN ACHILLES TENDINOPATHY

The exact biological mechanisms underlying the development of Achilles tendinopathy still remain poorly understood.[5] Mechanical overload is thought to be the primary extrinsic risk factor for development of tendinopathy.[119, 120] It is established that mechanical loading is required to maintain normal tendon homeostasis [121] and mechanotransduction of mechanical loading into cellular responses is an ongoing physiological process which allows for adaptation.[60] However, mechanical overload may compromise the natural tendon healing and adaptation processes leading to tendinopathy. This being said, preventing tendinopathy is not as simple as avoiding mechanical overloading since AT also occurs in sedentary individuals.[34] Therefore other intrinsic risk factors, including genetic risk factors, should be explored to possibly explain some of inter-individual variation in susceptibility to AT.[90]

Studies investigating gene expression in injured tendons reveal that chronic tendinopathy has a distinct mRNA expression signature, consistent with accelerated ECM remodelling.[22, 122, 123] Some of the major expression alterations observed in tendinopathic tendons are summarised in Table 2.1.

Table 2.1. Expression patterns of matrix proteins, cytokines and signalling factors and enzymes in degenerative tendons. Modified from Riley, (2008) [13] and Xu *et al.*,(2008) [122] supplemented by [15, 72, 123].

	Cytokines and signalling factors	Enzymes	Structural matrix components
Increased expression	TGF-β IGF-1 IL-6, 15, 18 PDGF-R VEGF-R COX-2 NOS Glutamate Substance P NMDAR TNFRSF8	MMP-1, 2, 9, 11, 13, 14, 16, 17, 19, 23, 25 Caspase 3, 8 ADAMTS-2, 3, 4 ADAM-8, 12	Collagen type I, III, IV, V, XVI, VIII Fibronectin Laminin Chondrointin sulphate Heparan sulphate Tenascin C Aggrecan Biglycan
Decreased expression	TGF-βR1	MMP-3, 10, 12, 24, 27, 28 TIMP-1, 2, 3, 4 ADAMTS-5, 7, 13	Dermatan sulphate Collagen type XI

Abbreviations: ADAM, a disintegrin and metalloproteinase; ADAMTS, ADAM with thrombospondin motifs; COX-2, cyclooxygenase-2; IGF-I, insulin like growth factor-1; IL, interleukin; MMP, matrix metalloproteinase; NMDAR, n-methyl-D-aspartate receptor; NOS, nitric oxide synthase; PDGF-R, platelet-derived growth factor receptor; TIMP, tissue inhibitors of metalloproteinases; TGF-β2, transforming growth factor beta-2; TGF-βR1, transforming growth factor beta receptor-1; TNFRSF8, tumor necrosis factor receptor superfamily, member 8; VEGF-R, vascular endothelial growth factor receptor.

Gene expression of tenocytes is modulated in response to mechanical loading.[22] It is therefore reasonable to hypothesise that tendon degeneration, characteristic of tendinopathy, may be secondary to the tenocytes inability to effectively regulate gene expression and maintain ECM homeostasis in response to repeated microinjury resulting from repetitive mechanical loading. While the expression of all these protein types; structural components, enzymes and cytokines, are altered in tendinopathy, it is only during surgery that histopathological tendon samples can be obtained and this is well into the chronic stage of tendinopathy. Therefore the expression signature of tendinopathy (Table 2.1) is a 'snap shot' view of the tendon, well into the chronic stage of the disease and it is not known whether the altered expression of these proteins is a consequence of the tendon injury or the cause. It should be noted that, while we assume the altered expression signature to be as a result of altered expression by tenocytes, since tenocytes are the main cell type in the tendon and end stage tendinopathy is normally void of inflammatory cells, we can't exclude the possibility that other cell types invading the tendon, such as endothelial cells from neovascularization, contribute to the altered expression signature of tendinopathy.

Studying the earlier phases of the condition may provide a better understanding of the underlying molecular mechanisms and provide insight into the sequence of molecular changes as the condition progresses. However, researching the early stages of tendinopathy poses many ethical challenges, such as obtaining tendon samples from early stage tendinopathic tendons in individuals who do not require surgery. In addition, pathophysiological changes in the tendon often precedes symptoms,[124] making it difficult to identify these individuals in the early stages of tendinopathy. Therefore animal models and other molecular approaches, such as genetics association studies, are currently two of the tools used to identify the underlying biological pathways.

Backman *et al*., (1990) [125] presented a rabbit model for Achilles tendinopathy. The degenerative histological changes of chronic tendinopathy, were induced in the rabbit tendons following chronic loading programmes,[126] thus providing a suitable animal model investigating the mechanisms underlying Achilles tendinopathy. Furthermore, if the tendons were examined after a single exercise session, evidence of inflammatory cell infiltration is observed.[125] The animal model has thereby provided evidence for an early inflammatory reaction and that degenerative processes occur later in the progression, however the steps in between and molecular mechanisms are still unclear and further research is required.

An advantage of using a genetic association study design, as employed in this dissertation, is that it exploits differences between the genetic profile of affected and unaffected individuals which are independent of the disease, unlike protein/mRNA expression studies in diseased tissue in which expression profiles may be as a consequence of the pathology and not the underlying cause. One limitation however of genetic association studies is that they cannot establish or infer causation. Genetic association studies are however a good starting point as they have the potential to allow one to more easily discriminate the proteins which are potentially directly involved in the pathogenesis from those which are upregulated as a consequence of the condition.

The studies in this dissertation exploit genetics as a tool to identify specific regions of the genome associated with tendinopathy, with the ultimate goal of elucidating the pathways underlying AT.[97] We propose that it is initially the expression of cytokines from tenocytes which are modulated in response to mechanical loading and this leads to altered expression of enzymes and structural proteins. Furthermore, microdamage caused by the mechanical loading, could result in infiltration of inflammatory cells which may aid tenocytes in their remodelling processes. The identification of components of the inflammatory pathway involved in tendinopathy would provide critical

insight into the mechanisms underlying the condition so that, in the future, steps can be taken towards the development of improved therapeutic and clinical management strategies.

There is a vast body of evidence to suggest that tendinopathy is a degenerative disorder characterised by degradation of the ECM.[43, 55, 127-130] In most cases, histological and microdyalisis analysis has revealed that this degeneration occurs in the presence of only a few or no inflammatory cells.[43, 55, 127-130] This being said, it should be noted that new immunohistochemical techniques have rendered interesting results: inflammatory cells have indeed been identified in degenerative tendons [66-69] and inflammation has been observed in tendinopathic Achilles tendons using colour Doppler imaging.[131] Specifically, in tendinopathy, increased presence of macrophages and lymphocytes were observed within early tendinopathic (symptoms for 5 – 12 months) Achilles tendons [66] and mast cell infiltration was observed in patellar tendinopathy (symptoms for >12 months).[68] The presence of macrophages, mast cells, and T cells in early (symptoms for average 8 months) tendinopathic supraspinitus tendons has also been observed.[69] In ruptured Achilles tendons, neutrophils were observed [66, 67] and the authors concluded that the infiltration of these inflammatory cells preceded the rupture and were not present as a result of the rupture because the rupture was only a few hours old at the time of surgery.[67] Neutrophils are however, acute inflammatory cells and hours is indeed sufficient time for recruitment of neutrophils to the acute injury, therefore it is still debatable whether neutrophil infiltration precedes rupture. Vascularity of the tendon is required for the infiltration of inflammatory cells and indeed neovascularisation of the tendon is considered an important aetiological factor in AT.[43, 56] Abnormal vessels accompanied by proliferating nerves have been observed in the ventral aspect of the tendon adjacent to Kager's triangle.[56, 132] These studies collectively suggest that inflammation may be implicated in tendinopathy, especially in the early stages. Further studies will have to be conducted to confirm these results.

Whether inflammatory cells are present in tendinopathy and play a critical role in its initiation or progression, is a concept which is only beginning to be accepted in a condition which is currently considered to be primarily degenerative (and not inflammatory). Irrespective of inflammatory cell presence, tendinopathy is unquestionably associated with the altered expression of many cytokines and signalling factors (Table 2.1). Inflammatory cells do not have to be present in order for inflammatory signalling cascades to be triggered.[14, 81, 133] Tenocytes themselves are able to upregulate and produce cytokines and inflammatory mediators in response to mechanical loading, which act in an autocrine and/or paracrine manner to further influence gene expression of the resident tenocytes.[14, 81, 133] It must be emphasised that the cytokines traditionally associated with the inflammatory response may also be part of the normal healing and adaptation strategy of the tenocytes to maintain ECM homeostasis. However, it can be propose that the upregulated expression of inflammatory mediators by tenocytes, in circumstances of repetitive mechanical loading, are able to drive the progression of tendinopathy.

There is also the question of the involvement of inflammatory mediators from adipose tissue which may infiltrate tendon tissue and act on tenocytes. Increased adiposity is associated with elevated proinflammatory cytokine levels resulting in a systemic chronic low-grade inflammation. This is thought to be a contributing factor in the development of cardiovascular disease and diabetes mellitus type II [134, 135] and could therefore also exacerbate inflammatory cascades triggered by loading in tenocytes.[136] Additionally, individuals with Achilles tendinopathy were found to have dyslipidaemia, similar to the lipid profile observed in insulin resistance.[137] Adiposity is therefore an important intrinsic risk factor to consider in the development of tendinopathy. The inflammatory effect (if there is one) of Kager's fat pad, which is situated within the Achilles tendon paratenon, also needs to be investigated, since it is a potential source of inflammatory mediators in close proximity to the Achilles tendon.

Since expression of inflammatory mediators is altered in the tendinopathic state (Table 2.1), the genes encoding the proteins involved in the tenocyte inflammatory pathway may be considered as candidate genes to investigate for associations to AT. Recently, variants within the *GDF5, IL-1β* and *IL-6* genes were identified to, independently or collectively, be associated with risk of AT,[111, 118] providing further evidence for the potential involvement of cytokines in the development of AT. The *IL-1β* and *IL-6* genes encode the cytokines IL-1β and IL-6, which are involved in the tendon ECM degradation and apoptosis inflammatory signalling cascades, respectively (Figure 2.1).[14, 15] These signalling cascades will be further explored.

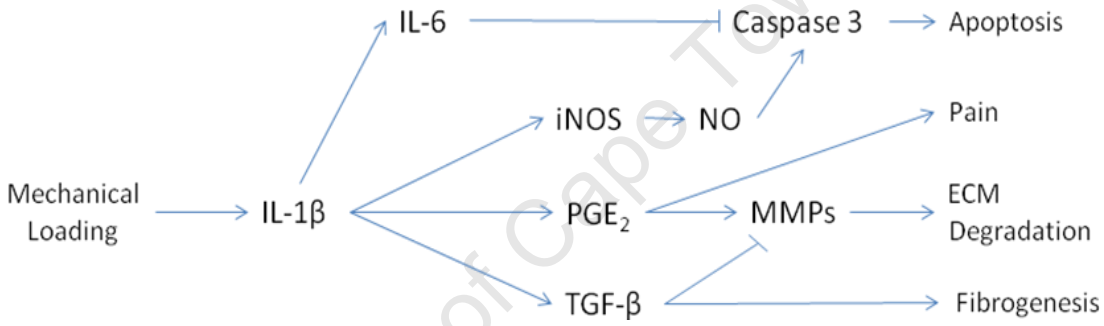

Figure 2.1. Schematic representation of the downstream effects of IL-1β and IL-6 activation in response to mechanical loading.[14, 81, 133, 138-148] Mechanical loading causes upregulation of cytokines such as IL-1β from tenocytes which initiates signalling cascades resulting in apoptosis, pain, ECM degradation and fibrogenesis. ECM, extracellular matrix; IL-1β, interleukin-1β; IL-6, interleukin-6; PGE_2, prostaglandin E_2; MMP, metalloproteinase; NO, nitric oxide; NOS, nitric oxide synthase; TGF-β, transforming growth factors-β.

2.1. **EXTRACELLULAR MATRIX DEGRADATION SIGNALING CASCADE**

The cytokine which has largely been implicated for the propagation of the tendon matrix degradation signalling cascade in tenocytes is IL-1β, via its inflammatory mediators, cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2). COX-2 expression is induced by pro-inflammatory mediators such as cytokines and growth factors.[149] This is in contrast to COX-1, which is constitutively expressed in most cells.[149] Microdialysis has failed to demonstrate an increase in PGE_2 in patellar or Achilles tendinopathy.[128, 130, 131] Immunohistochemistry has however confirmed the presence of elevated COX-2 in patellar tendinopathy [73] and also elevated levels of PGE_2 in mouse Achilles and patellar tendons, as well as human tenocyte culture following repetitive mechanical loading.[73, 150] COX-2 is of clinical significance as it is the target for non-steroidal anti-inflammatory drugs (NSAIDs), one of the major drugs which were prescribed for the short-term alleviation of tendinopathy-associated pain. NSAIDs are however no longer recommended for pain alleviation since their effectiveness in treating the symptoms of AT is less than desirable and they may interfere with the signalling pathways critical for tendon healing and adaptation.[151, 152] Polymorphisms within the *COX-2* gene have been shown to modulate response to NSAID treatment.[153] One of these polymorphisms, -765G>C was investigated in this dissertation for risk of AT (Chapter 3).

Tsuzaki *et al.*, (2003b) [14] proposed a model for cytokine-induced expression of inflammatory mediators: trauma or overuse causes tendon cells to respond to exogenous IL-1β by producing COX-2 and PGE_2 which leads to matrix metalloproteinase (MMP) production and subsequent ECM degradation. Endogenous interleukins such as IL-1β and IL-6 are also induced and act on tenocytes in a paracrine fashion.[14] IL-1β-mediated expression of endogenous IL-1β and IL-6, if chronically upregulated, could potentially result in a destructive positive feedback loop in which IL-1β

perpetuates its own expression resulting in excessive cytokine-mediated ECM degradation.

The model for cytokine-induced expression of inflammatory mediators was based on findings that (i) tenocytes possess the active IL-1 receptor, IL-1RI, (ii) repetitive strain capable of causing microinjury induces endogenous IL-1β in tenocyte culture models, (iii) IL-1β upregulates expression of cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) (iv) COX-2 and mPGES-1 are needed for the PGE_2 synthesis and (v) PGE_2 induces transcription of MMP-1 and MMP-3 and inhibits collagen type I transcription (Figure 2.2).[14, 133, 154] Taken together, these *in vitro* and *ex vivo* studies indicate that IL-1β is an important cytokine responsible for activating the signalling cascade leading to ECM degradation and tendinopathy *in vivo*. Furthermore, the progression of tendinopathy has been suggested to centre around the production of PGE_2 as it has been identified as an important mediator of pain and inflammation in tendons, both in rabbit and mechanically loaded human tendons.[82, 155, 156]

Figure 2.2. Schematic representation IL-1β inflammatory pathway leading to ECM degradation in tendons.[14, 81, 133, 138, 139, 142] COX-2; cyclooxygenase-2; ECM, extracellular matrix; IL-1β, interleukin-1β; PGE_2, prostaglandin E_2; MMP, matrix metalloproteinase.

Interestingly, IL-1β also specifically upregulates the PGE_2 receptor subtype EP_4.[133] Furthermore, blocking this receptor with an antagonist reverses the catabolic PGE_2 effects, namely the degradation of ECM components by MMPs and suppression of type I collagen synthesis.[133] This suggests that the EP_4 receptor is critical to PGE_2 signalling and subsequent matrix degradation.

Transforming growth factor-β (TGF-β) is another cytokine which could potentially be involved in tendon pathology since it regulates tissue repair [157] and is elevated in both Achilles and patellar tendinopathy in humans.[73, 158] The TGF-β superfamily include the bone morphogenic proteins (BMPs), growth differentiation factors (GDFs), anti-müllerian hormone (AMH) and TGF-β's.[159] There are three isoforms of TGF-β that are expressed in mammals; TGF-β1, TGF-β2, and TGF-β3. It has been suggested that TGF-β may play a pivotal role in switching the healing process from normal to pathological.[160] IL-1β is able to upregulate TGF-β and inhibit GDF-5 in chondrocytes and we propose, given the similarity in function within their respective tissues, that a similar mechanism is proposed to occur in tenocytes (Figure 2.3).[145, 161] The TGF-β isoforms interact with each other to regulate collagen deposition in healing tendons, with different isoforms causing expression or inhibition of different collagen types, including type I, type II and type V collagen.[140, 143, 162] Their regulation is therefore important in determining the balance between fibrosis and normal healing.[143, 162]

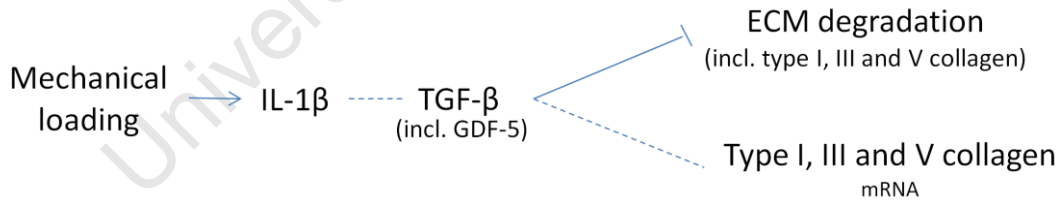

Figure 2.3. Schematic representation of the TGF-β signalling cascades contributing towards fibrosis, by the prevention of ECM degradation and the upregulation of collagen gene expression.[81, 140, 141, 143, 145, 161, 162] Hashed arrows indicate signalling pathways that are known to occur in chondrocytes or osteoblasts and proposed to occur in tenocytes.[140, 145, 161, 162] Lines without arrow heads or inhibitory heads indicate that some subtypes of the effector protein are upregulated and others inhibited. Details are provided in Figure 2.7. ECM, extracellular matrix; GDF-5, growth differentiation factor-5; IL-1β, interleukin-1β; IL-6, interleukin-6; TGF-β, transforming growth factors-β.

The upregulation of TGF-β in tendinopathy may seem counterintuitive because it initiates signalling cascades preventing ECM degradation and initiating synthesis of ECM components, in the midst of a degenerative condition (Figure 2.3). However, the increased TGF-β expression observed in late tendinopathy may be as a result of the tenocytes' response to the ongoing matrix degradation. It is the natural healing response to balance the ECM degradation with collagen synthesis to replace that which has been removed. It is hypothesised that the perpetuating IL-1β feedback loop as a result of repetitive loading, however, does not allow for the expected assembly of newly synthesised collagen fibrils into stronger tendon fibrils and disarrayed collagen deposition results, forming the fibrotic nodules characteristic of tendinopathy.[46] TGF-β contributes towards the fibrosis process via two proposed mechanisms (i) by activating expression of the major collagen types and (ii) by enhancing tissue inhibitors of metalloproteinase (TIMP) expression and inhibiting MMP expression (Figure 2.3).[22, 141] MMPs are catabolic enzymes that are responsible for tendon ECM degradation and TIMPs bind to the active site of MMPs, inhibiting the action of MMPs and therefore ECM degradation.[163] The effects of TGF-β on TIMP and MMP expression effectively shift the MMP/TIMP balance to net inhibition of ECM breakdown and together with the increased collagen synthesis, TGF-β expression may contribute towards fibrosis (Figure 2.4).[157] The TGF-β signalling cascade within tenocytes therefore counteract the catabolic downstream effects of PGE_2 signalling to maintain homeostasis of the ECM (Figure 2.1).

Therefore it is feasible that alterations in the expression of TGF-β could lead to disregulation of tendon ECM components and ultimately AT. This being said, the downstream consequence of elevated TGF-β, namely elevated TIMP expression, is however not observed in tendinopathy.[123, 164] It should be kept in mind that there is redundancy in the genome, meaning that different genes encode proteins with similar functions. In the case of cytokines, many cytokines have similar effects, so if there is a lack of a

certain element, it may be compensated for by a complementary signalling cascade. It is therefore possible that there are other regulators of TIMP and MMP expression which may have a greater effect, for example the PGE_2 signalling cascade, and perhaps the role of TGF-β in tendinopathy is rather its role in fibrosis via collagen synthesis.[143]

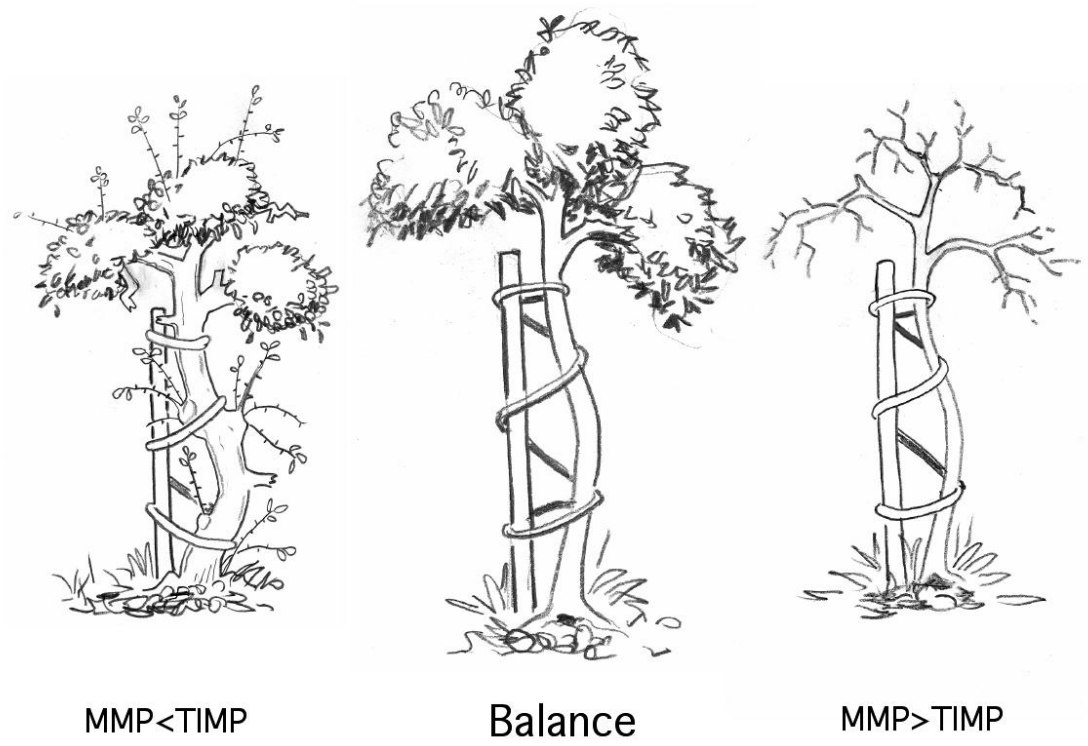

Figure 2.4. MMPs are primarily responsible for degradation of the tendon ECM and TIMPs counteract this degradation. If the balance is not tightly regulated, the equilibrium may shift leading to fibrosis (left) or degeneration (right). Taken from Pasternak *et al.*, (2009).[142]

The emphasis of this chapter has been pathological inflammatory cascades leading to abnormal degradation, however, it should be stressed that the production of inflammatory cytokines by tenocytes in response to mechanical loading is part of the normal healing response in reaction to mechanical loading. More specifically, the pro-inflammatory response causes upregulation of MMPs, via PGE_2, to degrade damaged collagen molecules

and upregulation of TGF-β to synthesise new replacement collagen. However, repetitive mechanical loading may lead to a failure to regulate the expression of inflammatory mediators. This may ultimately lead to a failed healing response due to extended periods of matrix degradation in the absence of appropriate adaptation and time to generate stronger fibrils.[57] Furthermore, since increased expression of inflammatory mediators can result in pathological ECM degradation, it is reasonable to propose that possession of genetic variants that result in increased expression of these inflammatory mediators in response to mechanical loading, may predispose an individual to AT.

It should be emphasised that AT is a multifactorial condition and therefore it is most likely that many biological mechanisms and pathways are involved in the predisposition to injury. The ECM degradation signalling pathway is therefore only one biological pathway which may play a role in the development of tendinopathy. Another signalling cascade of the inflammatory pathway, which may be involved in the development of tendinopathy, is the apoptosis signalling cascade.

2.2. **APOPTOSIS SIGNALING CASCADE**

Tendinopathy is also characterised by apoptosis of tenocytes, as observed in supraphysiologically loaded rat tibialis anterior tendons,[165] in the torn edges of the human rotator-cuff [78] and in patellar tendinopathy.[53] The main function of tenocytes is to regulate ECM turnover,[21] thus excessive apoptosis of these cells may place the tendon in a compromised state.

Millar *et al*, (2008) [166] found that the apoptotic genes, *CASP8* and *CASP3*, encoding caspase-8 and -3, were upregulated in the torn edges of rat supraspinatus tendons. Hypothesising that the upregulation of caspase genes was cytokine induced, in a subsequent study the authors investigated interleukin expression in tendinopathic tendons. The cytokines TNF-α , IL-18, IL-15 and IL-6 were shown to be upregulated in both a rat model of tendinopathy and human supraspinatus tendons.[15] In addition this study showed that the increase in inflammatory cytokine production was accompanied by an increase in the caspase-8 and -3 expression.[15] Caspase-8 is part of the death receptor apoptosis signalling cascade, which is activated by multimerization of the trans-membrane receptor Fas. Fas is part of the TNF receptor superfamily and is expressed by fibroblasts, amongst other cell types.[167]

Caspases or *c*ysteine-*asp*artic prote*ases*, are a family of cysteine proteases which mediate apoptosis by proteolysis of structural and enzymatic proteins.[168] Typically, the apoptosis cascade will contain an initiator caspase and an effector caspase.[168] Initiator caspases include caspase-8 and -10 which, once activated, activate other caspases in a cascade. The cascade eventually results in the activation of the effector caspases, such as caspase-3 and -6. Effector caspases are responsible for the cleavage of structural nuclear proteins, the inhibition of DNA repair enzymes and the activation of DNases, which leads to apoptosis.[168]

It is therefore proposed that during the tendon healing phase in response to normal physiological loading, TNF-α, IL-18, IL-15 and IL-6 are expressed by tenocytes or other cell types in the vicinity.[15] These cytokines are involved in apoptosis, either activating or inhibiting synthesising of caspases to regulate apoptosis.[169-172]

IL-6 activates signal transducer and activator of transcription-3 (Stat-3) in tenocytes.[173] The function of IL-6 in the apoptosis pathway in tenocytes has not been fully characterised, however, IL-6, via Stat-3, is known to have anti-apoptotic effects [144, 174-177] and may act through Bcl-2 and FLIP FLICE-like inhibitory protein (FLIP) to inhibit caspase-8 and caspase-3, respectively (Figure 2.5).[144] Furthermore, IL-6 can be induced by IL-1β following cyclic mechanical loading.[14]

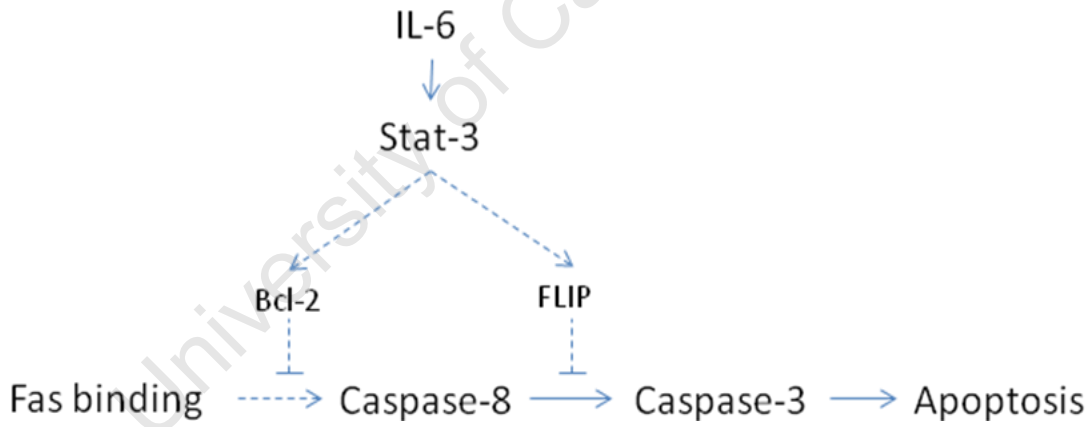

Figure 2.5. Schematic representation of the proposed inflammatory pathway leading to apoptosis indicating the anti-apoptotic effect of IL-6. [144, 166, 173] Bcl-2, B-cell lymphoma-2; FLIP, FLICE-like inhibitory protein, IL-6, interleukin-6; Stat-3, signal transducer and activator of transcription-3.

The function of tenocyte apoptosis following loading is thought to be for removal of partially damaged cells while the undamaged tenocytes proliferate [178] and secrete enzymes and structural protein to degrade and remodel the ECM; thus the natural healing process can occur. In situations of repetitive

loading the tenocytes reduce forces on the tendon by secreting ECM components which draw water (as described in Section 1.2.2).[63] However, this reduction of tension between the tenocytes and ECM may trigger apoptosis [65] and may therefore lead to an inability to complete the normal repair process effectively. In this way cytokine-mediated tenocyte apoptosis could contribute to development of tendinopathy. Identification of the factors driving tenocytes towards abnormal apoptosis are therefore of interest, especially since pro-apoptotic and anti-apoptotic factors are found simultaneously in tendinopathic tendons.[15]

Another apoptosis signalling cascade which may play a role in the aetiology of AT is the nitric oxide (NO) pathway.[179] NO is upregulated in response to cyclic strain of tendon explants.[180] This is expected because NO is important for healing of tendons [181-183] and can induce critical healing strategies such as angiogenesis, cell proliferation and collagen synthesis.[184-186] In contrast, high levels of NO are often associated with degradative processes. Increased NO levels are implicated in several degenerative conditions and as a mediator for apoptosis.[148, 187-189] NO can trigger apoptosis through several mechanisms, all of which act through release of cytochrome-c from mitochondria and subsequent activation of caspase-3.[147, 190] NO has also been implicated in the activation of IL-1 mediated MMP production in chondrocytes [146, 148] and perhaps a similar mechanism may occur in tenocytes (Figure 2.6).

NO synthase (NOS) is the enzyme which catalyses synthesis of NO from L-Arginine (Figure 2.6). Three isoforms have been described; inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS). iNOS produces high concentrations of NO in response to stimuli, while eNOS and nNOS isoforms are expressed constitutively to produce relatively low, stable amounts of NO.[191] Synthesis by NOS is the rate limiting step for the production of NO, therefore NOS plays a critical role in regulation of tendon

catabolism via this apoptosis signalling cascade. The expression of all three isoforms is elevated in tendinopathy, with eNOS and iNOS specifically being investigated and found to be elevated in AT.[179, 192]

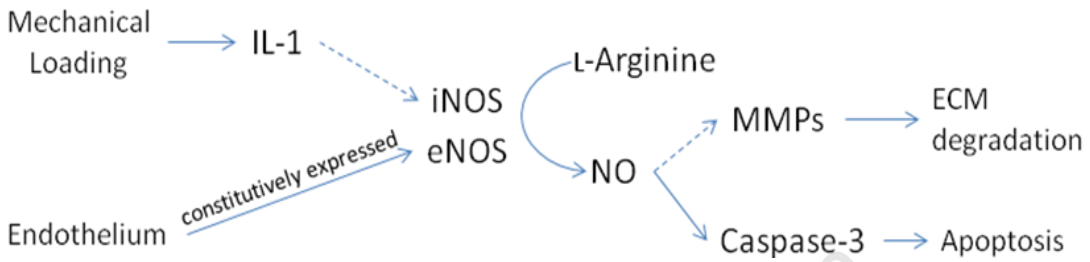

Figure 2.6. Schematic representation nitric oxide signalling cascade leading to apoptosis. [81, 146-148] Hashed arrows indicate pathways which have been observed in chondrocytes and are proposed to be similar in tenocytes. ECM, extracellular matrix degradation, IL, interleukin; eNOS, endothelium nitric oxide synthase; iNOS, inducible nitric oxide synthase; NO, nitric oxide.

2.3. CANDIDATE GENES WITHIN THE INFLAMMATORY PATHWAY

The research implicating loci within the *IL-1β* and *IL-6* genes as collective contributors to risk of AT,[118] substantiates further exploration of the inflammatory signalling cascades in the development of AT. The products of these genes, namely IL-1β and IL-6 respectively, are involved in the ECM degradation and apoptosis pathways.

The focus of this dissertation was therefore to investigate inflammatory mediators downstream of the IL-1β and IL-6 in the signalling cascades involved in ECM degradation and apoptosis [14, 15] However, we recognise that several other plausible candidate genes could be explored, which encode proteins that play a role in the pain, ECM degradation and apoptosis signalling cascades, such as tumor necrosis factor alpha (TNF-α), substance P, vascular endothelial growth factor (VEGF) and insulin-like growth factors-1 (IGF-1).[58, 62, 66, 193, 194] Furthermore, while signalling cascades within tenocytes were explored, other cell types resident in the tendon in the healing phases or chronic AT, may also be influenced by genetic variants within the genes encoding mediators within these important biological pathways. The candidate genes explored in this dissertation were however chosen because they function downstream of the IL-1β and IL-6 cytokines in these signalling pathways.

The aims of this dissertation were therefore to use a pathway-based genetic association approach following a case-control study design to implicate the inflammatory pathway in the predisposition to AT. Proposed functional variants within genes encoding proteins functioning in the ECM degradation and the apoptosis signalling cascades were explored for associations to AT (Figure 2.7). Candidate genes were selected based on their biological function in tendon ECM degradation or apoptosis signalling cascades and their chromosomal locations are presented in Figure 2.8.

The objectives of the specific genetic association studies forming part of this dissertation were as follows:

- to determine whether variants within genes encoding proteins involved in the ECM degradation signalling cascade (*COX-2* -765G>C, *PTGER4* gene desert A>C and *TGFB2* -876A>C) were associated with risk of AT and to assess the effectiveness of a risk model comprising of six variants within genes encoding proteins functioning within the ECM signalling cascade as predictors of risk of developing AT (**Chapter 3**).

- to determine whether variants within genes encoding proteins involved in the apoptosis signalling cascade (*CASP8* -652 6N del, *CASP8* Asp302His, *NOS3* Glu298Asp and *NOS2* -1026C>A) were associated with risk of AT and to assess the effectiveness of a risk model comprising seven variants within genes encoding proteins functioning within the apoptosis cascade as predictors of risk of developing AT (**Chapter 4**).

