Genetics of tendon properties: *In vivo* studies in asymptomatic men and women

Brandon Paul Foster

Institute for Performance Research

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List of abbreviations

(Those not widely used in scientific literature are listed)

ABO	Antibodies blood group
AGE	Advanced glycation end products
ATP	Achilles tendon pathology
BF	Biceps femoris
*Bst*UI	*Bacillus stearothermophilus* U458 restriction enzyme
BMI	Body mass index
cDNA	Complementary deoxyribonucleic acid
Col I	Collagen type 1
Col V	Collagen type V
COL1A1	Gene encoding collagen type 1 alpha 1 chain
COL1A2	Gene encoding collagen type 1 alpha 2 chain
COL5A1	Gene encoding collagen type V alpha 1 chain
COL5A2	Gene encoding collagen type V alpha 2 chain
COMP	Cartilage oligomeric matrix protein
CSA	Cross-sectional area
DNA	Deoxyribonucleic acid
DEXA	Dual X-ray absorptiometry
dNTP	Deoxyribonucleotide triphosphates
*Dpn*II	*Diplococcus pneumonia* type II
ECM	Extracellular matrix
EDTA	Ethylene diamino tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EMD	Electromechanical delay
EMG	Electromyography
E2	Oestradiol
GAG	Glycoaminoglycan
GAS	Genetic association studies
GWAS	Genome-wide association studies
GDF	Growth differentiation factor
GPa	GigaPascals
GT	Guanine-thymine
HRP	Horse-radish peroxidise
HWE	Hardy-Weinberg Equilibrium

IGF-1	Insulin-like growth factor-1
IL-6	Interleukin-6
IL-1ß	Interleukin-1beta
K	Stiffness
kΩ	kiloOhms
LOX	Lysyl oxidase
miRNA	Micro-ribonucleic acid
µSv	Micro-seivert
mm^3	Cubic millimetres
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MVC	Maximal voluntary contraction
$N \cdot mm^{-1}$	Newtons per millimetre
PCR	Polymerase chain reaction
PG	Proteoglycan
pg	Picogram
PTL	Patellar tendon length
PTMA	Patellar tendon moment arm
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROM	Range of motion
Rpm	Revolutions per minute
RTD	Rate of torque development
SNP	Single nucleotide polymorphism
Sp1	Specificity protein 1
TIMP	Tissue inhibitor metalloproteinases
TGF-ß	Transforming growth factor-beta
TGS	Total genotype score
TMB	Tetramethylbenzidine
TNC	Tenascin-C
US	Ultrasonography
UTR	Untranslated region
$VO_{2\ max}$	Maximal oxygen uptake
V_t	Volume of tendon

Publications

B.P.Foster, C.I.Morse, G.L. Onambele, I.I. Ahmetov and A.G.Williams. (2012). Genetic variation, protein composition, and potential influences on tendon properties in humans. *The Open Sports Medicine Journal* **6,** 1-14.

Abstract

In recent years, research interest has increased regarding the influence of genetic factors on health, physical activity, and sports research. This is achieved through research study designs including 'genetic variation' as an independent variable. These studies aim to link gene variants (common difference in the genome of a population) with a trait of interest (phenotype), known as 'genetic association'. Albeit, these genetic factors potentially only have a small influence. The aims of the present study were to determine the gene variants within genes that encode for proteins involved in homeostatic balance of tendon physiology, and the contribution to interindividual variability in patellar tendon structural and mechanical properties. Genotype and phenotype data was collected from 84 asymptomatic men and women (aged 18-39 years). Gene variants in the *COL5A1* and *MMP3* genes were not associated with the variability in the patellar tendon phenotypes, in either sex (*COL5A1* rs12722 –Male/Female - Volume, $P = 0.936/0.938$; Young's Modulus, $P = 0.897/0.227$; Z-scores, $P = 0.635/0.896$: *MMP3* 679620 & 591058 – Male/Female – Volume, $P = 0.796/0.532$; Young's Modulus, $P = 0.238/0.680$; Z-scores, $P = 0.346/0.862$: *MMP3 650108* – Male/Female – Volume, $P = 0.952/0.676$; Young's Modulus, $P = 0.170/0.557$; Z-scores, $P = 0.681/0.531$). Furthermore, a polygenic profile including these gene variants could not account for the interindividual variability in patellar tendon properties (Male/Female - Volume, $P = 0.359/0.949$; Young's Modulus, $P = 0.073/0.067$; Z-scores, $P = 0.110/0.579$). In conclusion, the data suggest that tendon properties are not influenced by the genetic variants studied here. In addition, there are no sex-specific associations. The research on gene variants and their influence on the risk of tendon injury and tendon properties remain quite limited, and the preliminary nature of this research, makes potential genetic influences difficult to quantify at this time. Continued investigations are encouraged into these genes/proteins named here (*MMP3*, *COL5A1*), as well as others that may influence the maintenance of tendon homeostasis. Future advances in determining the genetic components of tendon properties in an asymptomatic population may have implications for our understanding of the aetiology of tendinopathies, as well as physical performance potential.

Chapter 1.

Literature Review

1.1 Introduction

Historically, tendons were considered bands of connective tissue with relatively no dynamic function (Neuberger et al., 1951, Peacock, 1967). More recently, a plethora of studies have highlighted the importance of a tendon's ability to provide an integral interface for transmission of forces from muscle to bone in order to produce moments about joints. Tendons not only provide coordinated musculoskeletal movement but as a singularity are highly adaptable to this movement and therefore, a tendon's function can change as a result. Hence, the mechanical properties of tendon contribute to the degree of joint motion in direct response to this movement (McGinnis, 2005).

Biomechanically, internal structures such as tendon undergo deformation when resisting external loads that act on the body. The degree of deformation or strain produced is related to the stress caused by these external loads and the material that it acts upon (McGinnis, 2005). Thus, knowledge of the mechanical properties of tendon can assist in understanding injury risk and physical performance capabilities. The primary parameters describing the mechanical properties of relevance to human physical performance include tendon stiffness and tendon modulus (Heinemeier and Kjaer, 2011).

To further understand the mechanical properties of tendon, one needs to explore its structure, both its global characteristic dimensions (e.g. cross-sectional area and length) as well as its internal structural composition (e.g. cross-link density) whilst acknowledging its metabolically active state. Thus, an examination of its biochemical properties is necessary. The structure and biochemical dynamics of tendon and thus its function, is controlled by cells called tenocytes that maintain the extracellular matrix (ECM) (Arnoczky et al., 2008) and ultimately its material and mechanical properties.

In the past few decades considerable time and effort has been channelled into discovering how tendon responds to mechanical loading *in vivo* within human populations. However, response has shown to differ substantially among individuals. This difference is due in part to exogenous factors such as body mass, sex, age and training status but this difference can also remain, even when accounting for such factors (Roth, 2007, Spurway, 2006). Endogenous or biological factors such as genetics may contribute in some part to these observed/measurable differences in the material and mechanical properties of tendon.

1.2 Interindividual variability

For any physical trait/phenotype that can be observed or measured, for example tendon stiffness, there is likely to be a broad range of values within that population of individuals. A majority of the population will be represented within a narrow range surrounding the mean, which is the average value for the whole population. However, this average value does not represent each individual and so the standard deviation or variance observed between the two extremes of the phenotype is known as the 'interindividual variability'. This variability among individuals for such phenotype measures can be explained by three major factors, experimental error, environmental factors, and genetic factors (Roth, 2007).

Experimental error can be minimised by adopting both valid and reliable phenotype measurements that are established and follow strict operating procedures (Batterham and George, 2000, George et al., 2000), thus, the source of error variability can be controlled to a high degree. More importantly, environmental factors need careful consideration in the research design and/or in the statistical analysis, in order to minimise variability seen in measurement values across the population. However, measurement error and environmental factors may not completely explain all of the variability in the phenotype being measured. The remaining variability can be accounted for by the unique genetic profile of an individual. The focus of this thesis is on understanding the correlation of genetic variation and tendon material and mechanical properties.

1.3 Genetic influences

Genetic contribution to a phenotype can be explained through estimating heritability. In the 1970s, twin studies were conducted on identical (monozygous) and non-identical (dizygous) twins to investigate this phenomenon (Howald, 1976, Klissouras, 1971, Klissouras et al., 1973, Komi et al., 1977). The assumption was that identical twins have identical genetic profiles (genotypes) and reside in similar environments. Non-identical twins also share similar environmental experiences yet have genetic profiles no different to any other siblings. Hence, any variability still evident in phenotype measures between twin pairs was assumed to be a result of genetic influences (Babraj et al., 2005).

Educationalists in the 1920's outlined methods used to quantitatively calculate this variance (heritability estimate) manifesting itself as the difference between the intraclass correlation for absolute agreement, values of both twin pairs (Spurway, 2006). Almost 50 years later, Klissouras, (1971) and Klissouras *et al.* (1973) applied these same methods

when analysing the genetic contribution to human physical performance (VO_{2max}) in individuals, from very young adolescents to middle-aged, and found that the genetic influence is maintained throughout life, in the region of 80-93%. Such high values were attributed to the indirect measures of the phenotype, as well as the suboptimal precision of these measures, small sample size, issues with determining zygosity and systematic error related to more equal environmental influences on monozygotes than the dizygotes. More recent values of human physical performance are typically in the range of 40-80% (Babraj et al., 2005).

A recent twin study investigating the heritability of lumbar flexibility or range of motion which encompasses the muscle-tendon unit, found the proportion of variance attributable to genetic influences to be 47% (Battie et al., 2008). It is tempting to suggest genetic influence on the material and mechanical properties of tendon to have similar heritability estimates, leaving space for a significant amount of variability due to environmental influences.

As well as classic twin studies being conducted to estimate heritability in human performance related phenotypes, family studies have become more desirable in that they provide a more flexible statistical approach (path analysis) and allow for larger samples and combinations of family members to be compared, the HERITAGE Family Study being the first to do so. Moreover, identification of genomic regions that might contain specific genes, gene-gene interactions and dominant alleles important to the phenotype is also possible with this type of analysis. However, this has only been conducted in physiological parameters related to aerobic exercise and not for example, tendon properties *per se*. Nevertheless, both twin and family studies provide us with a 'top down' approach or indirect measurement of genetic influences within a population.

1.4 Genetic association studies

Once a significant genetic influence is established, it is prudent to identify what genes are involved in causing this interindividual genetic variation, in the hope of discovering the biochemical mechanism of the variations observed in the measurable phenotype. 'Bottom up' or direct measures of the genetic factors involved are popular means of identifying one or more gene variants that may change the structural and functional aspect of a phenotype, from the DNA sequence up to the whole body level or measurable phenotype (Spurway, 2006). Genetic association studies (GAS) allows such comparisons to be made.

Genetic association studies adopt cross-sectional, cohort, and case-control study designs of unrelated individuals in a population and arise from the fact that human populations share common ancestry. Moreover, GAS are a more powerful research tool in detecting variation explained by genetic factors than data from related individuals, but at the same time require sound physiological rationale for examining certain genetic markers (Langberg et al., 2000). It is natural to turn to testing a comprehensive catalogue of common gene variants across the entire genome to pinpoint key genes and shed light on underlying mechanisms. A popular approach used toward this goal is the use of genome-wide association studies (GWAS), made possible by advancing technology that allows genotyping of as many as two million variants simultaneously (O'Dea et al., 2004). A genetic association study design will be employed to document the research in this thesis and will be discussed in more detail in further sections.

1.5 Tendon properties

The overall aim of this thesis is to identify gene variants that are involved in the material and mechanical properties of tendon in humans, which may influence the rate of torque development (RTD) during physical activity. The following sections of this thesis will provide a detailed overview of tendon material and mechanical properties and importantly, the functional changes that occur as a result of mechanical stimulus. This will provide an opportunity to understand the dynamic nature of tendon homeostasis, so that possible associations can be made with genetics in subsequent sections.

1.5.1 Tendon structure and composition

A tendon's internal architecture is predominantly made up of insoluble fibrillar collagen comprising of molecules, fibrils, fibres and fascicles in a hierarchical manner and are aligned in parallel in accordance with the long axis of the tendon. These components of tendon have evolved to take the stresses of movement by resisting pulling forces (Sorensen et al., 2011), and it is thought that the mechanical behaviour of whole tendon derives from the arrangement of these fascicles (Sorensen et al., 2010).

Fibrillar collagen type I is the most abundant collagen type in the human body and forms 97-98% of the total portion of collagen in fibrils (Jozsa and Kannus, 1997), the most fundamental molecular structure of the tendon. The remaining portion is taken up by other types of collagen namely types II, III, V and XI (Eikenberry et al., 1984). Different collagen types can co-exist in the same fibril to form heterotypic fibres, for example, type I

and III (Surh et al., 2000) and type V and I, the latter combination is thought to regulate the diameter of these fibres (Svensson et al., 2012). At a molecular level fibrillar collagen is a trimeric molecule consisting of three polypeptide chains in a triple helix. These molecules are arranged end to end in a quarter-staggered array with an approximate length of 1nm, aggregating into fibrils with a length of about 300nm (Haraldsson et al., 2009). Finally, fibril formation is made possible by the biosynthesis of these collagens from procollagens, after the secretion into the ECM from inside the cell (e.g. tenocytes) (Haraldsson et al., 2006). Moreover, collagens in the ECM are covalently cross-linked (Haraldsson et al., 2005, Svensson et al., 2012) providing optimal force transmission between collagen molecules (intramolecular) (Eleswarapu et al., 2011). There are two mechanisms by which cross-linking occur, enzymatically and non-enzymatically. Enzymatic cross-linking involves the enzyme lysyl oxidase during development and maturation, to connect adjacent amino acids within the collagen molecule, whereas, non-enzymatic cross-linking occurs via the glycation of reducing sugars and matrix proteins, and is thought to be associated with increasing age (Doane and Birk, 1991, Bianchi, 1993).

In addition to the fibrils resisting pulling forces and providing intra- and intermolecular stability, there are interfibrillar soluble polymers that resist compressive forces, known as proteoglycans (PGs). PGs are capable of binding and interacting electrostatically with fibrillar collagen largely due to their respective large surfaces (Limper et al., 1991). Electron microscopy has demonstrated that these interactions occur in multiple locations on the surface of the fibrils, interconnected through glycoaminoglycan (GAG) side chains (Silver et al., 2002). There are numerous PGs namely aggrecan and decorin (Silverman, 2001), which in addition to resisting compressive forces may also facilitate further slippage during mechanical deformation, a finding associated with decorin. This behaviour essentially improves the tensile properties of collagen fibres (Linsenmayer et al., 1984). The remaining composites of tendon, such as glycoproteins (e.g. Tenascin-C and Fibronectin) as well as elastin, interact with collagen fibrils to provide elasticity (Fitch et al., 1988) and regenerative qualities when stretched (Birk et al., 1988).

It is important at this stage to briefly introduce the possible role genetics may have in influencing tendon composition and function. The ECM proteins alluded to previously (e.g. collagens, proteoglycans, glycoproteins, elastin) are controlled and synthesised by the dominant cell type in tendon called ‘tenocytes’. These cells are aligned in rows between collagen fibre bundles (Jozsa et al., 1991) and are thought to ‘sense’ and respond to

mechanical stimuli, via complex interactions between the cells' cytoskeleton and the ECM (Arnoczky et al., 2008, Lavagnino et al., 2008a, Eastwood et al., 1998a). This gives rise to gene regulation, expression and control of functional gene products or ECM proteins at a molecular level. All in all, these morphologies, arrangements and interactions that occur in tendon may contribute to its unique mechanical behaviour.

1.5.2 Mechanical properties

As tendon mediates force transmission of the musculoskeletal system, understanding tendon mechanical properties is essential, especially when alluding to the functional mechanisms by which physical performance can be enhanced (Gerecke et al., 1993, Linsenmayer et al., 1993, Chen et al., 1993), or when injury risk can be reduced (Hayashi et al., 1992, Speit et al., 1985, Drahovsky et al., 1985). Over the past two decades considerable effort has been channelled into assessing the mechanical properties of human tendon *in vivo* (Rees et al., 2009b, Rees et al., 2009a).

The parameters that describe tendon mechanical properties derive from the force-elongation relationship of tendon determined by mechanical testing. Traditionally, such testing was exclusively performed on *ex vivo* animal tendons (Forslund and Aspenberg, 2002), however, the introduction of ultrasonography (US) has provided an opportunity for real-time monitoring of tendon excursions in recent years and made investigations of human tendons possible. Ramped isometric loading to maximum force production (MVC) of the tendon, correcting for other relevant parameters (joint moment arm length and muscle antagonist activation), will provide an estimate of actual forces applied to the tendon (Robinson et al., 2004b). Together with the amount of deformation or elongation of tendon, the tendons resistance can be calculated, thus, measures of strain and stiffness are made possible. Strain (ε) describes the absolute elongation from its original length, with stiffness (K) describing the elongation relative to the corrected forces applied to the tendon, as represented by the force-displacement curve (Figure 1). Stiffness is dependent on the cross-sectional area (CSA) and length of an individual's tendon (i.e. a greater CSA and shorter tendon length equates to greater tendon stiffness). However, to account for interindividual dimensional variability, a reliable assessment of CSA and resting tendon length at a standardised joint angle using ultrasonography is necessary (Maganaris and Paul, 1999). This allows for a comparison of the material properties of tendon across a human population, independent of CSA and length, which are normalised to stress (σ) (Force/CSA) and strain values, respectively. The mechanical behaviour of the actual

tendon material properties is described by its modulus. Therefore, tendon modulus is defined as the relationship between stress and strain (Equation 1), and in effect represents the elasticity of the material or Young's Modulus (E). Greater tendon modulus equates to a greater structural stiffness.

Equation 1: $E = \sigma / \varepsilon$

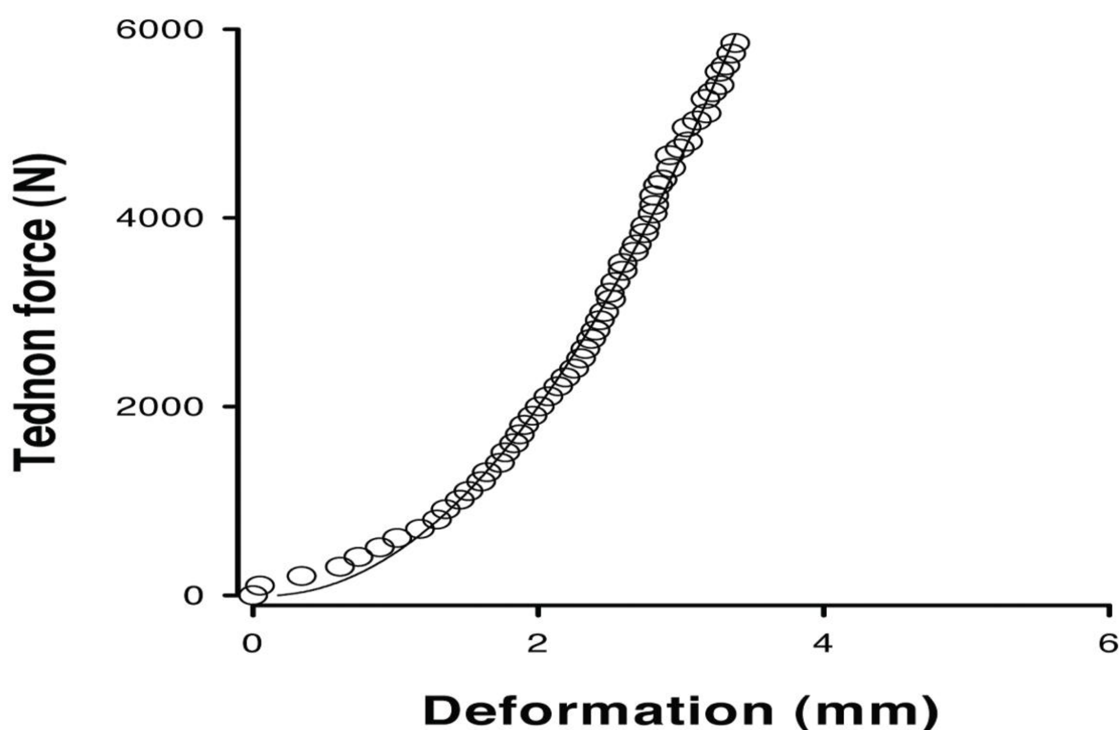


Figure 1. *In vivo* human patellar tendon force-elongation curve. Relationship between the force applied to tendon and the tendon elongation (Heinemeier and Kjaer, 2011)

Many *in vivo* studies have investigated tendon stiffness in the lower limbs in several populations, which differ in the methodologies and study designs implemented. The majority of which have assessed Achilles (Erickson, 1993a, Jones and Jones, 2000b, Fessel and Snedeker, 2009, Aparecida de Aro et al., 2012, Fessel and Snedeker, 2011, Joshi et al., 1993, Vollmer et al., 1993, Kannus et al., 1998, Martin et al., 2003a, Martin et al., 2003b, Riley et al., 1996) and patellar (Banes et al., 1999b, Ivanovic-Matic et al., 2000, Bergers et al., 2000, Yoshihara et al., 1995, Corps et al., 2005, Arnoczky et al., 2007, Storm et al., 1994) tendon stiffness using cross-sectional study designs. These types of studies

investigate differences between subject groups that vary either in age, sex, level of expertise or sporting activity. For example, Onambele et al. (2007) were novel in their investigations in that they were the first to assess the structural and mechanical properties of the patellar tendon, for direct comparisons between the sexes, who were age-matched (young participants), and exhibited similar activity levels (recreationally active). They observed significantly greater mean CSA (112.5 mm^2 vs. 88.6 mm^2), tendon stiffness (1940 $N \cdot mm^{-1}$ vs. 1080 $N \cdot mm^{-1}$) and Young's Modulus (1.2 GPa vs. 0.8 GPa) values in males, compared to females.

In subsequent sections, ways in which the mechanical properties of tendon can be modified are explored in great depth, relating to changes in structural and material properties. The following section however, will address the inherent complexity in measuring tendon mechanical properties *in vivo* in humans, which is characterised by the large variation in tendon stiffness measures, even across similar study designs and interventions.

1.5.3 Assessment of mechanical properties *in vivo*

The considerable variation in measuring tendon mechanical properties can be attributed to the different methodologies used by investigators in this area of study, owing to the fact that there is no agreed standard technique. Also, there is an inherent experimental error associated with instruments and techniques used to assess tendon behaviour *in vivo.*

The majority of studies assessing the mechanical properties of tendon *in vivo* have investigated the Achilles and patellar tendons, and to a lesser degree the quadriceps tendons (Stafilidis et al., 2005, Kubo et al., 2000b, Kubo et al., 2001c, Wilson et al., 2009) and tibialis anterior tendon (Maganaris and Paul, 2000c, Maganaris and Paul, 1999, Maganaris and Paul, 2000b). From this point on, studies that only investigated patellar tendon and reported an increase in stiffness with mechanical loading will be of primary focus. This is to allow a better comparison of methodologies between such studies, which will in turn bring attention to discerning differences.

Focusing on just one tendon type is particularly relevant when considering the following evidence. The collagen content has been reported to vary between different tendon types in normal tendon, albeit between patellar (~38%) and common biceps/supraspinatus (~ 65%) (Riley et al., 1994, Samiric et al., 2009), possibly due to the different habitual levels of mechanical stress placed upon. Therefore, as collagen type I fibrils and fibres are well

recognised to be involved in the mechanical stiffness of tendon matrix based on animal models (Birch, 2007, Eliasson et al., 2007, Reddy, 2004, Hansen et al., 2009c, Silver et al., 2001, Silver et al., 2003), as well as on *in vivo* studies in humans through patellar tendon biopsies (Couppe et al., 2009), it is prudent to associate mechanical properties such as stiffness with only one type of tendon. Furthermore, the differences in plasticity of tendon properties of the patellar and Achilles tendons as previously described, substantiate this reasoning for just focusing on one tendon type (Kubo et al., 2009, Kubo and Ikebukuro, 2012).

Methodological differences between studies that introduce varying degrees of measurement error include; methods of obtaining CSA by imaging techniques, methods for controlling for the viscoelasticity of tendon, methods used to precondition the tendon before assessment, methods for estimating actual tendon forces, and methods for computing stiffness estimates.

1.5.3.1 Tendon size

As alluded to previously, the CSA of a tendon is an integral characteristic for its mechanical properties; a tendon with a larger CSA will elongate less than a tendon with a smaller CSA at the same force and thus, it can be described as being 'stiffer', provided that the material properties of tendon are similar. Some studies have utilised magnetic resonance imaging (MRI) (Kongsgaard et al., 2007, Seynnes et al., 2009, Couppe et al., 2008, Kubo et al., 2012), whilst others have used US (Kubo et al., 2009, Pearson et al., 2007, Onambele-Pearson and Pearson, 2012, Reeves et al., 2003a) to assess CSA before and after a training regime. Both methods have been reported to be reliable when evaluating patellar tendons *in vivo* (Kartus et al., 2000) with US imaging providing higher reproducibility than MRI (Koivunen-Niemela and Parkkola, 1995). US is a well established 2D *in vivo* method for assessing fascicle movement and the CSA of tendon, with all internal threats to validity having been addressed and minimised (Magnusson et al., 2003b). Moreover, reliability of this method is maximised with an appropriate designed protocol and a single evaluator (Collinger et al., 2009). MRI mainly due to its capacity to image in 3D, provides a greater ability to image associated structures, which exhibit a non-homogeneous thickness throughout its length (Basso et al., 2001). It has been reported that CSA increases due to mechanical loading, occurs only at certain points across the tendon, thus, implying that hypertrophy occurs only regionally (Kongsgaard et al., 2007, Couppe et al., 2008, Seynnes et al., 2009). Therefore, it would be prudent to assess the CSA of tendon

at as many locations as possible to enhance the ability to detect such changes. However, most studies using MRI assessed CSA at only three sites (Kubo et al., 2012, Kongsgaard et al., 2007, Couppe et al., 2008), the same being true for studies adopting US (Reeves et al., 2003a, Kubo et al., 2009) with Onambele-Pearson and Pearson (2012) only measuring CSA at 50% of patellar tendon length. Further, Seynnes et al. (2009) assessed CSA at each 10% interval of tendon length and found a mean increase of 3.7%, with tendon hypertrophy being heterogeneous across its length. This highlights a possible explanation as to why in previous studies reporting an increase in stiffness, there was no concurrent increase in CSA, as at most, only three locations were recorded (Pearson et al., 2007, Kubo et al., 2009, Kubo et al., 2012, Reeves et al., 2003a). Incidentally, these studies used US with the exception of Kubo et al. (2012) who only assessed CSA proximally and with a low number of locations. Even though studies utilising MRI and assessing tendon CSA at only three regions (proximal, mid, distal), region-specific hypertrophy was reported (Kongsgaard et al., 2007, Couppe et al., 2008), thereby emphasising the need for imaging techniques that are able to capture the non-uniform thickness of tendon structure, of which MRI proves to be a more suitable option (Figure 2).