- to identify whether a polygenic risk model comprised of variants within genes from both the ECM degradation and apoptosis pathways is a better predictor of risk of AT than the respective independent models (**Chapter 5**).

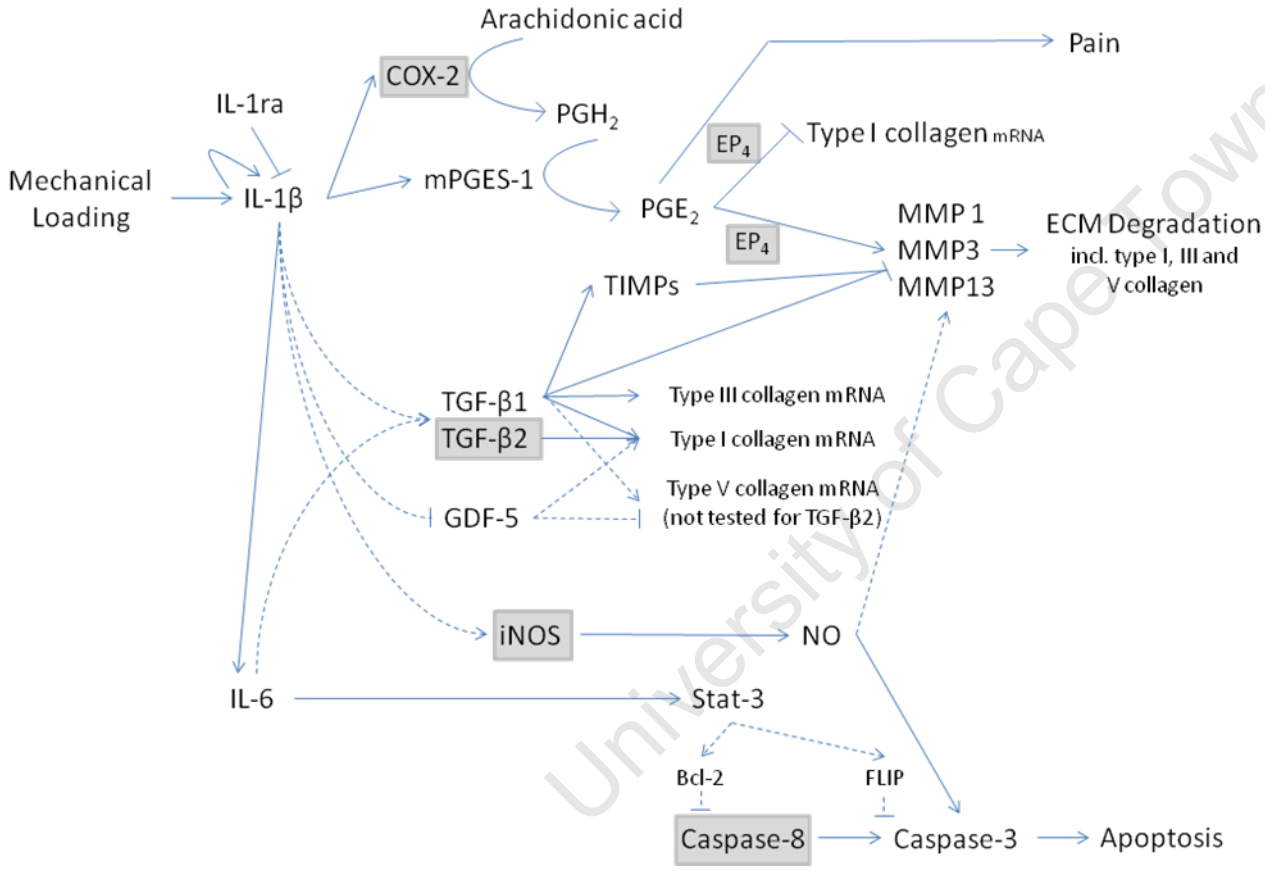

Figure 2.7. Schematic representation of the IL-1β and IL-6 signalling cascades resulting in matrix degradation and apoptosis, respectively.[14, 15, 81, 133, 138-148, 161, 162, 173] (This diagram depicts all the identified inflammatory signalling cascades downstream of IL-1β and IL-6, but this does not exclude the possibility that these cytokines and mediators may also be involved in other signalling pathways.) Hashed arrows indicate signalling pathways that are known to occur in chondrocytes or osteoblasts and proposed to occur in tenocytes.[138-140, 145, 146, 148, 161, 161, 162] The proteins of the genes which were investigated in this dissertation are boxed. Bcl-2, B-cell lymphoma-2; COX-2, cyclooxygenase-2; ECM, extracellular matrix; EP_4, prostaglandin receptor type EP_4; FLIP, FLICE-like inhibitory protein; GDF-5, growth differentiation factor-5; IL-1β, interleukin-1β; IL-1ra, interleukin-1 receptor antagonist; IL-6, interleukin-6; PGE_2, prostaglandin E_2; PGH_2, prostaglandin H_2; MMP, metallo-proteinase; mPGES-1, microsomal prostaglandin E synthase 1; NO, nitric oxide; NOS, nitric oxide synthase; Stat-3, signal transducer and activator of transcription-3; TIMPs, tissue inhibitors of metalloproteinases; TGF-β, transforming growth factors-β.

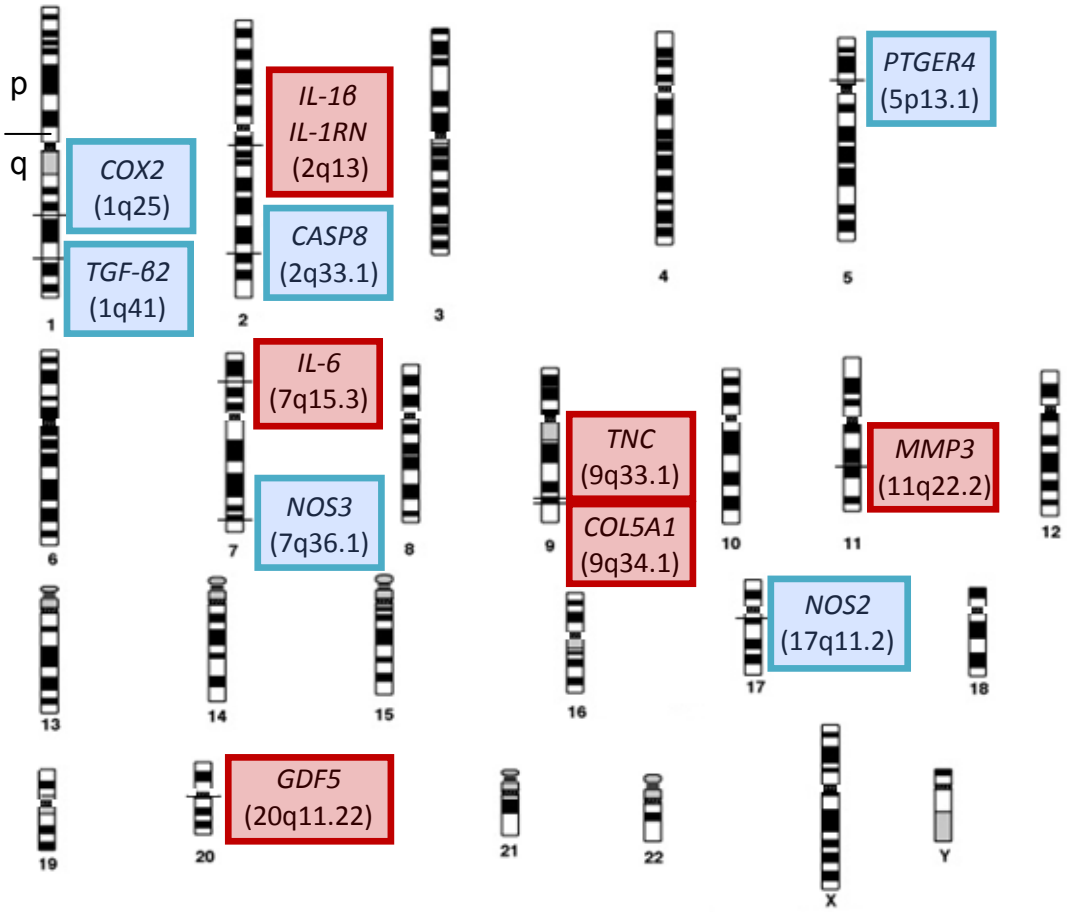

Figure 2.8. G-band ideogram for human chromosomes arrested in metaphase of mitosis. Chromosomal locations of genes (i) implicated with risk of AT in previous studies (red) and (ii) investigated in this dissertation (blue) are indicated, with chromosomal location in parentheses. The p and q arms are annotated, p indicating the short arm and q the long arm of the chromosome. *COL5A1*, gene encoding type V collagen; *COX2,* gene encoding COX-2; *CASP8,* gene encoding caspase-8; *GDF5*, gene encoding growth differentiation factor 5; *IL-1β*, gene encoding interleukin-1β; *IL-1RN,* gene encoding interleukin 1 receptor antagonist; *IL-6*, gene encoding interleukin-6; *MMP3*, gene encoding matrix metalloproteinases; *NOS3*, gene encoding endothelial nitric oxide synthase; *NOS2*, gene encoding inducible nitric oxide synthase; *PTGER4*, gene encoding PGE_2 EP_4 receptor; *TNC*, gene encoding tenascin C.

Chapter 3

EXTRACELLULAR MATRIX DEGRADATION SIGNALLING CASCADE IN TENDONS

GENETIC RISK FACTORS FOR ACHILLES TENDINOPATHY WITHIN THE EXTRACELLULAR MATRIX DEGRADATION SIGNALING CASCADE

3.1. **INTRODUCTION**

As discussed in the literature review (Section 2.1), central to the current view of the events leading to Achilles tendinopathy is the concept of mechanical overload [120] which results in ECM degradation.[43] IL-1β has been shown to be upregulated by mechanical loading [81] and induce expression of MMPs which are responsible for ECM degradation (Figure 3.1).[14] This inflammatory cascade is thought to be part of the normal response to mechanical load; to continually remodel the ECM so that damaged components are degraded and replaced by new ECM components to increase strength of the tendon components.[64] However, it is hypothesised that repetitive mechanical loading could result in a feedback loop perpetuating IL-1β expression (Figure 3.1) [14] which in turn could result in continual breakdown of ECM without adaptation, leading to the degeneration characteristic of AT.[40, 43] Identifying the inflammatory mediators which lead to ECM degradation is therefore critical in the elucidation of the biological pathways underlying AT.

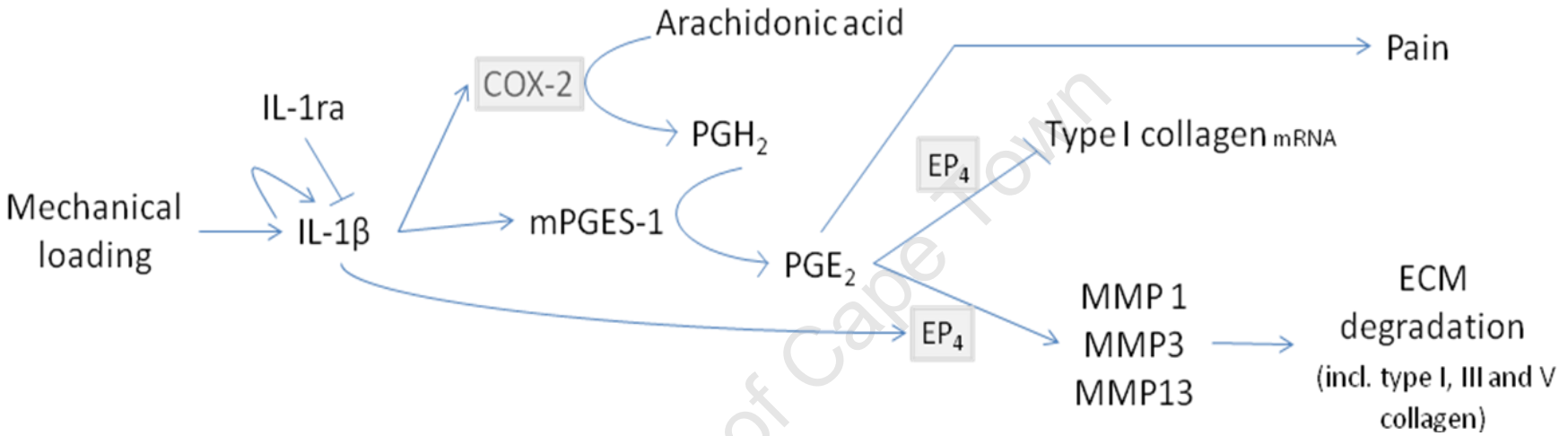

Figure 3.1. Schematic representation of the IL-1β signalling cascade in response to mechanical loading resulting in matrix degradation.[14, 81, 81, 133, 138, 139, 142] The *COX-2* and *PTGER4* genes encoding COX-2 and EP_4 receptor respectively were investigated in this dissertation and are boxed. ECM, extracellular matrix, EP_4, prostaglandin receptor type EP_4; IL-1β, interleukin-1β; IL-1ra, interleukin-1 receptor antagonist; PGE_2, prostaglandin E_2; PGH_2, prostaglandin H_2, MMP, metalloproteinase, mPGES-1, microsomal prostaglandin E synthase-1.

This study followed a pathway-based approach to identify whether the ECM degradation signalling cascade significantly contributes to risk of AT. One of the mediators of ECM degradation is PGE_2 (Figure 3.1). PGE_2 has been identified as an important mediator of pain and inflammation in tendons [82, 155, 156] and therefore genes encoding proteins involved in synthesis of PGE_2 and the propagation of PGE_2 signalling were explored.

COX-2 is one of the enzymes required for catalysing the conversion of arachidonic acid to PGE_2 (Figure 3.1), as described in detail in Section 2.1. COX-2 expression was found to be elevated in AT [92] and the expression of COX-2 can be induced by pro-inflammatory mediators such as IL-1β.[81] The gene encoding COX-2 (*COX-2,* also called *PTGS2*) was therefore investigated to determine whether polymorphisms within this gene may contribute to an intrinsic predisposition to developing AT. *COX-2* is mapped to 1q25.[195] The functional SNP -765G>C (rs20417) within the promoter region of *COX-2* is associated with increased risk of multifactorial conditions such as bronchial asthma and coronary heart disease.[196, 197] The C allele of *COX-2* -765G>C eliminates a stimulatory protein 1 (Sp1) binding site while creating an E2 promoter binding factor (E2F) binding element [149] and has been associated with 30% reduced COX-2 expression.[198] This *COX-2* variant was therefore investigated for an association to AT.

Interestingly, IL-1β also specifically upregulates the PGE_2 receptor subtype EP_4, crucial for PGE_2 signalling (Figure 3.1).[133] Any polymorphisms altering expression of EP_4 could affect the way that tenocytes respond to PGE_2. The gene encoding the EP_4 receptor, *PTGER4* is located on chromosome 5p13.1 (http://www.ncbi.nlm.nih.gov/gene/5734). Proximally from this gene is a 1.25 Mb gene-desert, which is a susceptibility locus for the multifactorial conditions Crohn's disease and asthma.[199, 200] Within the gene-desert, several SNPs have been identified. The A>C SNP, rs4495224, which is associated with Crohn's disease,[199] is situated within the gene-desert, approximately 270kb proximally from the *PTGER4* gene. The A allele of this

SNP is associated with increased expression of EP_4.[199] It is hypothesised that this region may contain *cis*-acting regulatory elements that control expression of neighbouring genes such as the *PTGER4* gene.[199, 201] The EP_4 receptor is crucial for the propagation of the PGE_2 signalling cascade and therefore altering the *PTGER4* gene expression may modulate an individual's susceptibility to AT.

TGF-β is another cytokine which alters ECM composition [143] and may therefore be involved in development of AT. TGF-β is upregulated by IL-1β [145] and contributes to fibrosis (Figure 3.2),[143] as was described in detail in Section 2.1. GDF-5 is part of a subclass of the TGF-β superfamily proteins and also activates collagen expression.[162]

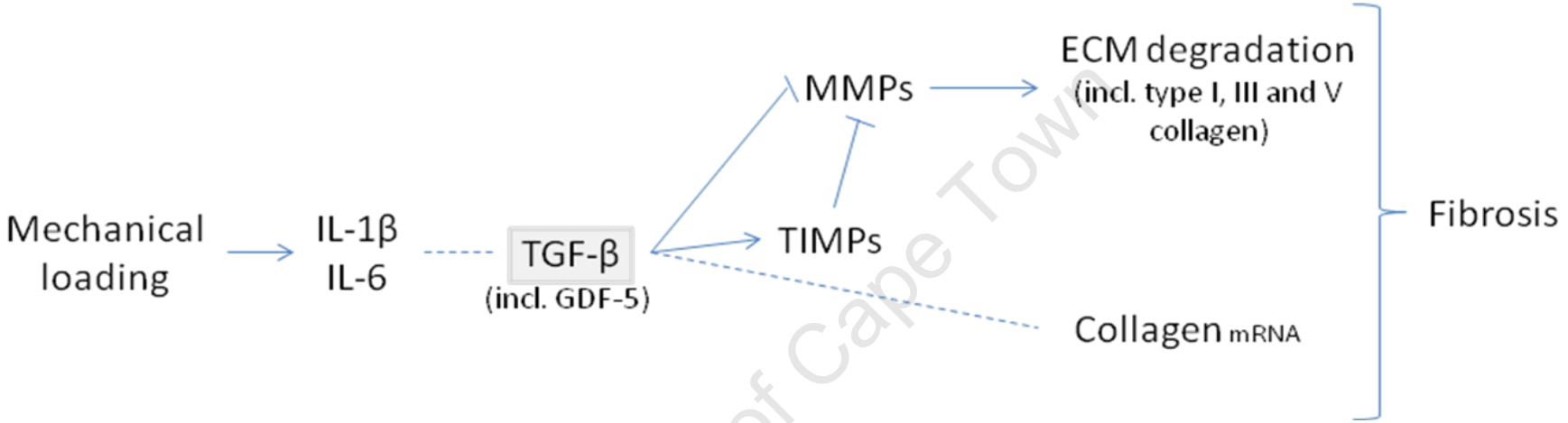

Figure 3.2. Schematic representation of the signalling cascades contributing towards fibrosis via TGF-β.[81, 140-143, 145, 161, 162] Hashed arrows indicate signalling pathways that are known to occur in chondrocytes or osteoblasts and proposed to occur in tenocytes.[140, 145, 161, 162] Lines without arrow heads or inhibitory heads indicate that some subtypes of the effector protein are upregulated and others inhibited. Details are provided in Figure 2.7. The *TGFB2* gene, encoding TGF-β2 was investigated in this study and is boxed. ECM, extracellular matrix; IL-1β, interleukin-1β; IL-6, interleukin-6; MMP, metalloproteinase; TIMPs, tissue inhibitors of metalloproteinases; TGF-β, transforming growth factors-β.

A recent genetic association study investigated polymorphisms within the *TGFB1* and *GDF5* genes for associations to AT, however no significant associations were observed.[111] TGF-β2 is however differentially expressed in human tendinopathic and normal tendons with expression of TGF-β2 being higher in tendinopathy,[158] and therefore warrants investigation. TGF-β2 is encoded by the *TGFB2* gene located on chromosome 1q41.[202] Polymorphisms within the promoter of a gene can often affect gene transcription rates and therefore the potentially functional -876A>C SNP (rs7550232) within the promoter region of the *TGFB2* gene was chosen for investigation.[203] This SNP was previously associated with the multifactorial condition myopia.[203] TGF-β plays a pivotal role in switching the healing process from normal to pathological states [160] therefore altering the expression of TGF-β2 may alter homeostasis within the ECM and thus TGF-β2 is an ideal candidate gene for investigation.

The aim of the study presented in this chapter of the dissertation was therefore to conduct a genetic association study following a pathway-based approach, investigating polymorphisms within several candidate genes in the inflammatory pathway leading to ECM degradation for associations to AT. Polymorphisms within three genes were investigated (i) the functional -765G>C variant within the promoter region of *COX-2*, (ii) the proposed functional A>C SNP in the intergenic region flanking *PTGER-4* and (iii) -876A>C variant within the promoter region of *TGFB2.* This study also aimed to assess the effectiveness of the variants within several genes encoding proteins functioning within the ECM signalling cascade using a polygenic profile to predict risk of developing AT.

3.2. **METHODS**

3.2.1. **Study design**

A pathway-based case-control genetic association study was conducted on Caucasian individuals from South Africa and Australia, investigating polymorphisms within selected candidate genes of the inflammatory pathway. This study forms part of a broader study to determine the genetic risk factors underlying tendon injury. Approval for the study was obtained from the Research Ethics Committee of the Faculty of Health Sciences within the University of Cape Town (reference number 172/2005) and Human Ethics Committees of La Trobe and Deakin Universities, Melbourne, Australia.

3.2.2. **Participants**

3.2.2.1. South African participants

A total of 256 physically active unrelated South African (SA) participants (93 clinically diagnosed with Achilles tendinopathy and 163 asymptomatic controls) were recruited, as previously described.[107, 118] The 93 participants with clinically diagnosed Achilles tendinopathy (SA TEN) were recruited from the Sports Medicine practice at the Sports Science Institute of South Africa and other clinical practices within the greater Cape Town area, South Africa. A clinician (Prof. Martin Schwellnus) reviewed the SA TEN group diagnoses, using the inclusion and exclusion criteria (Appendix A.1 – Inclusion and exclusion criteria) as described previously [107, 108] and using a checklist as presented in Appendix B – Clinical criteria. In addition to these clinical diagnostic criteria, soft tissue ultrasound examination was performed in a sub-group (31 of 93) of SA TEN participants to confirm the diagnosis in the affected Achilles tendon. The 163 asymptomatic control participants (SA CON), without history of tendon pathology, were recruited from various recreational sporting clubs.

All participants were of self-reported European Caucasian ancestry. The participants of the SA CON group were recruited such that the gender, country of birth and age of initial onset of AT were similar to that of the SA TEN group, to avoid possible confounding effects. All participants were given information regarding the study (Appendix B – Participant information) and gave their written informed consent (Appendix B – Informed consent). Participants also completed questionnaires regarding their medical history and involvement in physical activity (Appendix B – Questionnaires). The physical activity profile of the SA CON and SA TEN groups were previously described [107, 118] and are presented in Appendix A.2 – Physical activity.

3.2.2.2. Australian participants

A total of 280 Australian (AUS) participants, consisting of 198 AUS CON and 82 AUS TEN participants, were recruited from the Muskuloskeletal Research Centre at La Trobe University in Melbourne, Australia as previously described.[109, 118] The participants were recruited such that the country of birth and age of initial onset of AT were similar in the AUS CON and AUS TEN groups. All participants were of self-reported European Caucasian ancestry. A clinician (Prof. Jill Cook) reviewed the AUS TEN group diagnoses, using similar inclusion and exclusion criteria to the SA study (Appendix A.1 Inclusion and exclusion criteria), as previously described.[107, 108]

Participants all signed informed consent forms according to the Declaration of Helsinki and completed a questionnaire regarding medical history. All forms were similar to those used in the South African study (Appendix B). The Achilles tendons of all AUS TEN participants (n=85) and 116 AUS CON participants were examined by ultrasound to confirm diagnosis. Ultrasound investigation revealed that four symptomatic tendons were of normal architecture and four asymptomatic participants showed signs of AT.

3.2.3. **DNA extraction**

For the SA participants, approximately 4.5 ml of venous blood was collected into an EDTA Vacutainer® tube from the forearm vein of each participant. Blood samples were stored at 4^{o}C until total DNA extraction was carried out, following the procedure previously described by Lahiri and Nurnberger (1991), modified by Mokone *et al.*, (2005) (Appendix C – DNA extraction).[107, 204] The solutions used for blood extraction are presented in Appendix D (D.3, D.5, D.7, D.8 and D.9). For the Australian participants, approximately 4.5ml of venous blood was collected and DNA extracted using a sequence extraction technique (FlexiGene DNA Kit, Qiagen P/L, Valencia, California, USA) as per the manufacturer's recommendations.

3.2.4. **Polymorphism analysis**

Three genes, *COX-2*, *PTGER4* and *TGFB2*, were chosen for investigation because of their roles in the *IL-1β* inflammatory signalling cascade and their biological relevance to tendon pathology.[14, 133, 143, 145] Common clinically associated polymorphisms within these genes were annotated (Figure 3.4; Figure 3.5; Figure 3.7). To the authors knowledge no published studies had investigated polymorphisms within the *PTGER4* gene, however, a SNP within the gene desert proximal to the gene had been investigated.[199] There is also limited polymorphism analysis of the *TGFB2* gene in published genetic association studies. The only study investigating a functional polymorphism within Caucasian individuals investigated a novel functional -246ins polymorphism within the *TGFB2* promoter.[205] Originally we wanted to investigate this polymorphism for an association to AT. However, there were no endonucleases available which would differentially cleave at the polymorphism for RFLP analysis and the designing of primer and probe sets for TaqMan® analysis (Applied Biosystems, Foster City, California, USA) failed quality control, so other polymorphisms were considered for investigation. The -109 -/ACAA ins polymorphism

(rs10482719) within the 5'UTR of *TGFB2*, which was investigated in Japanese individuals,[206] was then selected for analysis, because, even though the frequency of the polymorphism was not determined in Caucasians, there was global data on the NCBI database (http://www.ncbi.nlm.nih.gov/) suggesting it was polymorphic. However, this too failed manufacturing for TaqMan® probe/primer sets and was unsuitable for RFLP analysis. Finally *TGFB2* -876A>C, which was previously investigated in Han Chinese,[203] was chosen for investigation. Although there was data on the NCBI database suggesting that this site was not polymorphic in European individuals, that data was generated from the analysis of only 22 people.

The three SNPs that were selected for investigation were (i) -765G>C within the promoter region of *COX-2* (rs20417),[196] (ii) the A>C SNP within the gene desert flanking *PTGER4* (rs4495224) [199] and (iii) -876A>C within the promoter region of *TGFB2* (rs7550232).[203]

Genotyping of *COX-2* -765G>C was conducted using PCR and restriction fragment length polymorphism (RFLP) analysis. The *PTGER4* gene-desert A>C SNP and *TGFB2* -876A>C were genotyped using fluorescence PCR. Polymorphism analysis was carried out following the same protocol for both the SA and AUS samples.

3.2.5. **Genotyping**

3.2.5.1. COX-2 -765G>C (rs20417)

The RFLP method was used for allelic discrimination of this SNP. Primer sets were designed for the PCR amplification of the promoter region containing COX-2 -765G>C, based on the genomic sequence as identified on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/). A *Bst*UI restriction site (CG'CG) was incorporated into the design of the primer to allow for allele discrimination (Figure 3.3). PCR amplification reactions were performed in

final volumes of 40µl containing approximately 100ng of genomic DNA, 20pmol of each primer (5'-CTCCTTGTTTCTTGGAAAGAGACG-3' and 5'-ATTCTGGCCATCGCCGCTTC-3'), 2.0mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl (pH 8.3), 200µM each dNTP and 0.5 U Taq DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA). PCR was conducted using a thermal cycler (Hybaid; PCR Express, Middlesex, UK), using the following cycling parameters: a denaturing step of 94°C for 5 minutes, 25 cycles of 94°C for 30s, 57°C for 30s and 72°C for 40s, and a final extension step of 72°C for 5 minutes. The 158bp PCR product was digested with the restriction endonuclease *Bst*UI, to manufacturer's recommendations (New England Biolabs, Ipswich, Massachusetts, USA). The resultant products were resolved on 6% polyacrylamide gels (Appendix D.4 and D.6), visualised by SYBER® Gold staining (Molecular Probe, Eugene, OR, USA), to produce one fragment (158bp) if the C allele was present or two fragments (133bp and 25bp) if the G allele was present (Figure 3.3). The localisation of the SNP within the *COX-2* gene and primer sequences were annotated (Figure 3.4).

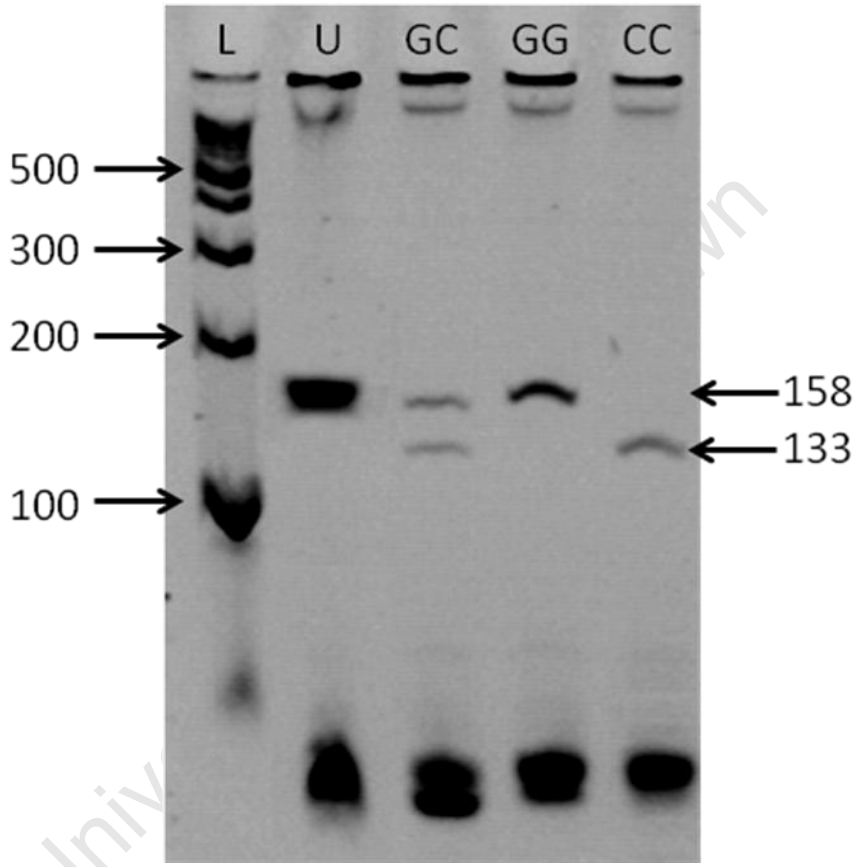

Figure 3.3. Typical 6% polyacrylamide gel of the *COX-2 Bst*UI restriction fragment length polymorphism (RFLP) genotypes. Sizes (in base pairs, bp) on the left are indicative of the 100bp DNA ladder (L) in lane 1. U in lane 2 is the uncut PCR product. Lanes 3 – 5 are samples which were digested with *Bst*UI. Fragment sizes (bp) are indicated on the right.

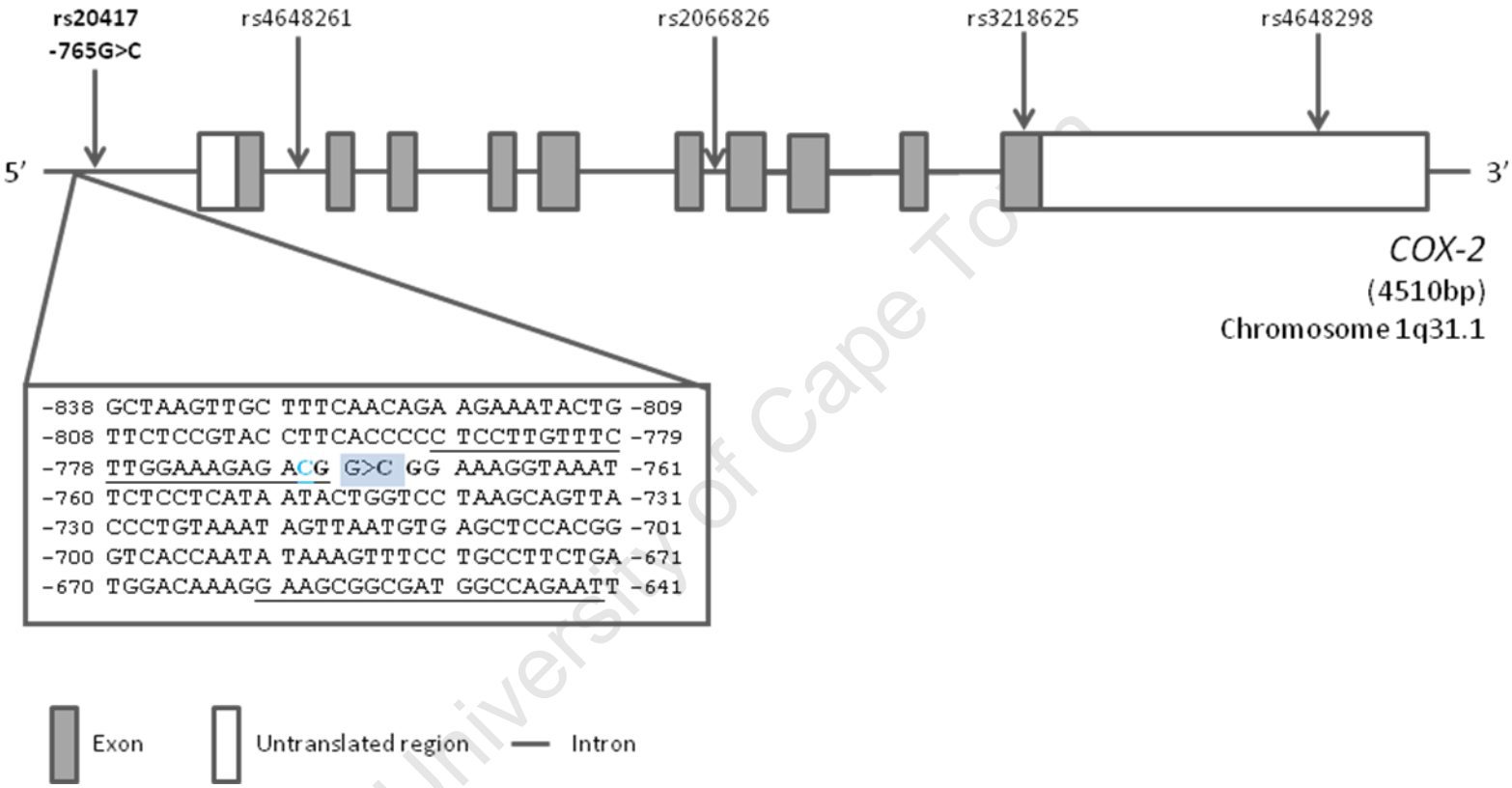

Figure 3.4. Schematic representation of the *COX-2* gene depicting some of the common clinically associated polymorphisms. The SNP chosen for investigation in this study is in bold typeset and the DNA sequence surrounding the SNP is given. Numbers refer to position relative to the transcription start site. Primer sequences are underlined and the SNP is highlighted. The sequence variant introduced into the primer sequence to create a cutting site for *Bst*UI is annotated in blue font. All the information used to construct this figure was obtained from databases hosted by the national centre for biotechnology information (NBCI) (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html).