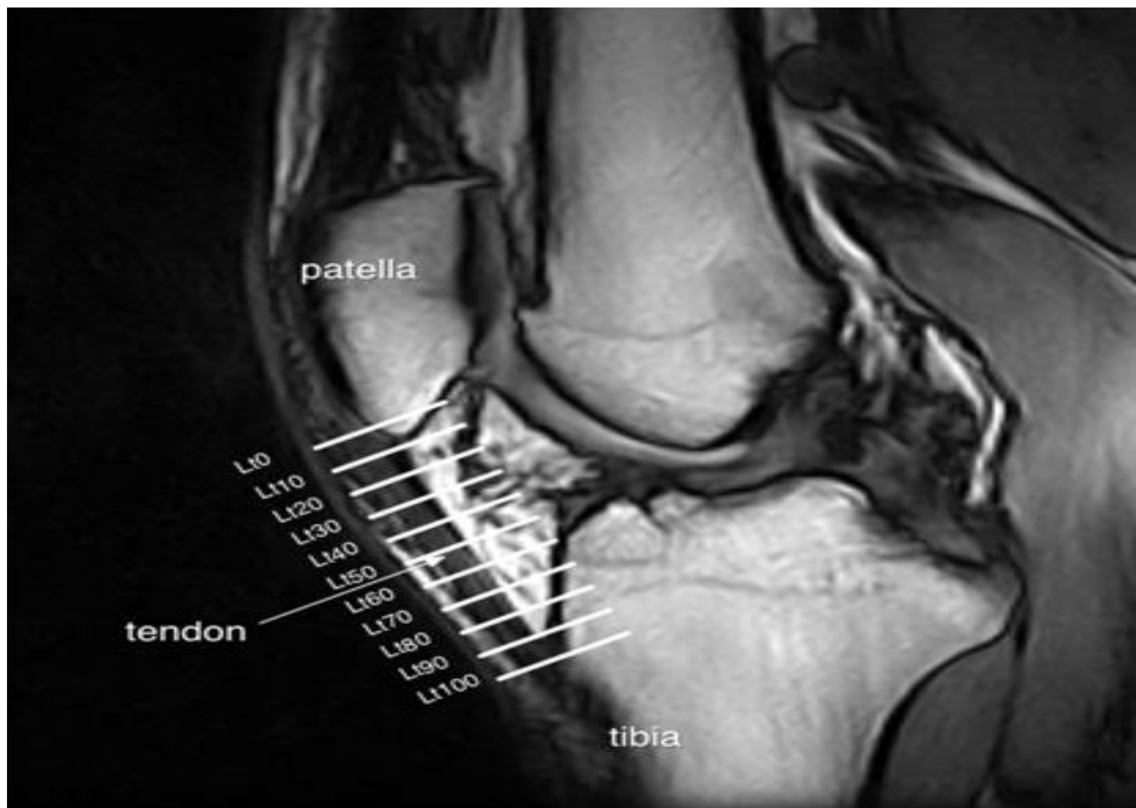


Figure 2. Sagittal magnetic resonance (MRI) scan of the patellar tendon (Seynnes et al., 2009)

1.5.3.2 Tendon elongation

Another possible methodological difference relates to how the elongation of tendon is assessed during the ramped isometric contractions using US imaging. When the patellar tendon is under investigation, some assessors position the ultrasound probe over both bony landmarks to which the patellar tendon is attached (the patella and the tibia), so that the entire length of the tendon is visible within the ultrasound image, throughout the contraction (Figure 3) (Kongsgaard et al., 2007, Couppe et al., 2008). Others make use of an external marker fixed on the skin distal to the origin of the tendon, in order to determine the proximal displacement relative to this particular bony end (patella) (Figure 4) (Reeves et al., 2003a, Storm et al., 1994, Li et al., 2004, Kubo et al., 2009, Reeves et al., 2003b). However, with the latter technique, there is no way of monitoring the degree of tendon displacement due to tibial movement in the longitudinal direction (Hansen et al., 2006). Therefore, tendon elongation would be underestimated and consequently tendon stiffness would likely be overestimated in these cases. An obvious way to correct this issue related to ultrasound probe length, would be to also assess the distal displacement of the tendon by applying an additional external marker, proximal to the tendons attachment to the tibial tuberosity (Onambele et al., 2007). However, two separate isometric contractions would be necessary to determine the tendon's true stiffness value during one movement.

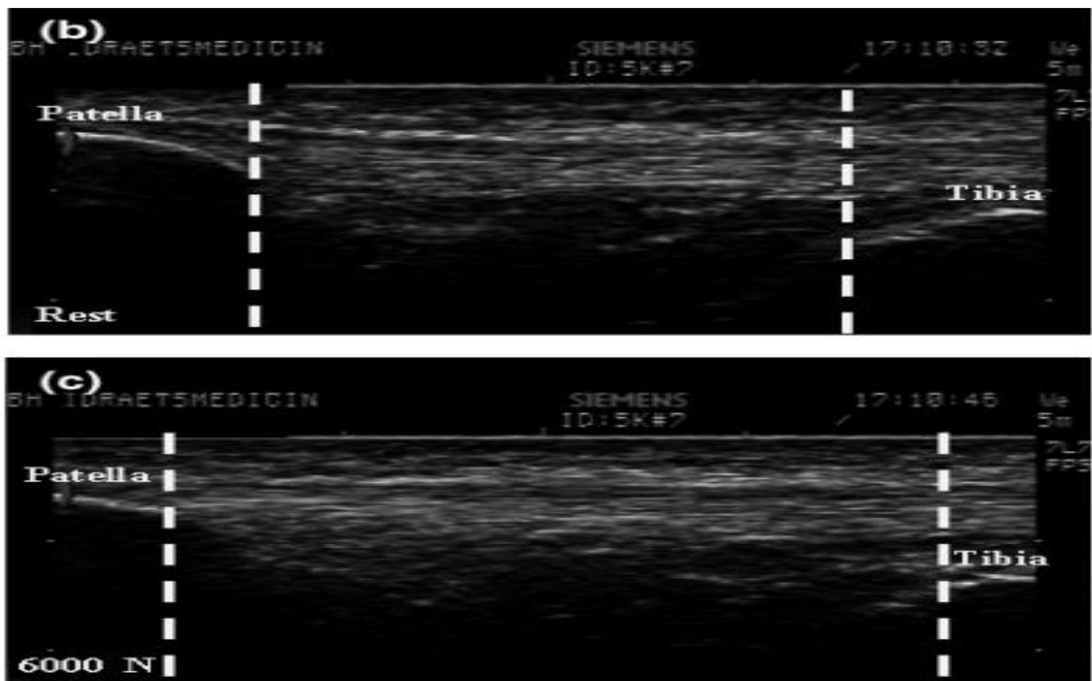


Figure 3. Sagittal-plane scans of the entire length of the patellar tendon at rest (top) and at maximal force exertion (bottom) in order to measure the displacement of the patella and tibia (Kongsgaard et al., 2007)

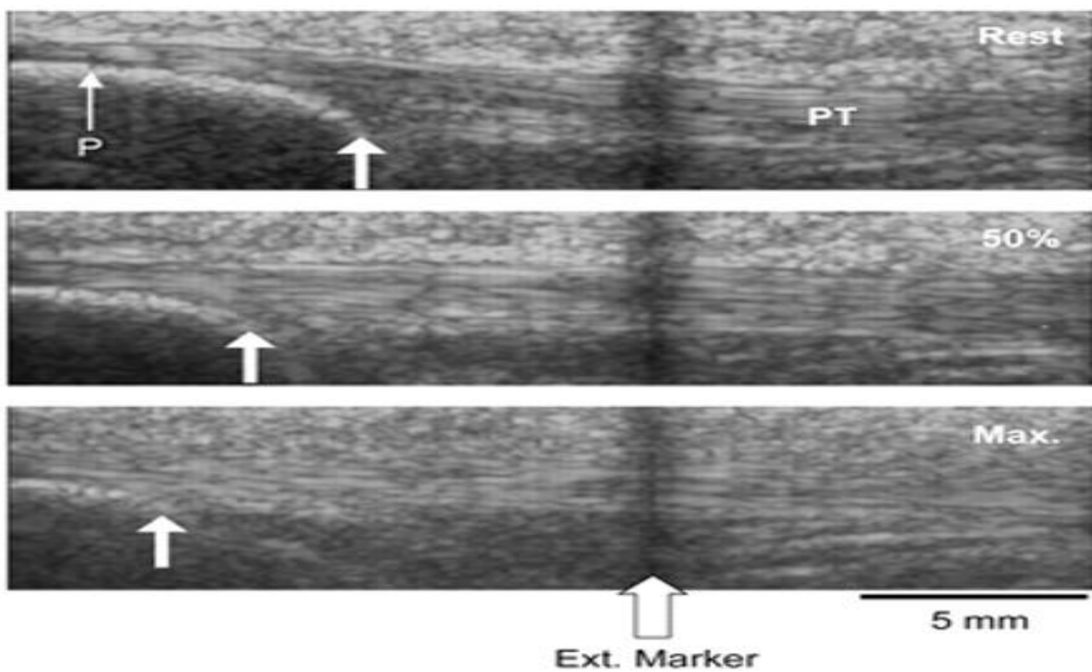


Figure 4. Sagittal-plane scans of the patella tendon (PT) at rest, during isometric contraction at 50 % of maximal and at maximal tendon force. Arrows indicate proximal displacement of the apex of the patella (P) during contraction with respect to an echo-absorptive external marker fixed on the skin distal to the displacement (Reeves et al., 2003a).

1.5.3.3 Viscoelasticity

Viscoelastic behaviour of the tendon during the ramped isometric contractions to maximal force levels, also contributes to the methodological disparity between studies. Not only does elongation depend on the degree of force application but also on the time and history of force applied, termed its 'viscoelastic' properties. The viscoelasticity of a material encompasses creep, stress-relaxation and hysteresis, with creep and stress-relaxation defining the time-dependent behaviour (Figure 5), and hysteresis defining the history-dependent behaviour (Figure 6) (Sorensen et al., 2010). Therefore, tendon is sensitive to varying strain rates with greater elongation occurring at low strain rates, with relatively less elongation occurring at high strain rates (greater stiffness) (Maniotis et al., 1997). Accordingly, the duration of the ramped isometric contractions used to quantify tendon elongation and the concurrent force levels, would need to be similar between studies to merit direct comparisons (Pearson et al., 2007), however, this is not the case with times varying from 4 s (Li et al., 2004, Reeves et al., 2003a) to 5 s (Kubo et al., 2009) to 6 s (Storm et al., 1994) to 10 s (Couppe et al., 2008, Kongsgaard et al., 2007). Pearson et al. (2007) investigated the *in vivo* effect of the duration of contraction on measured strain and stiffness of the patellar tendon, with particular emphasis being on the 'creep' characteristic of tendon, which describes the time-dependent increase in tendon elongation under constant force output. They found an increase in strain (~ 42%) with an associated decrease in stiffness (~ 77%), from 3-4 s contractions to 10-12 s contractions (Pearson et al., 2007). These results substantiate the importance of duration in respect to comparing mechanical properties within, or across studies.

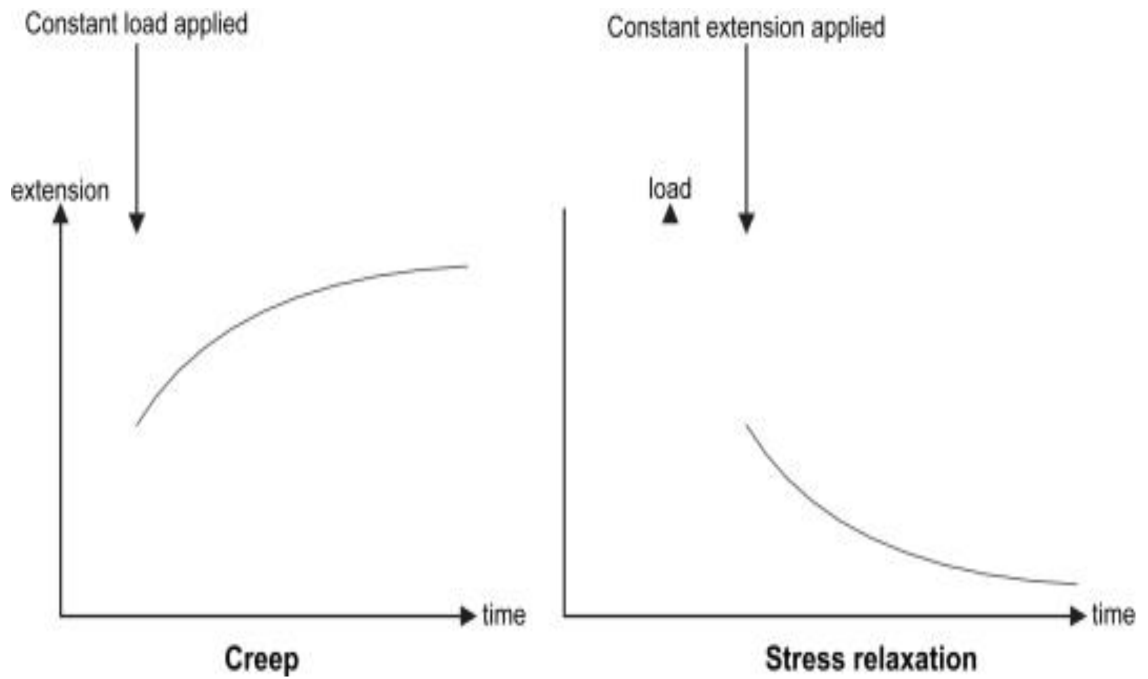


Figure 5. Creep (left) and stress-relaxation (right). In creep, constant compressive or tensile stress is applied to a tissue and corresponding strain is followed as a function of time. In stress-relaxation, predefined compressive or tensile strain is applied and corresponding stress is followed as a function of time (Smith et al., 2008).

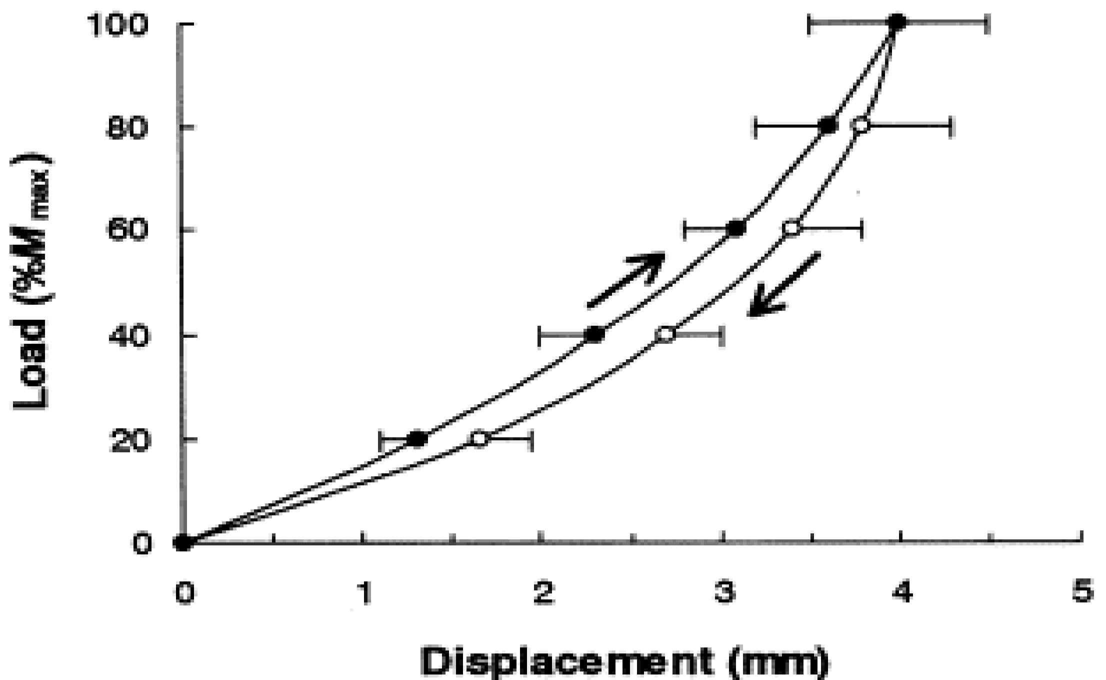


Figure 6. Load-displacement plots of the TA tendon. The arrows indicate loading and unloading directions. The area of the loop between the loading and unloading curves represents hysteresis, which is the strain energy dissipated by viscous damping (Maganaris and Paul, 2000a)

1.5.3.4 Preconditioning

Another related feature of the viscoelasticity of tendon is 'conditioning'. Conditioning is a time and history-dependent property resulting from creep, which acts on the material of tendon during the loading and residual unloading of the tissue (Cohen et al., 1976). Complex interactions of collagen, the surrounding proteins, and ground substance reflect the time and history dependent behaviour associated with conditioning (Kwan et al., 1993, Haut and Haut, 1997). It has been suggested that conditioning causes progressive collagen fibre recruitment, which increases the stiffness of the toe and linear regions of the force-elongation curves (Schatzmann et al., 1998). Conditioning also causes interstitial fluid loss, which in turn alters the PG's and hyaluronic acid concentrations, ultimately altering hydrogen and salt-like bonds between the ECM and fibrillar structures (Haut and Powlison, 1990, Yahia and Drouin, 1990) The conditioning phenomenon manifests itself in the methodologies of *in vivo* tendon studies as warm-up contractions or preconditioning, used to condition the tendon prior to assessment. Some studies do not report any preconditioning (Kubo et al., 2009, Reeves et al., 2003a, Kubo et al., 2012), whilst others do specify one (Kongsgaard et al., 2007, Couppe et al., 2008, Li et al., 2004, Pearson et al., 2007, Reeves et al., 2003b). Without any preconditioning it has been reported that the first five cycles of loading and unloading of the tendon *in vivo,* causes increased elongation as a function of contraction number. However, following these conditioning cycles tendon behaviour becomes more uniform between repeated loading cycles, as represented by similar force-displacement relationships (Figure 7) (Lavagnino and Arnoczky, 2005). Yet, even with a structured form of preconditioning or warm-up, the variability between the types of loading activity are likely to produce varying force-displacement curves and thus, stiffness values. Nonetheless, a preconditioning phase is an important step in producing reliable measures of mechanical properties for comparisons between studies.

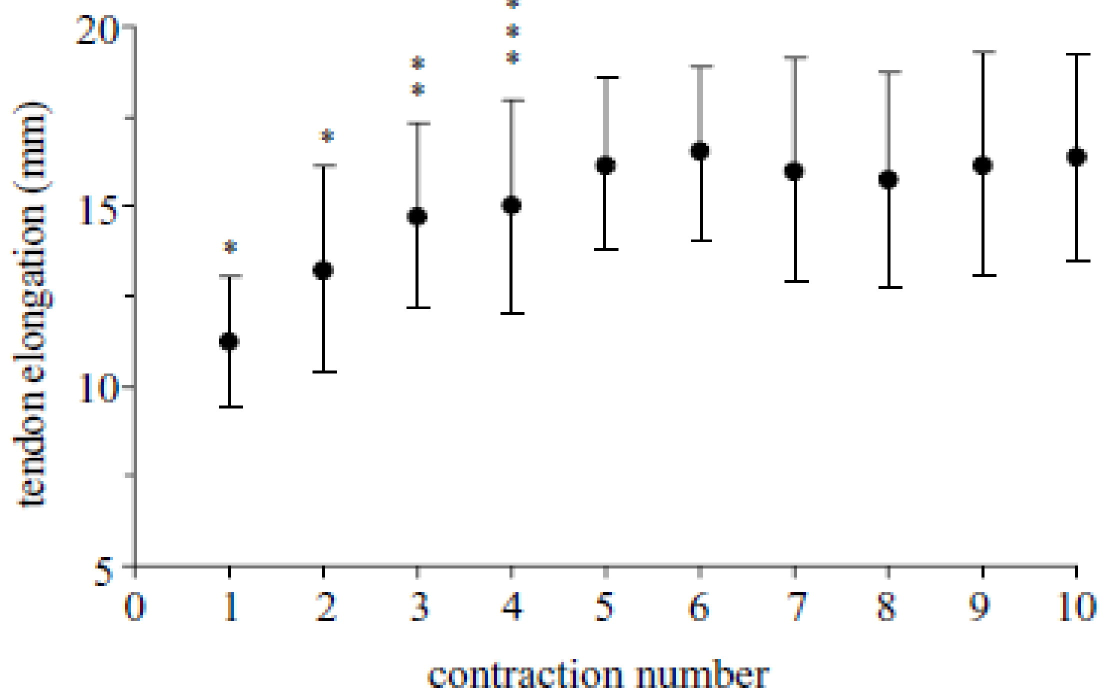


Figure 7. Tendon elongation at 80% MVC as a function of contraction number. No significant difference is observed in elongation following the fifth cycle. * $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$, compared with the tenth contraction (Maganaris, 2003)

1.5.3.5 Tendon force

In order to determine tendon stiffness *per se,* it is essential that the actual force transmitted through it is calculated. The calculation of tendon force from *in vivo* assessment is very much an estimated value, in that separate methods used to calculate tendon force, such as antagonist co-activation and joint moment arm length are measures that may introduce error, due to the complexity of related anatomical and physiological characteristics. Compounding these issues are the different approaches used by investigators to estimate, for example, antagonistic co-activation and joint moment arm length. Some studies estimated internal moment arm from individually measured femur lengths (Kongsgaard et al., 2007, Couppe et al., 2008, Kubo et al., 2012), derived from methods described by Visser et al. (1990). Others used sophisticated and more reliable methods such as MRI (Onambele-Pearson and Pearson, 2012, Reeves et al., 2003a, Seynnes et al., 2009, Reeves et al., 2003b), deriving from methods described in previous reports (Baltzopoulos, 1995, Tsaopoulos et al., 2006), with even some estimates originating from average values from previous reports, obtained from anatomy-based mathematical models (Pearson et al., 2007). These indirect methods of obtaining moment arm length from anthropometric variables, cannot explain the significant amount of inter-subject patellar tendon moment

arm length (PTMA) variation (Tsaopoulos et al., 2007). These estimates are used to quantify PTMA for calculations in determining musculotendinous forces (Maganaris et al., 2001) and ultimately tendon stiffness. Tendon stiffness may be underestimated if PTMA is overestimated, and so the accuracy in determining tendon mechanical properties is negatively affected.

With regards to the correction for antagonist activation, all studies utilised the force-electromyogram relationship to determine the net torque applied to the patellar tendon, during the isometric knee extension performances, by assessing the level of antagonistic muscle co-contraction of the biceps femoris (BF). Methods such as the placement of electrodes in relation to the BF for EMG measurements, can introduce measurement error (random error and systematic bias) (Batterham and George, 2000), and consequently makes comparisons difficult between studies, and thus, the extent to which the data generated is applicable to other populations, settings, or treatments, is made more difficult (George et al., 2000). Nevertheless, this technique for determining antagonist co-activation has been reproduced in several research papers (Reeves et al., 2004, Kellis and Baltzopoulos, 1997, Reeves et al., 2003a, O'Brien et al., 2009).

1.5.3.6 Tendon stiffness computation

There is a variety of calculation methods used in the research literature to ascertain tendon stiffness, which results in the inconsistencies evident between studies. In essence, tendon stiffness is determined from the gradient of the force-elongation relationship, in accordance with an appropriate function, generally a second degree polynomial function forced through zero. However, different studies utilise varying load levels as a proportion of the MVC, to which the stiffness function is adherent to. For example, Kongsgaard et al. (2007) adopted a MVC range of 90-100% linear, Reeves et al. (2003a), above 60% MVC linear, and Muraoka et al. (2005), 0-100% MVC linear, to which the gradient of the force-elongation curve is fitted (Figure 8).

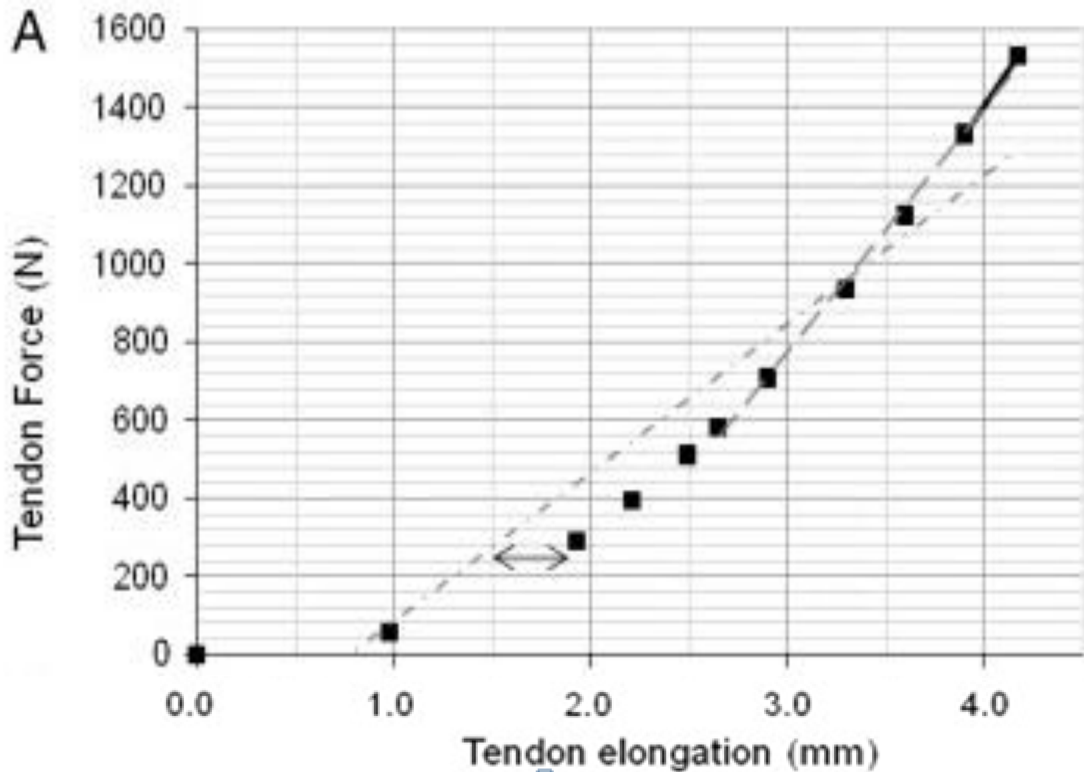


Figure 8. Computation methods that are commonly applied in determining *in vivo* tendon stiffness. 90-100% MVC-full black line. 50-100% MVC-short grey dashed line. 0-100% MVC- long grey dashed line (Pearson and Onambele, 2012)**.**

As a result of the differences in regression procedures (linear and tangential), different stiffness values become apparent for any given data set, since the gradient changes with the magnitude of load, owing to the curvilinear relationship between tendon force and tendon elongation. Numerically, this translates into a 26% underestimation and 51% overestimation of tendon stiffness, in relation to the gold standard methods (linear every 10% MVC and tangent every 10% MVC) (Pearson and Onambele, 2012) (Figure 9). It is therefore a requirement to adopt one methodology, in order to bring together the findings from the literature, preferably one of the gold standards.

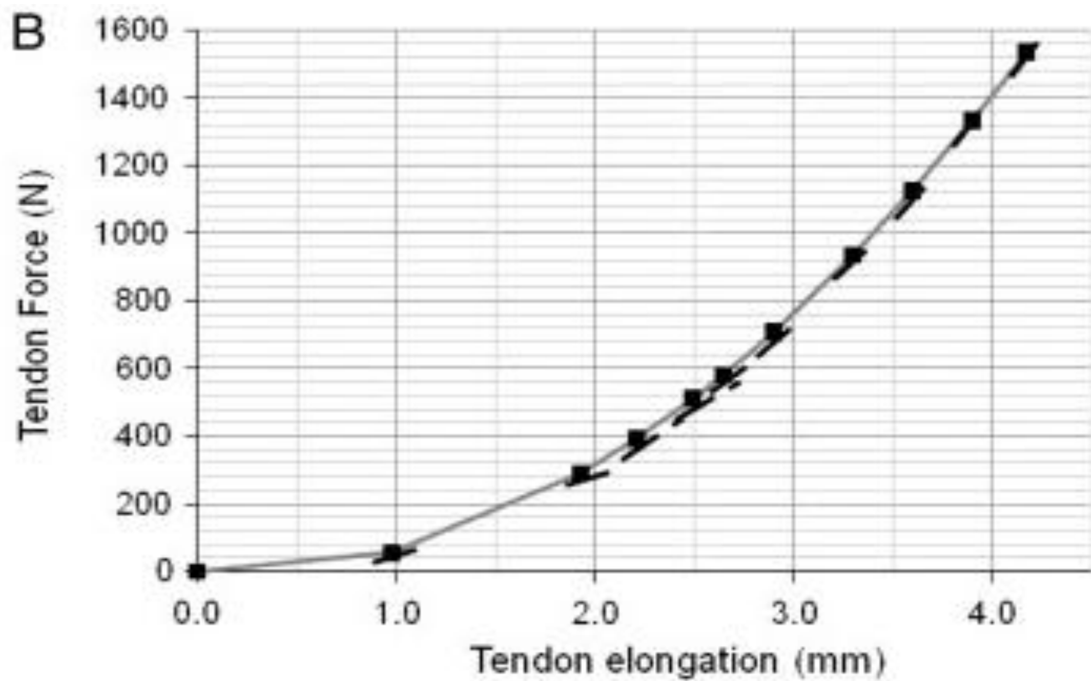


Figure 9. Gold standard methods of computing *in vivo* tendon stiffness. Every 10% MVC-full grey line. Tangent every 10% MVC-dashed black line (Pearson and Onambele, 2012).

1.5.4 Material properties

Not only are global characteristic dimensions (CSA) key in determining the mechanics of tendon, but also a change in the material properties is another way for tendon (see section 1.5.2, Mechanical properties) to adapt to external loads and influence mechanical properties. This has only been demonstrated indirectly in humans, yet it is pertinent to conceive this interpretation; firstly, due to changes in tendon stiffness without a concurrent change in CSA, and secondly, the research on animal models points toward a qualitative change in tendon in response to long-term, high frequency, cyclic exercise. Animal studies allow a direct measurement of CSA and tendon mechanics *in situ* (Buchanan and Marsh, 2001, Viidik, 1967, Woo et al., 1982), thus, supporting the generally agreed view that changes in material properties are valid with long-term mechanical loading, at least. The following section will review key structural and regulatory proteins at a microstructural level, studied intensively in animal models and scarcely in humans, which have shown to affect the mechanical properties of tendon.

1.5.4.1 Structural properties of ECM

The ability of tendons to resist mechanical stress is directly related to the structural organisation of the ECM (Aparecida de Aro et al., 2012). An increase in collagen content may explain the adaptive response of tendon to this mechanical loading stimulus, and as a result enhance the mechanical properties of tendon (Parry et al., 1978).

1.5.4.1.1 Collagen

It is appropriate to introduce the most fundamental component of tendon material properties at this point. Collagen type I molecules have been reported to constitute 65-75% of tendon dry mass in cadaver tissue (Elliott, 1965) and more recently ~ 90% from *in vivo* patellar tendon biopsies (Lemoine et al., 2009). These molecules have been shown to impact on tendon tensile strength and mechanical properties (Kjaer, 2004, Kjaer et al., 2005, Provenzano and Vanderby, 2006) and hence, have been shown to be of primary relevance to the material and mechanical properties of tendon.

The collagen type I molecule has been reported in past studies to possess little flexibility and to be rod-like in nature, which is why it was thought to carry high mechanical strength (Diamant et al., 1972, Fletcher, 1976, Silver et al., 1979, Thomas and Fletcher, 1979). However, it was later reported that collagen type I molecules possessed numerous bands or crimps and was not entirely rigid (Silver and Birk, 1984). Described from a molecular basis, the flexibility evident in collagen type I derives from sequences that lack the amino acids, proline and hydroxyproline, so these sites are typically characterised by bends in the triple helix structure (Figure 10) (Hofmann et al., 1984). Alternatively, sequences with glycine-proline-hydroxyproline contribute to a very rigid structure (Kjaer et al., 2005, Silver et al., 2003).

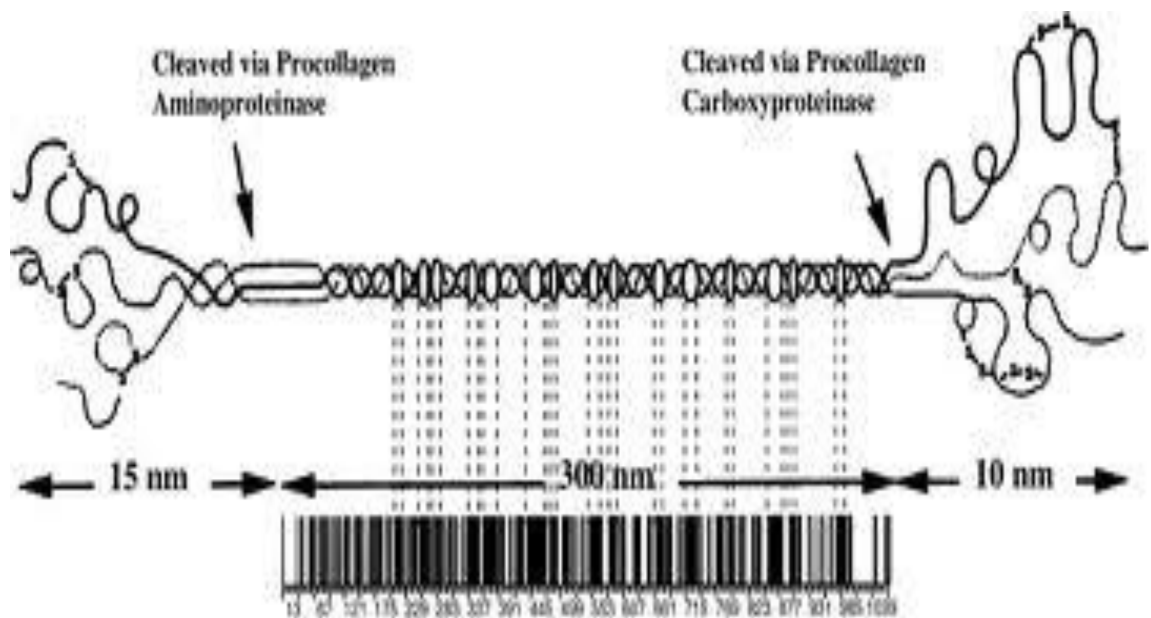


Figure 10. Diagram of procollagen type I molecule. The circles in the triple helix represent sequences lacking the amino acids proline and hydroxyproline. More flexibility is evident in these regions of the triple helix in what is otherwise a rigid helix (Silver et al., 2003)

There are a number of possible mechanisms in which collagen type I fibrils, the most fundamental force transmitting unit assembled from collagen molecules within the tendon matrix, can adapt morphologically to mechanical loading. These include changes to its diameter, length, intra- and inter- molecular cross-links, orientation and density (Parry et al., 1978). However, due to the complexity of tendon dynamics, as well as the effect of maturation and ageing, and form of fibril distribution may have on fibril morphology (Parry, 1988, Parry et al., 1978), a precise role for any one of these components is difficult to comprehend. It may be that interactions between the constituent components may influence tendon mechanics to a greater extent. Nevertheless, a better understanding of each component's role in tendon function and mechanics is required.

The diameter of collagen fibrils have been suggested to be associated with mechanical properties of tendon, in that fibril diameter appears to be inversely related to collagen molecule flexibility (Silver et al., 2001, Silver et al., 2003). Several *in vitro* animal studies support this association by reporting that the whole tendon Young's Modulus and stiffness increases, due to an increase in collagen fibril diameter (Parry and Craig, 1977, Parry, 1988, Diamant et al., 1972, Parry et al., 1978, Hansen et al., 2009c, Birch, 2007, Derwin and Soslowsky, 1999, Rigozzi et al., 2010, Patterson-Kane et al., 1997b). However, it has

also been reported in an *in vitro* animal study that fibril diameter is not the main predictor of tendon mechanical properties. Instead, fibril volume fraction which includes both fibril density and mean fibril area as contributing factors, as determined by transmission electron microscopy, is fundamentally more influential (Lavagnino et al., 2005, Robinson et al., 2004a). This discrepancy between these tendon components and tendon function may be explained by the lack of quantitative evidence that exists, correlating the exact roles and structural arrangement of distinct tendon components, and their effect on mechanical properties. These discrepancies are further compounded by the suggestion that there are complex structure-function relationships within the tendon fascicles (Robinson et al., 2004a).

A recent study on humans investigating the mechanical properties of tendon in a cohort with patellar tendinopathies, found that heavy slow resistance training for 12 weeks changed the collagen fibril morphology. Fibril density increased and mean fibril area decreased in patellar tendon biopsies, with a concurrent decrease of 9% in tendon stiffness (Kongsgaard et al., 2010). Interestingly, this suggests that mechanical loading actually decreases fibril diameter but at the same time increases the presence of more small-diameter fibrils, supporting the findings in several animal studies (Michna, 1984, Patterson-Kane et al., 1997b). Smaller fibrils have been suggested to provide a greater contact area between the fibrils and ECM where shear stresses are likely to be enhanced, thus, increasing the elastic properties of the tissue (Haraldsson et al., 2005). In support of this assumption, the mechanical properties of the patellar tendon stiffness and modulus decreased by 9% and 17%, respectively, following the resistance training protocol (Kongsgaard et al., 2010). This indirectly implies that there is a positive correlation between the diameter of the fibril and tendon stiffness/modulus. However, whether this is the case in a healthy tendon has yet to be resolved in humans. Furthermore, it must be noted that fibril diameter distribution has been shown to differ between individual tendons in different anatomical positions (Davankar et al., 1996) and within individual tendon regions, in animal (Patterson-Kane et al., 1997b, Williams et al., 2008) and human (Hansen et al., 2010) tendon studies, thus, compounding the lack of clarity on this topic.

Again, it can be inferred that variations in diameter of collagen type I fibrils between individuals, independent of mechanical loading and ageing for example, may be attributed to genetics. A possible mechanism by which collagen type I diameter may inherently vary may be the result of the temporal order of peptide removal of procollagen propeptides

during fibrillogenesis, as well as the amount of enzymatic activity (Birk et al., 1990), investigated *in vitro* using chick embryo tendons (Miyahara et al., 1984, Miyahara et al., 1982). Therefore, the processing of these peptides may be important in the control of fibril assembly and variations in diameter.

Collagen cross-links have been reported to play a crucial part in determining the stiffness of the fibrils when observing load-extension curves. An increased creep behaviour was evident in cross-link deficient collagen compared to in normal collagen (Puxkandl et al., 2002). Supporting this observation, it has been reported that cross-linking between collagen molecules provides an opportunity for increased cross-link density, and has a direct association with increased matrix stiffness of the tendon in animal models (Reddy, 2004, Eliasson et al., 2007, Reddy et al., 2002). More recently the importance of intra- and intermolecular covalent cross-linking, mediated by the enzymatically derived activity of lysyl oxidase (LOX) has been observed (Maruhashi et al., 2010a), which has shown to increase the modulus at the level of the collagen fibril (Hansen et al., 2009c). There is no evidence available from tendon biopsies of how a structured loading program affects cross-link levels and the impact this has on mechanical properties in humans. Conjointly, a recent study investigating collagen cross-linking of the patellar tendon between old and young men, indirectly emphasises the importance of these cross-links. Even though collagen concentrations were 74% lower in old compared to young men, mechanical properties such as stiffness, stress, strain and modulus were all similar between groups, due to the concurrent increase in the density of the cross-links as a function of age (Couppe et al., 2009). However, a recent study investigating region-specific differences in material properties of anterior and posterior fascicles of human patellar tendon, reports no association between the mechanical properties (stress, strain, modulus) of the fascicles and the concentration of mature cross-links (Hansen et al., 2010). Yet, mechanical properties were not examined *in vivo* but *in vitro,* further highlighting the inherent complexity of associating material properties with mechanical properties. Notwithstanding, several animal studies support the common belief that the material levels of cross-links, are the primary contributors to tendon mechanical properties (Patterson-Kane et al., 1997a, Avery and Bailey, 2005, Bailey, 2001, Barnard et al., 1987), which is in direct opposition with the belief that fibre and tendon size is the overriding mechanism, by which tendon mechanical properties change. A combination of both parameters is more likely.

Cross-linking of collagen molecules can also impact upon collagen fibril length as it permits end to end in series connections between collagen molecules within fibrils (Provenzano and Vanderby, 2006, Silver et al., 2000b, Silver et al., 2000a). It has been proposed that longer fibrils provide an increased ultimate tensile strength, as well as increased elastic contribution to the stress-strain behaviour (Silver et al., 2000b). However, the relative contribution of cross-linking between collagen molecules to tendon mechanical properties, for example, Young's Modulus, remains largely unclear (Eleswarapu et al., 2011).

As alluded to previously, different collagen types can co-exist or co-polymerise in the same fibril to form heterotypic fibrils in tendon, which may affect the mechanical properties of whole tendon, due to the role such interactions have in regulating the diameter of collagen type I. Collagen types III and V can be found in small quantities with collagen type I in a single fibril, suggesting that the mechanical properties may be different than that of unimodal fibril types (Silver et al., 2003). Moreover, it has been reported that the collagen type III molecule is more flexible than collagen type I, thus, the elastic modulus of such heterotypic fibrils may vary as a result (Silver et al., 2002). The interactions between collagen type I and V, have also been shown on *in vitro* self-assembly assays of chick cornea (Fitch et al., 1984, Fitch et al., 1988, Birk et al., 1988). These investigators report a decrease in fibril diameter of collagen type I with an increase in the ratio of collagen type V to I. High concentrations of collagen type V in this tissue are believed to result in small fibril diameters, possibly due to an increase in nucleation sites in the thin filaments of collagen type V, for a given quantity of collagen type I. These sites serve as steric hindrances for the addition of collagen type I molecules through amino-terminal domains which project out, thereby regulating lateral growth and diameter (Linsenmayer et al., 1993). Increases in collagen type V have shown to correlate with diminishing mechanical properties, such as maximum stress and linear modulus in rabbit patellar tendon *in vitro* (Dressler et al., 2002). Therefore, varying quantities of collagens within the same fibril can affect fibril diameter and consequently, has the potential to modify mechanical properties in humans.

1.5.4.1.2 Proteoglycans

Another notable structural property of tendon residing in the ground substance of ECM surrounding collagen, are proteoglycans (PGs). PGs are protein/polysaccharide complexes that consist of a protein core with attached glycoaminoglycans (GAGs). Tendon contains a wide variety of PGs, the vast majority of which are small-leucine rich PGs (Vogel and Heinegard, 1985). Decorin is the most abundant of these PGs (Scott, 1993). Transmission electron microscopy demonstrates that PGs attach to d-bands of type I collagen fibrils (Figure 11) (Scott and Orford, 1981), yet the amount of PGs decreases with increased collagen fibril diameter and age. Put another way, PGs may inhibit collagen fibril lateral growth through interference with cross-linking (Scott et al., 1981). The importance of PGs in relation to collagen fibrils has been demonstrated in animal models using gene-knockout of decorin in mice, whereby abnormal fibril morphologies were observed (Danielson et al., 1997). Also, when decorin was down-regulated, larger diameters of collagen fibrils were observable (Nakamura et al., 2000). These observations suggest that PGs are required for normal collagen fibril development and maturation, and on a wider scale, increased PG turnover within normal physiological ranges, is essential in maintaining normal tendon homeostasis (Rees et al., 2009b).

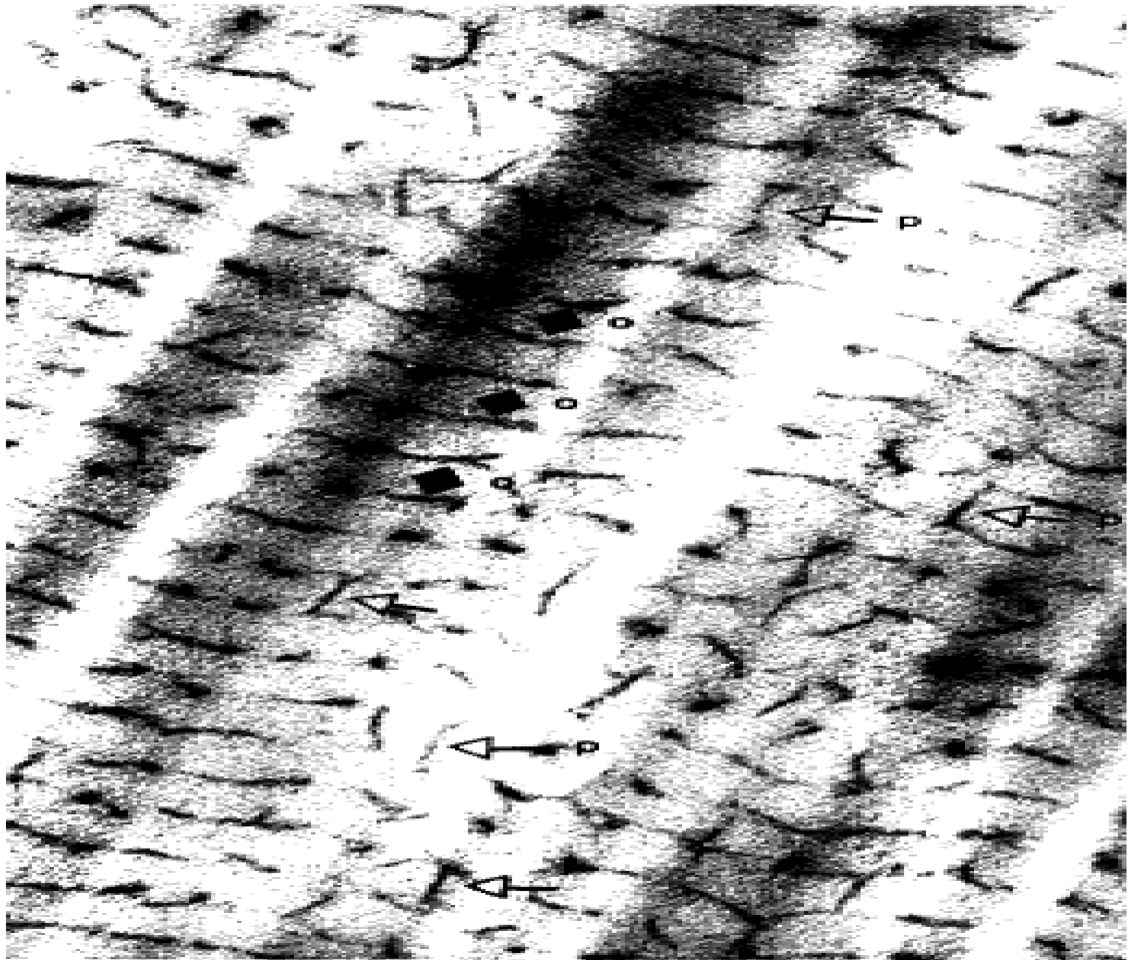


Figure 11. Electron micrograph of foetal calf flexor digitorum tendon. Lighter bands running from top to bottom are collagen fibrils. The orthogonal proteoglycans (o) and parallel proteoglycans (p) are 1 D-band apart (Scott, 1988).

Interestingly, when associating PGs to the mechanical properties of collagen fascicles, in tendon of transgenic mice models lacking decorin, there was no effect on maximum stress and modulus (Robinson et al., 2004b, Dourte et al., 2012). Consistent with this, adding decorin to self-assembled collagen fibrils does not significantly increase its maximum stress values, compared to controls (Pins et al., 1997). The absence of any significant effects on mechanical properties is also evident with other small leucine-rich PGs using similar animal models (Jepsen et al., 2002, Reuvers et al., 2011, Fessel and Snedeker, 2009, Fessel and Snedeker, 2011), although PGs may have a strain rate dependent on viscoelastic properties, possibly facilitating slippage between collagen fibrils in close proximity (Robinson et al., 2004b, Reuvers et al., 2011, Dourte et al., 2012). Therefore, PGs are unlikely to be involved in direct force transmission of tendon (Provenzano and Vanderby, 2006) and so will not be discussed further in this thesis, with regard to the

genetic variation contributing to such protein structures, and the possible structural and functional roles they might have on mechanical properties of tendon.

1.5.4.1.3 Glycoproteins

Glycoproteins such as tenascin-c (TNC), fibronectin and cartilage oligomeric matrix protein (COMP) are ECM constituents that are involved in providing structural resilience to the ECM, and in mediating mechanical interactions between cells, as well as participating in cell growth and differentiation during morphogenesis. These proteins are also responsible for repair, regeneration, and overall tissue remodelling in tendon (Jozsa et al., 1991, Sage and Bornstein, 1991). Because most of the functional and regulatory studies on glycoproteins have concerned TNC (Jones and Jones, 2000a), as well as it being potentially a prominent constituent of tendon undergoing high tensile stress, as with the collagen fibrils (Jarvinen et al., 2003, Jarvinen et al., 1999), it will be the major focus of discussion, particularly when selecting proteins and their associated genes and SNPs, known to have a genetic influence on tendon material properties.

1.5.4.2 Regulatory properties of ECM

Evidence is evolving that tendon and their associated ECM tissues are dynamic structures with plastic properties that adapts in a functional way to an external mechanical stimulus (Banes et al., 1999a, D'Souza and Patel, 1999, Langberg et al., 1999). This observation is typified by TNC for example, which is recognised to be involved in both structural and regulatory behaviours in tendon ECM, further substantiating its role in regulating the organisation of the most domineering constituent, collagen. The proteins described below have predominantly regulating roles within tendon.

1.5.4.2.1 Matrix Metalloproteinases

Matrix Metalloproteinases (MMPs), a family of enzymes are also highly influential in regulating intact fibrillar collagen and non-collagenous proteins within the ECM. More specifically, their functions are to degrade and remodel the ECM during development, adaptation and repair, and even activate other MMPs (Sternlicht and Werb, 2001, Vu and Werb, 2000, Murphy and Knauper, 1997, Somerville et al., 2003, Birkedal-Hansen et al., 1993). MMP-3, one of the most functionally diverse of the MMPs, will be discussed to a greater extent as to its possible role in genetic variation and tendon properties in subsequent sections.

1.5.4.2.2 Transforming Growth Factors-beta

Transforming growth factors-beta (TGF-ß) belongs to a superfamily of growth/differentiation factors (GDFs), which play an essential role in the maintenance, growth and repair of bones, cartilage and musculoskeletal soft tissues, including tendon (Hotten et al., 1994, Storm et al., 1994, Thomas et al., 1996). TGF-ß1, an isoform of TGF-ß has been identified as an important mediator of collagen synthesis, cross-link formation, and enhanced mechanical properties, following the introduction of mechanical stimuli to tendon fibroblasts *in vitro* (Yang et al., 2004, Keller et al., 2011), as well as in gene transfection studies on injured rat Achilles tendon (Rickert et al., 2005, Bolt et al., 2007, Heinemeier et al., 2007), rat supraspinatus tendon (Manning et al., 2011), and rabbit Achilles (Hou et al., 2009) and patellar tendons (Lyras et al., 2010). It must be affirmed that the levels of active TGF-ß are directly dependent on the levels of tensile loading, highlighting the critical role that mechanical loading plays in tendon homeostasis, as determined by a rodent model *in vitro* (Maeda et al., 2011). However, no correlation was found between circulating TGF-ß and tendon properties, at baseline and following a training stimulus, in human's *in vivo* (unpublished data from our labs). The lack of evidence *in vivo* on tendon, casts questions on the possible causal mechanisms that TGF-ß may have on stimulating collagen synthesis (Heinemeier et al., 2011) This may be because of the unknown extent to which these mechanisms control and interact with the biochemistry, via signalling systems such as GDF (Eliasson et al., 2008). GDF5, a member of the GDF subfamily of TGF-ß proteins will also be discussed for its potential role in influencing tendon properties at a genomic level.

It is rational to assume that regulating components of tendon ECM, which are highly involved in the adaptive responses of collagen structures to mechanical loading as discussed briefly above, are likely to have a defining role in modifying tendon structural and mechanical properties. These components are mediated via complex control centres of tendon, namely tenocytes.

1.5.5 Tenocytes

Tendon cells or 'tenocytes' are elongated fibroblast-like cells which are the principal cell type in tendon tissue (Jozsa et al., 1979). These cells are embedded within the ECM but are sparsely distributed in longitudinal rows, yet closely packed between collagen fibres (Figure 12) (Ross et al., 1989). There are cell extensions that extend into the ECM providing a three-dimensional network of cell processes, associated with the proliferation and maintenance of all the macromolecular components that make up the ECM, namely collagen, proteoglycans, and specialised non-collagenous proteins (McNeilly et al., 1996). As discussed in the preceding sections, global dimensions and material properties of tendon are altered in response to mechanical stimuli, by changing their structures, compositions, and mechanical properties (O'Brien, 1997, Vogel and Heinegard, 1985). This assumption is epitomised in a study by Ippolito et al. (1977) who discovered that within the cytoplasm of tenocytes there are actin filaments, suggesting that tendon cells have contractile activity, and thus, the ability to respond, process, and adapt to mechanical forces. More recently this assumption has been extended upon by the suggestion that actin stress fibres when mechanically loaded, transforms into contractile components that are involved in active recovery after stretch, maintain the integrity of longitudinal tendon rows, monitor tensile load, and contribute in the gene regulation of tendon cells during mechanical loading (Ralphs et al., 2002).

Figure 12. Tendon cells (tc) extend broad flattened lateral cell processes, meeting up with those from adjacent cells. Collagen fibre bundles (cf) are surrounded by the tendon cell processes (McNeilly et al., 1996)

Tenocytes are the major mechanoresponsive cells in tendon tissue (Yang et al., 2005), ultimately responsible for initiating changes within tendon by altering the expression and synthesis of ECM bio-molecules, involved in structure and regulation (Banes et al., 1999a, Benjamin and Ralphs, 2000, Kjaer, 2004, Sarasa-Renedo and Chiquet, 2005), such as collagen precursor molecules and growth factors (Lindahl et al., 2002, Schild and Trueb, 2002). The initiation of changes in response to mechanical loads has recently been found to be mediated by a 'mechanostat' set point *in vitro*. This preset threshold is governed by complex interactions between the cells cytoskeleton and the ECM (Arnoczky et al., 2008, Lavagnino et al., 2008a). Mechanotransduction is the mechanism by which tenocytes sense and respond to mechanical signals bi-directionally, via the cell nucleus, cytoskeleton and ECM (Banes et al., 1995b, Brown et al., 1998, Ingber, 1997, Banes et al., 1995a, Banes et al., 1999a, Wang and Ingber, 1994, Eastwood et al., 1998a, Janmey, 1998, Wang et al., 1993). The mechanostat set point relates to cytoskeletal tensional homeostasis, whereby the cell is thought to maintain its tensional integrity in response to differing force levels (Brown et al., 1998, Chicurel et al., 1998, Eastwood et al., 1998a, Ingber, 1997). The deformation of the cytoskeleton via membrane integrins and transmembrane proteins (G-

protein receptors and kinases) (Wang, 2006), induced by mechanical forces (stress and strain), is thought to up-regulate the gene expression of catabolic and/or anabolic mechanisms, which would ultimately provide the basis for changes in structure and function (Lavagnino and Arnoczky, 2005). The levels of gene expression have been shown to change depending on the strain and frequency of cyclic mechanical forces (Lavagnino et al., 2003, Arnoczky et al., 2002b, Arnoczky et al., 2002a, Maeda et al., 2007, Lavagnino et al., 2008a, Arnoczky et al., 2008, Legerlotz et al., 2011). Furthermore, it has been shown that by blocking signalling pathways, in particular gap junctions (intercellular communicators), results in a decrease of collagen type I production (Waggett et al., 2006, Banes et al., 1999b, Wall et al., 2007). This highlights the critical role that mediators of mechanotransduction play in providing cell-ECM interactions for tendon homeostasis and adaptive changes. However, gene expression and protein synthesis of various proteins involved in tendon homeostasis between, and within animal and human populations, can still vary greatly even if the size and frequency of mechanical loading is controlled. This is where genetic variation is likely to influence observed/measurable differences in protein content, and thus, material and mechanical properties.

1.5.6 Tendon function

The tendon's most fundamental function is the transmission of contractile forces from muscle to bone, in order to allow movement about joints. Tendons must be effective in resisting great tensile forces whilst being able to limit elongation (Jozsa and Kannus, 1997), a characteristic of an inextensible tissue structure with efficient force transfer. However, tendon cannot be described as being inextensible as its internal structures undergo deformation in response to an applied external load, hence, the mechanical properties of tendon contribute to the degree of joint motion (Elliott, 1965, Butler et al., 1978, Dunn and Silver, 1983, McGinnis, 2005), within anatomical constraints.

Tendons are also well known to operate as spring-like structures exhibiting elastic and force-dependent attributes, which may impact upon the function of the muscle-tendon complex as a whole, and provide additional important functional characteristics. Not only is force transmission influenced by muscle-tendon interactions (Ettema, 1996) but also energy storage and return for locomotion (Alexander, 1991, Voigt et al., 1995, Fukunaga et al., 2001, Maganaris and Paul, 2002, Ishikawa et al., 2005, Lichtwark and Wilson, 2005a), joint positional control (Loram et al., 2004, Loram et al., 2005b, Loram et al., 2005a), and protection from muscle fibre injury (Griffiths, 1991, Lieber et al., 2000).

It is generally accepted that tendons possess the ability to store strain energy upon stretching and subsequently released during recoil, supplying considerable mechanical work during locomotion. These observations have been demonstrated in animal models during walking and running (Griffiths, 1991, Roberts et al., 1997). The increased strain energy recovered during running over walking highlights the importance of tendon's elastic saving properties for the economy of movement (Cavagna, 1977, Magnusson et al., 2008).