3.2.5.2. PTGER4 gene desert A>C (rs4495224)

The A>C SNP 270kb proximal form the *PTGER4* gene was genotyped by the TaqMan™ allelic-discrimination method, using custom TaqMan™ SNP genotyping assays (Applied Biosystems, Foster City, California, USA). The master mix and assay mix containing the custom-designed primers (5'-GTCCAATCCCACCCACAGA-3' and 5'-GTAATTGGAGTCCAGGTCCT-CAG-3') and MGB-labelled probes (5'-VIC-TTTAAATTGGAACTTCCC-3' to recognise the A allele and 5'-FAM-ATTGGCACTTCCC-3' to recognise the C allele) were used as per manufacturer's recommendations to PCR amplify the region containing the SNP (Applied Biosystems, Foster City, California, USA). PCR reactions were performed on a thermal cycler (Hybaid; PCR Express, Middlesex, UK) in a final volume of 8µl using the following cycling parameters: an initial hold step at 95°C for 10 minutes and 40 cycles of 92°C for 15s, 60°C for 1 min (Applied Biosystems, Foster City, California, USA). Fluorescence in each sample well was measured after PCR using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, California, USA) (Figure 3.6). Data were analysed with SDS2.3 software (Applied Biosystems, Foster City, California, USA). The localisation of the SNP within the gene desert between the *PTGER4* and *DAB2* genes as well as primer sequences were annotated (Figure 3.5).

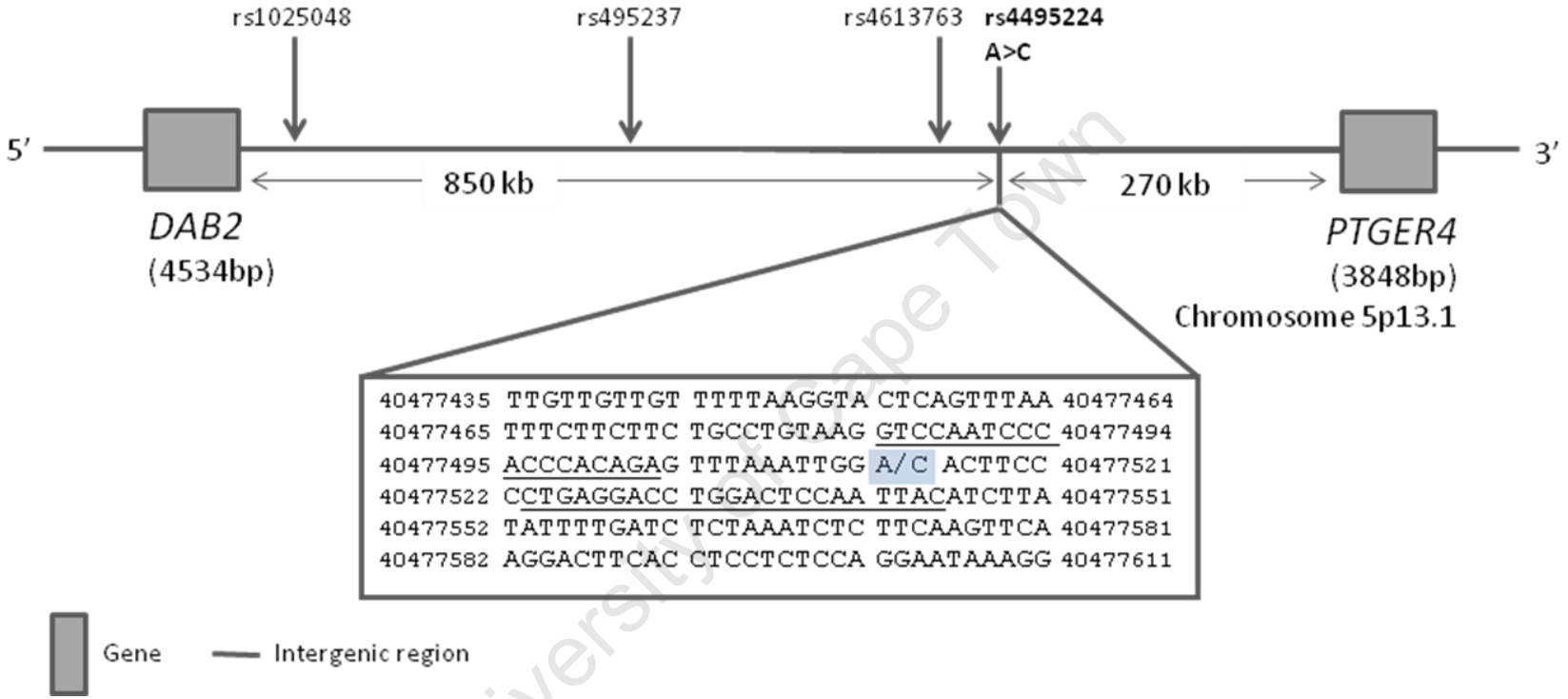

Figure 3.5. Schematic representation of the gene desert flanking the *PTGER4* gene depicting some of the clinically associated polymorphisms. *PTGER4* encodes the EP_4 receptor to which PGE_2 binds and *DAB2* encodes the mitogen-responsive phosphoprotein, disabled homologue 2. The sizes of the genes are in parentheses. The SNP chosen for investigation in this study (rs4495224) is in bold typeset and the DNA sequence surrounding the SNP is boxed. Numbers refer to genomic position on chromosome 5. Primer sequences are underlined and rs4495224 is highlighted. All the information used to construct this figure was obtained from databases hosted by the national centre for biotechnology information (NBCI) (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/ index.html).

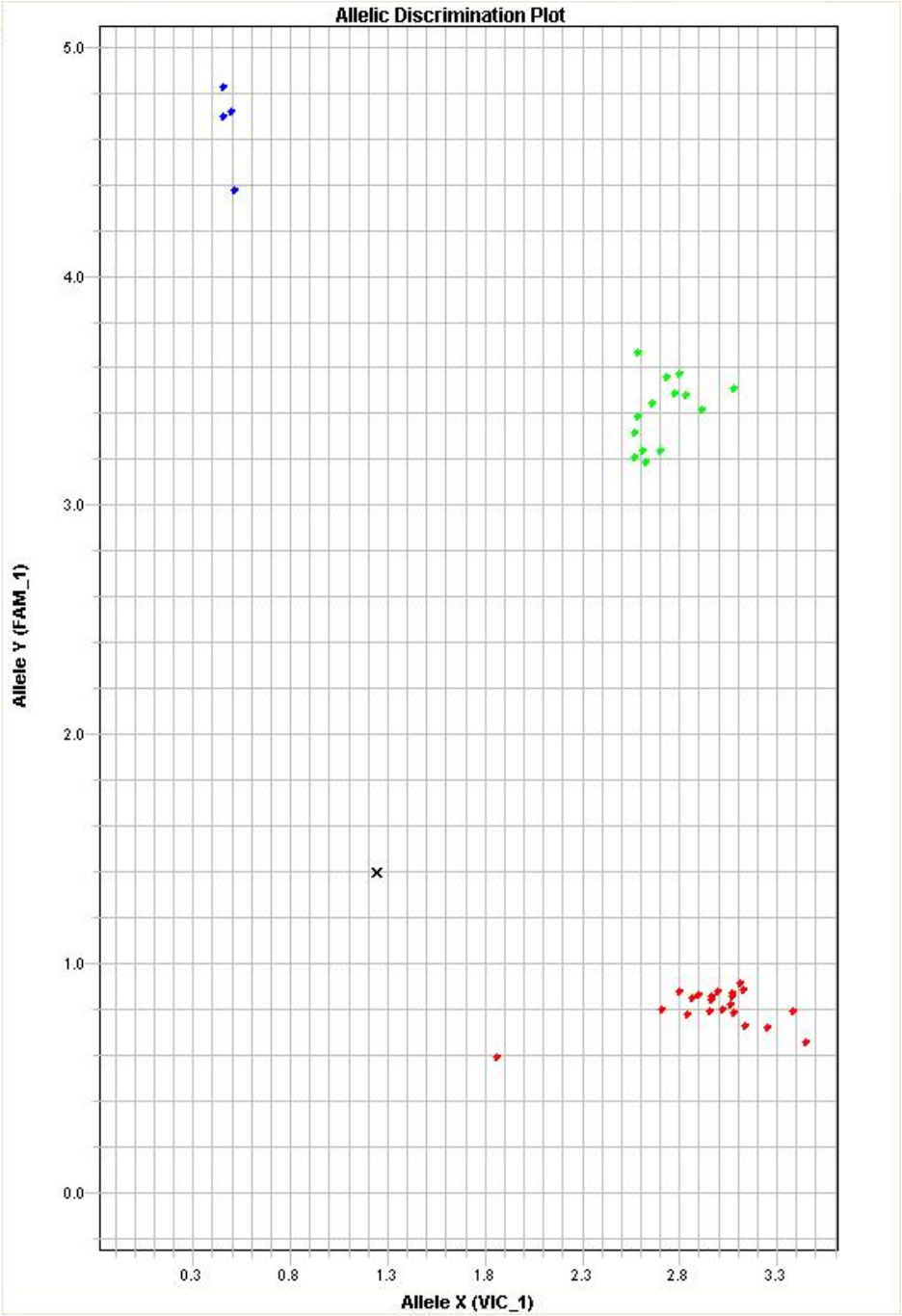

Figure 3.6. Typical scatter of VIC and FAM labelled alleles when performing real-time fluorescence PCR allele discrimination. The graph plots fluorescence of the FAM labelled allele vs the fluorescence of the VIC labelled allele for a sample. Genotypes are automatically called by the SDS3.2. software (Applied Biosystems, Foster City, California, USA). Blue data points are homozygous for the FAM-labelled allele, green data points are heterozygous, red data points are homozygous for the VIC labelled allele and the black cross data point indicates the no DNA control.

3.2.5.3. TGFB2 -876A>C (rs7550232)

TGFB2 -876A>C was genotyped by the TaqMan™ allele-discrimination assay (Applied Biosystems, Foster City, California, USA). Custom-designed primers (5'-TCAGGGCGCACATTCCA-3' and 5'-CGTTGAGGGAGTGTGG-AAATGAG-3') and MGB-labelled probes (5'-VIC-CCTTCCTCCCTTACCC-ACA-3' to recognise the A allele and 5'-FAM-CCTTCCTCCCTTCCCCACA-3' to recognise the C allele) were used as per manufacturer's recommendations, as described in Section 3.2.5.2 (Applied Biosystems, Foster City, California, USA). The localisation of the SNP within the *TGFB2* gene and primer sequences were annotated (Figure 3.7).

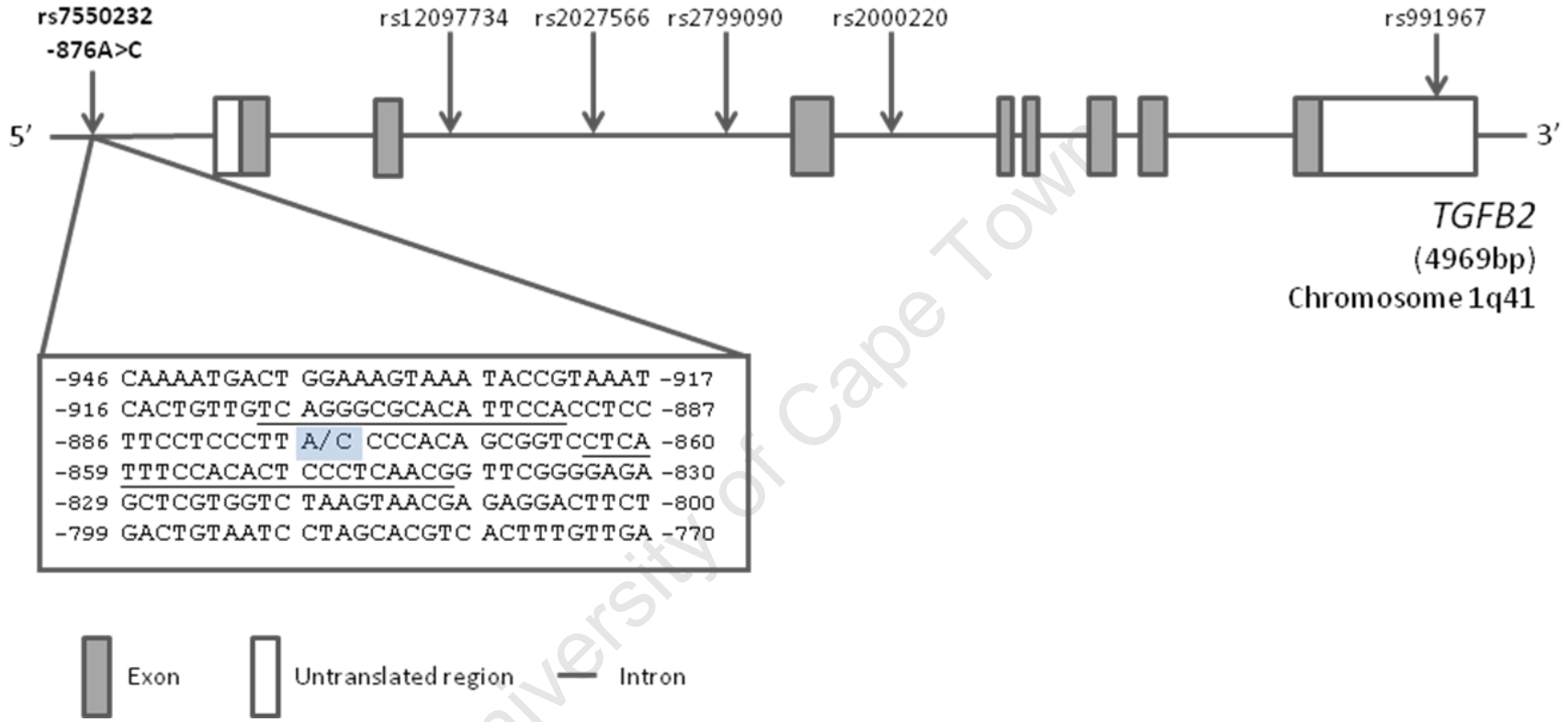

Figure 3.7. Schematic representation of the *TGFB2* gene depicting some of the common clinically associated polymorphisms. The size of the gene is in parenthesis. The SNP chosen for investigation in this study (rs7550232) is in bold typeset and the DNA sequence surrounding the SNP is boxed. Numbers refer to the base position relative to the transcription start site. Primer sequences are underlined and rs7550232 is highlighted. All the information used to construct this figure was obtained from databases hosted by the national centre for biotechnology information (NBCI)(http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html).

3.2.6. **ECM degradation risk model for AT**

The ECM degradation risk model for AT was designed, incorporating six loci within genes encoding proteins functioning in the ECM degradation pathway which were previously associated with risk of AT in either SA or AUS (*COL5A1 Bst*UI RFLP *MMP3* rs679620 A>G, *GDF5* rs143383 T>C, *IL-1β* -511C>T and *IL-1β* -31T>C) [107, 109-111, 118] or associated with AT in this study (*PTGER4* gene desert A>C), were investigated. The single signalling cascade polygenic risk model presented in this chapter was exploratory, in preparation for the combined risk model presented in the final experimental chapter (Chapter 5). The polygenic profile will be explored in detail in Chapter 5.

For the ECM degradation risk model in this study, the genotypes of each polymorphism were assigned a score of 2, 1 or 0; the genotype most closely associated with increased risk of AT was given a score of 2 and the genotype most closely associated with decreased risk of AT was given a score of 0. The scoring of each polymorphism is presented in Table 3.1.

Table 3.1. Genotype scores of the polymorphisms within genes encoding proteins involved in the ECM degradation signalling cascade which are associated with Achilles tendinopathy in Caucasian individuals.

Gene	Polymorphism	risk (2)	neutral (1)	protection (0)
COL5A1	rs12722 (T/C)	TT	TC	CC
MMP3	rs679620 (A/G)	GG	AG	AA
GDF5	rs143383 (T/C)	TT	TC	CC
IL-1β	rs16944 (T/C)	CC	CT	TT
	rs1143627 (T/C)	TT	TC	CC
PTGER4	rs4495224 (A/C)	CC	GC	GG

Only the participants who were successfully genotyped for all six loci within the ECM degradation signalling cascade were included for analysis. A total of

92 SA CON individuals and 65 SA TEN individuals as well as 99 AUS CON and 45 AUS TEN individuals were therefore included for this analysis.

For each participant, a total genotype score (TGS) was generated from the accumulative risk score at all six loci, as described by Williams and Folland, (2008).[207] TGSs were calculated by dividing the sum of the individual genotype scores by the maximum score obtainable (in this case 12) and multiplying by 100. A TGS of 100 represents the complete risk polygenic profile for AT and a TGS of 0 represents the perfect protection polygenic profile.

A receiver operating characteristic (ROC) curve [207, 208] was used to determine the power of the loci within the ECM degradation pathway to discriminate between cases and controls. The area under the ROC curve (AUC) was used to quantify the ability of the model to discriminate between diagnostic groups based on genotype risk, such that a perfect model gives an AUC of 1.0 and a model with no predictive power gives an AUC of 0.5. ROC curves were also plotted to graphically display the true positive rate (sensitivity) vs the false positive rate (1-specificity) across a range of TGS cut-offs. Sensitivity and specificity are used clinically to select an optimal cut-off. Sensitivity in this case indicates the frequency of TEN individuals that the test correctly identifies as “at risk” and specificity indicates the frequency of CON individuals that the test correctly identifies as protected.

3.2.7. **Statistics**

Analysis was performed using STATISTICA 9 (Statsoft inc., Tulsa, OK, USA) and Graphpad InStat Version 3 (Graphpad Software, San Diego, California, USA). Continuous variables (such as weight and age) between CON and TEN groups were compared with one-way analysis of variance (ANOVA), adjusting for age and gender where required. Pearson’s chi-square tests, or Fischer’s tests when necessary, were used to compare categorical variables

between CON and TEN groups, independently in both SA and AUS participants. Statistical significance was accepted when $p<0.05$. The results were not adjusted for multiple testing as no obvious appropriate method exists to date.[209, 210] The Bonferroni adjustment, requires that each p-value be multiplied by the total number of independent tests conducted (which is equivalent to dividing the significance level by the number of independent tests), was considered too conservative because the tests conducted in this study were hypothesis driven and were all conducted on the same group of individuals. GenePop was used to determine Hardy-Weinberg equilibrium (HWE) probabilities for the SNPs (http://genepop.curtin.edu.au/ genepop_op1.html). GraphPad Prism Version 5.02 (GraphPad Software, San Diego California, USA) was used for the risk model analysis. A D'Agnostino and Pearson's omnibus normality test was used to assess whether data was normally distributed. An unpaired t-test was used to determine whether the distribution of TGSs were different between CON and TEN. A ROC curve was plotted to determine the discriminating accuracy of the model. Statistical significance was accepted at $p<0.05$.

3.3. **RESULTS**

3.3.1. **Participants' characteristics**

The CON and TEN groups of both SA and AUS were similar in age, height and country of birth (Table 3.2). The age of the SA TEN group was 47.8 ± 11.2 years at recruitment which was on average 8.3 ± 9.2 years after the onset of symptoms and the age of the AUS TEN group was 49.1 ± 12.5 at recruitment which was 8.8 ± 9.9 years after the onset of symptoms. The TEN groups of both SA and AUS were significantly heavier and thus also had a higher mean body mass index (BMI) than the CON group. However, when weight and BMI were adjusted for age at recruitment or age at recruitment and/or gender, these differences were no longer significant (Table 3.2). The injury profile of the SA TEN and AUS TEN participants were previously described,[107, 118] and are presented in Appendix A.2 – Injury profile of SA TEN and AUS TEN groups. Furthermore there were no meaningful genotype effects on participant characteristics (Appendix A.3 – Genotype effects on descriptive measures).

Table 3.2. Characteristics of the participants within the control (CON) and Achilles tendinopathy (TEN) groups of South Africa (SA) and Australia (AUS).

		CON (161)	TEN (85)	p-value	Adjusted p-value
South Africa	Age (years)	36.3 ± 10.7 (156)	39.4 ± 14.6 (84) [a]	0.056	
	Height (cm)	175.0 ± 9.1 (157)	176.6 ± 9.0 (83)	0.197	
	Weight (kg)	72.2 ± 11.8 (161)	77.8 ± 13.5 (86)	**>0.001**	0.061[b]
	BMI (kg/cm^2)	23.6 ± 2.8 (157)	24.8 ± 3.3 (83)	**0.003**	0.156[b]
	Gender (% males)	64.2 (162)	72.8 (92)	0.160	
	Country of birth (% SA)	73.9 (161)	70.5 (88)	0.474	
		CON (205)	TEN (82)	p-value	Adjusted p-value
Australia	Age (years)	38.7 ± 12.4 (203)	40.3 ± 14.2 (83) [a]	0.336	
	Height (cm)	171.5 ± 9.2 (205)	173.9 ± 10.0 (81)	0.051	
	Weight (kg)	72.5 ± 14.0 (206)	80.4 ± 14.1 (84)	**>0.001**	0.102[c]
	BMI (kg/cm^2)	24.6 ± 3.9 (205)	26.5 ± 3.8 (81)	**>0.001**	0.194[d]
	Gender (% males)	40.1 (207)	72.6 (84)	**>0.001**	
	Country of birth (% AUS)	81.8 (203)	77.1 (83)	0.092	

Variables are expressed as mean ± standard deviation, except for gender and country of birth, which are represented as a percentage (%). Number of participants for which data was available is in parenthesis. Bold typeset denotes significant differences ($p<0.05$) between CON and TEN groups. BMI, Body Mass Index

[a] Age for the TEN group refers to age at onset of symptoms. The South African TEN group was on average 47.8 ± 11.1 years old at recruitment, which was 8.3 ± 9.2 years after onset of symptoms. Similarly the Australian TEN group was 49.1 ± 12.5 years old at recruitment, which was 8.8 ± 9.9 years after onset of symptoms. [b] Adjusted for age at recruitment. [c] Adjusted for gender. [d] Adjusted for age at recruitment and gender.

3.3.2. **Genotype of allele frequency distribution**

3.3.2.1. COX-2 -765G>C

There were no differences in genotype (p=0.141) or allele (p=0.126) frequency distribution of *COX-2* -765G>C between SA TEN and SA CON. Similarly there were no differences in genotype (p=0.914) or allele (p=0.898) frequency distribution in AUS participants (Table 3.3). All groups were in HWE (Table 3.3). Frequency distribution was comparable to previously published data of individuals of Caucasian ancestry (Table 3.4).

3.3.2.2. PTGER4 gene desert A>C

There were no differences in genotype (p=0.421) or allele (p=0.413) frequency distribution of the *PTGER4* gene desert A>C polymorphism in SA participants (Table 3.3). The genotype frequency distribution was however significantly different in AUS participants (p=0.010) (Table 3.3). The CC genotype was significantly overrepresented in the AUS CON group (14.4%) compared to the AUS TEN group (3.7%) (p=0.010, OR=4.4, 95%CI 1.3 – 15.0) (Figure 3.8; Table 3.3). Allele frequency distribution in AUS participants was not significantly different (p=0.109). All groups were in HWE (Table 3.3). This polymorphism has not been explored in many case-control genetic association studies, nevertheless, the frequency distribution was comparable to previously published data of individuals of Caucasian ancestry (Table 3.5).

3.3.2.3. TGFB2 -876A>C

There were no differences in genotype (p=0.060) or allele (p=0.066) frequency distribution of *TGFB2* -876A>C between SA TEN and SA CON. Similarly there were no differences in genotype (p=0.108) or allele (p=0.114) frequency distribution in AUS participants (Table 3.3). All groups were in HWE (Table 3.3). This polymorphism has not been explored in genetic association studies in Caucasian individuals and frequency distribution is very different to previously published data in other studies (Table 3.6).

Table 3.3. Genotype frequency distribution and minor allele frequency of COX-2 -765G>C (rs20417), PTGER4 gene desert A>C (rs4495224) and TGFB2 -876A>C (rs7550232) polymorphisms in control (CON) and Achilles tendinopathy (TEN) groups of South Africa and Australia.

	Genotype Frequency CON			CON n	CON HWE	Genotype Frequency TEN			TEN n	TEN HWE	Minor Allele	Minor Allele Frequency CON	Minor Allele Frequency TEN	Genotype p-value	Allele p-value
COX-2 -765G>C	GG	GC	CC			GG	GC	CC							
SA	74.1	22.8	3.1	158	0.330	64.7	30.6	4.7	85	0.735	C	14.6	20.0	0.141	0.126
AUS	68.6	27.8	3.6	169	0.600	69.6	27.9	2.5	79	1.000	C	17.5	16.5	0.713	0.943
PTGER4 A>C	AA	AC	CC			AA	AC	CC							
SA	45.6	43.0	11.4	158	0.723	53.6	34.5	11.9	84	0.186	C	32.9	29.2	0.421	0.413
AUS	42.8	42.8	14.4	180	0.333	46.9	49.4	3.7	81	0.097	C	35.8	28.4	**0.010**	0.109
TGFB2 -876A>C	AA	AC	CC			AA	AC	CC							
SA	93.5	6.5	0.0	155	1.000	85.7	14.3	0.0	84	1.000	C	3.2	7.1	0.060	0.066
AUS	92.0	8.0	0.0	200	1.000	97.6	2.4	0.0	82	1.000	C	4.0	1.2	0.108	0.114

Genotype and allele frequencies are expressed as percentages. HWE is the p-value for exact tests for Hardy-Weinberg equilibrium. Bold typeset denotes significant differences ($p<0.05$) in genotype or allele frequency between CON and TEN groups.

Table 3.4. Frequency distribution of the *COX-2* -765G>C genotypes in asymptomatic control participants of published case-control studies.

	n	GG	GC	CC	Reference
SA CON	158	74.1	22.8	3.1	
AUS CON	169	68.6	27.8	3.6	
European	174	70.0	25.0	5.0	NCBI
Chinese	1270	98.7	4.3	0.0	[149]
Nederland Caucasian	6147	73.8	23.9	2.3	[153]
Polish Caucasian	547	68.7	28.9	2.4	[196]

Table 3.5. Frequency distribution of the *PTGER4* gene desert A>C alleles in asymptomatic control participants of published case-control studies.

	n	A	C	Reference
SA CON	158	67.1	32.9	
AUS CON	180	64.2	35.8	
European	174	65.8	34.2	NCBI
Belgian and French Caucasian	926	65.1	34.9	[199]

Table 3.6. Frequency distribution of the *TGFB2* -876A>C genotypes in asymptomatic control participants of published case-control studies.

	n	AA	AC	CC	Reference
SA CON	155	93.5	6.5	0.0	
AUS CON	200	92.0	8.0	0.0	
European	22	100.0	0.0	0.0	NCBI
Chinese	94	67.0	32.0	1.0	[203]

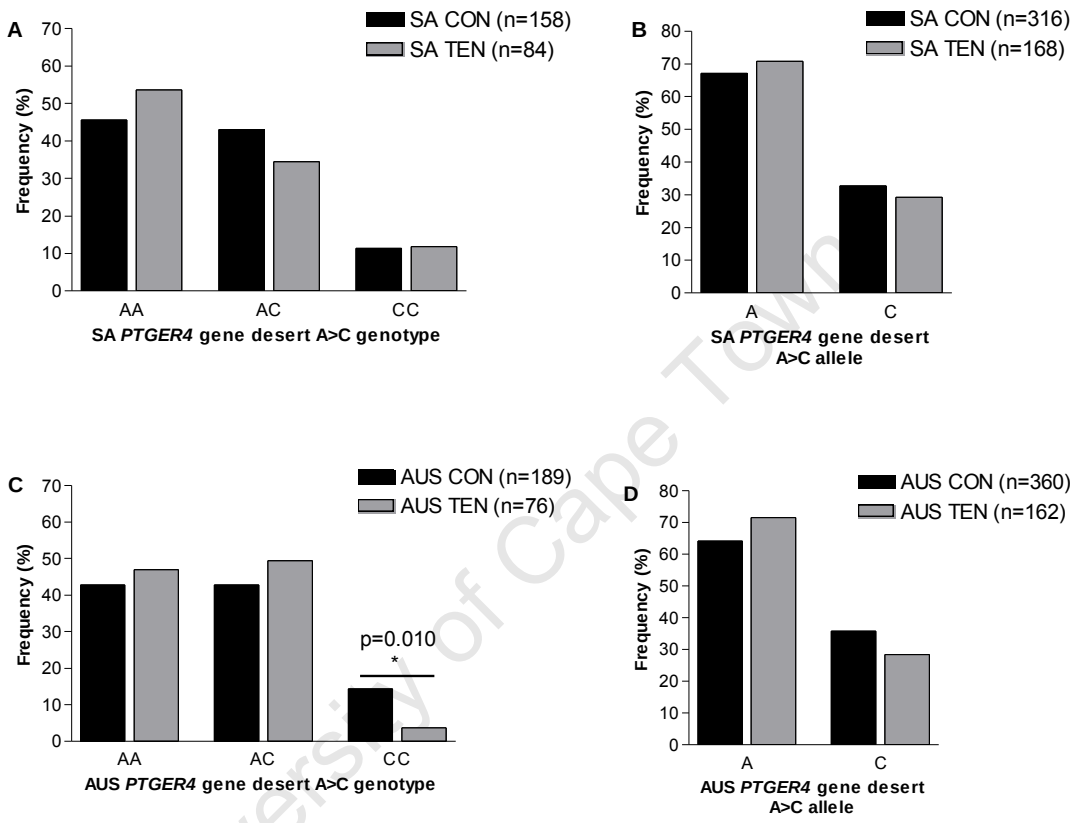

Figure 3.8. Genotype (A) and allele (B) frequency distribution of *PTGER4* gene desert A>C SNP within South African (SA) participants and genotype (C) and allele (D) frequency distribution within Australian (AUS) participants for asymptomatic control (CON, black bars) and Achilles tendinopathy (TEN, grey bars) groups. Asterisk (*) denotes significance between CON and TEN groups. (A) SA CON vs SA TEN, p=0.421; (B) SA CON vs SA TEN, p=0.413; (C) AUS CON vs AUS TEN, p=0.037; CC vs AA+AC p=0.010; (D) AUS CON vs AUS TEN, p=0.109. The number (n) of participants (A and C) or alleles (B and D) are in parentheses. Significance was accepted at p<0.05. SNP, single nucleotide polymorphism.

3.3.3. **ECM degradation risk model for AT**

A total genotype score (TGS) for each individual was calculated from the assigned risk scores at the *COL5A1 Bst*UI RFLP, *MMP3* rs679620 A>G, *GDF5* rs143383 T>C, *IL-1β* -511C>T, *IL-1β* -31T>C and *PTGER4* gene desert A>C loci, as described in Section 3.2.6. TGSs in all four diagnostic groups were normally distributed. The TGS was significantly higher in SA TEN (64.7±14.0) compared to SA CON (55.8±16.9) ($p<0.001$) (Figure 3.9 A). The TGSs of AUS CON (56.9±15.5) and AUS TEN (60.0±13.4) were however not significantly different ($p=0.249$) (Figure 3.9 A). However, the distribution of TGS scores were similar in SA CON and AUS CON ($p=0.638$) as well as in SA TEN and AUS TEN ($p=0.077$) and therefore data was combined for further analysis. The TGS in the combined [SA+AUS] TEN group (62.8±13.9) was significantly higher than the [SA+AUS] CON group (56.4±16.2) ($p<0.001$) (Figure 3.9 B). Furthermore, the polygenic profile incorporating loci from the ECM degradation signalling cascade in the [SA+AUS] group was a significant discriminator of risk of AT (AUC=0.62, 95%CI 0.55 – 0.67; $p<0.001$).

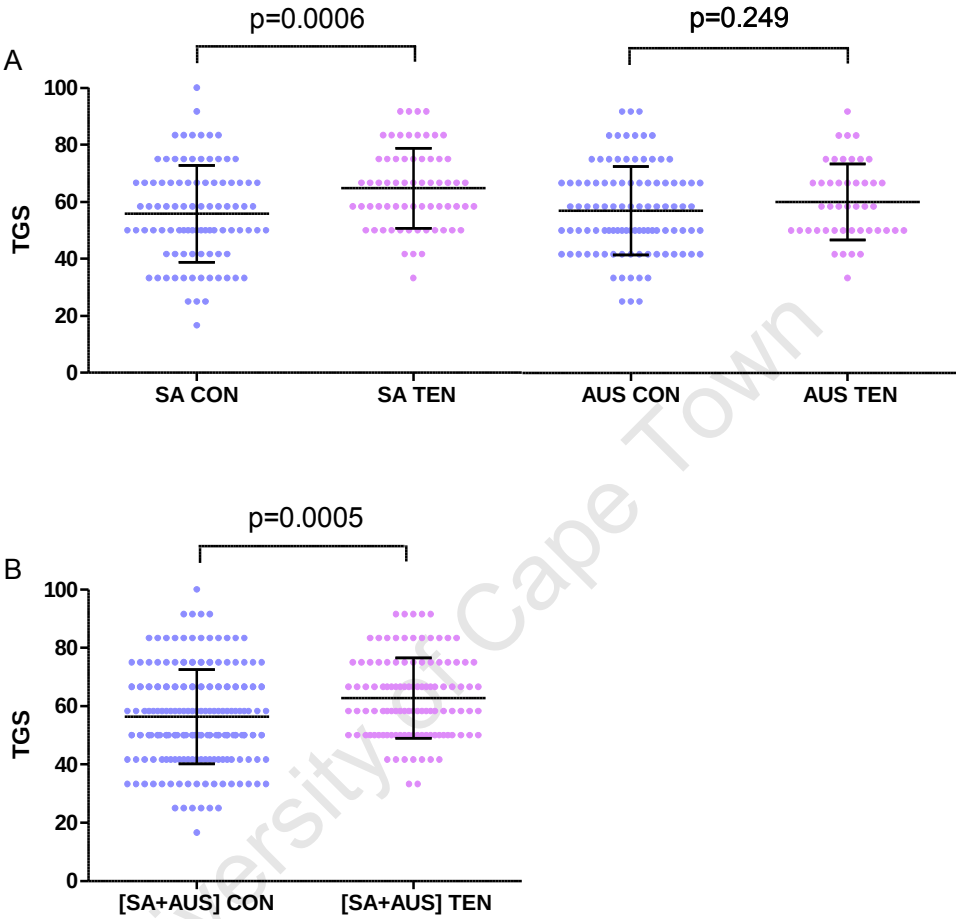

Figure 3.9. The distribution of total genotype scores (TGS) of (A) South African participants in the asymptomatic control (SA CON; n=92) and clinically diagnosed Achilles tendinopathy (SA TEN; n=65) group, as well as Australian control (AUS CON; n=99) and Australian Achilles tendinopathy (AUS TEN; n=45) group and (B) the TGS distribution in the combined South Africa and Australia groups ([SA+AUS] CON; n=191 and [SA+AUS] TEN; n=110). Significance was accepted at p<0.05.

3.4. **DISCUSSION**

The main findings of this study are the association of (i) the CC genotype at the *PTGER4* gene desert A>C locus with protection from developing AT in AUS and (ii) the identification of a polygenic risk profile implicating the ECM degradation pathway in SA and the combined [SA+AUS] group.

The strength of this study was the analysis of the cumulative effect of functional sequence variants within genes encoding proteins functioning in a common biological pathway to determine risk of AT. There is evidence in the literature which suggests that exploring multiple genetic factors from a common biological pathway, is more effective at capturing disease predisposition of multifactorial conditions.[95, 96] A pathway-based approach has been suggested to be a more effective tool to highlight underlying biological pathways and mechanisms relevant at a population level.[96]

The single SNP analysis revealed that *COX-2* -765G>C and *TGFB2* -876A>C were not significantly associated with risk of AT in either SA or AUS. However, the *PTGER4* gene desert CC was found to confer a 4.4 fold decreased risk of AT in AUS, but not in SA. This association is in accordance with the hypothesis that the AA genotype of *PTGER4* gene desert A>C would increase an individual's risk of AT, since the A allele is associated with increased expression of EP_4.[199]

No associations were observed between *COX-2* -765G>C and AT. This does not exclude the possibility that *COX-2* may play a role in the development of AT, since COX-2 does play an important role in catalysing the production of PGE_2. Other polymorphisms in the *COX-2* gene should be explored to establish the involvement of COX-2 in AT predisposition.

The *TGFB2* -876A>C locus was polymorphic (minor allele frequency of >1%) in the two Caucasian populations investigated in this study, which is contrary

to the genotype frequency data of 22 European Caucasian individuals in the database hosted by NCBI. However, the minor allele frequency was <5%, which is considered undesirable for genetic association studies.[211] The genotype analysis of *TGFB2* -876A>C in this study provides evidence (n=355) suggesting that this variant is polymorphic in Caucasian populations, however the CC genotype is rare. *TGFB2* remains an important candidate gene based on its biological function and needs to be further explored using more informative variants.

The most interesting finding of this study was the identification of a polygenic risk model incorporating functional sequence variants within the *COL5A1*, *MMP3*, *GDF5*, *IL-1β* and *PTGER4* genes, encoding proteins involved in the ECM degradation pathway. While single SNP associations were not able to capture associations in both populations, the polygenic profile incorporating six loci was similar in SA and AUS and was able to discriminate between CON and TEN groups in a combined [SA+AUS] group. The model in this study with an AUC of 0.62 is comparable with models of other multifactorial conditions.[207, 208] The identification of this risk profile emphasises the strength of pathway-based approaches to studying multifactorial conditions as opposed to single SNP associations to effectively capture a risk profile.

This study is not without limitations. Notably, the physical activity of the AUS TEN and AUS CON participants were not documented. Nevertheless, care was taken to recruit physically active individuals. Physical activity was however, well documented in SA. The SA CON and SA TEN groups had participated in a similar number of years in running as well as participated in similar amount (hours/week) of high impact sports for the two years prior to recruitment (Appendix A.3 – Physical activity of SA and AUS participants).

Another limitation is the TEN participants in both SA and AUS were heavier at recruitment than the CON participants. Adiposity is a possible contributing risk factor for the development of AT.[136] However, accurate weight

measurements could not be attained for TEN participants prior to injury since SA TEN were recruited 8.3 ± 9.2 years after onset of symptoms and AUS TEN 8.9 ± 9.8 years after onset of symptoms. However when weight was adjusted for age at recruitment, the weights of the diagnostic groups were similar and TEN participants anecdotally reported gaining weight after injury. Furthermore no genotype effects on weight were observed in either SA or AUS.

In conclusion, this study has identified a polygenic profile, incorporating six loci from the ECM degradation signalling cascade, able to discriminate between diagnostic groups. This study also identified a single SNP association, specifically the CC genotype of the A>C polymorphism within the gene desert flanking *PTGER4* gene being associated with protection against developing AT. The study provides additional evidence for the involvement of the inflammatory pathway as an important biological mechanism leading to ECM degradation in the development of chronic Achilles tendinopathy.

Chapter 4

APOPTOSIS SIGNALLING CASCADE IN TENDONS

GENETIC RISK FACTORS FOR ACHILLES TENDINOPATHY WITHIN THE APOPTOSIS SIGNALING CASCADE

4.1. **INTRODUCTION**

The cytokines expressed by tenocytes in response to mechanical loading not only initiate ECM degradation, but also have the potential to initiate the apoptosis signalling cascade. As described in Section 2.2, cytokines involved in the regulation of apoptosis include IL-6, which is upregulated by IL-1β following repetitive loading (Figure 4.1) [14, 15, 81, 144] and variants within the *IL-6* and *IL-1β* genes were previously collectively found to contribute to the risk of AT.[118]

It is proposed that the normal tendon healing process, in response to overload, involves the removal of damaged tenocytes by cytokine-mediated apoptosis.[15] Repetitive mechanical loading may however change the ECM composition and result in excessive tenocyte apoptosis.[15, 65, 78, 166] Tenocytes are required to maintain homeostasis of the ECM by regulating the balance between ECM synthesis and degradation,[57] therefore excessive tenocyte apoptosis may compromise the ability of the tendon to optimally regulate repair processes. Excessive apoptosis has been observed in supraphysiologically loaded rat tibialis anterior tendons,[165] in the torn edges of the human rotator-cuff [78] and in athletes with patella tendinopathy.[53] Identification of the biological mechanisms driving tenocytes towards abnormal apoptosis are therefore of interest in understanding the pathophysiology of tendinopathy.

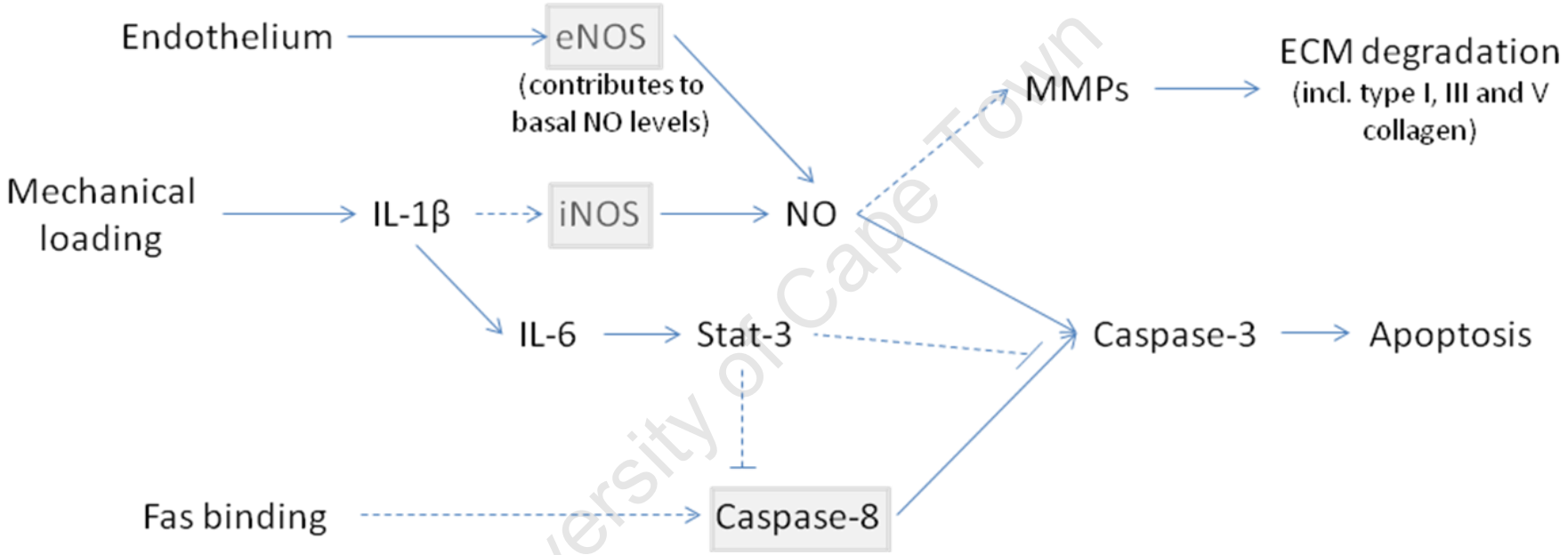

Figure 4.1. Schematic representation of the apoptosis signalling cascades in tendons.[15, 81, 146-148] Hashed arrows indicate signalling pathways that are known to occur in chondrocytes or osteoblasts and proposed to occur in tenocytes. [146, 148] The proteins of the *CASP8*, *NOS3* and *NOS2* genes, encoding capsase-8, eNOS and iNOS respectively, were investigated in this dissertation and are boxed. ECM, extracellular matrix; eNOS, endothelial nitric oxide synthase; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; MMPs, matrix metalloproteinases; NO, nitric oxide; Stat-3, signal transducer and activator of transcription-3.

The study presented in this chapter of the dissertation also followed a pathway-based candidate gene approach to investigate the apoptosis signalling cascade, specifically investigating *CASP8*, *NOS3* and *NOS2* (Figure 4.1).

The expression of capsase-8, an apoptotic mediator, was found to be elevated in tendinopathy, together with TNF-α, IL-6 and caspase-3 [15] as was discussed in detail in Section 2.2. Two polymorphisms within *CASP8*, mapped to chromosome 2q33.1,[212] were chosen for investigation, (i) -652 6N del (rs3834129) and (ii) Asp302His (rs1045485). Both these variants were previously associated with reduced risk of other multifactorial conditions. [213-218] The functional *CASP8* -652 6N del polymorphism is a six nucleotide deletion (CTTACT) within the promoter of the *CASP8* gene. The del allele destroys a Sp1 binding element, therefore resulting in decreased caspase-8 expression.[215] A missense polymorphism, 51423G>C (rs1799983) within exon 9 of *CASP8* results in an Asp302His substitution. The functional significance of the Asp302His substitution is not known, however it is proposed that the substitution of the acidic Asp residue on the surface of the caspase-8 protein with the basic His amino acid may interfere with the processing of procaspase-8 and alter interactions with anti-apoptosis molecules.[219]

Nitric Oxide (NO) can also trigger apoptosis [148, 187-189] as discussed in Section 2.2 and forms part of the IL-1 signalling cascade (Figure 4.1).[148] The enzyme that catalyses the formation NO is known as NOS and all three isoforms of NOS, namely eNOS, iNOS and nNOS, were found to be elevated in tendinopathy.[179, 192] More specifically, eNOS and iNOS were found to be elevated in AT and modulate the inflammatory status of the tendon.[179, 192]

eNOS is encoded by the *NOS3* gene, which was mapped to chromosome 7q36.1.[220] An increase in eNOS expression was observed in an *in vivo* rat

tendon overuse model and in AT.[179, 192] A functional missense polymorphism, +894G>T (rs1799983) within exon 7 of *NOS3* results in a Glu298Asp substitution. Studies have determined that the T allele of *NOS3* 894G>T, causing the Asp substitution, results in reduced eNOS activity and therefore reduced basal NO productions.[221, 222] The T allele was associated with exercise performance and negative outcomes in multifactorial conditions such as psoriasis and cardiovascular disease.[223-229]

iNOS is encoded by the *NOS2* gene, situated on chromosome 17q11.2.[230] The functional -1026C>A SNP (rs2779249) within the promoter of the *NOS2* gene, increases the binding affinity of transcription factor Ying Yang 1 (YY1) to the promoter.[231] YYI has been shown to activate transcription or repress transcription, due to its ability to interact with other proteins.[232] Thus under different transcriptional control in different cell types, increasing YY1 binding affinity may have different consequences. Although it is predicted from functional studies in hepatoma bel cells that the A allele results in increased iNOS levels,[231] this is yet to be confirmed in tenocytes. The C allele was associated with increased risk of hypertension and tuberculosis.[231, 233]

The aim of the study presented in this chapter of the dissertation was therefore to investigate polymorphisms within several candidate genes in the inflammatory pathway leading to apoptosis for associations to AT. Polymorphisms within three genes were investigated (i) the functional -652 6N del within the promoter region of *CASP8*, (ii) Asp302His within exon 9 of *CASP8,* (iii) the proposed functional Glu298Asp variant within exon 7 of *NOS3* and (iv) the functional -1026C>A variation within *NOS2*. This study also aimed to assess the effectiveness of a genetic risk model for a single signalling cascade, the apoptosis cascade, to predict risk of developing AT, using a polygenic profile.

4.2. METHODS

4.2.1. Participants

The South African and Australian participants, previously described in Section 3.2.2 and Appendix A.2, A.3 and A.4, were used in this study.

4.2.2. DNA extraction

DNA extraction for South African and Australian participants were described in Section 3.2.3.

4.2.3. Polymorphism analysis

The three genes, *CASP8, NOS2* and *NOS3*, were chosen for investigation because of their role in the apoptosis signalling cascades and their biological relevance to tendon pathology.[15, 179, 192] Common clinically associated polymorphisms within these genes were annotated (Figure 4.2; Figure 4.4; Figure 4.5). Four functionally relevant polymorphisms were selected for investigation: -652 6N del (rs3834129) and Asp302His (rs1045485) within the promoter region and exon 9 of *CASP8* respectively,[213-218] the missense Glu298Asp mutation within exon 7 of *NOS3* (rs1799983) [227-229] and -1026C>A within the promoter region of *NOS2* (rs2779249).[231, 233]

Genotyping of *CASP8* -652 6N del, *CASP8* Asp302His and *NOS2* -1026C>A was conducted using fluorescence PCR and *NOS3* Glu298Asp was genotyped using PCR and RFLP analysis. Genotyping was carried out following the same protocol for SA and AUS samples.

4.2.4. **Genotyping**

4.2.4.1. CASP8 -652 6N del (rs3834129)

The TaqMan™ allele-discrimination assay (Applied Biosystems, Foster City, California, USA) was used to genotype the *CASP8* -652 6N del polymorphism. Custom-designed primers (5'-TTGATTCTTTCAGACTTT-TTCCTAGGCTT-3' and 5'-GGAAGGCACTGAGACGTTAAGTAAC-3') and MGB-labelled probes (5'-VIC-TTGCTCTGCCACTTACT-3' to recognise the insertion allele and 5'-FAM-CTCTGCCAAGCTGC-3' to recognise the deletion allele) were used as per manufacturer's recommendations, as described in Section 3.2.5.2. (Applied Biosystems, Foster City, California, USA) The localisation of the polymorphism within the *CASP8* gene and primer sequences were annotated (Figure 4.2).

4.2.4.2. CASP8 Asp302His (rs1045485)

CASP8 Asp302His was genotyped by the TaqMan™ allele-discrimination assay (Applied Biosystems, Foster City, California, USA). Custom-designed primers (5'-ACCACGACCTTTGAAGAG-CTT-3' and 5'-TCCATGAGTTGG-TAGATTTTCAAAATCTCA-3') and MGB-labelled probes (5'-VIC-AAGCCC-CACCATGACT-3' to recognise the C allele and 5'-FAM-AGCCCCACGAT-GACT-3' to recognise the G allele) were used as per manufacturer's recommendations, as described in Section 3.2.5.2. (Applied Biosystems, Foster City, California, USA). The localisation of the SNP within the *CASP8* gene and primer sequences were annotated (Figure 4.2).

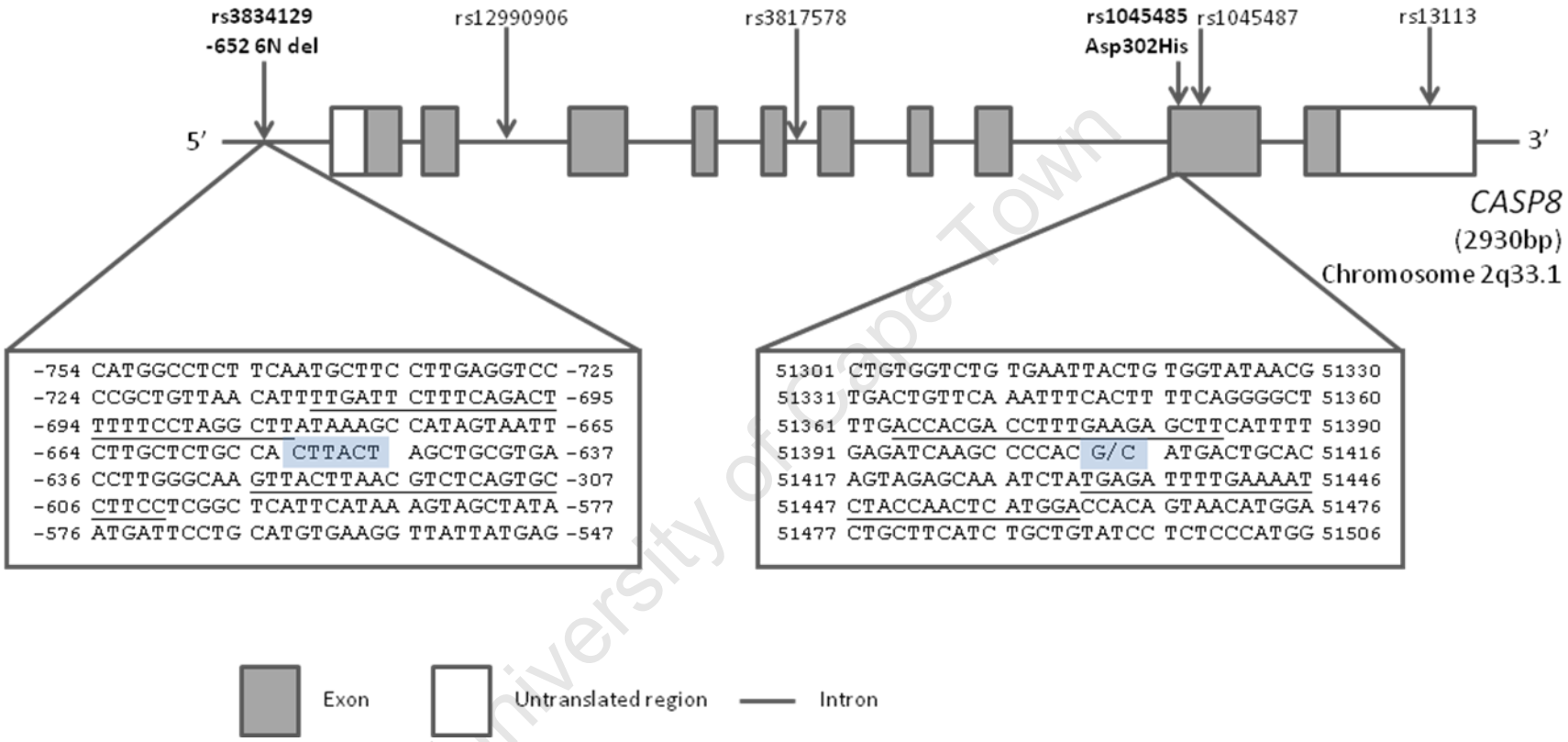

Figure 4.2. Schematic representation of the CASP8 gene depicting some of the common clinically associated polymorphisms. The size of the gene is given in parenthesis. The polymorphisms chosen for investigation in this study (rs3834129 and rs1045485) are in bold typeset and the DNA sequence surrounding these polymorphisms are boxed. Numbers refer to the base position relative to the transcription start site. Primer sequences are underlined and polymorphisms highlighted. All the information used to construct this figure was obtained from databases hosted by the national centre for biotechnology information (NBCI) (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html).

4.2.4.3. NOS3 Glu298Asp (rs1799983)

RFLP analysis was used for allelic discrimination of *NOS3* Glu298Asp. Primer sets were designed for the PCR amplification of the exonic region containing *NOS3* Glu298Asp, using genomic sequences in the region of the polymorphism as identified on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/). PCR amplification reactions were performed in final volumes of 40µl containing approximately 100ng of genomic DNA, 20pmol of each primer (5'-CAGGAGACAGTGGATGGAGG-3' and 5'-CTCATGTACCAGCCACTG-AAGG-3'), 2.0mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl (pH 8.3), 200µM each dNTP and 0.5 U Taq DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA). PCR was conducted using a thermal cycler (Hybaid; PCR Express, Middlesex, UK), using the following cycling parameters: a denaturing step of 94^{o}C for 5 minutes, 25 cycles of 94^{o}C for 30s, 61^{o}C for 30s and 72^{o}C for 40s, and a final extension step of 72^{o}C for 5 minutes. The 443bp PCR product was digested with the restriction endonuclease *Dpn*II following manufacturer's recommendations (New England Biolabs, Ipswich, Massachusetts, USA). The resultant products were resolved on 6% polyacrylamide gels (Appendix D.4 and D.6), visualised by SYBER® Gold staining (Molecular Probe, Eugene, OR, USA), to produce two fragments 144 and 299bp (G allele) or three fragments 144, 145 and 154bp (T allele) (Figure 4.3). The localisation of the SNP within the *NOS3* gene and primer sequences were annotated (Figure 4.4).

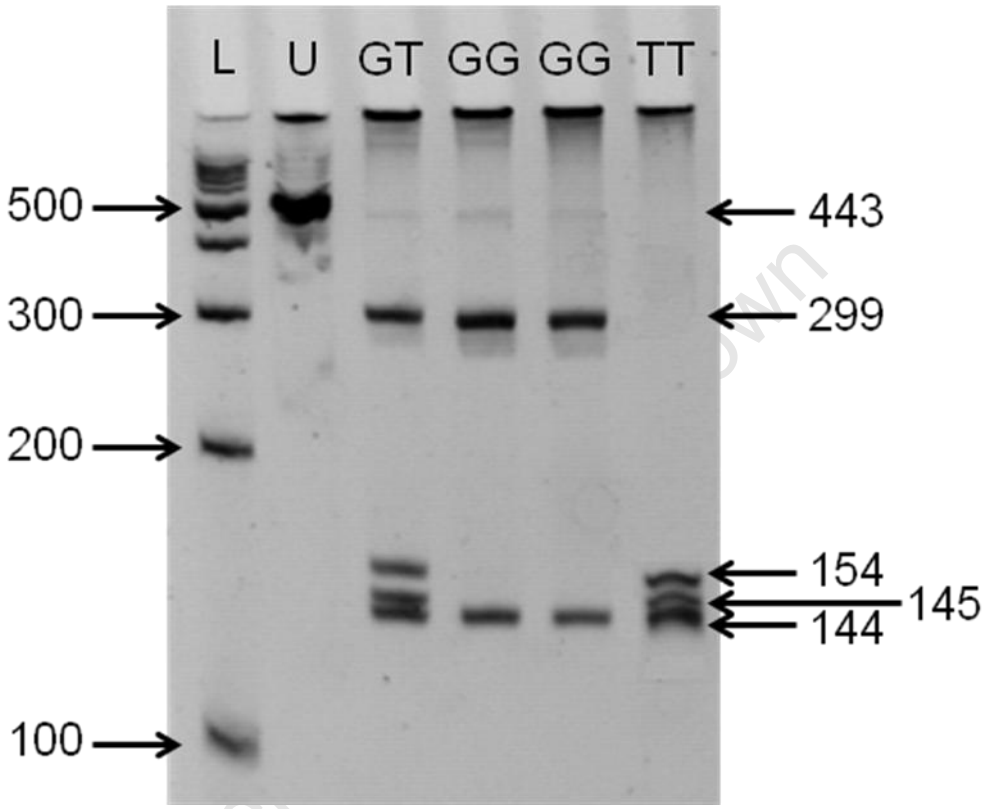

Figure 4.3. Typical 6% polyacrylamide gel of the *NOS3 Dpn*II restriction fragment length polymorphism (RFLP) genotypes. Sizes (in base pairs, bp) on the left are indicative of the 100bp DNA ladder (L) in lane 1. U in lane 2 is the uncut PCR product. Lanes 3 – 6 are samples which were digested with *Dpn*II. Fragment sizes (bp) are indicated on the right.

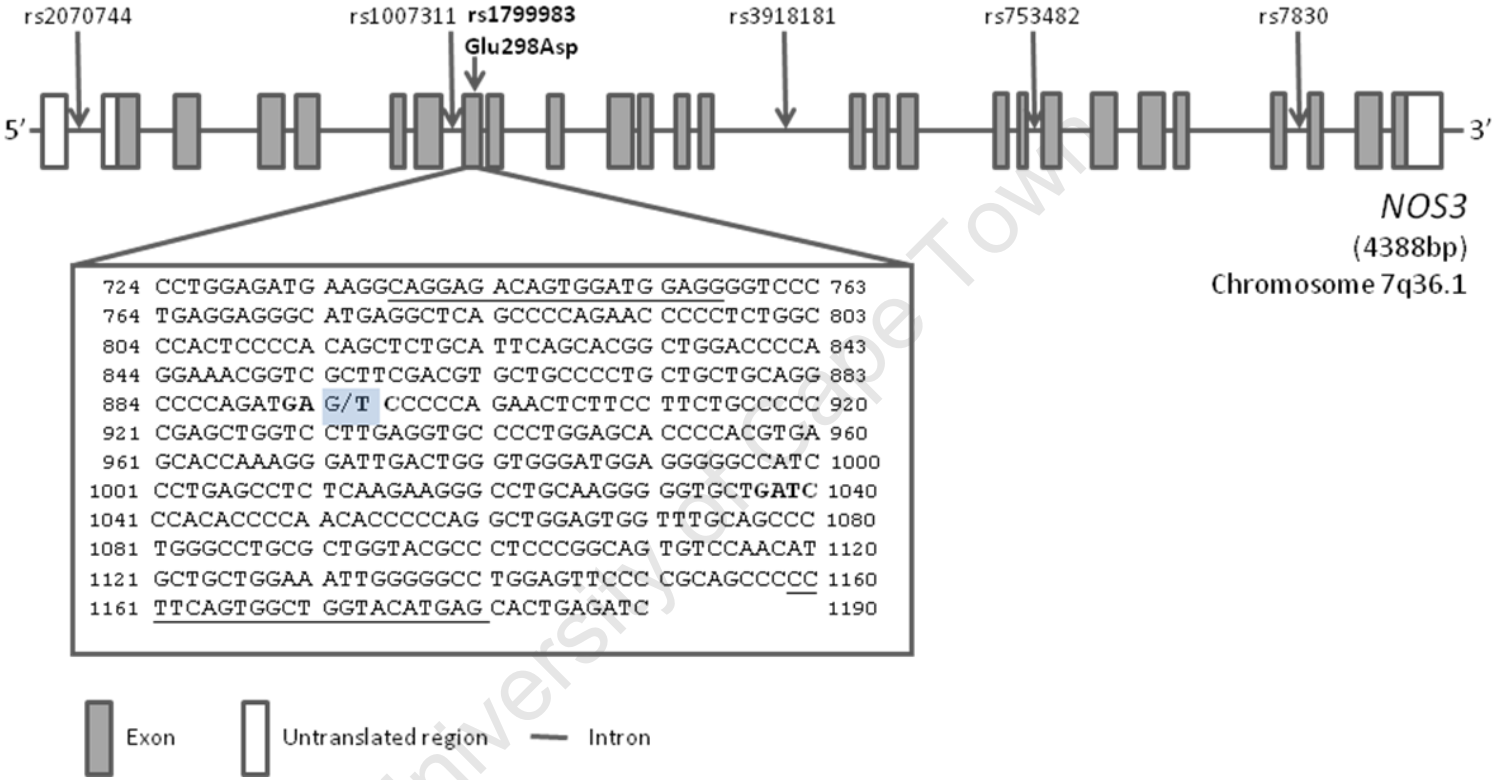

Figure 4.4. Schematic representation of the *NOS3* gene depicting some of the common clinically associated polymorphisms. The size of the gene is given in parenthesis. The SNP chosen for investigation in this study (rs1799983) is in bold typeset and the DNA sequence surrounding the SNP is boxed. Numbers refer to the base position relative to the transcription start site. Primer sequences are underlined. The position of rs1799983 is highlighted and recognition sequence of the restriction endonuclease, *Dpn*II, used for RFLP analysis is in bold type set. All the information used to construct this figure was obtained from databases hosted by the national centre for biotechnology information (NBCI)(http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html).

4.2.4.4. NOS2 -1026C>A (rs2779249)

NOS2 -1026C>A was genotyped by the TaqMan™ allele-discrimination assay (Applied Biosystems, Foster City, California, USA). Custom-designed primers (5'-GCCTCTCAAAGTGCTAGGA-TTACAA-3' and 5'-GGGAATAC-TGTATTTCAGGCATTATAAGGA-3') and MGB-labelled probes (5'-VIC-CGGGCATGGTGGCTA-3' to recognise the C allele and 5'-FAM-CGGGC-ATTGTGGCTA-3' to recognise the A allele) were used as per manufacturer's recommendations, as described in Section 3.2.5.2. (Applied Biosystems, Foster City, California, USA) The localisation of the SNP within the *NOS2* gene and primer sequences were annotated (Figure 4.5).

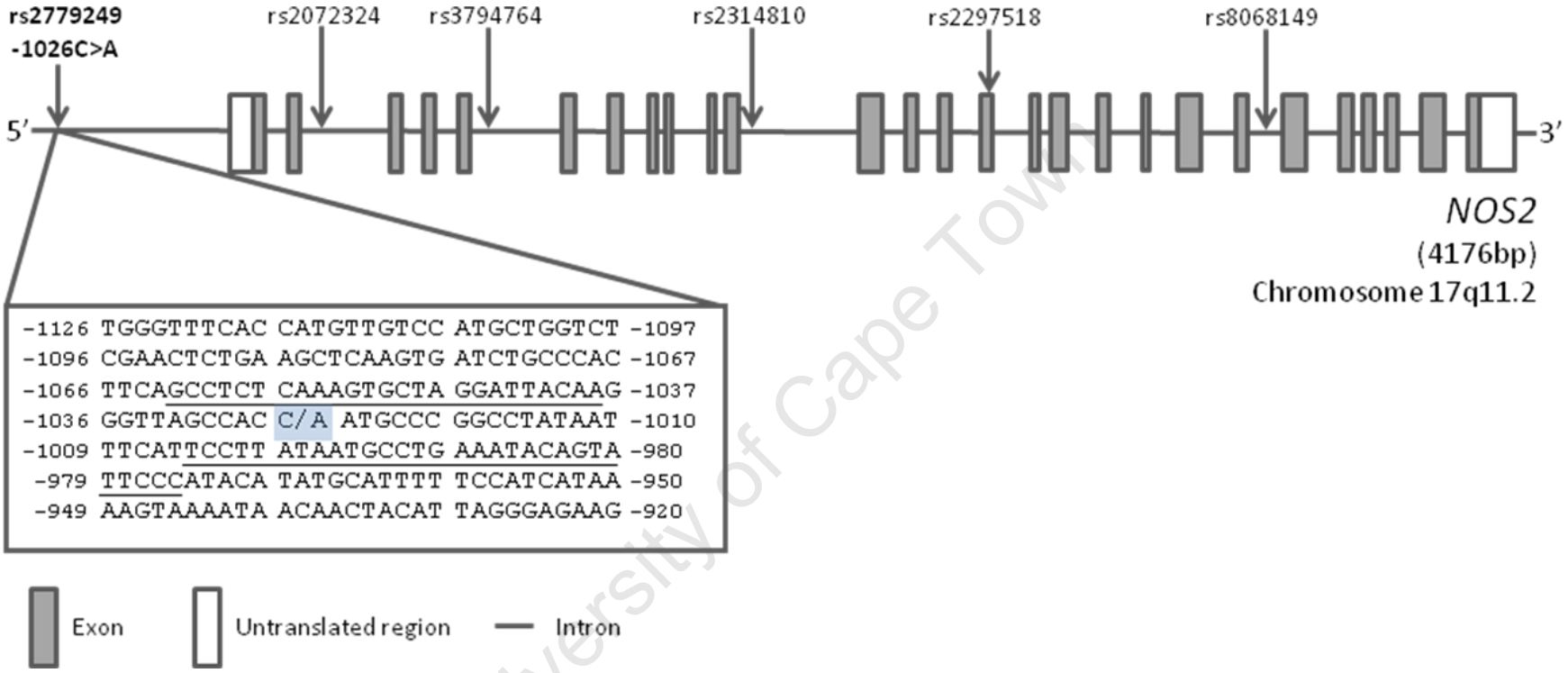

Figure 4.5. Schematic representation of the *NOS2* gene depicting some of the common clinically associated polymorphisms. The size of the gene is in parenthesis. The SNP chosen for investigation in this study (rs2779249) is in bold typeset and the DNA sequence surrounding the SNP is boxed. Numbers refer to the base position relative to the transcription start site. Primer sequences are underlined and the position of rs2779249 highlighted. All the information used to construct this figure was obtained from databases hosted by the national centre for biotechnology information (NBCI) (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html).

4.2.5. **Inferred haplotype construction**

CASP8 haplotype pairs were inferred using the genotypes at the *CASP8* -652 6N del and *CASP8* Asp302His loci obtained for the SA and AUS participants.