As alluded to in preceding sections, US has provided a means of observing human tendon *in vivo*. Such studies have indicated that tendon can store and release elastic energy in accordance with the aforementioned animal studies (Kubo et al., 2000c, Fukunaga et al., 2001, Kawakami et al., 2002b, Kurokawa et al., 2003, Lichtwark and Wilson, 2005a, Muraoka et al., 2005, Lichtwark and Wilson, 2005b, Ishikawa and Komi, 2004, Ishikawa et al., 2005), the majority of which included stretch-shortening cycles. For example, in a study by Lichtwark and Wilson, (2005b), they were able to calculate the average peak tendon strain (8.3%) during one-legged hopping in the Achilles tendon of humans *in vivo*, with the tendon contributing 16% of the total average mechanical work to the activity. However, the investigators did report that the individual variation across measures such as stiffness and elastic modulus did vary greatly pertaining to the individual's variation in material properties, thus, the energy storing capacity of the structure is likely to differ as a direct result. Despite this discrepancy, the material properties of the group of participants were within previously published ranges, and therefore their results are viable. Moreover, modelling work involving the muscle and tendon in series, approximates the force delivered to an inertial load to be 40% greater than muscle alone, asserting once again the importance of tendon elastic recoil for mechanical efforts (Galantis and Woledge, 2003).

The animal models are further substantiated by the findings that greater stretch and therefore strain energy storage, is achieved with a greater amount of active muscle fibres working isometrically during more dynamic tasks (Kubo et al., 2000c, Ishikawa and Komi, 2004). For example, Kubo et al. (2000c) reported that during an isolated countermovement task involving a dorsiflexion followed by a plantarflexion of the ankle joint, tendon elongation was significantly greater during the fast countermovement (1.0 Hz) than the slow (0.3 Hz). Subsequently, a rapid shortening of the tendon occurred with little shortening of the muscle fascicle in the fast movement, with tendon structures contributing to 42.5% of the total amount of mechanical work, compared to 20.2% in the slow

movement. This highlights how the rate of force transmission contributes to the mechanics of tendon.

The mechanical properties of tendon will impact upon the overall muscle-tendon unit to produce functional movements. Indeed there is substantial evidence that tendon mechanical properties such as stiffness influence the capacity of the muscle-tendon unit to produce force during activities of various kinds (Wilson et al., 1994, Wilson et al., 1992, Walshe and Wilson, 1997, Kubo et al., 1999, Lichtwark and Wilson, 2008, Lichtwark and Barclay, 2010, Kubo et al., 2007a, Stafilidis and Arampatzis, 2007). For example, it has been interpreted that the optimal musculotendinous stiffness for maximal concentric and isometric muscle performance is toward the stiff end of the elastic continuum, as it may be more beneficial from a force-velocity perspective (Wilson et al., 1994, Burgess et al., 2007), as well as improved running economy and low energy costs (Fletcher et al., 2010), by increasing the length and rate of shortening of the muscle fascicle. On the other hand, during cyclical contractions, low tendon stiffness (high tendon compliance) has been shown to improve muscle power and efficiency *in vitro* (Lichtwark and Barclay, 2010). Even though Lichtwark and Barclay (2010) use artificial tendons in their methodology, the compliance of the tendons can be fine-tuned by the preliminary assessment of the force-extension properties of the latex strips (acting as artificial tendons), which vary in compliance, by the use of different widths or multiple strips in parallel. The power output and mechanical efficiency measures can then be directly related to tendon compliance. This is somewhat a limitation of *in vivo* studies, whereby separation of the human *in vivo* tendon and muscle behaviour is difficult to investigate. Therefore, the optimal level of tendon stiffness for functional performance on the contractile requirements of the activity has yet to be determined *in vivo*, due to the underwhelming evidence of the state dependent properties of muscle, as well as the complex interactions of the muscle-tendon unit in series (Lichtwark and Wilson, 2008).

The mechanical properties of tendon can firstly be understood in relation to its global characteristic dimensions (CSA and length), as it can be theorised that a long, thin tendon exhibiting low stiffness will be advantageous for activities requiring stretch shortening cycles. This is because they will experience greater stress for a given load because of a small CSA, but also greater strain will favour increased storage and recovery of elastic strain energy (Cavagna, 1977, Morgan et al., 1978). It has been reported that tendons can store up to ten times more elastic strain energy than muscles in human running (Alexander

and Bennet-Clark, 1977), but it may be that a stiff muscle in series with the tendon exhibiting low stiffness, will optimise the utilisation of elastic energy (Hof et al., 1983). On the other hand, when force transmission is essential in activities not requiring a pre-stretch, a short, thick tendon exhibiting high stiffness is desirable for joint movement (Alexander, 1974, Biewener and Roberts, 2000).

An increase in tendon stiffness acts to improve the rate of torque development (RTD), which is indirectly supported by previous studies indicating an increase in stiffness with strength training (Hakkinen et al., 1985, Reeves et al., 2003a), and a decrease in electromechanical delay (EMD) (Cavanagh and Komi, 1979). Tendon stiffness also alters the joint angle during isokinetic and isometric maximal knee extensions (Kawakami et al., 2002a), and the force-velocity characteristics (Wilson et al., 1994) of the contractile elements of muscle. Increased stiffness favours force transmission, as less muscle fibre shortening is needed for a given amount and rate of overall muscle-tendon shortening, shifting the length-tension relationship to the right, allowing muscle fibre contractile units to operate closer to the plateau region or resting muscle length (Cutts, 1988, Lieber and Friden, 2000, Lieber et al., 1992). Concurrently, the optimal angle of the functioning muscle is also shifted.

The mechanical properties of tendon may also play a critical role in providing a protective mechanism against musculotendinous injury. An increase in tendon CSA and stiffness will decrease the stress and strain for a given magnitude of force per unit area, thus, dissipating the stress imposed on the structures, and potentially reducing injury risk (Couppe et al., 2008, Ker et al., 1988, Seynnes et al., 2009). Alternatively, it has been theorised that a stiffer tendon will induce a relative increase in elongation of the muscle fibre in response to mechanical loading, and hence, the muscle fibre will potentially be at a greater risk of strain overload (Onambele et al., 2006). Whereas, a more compliant tendon has the capacity to act as a mechanical buffer and protect the muscle fibre from strain overload (Griffiths, 1991). However, it is too early to link tendon stiffness to musculotendinous injury susceptibility, until the exact mechanisms of such injuries have been determined. This ambiguity may originate from the inherent difficulty in defining muscle and tendon behaviour independently. For example, there are different muscles that contribute to the forces that are experienced by the Achilles tendon *in vivo,* which may be a source of non-uniform stress distribution, and hence, the complexity of tendon injury aetiology is compounded (Arndt et al., 1998)

1.5.7 Tendon pathology

Tendon pathologies or tendinopathies (including tendinosis and tendinitis) are primarily degenerative conditions that may or may not be associated with signs of inflammation (Maffulli et al., 1998, Khan et al., 1999). Tendon pathologies can be a result of acute injury (e.g. laceration or single trauma) or chronic impairment (overuse injury or degeneration) (Killian et al., 2012), and are common in both recreational and elite athletes as well as in the general population (Almekinders and Temple, 1998). In acute injuries, extrinsic factors are dominant, such as excessive load on the body, training errors, and environmental substandard conditions (Kannus, 1997). In overuse injuries, the reasons are multifactorial, with intrinsic and extrinsic factors contributing to the pathogenesis (Kannus, 1997). For example, patellar tendinopathies in athletic or elite populations have been reported to be linked to normal foot posture (de Groot et al., 2012), low range of ankle dorsiflexion (Backman and Danielson, 2011), high total amount of exposure (Hagglund et al., 2011, Ferretti, 1986, Gaida et al., 2004, Crossley et al., 2007), eccentric overload of leg extensors (Boublik et al., 2011, Lian et al., 2005b), and knee joint angle, which was determined using computational models of cadaveric human tendon fascicles (Lavagnino et al., 2008b). Major limitations associated with these study designs is the unspecific diagnostic criteria used to define a tendinopathy, as well as the lack of direct assessment on tendon *per se*. Therefore, the precise mechanism for tendon pathologies remains poorly understood, most likely due to the complex composition, structure and mechanical behaviour of the tendon, as well as its interaction in series with muscle dynamics.

It is commonly reported that repetitive tissue micro-trauma resulting in injury to the material properties at a microscopic level, may be a major causative factor in the pathogenesis of overuse injury. Overuse or repeated overloading of tendon has been demonstrated in overuse animal models to lead to tendinopathies and microtearing of collagen fibrils, as well as weakening the tensile properties (Glazebrook et al., 2008, Huang et al., 2004, Perry et al., 2005, Soslowsky et al., 2002, Soslowsky et al., 2000, Neviaser et al., 2012, Andarawis-Puri et al., 2012, Andarawis-Puri and Flatow, 2011), which will consequently lead to rupture. It is believed that repetitive strains in the region of 4-8% which are below the failure threshold of the tendon cause such damage to tissue at a microscopic level (Curwin and Stanish, 1984, Kannus, 1997). Specifically, it is assumed that tendon matrix damage is the major event resulting from this repetitive loading, preventing the ability of the tenocytes and associated cell population from repairing structural defects (Riley, 2004). From microscopic observations, histopathology indicates

changes in cellularity, cell rounding, decreased matrix organisation, (including collagen fascicles and proteoglycans) and neovascularisation (Astrom and Rausing, 1995, de Mos et al., 2007, Riley, 2005). Although the nature of these degenerative changes may vary because of different sites and tendon types (Kannus and Jozsa, 1991, Samiric et al., 2009). Homeostasis within connective tissues such as tendon requires cell activity, so it is possible that disturbances in cell metabolism will cause imbalances between the synthesis and degradation of the ECM, thus, influencing structural properties and ultimately mechanical properties.

It is prudent to assume that by exploring tendon tissue at a biochemical and molecular level, a greater understanding of its dynamic function is possible. Indeed, during the last 20 years much research has focused on increasing our understanding of the underlying pathology in this respect. Studies investigating major changes in the molecular structure of the ECM have used techniques allowing for the quantitative assessment of protein analysis and gene-expression levels (Riley, 2008). In the instance of human pathologic tendon, matrix proteins such as the collagens (collagen type I and collagen type III), glycoproteins (TNC and fibronectin) and the majority of proteoglycans, have exhibited an increased expression of messenger RNA (mRNA) (Riley, 2004). Another pattern of increased expression is evident with growth factors such as TGF-ß (Fu et al., 2002, Fenwick et al., 2001), and cytokines, such as IL-6 (Legerlotz et al., 2011) and IL-1ß (Gotoh et al., 2001, Sun et al., 2008) involved in inflammatory responses, and glutamate (Schizas et al., 2010, Schizas et al., 2012), which is involved in the peripheral nervous system regulation of tendon homeostasis.

In terms of ECM regulatory processes, high levels of matrix remodelling indicative of increased collagen turnover and expression of associated proteins (collagen type I and III), are consistent with the onset of degenerative pathology, which may be associated to a high degree with the increased expression and proteolytic activity of the regulatory enzymes of the MMP family (Riley, 2004). For example, the expression levels of MMP3 appear to be elevated in highly stressed tendons as well as tendons displaying pathological characteristics, compared to normal tendons, as determined by histological analyses in animal models *in vitro* (Maeda et al., 2009, Asundi and Rempel, 2008, Birch et al., 2008). This observation is thought to represent a repair or maintenance function that may be associated with an underlying degenerative process (Jones et al., 2006). In addition, 'stress-shielding' or load deprived tendon has shown to increase the expression of MMP3 mRNA

in relation to normal tendon tissue samples (Asundi and Rempel, 2008, Leigh et al., 2008, Thornton et al., 2010). Collectively, these studies point toward a 'U' shape relationship between load and MMP3 expression levels. A loss of mechanical function may result from the extremes of this relationship which may relate to the subtle degradation of ECM components, notably those involved in cross-linking and/or stabilisation of the tendon structure (Leigh et al., 2008), such as minor collagens including collagen type V, as well as proteoglycans.

In contrast to high levels of MMP3 expression in pathological tendon, lower levels of MMP3 expression compared to normal tissue samples, have also been reported in human tendon pathologies (Ireland et al., 2001, Jones et al., 2006, Parkinson et al., 2010, de Mos et al., 2007). These observations may represent a failure of the normal matrix remodelling process (Riley et al., 2002). It should also be noted that even in normal tendon there is a significant difference between sexes, where males have twice the amount of resting mRNA expression levels of MMP3, compared to females (Sullivan et al., 2009). This may indicate an impaired ECM maintenance and weakening of the material properties in females, leading to an increase in injury susceptibility (Gray et al., 1985).

The ambiguity of MMP3 expression levels (increased and decreased in pathologies) evident between studies, may lie in complexities of controlling for the levels of mechanical loading, in particular, between animal and human studies, as well as the retrospective or prospective nature of these studies (whether the pathology was induced or not). Also, investigations into determining the expression of MMPs have elicited excessive strain directly on the tenocytes *in vitro,* which is likely to trigger high levels of proteolytic activity (Archambault et al., 2002a). These strain levels are potentially much higher than the strains experienced by cells *in vivo* (Riley, 2008), so it is uncertain to what extent these observations can apply to the aetiology of human tendon pathologies. Furthermore, it may be that increased MMP3 mRNA expression does not mean that a given amount of MMP3 protein will be produced, due to post-transcriptional and post-translational regulation (Matrisian, 1990, Riley et al., 2002). Therefore, the cellular and molecular processes associated with remodelling the ECM in tendinopathies remains largely elusive.
There is conflicting evidence as to whether pathological tendons affect mechanical properties in humans *in vivo,* even though it is widely documented that there are material changes of tendon tissue at a macroscopic and microscopic level. Some studies report no significant difference in mechanical properties in patients with tendon pathologies from

healthy subjects (Kongsgaard et al., 2005, Kongsgaard et al., 2009, Kongsgaard et al., 2010), possibly due to no change in fibril volume fraction. This assumption is supported by studies reporting no significant difference in total collagen content between normal and pathologic tendons (de Mos et al., 2007, Samiric et al., 2009). Other studies report a significant decrease in stiffness and an increase in compliance of the Achilles tendon, between middle-aged male running athletes with and without Achilles tendinopathies (Child et al., 2009), in similarly aged recreational active males (Arya and Kulig, 2010), and within elite athletes with unilateral tendinosis (Wang et al., 2012), as well as young healthy males with and without patellar tendinopathies (Liu et al., 2008). The decrease in stiffness observed in these studies may have negative implications for tendon function (refer to section on 'tendon function' 1.5.6) and movement production associated with the whole musculotendinous complex. However, the magnitude of stiffness changes in tendinopathies has yet to be determined particularly for clinical applications. Due to the cross-sectional design of these studies, no assumptions can be made about the temporal sequence of increased tendon compliance and tendon pathologic abnormalities, or the cause-effect relationship between mechanical properties and tendinopathies. For instance, whether the tendinopathy leads to decreased stiffness or alternatively, the decreased stiffness observed at region-specific lengths of the tendon (myotendinous junction) contributed significantly to the onset of the tendinopathy, remains to be determined. Also, the inconsistent results regarding human *in vivo* mechanical properties between studies on tendon pathology may be a consequence of different tendon types under investigation (total collagen levels vary between tendon types in normal tendons) (Riley et al., 1994, Samiric et al., 2009), activity levels in terms of degree of mechanical loading placed on tendon (higher in athletic populations) (Hansen et al., 2003, Kubo et al., 2002, Kubo et al., 2004, Reeves et al., 2005a), and sex (Kubo et al., 2003a, Magnusson et al., 2007, Onambele et al., 2007, Onambele-Pearson and Pearson, 2012), all of which are frequently reported to be highly influential on tendon material and mechanical properties.

Differential expression and/or changes in the metabolic turnover of specific macromolecules, known to serve important structural and functional roles in tendon homeostasis, are likely to influence changes in the mechanical properties of tendon, either with or quite separately from their influence on the incidence of pathologies. As alluded to in previous sections (1.5.4-material properties), TNC has both structural and regulatory roles within tendon tissue and may also be a strong candidate for involvement in the aetiology of tendinopathies and musculotendinous injuries. The expression levels of TNC

are altered with human tendinopathies, as determined by immunoblotting (Riley et al., 1996) and cDNA expression arrays (Ireland et al., 2001), although as with MMP-3, levels of new TNC protein synthesis may not directly follow mRNA expression levels. Nonetheless, the role of TNC may be particularly relevant when engaging in intensive activity following prolonged periods of inactivity. Inactivity has been reported to decrease tendon stiffness *in vivo,* particularly at the myotendinous junction (Kubo et al., 2000a), yet TNC expression relies on mechanical loading, and as it is an 'elastic' protein, a relative decrease in stiffness due to inactivity makes intuitive sense, if TNC is more highly expressed. Indeed, the myotendinous interface has been reported to be mechanically the most vulnerable site for injury (Kaariainen et al., 2000a, Kaariainen et al., 2000b), thus, during reloading after inactivity the overall extensibility (strain to failure) would decrease, increasing the risk of tendon rupture due to experiencing greater strains for a given load (Onambele et al., 2006). It remains to be determined whether the change in TNC expression is part of the degenerative processes in tendon pathologies or is the consequence of microtears or rupture, which may indicate a repair and remodelling response. In addition, its role in normal and pathologic, animal and human tendon *in vivo,* in terms of modifying mechanical properties, has yet to be affirmed.

1.5.8 Mechanical loading

1.5.8.1 Short-term loading

Numerous studies have reported the effects of short-term mechanical loading on observed changes in tendon stiffness in similar populations, with the same studies using different modes of mechanical loading to one another, namely static stretching, isometric plantarflexions, and two-legged hopping. Furthermore, these studies have assessed tendons in different anatomical positions; Achilles (Lin et al., 1996, Magnan et al., 1996, Liu et al., 2004b, Yang et al., 2004) and patellar (Wang et al., 2004) tendon. Comparisons between these studies are therefore not possible. For example, even though Kubo et al. (2001d) and Kay and Blazevich (2009) reported significant decreases in tendon stiffness with static stretching of the Achilles tendon, in age and level of activity-matched individuals, of independent populations, closer examination of their respective methods reveals entirely different static stretching protocols. Intermittent stretching for a total of 180s in full dorsiflexion was utilised by Kay and Blazevich (2009), compared to 10 minutes of stretching at 35° dorsiflexion, in the study by Kubo et al. (2001d).

Comparing studies that investigate tendons in different anatomical positions is of particular relevance when considering that, markedly lower increases in tendon stiffness of the

Achilles tendon compared to the patellar tendon, have been reported with plantarflexion and knee extension training, respectively ((36% (Arampatzis et al., 2007), 16% (Kubo et al., 2002), and 29% (Kubo et al., 2007b) versus 83% (Kubo et al., 2009), 58% (Kubo et al., 2001a), and 65% (Reeves et al., 2003a), respectively)). These differences have been assumed to be related to differences in the plasticity of tendon properties of these tendons. The mechanisms for these results are currently unknown, however, greater increases in blood circulation from rest to after exercise within the patellar tendon, suggests a higher metabolism over the Achilles tendon (Kubo and Ikebukuro, 2012). Hence, it may explain in part the differing adaptational changes in material and mechanical properties between these tendons.

It appears that tendon stiffness does change in response to short-term mechanical loading of different durations and tensions, however, the functional implications of this form of loading remains unclear. This is mainly due to the ambiguity across the methodologies between studies, as well as the lack of research in this area.

1.5.8.2 Long-term loading

When reviewing the mechanical properties of tendon in response to long-term loading, the ambiguity between studies still remains, and comparison of results between studies remains a difficult task, even with a greater amount of research having been conducted in this area (Table 1). These discrepancies are typified by the variations in the length of study (3-14 weeks), the number of participants undertaking the training protocol (7-33), and the modality of the training intervention.

One notable discrepancy in a number of these studies' designs is the combination of male and female subjects. This is problematic when attempting to interpret the results, considering that hormonal factors in females such as high oestrogen levels have shown to blunt the mechanical related adaptation of tendon tissue (Jemth et al., 2002, Rolny et al., 2002, Lindahl et al., 2002, Siems et al., 2002). Studies not directly assessing oestrogen levels also support these sex-specific differences (Kubo et al., 2003a, Magnusson et al., 2007, Onambele et al., 2007). Sex-specific factors will be discussed in more detail in a subsequent section of this thesis.

On a positive note, two studies by Arampatzis and co-authors (Arampatzis et al., 2007, Arampatzis et al., 2010) are of notable significance, as they adopt very similar research

designs, especially in terms of the training volume. They were able to replicate their findings, thus, allowing a direct comparison of the results. Both studies demonstrated an increase in stiffness with isometric, plantar flexion contractions. Moreover, what was more enlightening about their findings was that they were able to provide evidence *in vivo* of a threshold in strain magnitude (4.55-4.72%) that corresponded to the mechanical stimulus, required to increase tendon stiffness and modulus, as well as the frequency of contractions (3 s loading, 3 s relaxation) that support these adaptations. Lower frequencies equate to an increase in stiffness due to higher tendon strain duration per contraction. Therefore, further research is needed to understand the different loads and frequencies of contraction at which mechanical properties are modified. The *in vivo* studies reporting no change in tendon stiffness with training, may not have adapted a training intervention that applied a strain magnitude in excess of those experienced during habitual activity, in order to trigger adaptational effects on the morphological and mechanical properties of tendon. For example, Mathieu et al. (2007) and Kay and Blazevich (2009) adopted static stretch protocols, which exerted a low magnitude of strain over 20 s and 60 s, respectively. Replication of research designs is crucial in substantiating findings between studies, and therefore should be considered in future research in this area, in order to better our understanding of the mechanics of tendon in humans.

Table 1. Longitudinal studies outlining the effect of a training intervention on tendon stiffness *in vivo* in humans

Study	Length/ frequency of study	Participant numbers (intervention group)	Demographic	Training intervention	Tendon	Outcome measure (significance $P < 0.05$ for change)
(Kubo et al., 2012)	12 weeks/4 times/wk	9	Young active males	Unilateral isometric plantarflexions	Achilles	Increase
Arampatzis et al., 2010	14 weeks/4 days/wk	11	Young active males	Unilateral isometric plantarflexions	Achilles	Increase
Foure et al., 2010	14 weeks/34 one-hour sessions	9	Young active males	Plyometric training (~ 6,800 jumps)	Achilles	Increase
Seynnes et al., 2009	9 weeks/ 3 times/wk	15	Young active males	Unilateral resistance knee extensions	Patellar	Increase
Kubo et al., 2009	12 weeks/4 days/wk	10	Young active males	Unilateral isometric knee extensions	Patellar	Increase
Burgess et al., 2007	6 weeks/2/3 times/wk	13	Young active males	Unilateral explosive isometric, Unilateral straight-legged jump	Achilles	Increase for both interventions
Arampatzis et al., 2007	14 weeks/4 times/wk	11	Young active/ 8 females/3 males	Unilateral isometric plantarflexions (low strain magnitude), Unilateral isometric plantarflexions (high strain magnitude)	Achilles	Increase in high strain intervention only
Kubo et al., 2007d	12 weeks/4 days/wk	10	Young active males	Unilateral weight training, Unilateral plyometric training	Achilles	Increase in both interventions
Kongsgaard et al., 2007	12 weeks/ 3 times/wk	12	Young untrained males	Heavy resistance knee extension training	Patellar	Increase
Reeves et al. 2003a	14 weeks/3 times/wk	9	Elderly active/5 females/4 males	Isotonic resistance leg exercises	Patellar	Increase
Reeves et al., 2003b	14 weeks/3 times/wk	7	Elderly active/3 males/ 4 females	Isotonic resistance leg exercises	Patellar	Increase
Kubo et al.,	8 weeks/4	8	Young active	Resistance	Achilles	Increase in

2002b	times/wk		males	training with static stretching, Resistance training only		both interventions
(Foure et al., 2011	14 weeks/34 one-hour sessions	9	Young active males	Plyometric training (~ 6,800 jumps)	Achilles	Decrease
Mahieu et al., 2007	6 weeks/everyday	21	Young active/8 males/13 females	Ballistic stretching	Achilles	Decrease
Morrissey et al., 2011	6 weeks/ 3 times/wk	19	Young active/6 males/13 females	Eccentric training	Achilles	Decrease
Morrissey et al., 2011	6 weeks/ 3 times/wk	19	Young active/8 males/11 females	Concentric training	Achilles	No change
Fletcher et al., 2010	8 weeks/3 times/wk	12	Young highly trained distance runners	Isometric plantarflexions	Achilles	No change
Mahieu et al., 2009	6 weeks/everyday	33	Young active/19 males/14 females	PNF stretching	Achilles	No change
Kubo et al., 2009	12 weeks/4 days/wk	10	Young active males	Unilateral isotonic knee extensions	Patellar	No change
Mahieu et al., 2007	6 weeks/everyday	31	Young active/21 males/10 females	Static stretching	Achilles	No change
Kubo et al., 2006	12 weeks/4 days/wk	8	Young active males	Isometric squat training	Patellar	No change
Kubo et al., 2002a	3 weeks/everyday	8	Young active males	Unilateral static stretching	Achilles	No change

1.5.8.3 Effect of mechanical loading on structural and material properties

Having highlighted the variability in tendon responses to mechanical loading *in vivo,* between populations, and in the short and relatively long-term, described by tendon stiffness and modulus, it is important to try to understand how the mechanical properties change as a result. There is relatively strong evidence that tendons undergo hypertrophy or an increase in CSA with mechanical loading (Wang et al., 1994, Eastwood et al., 1998a, Eastwood et al., 1998b), with stiffness being directly dependent on CSA. Therefore, an increase in stiffness may be a result of increased CSA or tendon size (Arampatzis et al., 2007, Kongsgaard et al., 2007, Li et al., 2004). A recent study typifies this association by reporting a significant increase in tendon stiffness (36%) (Figure 13) with an increase in CSA (20-28%) of the patellar tendon in the lead leg, compared with the non-lead leg of elite fencers and badminton players (Couppe et al., 2008). By investigating such a population, the authors were able to adopt a unilateral training intervention to demarcate issues such as interindividual variations, associated with cross-sectionally designed studies.

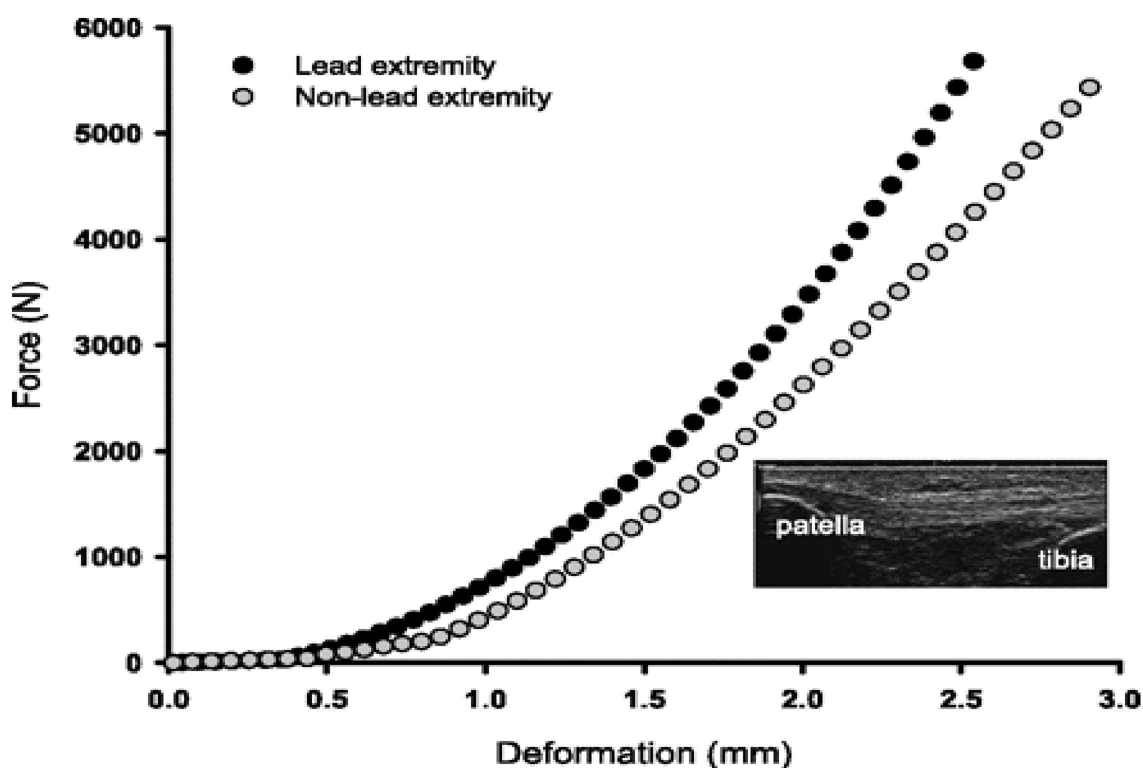


Figure 13. Patellar tendon force-elongation relationship to a common force. Values are means of all subjects. Stiffness was higher on the lead extremity compared with the non-lead extremity ($P < 0.05$), (Couppe et al., 2008).