4.2.6. **Apoptosis risk model for AT**

Four apoptosis risk models for AT were constructed. The models incorporated seven loci within genes encoding proteins functioning in the apoptosis signalling cascade, associated with risk of AT either previously (*IL-6* -172G>C, *IL-1β* -511C>T, *IL-1β* -31T>C) [118] or in this study (*CASP8* 6Ndel, *CASP8* Asp302His, *NOS3* Glu298Asp, *NOS2* -1026A>C). Only the participants who were successfully genotyped for all loci of a model were included for analysis. The single signalling cascade polygenic risk model presented in this chapter was also exploratory, in preparation for the integrated risk model presented in the final experimental chapter (Chapter 5).

For the apoptosis risk models presented in this chapter, the genotypes of each polymorphism were assigned a score on a 0 – 2 risk scale; the genotype associated with risk was assigned a score of 2 and the genotype associated with protection was assigned a score of 0. The loci within the *IL-1β* gene did not display the heterozygous protection observed at loci within the apoptosis mediator genes, but IL-1β is able to upregulate apoptosis regulators (IL-6 [14] and iNOS [148]) and was therefore included in the apoptosis risk models. The loci within *IL-1β* were scored as previously described (Section 3.2.6) in all four models. The four models were designed to differentiate between the different ways to score the loci which displayed heterozygous protection and evaluate which model best predicts risk of AT.

Briefly, the genotypes of the loci displaying heterozygous protection for the four models were assigned as follows:

- *A priori* model: An additive model scoring system was employed and the genotypes were scored according to an *a priori* hypothesis based on the functional effect of the polymorphisms as determined from previously published functional assays. [215, 219, 221, 222, 231, 234-236]
- Allele-dose model: An additive model was again assumed, but the genotypes were scored based on the association to risk of AT conducted in SA and AUS.
- Heterozygous protection model: The heterozygous protection phenomenon observed in this study was incorporated into the model scoring system. The heterozygous genotypes associated with protection were scored 0 and the homozygous genotype most closely associated with risk of AT was assigned a score of 2.
- Recessive model: A recessive model was assumed and the heterozygous protective genotypes were assigned a score of 0 as were the homozygous genotypes most closely associated with protection.

The scoring of each polymorphism in the different models is presented in Section 4.3.2 of the Results since the scoring is dependent on the results from this chapter.

For each participant, a total genotype score (TGS) was generated from the accumulative risk score at all six loci, as described by Williams and Folland, (2008).[207] TGSs were calculated by dividing the sum of the individual genotype scores by the maximum score obtainable and multiplying by 100. A TGS of 100 represents the complete risk polygenic profile for AT and a TGS of 0 represents the perfect protection polygenic profile.

The area under the ROC curve quantifies the overall ability of the test to discriminate between affected and unaffected individuals. ROC analysis [207,

208] was therefore used to determine the power of the loci within the apoptosis pathway to discriminate between cases and controls, as described in Section 3.2.6.

4.2.7. **Statistics**

Analysis was performed using STATISTICA 9 (Statsoft inc., Tulsa, OK, USA), Graphpad InStat Version 3 (Graphpad Software, San Diego, California, USA) GenePop (http://genepop.curtin.edu.au/genepop_op1.html), Chaplin 1.2.3 (http://www.genetics.emory.edu/labs/epstein/software/chaplin/index.html; Dept of Human Genetics, School of Medicine, Emory University) and GraphPad Prism Version 5.02 (GraphPad Software, San Diego California, USA) as described in Section 3.2.7. Haplotype frequencies were inferred and analysed using Chaplin 1.2.3 (http://www.genetics.emory.edu/labs/epstein/software/chaplin/index.html; Dept of Human Genetics, School of Medicine, Emory University). Statistical significance was accepted when $p<0.05$.

4.3. **RESULTS**

4.3.1. **Genotype and allele frequency distribution**

4.3.1.1. CASP8 -652 6N del

There were no significant differences in genotype (p=0.576) or allele (p=0.700) frequency distribution of *CASP8* -652 6N del in SA participants. The genotype frequency distribution was significantly different in AUS CON compared to AUS TEN (p=0.042). The del/del genotype was significantly underrepresented in AUS CON (20.6%) compared to AUS TEN (34.2%) (p=0.027, OR=2.0, 95%CI 1.1 – 3.6) (Figure 4.6; Table 4.1). Furthermore, the heterozygous genotype, ins/del, was significantly overrepresented in AUS CON (59.8%) compared to AUS TEN (44.7%) (p=0.029, OR=1.8, 95%CI 1.1 – 3.1). All groups except for AUS CON were in HWE (Table 4.1). Frequency distribution was comparable to previously published data of individuals of Caucasian ancestry (Table 4.2).

Table 4.1. Genotype frequency distribution and minor allele frequency of *CASP8* -652 6N del (rs3834129), *CASP8* Asp302His (rs1045485), *NOS3* Glu298Asp (rs1799983) and *NOS2* -1026C>A (rs2779249) polymorphisms in control (CON) and Achilles tendinopathy (TEN) groups of South Africa (SA) and Australia (AUS).

	Genotype Frequency CON			CON n	CON HWE	Genotype Frequency TEN			TEN n	TEN HWE	Minor Allele	Minor Allele Frequency CON	Minor Allele Frequency TEN	Genotype p-value	Allele p-value
CASP8 -652 6Ndel	II	ID	DD			II	ID	DD							
SA	25.6	48.1	26.3	156	0.637	26.8	41.5	31.7	82	0.127	del	50.3	52.4	0.576	0.700
AUS	19.6	59.8	20.6	189	**0.009**	21.1	44.7	34.2	76	0.486	del	50.5	56.6	**0.042**	0.213
CASP8 Asp302His	GG	GC	CC			GG	GC	CC							
SA	70.4	26.4	3.2	159	0.573	79.8	19.0	1.2	84	1.000	C	16.4	10.7	0.128	0.092
AUS	53.8	44.2	2.0	199	**0.003**	67.1	30.4	2.5	79	1.000	C	22.1	17.7	**0.042**	0.102
NOS3 Glu298Asp	GG	GT	TT			GG	GT	TT							
SA	41.7	47.7	10.6	151	0.593	39.1	54.0	6.9	87	0.091	T	34.4	33.9	0.508	0.921
AUS	40.8	50.6	8.6	174	0.128	42.1	52.6	5.3	76	0.106	T	33.9	31.6	0.654	0.680
NOS2 -1026C>A	CC	CA	AA			CC	CA	AA							
SA	50.7	42.2	7.1	154	0.697	48.8	40.2	11.0	82	0.612	A	28.2	31.1	0.602	0.525
AUS	37.7	55.0	7.3	191	**0.004**	47.4	38.5	14.1	78	0.305	A	34.8	33.3	**0.030**	0.765

Genotype and allele frequencies are expressed as percentages. HWE is the p-value for exact tests for Hardy-Weinberg equilibrium. Bold typeset denotes significance. I, insertion allele; D, deletion allele.

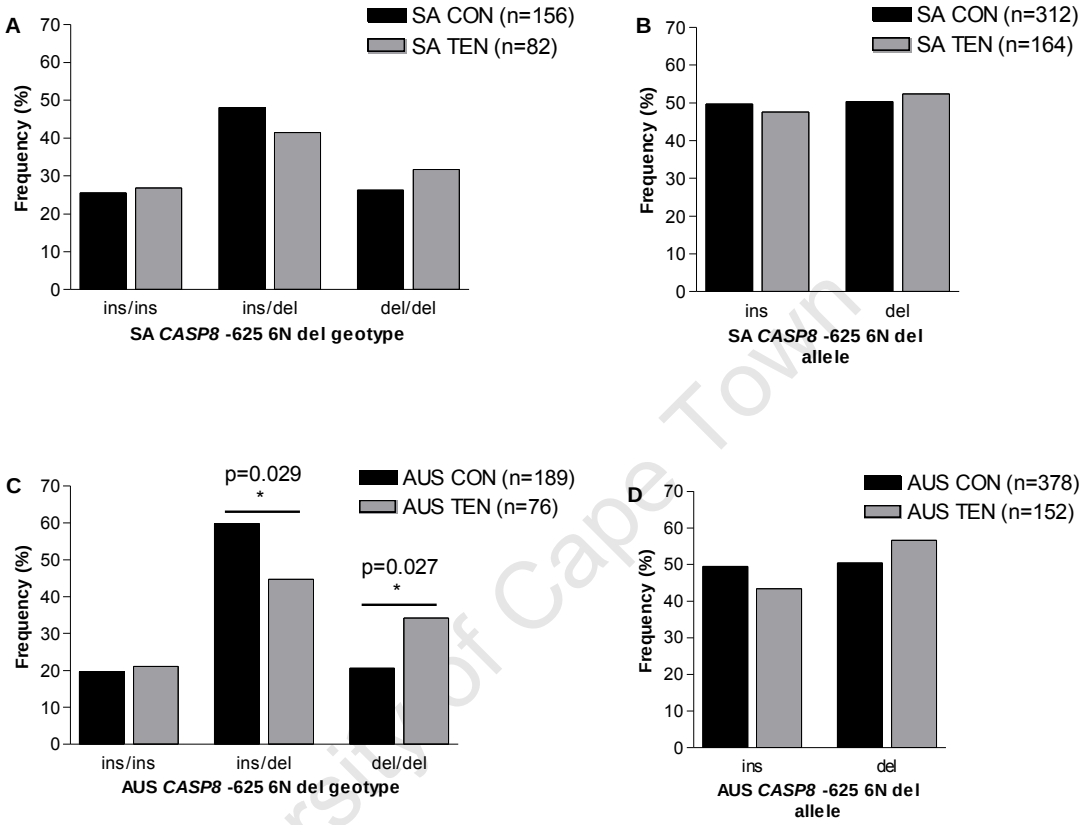

Figure 4.6. Genotype (A) and allele (B) frequency distribution of *CASP8* -652 6N del within South African (SA) participants and genotype (C) and allele (D) frequency distribution within Australian (AUS) participants for asymptomatic control (CON, black bars) and Achilles tendinopathy (TEN, grey bars) groups. Asterisk (*) denotes significance (p<0.05) between CON and TEN groups. (A) SA CON vs SA TEN, p=0.576; (B) SA CON vs SA TEN, p=0.700; (C) AUS CON vs AUS TEN, p= 0.009; ins/del vs ins/ins+del/del, p=0.029; del/del vs ins/ins+ins/del, p=0.027; (D) AUS CON vs AUS TEN, p=0.213. The number (n) of participants (A and C) or alleles (B and D) are in parentheses. ins, insertion allele; del, deletion allele.

Table 4.2. Frequency distribution of the *CASP8* 6N del genotypes in asymptomatic control participants of published case-control studies.

	n	ins/ins	ins/del	del/del	Reference
SA CON	156	25.6	48.1	26.3	
AUS CON	189	19.6	59.8	20.6	
European	24	19.0	52.4	28.6	NCBI
UK	3749	24.0	51.0	25.0	[237]
Caucasian Americans	835	24.8	52.7	22.5	[216]
Chinese	907	57.4	35.6	7.0	[238]

4.3.1.2. CASP8 Asp302His

There were no significant differences in genotype (p=0.128) or allele (p=0.092) frequency distribution of *CASP8* Asp302His in SA participants. The genotype frequency distribution was however significantly different in AUS CON compared to AUS TEN (p=0.042). The GG genotype was significantly underrepresented in AUS CON (53.8%) compared to AUS TEN (67.1%) (p=0.045, OR=1.8 95%CI 1.0 – 3.0) and the GC genotype was significantly overrepresented in AUS CON (44.2%) compared to AUS TEN (30.2%) (p=0.042, OR=1.8 95%CI 1.0-3.2) (Figure 4.7; Table 4.1). No differences in allele frequency distribution were observed in AUS participants (p=0.102) (Table 4.1). All groups except for AUS CON were in HWE (Table 4.1). Frequency distribution was comparable to previously published data of individuals of Caucasian ancestry (Table 4.3).

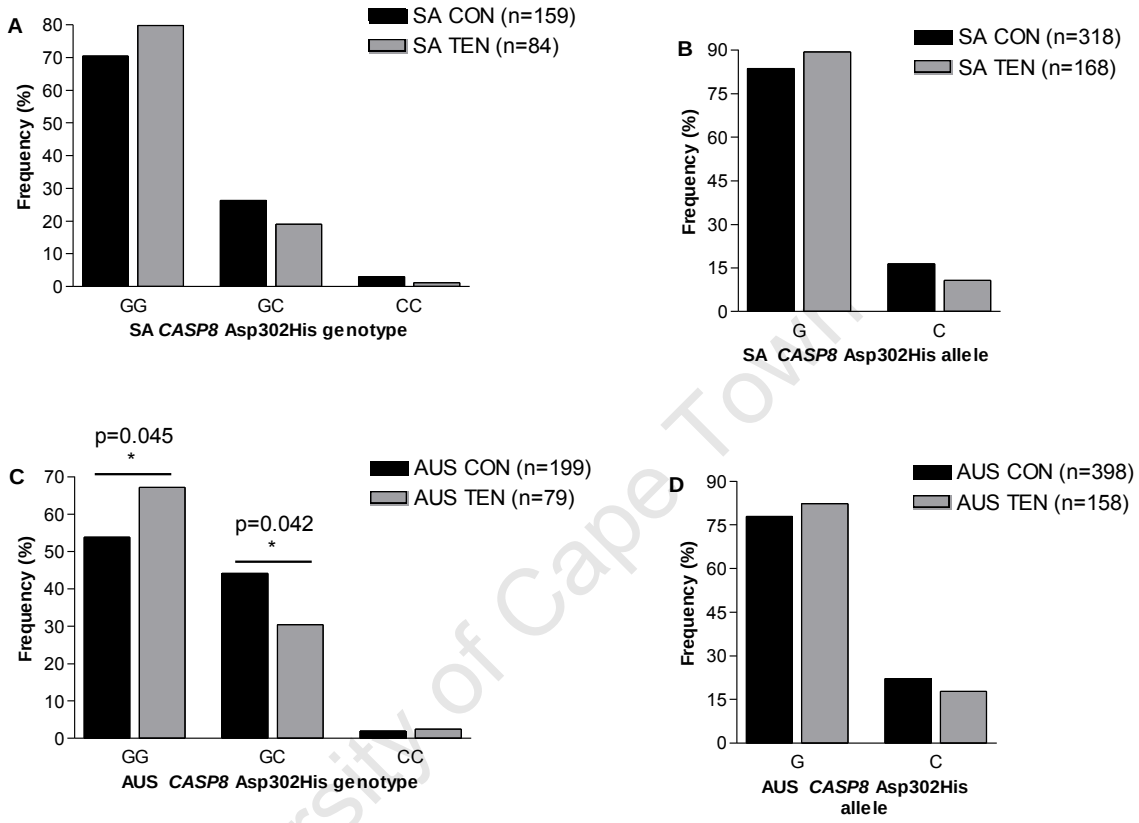

Figure 4.7. Genotype (A) and allele (B) frequency distribution of *CASP8* Asp302His within South African (SA) participants and genotype (C) and allele (D) frequency distribution within Australian (AUS) participants for asymptomatic control (CON, black bars) and Achilles tendinopathy (TEN, grey bars) groups. Asterisk (*) denotes significance ($p<0.05$) between CON and TEN groups. (A) SA CON vs SA TEN, p=0.128; (B) SA CON vs SA TEN, p=0.092; (C) AUS CON vs AUS TEN, p= 0.042; GG vs GC+CC, p=0.045; GC vs GG+CC, p=0.042; (D) AUS CON vs AUS TEN, p=0.102. The number (n) of participants (A and C) or alleles (B and D) are in parentheses.

Table 4.3. Frequency distribution of the *CASP8* Asp302His genotypes in asymptomatic control participants of published case-control studies.

	n	GG	GC	CC	Reference
SA CON	159	70.4	26.4	3.2	
AUS CON	199	53.8	44.2	2.0	
European	84	76.7	21.7	1.7	NCBI
Spanish Caucasian	716	68.0	30.0	2.0	[214]
Caucasian Americans	835	73.6	24.8	1.6	[216]
Indian	240	92.2	7.4	0.4	[217]

4.3.1.3. NOS3 Glu298Asp

There were no significant differences in genotype (p=0.508) or allele (p=0.921) frequency distribution of *NOS3* Glu298Asp in SA participants. Similarly, no differences genotype (p=0.654) or allele (p=0.680) frequency distribution were observed in AUS participants (Table 4.1). All groups were in HWE (Table 4.1). Frequency distribution was comparable to previously published data of individuals of Caucasian ancestry (Table 4.4).

Table 4.4. Frequency distribution of the *NOS3* Glu298Asp genotypes in asymptomatic control participants of published case-control studies.

	n	GG	GT	TT	Reference
SA CON	151	41.7	47.7	10.6	
AUS CON	174	40.8	50.6	8.6	
European	60	40.7	50.8	8.5	NCBI
Czech Caucasian	22	33.3	58.3	8.3	[229]
Brazilian Caucasian	41	58.5	34.1	7.3	[236]
South African Caucasian	163	39.3	44.2	16.6	[227]

4.3.1.4. NOS2 -1026C>A

The difference in the genotype (p=0.602) and allele (p=0.525) frequency distributions of *NOS2* -1026C>A between SA CON and SA TEN were not significant. The genotype frequency distribution was significantly different in AUS (p=0.030). The heterozygous CA genotype was significantly overrepresented in AUS CON (55.0%) compared to AUS TEN (38.5%) (p=0.016, OR=2.0, 95%CI 1.1–3.3) (Figure 4.8; Table 4.1). All groups except for AUS CON were in HWE (Table 4.1). This polymorphism has not been explored much in case-control genetic association studies, nevertheless the frequency distribution was comparable to previously published data of individuals of Caucasian ancestry (Table 4.5).

Table 4.5. Frequency distribution of the *NOS2* -1026C>A genotypes in asymptomatic control participants of published case-control studies.

	n	CC	CA	AA	Reference
SA CON	154	50.7	42.2	7.1	
AUS CON	191	37.7	55.0	7.3	
European	22	65.0	25.0	10.0	NCBI
Japanese	95	73.0	24.0	3.0	[239]
Chinese	432	41.7	46.7	11.6	[231]

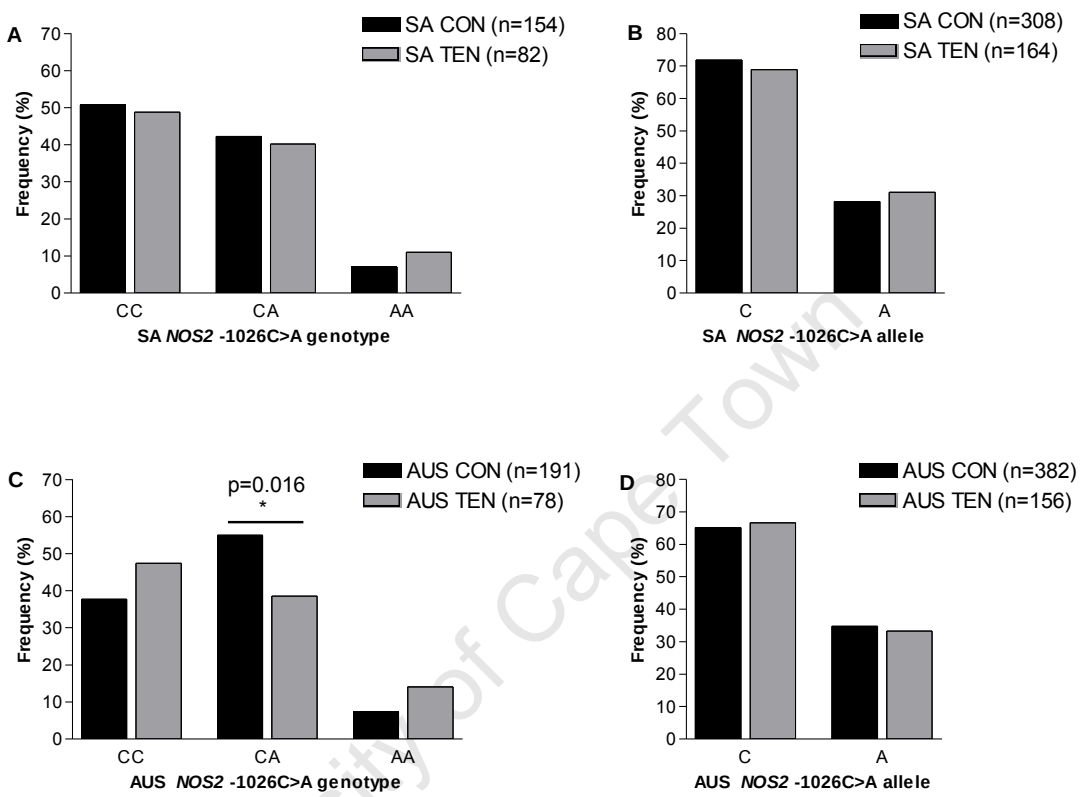

Figure 4.8. Genotype (A) and allele (B) frequency distribution of *NOS2* -1026A>C within South African (SA) participants and genotype (C) and allele (D) frequency distribution within Australian (AUS) participants for asymptomatic control (CON, black bars) and Achilles tendinopathy (TEN, grey bars) groups. Asterisk (*) denotes significance ($p<0.05$) between CON and TEN groups. (A) SA CON vs SA TEN, p=0.602; (B) SA CON vs SA TEN, p=0.525; (C) AUS CON vs AUS TEN, p= 0.030; CA vs CC+AA, p=0.016; (D) AUS CON vs AUS TEN, p=0.765. The number (n) of participants (A and C) or alleles (B and D) are in parentheses.

4.3.1.5. CASP8 Inferred Haplotype

Inferred haplotypes were constructed for *CASP8* using genotypes obtained at the -652 6N del and Asp302His loci for SA and AUS participants. There were no differences in the frequency distribution of the *CASP8* inferred haplotypes within SA participants (Table 4.6). The *CASP8* del/G inferred haplotype was significantly underrepresented ($p<0.001$, LR=11.3) in AUS CON (31.3%) compared to AUS TEN (45.6%) (Table 4.6). No significant differences in the distribution of inferred haplotypes were noted between SA and AUS (Table 4.6) and therefore SA and AUS participants were combined and reanalysed for the inferred haplotype. The del/G inferred haplotype remained significantly overrepresented in [SA+AUS] TEN (45.3%) compared to [SA+AUS] CON (35.2%) ($p<0.001$, LR=11.5). Furthermore, the del/C inferred haplotype was found to be significantly overrepresented in [SA+AUS] CON (15.4%) compared to [SA+AUS] TEN (9.5%) ($p=0.017$, LR=5.7) (Table 4.6).

Table 4.6. Frequency distribution of the inferred haplotypes constructed from the *CASP8* -652 6Ndel and *CASP8* Asp302His polymorphisms, within the control (CON) and Achilles tendinopathy (TEN) groups of independent South African (SA) and Australian (AUS) participants and in the combined [SA+AUS] group.

CASP8 -652 6Ndel	*CASP8* Asp302 His	SA				AUS				[SA+AUS]			
		CON	TEN	p-value	LR	CON	TEN	p-value	LR	CON	TEN	p-value	LR
ins	G	44.7 (72)	43.9 (40)	0.473	-	44.5 (93)	36.6 (31)	0.117	-	44.1 (164)	40.6 (72)	0.111	-
ins	C	4.8 (7)	3.4 (3)	0.293	-	4.9 (10)	6.6 (5)	0.304	-	6.3 (19)	4.7 (8)	0.103	-
del	G	38.9 (63)	45.4 (42)	0.149	-	31.3 (65)	45.6 (38)	**0.0008**	11.3	35.2 (131)	45.3 (80)	**0.0007**	11.5
del	C	11.7 (18)	7.4 (6)	0.122	-	19.3 (40)	11.2 (9)	0.076	-	15.4 (57)	9.5 (16)	**0.017**	5.7

Frequencies are expressed as percentages with number of participants in parentheses. Bold typeset denotes significant differences ($p<0.05$) between CON and TEN groups. Frequency distribution is similar in SA and AUS (SA CON vs AUS CON, $p=0.646$; SA TEN vs AUS TEN, $p=0.362$)

4.3.2. **Apoptosis risk model for AT**

Four models were designed for loci within genes encoding proteins functioning in the apoptosis signalling cascade. The four models differed in the genotype scoring system and (i) an *a priori* model, (ii) an allele-dose model (iii) a heterozygous protection model and (iv) a recessive model were constructed. Participants were only included if they were successfully genotyped at all loci included in the respective models. The number of participants included for analysis of each model is presented in Table 4.7. TGSs of all four diagnostic groups were normally distributed in all four models.

Table 4.7. Number of asymptomatic control participants (CON) and participants with diagnosed Achilles tendinopathy (TEN), from South Africa (SA) and Australian (AUS) which were included in the analysis of the four apoptosis risk models.

	SA CON (161)	SA TEN (85)	AUS CON (205)	AUS TEN (82)
A priori model	121	66	135	57
Allele-dose model	128	66	149	59
Heterozygous protection model	128	66	149	59
Recessive model	128	66	149	59

The total number of participants genotyped in each diagnostic group is presented in parentheses.

4.3.2.1. A priori model

Total genotype scores (TGS) were calculated according to the *a priori* model scoring system for all participants that were genotyped for the seven loci within the apoptosis signalling cascade (Table 4.8).

Table 4.8. The *a priori* model genotype scoring system at the seven loci within the apoptosis signalling cascade.

Gene	Variant	scoring system			Rationale
		2	1	0	
IL-1β	rs16944 (T/C)	CC	CT	TT	C allele ↑ *IL-1β* expression [240]
	rs1143627 (T/C)	TT	TC	CC	T allele ↑ *IL-1β* expression [240]
IL-6	rs1800795 (G/C)	GG	GC	CC	G allele ↑ *IL-6* expression [236]
CASP8	rs3834129 (I/D)	II	ID	DD	D allele ↓ *CASP8* expression [215]
	rs1045485 (G/C)	GG	GC	CC	C allele proposed to ↓ capspase-8 function [219]
NOS2	rs2779249 (C/A)	AA	CA	CC	A allele ↑ *NOS2* expression [231]
NOS3	rs1799983 (G/T)	GG	GT	TT	T allele ↓ eNOS activity [221, 222]

The accession numbers of single nucleotide polymorphisms (SNPs) were identified from databases hosted by the national centre for biotechnology information (NCBI) (http://www.ncbi.nlm.nih. gov/). Major and minor alleles of SNPs are reported in parenthesis, respectively. ↑, increase; ↓, reduce; AT, Achilles tendinopathy; D, deletion; I, insertion.

There was no difference in the TGSs between SA CON (58.2±13.7) and SA TEN (61.5±16.1) (p=0.145). The TGS was significantly higher in AUS TEN (59.4±13.9) compared AUS CON (55.1±12.7) (p=0.037) (Figure 4.9 A). The *a priori* model of AUS data did however not discriminate between diagnostic groups (AUC=0.57, 95%CI 0.48 - 0.66; p=0.107). The distribution of TGSs in SA CON (58.2±13.7) and AUS CON (55.1±12.7) were similar (p=0.059), as were the TGS distribution in SA TEN (61.5±16.1) and AUS TEN (59.4±13.9) (p=0.450) and therefore the groups were combined and reanalysed as a combined [SA+AUS] group for statistical power. The TGS of [SA+AUS] TEN (60.5±15.1) was significantly higher than [SA+AUS] CON (56.6±13.2) (p=0.010) (Figure 4.9 B). Furthermore, the *a priori* model in the combined [SA+AUS] group was a significant discriminator between diagnostic groups (AUC=0.58, 95%CI 0.51 – 0.64; p=0.015) however, a model with an AUC of 0.58 does not have very strong predictive power.

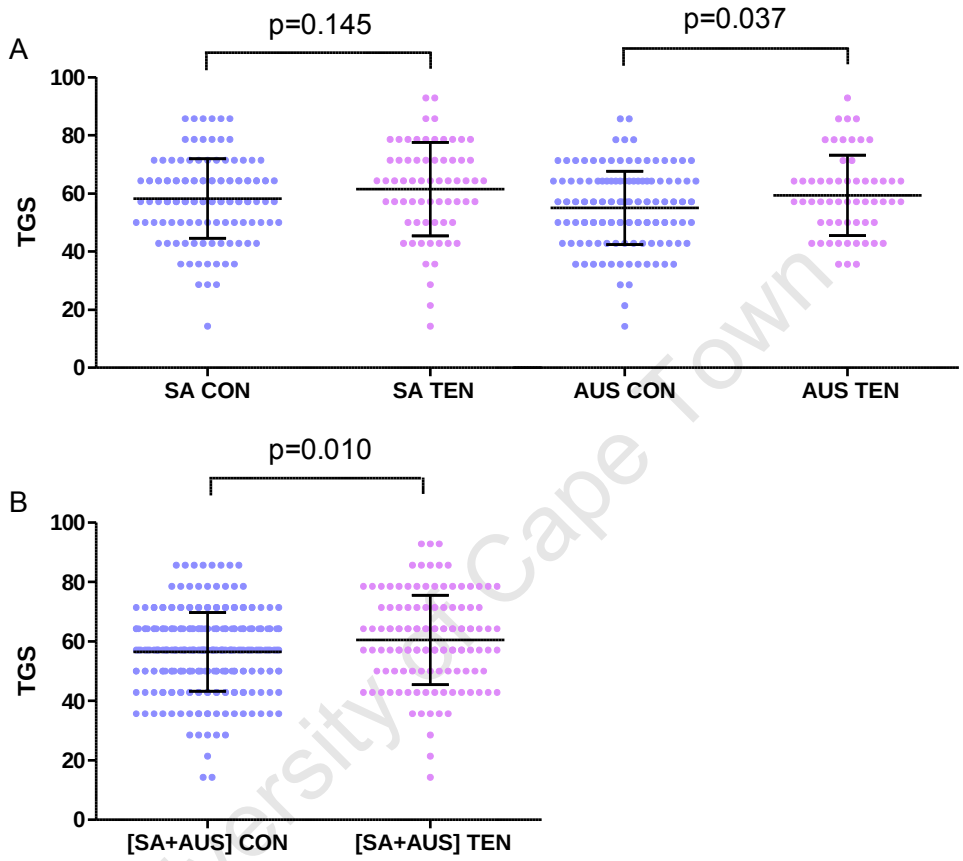

Figure 4.9. The distribution of total genotype scores (TGS), as calculated using an *a priori* model, of (A) South African participants in the asymptomatic control (SA CON; n=121) and clinically diagnosed Achilles tendinopathy (SA TEN; n=66) groups, as well as Australian control (AUS CON; n=135) and Australian Achilles tendinopathy (AUS TEN; n=57) groups and (B) the TGS distribution in the combined South Africa and Australia groups ([SA+AUS] CON; n=256 and [SA+AUS] TEN; n=123). Significance was accepted at p<0.05.

4.3.2.2. Allele-dose model

TGSs were calculated according to the allele-dose model scoring system for all participants that were genotyped for the six loci within the apoptosis signalling cascade (Table 4.9).

Table 4.9. The allele-dose model genotype scoring system at the six loci within the apoptosis signalling cascade.

Gene	Variant	scoring system			Rationale
		2	1	0	
IL-1β	rs16944 (T/C)	CC	CT	TT	TT genotype associated with ↓ risk of AT in SA males [113]
	rs1143627 (T/C)	TT	TC	CC	C allele associated with ↓ risk of AT in SA males [113]
IL-6	rs1800795 (G/C)	GG	GC	CC	GG genotype and G allele associated with ↑ risk of AT in AUS [112]
CASP8	rs3834129 (I/D)	DD	ID	II	The DD genotype was associated with ↑ risk of AT in AUS (Section 4.3.1.1)
	rs1045485 (G/C)	GG	GC	CC	The GG genotype was associated with ↑ risk of AT in AUS (Section 4.3.1.2)
NOS2	rs2779249 (C/A)	AA	CA	CC	The AA genotype was most closely (although not significantly) associated with ↑ risk of AT in SA and AUS (Section 4.3.1.4)

The accession numbers of single nucleotide polymorphisms (SNPs) were identified from databases hosted by the national centre for biotechnology information (NCBI) (http://www.ncbi.nlm.nih. gov/). Major and minor alleles of SNPs are reported in parenthesis, respectively. ↑, increase; ↓, reduce; AT, Achilles tendinopathy; D, deletion; I, insertion.

The TGS of SA TEN (61.2±15.5) was significantly higher than SA CON (56.2±15.3) (p=0.031). Similarly, the TGS of AUS TEN (59.6±16.7) was significantly higher than AUS CON (53.1±14.6) (p=0.006) (Figure 4.10 A). The TGS of SA CON (56.2±15.3) and AUS CON (53.1±14.6) were similar (p=0.091), as were SA TEN (61.2±15.5) and AUS TEN (59.6±16.7) (p=0.572) and therefore the groups were combined and reanalysed as a combined

[SA+AUS] group. The TGS of [SA+AUS] TEN (60.5±16.1) was significantly higher than [SA+AUS] CON (54.5±15.0) (p<0.001) (Figure 4.10 B). Furthermore, the allele-dose model in this combined [SA+AUS] group was a significant discriminator between diagnostic groups (AUC=0.60, 95%CI 0.54 – 0.66; p<0.001).

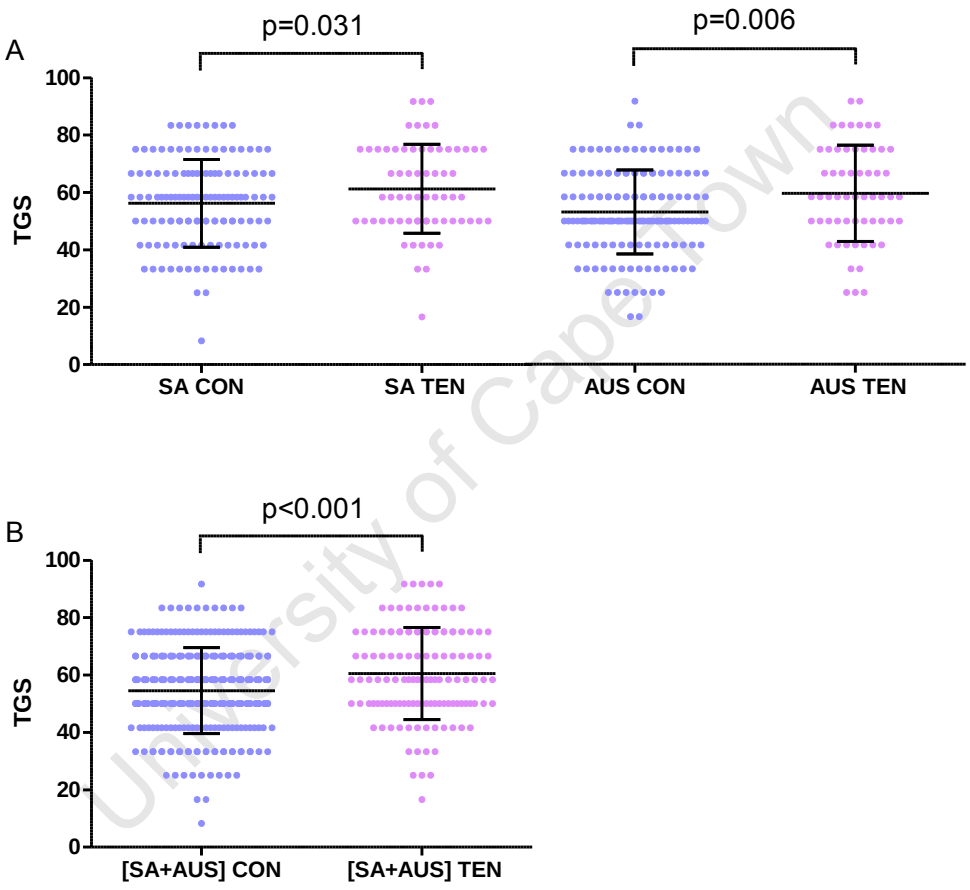

Figure 4.10. The distribution of total genotype scores (TGS), as calculated using an allele-dose model, of (A) South African participants in the asymptomatic control (SA CON; n=128) and clinically diagnosed Achilles tendinopathy (SA TEN; n=66) groups, as well as Australian control (AUS CON; n=149) and Australian Achilles tendinopathy (AUS TEN; n=59) groups and (B) the TGS distribution in the combined South Africa and Australia groups ([SA+AUS] CON; n=277 and [SA+AUS] TEN; n=125). Significance was accepted at p<0.05.

4.3.2.3. Heterozygous protection model

TGSs were calculated according to the heterozygous protection model scoring system for all participants that were genotyped for the six loci within the apoptosis signalling cascade (Table 4.10).

There was no difference in TGS distribution between SA CON (50.6±17.8) and SA TEN (55.7±16.2) (p=0.056). The TGS was significantly higher in AUS TEN (54.7±18.4) compared to AUS CON (42.8±19.1) (p<0.001) (Figure 4.11). The heterozygous protection model of AUS data had significant discriminating accuracy in assessing risk of AT (AUC=0.67, 95%CI 0.59 – 0.75; p<0.001). While the TGSs of SA TEN (55.7±16.2) and AUS TEN (54.7±18.4) groups were similar (p=0.742), SA CON (50.7±17.8) and AUS CON (42.9±19.1) groups were not similar (p<0.001) and therefore SA and AUS were not combined for analysis.

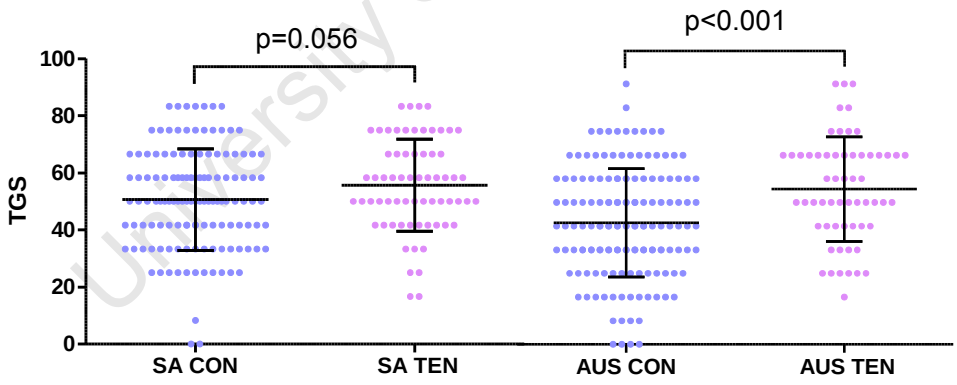

Figure 4.11. The distribution of total genotype scores (TGS), as calculated using the heterozygous protection model, of South African participants in the asymptomatic control (SA CON; n=128) and clinically diagnosed Achilles tendinopathy (SA TEN; n=66) groups, as well as Australian control (AUS CON; n=149) and Australian Achilles tendinopathy (AUS TEN; n=59) groups. Significance was accepted at p<0.05.

Table 4.10. The heterozygous protection model genotype scoring system at the six loci within the apoptosis signalling cascade.

	Gene	Variant	scoring system			Rationale
			2	1	0	
Loci NOT displaying heterozygous protection	*IL-1β*	rs16944 (T/C)	CC	CT	TT	As for allele-dose model
		rs1143627 (T/C)	TT	TC	CC	
Loci displaying heterozygous protection	*IL-6*	rs1800795 (G/C)	GG	CC	GC	GC genotype associated with ↓ risk of AT in AUS [112] GG genotype and G allele associated with ↑ risk of AT in AUS [112]
	CASP8	rs3834129 (I/D)	DD	II	ID	The ID genotype was associated with ↓ risk of AT in AUS (Section 4.3.1.1) The DD genotype was associated with ↑ risk of AT in AUS (Section 4.3.1.1)
		rs1045485 (G/C)	GG	CC	GC	The GC genotype was associated with ↓ risk of AT in AUS (Section 4.3.1.2) The GG genotype was associated with ↑ risk of AT in AUS (Section 4.3.1.2)
	NOS2	rs2779249 (C/A)	AA	CC	CA	The CA genotype was associated with ↓ risk of AT in AUS (Section 4.3.1.4) The AA genotype was most closely (although not significantly) associated with risk of AT in SA and AUS (Section 4.3.1.4)

The accession numbers of single nucleotide polymorphisms (SNPs) were identified from databases hosted by the national centre for biotechnology information (NCBI) (http://www.ncbi.nlm.nih. gov/). Major and minor alleles of SNPs are reported in parenthesis, respectively. ↑, increase; ↓, reduce; AT, Achilles tendinopathy; D, deletion; I, insertion.

4.3.2.4. Recessive model

TGSs were calculated according to the recessive model scoring system for all participants that were genotyped for the six loci within the apoptosis signalling cascade (Table 4.11).

The TGS of SA TEN (48.5±17.5) was significantly higher than SA CON (42.8±17.6) (p=0.033). Similarly, the TGS of AUS TEN (47.3±19.2) was significantly higher than AUS CON (35.7±18.1) (p<0.001) (Figure 4.12). While SA TEN and AUS TEN groups were similarly distributed (p=0.722), SA CON and AUS CON groups were not similar (p=0.001) and therefore SA and AUS were not be combined for analysis. The recessive model of both SA and AUS data had significant discriminating accuracy in assessing the risk of AT; with AUS data (AUC=0.67, 95%CI 0.58 – 0.75; p<0.001) having greater discriminating power than SA data (AUC=0.60, 95%CI 0.51 – 0.68; p=0.027).

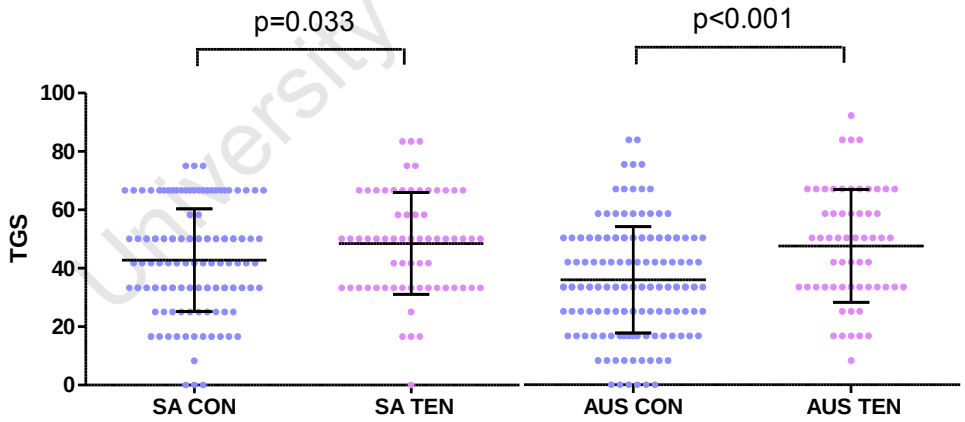

Figure 4.12. The distribution of total genotype scores (TGS), as calculated using a recessive model, of South African participants in the asymptomatic control (SA CON; n=128) and clinically diagnosed Achilles tendinopathy (SA TEN; n=66) group, as well as Australian control (AUS CON; n=149) and Australian Achilles tendinopathy (AUS TEN; n=59) group. Significance was accepted at p<0.05.

Table 4.11. The recessive model genotype scoring system at the six loci within the apoptosis signalling cascade.

	Gene	Variant	scoring system			Rationale
			2	1	0	
Loci NOT displaying heterozygous protection	*IL-1β*	rs16944 (T/C)	CC	CT	TT	As for allele-dose model
		rs1143627 (T/C)	TT	TC	CC	
Loci displaying heterozygous protection	*IL-6*	rs1800795 (G/C)	GG	-	GC/CC	GC genotype associated with ↓ risk of AT in AUS [112] GG genotype and G allele associated with ↑ risk of AT in AUS [112]
	CASP8	rs3834129 (I/D)	DD	-	ID/II	The ID genotype was associated with ↓ risk of AT in AUS (Section 4.3.1.1) The DD genotype was associated with ↑ risk of AT in AUS (Section 4.3.1.1)
		rs1045485 (G/C)	GG	-	GC/CC	The GC genotype was associated with ↓ risk of AT in AUS (Section 4.3.1.2) The GG genotype was associated with ↑ risk of AT in AUS (Section 4.3.1.2)
	NOS2	rs2779249 (C/A)	AA	-	CA/CC	The CA genotype was associated with ↓ risk of AT in AUS (Section 4.3.1.4) The AA genotype was most closely (although not significantly) associated with risk of AT in SA and AUS (Section 4.3.1.4)

The accession numbers of single nucleotide polymorphisms (SNPs) were identified from databases hosted by the national centre for biotechnology information (NCBI) (http://www.ncbi.nlm.nih. gov/). Major and minor alleles of SNPs are reported in parenthesis, respectively. ↑, increase; ↓, reduce; AT, Achilles tendinopathy; D, deletion; I, insertion.

4.4. **DISCUSSION**

This study investigated four polymorphisms within three genes of the apoptosis signalling cascade, following a case control genetic association design. The main findings of the study are (i) the association of *CASP8* -652 del/del and *CASP8* Asp302His GG genotypes with increased risk of AT in AUS and the identification of a risk haplotype using these two loci in AUS and the combined [SA+AUS] group (ii) the association of heterozygosity at *CASP8* -652 6Ndel, *CASP8* Asp302His and *NOS2* -1026A>C loci with protection from developing AT in AUS and (iii) the identification of a polygenic profile, using genetic markers within the apoptosis pathway, to capture the risk of AT in both SA and AUS.

The *CASP8* -652 del/del genotype was associated with a 2 fold increase risk of AT in AUS. This finding is interesting and unexpected, since the del allele destroys a Sp1 binding element, therefore resulting in decreased caspase-8 expression.[215] One would expect that reduced caspase-8 expression would protect against excessive apoptosis and thus that the deletion allele would protect from developing AT. A possible explanation for this observation is that Sp1 can have different effects under different transcriptional control in different cell types, as Sp1 has been identified to both repress and activate transcription.[241] The effect of this deletion of caspase-8 expression has only been investigated in lymphocytes [215] and the functional effect in tenocytes is not known. Future studies should explore the effect of this polymorphism in tenocytes to aid the understanding of this association. Another explanation for this unexpected finding is that caspase expression within the inflammatory cells that may infiltrate the tendon tissue in response to microtrauma, is reduced and the effect the inflammatory cells in the tendon is prolonged, which may lead to an increase in apoptosis.

The *CASP8* Asp302His GG genotype was associated with a 1.8 fold increased risk of developing AT in AUS. The *CASP8* Asp302His GG genotype codes for the wildtype Asp (D) amino acid, predicted to be the more stable form of the caspase-8 enzyme,[219] and thus the association of *CASP8* Asp302His GG with risk for AT, makes biological sense.

The independent *CASP8* polymorphism associations were mirrored in the *CASP8* inferred haplotype. The *CASP8* del/G inferred haplotype was associated with increased risk of AT in AUS and [SA+AUS] and the *CASP8* del/C was associated with protection from developing AT in [SA+AUS]. Genotyping other SNPs in the region implicated by the haplotype may provide more informative haplotypes in identifying the critical region within the *CASP8* gene involved in AT predisposition.

There were no associations observed for *NOS3* Glu298Asp. This does however not exclude the possibility that eNOS may play a role in the development of AT, since it does contribute to the basal inflammatory status of the tissue. *NOS3* therefore remains a good candidate gene and requires further exploration.

An interesting finding of this study was the heterozygosity phenomenon noted at the *CASP8* and *NOS2* loci to protect from developing AT. When investigating associations of functional genes variants with a clinical phenotype, it is not uncommon to observe, if a particular genotype/allele confers risk, such as *CASP8* -652 del/del, that the opposite genotype/allele (in this case *CASP8* -652 ins/ins) would confer protection, or at least be overrepresented in CON. However, the possession of the *CASP8* -652 ins/del, *CASP8* Asp302His GC and *NOS2* -1026GC heterozygous genotypes were found to confer protection against AT in AUS. These findings suggests a "*Goldilocks effect*": that within the apoptosis pathway, both too much and too little expression of apoptotic mediators may be detrimental [242] and lead

to pathology and perhaps this is synonymous with MMP/TIMP balance of the extracellular matrix degradation signalling cascade.[142]

Heterozygous protection has not been observed in the genetic predisposition to injury, however, this phenomenon is not uncommon in susceptibility to infectious disease.[242-246] The inflammatory pathway plays a critical role in the body's defence mechanism against pathogens and the protective heterozygosity observed at apoptotic loci in this study has also been observed at loci within other pro-apoptosis genes in other multifactorial conditions, such as invasive pneumococcal disease.[246] More specifically, for the polymorphisms within *NFKBIA*, the gene encoding nuclear factor-kappaβ (NF-κβ) inhibitor α, which were associated with risk of developing pneumococcal disease; at the four loci in the Kenyan individuals (n=550) and the nine loci in the Caucasian individuals (n=743) associated with risk, heterozygosity was found to be protective.[246] NF-κβ prevents entry into apoptosis and therefore *NFKBIA* also promotes apoptosis. These findings therefore collectively suggest that the heterozygous genotypes at the specific loci within genes encoding for components of the apoptosis pathway may provide the tenocytes with versatility to respond more effectively to the changing cellular environment. Therefore the hypothesis that heterozygosity may be more favourable at the loci within the *CASP8* (-652 6N del; Asp302H) and *NOS2* (-1026G>C) genes, protecting an individual from risk of development of AT, needs to be validated in independent populations.

Lastly, polygenic risk profiles were constructed from the cumulative effect of variants within several genes encoding proteins involved in the apoptosis signalling cascade. Four different models were constructed, (i) an *a priori* model, (ii) an AT risk model (iii) a heterozygous protection model and (iv) a recessive model, differing in how the loci displaying heterozygous protection were scored. A summary of results obtained for the four models are presented in Table 4.12 for ease of comparison.

Table 4.12. A summary of the results from the four apoptosis polygenic profile risk models.

	TGS different between SA diagnostic groups	TGS different between AUS diagnostic groups	TGSs comparable in SA CON and AUS CON	TGSs comparable in SA TEN and AUS TEN	TGS different between [SA+AUS] diagnostic groups	ROC AUC*
A priori model	✗ (p=0.145)	✓ (p=0.037)	✓ (p=0.059)	✓ (p=0.450)	✓ (p=0.010)	0.58 in [SA+AUS]
Allele-dose model	✓ (p=0.031)	✓ (p=0.006)	✓ (p=0.091)	✓ (p=0.572)	✓ (p<0.001)	0.60 in [SA+AUS]
Heterozygous protection model	✗ (p=0.056)	✓ (p<0.001)	✗ (p<0.001)	✓ (p=0.742)	-	0.67 in AUS
Recessive model	✓ (p=0.033)	✓ (p<0.001)	✗ (p=0.001)	✓ (p=0.722)	-	0.60 in SA 0.67 in AUS

* AUC of combined [SA+AUS] group only presented if TGSs were similar in SA CON and AUS CON, as well as in SA TEN and AUS TEN. ✓, desired results; ✗, undesired result; AUS, Australia; CON, asymptomatic control group; SA, South Africa; TEN, clinically diagnosed Achilles tendinopathy group; TGS, Total genotype score.

The recessive model scoring system displayed the best predictive power. Even though the heterozygosity phenomenon of single SNP associations was only observed in AUS, the distribution of TGSs incorporating several loci using the recessive model scoring system was significantly different in both SA and AUS. This finding adds strength to the hypothesis that heterozygosity may be protective, even if in a recessive manner (both risk alleles are required to increase risk of AT) and should be further explored.

Two of the polygenic risk models that were constructed incorporated the heterozygous protection phenomenon: the heterozygous protection model and the recessive model. The heterozygous protection model was a reasonably good predictor of risk of AT in AUS,[207, 208] however, the distribution was not significantly different in SA, whereas the recessive model TGS distribution was significantly different in both SA and AUS. Therefore of the two models that incorporate heterozygous protection into the scoring system, the recessive model may have a greater discriminating power, since TGS distributions were different between CON and TEN in both SA and AUS.

Models which did not incorporate heterozygous protection, namely the *a priori* and allele-dose models, were also evaluated using a more conservative, additive, scoring system; the heterozygous genotype always being assigned 1 on the 0 - 2 risk scale. The distributions of TGSs were similar in SA and AUS for the *a priori* model, however, the model was a weak predictor of risk of AT, with an AUC of 0.58. This finding is to be expected as the scoring in the *a priori* model was assigned purely based on published functional studies and did not take into consideration the effects of genotype associations with risk of AT as observed in this study. The allele-dose model took into account the risk genotype as determined from SNP associations while still assigning the conservative heterozygous genotype a score of 1. The distribution of TGSs were similar in SA and AUS for the allele-dose model and the model had greater discriminating power to predict risk of AT,

with an AUC of 0.60, comparable with published genetic risk models of other multifactorial conditions. [207, 208] Still higher AUCs were achieved with the recessive model (AUS AUC=0.67 and SA AUC=0.60) and we therefore hypothesise that the recessive model scoring system may be the appropriate apoptosis model.

The AUS CON group was not in HWE for all the SNPs that were significantly associated with risk of AT. HWE evaluates the relationship between allele and genotype frequencies at a locus. Genotypes out of HWE may be an indication of genotyping errors or small sample size or that a group may be subject to selection bias, population stratification or selective survivorship among genotypes.[247] To validate our findings, genotyping analysis was repeated in a subset of samples and consistent results were obtained. In all PCR reactions, several negative and positive control DNA samples were included on each plate and all plates passed this quality control measure, ruling out genotyping errors as a confounder. Indeed the sample size was small for a genetic association study, but power evaluation suggested that the SA and AUS sample sizes were large enough to detect significance. The CON and TEN individuals in this study had to meet strict criteria for inclusion and therefore represent a highly selective group of individuals.[248] It is therefore proposed that the deviation from HWE as noted in the CON participants is a reflection of the apparent selection of the protective heterozygous genotypes.[249]

In conclusion, this genetic association study provides an additional level of evidence for the involvement of the apoptosis signalling cascade in AT. This study also provides evidence to suggest that even if single SNP associations are only observed in individuals representative of one population, investigating the cumulative effect of multiple loci can be similar in different populations. Additionally, this study presented a novel heterozygosity phenomenon suggesting that heterozygosity is more favourable at specific

loci *CASP8* -652 6N del, *CASP8* Asp302His and *NOS2* -1026C>A and this interesting finding should be further explored for validation. The associations observed in this study should be further investigated in larger independent populations to highlight the biological significance of the apoptosis signalling cascade in the pathophysiology AT.

Chapter 5

AN INFLAMMATORY POLYGENIC PROFILE TO DETERMINE RISK OF DEVELOPING ACHILLES TENDINOPATHY

AN INFLAMMATORY POLYGENIC PROFILE TO DETERMINE RISK OF DEVELOPING ACHILLES TENDINOPATHY

5.1. **INTRODUCTION**

Achilles tendinopathy is a multifactorial condition resulting from the complex interactions of several environmental and intrinsic risk factors, including genetic factors (Section 1.2.3). Furthermore, risk of AT is regarded as polygenic and is most likely the result of the cumulative effect of several loci in biologically significant pathways.[90, 250] Several genetic risk factors have been identified in previous published studies and in this dissertation and are summarised in Table 5.1.

AT is a debilitating painful chronic condition often requiring early retirement from sport or change of occupation. Treatment for recalcitrant AT have unsatisfactory success rates and can be a lengthy, taxing process.[251] The lifetime prevalence of AT has been reported to be as high as 52% in runners.[39] Preventing the condition should therefore be the priority. In order for this to be achieved effectively, a risk model is required to identify high risk individuals so that targeted preventative strategies can be prescribed and applied more effectively.[252]

Williams and Folland, (2008) [207] have investigated the concept of a polygenic profile to determine elite human performance and the polygenic profile approach has also been applied in clinical medicine to predict the risk of cardiovascular events.[253] Kathiresan *et al.*, (2008) [253] evaluated the cumulative effect of nine validated SNPs that were associated with

modulation in levels of LDL or HDL cholesterol and showed that this polygenic profile was an independent risk factor for incident cardiovascular disease.

The important factors which would need to be identified to achieve a risk model for AT, at least from the genetic predisposition perspective, would be (i) to identify the key biological sequence variants underpinning risk of developing AT and (ii) to determine how these variants interact. In the two preceding experimental chapters of this dissertation, polygenic profiles of loci within the ECM degradation signalling cascade (Chapter 3) and apoptosis signalling cascade (Chapter 4) were constructed and the discriminating power of the risk models assessed.

The aim of the final study of this dissertation was therefore to evaluate all loci in an all-encompassing inflammatory polygenic risk profile, to determine whether the cumulative effect of all the variants improve the power of the model to predict risk of AT. The area under the ROC curve was therefore used to quantify the ability of TGSs to discriminate between unaffected (CON) and affected (TEN) individuals based on their inflammatory pathway genotype profile.

Table 5.1. Polymorphisms which are associated with Achilles tendinopathy in Caucasian individuals.

Gene	Polymorphism	Association (↓ or ↑ risk of AT)	Population group	Reference
TNC	GT repeat in intron 17	13 and 17 repeats ↓ risk 12 and 14 repeats ↑ risk	SA	[107]
COL5A1	rs12722 (T/C)	CC genotype ↓ risk	SA and AUS	[108, 109]
	rs591058 (T/C)	CC genotype ↑ risk		
MMP3	rs679620 (A/G)	GG genotype and G allele ↑ risk	SA	[110]
	rs650108 (G/A)	AA genotype ↑ risk		
GDF5	rs143383 (T/C)	TT genotype ↑ risk	AUS and [SA+AUS]	[111]
IL-1β	rs16944 (T/C)	modulates risk (TT genotype ↓ risk in SA males[113])	[SA+AUS]	[118]
	rs1143627 (T/C)	modulates risk (C allele ↓ risk in SA males[113])		
IL-1RN	rs2234663 (VNTR)	modulates risk	[SA+AUS]	[118]
IL-6	rs1800795 (G/C)	modulates risk (GG genotype and G allele ↑ risk in AUS[112])	[SA+AUS]	[118]
COX-2	rs20417 (G/C)	GC modulates risk (↑) GG modulates risk (↓)		Chapter 3
PTGER4	rs4495224 (A/C)	CC genotype ↑ risk	AUS	Chapter 3
CASP8	rs3834129 (ins/del)	ins/del genotype ↓ risk del/del genotype ↑ risk	AUS	Chapter 4
	rs1045485 (G/C)	GC modulates risk (↓) GG modulates risk (↑)	AUS and [SA+AUS]	
NOS2	rs2779249 (C/A)	CA genotype ↓ risk	AUS	Chapter 4

The accession numbers of single nucleotide polymorphisms (SNPs) were identified from databases hosted by the national centre for biotechnology information (NCBI) (http://www.ncbi.nlm.nih. gov/). Major and minor alleles of SNPs are reported in parenthesis, respectively. Modulation of risk refers to modification of risk when analysed in combination with other associated variants, from September *et al.*, 2010.[118] Variants highlighted in grey were included in the polygenic profile analysis. AT, Achilles tendinopathy.

5.2. **METHODS**

The ten polymorphisms identified to contribute towards risk of AT (*COL5A1 Bst*UI RFLP, *MMP3* rs679620 A>G, *GDF5* rs143383 T>C, *IL-1β* -31T>C, *IL-1β* -511C>T, *IL-6* -172G>C, *PTGER4* -270kb A>C, *CASP8* 6Ndel, *CASP8* Asp302His, *NOS2* -1026A>C [108-111, 118] (Chapter 3 and Chapter 4)) were used for the construction of an inflammatory risk model (Table 5.2). The TGS was calculated from the individual genotype scores as described in Section 3.2.6 and Section 4.2.6 (recessive model). The recessive model for loci displaying heterozygous protection was chosen as the appropriate scoring system because (i) the recessive model (SA AUC 0.60 and AUS AUC 0.67) had greater discriminating power than the *a priori* model ([SA+AUS] AUC 0.58) and allele-dose model ([SA+AUS] AUC 0.60) and (ii) the distribution of TGSs scored according to the recessive model was significantly different in both SA and AUS while the TGS distribution scored according to the heterozygous protection model was only significantly different in AUS (Section 4.3.2 and Table 4.11).

For loci that did not display heterozygous protection the standard scoring system was applied in which the genotype most closely associated with risk was assigned a score of 2 and the genotype most closely associated with protection was assigned a score of 0. The scoring of each polymorphism is presented in Table 5.2.

Only participants who were successfully genotyped at all ten loci were included for analysis. A total of 87 SA CON and 57 SA TEN participants as well as 88 AUS CON and 39 AUS TEN participants were included for analysis. For each participant, a TGS was calculated from the accumulative risk score at all ten loci, as described by Williams and Folland, (2008).[207] TGSs were calculated by dividing the sum of the individual genotype scores by the maximum score obtainable (in this case 20) and multiplying by 100. A

TGS of 100 represents the complete risk polygenic profile for AT and a TGS of 0 represents the perfect protection polygenic profile.

Table 5.2. Genotype scores of the polymorphisms which are associated with Achilles tendinopathy in Caucasian individuals.

Gene	Polymorphism	risk (2)	neutral (1)	protection (0)
COL5A1	rs12722 (T/C)	TT	TC	CC
MMP3	rs679620 (A/G)	GG	AG	AA
GDF5	rs143383 (T/C)	TT	TC	CC
IL-1β	rs16944 (T/C)	CC	CT	TT
	rs1143627 (T/C)	TT	TC	CC
PTGER4	rs4495224 (A/C)	AA	AC	CC
IL-6	rs1800795 (G/C)	GG	-	GC and CC
CASP8	rs3834129 (ins/del)	del/del	-	ins/del and ins/ins
	rs1045485 (G/C)	GG	-	GC and CC
NOS2	rs2779249 (C/A)	AA	-	CA and CC

The accession numbers of polymorphisms were identified from databases hosted by the national centre for biotechnology information (NCBI) (http://www.ncbi.nlm.nih. gov/). Major and minor alleles of SNPs are reported in parenthesis, respectively. del, deletion allele; ins, insertion allele.

GraphPad Prism Version 5.02 (GraphPad Software, San Diego California, USA) was used for statistical analysis. A D'Agnostino and Pearson's omnibus normality test was used to assess whether data was normally distributed. An unpaired t-test was used to determine whether the distribution of TGSs were different between CON and TEN.

ROC curve analysis [207, 208] was used to determine the power of the loci within the inflammatory pathway to discriminate between cases and controls (Section 3.2.6). ROC curves were also plotted to graphically display the true positive rate (sensitivity) vs the false positive rate (1-specificity) across a range of TGS cut-offs. Sensitivity and specificity are used clinically to select

an optimal cut-off. Sensitivity in this case indicates the frequency of TEN individuals that the test correctly identifies as “at risk” and specificity indicates the frequency of CON individuals that the test correctly identifies as protected. Statistical significance was accepted at $p<0.05$.

5.3. **RESULTS**

The model that was explored was the inflammatory polygenic profile incorporating ten loci within eight genes. Some of these loci were not independently associated with risk of AT, specifically *IL-1β* -511C>T, *IL-1β* -31T>C and *IL-6* -172G>C [118], however they did contribute meaningfully towards the risk model. TGSs of all four diagnostic groups were normally distributed. The TGS of SA TEN (55.4±12.8) was significantly higher than SA CON (47.8±13.3) ($p<0.001$). Similarly, the TGS of AUS TEN (51.5±12.9) was significantly higher than AUS CON (44.4±12.9) ($p=0.005$) (Figure 5.1 A). The distribution of TGS scores were similar in SA CON and AUS CON ($p=0.084$) as well as in SA TEN and AUS TEN ($p=0.145$) and therefore the data was combined for further analysis for statistical power. The TGS of [SA+AUS] TEN (53.9±12.9) was significantly higher than [SA+AUS] CON (46.1±13.2) ($p<0.0001$) (Figure 5.1 B). The inflammatory polygenic profile incorporating all ten loci within the ECM degradation and apoptosis signalling cascades was a significant predictor of risk of AT (AUC=0.66; 95%CI 0.59-0.72; $p<0.0001$) (Figure 5.2) (see Box 1 for explanation of ROC curves).

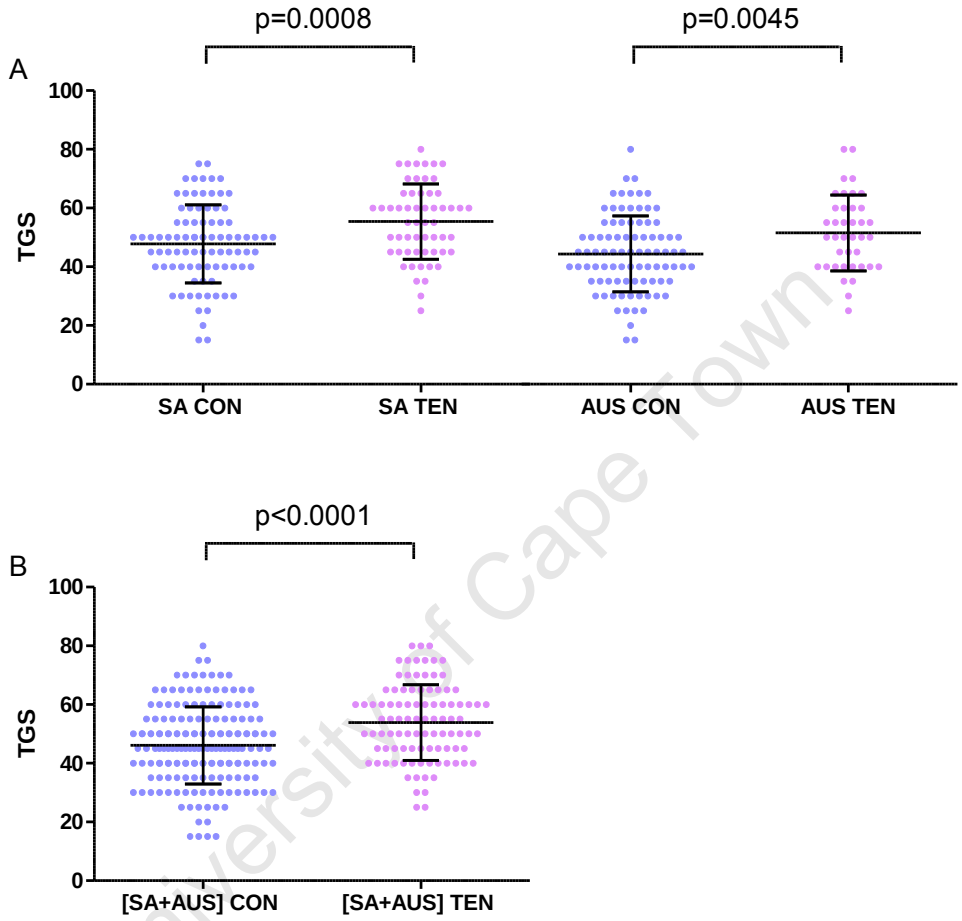

Figure 5.1. The distribution of total genotype scores (TGS) of (A) South African participants in the asymptomatic control (SA CON; n=87) and clinically diagnosed Achilles tendinopathy (SA TEN; n=57) group, as well as Australian control (AUS CON; n=88) and Australian Achilles tendinopathy (AUS TEN; n=39) participants and (B) the TGS distribution in the combined South Africa and Australia groups ([SA+AUS] CON; n=175 and [SA+AUS] TEN; n=96). Significance was accepted at p<0.05.

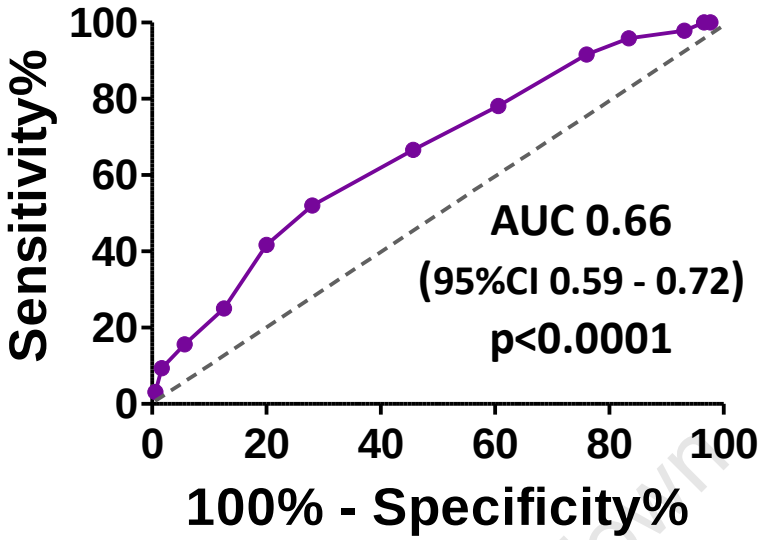

Figure 5.2. Receiver operating characteristic (ROC) curve of the inflammatory polygenic profile (solid line) to determine the true positive (sensitivity) versus true negative (100 - specificity) rate for various cut-offs total genotype score (TGS) in determining risk of Achilles tendinopathy. Dashed line indicates the 0.5 AUC line.