As well as CSA contributing to the mechanical properties of tendon, the majority of evidence indicates that stiffness can increase without changes in CSA, in response to relatively long-term loading (Banes et al., 1995a, Pearson et al., 2007, Staufenbiel et al., 1989, Manning et al., 2011, Kubo et al., 2009, Pozio et al., 1989, Kabala et al., 1989, Kubo et al., 2002, Giaccari and Rossetti, 1989, Reeves et al., 2003a). Prime examples that highlight the evidence that CSA does not change in response to long-term loading include; Kubo et al. (2009) who found a substantial increase in stiffness of the patellar tendon of 83% in young men, partaking in a static resistance training programme over 12 weeks. Also, Reeves et al. (2003a) reported an increase of 65% in patellar tendon stiffness, in older individuals who undertook a strength training programme lasting 14 weeks (Reeves et al., 2003a). However, other evidence suggests only small, yet significant increases in tendon stiffness without a marked increase in CSA. For example, Arampatzis et al. (2010) report an increase in stiffness of less than 15% of the Achilles tendon in young men, once a static resistance training programme of 14 weeks' duration had been completed. These changes in stiffness without detectable changes in CSA may be attributed to the region-specific hypertrophy, which could not be detected in the aforementioned studies, due to the low number of locations assessed.

Qualitative changes to tendon tissue or material properties (modulus), reflecting adaptations of the underlying tendon microstructure to mechanical loading, may also be prominent in explaining changes in stiffness without marked changes in CSA. Numerous *in vivo* human studies indicate a net increase in collagen synthesis with exercise (Miller et al., 2005, Kjaer, 2004, Langberg et al., 1999, Langberg et al., 2000, Langberg et al., 2001, Langberg et al., 2007, Miller et al., 2007), which may in part contribute to the tendon's immediate adaptational response to mechanical loading. Young's Modulus (E) describes the mechanical properties of tendon when referring to the actual material properties, independent of CSA. Furthermore, a combination of changes in CSA and material properties are possible. Indeed, the studies that report an increase in CSA with a concurrent increase in stiffness cannot directly impute the increase in stiffness with an increase in CSA. The remaining variance observed once mechanical properties are calculated can be attributed to the modulus of the tendon (Equation 1) (Hou et al., 2009, Kongsgaard et al., 2007, Li et al., 2004). Incidentally, no studies have reported an increase in stiffness entirely explained by tendon hypertrophy, further underlining the importance of the material properties adapting to mechanical loading.

1.5.9 Sex-specific influences

It has been identified that female hormones may negatively affect collagen protein synthetic response in connective tissue, a possible underlying reason why there are sex-specific differences in injury rates and rates of healing, associated with exercise related musculoskeletal injuries (Jones et al., 1993, Geary et al., 2002, Gray et al., 1985, Kannus et al., 1987). In the past before US was applied to quantifying *in vivo* human tendon mechanics, and the introduction of validated techniques used to measure physiological markers of collagen synthesis in humans, it was believed that the higher incidence of overuse injuries among females was primarily due to intrinsic factors, related to a weaker musculoskeletal system, compared to males of equal body mass (Kannus, 1997). These intrinsic factors include an average higher body fat percentage, less muscle mass per unit body weight, overall lower muscle strength, lower bone mass, and risk factors associated with body anatomy and biomechanics.

In tendon, there are oestrogen receptors that are responsive to female sex hormones (Hart et al., 1998, Wentorf et al., 2006). Studies in animals have demonstrated that oestrogen may have an inhibiting effect on collagen synthesis (Fischer, 1973, Liu et al., 1997b, Irie et al., 2010, Yu et al., 1999), and indeed more recently, direct measurements of collagen synthesis at rest and after a single bout of mechanical loading in human tendon, have shown a significant blunting effect of oestrogen in young women administered synthetic oestrogen, over controls (Hansen et al., 2009b, Hansen et al., 2008). Indirectly, the negative relationship between oestrogen and collagen synthesis is emphasised in a cross-sectional study that compared tendon collagen synthetic rates between men and women (Miller et al., 2007). This study shows that women had an approximately 50% lower collagen synthetic production at rest and after exercise compared to men, and is partly supported at a molecular level by reduced levels of regulatory mRNA expression (MMP-3). In addition, the levels of resting collagen type III mRNA expression in women were greater than those of men. Enhanced production of collagen type III may be indicative of a reparative type response (Eriksen et al., 2002, Maffulli et al., 2000, Williams et al., 1984) with the tendency to produce smaller and less organised fibrils than collagen type I (Lapiere et al., 1977). These fibrils have found to exhibit inferior mechanical properties compared to normal healthy tendon (Tohyama et al., 2003). Therefore, this observation may be a possible explanation as to why women are more susceptible to tendinopathies than men (Riley et al., 1994).

From a functional perspective, mechanical properties of isolated collagen fascicles in women showed a reduced stress-to-failure and less than half the elastic modulus of that of men (576 MPa vs. 1231 MPa) (Magnusson et al., 2007). This finding correlates well with the sex-specific differences in the metabolic activity of tendon collagen and points toward a compromised collagen fibril, or a reduced capacity to regulate fibril diameter distribution. It has been reported that endogenous levels of oestrogen do not affect the strain behaviour of Achilles tendon, however long-term exposure to reduced oestrogen in females administered monophasic oral contraceptive pills, decreased tendon strain by 25.5% (Bryant et al., 2008). The authors suggested that this was because collagen synthetic rates would be augmented following the habitual training loads of these females, who were highly trained runners, so it may be that oestrogen has a minimal effect on the material properties of tendon within normal physiological limits.

In post-menopausal women there is a rapid decline in oestrogen levels (Bjornerem et al., 2004), so it is assumed that sex-specific differences if any, on the mechanical properties of tendon in age and lifestyle matched males, would not be induced directly by oestrogen or indirectly by its effect on reducing the bioavailability of IGF-1 (Hansen et al., 2009b). Indirect support of these findings can be found by studies that reported no significant differences in tendon mechanical properties between sexes *in vivo*, when normalising for force output (Burgess et al., 2009b, Carroll et al., 2008, Carroll et al., 2011). Interestingly, a study by Onambele-Pearson and Pearson (2012) reported a reduced capacity of female tendon to adapt to a standardised resistance training protocol, than that of aged-matched males. Males exhibited a more dramatic increase in patellar tendon stiffness when normalising force output (317 to 580 $N \cdot mm^{-1}$ vs. 380 to 402 $N \cdot mm^{-1}$) and in addition, a sex-specific pattern of changes in tendon stiffness were identified, with males exhibiting greater increases above 40% MVC, and females below this level. Tendon stress, strain and Young's Modulus showed similar changes between sexes. The authors suggested that sex-specific differences in tendon stiffness changes may be a result of preferential adaptive responses to lower loads in females, possibly linked to the mechanotransduction mechanism. Seynnes et al. (2011) also reported a blunted increase in patellar tendon stiffness in post-menopausal women, following 12 weeks of alpine skiing training. A 7% increase in Young's Modulus for women was demonstrated, whilst age and trained matched males demonstrated a 17% increase. However, sex-related differences in Young's Modulus did not reach significance ($P = 0.12$). Therefore, it can be assumed from this particular study, that there are no sex-specific differences or adaptations.

In contrast to the reviewed studies previously, another study investigating the effect of stretching on the mechanical properties of Achilles tendon between males and females, found that females were more responsive, with a 22.4% and 20.5% decrease in stiffness and Young's Modulus, respectively, versus 8.8% and 8.4% for males (Burgess et al., 2009a). The mechanisms for this change were unidentified, although structural dimensions were ruled out due to no change in CSA or length after this intervention. Alterations in the material properties of tendon were proposed, with the effect of oestrogen being suggested as an influential factor on tendon tissue quality, although hormonal factors were not directly measured. Moreover, the shorter tendon moment arm length in females was also suggested to contribute to this disparity, with greater force being experienced in the tendon of females in the region of 13%.

Interestingly, a study by O'Brien et al. (2010) found no difference in patellar tendon stiffness between adult males and females as well as pre-pubertal boys and girls, and so acknowledged that sex hormones are not playing an influential role in this case. Instead, it was proposed that the indifferent development of the tendon with maturation between males and females, may contribute to a greater extent to the mechanical properties, than mechanical loading or sex hormones, for example. Young's Modulus was shown to increase in males, but both CSA and Young's modulus increased in females.

There appears to be a lack of unequivocal evidence for sex-specific differences on the mechanical properties of tendon in response to mechanical loading, and it is still unclear as to the extent sex hormones such as oestrogen can induce on its own, or in combination with mechanical loading, on the global dimensions (Finni et al., 2009, Cook et al., 2007), and material and mechanical properties of tendon.

1.5.10 Ageing

Ageing can be defined as a progressive functional decline or a gradual deterioration of physiological function with age (Partridge and Mangel, 1999). Ageing has been reported to have an adverse effect on tendon tissue, particularly on its associated collagenous and non-collagenous structures (Thorpe et al., 2010, Butler et al., 1978, Diamant et al., 1972, Tuite et al., 1997, Kjaer, 2004). Further, biological maturation is any process that marks progress toward the adult (mature) state (Beunen et al., 2006).With increasing age or biological maturation, tendon collagen content increases and then plateaus after maturity, while proteoglycan content decreases and elastin increases (Elliott, 1965, Ippolito et al., 1980,

Vogel, 1991). Ageing and biological maturation are therefore separate processes that need to be examined independently, in order for comparisons between studies to be consistent.

With ageing, collagen fibril distribution may change with an increase in the ratio of collagen type III and V to collagen type I (Riley et al., 1994, Kjaer, 2004), as well as a decrease in collagen fibril diameter (Nakagawa et al., 1994, Dressler et al., 2002, Gillis et al., 1997). The slower rate of turnover of collagen after maturity results in the accumulation of a number of irreducible cross-links. This occurs via the process of non-enzymatic glycation which produces advanced glycation end-products (AGEs), such as pentosidine, of which is a widely accepted marker of tendon matrix age (Paul and Bailey, 1996, Moriguchi and Fujimoto, 1978, Bank et al., 1999, Couppe et al., 2009). Consequently, the material properties are altered in ageing tendon, synonymous with reduced elasticity, increased stiffness, decreased solubility and increased thermal stability (Vogel, 1983). In addition, because of an increased collagen and decreased water content, the permeability of the matrix is reduced, which negatively affects tenocytes nutrition and energy production pathways (O'Brien, 1992). Therefore, there is a decrease in collagen turnover and cell-matrix activity, which reduces the capacity of the tendon to repair (Astrom and Rausing, 1995). Recent evidence suggests that there is an increase in partially cleaved collagen within the matrix with mechanically induced micro-damage with age (Thorpe et al., 2010). As a result, the functional capacity and material and mechanical integrity of tendon is compromised, which may explain the increased prevalence of degenerative changes and injury (Hess et al., 1989, Hess, 2010).

When relating these findings to the mechanical properties of tendon, there are inconsistencies in the evidence provided. It appears that many animal studies investigating the role of ageing on tendon, use cross-sectional designs that include very young animals (Shadwick, 1990, Nakagawa et al., 1996, Ensey et al., 2009). Therefore, when comparing data from such populations, it is possible that maturation and the ageing process were not independently examined, thus, introducing confounding effects (Narici et al., 2008). When comparing mature tendon with tendons from older animals, such confounding effects are avoided and the general consensus is that older tendon tissue is less stiff and more compliant than mature tendon (Vogel, 1991, Vogel, 1980, Blevins et al., 1994, Nakagawa et al., 1996). However, relating *in vitro* mechanical tests to *in vivo* function with ageing should be interpreted with caution.

Recently, findings based on US techniques show that mechanical properties of human tendons *in vivo,* change considerably with the ageing process, with age associated reductions in stiffness (Reeves et al., 2003a, Reeves et al., 2005b, Magnusson et al., 2003a, Kubo et al., 2003b, Karamanidis and Arampatzis, 2006, Karamanidis and Arampatzis, 2005, Narici et al., 2005, Maganaris et al., 2006, Morse et al., 2005, Onambele et al., 2006, Mian et al., 2007, Mademli et al., 2008, Baudry et al., 2012). For example, Onambele et al. (2006) reported a 48% decrease in Young's Modulus of the Achilles tendon, in a cross-section of old and young individuals (average age of 68 and 24 years, respectively). This decrease in Young's Modulus was not attributed to the ageing process but instead a reduction in CSA of tendon, possibly induced by mechanical unloading or disuse. Other studies attribute the decreased stiffness with ageing to material changes, for example, a 15% decrease in stiffness of the Achilles tendon was reported by Narici et al. (2008), yet no differences in tendon dimensions were detected. In contrast, a 22% thicker tendon was reported in elderly women compared to young women (Magnusson et al., 2003a). However, the exact mechanisms accounting for the compromised mechanical properties in tendon, cannot be assessed without analysing the composition of tendon at a microstructural level, and in a hierarchical order, which is not possible with current *in vivo* technology (Narici et al., 2008).

A recent cross-sectional study examining the mechanical properties of patellar tendons *in vivo* in old and young men, found that neither the dimensions nor the mechanics changed with ageing, yet there was a marked difference in the material properties (Couppe et al., 2009). Specifically, a seven-fold increase in AGEs cross-link density in older individuals was reported. An increase in cross-link density has shown to increase tensile stress and tendon stiffness (Andreassen et al., 1988, Andreassen et al., 1981, Bai et al., 1992, Galeski et al., 1977, Verzar, 1963, Reddy, 2004, Reddy et al., 2002), yet this was not the case. Furthermore, a 33% decrease in collagen concentration was present in older men, possibly indicating age-related mechanical unloading and signifying that other non-collagenous components of tendon may increase to maintain CSA. From a functional prospective, maintaining tendon stiffness would serve to maintain effective execution of motor tasks, through faster transmission of contractile forces.

There is a plethora of evidence indicating that tendon stiffness is markedly reduced in old age, yet in this demographic, tendon still retains the plasticity to adapt to resistive loads by increasing tensile stiffness (Reeves et al., 2003a, Reeves et al., 2003b, Reeves et al., 2005b,

Onambele et al., 2008, Onambele-Pearson and Pearson, 2012). However, the underlying causal mechanism for alterations with ageing have not been defined, particularly at microstructural and molecular levels, and it remains to be determined whether ageing alone is associated with these observations.

In summary, intrinsic factors such as age, sex and mechanical loading history as discussed in the preceding sections of this review, may be heavily implicated in the relationship between material and mechanical properties of tendon, via the control hub of tendon tissue, the tenocytes. Mechanical signalling from external loads related to training volumes, oestrogen's effect on collagen protein synthesis, and age-related changes in enzymes regulating cross-linking, are notable mechanisms by which gene expression can possibly vary greatly in humans, regulated at a genomic level within the tenocytes' nucleus. However, even if these intrinsic factors can be controlled so that they can be quantified and normalised to a baseline value, the associated gene products at a protein level can still vary. This is where genetic variation can potentially contribute to observed/measurable differences on complex traits, such as the material and mechanical properties of tendon.

1.6 Genetics of tendon

A strong genetic component may influence tendon mechanical properties such as stiffness at a phenotypic level, through the functional capacity of the tendon's material properties. Although to date, no genetic association has been reported on tendon properties *per se*. Recent work has associated tendinopathies with genetic variants in proteins that serve important structural and functional roles in tendon (Mokone et al., 2005, Mokone et al., 2006, September et al., 2008, Raleigh et al., 2009, Posthumus et al., 2010a), so it is plausible that these same molecular characteristics may influence tendon mechanical properties *per se*. That is to say, the same gene variants and differential gene expression of these same proteins may directly influence tendon mechanical properties. To a lesser extent gene variants linked to the predisposition of incurring tendinopathies have also been reported to be influential on musculotendinous range of motion (Collins et al., 2009, Brown et al., 2011b) as well as endurance running performance (Brown et al., 2011a, Posthumus et al., 2011), which will be discussed later in this thesis.

1.6.1 Genetic variation

Generally, genetic association studies link a phenotype of interest (e.g. tendon mechanical properties) with genetic factors. Differences between individuals for a particular trait when referring to genetic influences may be a result of genetic variability somewhere in the genome. Simply put, nucleotide differences among the DNA sequence of individuals can influence function.

Many types of genetic variation exist, though the most common form of genomic variation are single-nucleotide polymorphisms (SNPs), of which 11 million are estimated to exist in the human genome with a frequency of greater than 1% (Kruglyak and Nickerson, 2001). The SNPs within DNA coding regions are transcribed into their analogous mRNAs and these mRNAs are translated into their associated proteins, thus, SNP information is carried through three levels of information, from DNA to RNA to protein, by transcription and translation. Within these SNPs, different alleles are found in different individuals and these alleles can influence the three levels of information, by altering amino acid sequences or by influencing how the gene is regulated. This can have consequences for the type or amount of protein produced, potentially altering one or more phenotypes. However, these sequences of events are not straightforward, as the pathway from genotype to the phenotype can be complex. For instance, at the DNA level, methylation, and chromatin structural and biochemical changes may influence how the gene is transcribed to RNA. Indeed, this is possible at the transcription level where expression, microRNA (miRNA),

and post-transcriptional modifications can occur. Finally, at the translational level, splice variants, isoforms and tertiary/quaternary structural changes that occur via synonymous SNP variants, can affect the outcome or phenotype (Shapshak, 2012). For example, the activity of proteinases such as the MMPs that are primarily involved in the degradation of tendon ECM (refer to section 1.5.4.1-Regulatory properties of ECM), may be regulated at transcriptional or translational or post-translational levels (Jones et al., 2006). It is doubtful as to whether corresponding variation occurs at each level. Fundamentally the structure and function of the ECM, and consequently the end result mechanical properties at the phenotypic level, may not be directly related to the SNP information. Therefore, the heterogeneity of the SNPs can reduce the sensitivity required to detect gene associations in such studies, and ideally analysis at all three levels (DNA, RNA and protein) is warranted (Shapshak, 2012).

1.6.2 Identifying candidate genes

Genetic association studies in an exercise and health-related setting aim to investigate the correlation between the phenotype of interest and genetic variation, by identifying candidate genes that contribute to the variability within the phenotype. Ultimately, key gene variants may eventually be used clinically to improve the prevention and treatment of various diseases, by prescribing exercise, diet, and pharmacological interventions, based on an individual's genetic profile (Roth, 2007). However, genetic research and its applications can potentially cause ethical concerns, in that genetic research can be used for early talent identification and screening for risks of sudden death, which in turn may have severe consequences for that individual, upon receiving such a diagnosis (Wackerhage et al., 2009). In terms of genetics of tendon properties, candidate genes that relate to improved performance capacity and risk of incurring tendon pathologies is of primary importance.

Discussed below are the rationales for determining the importance of genetic contribution to the phenotype, so that the result will be a specific testable hypothesis-driven research question (s). Three key components need considering when identifying candidate genes for a genetic association study; the phenotype, subject recruitment and of course candidate gene/SNP selection.

1.6.2.1 Phenotype

Firstly, it is important to define the phenotype under investigation, whether it is to be discretely or continuously measured. For example, tendon stiffness is a quantitative trait that can have a range of values determined by force-displacement curves. By correctly defining the phenotype (s) of interest, the importance of genetic factors can be determined, as endpoint assessment may be too remote to detect the modifying genetic effects of the gene variants, if the phenotype was too generalised. Secondly, heritability has been reported for the specific phenotype that was previously defined. Low heritability suggests that there is a strong non-genetic contribution to the phenotype, which in essence decreases the power of genetic association studies (Sham et al., 2000). Heritability can be estimated using twin studies by looking for greater similarities of the phenotype measurement values, in related individuals (Kang et al., 1978) compared to between families, known as familial aggregation (Bouchard et al., 2000). Therefore, when embarking on an association study, prior evidences that genetic variation plays some role in determining the phenotype is desirable.

No direct association has been reported for heritability and its effect on structural and mechanical properties of tendon, yet interpretation of research literature relating to this domain provides evidence for the importance of genetic factors. For example, genetic variants that have been reported in recent tendinopathy studies, encode for proteins directly involved in biological processes within tendon. Moreover, there are studies reporting a moderate to strong genetic component with measures of flexibility, 70% (Hakim et al., 2004) and 47% (Battie et al., 2008), respectively. Flexibility is a composite measure of muscle-tendon joint dynamics and it has been suggested that candidate genes with respect to flexibility, include those related to structure and function in tendon, such as collagens and tenascins (Hakim et al., 2004). Therefore, these genes can also be considered as candidate genes for association with fundamental tendon properties.

The ease and accuracy of measurement in large numbers of individuals would minimise measurement error, thus, the noise found to contribute to total variability can also be minimised (Newton-Cheh and Hirschhorn, 2005). Accurate and reproducible assessments of tendon properties *in vivo* are available, although as of yet these techniques have not been applied to genetic association studies in humans.

1.6.2.2 Subject recruitment

Once the phenotype (s) has been clearly identified and genetic influences have been reported previously, the population sample can be selected. Within an exercise and health-related domain, quantitative traits are common, whereby large sample sizes can be used to ensure the answers to the hypothesis-driven research question (s) are statistically robust, and numerical data can be attributed to the discrete genotypes. The classic 'stress-the-genotype' approach has been popular when recruiting a particular group of subjects, as it aims to optimise the association of complex traits with genetic variation (SNPs), by virtue of enlisting a high proportion of homozygote individuals (Montgomery et al., 2002). Also, recruiting subjects based on randomised sampling that is prospective in nature is advantageous, as it permits the statistical control of environmental factors, reported in the research literature to affect the phenotype, as well as allowing the investigation of specific genetic factors (Kavvoura and Ioannidis, 2008). A vast majority of the genetic association studies, particularly in tendinopathies, have been retrospective and case-control type study designs. In these cases, knowledge of the pathology may influence recall of exposures to environmental factors and this can introduce responsive bias (Kopec and Esdaile, 1990). Randomised sampling of unrelated subjects is desirable as it avoids the difficulties in recruiting related individuals, but also avoids the possibility of committing ascertainment bias, if different generations of the family are included (Liu et al., 2011). However, recruiting unrelated subjects in this manner can introduce false-positives from admixture and population stratification, by misidentifying a genetic association with a trait. The main issue that arises is that populations with different geographic ancestries (e.g. African, Asian, Northern European), may have different allele and genotype frequencies for the polymorphisms under investigation, and so this can affect gene-gene interactions (alleles in one gene interact with alleles in another gene), and consequently the resulting combination can have unique influences on the phenotype (Lewis and Knight, 2012, Freedman et al., 2004). Therefore, it is important to prospectively control for these factors, in order to detect true associations, by focusing on one specific ethnic group of origin. Besides, because genetic association studies only focus on one or two genes, mainly due to logistics of time and limited technologies used for genotyping, statistical power is also limited. This is because most genes contributing to a phenotype confer only a very modest effect, and to detect these associations with high power, requires large sample sizes (Lewis and Knight, 2012, Newton-Cheh and Hirschhorn, 2005, Ioannidis et al., 2003, Long and Langley, 1999).

1.6.2.3 Candidate gene selection

Identifying potential candidate genes begins by identifying the proteins that are critical to the structural and regulatory systems that underlie the physiology of the phenotype. These proteins are coded for by genes, which become the candidate genes of interest (Roth, 2007). Prioritising these candidate proteins is the next phase toward selecting the most appropriate candidate genes. This can be achieved by reviewing the existing literature that intensely focuses on these proteins, and their involvement in key physiological pathways, either in homeostatic or pathologic states. As a result, these proteins can then be paired with their coding gene (s) and further prioritising can be applied to the candidates at the level of the gene and known genetic variants. Genes that have been intensively studied for their importance to the underlying physiology of the trait should be considered first. Animal models that characterise the genes and their associated pathways should be established, for instance, genetic manipulations within the same models that use gene knockout methods, can be used to assess their importance functionally. Furthermore, genes that are expressed only within the tissues of interest for the phenotype should be considered priorities as well, above genes that are ubiquitously expressed in many tissues (Roth, 2007).

The next step is to identify functionally significant SNPs within these genes that are likely to modify the protein. These include missense SNPs which have been intensively studied for their beneficial, as well as deleterious effects on the phenotype (Fay et al., 2001, Cargill et al., 1999), but also non-coding SNPs involved in regulating sequences, are becoming more appreciated for their role in complex traits (Newton-Cheh and Hirschhorn, 2005). Moreover, including multiple SNPs and indeed those involved in the same regulatory pathways for the phenotype, is likely to be a preferable approach in addressing their potential genetic influence in genetic association studies. Technological advancements in genotyping (SNP chips) have the potential to detect the vast majority of genomic variation for the phenotype, by assaying all gene variants known to be involved. This is made possible by the tightly correlated structures for which these gene variants reside in, termed haplotype blocks. Haplotype blocks are part of a classic concept known as linkage disequilibrium which describes the extent to which polymorphisms or alleles travel together during recombination events (Daly et al., 2001). However, the cataloguing of common gene variants involved in all exercise and health-related traits is very much in its infancy, and so currently genetic association studies investigating SNPs singularly, or as multiples, are of upmost importance, so that appropriate interventions can be developed

(Manolio, 2010). In addition, determining the functional basis for these associations is essential.

1.7 Proteins, genes and SNPs of interest

In the following section there will be a detailed review of candidate genes, as well as their respective SNPs, and the potential influences they may have on tendon properties at a protein level. Specifically, seven genes will be discussed together with the five tendon proteins they produce. Table 2 summarises this relationship. Four of the genes listed in Table 2 *(COL5A1, TNC, MMP3, GDF5)* have genetic variants reported in recent tendinopathy studies, that may predispose individuals to such conditions (Posthumus et al., 2010a, Mokone et al., 2005, September et al., 2008, Mokone et al., 2006, Raleigh et al., 2009). Figure 14 shows the key structural and regulatory proteins found in tendon, including those associated with tendon pathologies, musculotendinous ROM, and endurance running performance. These genes can be considered candidates for their association with fundamental tendon properties. In fact, two studies have recently reported an association between a SNP in one of these genes and measures of musculotendinous ROM in humans (Collins et al., 2009, Brown et al., 2011b). One study reports an association with musculotendinous ROM as well as endurance running performance (Brown et al., 2011a) with one similar study using an independent population, reporting an association with endurance running performance only (Posthumus et al., 2011). Table 3 summarises the genetic association studies that have identified a polymorphic association with tendon pathologies, musculotendinous ROM, and endurance running performance in humans. In addition to the four genes associated with these phenotypes (*COL5A1, TNC, MMP3, GDF5), COL5A2* will also be discussed because like *COL5A1,* it encodes for a protein which is a fundamental component of the collagen type V molecule (Col V) (quaternary protein). Collagen type I (Col I) and its two coding genes *COL1A1* and *COL1A2,* will also be included in the proceeding discussion, because it forms the major structural component of tendon, even though genetic variation has not yet been associated with these phenotypes or tendon properties. Associations have however been observed between genetic variation in *COL1A1,* and risk of ligament injury, and so this rationale justifies its inclusion and further discussion.

Table 2. Tendon proteins, genes of focus, and abbreviations addressed in this thesis.

Protein	Abbreviation of protein used in this review	Genes of focus in this review	Abbreviation of gene of focus
Type V collagen	Col V	collagen, type V, alpha 1 collagen, type V, alpha 2	*COL5A1* *COL5A2*
Tenascin C	TNC	tenascin C	*TNC*
Matrix metalloproteinase-3	MMP3	matrix metalloproteinase 3 (stromelysin 1, progelatinase)	*MMP3*
Growth/differentiation factor 5	GDF5	growth differentiation factor 5	*GDF5*
Type I collagen	Col I	collagen, type I, alpha 1 collagen, type I, alpha 2	*COL1A1* *COL1A2*

Note: the two collagen proteins comprise numerous protein chains and therefore are dependent on more than one gene.

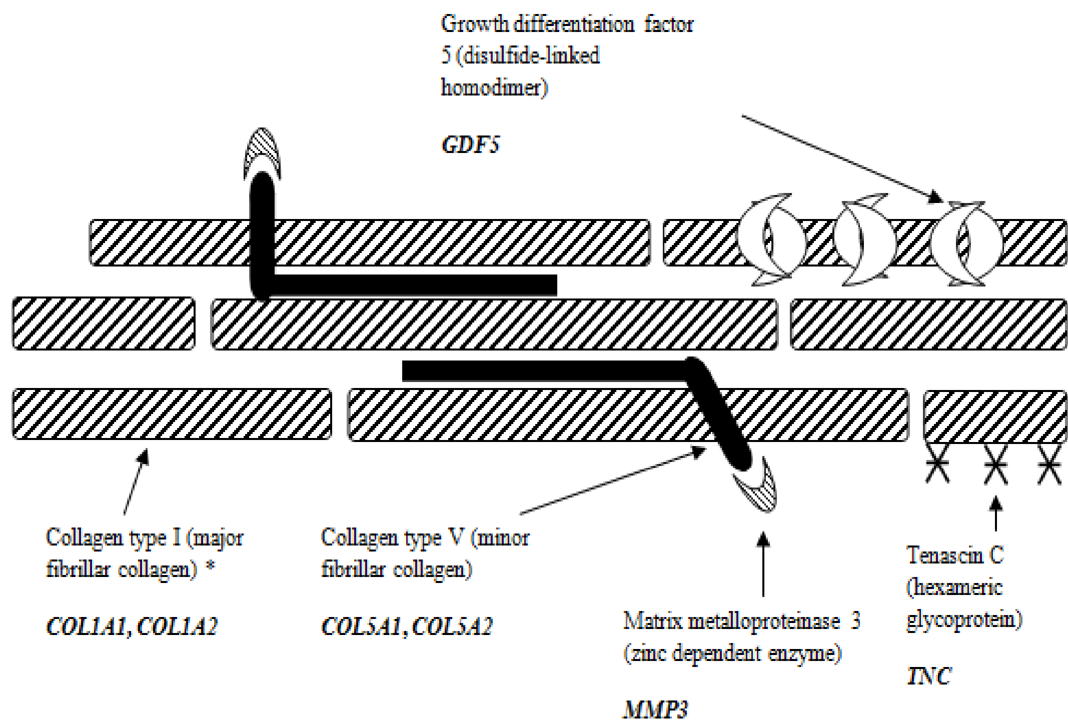


Figure 14. Major microstructural components of tendons associated with tendon pathologies/musculotendinous range of motion/endurance running performance, identifying related genes. * No genetic association with tendon pathologies/tendon properties yet reported, but its presence in this figure is warranted because it is the major structural component of tendon. Adapted from Collins and Raleigh (2009).

Table 3. Summary of genetic association studies that have identified a polymorphic association with tendon pathologies/musculotendinous range of motion and endurance running performance in humans.

Gene	Participants	Gene variant	Phenotype	Findings/Observations	Study
	White Caucasian. 72 with chronic ATP, 39 with acute Achilles tendon rupture. 129 control.		Chronic ATP.	Individuals with A2 (C) allele gene variant of this gene are less likely of developing symptoms of chronic Achilles tendinopathies	(Mokone et al., 2006)
	White Caucasian. 85 Australian and 93 South African patients with ATP, respectively. 210 Australian and 132 South African control subjects.			Individuals possessing 'CC' genotype had decreased risk of developing chronic ATP compared with those individuals with T allele (TC or TT genotypes) in both populations	(September et al., 2008)
COL5A1	White Caucasian. 50 with chronic ATP, 35 with acute Achilles tendon rupture. 34 control.	*Bst UI* RFLP within 3' untranslated region (UTR) (rs12722 C/T)	Standing leg raise, sit-and-reach.	Individuals with CT genotype were found to be less flexible than homozygous individuals	(Collins et al., 2009)
	White Caucasian. 325 healthy and physically active cohort.		Sit-and-reach.	Older individuals (≥35 years) homozygous for the C allele showed greater flexibility	(Brown et al., 2011b)
	White Caucasian. 72 runners (52 males,		Sit and-reach Time to completion	TT individuals were less flexible than CC individuals but TT	(Brown et al., 2011a)

	20 females)			individuals were faster than CC individuals	
	White Caucasian. 313 male triathletes		Time to completion of running component	Individuals with TT genotype completed running component faster than CC individuals	(Posthumus et al., 2011)
TNC	White Caucasian. 72 with chronic ATP, 42 with acute Achilles tendon rupture. 127 control	GT dinucleotide repeat polymorphism within intron 17	Chronic ATP. Acute Achilles tendon rupture.	Individuals with 12 and 14 GT repeats appear to have 6-fold risk of developing Achilles tendon injuries. 13 and 17 repeats were underrepresented	(Mokone et al., 2005)
MMP3	White Caucasian. 75 with chronic ATP, 39 with acute Achilles tendon rupture.	(rs679620) A/G transition at nucleotide position 28 within exon 2, (rs591058) T/C transition at nucleotide position 1547 within intron 4, (rs650108) G/A transition at nucleotide position 495 within intron 8.	Chronic ATP.	GG of rs679620, CC of rs591058, AA of rs650108 genotypes overrepresented in individuals with ATP but no association found independently with individuals with acute Achilles tendon rupture. Additional observation- inferred haplotype ATG greater in control subjects	(Raleigh et al., 2009)
GDF5	White Caucasian. 171 recruited. Australian population- 59 with chronic ATP. South African population- 73 with chronic ATP, 39 with acute Achilles tendon rupture. Australian population-	(rs143383) T/C substitution of functional promoter in 5' UTR	Chronic ATP. Acute Achilles tendon rupture.	Individuals with TT genotype have twice the risk of developing both chronic ATP and acute Achilles tendon rupture	(Posthumus et al., 2010a)

	142 control, South African population- 96 control.				

* ATP= Achilles Tendon Pathology

1.7.1 *COL5A1* as a candidate gene

Structure and function of protein and genes

Col V is a widely distributed quantitatively minor fibrillar collagen forming between 1-3% of total collagen content of tendon ECM (Chanut-Delalande et al., 2004), although evidence suggests that in functional terms it is a major collagen of developing connective tissues (Roulet et al., 2007). Col V can assemble into a diverse number of molecular forms but all contain a pro α1(V) chain. This pro α1(V) chain is encoded for by the *COL5A1* gene (9q34.3) and comprises 66 exons, distributed over 203.07 kilobases (kb) of genomic DNA. There is a pro α2(V) chain which is encoded for by the *COL5A2* gene (2q32.2), comprising of 54 exons and 147.98kb (Birney et al., 2004). Together these chains form the heterotrimer protein structure of Col V ($[\alpha1(V)]_2\alpha2(V)$), which is ubiquitous in human tendons.

Col V plays a functionally important role in tendon via its relationship with Col I fibrils. It is thought to co-polymerise with Col I fibrils to form heterotypic fibres, and thereby organises and regulates the diameter of these fibres (Birk et al., 1988, Birk et al., 1990, Linsenmayer et al., 1990, Marchant et al., 1996) as well as forming intermolecular cross-links with Col I fibrils (Niyibizi and Eyre, 1993). Both a decrease (Birk, 2001, Wenstrup et al., 2004) and an increase (Dressler et al., 2002) in Col V content, has been reported to decrease the diameter of the collagen fibril *in vitro*, which suggests there is an optimum amount of Col V for normal tendon function. Outside this optimum physiological range, a weakening of the material properties is possible, causing reductions in maximum stress and linear modulus (Dressler et al., 2002).

Focusing attention to a molecular level, the *COL5A1* gene and in particular a specific SNP in the 3' untranslated region (UTR), thought to regulate gene expression, will subsequently be discussed in relation to its association with complex exercise-related phenotypes. Recently, the biological function of this region of the gene has been investigated to enhance our understanding of the molecular basis of such phenotypes (Laguette et al., 2011). The investigators report novel findings that the mRNA stability is affected by gene

variability in this region, including that of the *COL5A1* rs12722 gene variant, which will be introduced in the following section. An increase in mRNA stability associated with the T allele variant was proposed to produce more α1(V) chain protein, and thus, increased Col V production. Subsequently, a possible direct link can be established between this gene variant and the tendinopathic phenotype.

Evidence of polymorphic associations with tendon pathologies

The first study to report an association between variation in the *COL5A1* gene and tendon pathology (Mokone et al., 2006), identified the *COL5A1* gene as an ideal candidate genetic marker of Achilles tendinopathies, because it is on the same locus of genomic DNA as the ABO gene, which encodes for transferases as part of the structure of glycoprotein antigens in red blood cells (Jozsa et al., 1989). It has been reported to be associated with tendon injuries in Hungarian and Finnish patients with the blood group O, and it may also determine the structure of ECM proteins in tendon (Jozsa et al., 1989, Kujala et al., 1992). Two restriction fragment length polymorphisms (RFLPs) were identified within the 3' UTR of the *COL5A1* gene (*BstUI* and *DpnII*) that had no known role in the expression or function of Col V. An association was found between the *BstUI* RFLP (rs12722) and Achilles tendon pathology (ATP), and more specifically, chronic tendinopathy without rupture, with the 'C' allele being protective against ATP (control group-29.8% vs. ATP-18%) (Mokone et al., 2006). As the authors rightly stated, this association does not show conclusively that Col V is involved in the development of these pathologies, and it is of course likely that there are numerous genetic variants that contribute to the overall heritability of such conditions (Magra and Maffulli, 2008). Magra and Maffulli (2008) did however emphasise that genes encoding for other collagens, known to interact with Col V during fibrillogenesis, such as *COL1A1* and *COL3A1*, show variable levels of expression in normal tendons, and significantly increased levels of expression in tendinopathies. Therefore, further studies investigating these correlations as well as how they interact at a genomic level with *COL5A1,* is warranted. It is also possible that non-genetic factors influenced the results of the study described in this paragraph (Mokone et al., 2006), as body mass and physical activity were not controlled participant selection criteria, primarily due to the retrospective nature of such case-control studies.

A subsequent study investigated the same variant of the *COL5A1* gene but in two independent Caucasian populations, in South Africa and Australia (September et al., 2008) and the results generally concurred with the initial association study (Mokone et al., 2006),

in that *COL5A1* rs12722 gene variant within the 3' UTR, was associated with Achilles tendinopathies, and associated with individuals who possess a 'T' allele at this locus. Thus, individuals who were homozygous for the 'C' allele were apparently less likely to develop the condition. The authors investigated a combination of additional markers (inferred haplotypes) or neighbouring alleles in the same sequence region of the 3' UTR of the *COL5A1* gene, in order to provide more information as to the predisposing causative factor. They found that the haplotype consisting of markers rs12722 and rs3196378 (alleles 'T' and 'C' respectively) was significantly overrepresented in the South African tendinopathy group, but not in the Australian group. The DNA sequence that contains the 'C' allele at the rs3196378 marker forms part of a miRNA recognition sequence. miRNA are key regulators of gene expression at a posttranscriptional level, by inhibiting translation or inducing mRNA cleavage (Ambros, 2004, Carthew, 2006). Consequently, protein expression may be modified and in this instance one could speculate that *COL5A1* expression may be altered, leading to suboptimal levels of Col V protein and ultimately a compromised collagen fibre and healing process. Regarding *COL5A2*, no polymorphisms as of yet have been associated with tendon pathologies or function.

Possible influences on tendon properties

Two recent studies considered a measurable *in vivo* phenotype that may link the *COL5A1* gene variant to tendon injuries, by investigating flexibility – a possible intermediate phenotype (Collins et al., 2009, Brown et al., 2011b). Flexibility is an established determining factor for patellar tendinopathies in active populations (Witvrouw et al., 2003, Witvrouw et al., 2001). As alluded to previously, genetics has been reported to contribute substantially to the variability of certain flexibility phenotypes (Hakim et al., 2004, Battie et al., 2008), and although that is not the case in other studies (Maes et al., 1996, Chatterjee and Das, 1995), associations between gene variants and flexibility are therefore plausible.

COL5A1 was hypothesised to be associated with flexibility following reports that mutations in the *COL5A1* gene have been implicated in Ehlers Danlos syndrome, a condition characterised by joint hypermobility and laxity, possibly due to disturbed fibrillogenesis of the collagen fibril containing Col I and Col V (Malfait and De Paepe, 2005). These disease-associated rare mutations may produce non-functional *COL5A1* and *COL5A2* alleles leading to haploinsufficiency of *COL5A1* and *COL5A2* mRNA, predictably resulting in the synthesis of around half the amount of normal Col V protein (Malfait et al., 2005, Malfait and De Paepe, 2005, Mitchell et al., 2009, Schwarze et al.,

2000, Symoens et al., 2012). Phenotypically, this may result in abnormally large collagen fibrils (Vogel et al., 1979, Wenstrup et al., 2006) and impaired mechanical properties of tendon.

An association has been reported between the common gene variant *COL5A1* rs12722, and flexibility (Collins et al., 2009). Individuals heterozygous (CT) for this genotype were less flexible than homozygous individuals of either allele, however the study sample contained significant heterogeneity in terms of tendon injury history – i.e. participants with tendinopathies, history of rupture, and no history of tendon injury were combined in the genotyping results. Furthermore, measures used to quantify flexibility, such as an instrumental standing leg raise (Goeken and Hof, 1993) and a trunk flexion sit and reach test (2006), are rather crude measures of the function of the muscle-tendon unit as a whole, and certainly do not provide precise data on the mechanical properties of the tendon *per se*. Regarding the instrumental standing leg raise test, subjectivity (Goeken and Hof, 1991) and specifically the perception of pain onset, as well as abnormal defence reactions, (Goeken and Hof, 1994) could be particularly relevant to the participants with history of Achilles problems. However, the main critical comment on the instrumental standard leg raise test is that the measured phenotype is a composite of various factors including muscle and tendon stiffness, and does not take into account the dimensions of the structures, so the mechanical properties cannot be determined. Additionally, any kind of sit-and-reach test is a composite measure of various factors contributing to 'flexibility', including muscle-tendon unit stiffness, (McHugh et al., 1998) limb lengths, and proportions (Fernandez and Stubbs, 1989). Furthermore, it has been reported that the mechanical properties of the series elastic component (tendon-aponeurosis) are independent of the parallel elastic component (passive muscle stiffness) *in vivo* (Kubo et al., 2001b). While their approach was a useful step in the study of the genetics of flexibility and range of motion, the mechanics of the tendon *per se,* were clearly not determined by Collins et al. (2009), so potential associations between tendon properties and genes coding for proteins expressed in tendon, could not be investigated directly in that study, or indeed any other study to date.

With a similar study design to Collins et al. (2009), Brown et al. (2011b) investigated the *COL5A1* rs12722 variant and sit-and-reach performance, in a healthy and physically active cohort (325 Caucasian subjects). Individuals homozygous for the 'C' allele had greater flexibility, but this was only observed in the older (≥ 35 years) subjects, where sex and

COL5A1 genotype accounted for approximately 23% of the variance. As per the previous study, some factors which may affect the material and mechanical properties of the tendon were not considered. For example, circulating oestrogen was not assessed in the female subjects – chronic oestrogen levels can influence tendon stiffness (Burgess et al., 2010). Also, a lack of detailed information regarding the habitual physical activity levels of the older subjects was another limitation noted by Brown et al. (2011b) themselves, because tendon stiffness can increase (Couppe et al., 2008, Kubo et al., 2001a) with higher physical activity and decrease (Kubo et al., 2000a, Reeves et al., 2005a) with lower activity. The issue of age and sex-related changes in the *COL5A1* rs12722 variant genotype frequency has been explained by Collins and Posthumus (2011). Specifically, these authors highlighted the importance of controlling for these non-genetic factors in the methodologies of such association studies, by carefully selecting an asymptomatic control group, in order to better identify individuals at risk of injury.

More recent studies have been conducted by the same group of investigators reporting an association between the *COL5A1* rs12722 gene variant and endurance running performance, in two independent populations (Brown et al., 2011a, Posthumus et al., 2011). Both studies found that individuals with a TT genotype were significantly faster than those individuals with a CC genotype. The investigators suggest that ROM of the lower limbs directly influenced endurance running performance, measured by time to completion. Previous research associating this SNP with ROM (Collins et al., 2009, Brown et al., 2011b) report that individuals with a TT genotype were less flexible than those with a CC genotype, with the investigators stipulating that a decrease in lower body flexibility has been associated with improved running economy. Therefore, they propose that the *COL5A1* rs12722 gene variant is associated with endurance running performance. They also speculate that the TT genotype increases musculotendinous stiffness and thus, accommodates greater running economy. This assumption is supported by substantial evidence in humans relating muscle-tendon unit function to performance parameters, reviewed previously in this thesis (refer to 1.5.6-Tendon function). However, due to the multifactorial nature of endurance performance and the crude measures of intermediate phenotypes, as alluded to previously, it is less likely that a single genetic variant can explain this genotype-phenotype association. The complexity of such associations reinforces the need to comprehensively assess the intermediate phenotypes (separate *in vivo* assessment of composites of ROM, e.g. tendon) and careful consideration of the non-genetic factors and variables known to contribute to the variability in the phenotype, so to

enhance our understanding of the link between genetic variation and exercise-related phenotypes.

A biological link has recently been proposed between the *COL5A1* rs12722 gene variant and the gene product (Col V) (Laguette et al., 2011), which goes some way in validating genetic association studies investigating this gene variant, although just how this directly or indirectly affects the mechanical properties of tendon remains to be tested. Collins and Posthumus (2011) proposed that increased Col V content within the normal physiological range as a result of the function of the TT genotype of the *COL5A1* rs12722 variant, results in smaller fibrils with increased surface area (conducive to increased electrostatic interactions with ground substance) (Ottani et al., 2001). Consequently, these attributes may lead to increased creep resistance and increased stiffness, which describe the mechanical behaviour of tissue (Silver et al., 2003). Conversely, the functional significance of the CC genotype may translate into an increased collagen fibril diameter and increased intrafibrillar cross links at the phenotypic level, with the mechanical properties of the tissue displaying a greater ultimate tensile strength (Ottani et al., 2001). Due to the lower Col V content, the inhibition of collagen molecule slippage is likely to be reduced between collagen molecules, and thus, stiffness of the tissue is reduced (Silver et al., 2003).

In conclusion for Col V, it is indeed possible that variations within the genes that encode for the molecular components of Col V may influence a tendon's material and mechanical properties, although such gene variants have not yet been shown to influence tendon properties *per se*. Since Col V expression levels appear critical in determining a tendon's fibre structure through diameter and fibril cross-linking (Wenstrup et al., 2004, Niyibizi and Eyre, 1993, Ottani et al., 2001), several testable hypotheses regarding genetic variants and mechanical properties of tendon such as stiffness, maximal strain, and elastic modulus are likely to be tested in the coming years.

1.7.2 *TNC* as a candidate gene

Structure and function of protein and gene

The TNC isoform which comprises of 6 monomers is the best understood of the family of tenascins. After maturation, TNC expression is only detectable in tendon-associated tissues but is rapidly unregulated in tendon ECM, undergoing remodelling processes (Hsia and Schwarzbauer, 2005). It is specifically expressed in the myotendinous and osteotendinous junctions of tendons in response to mechanical stress (Erickson, 1993b, Riley et al., 1996,

Kannus et al., 1998, Jarvinen et al., 1999, Jarvinen et al., 2000, Ireland et al., 2001, Martin et al., 2003b). It is encoded by the *TNC* gene (9q33.1), which comprises 28 exons spanning 97.63kb of genomic DNA (Birney et al., 2004).

Intriguingly, tenascins such as TNC have been described as 'elastic' proteins, deduced using atomic-force microscopy techniques (Oberhauser et al., 1998). As they are expressed in mechanically loaded tendons, it may contribute to increased elasticity of the ECM *in vitro* (Oberhauser et al., 1998) and *in vivo* (Eliasson et al., 2009, Jarvinen et al., 2003), through mechanisms relating to the stretch of single molecules of tenascin (Chiquet, 1999).

In addition to its structural roles, TNC performs various regulatory roles within the ECM. Due to its modular structure, the protein is able to interact with various other proteins involved in ECM integrity, as well as playing an important role in regulating cell-matrix interactions (Chiquet-Ehrismann and Tucker, 2004). It is also believed that TNC plays an invaluable role in regulating proper alignment and organisation of collagen fibrils (Mackie and Ramsey, 1996). Therefore, it could be postulated that an increase in TNC protein may contribute to an increased crimp angle, a region-specific morphological feature of collagen fibrils associated with the mechanical properties of tendon, due to the elastic properties of the TNC molecule. Specifically, alterations in crimp angle of collagen fibrils have been reported to affect tendon stiffness in trained rat tendons (Wood et al., 1988). It is perceptible to suggest that an increased crimp angle would reduce tendon stiffness.

Evidence of polymorphic associations with tendon pathologies

One study to date has investigated a guanine-thymine (GT) dinucleotide repeat polymorphism within the TNC gene, for potential association with the risk of incurring both chronic Achilles tendinopathies and Achilles tendon rupture (Mokone et al., 2005). Coincidentally, it was the first study to associate a gene variant with tendon-related phenotypes. This polymorphism is a tandem repeat, consisting of a two base pair sequence repeated a varying number of times within a non-coding region (intron 17). Variants containing 12 and 14 GT repeats were overrepresented in subjects with tendinopathies, while variants containing 13 and 17 repeats were underrepresented. The control group had been active in high impact sports for a considerable time (11.5 years) and were currently engaging in ~ five hours per week, and so their apparent resistance to tendinopathies was unlikely to be due to a significantly lower exposure to high impact loading. Thus, a genetic influence may indeed exist, although replication of these data would be a valuable

development. Furthermore, whether the *TNC* polymorphism is involved in causative mechanisms is still debatable.

Possible influences on tendon properties

Even though the GT repeat polymorphism in intron 17 is not part of the coding sequence, intronic variations may influence the binding of proteins involved in gene transcription, thus affecting gene expression. As TNC expression has been reported to be up-regulated in certain pathological conditions, (Chiquet-Ehrismann and Chiquet, 2003, Jones and Jones, 2000a, Jones and Jones, 2000b) it could be postulated that the 12 and 14 GT repeats within intron 17 of the *TNC* gene may overexpress TNC, increasing the elastic properties of the myotendinous unit, as well as reducing the ultimate tendon strain to failure for a given load (Eliasson et al., 2009). Thus, *TNC* is a candidate gene with regards to determining the degree of passive stiffness/compliance of tendon.

1.7.3 *MMP3* as a candidate gene

Structure and function of protein and gene

MMP3 (otherwise known as stromelysin-1) is part of a group of five domain structures of zinc-dependent enzymes known as Matrix Metalloproteinases (MMPs), characterised according to the type of zinc binding. Structurally, the MMP3 protein constitutes a multi domain structure made up of a propeptide, a catalytic N-terminal domain and a haemopexin-like C-terminal, all of which combine to form functional MMP3 which has the capacity to interact with its substrates (Murphy and Knauper, 1997). The MMP3 protein is encoded for by the *MMP3* gene (11q22.2) which is 10 exons in length and covers 7.79kb (Birney et al., 2004).

MMP3 is one of the most functionally diverse of the MMPs, hydrolysing multiple substrates such as all types of collagens except collagen type I, the proteoglycans, and a wide range of ECM components (Matrisian, 1990), as well as activating several MMPs by cleaving the propeptide from the pro-MMP, the precursor molecule (Shapiro et al., 1995, van Meurs et al., 1999, Somerville et al., 2003, Visse and Nagase, 2003). MMP3 gene expression may be regulated at the transcriptional, translational or post-translational levels by interaction with inhibitors (Jones et al., 2006) and has shown to be increased by mechanical loading *in vitro* (Thornton et al., 2010, Tsuzaki et al., 2003, Archambault et al., 2002b, Archambault et al., 2001). Recently, ECM regulation and MMP3 up-regulation in tendon, has shown to be determined by a combination of duration and magnitude of the

mechanical stimulus, *in vitro* (Maeda et al., 2009) and *in vivo* (Sun et al., 2010) in rodent models. This potentially represents the impact of differing forms of voluntary exercise in tissue remodelling processes in humans.

Evidence of polymorphic associations with tendon pathologies

Gene variants have been investigated in the *MMP3* gene which have the potential to substantially alter its expression (Koch et al., 2010), particularly the 5A/6A polymorphism within the promoter region of human *MMP-3*. This polymorphism has been associated with a number of pathological states (Beyzade et al., 2003, Ye et al., 2007, Samnegard et al., 2005). The association between gene variants in *MMP3* and tendon pathology was first postulated when immunochemically detectable MMP3 protein, was lower in a 'normal' region of Achilles tendon tissue, in patients with a degenerate core region nearby, compared to normal control tissue (Ireland et al., 2001). This suggests these patients with tendinosis were predisposed to developing the condition, due to inherently reduced MMP3 protein levels.

One study to date has reported an association between variation in the *MMP3* gene and Achilles tendinopathy (Raleigh et al., 2009). Three SNPs spanning most of the gene were identified as being potentially informative, as they are part of all four major haplotypes within the *MMP3* gene (one exon SNP – rs679620, two intron SNPs – rs591058, rs650108). All three *MMP3* variants were found to be associated with Achilles tendinopathy individually, and as inferred haplotypes – particularly between the rs679620 and rs591058 gene variants. These two variants were found to be in almost perfect linkage disequilibrium. In contrast, the 'ATG' inferred haplotype containing all three SNPs were significantly underrepresented in the tendinopathy group compared to the control group, suggesting this combination has a protective effect against the development of Achilles tendinopathy.

Raleigh et al. (2009) were the first to demonstrate an interaction between variants on two different genes, vis-à-vis the development of Achilles tendinopathy (all three SNPs of the *MMP3* gene and the marker rs12722 of the 3' UTR region of *COL5A1* gene). The rs679620 marker of the *MMP3* gene and the rs12722 marker of the 3' UTR region of *COL5A1* gene, represent the best pair of genotypes for estimating the risk for Achilles tendinopathy, with the 'G+T' allele combination associated with tendinopathies. However, the authors do not address how the *MMP3* variants alone, or as haplotypes and inferred

haplotypes between different genes, cause an increased/decreased risk of tendinopathies. Nevertheless, they do suggest that the rs679620 variant of *MMP3*, which is a non-synonymous polymorphism, may influence the downstream function of the mature MMP3 enzyme and its activation (Beyzade et al., 2003), due to the subtle change in the amino acid coding and its interaction with other amino acids ('G' allele = glutamate, 'A' allele = lysine). The 'G' allele may encourage elevated levels of MMP3 expression via increased MMP3 activation, as a result of altered interaction with other amino acids in the propeptide region.

Possible influences on tendon properties

As the *COL5A1* rs12722 gene variant was shown to be associated with human flexibility, the *MMP3* rs679620 variant was investigated for this same association, though no association was evident (Posthumus et al., 2010b). The precise rationale for investigating a link between this gene variant and flexibility is unclear, although as a link was previously identified between the *MMP3* gene variant and Achilles tendon injuries (Raleigh et al., 2009) and as flexibility has been reported to be a possible risk factor for these injuries, (Witvrouw et al., 2007) the investigation seems justified. It must be noted that the flexibility phenotype assessed was a measure of musculoskeletal passive flexibility, which encompasses tendons, ligaments, joint capsules, aponeuroses and fascia sheaths, as well as the muscle and not necessarily just the tendon. Thus, as previously mentioned in section 1.7.1, there are limitations to the techniques used for measuring flexibility in these studies.

As the mechanical properties of tendons are primarily a function of the ECM and because a majority of ECM components are substrates for the proteolytic activities of MMP3 (Sternlicht and Werb, 2001), it may be that MMP3 expression would contribute to the material integrity and thus, tendon mechanical properties. It can be postulated that elevated expression levels of the MMP3 protein may put the ECM in a state of imbalance with greater degradation compared to synthesis, thus, substrates involved in cross-linking and stabilisation of intact fibrillar collagen may be degraded. This may ultimately weaken the material properties and result in a reduction in matrix stiffness (Eliasson et al., 2007, Reddy, 2004). However, within normal physiological ranges the activity of MMP3 is tightly controlled by tissue inhibitors of MMPs (TIMPs) (Riley, 2005), thus, inhibiting the degeneration of the ECM and loss of material properties. This has been reported in stress-deprived tendons *in vitro,* subjected to inhibitors to prevent the activation of MMP activity (Arnoczky et al., 2007).

1.7.4 *GDF5* as a candidate gene

Structure and function of protein and gene

GDF5 is a member of the transforming growth factor (TGF) super-family, encoded for by the *GDF5* gene (20q11.22) of which its entire coding region comprises 4 exons and is approximately 21.42kb in length (Birney et al., 2004). Structurally, it is a 'dimer' consisting of two monomers interlinked by disulfide bonds. Mature forms of the protein are approximately 110-140 amino acids in length, and seven cysteine amino acid residues are involved in creating its rigid structure (Schreuder et al., 2005).