BOX 1: ROC CURVES EXPLAINED:

The area under the ROC curve quantifies the overall ability of the model to discriminate between affected and unaffected individuals, based on genotype risk (TGS), such that a perfect model has an AUC of 1.0 (zero false positives and zero false negatives) and a model with no predictive power has an AUC of 0.5 (no better than a coin toss), indicated by the dashed line in Figure 5.2. In this dissertation, the AUC represents the probability that a randomly selected TEN individual will have a higher TGS than a randomly selected CON. The AUC achieved in the inflammatory risk model was 0.66, meaning that, on average, TEN individuals will have higher TGSs than 66% of CONs. If the model was perfect (AUC=1.0), every TEN individual would have a higher TGS than every CON. Other risk factors should be included into the model to improve the AUC, since AT is a multifactorial condition and not only polygenic.

The distribution of the TGSs revealed three peaks, indicated as a, b and c in Figure 5.3. The left hand peak (Figure 5.3 a) was overrepresented by individuals who were protected from developing AT and the right hand peak (Figure 5.3 c) was overrepresented by individuals who were at risk of developing AT.

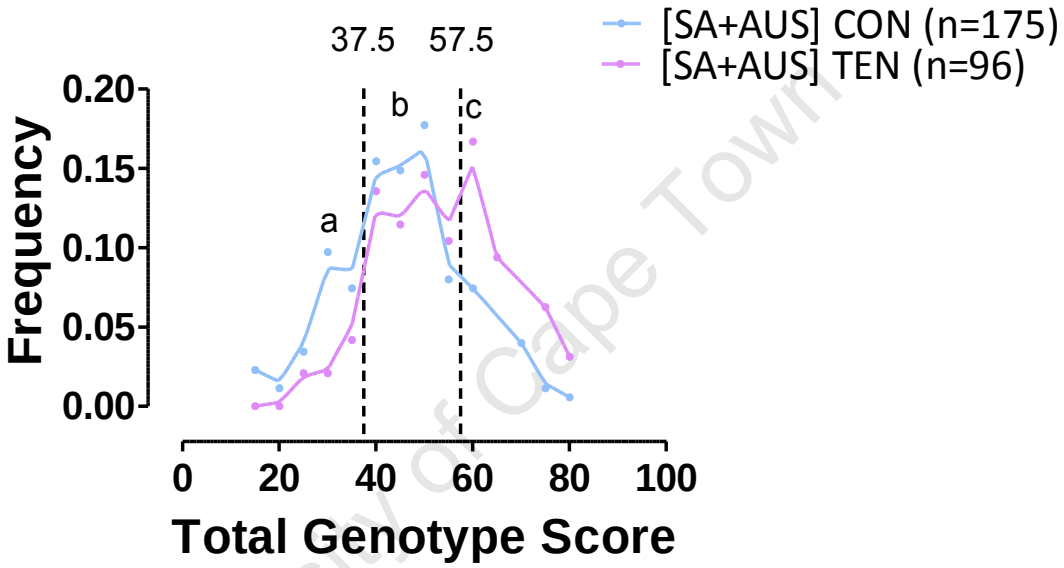

Figure 5.3. Frequency distribution of total genotype score (TGS) in the combined South Africa and Australia control ([SA+AUS] CON) and Achilles tendinopathy ([SA+AUS] TEN) groups. The dashed lines indicates the TGSs which were used as cut-offs due to the presence of three peaks in the distribution (a, b and c).

Conventionally, the cut-off which maximizes sensitivity and sensitivity (Box 2) is chosen as the best cut-off to discriminate between diagnostic groups, in a model that has two peaks in the score distribution (Figure 5.4). Using this method, the best TGS cut-off of the inflammatory polygenic risk model was 52.5, with a sensitivity of 52.1% and a specificity of 72.0% (Table 5.3).

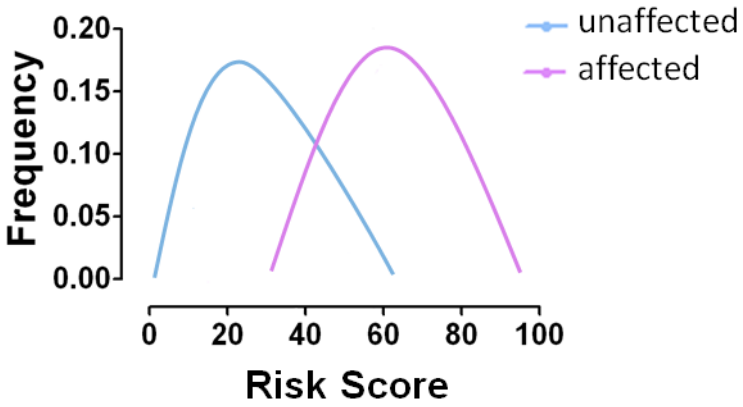

Figure 5.4. Typical (hypothetical) distributions of scores obtained in affected and unaffected individuals, in which better models will have reduced overlap between the two distributions and a single cut-off score is chosen to minimize false negatives and false positives.

However, the TGS distribution of the inflammatory polygenic profile revealed three peaks (Figure 5.3) and therefore the troughs between peaks were used as cut-offs. These troughs were at 35.5 and 55.2, therefore the closest cut-offs with sensitivity and specificity values (Table 5.3), namely 37.5 and 57.5 respectively, were chosen as the appropriate cut-offs (Figure 5.3).

An individual having a polygenic profile with a TGS of <37.5, was at a 2.9 fold reduced risk of developing AT (Table 5.3). The 37.5 TGS cut-off had a specificity of 24.0%, meaning that only 24.0% of individuals not at risk will be recognized as such, but more importantly this TGS cut-off had a sensitivity of 91.7%, meaning that 8.2% of individuals will be falsely classified as not at risk. Furthermore, an individual with a TGS of >57.5 was at a 2.1 fold increased risk of developing AT (Table 5.3). The 57.5 TGS cut-off had a sensitivity of 41.7%, meaning that 41.7% of individuals at risk of developing AT would be recognized at risk and the cut off had a specificity of 80.0%, meaning that 20.0% of individuals were falsely classified as at risk and (Table 5.3) (see Box 2 for an explanation of sensitivity and specificity).

Table 5.3. Frequencies of the sensitivity and specificity of various total genotype scores (TGS) cut-offs, as determined by receiver operating characteristic (ROC) analysis.

TGF cut-off	Sensitivity (95% CI)	LR not at risk of AT	Specificity (95% CI)	LR at risk of AT
17.5	100.0 (96.2 - 100.0)	-	2.3 (0.6 - 5.7)	1.0
22.5	100.0 (96.2 - 100.0)	-	3.4 (1.3 - 7.3)	1.0
27.5	97.9 (92.7 - 99.8)	3.3	6.9 (3.6 - 11.7)	1.1
32.5	95.8 (89.7 - 98.9)	4.0	16.6 (11.4 - 22.9)	1.2
37.5	91.7 (84.2 - 96.3)	2.9	24.0 (17.9 - 31.0)	1.2
42.5	78.1 (68.5 - 85.9)	1.8	39.4 (32.1 - 47.1)	1.3
47.5	66.7 (56.3 - 76.0)	1.6	54.3 (46.6 - 61.8)	1.5
52.5	52.1 (41.6 - 62.4)	1.5	72.0 (64.7 - 78.5)	1.9
57.5	41.7 (31.7 - 52.2)	1.4	80.0 (73.3 - 85.7)	2.1
62.5	25.0 (16.7 - 34.9)	1.2	87.4 (81.6 - 92.0)	2.0
67.5	15.6 (9.0 - 24.5)	1.1	94.3 (89.7 - 97.2)	2.7
72.5	9.4 (4.4 - 17.1)	1.1	98.3 (95.1 - 99.7)	5.5
77.5	3.1 (0.6 - 8.9)	1.0	99.4 (96.9 – 100.0)	5.5

Likelihood ratios (LR) of being protected for individuals possessing a “not at-risk” polygenic profile and LR of being at risk of AT for individuals possessing an “at-risk” polygenic profile, are presented respectively. The highlighted rows indicate the cut-off as determined by the three peaks in the TGS distribution.

BOX 2: SENSITIVITY AND SPECIFICITY EXPLAINED:

ROC curves are used to decide the appropriate cut-off of a model to discriminate between affected and unaffected individuals. Choosing the appropriate TGS cut-off would be easy if all the TEN values were higher than CON values. However, the distributions overlap (Figure 5.3). If the cut-off is high, unaffected individuals will not be mistakenly diagnosed as at risk (high specificity), but some affected individuals may be missed (low sensitivity). Conversely, if the cut-off is low, all affected individuals will be identified (high sensitivity), but unaffected individuals will also be falsely diagnosed as at risk (low specificity). Finding the correct cut-off is a tradeoff between sensitivity and specificity. For the data in this study, an unusual case of three peaks in TGS distribution were observed and we therefore decided to choose two cut-offs. While the model is uninformative for TGSs between the two cut-offs, the <37.5 TGS captures as many CONs as possible, while maintaining a low false negative rate in TENs (high specificity) and the >57.5 TGS captures as many TENs as possible, while maintaining a low false positive rate in CONs (high sensitivity).

5.4. **DISCUSSION**

This study demonstrated that an inflammatory polygenic profile to determine risk of developing AT is feasible. The TGS is an effective method to identify at-risk individuals before they develop AT based on their genotype profile at ten loci.

The TGSs of the TEN groups were significantly higher than the TGSs of the CON groups in both SA and AUS and the combined [SA+AUS] group. The distribution of TGSs in the combined [SA+AUS] CON and [SA+AUS] TEN participants revealed that individuals having a polygenic profile with a TGS of <37.5 were at a 2.9 fold decreased risk of developing AT while those individuals with a TGS of >57.5 were at a 2.1 fold increased risk of developing AT. We hypothesise that for individuals with a TGS of between 37.5 and 57.5, extrinsic risk factors may play a larger role in determining whether an individual may develop AT, while at the extremes of the spectrum, the individual's genetic profile may play a larger role.

The inflammatory model which incorporated all ten loci within the inflammatory pathway had greater discriminating accuracy in determining risk of AT than the ECM degradation model (Chapter 3) and the apoptosis model (Chapter 4). The inflammatory model had greater power (AUC=0.66) than the ECM degradation model (AUC=0.62) (Section 3.3.3). This model was also more effective in discriminating risk than the apoptosis (recessive) model because, the distribution of the apoptosis (recessive) model was not similar in SA and AUS (Section 4.3.3.2), whereas the distribution for this inflammatory risk model was comparable in SA and AUS. The strength of this inflammatory model was that the distributions of TGS were comparable in the two populations and a relatively high discriminating accuracy for a genetic risk model was achieved.[207, 208, 254] It has been proposed that the identification of disease susceptibility across different populations is possibly

of more value than the identification of population-specific effects as they potentially highlight significant biological pathways in the disease pathogenesis.[255] Collectively, these results further implicate the inflammatory pathway as one of the biological pathways involved in the development of AT.

This study also suggests that the more biomarkers incorporated into the design, the greater the power to capture and predict risk, as would be expected in a polygenic, multifactorial condition. Exactly how many biomarkers are required to achieve a feasible predictive rate for diagnostic purposes depends on the cost of intervention and the consequence of injury.

However, this model is still far from achieving the scores necessary for clinical application. At the cut-off of 37.5, the sensitivity was 91.7%, which means that 8.3% of individuals who are at risk, preventative strategies would not be prescribed and at the cut-off of 57.5 the specificity was 80.0%, meaning that 20.0% of individuals who are not at risk of developing AT, would unnecessarily be exposed to these preventative strategies. To the authors knowledge, this is the first attempt at a genetic risk model for any sporting injury and subsequent models should strive to attain higher sensitivity and specificity scores, so that the model could be used as an efficient screening tool for individuals at risk of AT.

Several new polymorphisms are likely to be associated with risk of AT in the near future and these would need to be incorporated to determine whether they improve the discriminating power of the model. Furthermore, models should also be designed which take into account the interaction of other nongenetic risk factors, such as age, gender and adiposity, to more accurately discriminate between “at risk” and “not at risk” individuals. The models constructed in this study did unfortunately not take into account the differences in the country of birth of the two populations investigated or the

differences in the gender distribution between the individuals studied. This is a limitation in the model presented and more sophisticated statistical models should be explored to account for potential confounders. In addition, a more effective model to predict risk should also evaluate differences in the weighting of each individual genotype on their relative effective risk on developing AT, perhaps by taking into account the OR of the association. However, prospective studies would also need to be conducted to determine the true population effect size of the polygenic risk profile and this should be more reliable in discriminating between “at risk” and “not at risk” individuals as such studies have a more reliable confidence level.

In conclusion, this study has identified that the polygenic profile incorporating ten loci within the inflammatory pathway was a significant discriminator between CON and TEN individuals in both SA and AUS and the combined [SA+AUS] group. This study also found the discriminating ability of the polygenic profile increased by the addition of more genes. Further research is however required into evaluating the risk factors which can be added to improving the model, before the polygenic risk profile of AT can be used as a screening tool.

Chapter 6

CONCLUSIONS

CONCLUSIONS

Achilles tendinopathy is a multifactorial condition and therefore the development of AT is influenced by the complex interactions of several intrinsic and extrinsic risk factors (Section 1.2.3).[22, 85] AT is characterised primarily as a degenerative condition of tendon ECM thought to be triggered by excessive or repetitive mechanical loading.[43] Furthermore, clinical and experimental evidence also supports the involvement of the inflammatory pathway in the development of AT (Section 2).[14, 15, 69]

The primary aims of this dissertation were therefore to use a pathway-based approach to explore genetic variants within genes encoding proteins functioning in the ECM degradation and apoptosis signalling cascades, downstream of IL-1β and IL-6, in the predisposition to AT and to evaluate the power of these loci to predict risk of AT in various polygenic risk models.

6.1. NOVEL FINDINGS OF THIS DISSERTATION

This dissertation identified the following independent associations in Australia, but not in South Africa:

- The CC genotype of the proposed functional A>C polymorphism within the gene desert flanking *PTGER4* gene, was associated with decreased risk of developing AT.
- The functional *CASP8* -652 del/del and proposed functional *CASP8* Asp302His GG genotypes were associated with increased risk of Achilles tendinopathy.

- The functional *NOS2* -1026 CA, *CASP8* ins/del and *CASP8* Asp302His GC heterozygous genotypes were associated with decreased risk of developing Achilles tendinopathy.

The heterozygous protection observed at the loci within genes encoding proteins functioning in the apoptosis signalling cascade (*CASP8* -652 del/ins, *CASP8* Asp302His GC and *NOS2* -1026 CA) is a novel finding of this dissertation as heterozygous protection has not previously been observed in susceptibility to soft tissue injury. The phenomenon is however common in infectious disease susceptibility [242-246] and since similar inflammatory pathways are involved, the finding is noteworthy. Loci within the genes that encode apoptotic mediators suggest a "Goldilocks effect"; that both too much and too little functional protein may increase risk of disease, while heterozygous co-dominantly expressed alleles allow for diversity in response to stresses and the optimal dose of apoptosis mediator (Figure 6.1).

This hypothesis should however, be considered with caution because if the minor allele frequency driving the protection is low, statistical significance may not be achieved in homozygotes. However, the allele frequencies obtained at the loci displaying heterozygous protection in this study, ranged from 16.4 – 34.8, which is not considered rare. These associations should be repeated in larger studies to verify the "Goldilocks" hypothesis and clarify whether it is truly a heterozygous advantage being observed, especially because the apoptosis polygenic profile with a recessive scoring system (discussed later) was the best model to determine risk of Achilles tendinopathy. Further functional studies may also help to elucidate the effect of these polymorphisms on the expression and functionality of apoptotic mediators in tenocytes in response to mechanical loading.

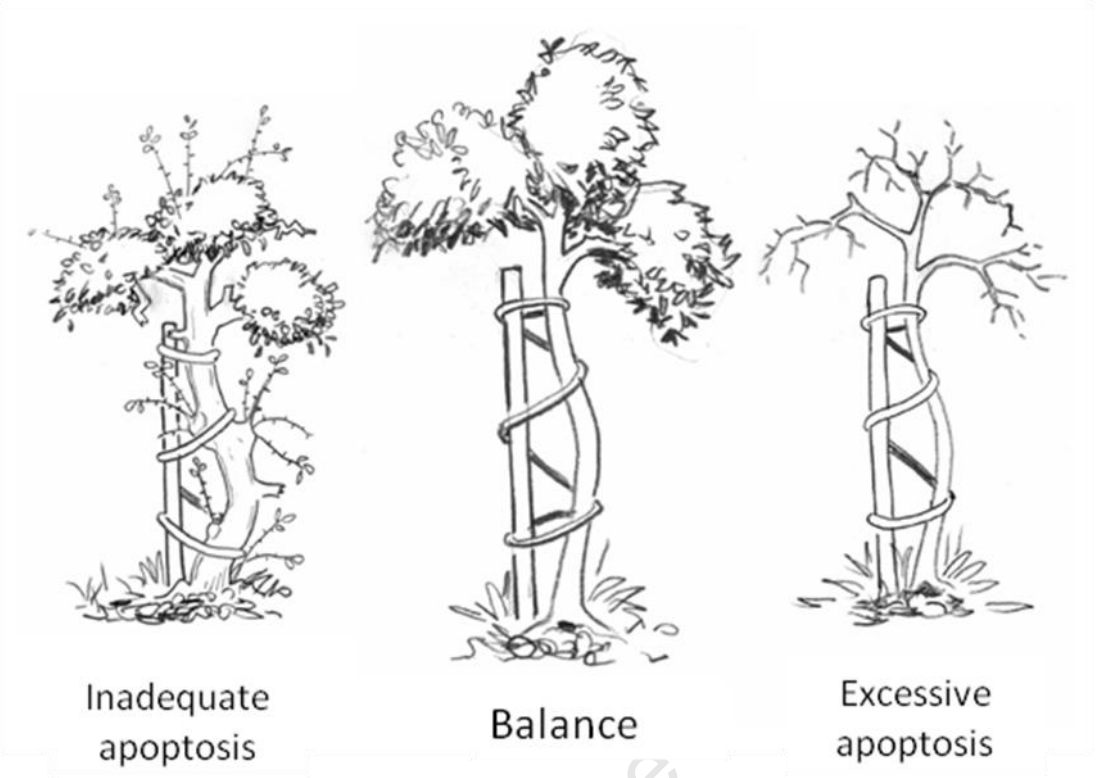

Figure 6.1. An illustration of the "Goldilocks effect" pertaining to apoptosis, as modified from the original MMP/TIMP balance illustration by Pasternak *et al.*, (2009).[142] Both too little apoptosis (left) and too much apoptosis (right) is detrimental to the tendon homeostasis. However, when the amount of apoptosis is "just right", the tendon homeostasis is in balance.

Another novel finding of this dissertation was the identification of polygenic profiles, incorporating variants of the ECM degradation signalling cascade, apoptosis signalling cascade and the combined inflammatory pathways, which were able to predict risk of Achilles tendinopathy (Figure 6.2). The total genotype score as a means to quantify the accumulative risk of several genetic risk factors in a polygenic profile has previously been described as an effective tool to discriminate between unaffected and clinically affected individuals.[256] This dissertation identified that while the pathway-specific polygenic profiles were statistically significant, they were limited in discriminating power and therefore were not suitable for clinical application. However, when the combined inflammatory polygenic profile was explored, greater discriminating power was achieved than the respective single

signalling cascades, therefore highlighting that the more loci incorporated into the polygenic profile, the greater the power to predict risk of Achilles tendinopathy. A more powerful polygenic profile is more likely to be clinically relevant when added into a model with other risk factors.

A strength of this dissertation was therefore that the inflammatory polygenic profile was comparable in both South Africa and Australia despite the independent SNP associations only being observed in Australia. It has been proposed that the identification of disease susceptibility across different populations is possibly of more value than the identification of population-specific effects as they highlight significant biological pathways in the disease pathogenesis.[255] Furthermore, this finding adds strength to the rationale of conducting a pathway-based genetic association study, investigating several loci within genes in a pathway, since single SNP associations could miss loci within genes encoding proteins in critical underlying pathways. Another strength of this dissertation was that the variants which were explored in these studies were biologically relevant; only functional or proposed functional variants within hypothesis-driven candidate genes, which were furthermore associated with other multifactorial conditions, were chosen for investigation.

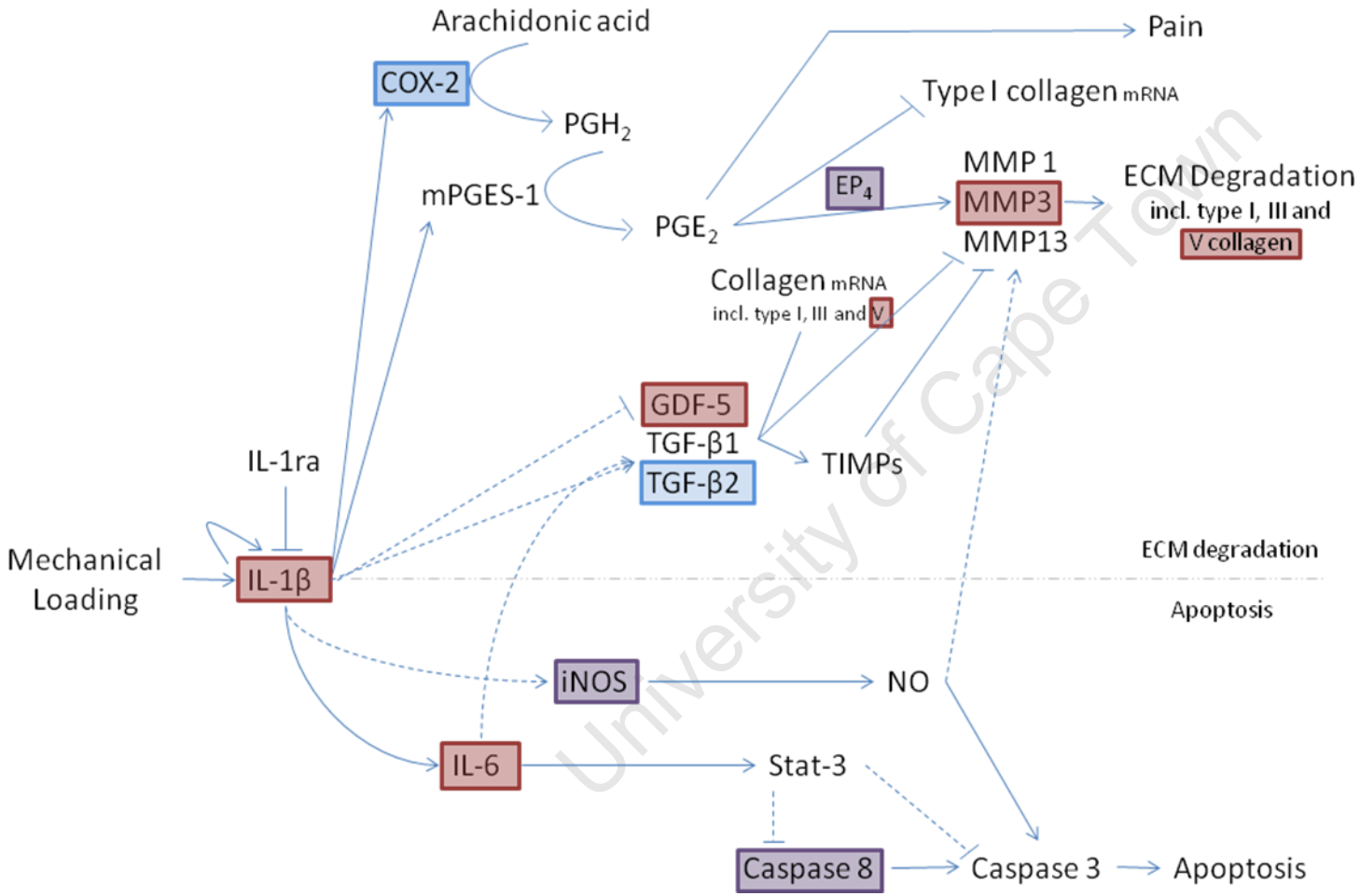

Figure 6.2. Schematic representation of the IL-1β and IL-6 signalling cascades influencing matrix degradation and apoptosis, modified from Figure 2.7.[14, 15, 81, 133, 138-148, 161, 162, 173] Proteins involved in the 2 pathways have been separated, with IL-1β initiating both cascades. Blue box: Investigated in this dissertation. Red box: Included in the polygenic profile. Purple box: investigated in this dissertation and included in the polygenic profile. Lines without arrow heads or inhibitory heads: upregulation or inhibition of the effector protein subtypes – see Figure 2.7 for details.

The inflammatory polygenic risk model explored in this dissertation had an interesting distribution. Such models are designed for clinical application of "biomarker scores" and typically, one cut-off is chosen, to capture as many affected individuals above that threshold and as many unaffected individuals below that threshold as possible (Figure 6.3 A), however the sensitivity (52.1%) and specificity (72.0%) scores obtained for this model, using this method, were not very informative. The TGS distribution of the inflammatory polygenic profile revealed three peaks (Figure 6.3 A/B). This interesting finding suggests that an individual's genetic profile may play a large role in determining risk of Achilles tendinopathy at the extremes of the TGS distribution, but individuals with middle range TGSs (between 37.5 and 57.5 – Section 5.3), other intrinsic and extrinsic risk factors may have a larger role in determining their risk. Therefore, the three peak TGS distribution lead to the unconventional choice of two TGS cut-offs (Figure 6.3 B), to improve sensitivity and specificity scores, because other factors may influence risk between the cut-offs. The <37.5 TGS captured as many CONs as possible (sensitivity of 24.0%), while maintaining a high specificity to limit false negatives (specificity was 91.8%). Similarly, the >57.5 TGS captured as many TENs as possible (sensitivity was 41.7%), while maintaining a low false positive rate in CONs (specificity was 80.0%). The limitation of the two cut-off system is that the model is uninformative for TGSs between the two cut-offs. The addition of other risk factors into the model may lead to a clearer two-peak risk score distribution so that one cut-off can be chosen and the model is informative at all points on the spectrum (Figure 6.3 C). One key research question which remains is to determine the minimum number of biomarkers, genetic and nongenetic, which are required to effectively capture all individuals (or at least the vast majority) who are at risk of developing Achilles tendinopathy.

Collectively, the results of this dissertation in the context of other published data implicate the inflammatory pathway as one of the biological pathways

involved in the development of Achilles tendinopathy. Additionally, this dissertation has highlighted that risk of Achilles tendinopathy is polygenic and several loci interact together to modulate the Achilles tendinopathy phenotype.

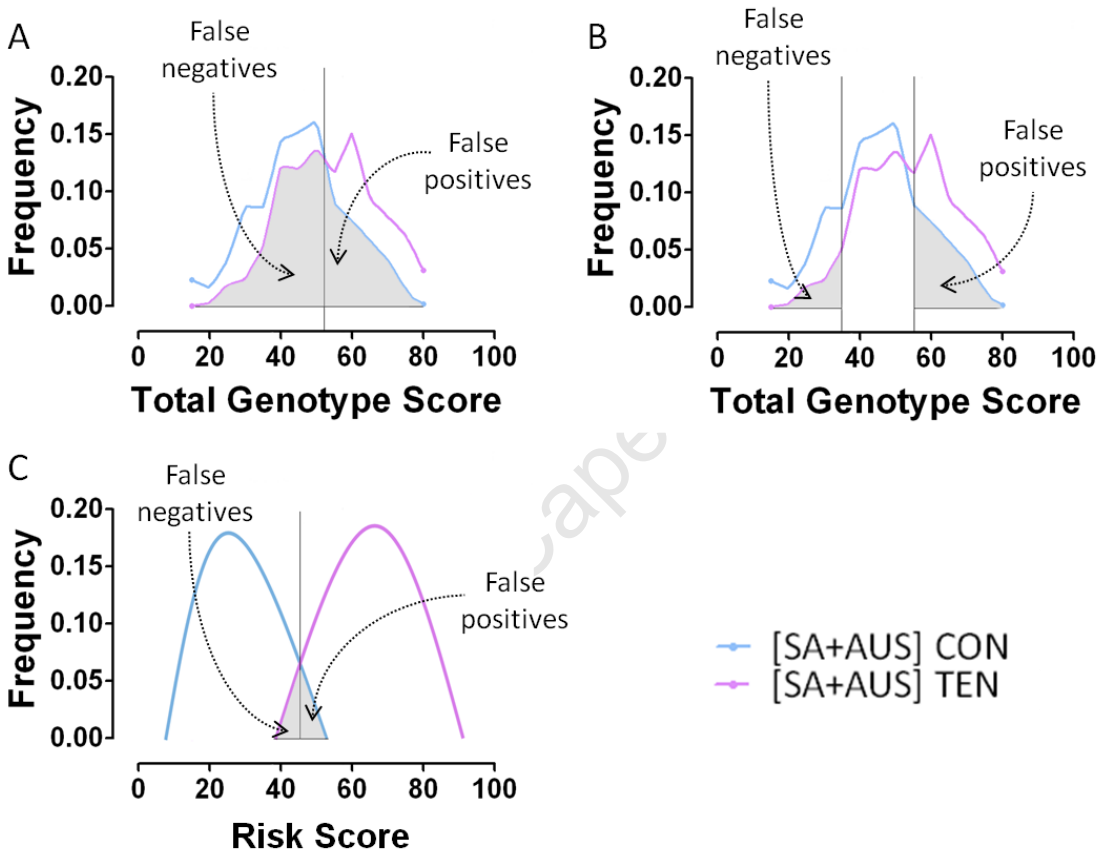

Figure 6.3. Total genotype score (TGS) distributions obtained for the inflammatory polygenic risk model in Section 5.3 (A and B) and the hypothesised score distribution for a risk model incorporating a polygenic profile as well as intrinsic and extrinsic risk factors (C). False positives and false negatives are indicated as shaded regions for (A) the typical one cut-off systems (at 52.5), with lower sensitivity and specificity scores, as indicated by larger shaded regions, (B) the unconventional two cut-off system (at 37.5 and 57.5), as used in this dissertation, to limit false positives and negatives and (C) a model with better sensitivity and specificity scores, due to incorporation of intrinsic and extrinsic risk factors into the model.

6.2. **LIMITATIONS**

There are limitations to the studies within this dissertation. Noteably, while South African participants were required to fill out a physical activity questionnaire and physical activity in the SA CON and SA TEN groups were similar, the physical activity of the AUS TEN and AUS CON participants were not documented. Nevertheless, care was taken to recruit physically active individuals in both South Africa and Australia and repeatable results were obtained between the two populations.

Another limitation is that the TEN participants were significantly heavier at recruitment than CON participants and it is acknowledged that there is strong evidence for adiposity as a contributing factor to risk of Achilles tendinopathy.[136] However, when weight was adjusted for age at recruitment and/or gender, the weight between diagnostic groups was no longer significant. There were also no genotype effects on weight and although accurate weight measurements were not available for TEN participants at time of injury, the TEN participants had reported gaining weight after their injury.

We acknowledge that this dissertation has only investigated a subset of the genes and sequence variants that may play a role in the inflammatory pathway and therefore more loci within these genes, as well as other genes encoding proteins that have been implicated in Achilles tendinopathy, should be explored and potentially included into the polygenic profile to improve the power of the risk model.

The polygenic risk models within this dissertation also did not take into account potential differences in the functional effect of the variants by weighting genotypes, or the differences in gender, age and country of birth between diagnostic groups. Future models could weight the genotype scoring according to the effect size of the genotype association (determined by the

OR) and should aim to use more sophisticated statistical models in larger sample populations so that the predictive power of the polygenic profile can be determined accurately, independent of potential confounders.

Lastly, the AUS CON group was not in HWE at the *CASP8* -652 6N del, *CASP8* Asp302His and *NOS2* -1026C>A loci. Precautions were taken to (i) limit the effects of population stratification by matching diagnostic groups for country of birth and (ii) assure correct, repeatable genotyping by including control samples. Therefore we proposed that the deviation from HWE was a reflection of the apparent selection of the protective heterozygous genotypes in the highly selective group.[248, 249] However, the associations within these studies should be repeated in a larger sample size to validate the findings.

6.3. **GENETICS AND TENDINOPATHY**

The genome holds the information for the production of all proteins in the body and therefore it is logical that a person's genetic architecture underpins all biological processes and would therefore also determine their likelihood of developing Achilles tendinopathy. Even though the same genetic code is found throughout the cells of the body, the tenocytes are unique in their ability to respond to mechanical stimuli by altering gene expression of crucial proteins to attain a stronger, homeostatically balanced tendon ECM. The human genome is 99.9% identical between individuals, with the 0.1% difference leading to all the variation seen in the human race, including susceptibility to disease and injury.[90] These small differences at specific loci within genes has the potential to influence the rate of gene expression and functionality of proteins and thus the small differences in genetic sequence determines how an individual responds to mechanical loading and how effectively their tenocytes are able to restore ECM homeostasis.

In addition, immerging research has demonstrated that epigenetics may also have a role in disease susceptibility.[257, 258] Recently, it has been discovered that it is not only the coding regions which are important, but that the noncoding regions of the genome are of great significance and contain regulatory sequences including non-protein coding RNAs. These noncoding RNAs are able to direct the cytosine methylation of DNA and histone modifications; two of the mechanisms of gene expression regulation.[259, 260] Such changes in gene expression, which are not directly caused by changes in the DNA sequence and are reversible, are known as epigenetic changes.[259] Recently it has also been proposed that epigenetics may play a role in injury susceptibility,[90] in light of a DNA methylation site within the *MMP3* gene, a gene which has been associated with Achilles tendinopathy.[110] Abnormal methylation at this site has furthermore been associated with risk of osteoarthritis.[257] To the authors knowledge, there are no published studies which have investigated the role of epigenetics in tendinopathy. However, we hypothesise that, since epigenetic changes are reversible, the ease with which an individual's epigenetic markers are altered could also provide the genome with greater plasticity to respond more effectively to changing cellular environments.[260] Furthermore, we hypothesise that individuals with genomes displaying more plasticity may be better equipped genetically to respond to loading and therefore more protected from developing injury. It is therefore important that future studies explore the sequence variants within noncoding regions. Indeed, in this dissertation, the CC genotype of the A>C polymorphism within the gene desert flanking *PTGER4* gene, which encodes the PGE_2 EP_4 receptor functioning in the ECM degradation pathway (Figure 6.2), was found to decrease the risk of developing Achilles tendinopathy. Thus important biological mechanisms for development of multifactorial diseases such as Achilles tendinopathy may be missed if the impact of polymorphisms within noncoding regions are ignored.