GDF5 is involved in a variety of musculoskeletal processes including the growth and repair of tendon tissue (Aspenberg, 2007). When, GDF5 was first investigated for its possible role in tendon biology, it was found to possess a unique ability to induce a tendon-like tissue rather than cartilage and bone, when implanted intramuscularly in rats (Wolfman et al., 1997). Further investigations found a significant role of GDF5 in rodent models with induced Achilles tendon injuries. Firstly, GDF5 was found to enhance tendon healing and tensile strength of the tendon when implanted on collagen sponges, in a dose-dependent manner (Aspenberg and Forslund, 1999), a recent review article supports the notoriety of this significant dose-dependency of GDF5 (Moore et al., 2010). Also, more recently GDF5 has shown to enhance tendon healing and maximum load to failure in flexor tendons of rabbits *in vivo* (Henn et al., 2010). Further studies examined the ultrastructural, compositional and mechanical characteristics of the Achilles tendon in rodents deficient in GDF5. The maximum load to failure was found to decrease, possibly due to significantly less collagen, an increase in irregularly shaped collagen type I fibrils and compromised material behaviour (decrease in strength and stiffness) (Mikic et al., 2001, Clark et al., 2001, Chhabra et al., 2003). Therefore unsurprisingly, GDF5 has been shown to increase mechanical strength in these rodent models (Loiselle et al., 2009, Rickert, 2008, Dines et al., 2007, Bolt et al., 2007). These studies are further supported at a cellular and gene level which show an improved collagen organisation with GDF5 treatment (Hogan et al., 2010, Henn et al., 2010), as well as an increased expression of genes and synthesis of the components of tendon ECM discussed previously, in particular, collagen type I, TNC and MMP3 (Keller et al., 2011). GDF5 may also be involved in collagen cross-linking by promoting the proteolytic activation of LOX (Maruhashi et al., 2010a), as well as mediating the collagen structure and organisation by increasing the thickness of collagen fibrils (Mikic et al., 2001). Again, it must be maintained that collagen cross-linking and diameter *per se,* have shown to increase tendon matrix stiffness in animal

models (Birch, 2007, Eliasson et al., 2007, Reddy, 2004), so all things considered, GDF5 may influence tendon material and mechanical properties via mediating the growth and development of other structures, in particular collagen type I fibrils.

Evidence of polymorphic associations with tendon pathologies

The human *GDF5* gene contains mutations known to cause a number of rare inherited disorders, including acromesomelic chondrodysplasia of the Hunter-Thompson and Grebe types as well as Du Pan Syndrome, all of which are characterised by musculoskeletal abnormalities, including shortened limb bones, brachydactyly and severe joint dislocations (Douzgou et al., 2008, Faiyaz-Ul-Haque et al., 2008, Schwabe et al., 2004). The hypothesised involvement of GDF5 in tendon pathologies derives from this evidence, as it was postulated that the observed joint dislocations may be attributed to abnormalities in tendons (Mikic et al., 2001). As well as genetic mutations within the *GDF5* gene, a functional promoter SNP (rs143383; T/C) of the 5' UTR of the *GDF5* gene, has been associated with multifactorial disorders, such as osteoarthritis at different joint locations, across different ethnic groups, (Chapman et al., 2008, Egli et al., 2009, Waarsing et al., 2011, Ji et al., 2010) congenital dislocation of the hip, (Rouault et al., 2010) as well as total body height, hip axis length and fracture risk, (Vaes et al., 2009, Sanna et al., 2008) and lumbar disc degeneration (Williams et al., 2011). In articular cartilage of individuals with osteoarthritis, there was a 12% lower expression of GDF5 associated with the 'T' allele at this SNP marker, compared to the 'C' allele (Southam et al., 2007). So, a reduction in the expression of the *GDF5* gene associated with the 'T' allele, may contribute to tendon pathologies, and this has been investigated by one study.

In a case-control study, an association was reported between the *GDF5* SNP rs143383 referred to above, and the risk of ATP (Posthumus et al., 2010a). Individuals of 'TT' genotype were found to have approximately twice the risk of developing ATP within an Australian population independently, and when combined with a South African population, which probably means it is less likely to be a false positive observation. No significant association between genotype and higher risk was shown in the South African cohort alone, although the observed odds ratio was still similar (~1.7). The relatively small sample size of the Australian tendinopathy group (n = 59) as well as the different physical characteristics (body mass and BMI), between the ATP and control groups of both populations, are perhaps limitations of the study. However, the odds ratios and confidence intervals observed suggest a robust association, and these findings complement those

studies demonstrating the impact on gene expression of the *GDF5* variant in question (Southam et al., 2007, Miyamoto et al., 2007, Valdes et al., 2010).

Possible influences on tendon properties

It may be hypothesised that the material properties of the tendon are compromised in the presence of the rs143383 'T' allele variant, i.e. a reduction in tensile strength and stiffness. GDF5 may be involved in collagen cross-linking by promoting the proteolytic activation of lysyl oxidase (Maruhashi et al., 2010a) as well as mediating the collagen structure and organisation, by increasing the thickness of collagen fibrils (Mikic et al., 2001). Collagen cross-linking and diameter *per se*, have been shown to increase tendon matrix stiffness in animal models (Birch, 2007, Eliasson et al., 2007, Reddy, 2004) and humans, (Hansen et al., 2009a) so the 'T' allele variant of *GDF5* may hinder these processes, and thus, reduce tendon stiffness. Therefore, if gene variants within the *GDF5* gene influence tendon material and mechanical properties, it is likely to be via mediating the growth of other structures, in particular Col I fibrils and TNC (Tan et al., 2012).

1.7.5 *COL1A1* as a candidate gene

Structure of protein and genes

Collagen comprises fibrillar collagen molecules containing more than 95% Col I (Riley et al., 1994). This collagen protein is encoded for by the *COL1A1* gene (17q21.33), which constitutes 52 exons and is 18.34kb in length, and to a lesser extent by the *COL1A2* gene (7q21.3), which constitutes 52 exons also, and is 36.67kb in length (Birney et al., 2004). The *COL1A1* gene encodes for the alpha (α) 1 chain, while the *COL1A2* gene encodes for the α 2 chain. Two α 1 chains and one α 2 chain combine to form a heterotrimer protein structure.

Col I is a major protein constituent contributing significantly to the structural integrity of soft tissues such as cruciate ligaments, joint capsules and tendons, via the formation of strong parallel bundles of fibres. Col I fibrils and fibres are well recognised to be involved in tensile strength and the stiffness of tendon matrix, based on its intra- and intermolecular cross links, orientation, density, diameter and length, all of which have been shown to affect the mechanical properties of the tendon as a whole in animal models (Eliasson et al., 2007, Reddy, 2004, Maruhashi et al., 2010a, Hansen et al., 2009c, Hansen et al., 2009a, Silver et al., 2001, Silver et al., 2003, Birch, 2007).

Possible polymorphic associations with tendon pathologies

Mutations as well as single nucleotide polymorphisms in the *COL1A1* gene, particularly a SNP affecting the Sp1 binding site in the first intron of the *COL1A1* gene (+1245; G/T; rs1800012), have been associated with lower bone mineral density and osteoarthritis (Lian et al., 2005a, Kuivaniemi et al., 1997, Jin et al., 2009, Liu et al., 2004a, McGuigan et al., 2000, Tran et al., 2009, Van Pottelbergh et al., 2001, Jin et al., 2011), as well as being implicated in the disease *osteogenesis imperfecta (OI),* which is characterised by fragile collagen structures (Hasegawa, 2010, Mann et al., 2001, Barbirato et al., 2009). Additionally, a point mutation in the *COL1A2* gene (nucleotide position 1121) that substitutes serine or cysteine for glycine residues (C-to-T transition and G-to-T transversion, respectively), also leads to the OI phenotype (Trummer et al., 2001). Consequently, sequence variants such as these and others, may be less clinically applicable but may still be associated with the risk of incurring soft tissue injuries. Indeed, associations have been reported between SNPs in the *COL1A1* gene at the intronic Sp1 transcription factor binding site, and the risk of cruciate ligament ruptures and shoulder dislocations, (Khoschnau et al., 2008, Posthumus et al., 2009), as well as upper limb muscle strength in elderly men (Van Pottelbergh et al., 2001). These associations may be mediated through reduced Col 1 content or a weaker form of Col 1, but as of yet no genetic association has been made with tendon pathologies or tendon properties.

Possible influences on tendon properties

No association has yet been reported between variation in the *COL1A1* gene and tendon pathologies or properties. It is known that a SNP within the intronic Sp1 binding site (rs1800012) increases transcriptional activity of the *COL1A1* gene, resulting in abnormal ratios of the α1 (1) protein relative to α2 (1), which possibly gives rise to weaker homotrimers being formed (three α1 (1) chains) instead of the conventional heterotrimers (two α1 (1) and one α2 (1) chains) (Mann et al., 2001). It has also been reported that two polymorphisms in the proximal promoter region of *COL1A1* are in linkage disequilibrium with the Sp1 polymorphism, (Garcia-Giralt et al., 2002) and in fact form an extended haplotype with the Sp1 polymorphism to regulate *COL1A1* transcription. This is achieved by affecting the binding affinity of important regulating factors, such as Sp1, with the 'T' allele at the Sp1 binding site, found to have a higher DNA binding affinity than the 'G' allele (Jin et al., 2009). Consequently, individuals who carry a 'T' allele instead of a 'G' at this SNP, highly express *COL1A1,* and thus, possess a greater proportion of the weaker α1 homotrimers, and so may be more likely to have a compromised tendon internal structure.

It has also been suggested that overproduction of Col 1 α1 chains in tendon might result in a higher tensile strength, (Khoschnau et al., 2008) although that statement contradicts the mechanism just outlined and is not expanded upon by the authors.

It is unlikely that tendon properties are affected solely by the Sp1 polymorphism of intron 1 of the *COL1A1* gene. It is more likely that the extended haplotype influences the transcription of *COL1A1* (Jin et al., 2009) and the material quality of the Col I fibril, with individuals carrying a 'T' allele at the Sp1 polymorphism ultimately producing higher gene activity, which might contribute to a more adversely affected Col I fibril. In consummation, other genes and their respective SNPs such as those already reviewed in this thesis need to be considered at the same time.

1.8 Overview

The majority of research in human genetics and its association with tendon phenotypes focuses on tendinopathies. One research group from South Africa led by Malcolm Collins, have been working on identifying genes and alleles important for such phenotypes, including ROM and endurance running performance. However, on one hand it must be emphasised that few genes/gene variants have been conclusively identified as key contributors to these traits, and indeed this is true for all health and fitness phenotypes (Roth, 2007). This suggests that tendon phenotypes are likely to be polygenic and concurs with the expectation that a multitude of genes, their associated proteins, and their complex heterogeneous interactions are required to maintain normal tendon structure and homeostasis, through development and regeneration. Therefore, it is likely, even after controlling for other non-genetic parameters such as sex, age and habitual physical activity, that the intrinsic material (structural and regulatory) and mechanical properties of a tendon are influenced by polygenics. On the other hand, association studies are a major tool for identifying gene/gene variants for complex traits, particularly when constructing multifactorial models. These models will not only better our understanding of molecular mechanisms involved in such phenotypes, but could also be of practical importance for clinicians and other health and fitness-related professionals, to develop personalised interventions. However, investigations into genetic factors involved in their aetiology, thus far, are very much in their infancy (Lippi et al., 2010).

The efficacy of association studies is undermined mainly due to pitfalls and problems in the design of such studies, which translates into the non-replication of significant findings.

These inherent issues include systematic errors leading to false-positive results, lack of power to detect true associations due to inadequate sample sizes, heterogeneity between studies in terms of imprecise classification of subjects and phenotypes, as well as heterogeneity across studies relating to population stratification (Lewis and Knight, 2012). Therefore, caution must be taken when interpreting the findings of these genetic association studies, yet these issues can be addressed sufficiently to justify the continual preferences of conducting such studies. For example, by beginning to assess the heritability of specific tendon phenotypes intra-ancestrally by conducting twin-family studies, the strength of these relationships can be enhanced and quantified, which has not yet been done.

Regarding the ECM proteins reviewed extensively within this thesis at a protein, gene and SNP level, it appears they do not act as one single entity but subtly interact to form interlinked structures (refer to Figure 14) and govern dynamic processes within the ECM. Notably, Col V fibrils combine with Col I fibrils to regulate the diameter of the fibres (Birk et al., 1990) with TNC playing an invaluable role in regulating the proper alignment and organisation of the collagen fibres (Jarvinen et al., 2000). MMP3 may degrade minor collagens such as Col V, which may alter the cross-linking and stabilisation of the tendon structure (Matrisian, 1990). And lastly, an improved collagen organisation and cross-linking density was observed with the addition of GDF5 (Maruhashi et al., 2010b, Hogan et al., 2010). The common theme in these associations is the integrity of the collagen fibre in conjunction with organisation, diameter and cross-linking, all of which have been linked to tendon properties such as tendon matrix stiffness (Parry, 1988, Birch, 2007).

It is therefore reasonable to suggest that variants in genomic DNA sequence within these and other relevant proteins are likely to contribute to observed phenotypic variations in the tendon, most notably the mechanical properties, which may have implications for physical performance capabilities and the risk of incurring musculoskeletal injuries. However, no study has yet attempted to investigate genetic influences upon tendon properties *per se* in an asymptomatic population.

1.9 Future considerations for thesis

When investigating a gene variant's influences on tendon properties, it is important to negate factors other than genetics that are likely to contribute to tendon phenotypes. To establish a valid and reliable association between a gene variant and tendon mechanical properties, experimental error must be minimised and appropriate phenotype measurements utilised, which has not been adequately achieved in the previous genetic association studies investigating flexibility (Collins et al., 2009, Brown et al., 2011b). It would be more appropriate to measure the overall stiffness of the muscle-tendon unit to assess flexibility using for example, passive isokinetic dorsiflexion adopted by Morse et al. (2008) to assess the human gastrocnemius muscle-tendon unit. This comprehensive *in vivo* assessment utilises techniques such as dynamometry, electrogoniometry, electromyography (EMG) and ultrasonography. The mechanical properties of the tendon itself can be assessed *in vivo*, which would be in line with the objectives of associating genetics with tendon properties. A thorough and highly reliable assessment is detailed by Pearson and Onambele (2006) with respect to the tendon compliance of the patella tendon, in that dynamometry, EMG and ultrasonography were utilised as well as the force-displacement relationship to calculate tendon mechanical stiffness. Therefore, an accurate, reproducible and non-invasive assessment of tendon properties *in vivo* is required to maximise the ability to detect a genetic contribution to the interindividual variability in mechanical properties of human tendon.

From a genetic perspective, genotyping all candidate genes and gene variants discussed above would be ideal, although this is not possible due to logistics relating to time, expense and technologies available. The highest priority candidates are *COL5A1* rs12722 and *MMP3* rs679620, rs591058, and rs650108, for examination in relation to the main phenotypes ((tendon structural properties (volume), tendon functional properties (Young's Modulus)). The selection of these specific polymorphisms was based on; (1) greater frequency of association with tendon phenotypes, particularly true of *COL5A1* rs12722, with tendinopathies, ROM, and endurance running performance. All three *MMP3* polymorphisms were independently associated with chronic tendinopathies; (2) increased probability of them being functional, a higher number of gene transcription analyses have been conducted for *COL5A1* and *MMP3* genes; (3) the correlation with potential causal variants (linkage disequilibrium), high genetic linkage between the three *MMP3* polymorphisms, as well as between other polymorphisms in the *MMP3* gene and flanking

sequences; (4) novel gene-gene interactions relating to tendinopathies between *COL5A1* rs12722 and *MMP3* rs679620 polymorphisms.

All in all, testable hypothesis-driven research questions can be established in relation to these SNPs independently, and when combining the alleles to form inferred haploypes, with tendon properties, based on established physiological theory discussed in great detail, in the preceding sections of this literature review. Statistical approaches will determine whether the SNPs independently or combined, are associated with the independent parameters, describing the structural and mechanical properties of tendon.

1.10 Aims of the thesis

A clearer picture of how to go about investigating the potential associations of key gene variants within genes which perform structural and regulatory functions in tendon, and definitive tendon phenotypes (i.e. *in vivo* structural and mechanical properties) was established via the interpretation of the literature in both research areas. Therefore, the overall aim of the work described in this thesis was to determine some of the genetic factors that contribute to independent parameters describing the structural and mechanical properties of tendon. Specific aims were:

1. To determine whether the *COL5A1* rs12722 gene variant and *MMP3* rs679620, rs591058 and rs650108 gene variants are associated with tendon properties in a asymptomatic male and female population

2. To determine whether allele combinations deriving from these gene variants associate with tendon properties

These aims are integrated into the following section which provides an overview of each of the experimental chapters in this thesis.

1.11 Overview of the experimental chapters

In chapter 3, the main aim of the study was to investigate the discrete associations between genetic variations in the *COL5A1* (rs12722) gene on tendon structural (volume) and functional (modulus) properties independently, and combined (z-scores), in asymptomatic men. It was hypothesised that an association would exist between the genetic variation and tendon properties.

The main purpose of the study described in chapter 4, was to determine whether sex (via the influence of relatively higher oestrogen in females) influences the ability to conduct research into tendon properties in asymptomatic individuals, and whether similar genotype-phenotype associations exist between the *COL5A1* rs12722 gene variant and parameters of tendon properties described for chapter 3, in women as in men. It was hypothesised that research of this kind is possible in women and that similar genotype-phenotype relationships would exist in women as in men.

In chapters 5 and 6, the research design was applied in a similar manner to that in chapters 3 and 4, although the genetic variation under investigation was within the *MMP3* gene and included three common gene variants, one within exon 2 (rs679620) and two, within introns 4 (rs591058) and 8 (rs650108). Chapter 5 was exclusively investigating these associations in males, and chapter 6, in females only.

In chapter 7, a polygenic profile and its association with tendon properties, including structure (volume), mechanics (modulus), and both in combination (z-scores) were assessed. It was hypothesised that a polygenic approach that included gene variants within the *COL5A1* and *MMP3* genes investigated in the preceding chapters, would collectively account for a greater proportion of the interindividual variability in tendon properties, than would be possible via a single gene variant approach.

Chapter 2.

General Materials & Methods

2.1 Participants

From the initial screening phase (See Appendix 4 for methods utilised for subject recruitment), 160 recreationally active volunteers were recruited (100 males and 60 females). A general health questionnaire was used to screen volunteers, and they were excluded for the purposes of the study if they: were not of white Caucasian origin; were highly trained (over three structured training sessions on the lower limbs a week); were sedentary with no or irregular activity; had any current musculoskeletal problems especially of the knee; were aged under 18 or over 40 years; were using local or systemic steroids; were diabetic; smoke the equivalent of more than 10 cigarettes a day for at least one year; regular users of medication including anti-inflammatory drugs; had blood disorders; or had a body mass index (BMI) under 18.5 or over 30. With female participants, they were excluded from the study if they were pregnant or were using any form of hormone-based contraception. Accounting for as many non-genetic or environmental factors as possible, was crucial in allowing the greatest opportunity for defining phenotype differences due to genetic factors. The final screening phase provided a selective group of individuals based on their genetic profiles.

The final screening phase related to the genotyping of the four gene variants (*COL5A1* rs12722, *MMP3* rs679620, rs591058, rs650108) for each participant. For inclusion in the full range of tests of tendon properties, the participants were selected based on their genetic profiles for these four gene variants. By 'stressing the genotype' (Montgomery et al., 2002), the genetic and potentially the phenotypic differences between the groups of individuals, could be maximised. This approach provided a model that may optimise the study of genetic variation at the SNPs in the *COL5A1* and *MMP3* genes. An algorithmic model provided a prediction of the number of participants from the larger groups (100 males and 60 females), optimised for the phenotypic tests (refer to Appendix 1). Approximately 45 participants in each group of males and females would provide a high proportion of homozygotes (3 to 4 out of a total of 4 gene variants).

Forty-five males and thirty-nine females took part in the full range of tests for tendon properties (characteristics shown in Table 4) by virtue of their high proportion of homozygosity for the four gene variants under investigation (see Table 5). Due to the initial sample size of the females being significantly lower than anticipated (n = 60), females displaying homozygosity for two gene variants were also recruited for the phenotype measures (n = 10), so to meet the minimum sample sizes required to detect

significant differences in phenotype measures, as determined by power calculations described above.

Participants gave their written consent to participate in the study which conformed to the latest revision of the Declaration of Helsinki and was approved by the Manchester Metropolitan University Ethics Committee (Refer to Appendix 5).

Table 4. Physical characteristics of participants

	Males (N = 45)		**Females (N = 39)**	
	Mean (SD)	**Range**	**Mean (SD)**	**Range**
Age (years)	22.9 (3.3)	19-32	22.4 (4.8)	18-39
Height (cm)	179 (7)	157-191	166 (6)	153-183
Body mass (kg)	78.3 (10.9)	58.4-99.0	63.8 (9.0)	47.4-80.1
BMI (kg/m^2)	24.6 (2.6)	20.4-29.8	23.2 (2.8)	18.8-29.8

Data is expressed as mean (standard deviation)

Table 5. Proportion of homozygosity of the participants involved in genotype-phenotype investigations for the four gene variants

Males (N = 45)		**Females (N = 39)**		
Proportion of homozygosity		**Proportion of homozygosity**		
Homozygote for all '4' gene variants	**Homozygote for '3' gene variants**	**Homozygote for all '4' gene variants**	**Homozygote for '3' gene variants**	**Homozygote for '2' gene variants**
14	31	10	19	10

2.2 Measurement of tendon structural and mechanical properties

Measurement of maximal patellar tendon isometric force

All measurements of torque were carried out on an isokinetic dynamometer (Cybex, Phoenix Healthcare, UK). The knee was fixed at 90º flexion (full extension = 0º) and hip angle at 85º (supine position = 0º). The centre of rotation of the dynamometer lever arm was aligned with the knee joint centre and straps were positioned at the hip, shoulders and over the left thigh to prevent any extraneous movement. All measurements were performed on the left lower limb. A lever attachment cuff was placed on the lower leg above the ankle joint at a length corresponding to 15% the distance from the lateral tibial condyle to the lateral malleolus, for each participant. Participants were instructed to perform ramped

isometric knee extensions to maximum over a 5-7 s period. Maximal tendon force was calculated as described previously (Equation 2), (Pearson and Onambele, 2006, Onambele-Pearson and Pearson, 2007).

$$\text{Equation 2: } F_{Max} = (OT + CcT) / PTMA$$

Where F_{Max} is the maximal tendon force, OT the observed maximal isometric knee-extensor torque (i.e. the measured torque during testing), CcT the knee flexion torque of the hamstrings during knee extension (antagonist co-contraction torque) (see section immediately below for the calculation of the latter), and PTMA the patellar tendon moment arm (see sections below for measurement).

Estimation of co-contraction using electromyography

Electromyographic (EMG) activity was assessed from the long head of the biceps femoris (BF) muscles (representative muscle of the knee flexors) to correct for co-activation during the isometric knee extension excursions. A pair of self-adhesive Ag-AgCl electrodes ~15 mm in diameter (Ambu Neuroline 72000-S/25, Ballerup, Denmark) was placed in a bi-polar configuration with a constant inter-electrode distance of ~20 mm, at a site corresponding to the distal one-third of the length (Zipp, 1982) in the mid-sagittal plane of the BF muscle. The reference electrode was placed on the lateral tibial condyle. Electrode placement was always preceded by shaving, abrading and cleansing with an alcohol-based tissue pad to minimise skin impedance to values below 5 kΩ. The raw EMG signal was collected at a frequency of 2000 Hz, pre-amplified (x 2000) and band pass filtered between 500 and 10 Hz by the same system that processes the torque data (Acknowledge, Biopac Systems, Santa Barbara, CA, USA), and displayed in real-time on the same output graph (iMac, Apple, California). The video, EMG and torque traces were time locked with a synchronising signal triggered at the start of each measurement. The reported EMG activity corresponds to the root mean square (RMS) after correcting for baseline values. A series of three maximal isometric flexion contractions were carried out to obtain the EMG at a knee joint angle of 90º. The RMS EMG activity corresponding to the peak torque period was analysed and averaged for a 500 ms period during the plateau of peak torque (i.e. 250 ms either side of the instantaneous peak torque). As alluded to previously, the EMG of the long head of the BF was measured to ascertain the level of antagonist muscle co-contraction during isometric knee extension performances. The maximal activation of the BF when acting as an agonist during the knee-flexion contraction was calculated in a

manner similar to the methods described by Reeves et al. (2003), where the BF EMG activity during knee extension is divided by the BF peak flexor EMG at 90º knee flexion. The maximal flexor torque of the hamstrings was then multiplied by this value to determine co-contraction torque (Equation 3). These calculations are based on the assumptions that BF is representative of its constituent muscle group (Carolan and Cafarelli, 1992) and BF EMG relationship with knee flexor torque is close to linear (Lippold, 1952). The co-contraction torque values can then be used to correct the maximum voluntary knee extension torques (Equation 4) during the ramped knee extension contractions.

$$\text{Equation 3: } CcT = (MaxT_{ham} \times EMG_{ham_KE}) / EMG_{ham_KF}$$

CcT is the co-contraction torque, $MaxT_{ham}$ is the maximum torque of the hamstrings, $EMG_{ham\ KE}$ is the EMG of the BF during knee extension MVC, and $EMG_{ham\ KF}$ is the EMG of the BF during knee flexion MVC.

$$\text{Equation 4: } CT = MVC_{KE} + CcT$$

Where CT equates to corrected knee-extensor torque, MVC_{KE} is the maximum torque of the knee-extensors, and CcT the co-contraction torque (CcT is calculated above).

Measurement of patellar tendon displacement

Patellar tendon displacement was determined using real-time B-mode ultrasonography (AU5, Esaote, Biomedica, Italy) set to a depth resolution of 49.3 mm, during a ramp isometric knee extension performed over 5-7 s with the knee fixed at 90º flexion, and either a) patella proximal (inferior pole of the patella) or b) tibia distal (tibial tuberosity) excursions. Measurements were taken at a consistent time of day, i.e. early afternoon, to avoid confounding the measures between subjects, as it has been reported that there are trends for time-of-day variability in tendon stiffness (Pearson and Onambele, 2006). Measurements were also taken after five preconditioning contractions to ensure reproducibility (Maganaris, 2003). The ultrasound probe (7.5 MHz linear array probe, 40 mm wide) was positioned in a sagittal plane over the patellar tendon at the above mentioned anatomical points of interest, on an echo-absorptive external marker fixed on the skin, which acted as a fixed reference from which relative measures of displacement

could be made. Any movement of the line cast by the external marker during contraction-relaxation on the ultrasound image, indicated movement of the probe with respect to the patellar tendon as well as the skin (see Figure 15 A and B), and this data would therefore be omitted from further analysis. This method has been used in numerous research papers and is reported to have high reliability (e.g. (Pearson and Onambele, 2006, Kubo et al., 2001d, Reeves et al., 2003a). Tendon displacements were determined at intervals of 10% of the maximal force (from 10-100%) using digitising software (Kinovea, version 0.8.15, Joan Charmant & Contributors, France), consistent with others (Onambele et al., 2007) Three MVC of the knee extensors were recorded for both the proximal (patella) and distal (tibia) displacements, but only the highest force excursions of each were utilised for the calculations of total tendon displacements (sum of tibial and patellar displacements) and subsequent tendon stiffness measures for each participant.