While the degenerative changes observed in tendinopathy are similar in all individuals, their experience of symptoms varies considerably. If this variability in symptoms exists, how much more variability would exist in response to mechanical loading and in the individual progression rate into nonresponsive tendinopathy? It is therefore not only the identification of variants which increase risk which are important, but also understanding (i) the effects of these variants on protein function within a dynamic cellular environment and (ii) the functional relationship of the genome in influencing the individual progression of the clinical condition and response to treatment. Comprehension of the functional consequences of an individual's genetic profile may facilitate the design of targeted therapeutic prescription to either silence or upregulate certain biologically relevant pathways or proteins.

6.4. **CLINICAL SIGNIFICANCE**

The exact aetiology of tendinopathy remains unclear [5] and it should be emphasised that understanding the intricacies of the biological mechanisms underlying the condition is critical for the development of effective biology-based therapeutic interventions for more effective prevention, treatment and rehabilitation strategies for tendon injuries.[99] The application of genetic association studies to identify risk profiles represents an exciting new direction which, confirmed in multiple populations, promises to highlight biologically significant pathways in the disease mechanisms for Achilles tendinopathy.[97] Once the pathways and mechanisms are identified, these genetic risk factors as a polygenic profile, together with other intrinsic and extrinsic risk can be incorporated into a risk model which can be used as a clinical screen to determine risk and prognosis in individuals.

It has been suggested that preventing tendinopathy may be more beneficial given the lengthy and frustrating course of tendinopathy treatment [59, 251] and a risk model seems the most likely way of identifying the "at risk"

individuals so that these individuals can receive preventative strategies. The inflammatory risk model presented in this dissertation was based on a statistical algorithm and only incorporated genetic factors. Improvements to the inflammatory polygenic profile as a risk model should be explored, perhaps by including important nongenetic risk factors as well as non-inflammatory genetic risk factors to achieve the best predictive model statistically. We also suggest using a more sophisticated statistical model which is able to determine the minimum number of risk factors to capture the most affected individuals, so that the model can be applied clinically.

However before risk model can be applied clinically, prospective studies should be conducted to evaluate the risk of the genotypes and determine risk and prognosis together with the collection of, amongst others, training, diet and gender data, to confirm the applicability of the risk model. Furthermore, a consortium study would go a long way in generating a database of polymorphisms in several populations to identify common loci and biological pathways across populations, thereby improving the understanding of injury risk. This multidisciplinary approach would not only allow for the comprehensive examination of genetic, environmental, pathological and clinical variables, but importantly also elucidate how the risk factors interact. This understanding is critical before effective targeted therapeutic strategies can be developed.

While prevention is ideal, for individuals that have already developed the condition, we hypothesise that the polygenic profile can also be incorporated into models to discriminate between responders and nonresponders to certain treatment regimes, before unnecessary time and money is wasted on treatments that will not be effective, as it is very possible that the correct preventative/treatment strategies are being targeted at the wrong patients.[252] A clearer understanding of the disease progression of tendinopathy, specifically the extent to which the inflammatory pathway is involved, may also lead to new treatment strategies aimed at modulating the

matrix degradation and apoptosis processes underlying Achilles tendinopathy. There are several therapeutic strategies that target protein expression within tenocytes which are currently used or being developed to treat Achilles tendinopathy. These include traditionally prescribed NSAIDs, recombinant protein administration and gene therapy and while the details of these strategies are beyond the scope of this dissertation they have been extensively reviewed [94, 151, 152, 261] and will briefly be described.

Oral NSAIDs have been used extensively for decades to treat pain associated with tendon overuse. NSAIDs target COX-2 within the inflammatory pathway to reduce the production of PGE_2 and therefore pain. Overall, the evidence suggests both oral and local topical NSAIDs are effective in relieving the pain associated with tendinopathy in the short term (7–14 days), especially regarding the shoulder (reviewed in [151, 152]). The effectiveness of NSIADs to alleviate symptoms of Achilles tendinopathy is however, far less.[152] In the long term, there is no evidence that NSAIDs are effective for treating Achilles tendinopathy, whereas the risk of adverse effects, such as gastrointestinal, cardiovascular and renal complications, rises.[151, 152] NSAIDs may therefore be an effective short term analgesic, but does not aid healing of tendinopathy.[262] This is possibly because COX-2 inhibitors not only block pain pathways, but also block the signalling pathways critical for attaining ECM homeostasis and shift the equilibrium toward the leukotrine pathway,[156] which may exacerbate inflammatory and degenerative processes.

Another treatment option which has been explored to promote tendon healing, is the administration of recombinant proteins. The recombinant growth factors that are currently administered to promote tendon repair include IGF-I, TGF-β, GDF-5, PDGF and VEGF.[263] However, pharmacological doses are often different to physiological doses and more research is needed to determine the effects and effectiveness of exogenous administration of growth factors. Furthermore, the short transient residence

time of these recombinant proteins does little to control proinflammatory catabolic cytokines that continue to degrade the ECM.[264] Gene therapy would allow for an extended period of protein synthesis by introducing a gene or coding sequences that prolong synthesis or increases synthesis of the target gene.[261]

Gene therapy relies on a delivery mechanism to incorporate a coding sequence into the recipient's target tissue cells. Viral vectors are most commonly and successfully used for this task.[265, 266] This technique has already been explored in rheumatoid arthritis and osteoarthritis to modulate the inflammatory response. The delivery of the coding sequence of IL-1ra to compete with pro-inflammatory cytokine IL-1β [267-270] and delivery of TNF-α soluble receptor [267] to bind TNF-α has successfully been demonstrated to reduce the amount of biologically active protein.

Recently, the development of novel biotechnological tools such as synthetic genetic interference has led to a whole new field that can be explored for therapeutic intervention of multifactorial conditions in combination with gene therapy. Posttranslational knockdown of gene expression is more desirable than delivery of a protein coding sequence because of the inherent stringency of silencing RNA (siRNA) and it moves the level of control upstream to noncompetitive gene silencing. There is already promising work being done in tenocytes with regard to gene silencing. The effect of silencing of the *TGFB1* gene has been explored by delivery of a vector containing complementary miRNA posttranslationally diminishing TGF-β1 mRNA, to minimize adhesions after tendon rupture/surgery.[271] Indeed, TGF-β1 was significantly downregulated, as was collagen type III.[271] IL-1 translation has also been knocked down in chondrocytes using siRNA techniques, leading to decreased IL-1 and MMP-13 levels.[263] The techniques used in these studies could easily be applied to the treatment of tendinopathy once the critical genes and biological mechanisms are identified.

Inflammatory mediators modulate the inflammatory status of the tendon milieu and thereby the proteins which result in ECM degradation and apoptosis, making the inflammatory pathway an ideal candidate to be targeted. Some of the therapies which could be administered include (i) the local administration of anticytokine antibodies, soluble cytokine receptors or cytokine antagonists, (ii) introduction of genes encoding the antagonist of these inflammatory mediators and (iii) knockdown of overexpressed cytokine genes using siRNA gene therapy. All three strategies would aim to decrease the biological effect of the disease causing cytokines and thus potentially reduce the ECM degradation and apoptosis, preventing pathological tendon degeneration. In addition, many of the growth factors and catabolic cytokines converge on similar pathways and it has been suggested that targeting key components in common signalling pathways, instead of individual growth factors may be more effective.[272]

Also it is important to remember that these pathways which are being altered by treatment strategies are part of the normal healing and adaptation strategies in tenocytes and therefore the most effective treatments will most probably be combination treatments so that one aspect of tendon homeostasis (ECM degradation, collagen synthesis, cell proliferation and apoptosis, being examples from this dissertation) is always balanced with its counter-process. In addition, an individual's genetic architecture may highlight the factors which are lacking and need to be targeted for that individual. The Achilles tendon is also conveniently situated superficially allowing easy access for the administration of treatment strategies in cases of tendon injury. Thus treatment can be administered locally to minimise negative systemic effects that the treatment may have, especially if the levels of growth factors and catabolic cytokines, which bind to receptors on all body cell types, are being altered.

6.5. **A ROLE FOR INFLAMMATION IN ACHILLES TENDINOPATHY: A HYPOTHESIS**

Tendinopathy is considered to be a degenerative condition occurring in the absence of inflammation. However, there is increasing evidence to suggest that microtrauma in response to loading may trigger infiltration of inflammatory cells into the tendon tissue.[43, 55, 127-129, 273] Mechanical loading also directly stimulates gene expression in tenocytes and instigates cytokine mediated ECM remodelling (Figure 6.4).[60, 62] Furthermore, a bout of mechanical loading causes both cell death, to remove damaged tenocytes, and cell proliferation, to restore tenocyte numbers (Figure 6.4).[178] We hypothesise that inflammatory cells release cytokines to amplify the tenocytes' endogenous inflammatory response, aiding the tenocytes in maintaining ECM homeostasis (Figure 6.4).[14] It should once again be emphasised that the inflammatory response is not inherently a negative process, but forms part of the tenocytes' natural healing and adaptive response to mechanical loading. Furthermore, the presence of inflammatory mediators are not "on" or "off", but rather modulated in intensity depending on the tenocyte environment.[274] If loading is excessive or there is not sufficient time between loading sessions to allow for adequate healing and adaptation, these inflammatory signals are not effectively regulated and abnormalities in the tendon tissue accumulate eventually resulting in pathological collagen disorganisation.[59, 61] We hypothesise that by this stage in the progression of the disease that inflammation has subsided, being long after the initial trigger for inflammation and this could explain the absence of inflammatory cell presence in late tendinopathy.

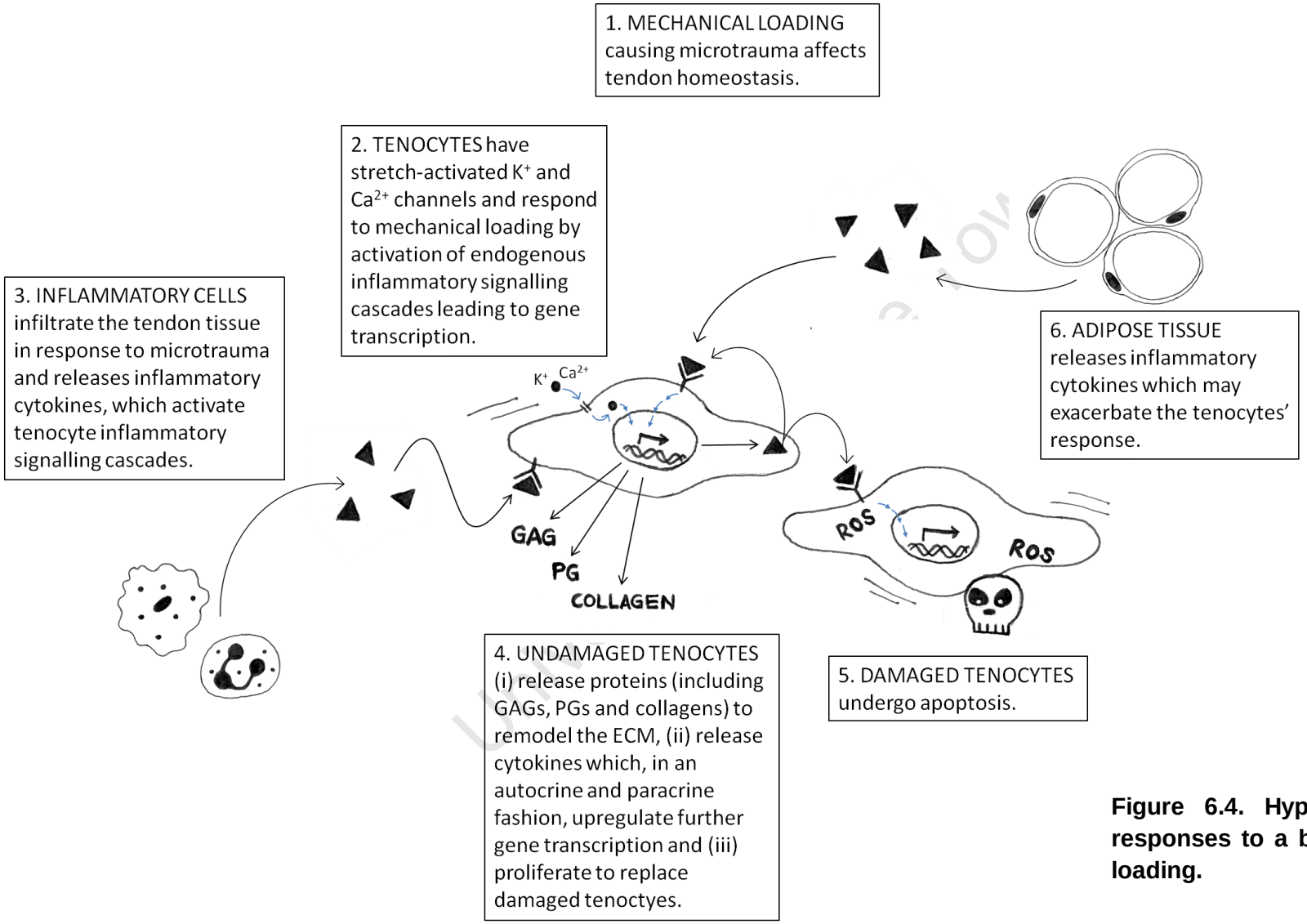

Figure 6.4. Hypothesised cellular responses to a bout of mechanical loading.

Furthermore, Scott *et al.*, (2005) [275] suggested that tenocyte apoptosis is only increased in end stage tendinopathy possibly as a result of a hypoxic gradient originating at the tendon lesion due to compromised blood supply at the affected region.[62] While tenocytes are able to cope with loading-induced hypoxia, due to a developed anaerobic capacity,[24] prolonged periods of hypoxia due to collagen disorganisation and matrix expansion may trigger apoptosis.[275] This would account for the regions of cell death observed in tendinopathy which are bordered by cell proliferation.[59, 276] Hypoxia may also account for the altered gene transcription in tendon cells, perhaps in the presence of PGE_2, resulting in matrix abnormalities such as calcification and adipogenesis,[150, 277] which accompanies apoptosis in late tendinopathy.[44]

In addition, it is tempting to speculate that the inflammatory response in tenocytes can be exacerbated by inflammatory adipose tissue (Figure 6.4), especially since Kager's fat pad is situated in such close proximity to the Achilles tendon.[27]

Further studies are needed to explore these hypotheses, however they are built on the premise of the involvement of an inflammatory response in tendinopathy and this dissertation has added novel evidence for a genetic predisposition to Achilles tendinopathy in genes of the ECM degradation and apoptosis signalling cascades.

6.6. **FINAL REMARKS**

In conclusion, this dissertation has added an additional level of evidence suggesting the involvement of the inflammatory pathway, including the ECM degradation and apoptosis signalling cascades, in the development of Achilles tendinopathy. Moreover, this dissertation has shown that the polygenic risk models of a single signalling cascade does not have sufficient power to discriminate between affected and unaffected individuals and that the polygenic profile incorporating several biomarkers in the collective inflammatory pathway is a more effective discriminator with greater clinical relevance.

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Appendix A

PARTICIPANT CHARACTERISTICS

A.1. Inclusion and exclusion criteria

Inclusion Criteria

A clinician reviewed the clinical diagnosis for every participant (Appendix B – Clinical criteria assessment form), as previously described.[107, 108] Briefly, participants had to have experienced gradual, progressive pain in the region of the Achilles tendon for more than 6 months (n=93)*. Furthermore, at least one other of the following criteria had to be fulfilled regarding the Achilles tendon region: (1) early morning pain (n=32)*, (2) early morning stiffness (n=53)*, (3) history of swelling (n=36)*, (4) tenderness to palpation (n=74)*, (5) palpable nodular thickening (n=28)* and (6) movement of the affected area with plantar dorsiflextion (positive shift test) (n=22)*.

*n refers to the number of SA TEN participants fulfilling the criteria

Exclusion Criteria

Any participants with known connective tissue disease or systemic disease known to be linked to tendon pathology were excluded from this study.[278] Such conditions include Ehlers–Danlos syndrome, benign hypermobility joint syndrome, rheumatoid arthritis, systemic lupus erythematosus, hyperparathyroidism, renal insufficiency, diabetes mellitus and familial hypercholesterolaemia. [278] Furthermore individuals that made use of fluoroquinolone antibiotics or corticosteroid injections prior to injury were also excluded, due to the known increased risk of tendon rupture with use of these drugs.[278, 279]

A.2. Injury profile of SA TEN and AUS TEN groups

Table A.1. Injury profile of SA TEN group (8.3 ± 9.2 years after initial onset of symptoms) and AUS TEN group (8.9 ± 9.8 years after initial onset of symptoms).

	SA TEN	AUSTEN
	percent (n)	percent (n)
Bilateral Injury	41.2 (85)	52.4 (84)
Multiple Injuries	25.6 (86)	37.9 (58)
Other Tendon Injuries	No Data	38.1 (84)
Early Onset (<30 yrs old)	26.4 (87)	27.7 (83)
At least one of the above	55.9 (93)	81.0 (84)

A.3. Genotype effects on descriptive measures

There were no meaningful genotype effects on the descriptive measures in either SA or AUS (Table A.2).

Table A.2. Genotype effects on descriptive measures for COX-2 -765G>C (rs20417), PTGER4 gene desert A>C (rs4495224), TGFB2 -876A>C (rs7550232) CASP8 -652 6N del (rs3834129), CASP8 Asp302His (rs1045485), NOS3 Glu298Asp (rs1799983) and NOS2 -1026C>A (rs2779249) polymorphisms in South Africa (SA) and Australia (AUS).

	SA							AUS						
	COX-2 -765 G>C	*PTGER4* gene desert A>C	TGFB2 -876 G>T	CASP8 -652 6N del	*CASP8* Asp302 His	*NOS3* Glu298 Asp	NOS2 -1026 A>C	COX-2 -765 G>C	*PTGER4* gene desert A>C	TGFB2 -876 G>T	CASP8 -652 6N del	*CASP8* Asp302 His	*NOS3* Glu298 Asp	NOS2 -1026 A>C
Age	0.847	0.570	0.120	0.388	0.388	0.217	0.634	0.234	0.822	0.940	0.600	0.535	0.234	0.053
Height	0.444	0.408	0.100	0.845	0.845	0.553	0.620	0.308	0.943	0.517	0.067	0.480	0.308	0.073
Weight	0.882	0.722	**0.002**	0.475	0.475	0.538	0.687	0.655	0.363	0.134	0.415	0.457	0.655	0.650
BMI	0.706	0.242	**0.007**	0.311	0.311	0.762	0.852	0.226	0.184	0.104	0.490	0.793	0.226	0.720
Gender	0.383	0.667	0.156	0.719	0.719	0.293	0.233	0.294	0.447	0.140	0.288	0.676	0.294	**0.021**

P-values are tabulated. Bold values indicate significant genotype effects ($p<0.05$), however the significant findings in one population are not meaningful, as there is no plausible biological explanation for significance.

A.4. Physical activity of SA and AUS participants

All SA participants were required to complete a detailed physical activity questionnaire. The physical activity questionnaire was composed of a section detailing the current and past participation in different sporting codes and the number of years of participation in these sports. In addition, the hours of training per week during the two years prior to recruitment were also documented.

The majority of SA TEN participants reported that they injured their Achilles tendon while running (69.1%). The number of years that the SA CON and SA TEN groups participated in running were similar (Table A.3). In the two years prior to recruitment, the SA CON group ran for more hours a week than the SA TEN group, but this may be because of reduced physical activity in the SA TEN group in response to pain. The SA TEN group participated for significantly more years in high impact sports than the SA CON group (Table A.3). There were no significant differences in the hours of training in high impact sports over the two years prior to recruitment between the groups (Table A.3).

The authors do recognize that the physical activity of the AUS TEN and AUS CON participants were not documented and is therefore a limitation of this study.

Table A.3. Participation in physical activity and training of the control (CON) and the Achilles tendinopathy (TEN) groups.

	SA CON	SA TEN	p-value
Running (yrs)	8.3 ± 8.1 (139)	9.0 ± 10.3 (75)	0.549
Running in the 2 years prior to recruitment (hrs/week)	3.3 ± 2.9 (125)	2.5 ± 2.8 (66)	**0.046**
High Impact Sports (yrs)	16.5 ± 16.1 (129)	31.1 ± 29.5 (75)	**<0.001**
High Impact Sports in the 2 years prior to recruitment (hrs/week)	5.1 ± 4.8 (129)	4.4 ± 5.2 (75)	0.321

Values are expressed as mean ± standard deviation. Number of participants (n) is in parentheses. Bold typeset denotes significant differences ($p<0.05$) between CON and TEN groups.

Appendix B

FORMS

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PARTICIPANT INFORMATION

The identification of genetic risk factors underlying Achilles tendon injuries

Although there is a high incidence of tendon overuse injuries as a result of participation in exercise and sporting activities, the cause(s) of these injuries are poorly understood. Some researchers have suggested that there is a genetic component to exercise-induced tendon injuries. In an attempt to determine whether there is a genetic basis for tendon pathology, we are interested in studying whether certain genes are associated with chronic tendinopathies. This project is being done in the UCT/MRC Research Unit for Exercise Science and Sports Medicine within the Department of Human Biology at the University of Cape Town.

You will be required to visit the Sports Science Institute of South Africa (SSISA) in Boundary Road, Newlands. During the visit, which should take at least 1 hour, you will be asked to donate 5 ml (1 teaspoon) of a blood sample for DNA analysis. You will also be required to complete personal particulars, sporting details, medical history and stretching and warm up questionnaires.

At a later stage, some participants will be asked to visit a doctor (radiologist) for a tendon scan at no cost to themselves.

All the information retrieved from this study will be treated with the strictest confidentiality and will be used only for scientific research purposes. Your name and personal particulars will not be released under any circumstances

and all data will be analysed anonymously. Your DNA sample will be destroyed on completion of the study on the genetic basis of tendon pathology. You are also free to request that your DNA sample be destroyed before the completion of the study.

If you are part of the tendon pathology group, we would appreciate it if you could help us by recruiting two other people of same (or similar) age whom you know and who has trained without suffering any tendon injuries for the control group.

We will keep you informed about the outcomes of this study and look forward to working together with you. If you have any questions about this study, please feel free to contact us at:

Dr Alison September, PhD (021) 650 4559 alison.september@uct.ac.za	Erica-Mari Nell, BSc (Med) Hons 072 432 2810 erica-mari.nell@uct.ac.za	Prof. Malcolm Collins, PhD (021) 650 4574 malcolm.collins@uct.ac.za

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CLINICAL CRITERIA ASSESSMENT FORM

FOR DIAGNOSIS OF ACHILLES TENDINOPATHY

SUBJECT NUMBER/CODE _____

Clinical criteria [1; 2]	Present
Gradual progressive pain over the posterior lower leg - Achilles tendon area (> 6 weeks)	
Early morning pain	
Early morning stiffness	
History of swelling over the Achilles tendon area	
Tenderness to palpation over the Achilles tendon	
Palpable nodular thickening over the affected Achilles	
Positive "shift" test (movement of the nodular area with plantar- /dorsi-flexion)	

Other criteria	Present
Confirmation of the diagnosis by ultrasound *	
Confirmation of the diagnosis by MRI *	
Confirmation of the diagnosis by CT scan *	

*: One of these criteria must be present to confirm the diagnosis

Date: _____ / _____ / 20_____
Investigator: Prof M Schwellnus
Signature: _____

References:

1. Schepsis AA, Jones H, Haas AL. Achilles tendon disorders in athletes. *Am.J Sports Med* 2002;**30**:287-305.
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INFORMED CONSENT

I, (the participant), have been fully informed about this study on the genetic basis of Achilles tendinopathy to be conducted by the UCT/MRC Research Unit for Exercise Science and Sports Medicine at the University of Cape.

I have agreed to donate five millilitres of venous blood, which will be used for the extraction and analysis of genetic material (DNA), and will be taken by a phlebotomist. The DNA will only be used for scientific research purposes relating to the identification of genetic risk factors underlying Achilles tendinopathy. I agree to my height, weight and waist circumference measured. I have also agreed to complete questionnaires relating to personal particulars, sporting participation, medical history, stretching and warm up exercise and understand that all the information that is collected during the study will be treated with the strictest confidentiality and will only be used for scientific research purposes. I also understand that all data will be analysed anonymously and my DNA sample will be destroyed on completion of the study. I understand that the DNA will be genotyped (analysed) for variations (polymorphisms) within genes relating to the genetic basis of Achilles tendinopathy.

If requested, I am also prepared to visit a doctor (radiologist) at a later stage for a tendon scan at no cost to myself (please delete this sentence if not applicable). If requested, I am also prepared to visit the Sports Science Institute of South Africa (SSISA) in Boundary Road, Newlands for measurements to determine musculotendinous stiffness.

The potential risks associated with blood collection technique from the ante-cubital veins are: infection, delayed healing, haematoma, physical pain, mental discomfort and injury to a nerve or a vessel. These risks are small and will be minimized by the use of trained phlebotomists, use of sterile techniques and the use of disposable, single use materials.

I understand that whilst there is no direct benefit to myself, a genetic predisposition to Achilles tendinopathy can be established. I have read (or, where appropriate, have had read to me) and understood the information about this study, and any questions I have asked have been answered to my

satisfaction. I agree to participate in the study, realising that I have the right to request that my DNA sample be destroyed at anytime and, further, to demand that data arising from my participation is not used in the research project provided that this right is exercised within four weeks of the completion of my participation in the project. I agree that research data provided by me or with my permission during the project may be included in a dissertation, presented at conferences and published in journals on the condition that neither my name nor any other identifying information is used.

Any questions regarding this project may be directed to the co-investigator **Dr Alison September** on telephone number **021 650 4559** or e-mail **alison.september@uct.ac.za** or to the principle investigator: **Prof. Malcolm Collins** on telephone number **021 650 4574** or e-mail **malcolm.collins@uct.ac.za**.

If you have any complaints or queries that the investigator has not been able to answer to your satisfaction, you may contact the Faculty of Health Sciences Human Research Ethics Committee at the University of Cape Town **Prof. Marc Blockman** on telephone number **021 406 6452**.

Name of Participant: _____

Signature:_____
Date:_____

Name of Researcher:_____
Signature:_____
Date:_____

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GENETIC BASIS OF TENDON INJURY QUESTIONNAIRES

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

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

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

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

C. FLEXIBILITY TRAINING HISTORY	
Do you perform flexibility training (regular stretching exercises)?	Yes ☐ No ☐
<u>If YES,</u> please complete the rest of the flexibility training history section below:- If NO, continue completing the questionnaire from the top of page 5 (Equipment use history).	

On average, how many days a week do you perform a stretching session?	days/week
On average, how times a day do you perform a stretching session?	times/day
Please tick which muscle groups do you include in your stretching session?	☐ Hamstrings ☐ Quadriceps ☐ Calf (gastrocnemius) ☐ Calf (soleus) ☐ Groin (inner thigh) ☐ Upper body limbs ☐ Other: _____
Please tick when you stretch? (before, during and/or after exercising. You can tick more than one box)	☐ Before Exercise ☐ During Exercise ☐ After Exercise
When you stretch an individual muscle group, on average, **how long do you hold the stretch** for?	seconds
When you stretch an individual muscle group, on average, **how many times do you stretch the muscle for**?	☐ Once ☐ Twice ☐ 3 times ☐ 4 times ☐ 5 times ☐ 6 or more times

D. TENDON INJURY - MEDICAL DETAILS

Symptoms

How many times have you had tendon injuries?

[1]Sudden onset is within a few seconds or minutes

[2]Gradual onset is over days or weeks

Tendon Injured	Date of Injury	Acute or Chronic Injury	Sudden[1] or Gradual[2] Onset
1			
2			
3			
4			
5			

Please complete a **separate form**, Part D only, for each Tendon Injury you have had

Injury Number (1,2,3,4,or 5)	☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ _____
Which tendon did you injure?	Rotator cuff tendon ☐ • Supraspinatus ☐ • Infraspinatus ☐ • teres minor ☐ Patellar tendon ☐ Wrist extensor tendons ☐ Achilles tendon ☐
Which side was injured?	☐ Left ☐ Right ☐ Both
Which region of tendon was injured?	☐ Upper 1/3 ☐ Middle 1/3 ☐ Lower 1/3

To what extent was your Tendon ruptured?	☐ Complete ☐ Partial ☐ None
How were you injured? (e.g. sport, walking)	
Grade of injury **at the time** of injury	☐ pain only after exercise ☐ pain during exercise, but did not cause you to alter training ☐ pain during exercise, which causes you to alter training ☐ pain which causes you to stop training ☐ no pain ☐ not sure ☐ Other (Specify _____)
Grade of injury **currently**	☐ pain only after exercise ☐ pain during exercise, but did not cause you to alter training. ☐ pain during exercise, which causes you to alter training ☐ pain which causes you to stop training ☐ no pain ☐ not sure ☐ Other (Specify _____)
Which of the following symptoms were present **before** the injury	☐ Pain (less than 1 week) ☐ Stiffness ☐ Pain (1-4 weeks) ☐ Swelling ☐ Pain (> 4 weeks) ☐ None
Which of the following symptoms were present **after** the injury	☐ Pain (less than 1 week) ☐ Stiffness ☐ Pain (1-4 weeks) ☐ Swelling ☐ Pain (> 4 weeks) ☐ None
If you have or had chronic tendon pain, what seems to alleviate the pain?	

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

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

F. MEDICAL HISTORY

Do you currently suffer from any of these medical conditions:

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

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

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

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

What surgical operations have you had? (please list and give dates)	Operation	Date
If female:		
At what age did you start menstruating? (years)		
Are you currently using any type of contraception?	☐ Yes ☐ No	
If Yes, what type of contraception are you using?	☐ Pill ☐ Injection ☐ IUD	
Are you currently?	☐ Pre-menopausal (±12 cycles per year at intervals of 23–33 days & bleeding lasts 3-7 days) ☐ Menopausal (cycles are irregular and less frequent) ☐ Post-menopausal (no longer menstruating)	
Family History		
Do any other members of your family suffer from elevated blood cholesterol?	Yes ☐ No ☐	If Yes, which relative? ☐ Mother ☐ Father ☐ Sibling ☐ Son / daughter ☐ Other relative:…….……..…….
Is there any history of arthritis in your family?	Yes ☐ No ☐	If Yes, which relative? ☐ Mother ☐ Father ☐ Sibling ☐ Son / daughter ☐ Other relative:…….……..……. & What type of arthritis? Rheumatoid ☐ Osteoarthritis ☐ Other ☐

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

If yes, please select from the list below:

☐ ADCO-CIPRIN	☐ CIPROBAY	☐ SANDOZ CIPROFLOXACIN
☐ AVELON	☐ CIPROGEN	☐ TAFLOC
☐ BACTIDRON	☐ CPL ALLIANCE CIPROFLOXACIN	☐ TARIVID
☐ CIFLOC	☐ DYNAFLOC	☐ TAVANIC
☐ CIFRAN	☐ FLOXIN	☐ TEQUIN
☐ CIPLA-CIPROFLOXACIN	☐ MAXAQUIN	☐ UNIQUIN
☐ CIPLOXX	☐ NOROXIN	☐ UTN-400
☐ CIPRO-HEXAL	☐ ORPIC	☐ ZANOCIN
☐ Other_____		

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

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

Appendix C

TECHNIQUES

DNA extraction as described in Lahiri and Nurnberger, (1991), modified by Mokone *et al.*, (2005)

Blood samples were transferred to 15ml centrifuge tubes and to that was added 10ml TKM-1 buffer (Appendix D.8) containing 2.5 % Nonidet P-40 to lyse erythrocytes. Tubes were centrifuged at 220rpm for 10 minutes and supernatant poured off. The pellet was washed in 5ml of TKM1 buffer and centrifuged again at 200rpm for 10 min. The pellet was then resuspended in 0.8ml of TKM-2 buffer (Appendix D.9) and 50µl of 10% SDS (Appendix D.5). Samples were incubated for at least 60 minutes at 55^{o}C to lyse leukocytes. To each sample, 150µl of 5M $NaClO_4$ and 500µl of chloroform was added. Samples were vortexed for 15 – 20 seconds and transferred to 1.5ml microfuge tubes. Protein was precipitated by centrifugation at 13000rpm for 5 minutes at room temperature. 0.5ml of the supernatant, containing the DNA was then pipetted into another microfuge tube and 2 volumes of 100% ethanol was added to the supernatant. The tubes were centrifuged at 13000rpm for 2 minutes and supernatant discarded. The tube was inverted and pellet was allowed to dry for at least 30 minutes before being resuspended in 200µl of TE buffer (Appendix D.7) at 65^{o}C for 15 minutes.

Appendix D

SOLUTIONS

D.1. 40% Acrylamide-Bisacrylamide (PAGE Stock)

40g Acrylamide

2.11g Bisacrylamide

make up to 100ml with dH_2O

D.2. 2% Agarose gel

2g Agarose

100ml 1X TBE buffer (see below)

D.3. 5M $NaClO_4$

61.2g $NaClO_4$

make up to 100ml with dH_2O

D.4. 6% Polyacrylamide gel

100ml PAGE stock

67ml 10X TBE buffer (see below)

500ml dH_2O

D.5. 10% SDS

10g SDS (Sodium dodecyl sulphate)

make up to 100ml with dH_2O

D.6. 10X TBE buffer

216g Tris-HCl

110g Boric Acid

14.9g EDTA

make up to 2l with dH_2O

D.7. TE buffer

121mg Tris-HCl

37mg EDTA

make up to 100ml with dH_2O

D.8. TKM-1 buffer

605.6mg Tris-HCl

372.8mg KCl

1016mg $MgCl_2.6H_2O$

372mg EDTA

make up to 500ml with dH_2O

D.9. TKM-2 buffer

242mg Tris-HCl

149mg KCl

406mg $MgCl_2.6H_2O$

148.8mg EDTA

4675mg NaCl

make up to 200ml with dH_2O