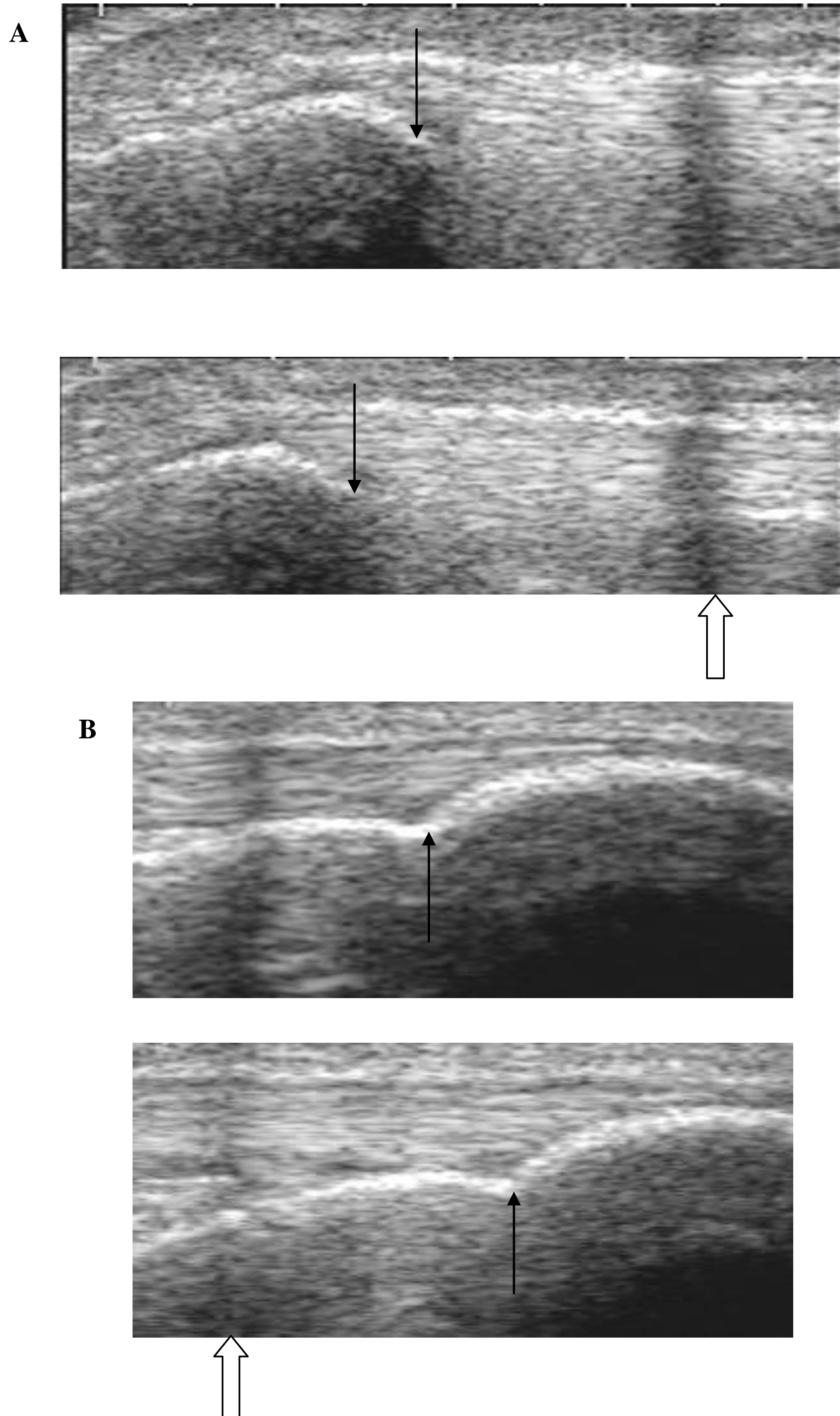


Figure 15. Sagittal-plane scans of the patella tendon at rest (above), and at maximal tendon force (below). Arrows indicate; **A:** proximal displacement of the apex of the patella. **B:** distal displacement of the tibial tuberosity, during contraction with respect to an echo-absorptive external marker fixed on the skin; **A:** distal to **B:** proximal to, the displacement (large arrow).

Measurement of patellar tendon moment arm length

Patellar tendon moment arm length (PTMA) was measured from an 11 s sagittal scan of the left leg of each participant at rest, using a single, low-energy x-ray beam (0.9μSv), DEXA (Dual X-ray Absorptiometry) scan (Hologic QDR, Vertec, Reading, UK). The scan penetrates bone and soft tissue areas to a depth of approximately 30 cm. For the imaging limb, the participant lay on their side with the hip and knee flexed at 90° aided by a goniometer, whilst the contralateral limb was placed straight, so that the source detector probes could pass across the knee within a 20 cm scanning window. The PTMA was defined as the perpendicular distance from the patellar tendon to the midpoint of the distance between the estimated tibio-femoral contact points in the lateral and medial femoral condyles (See Figure 16 A and B) (Baltzopoulos, 1995, Tsaopoulos et al., 2006). Reliability measurements with the previously validated MRI for measuring PTMA indicates a very strong relationship with DEXA ($r^2 = 0.962$, $P = 0.001$, unpublished data from our labs).

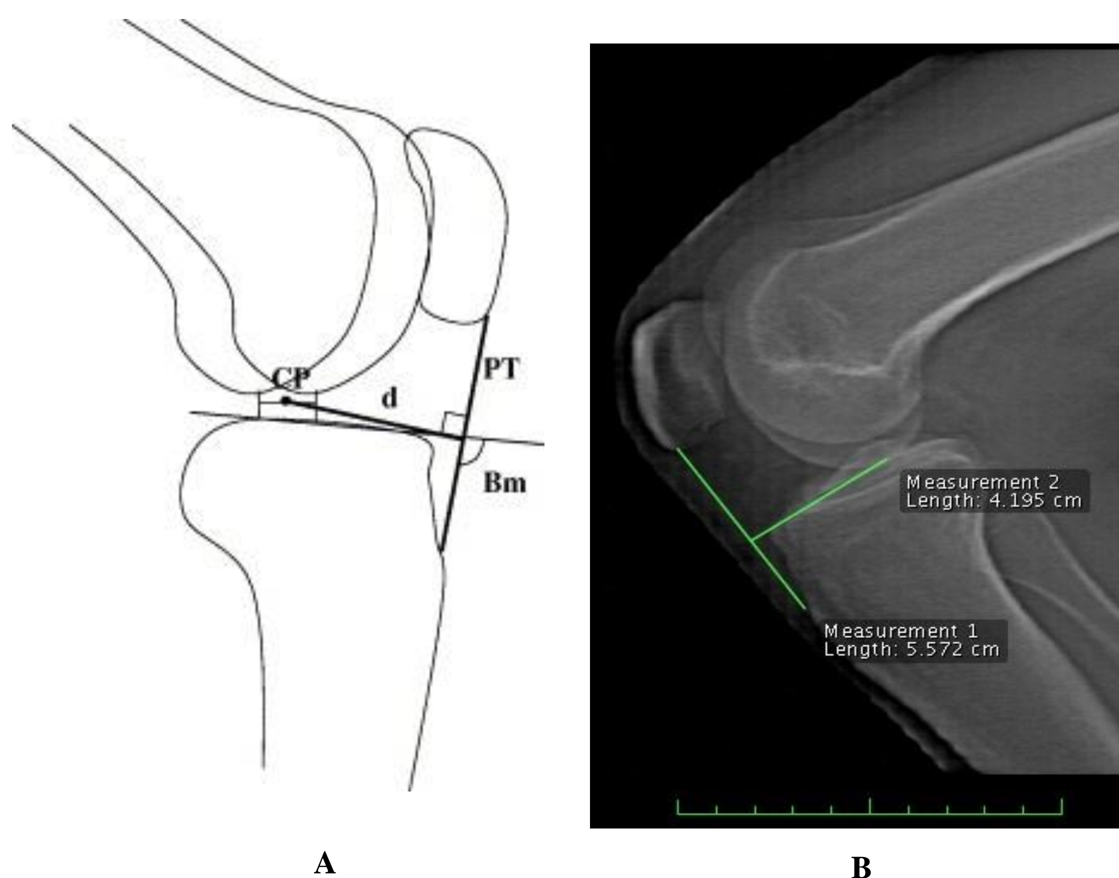


Figure 16. A: Diagrammatic representation of the measurement for PTMA using the tibio-femoral “contact point”. TFCP is the tibio-femoral contact point, *d* is the PTMA (Tsaopoulos et al., 2006). **B:** DEXA scan with measurement 1 showing the length of the patellar tendon, and measurement 2 is the PTMA (unpublished figure from our laboratory)

Calculation of patellar tendon stiffness

Patellar tendon stiffness (K, N·mm^{-1}), was calculated from the slope of the tangents of the force-displacement relations (at 10% force intervals), which were fitted with a second-order polynomial function forced through zero (see Figure 17). The 10% force intervals derive from the estimated maximum force (F_{Max}) experienced by the tendon during the ramped MVC (See Equation 2). The displacement of the tendon was measured as described previously. In addition, to allow for stiffness comparisons at an absolute load across populations, tendon stiffness was also calculated at a standardised force level which corresponded to just under the maximum baseline value of the weakest person (male = 1067.2 N, female = 1033.9 N).

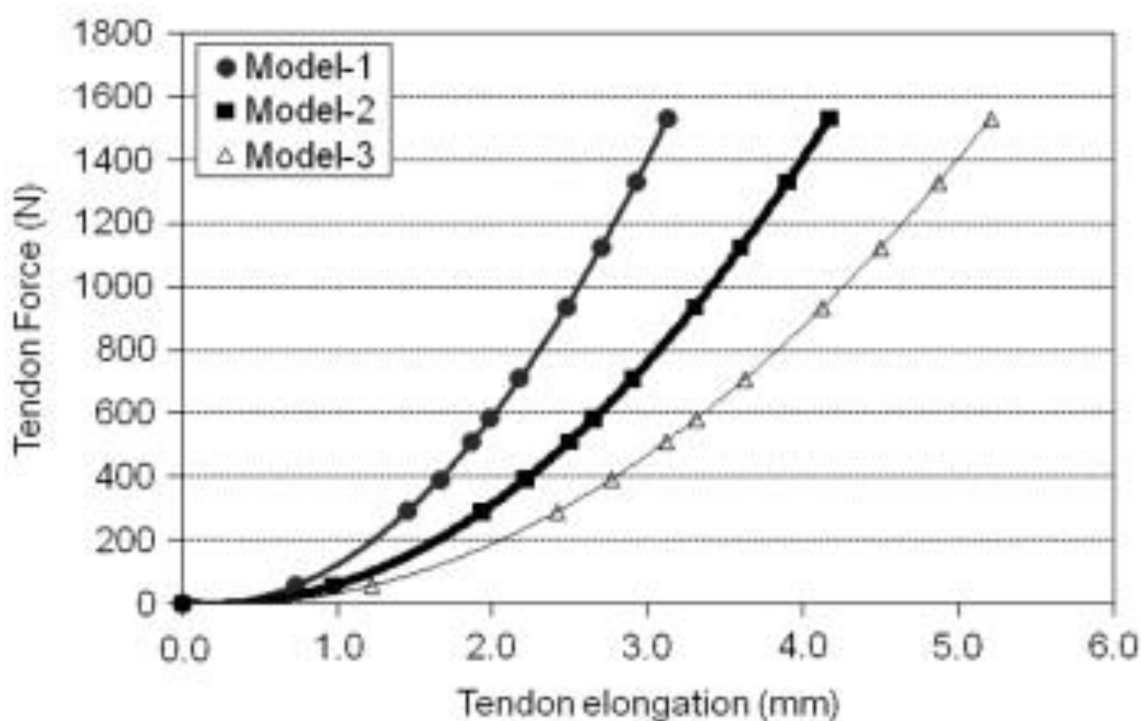


Figure 17. Model data sets described by a second degree polynomial curve. Tendon stiffness can be calculated at every 10% force interval from the force-displacement relations. Model 1 represents a stiff tendon, Model 2 - a mid-range stiffness tendon, and Model 3 - a compliant tendon (Pearson and Onambele, 2012)

Calculation of Young's Modulus

Patellar tendon cross-sectional area (PTCSA) and patellar tendon length (PTL) were measured in the resting state at a knee joint angle of 90º. PTCSA was determined from the mean of transverse-plane ultrasound images taken at 25, 50 and 75% of patellar tendon length, and processed using digitising software (Image J, National Institute of Health, Bethesda, MD, USA). PTL was assessed from sagittal-plane ultrasound images and measured from the inferior pole of the patella to the superior aspect of the tibial tuberosity. Young's Modulus (GPa) was calculated by multiplying the calculation of K by the ratio of PTL to PTCSA ($E = K \times$ (PTL ÷ PTCSA).

Calculations of tendon strain and tendon stress

Tendon strain (%) was calculated as the ratio of tendon displacement to PTL. Tendon stress (MPa) was calculated by dividing tendon force by PTCSA.

Calculation of tendon volume

Patellar tendon volume was calculated by geometric principles assuming a uniformly tapering truncated cone between measurement positions (i.e. the product of PTCSA at the three sections of the tendon, 25, 50, 75%, and PTL). Because three sections of the tendon are included in the measurement, separate measurements were made for top and bottom cones but overall volume is the sum of both calculations. Equation 5 was used to calculate tendon volume (V_t):

$$\text{Equation 5: } V_t = [(A_{25} + A_{50}) + (A_{25} \times A_{50})^{0.5} + (A_{50} + A_{75}) + (A_{50} \times A_{75})^{0.5}] \times h / 2$$

Where A_{25} and A_{50} are areas at the 1st and 2nd sections of the tendon (first cone) and A_{50} and A_{75} are the areas at the 2nd and 3rd sections of the tendon (second cone), and h is the height of each cone (i.e. ¼ of the total length of tendon). Anthropometric measures of muscle and tendon tissue have been modelled using truncated cone geometry in previous studies (Jones and Pearson, 1969, Fuller et al., 1999, Tothill and Stewart, 2002).

2.3 Genetic analysis

Sample collection

To obtain large amounts of genomic DNA, buccal cell samples were collected in duplicate from each participant. This method of collection brings a new perspective to obtaining DNA (Saab et al., 2007), it is not as invasive as blood-taking so is better tolerated by participants, and the procedure has low cost. The participants providing the buccal cell samples were instructed to refrain from eating or drinking within one hour of giving the sample. The investigator wore biohazard-barrier gloves and care was taken to avoid contact with the OmniSwab collection tip (Whatman Sterile OmniSwab, GE Healthcare, USA). Participants were instructed to brush the swab firmly against the inside of the cheek for 30 s. The second swab was collected from the opposite side of the mouth. After each sample was taken, the collection tip was ejected by firmly pressing the plunger at the end of the handle into a 2 mL microcentrifuge tube. Tubes were labelled and coded to ensure participant anonymity. The samples were immediately stored in a freezer at -20ºC until DNA extraction.

DNA extraction

Genomic DNA was extracted from buccal swab samples using the Qiagen QIAcube spin column protocol in accordance with the manufacturer's instructions (Qiagen, West Sussex, UK). All necessary buffers for DNA extraction were supplied in the Qiagen DNA blood Mini kit (Qiagen, West Sussex, UK). Briefly, buccal cells from the collection tip were heated to 56ºC in the QIAcube incubator for 10 min and lysed using Qiagen protease enzyme and briefly centrifuged, leaving ~ 900 µL of buccal swab lysate. The lysate was transferred to fresh 2 mL microcentrifuge tubes so that the purification phase could follow. This involved further centrifugation of the lysate at 8000 rpm for 60 s, and the addition of 200 µL of ethanol. The DNA was then free to bind to a silica-gel membrane and impurities were washed away following three further buffer centrifugation cycles, and the remaining was eluted into 200 µL low salt buffer within a 1.5 mL collection tube (Microtube 1.5 mL Safety Cap, Sarstedt AG & Co, Numbrecht, Germany) (See Figure 18). A maximum of 12 samples could be processed in this automated Qiagen QIAcube process.

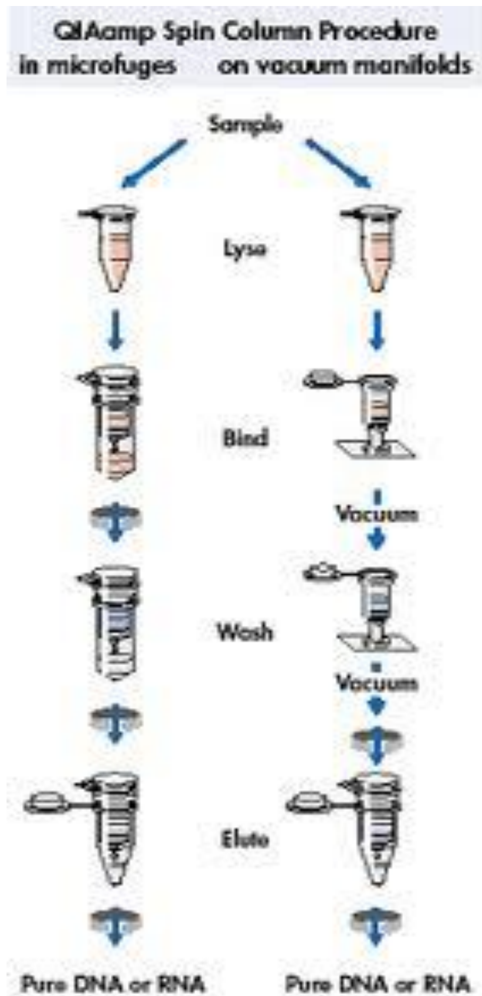


Figure 18. Summary of processes involved in DNA extraction from lysis to elution (QIAamp DNA Mini and Blood Mini Handbook, 2010)

Quantification of DNA

The concentration and purity of the sample (i.e. successful removal of protein contaminants) was evaluated using a biophotometer (WPA UV1101, Biochrom, Cambridge, UK). Briefly, ~12 µL of the DNA sample was pipetted into a glass cuvette, the absorbance readings of ultra-violet light at wavelengths of 260 nm (optimal absorption wavelength of DNA) and 280 nm (optimal absorption wavelength of aromatic amino acids present in protein) were performed and the ratio of absorbance at 260 nm divided by the reading of 280 nm was determined. Good quality DNA will have a ratio of 1.7-2.0 (Glasel, 1995); however any samples that were within 0.1units outside of this ratio range were also used for processing. Nevertheless, the best test of DNA quality is whether you achieve good results when determining the genotypes (e.g. real-time PCR-see below), so the

remaining ~ 190 µL DNA sample was stored at 4ºC until genotyping analyses were performed.

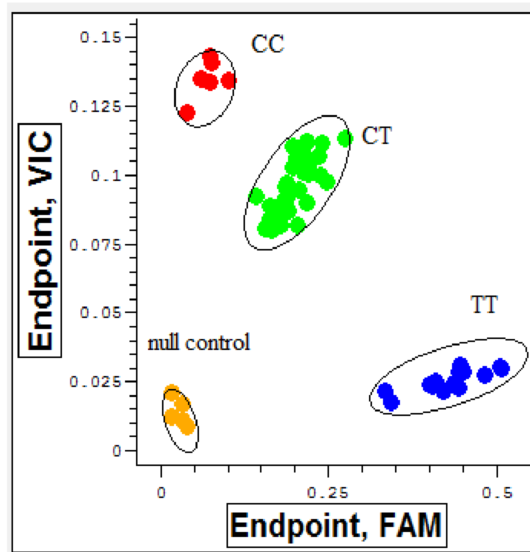
Genotyping

COL5A1 genotyping

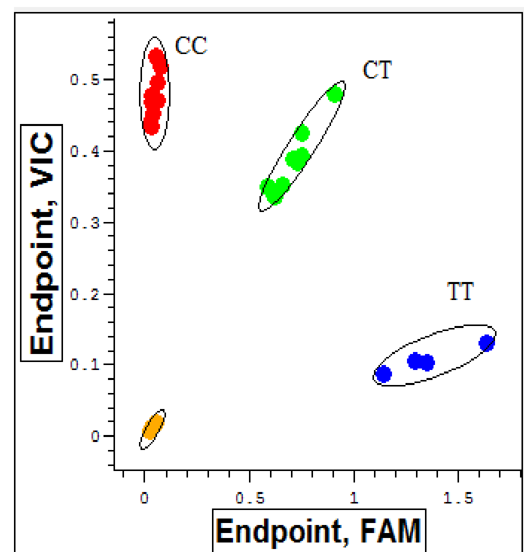
The *COL5A1* rs12722 genotype was determined using fluorescence-based TaqMan technique of polymerase chain reaction (PCR), based on the amplification of a fragment of genomic DNA overlapping the *COL5A1* rs12722 polymorphism, within the 3' untranslated region (UTR) of the *COL5A1* gene. Flanking primers and allele-specific probes specific to this SNP were used (obtained from Applied Biosystems Inc, UK). Forward primers were used to identify the starting point of the fragment and the reverse primer was used to identify the end point of the fragment of DNA to be amplified. Allele-specific probes to the C allele (VIC) and T allele (FAM) (obtained from Applied Biosystems Inc, UK) attached to their respective complementary sequences and emitted a fluorescent dye signal that could be recognised by the PCR machine.

The PCR assay volume within any given well of a 96 well PCR plate (Bio-Rad Laboratories Ltd, Herts, UK) was 10 µL, which contained 1 µL of purified DNA sample, and 5 µL of 2X TaqMan genotyping master mix. The master mix contained AmpliTaq Gold DNA polymerase, deoxyribonucleotide triphosphates (dNTPs), ROX Passive reference (an internal reference for reporter dye signal) and buffers (optimised for tight endpoint allelic discrimination) (Applied Biosystems Inc, UK). In addition, 0.5 µL of 20X SNP genotyping assay (Applied Biosystems Inc, UK) containing two primers and two probes (specific for the *COL5A1* rs12722 polymorphism), and 3.5 µL of nuclease-free H_2O (Qiagen, West Sussex, UK), contributed to the total PCR assay volume. Samples were run in duplicate to ensure minimal risk of genotyping errors, which can otherwise negatively affect the statistical power of genetic-association studies (Tintle et al., 2009). The PCR plate was sealed using Microseal 'B' Adhesive seals (Bio-Rad Laboratories Ltd, Herts, UK) and run on a Chromo4 Real-Time PCR Detection System (BioRad Laboratories Ltd, Herts, UK) for a total of 40 cycles of: 10 min at 95ºC to activate the DNA polymerase, denaturing at 95ºC for 15 s, primer annealing and extension at 60ºC for 60 s, and plate read. Genotypes were determined by endpoint fluorescence of VIC and FAM signals using the Chromo4 PCR machine, and results were analysed using Opticon Monitor Software (3.1.32: BioRad Laboratories Inc, Herts, UK). An example result is displayed in Figure 19A, with a high VIC/FAM ratio indicating a CC homozygote.

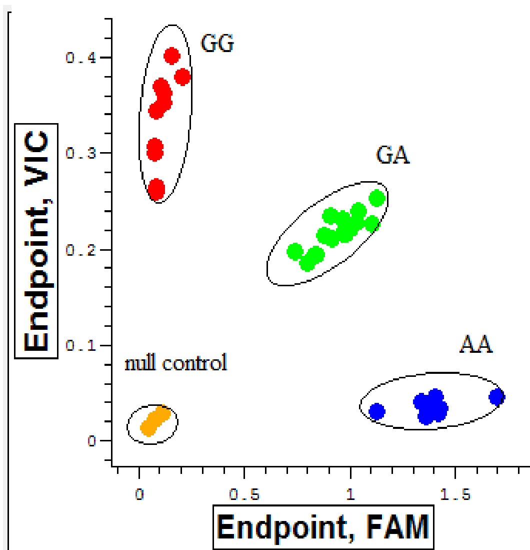
A: *COL5A1* genotype



B: *MMP3* rs591058 genotype



C: *MMP3* rs679620 genotype



D: *MMP3* rs650108 genotype

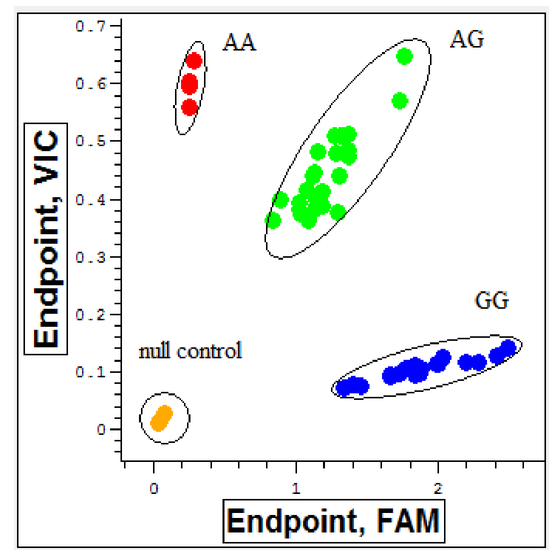


Figure 19. Example results of *COL5A1* rs12722 **(A)**, *MMP3* rs591058 **(B)**, *MMP3* rs679620 **(C)**, and *MMP3* rs650108 **(D)** genotypes, following PCR. Values for VIC and FAM (each emits a fluorescent dye signal when bound to complementary sequences) represent end-point values after 40 PCR cycles.

MMP3 genotyping

The three *MMP3* variants were genotyped using a modified version of the TaqMan PCR technique described above for the *COL5A1* rs12722 assay. Briefly, the PCR reaction volume was 10 µL, containing 1 µL of purified DNA sample, 5 µL master mix, 3.5 µL H_2O and 0.5 µL 20X SNP genotyping assay containing primers and probes, specific to the *MMP3* rs591058, rs679620 and rs650108 polymorphisms (Applied Biosystems Inc, UK). Amplification of the samples was determined as described for the *COL5A1* genotyping and example results are shown in Figure 19. B, C, D. A high VIC : FAM ratio indicates a CC homozygote for *MMP3* rs591058, GG homozygote for *MMP3* rs679620, and AA homozygote for *MMP3* rs650108.

2.4 Oestradiol measures

Following the measures of tendon properties, female participants only, reported to the biochemistry laboratory. A trained phlebotomist inserted a 21-gauge 25mm ultrathin wall needle (Terumo Medical Corporation, New Jersey, USA), into a superficial forearm vein. Using a syringe and serum separator tubes containing anti-coagulant (EDTA) (Sarstedt Monovette-Red cap, Numbrecht, Germany), 5 mL blood samples were collected. After being kept on an ice-bed for approximately 30 minutes, the sample was then centrifuged at 2-5°C for 10 min at 4100 rpm, with the supernatant of whole blood being extracted (~ 2 mL) and stored in two separate 1.5 mL microcentrifuge tubes (~ 1 mL in each) at -20°C, for later analyses. 17ß-oestradiol (E2) in the serum of the females was quantitatively determined using standard enzyme-linked immunosorbent assay (ELISA) procedures (Alpha Diagnostic International, San Antonio, USA; minimal detectable conc. of ~ 10 pg/mL, intra-assay precision of 9.87%, inter-assay precision of 10.11%).

Principle of the test and assay procedure

ELISA is based on the principle of competitive binding between endogenous E2 of the participants' samples and E2-Biotin-Avidin Horse-Radish Peroxidise (HRP) conjugate, for binding to a constant amount of an antibody (anti-Oestradiol polyclonal rabbit antibody), directed towards a unique E2 antigenic site on the molecule.

A 96 microwell plate (Alpha Diagnostic International, San Antonio, USA) was used to secure 39 samples, 6 standards of known quantity (ranging from 0-3200 pg/mL), 2 controls ('LOW'=230-450 pg/mL and 'HIGH'=750-1250 pg/mL) and 1 blank (dH_2O), all ran in duplicate concurrently so that all conditions of testing were the same. Firstly, 200-300 µL

of wash buffer (Alpha Diagnostic International, San Antonio, USA) was dispensed into all wells and then removed, so that 50 µL of the E2 standards, controls and samples could be dispersed into the appropriate wells coated with the antibody. An incubation period of 60 min at room temperature followed to allow 100 µL of the HRP labelled E2 (Alpha Diagnostic International, San Antonio, USA) to compete with the endogenous E2 in the standard, sample, and control serum, for a fixed number of binding sites of the specific E2 antibody. After incubation, unbound E2-HRP conjugate was removed by briskly shaking out the contents of the wells and rinsing the wells three times with diluted wash solution, interspersed each time with the sharp striking of the wells on absorbent paper to remove residual droplets. The premise in this instance is that the amount of the E2-HRP conjugate immunologically bound to the well progressively decreases as the concentration of E2 in the samples increases. Next, 150 µL of TMB HRP substrate solution (Alpha Diagnostic International, San Antonio, USA) was added to each well and a further incubation of 15 min at room temperature followed, which resulted in the development of blue colour from the enzymatic reaction. The colour development was stopped with the addition of 50 µL of TMB stop solution containing 0.5 mol/L of H_2SO_4 (blue colour turns yellow) (Alpha Diagnostic International, San Antonio, USA). The absorbance of each well was measured spectrophotometrically using a plate reader (Biotek Instruments Inc, Winooski, USA) at 450 nm within 10 min of adding the stop solution. The intensity of the colour formed (yellow colour) is inversely proportional to the amount of E2 in the samples.

Calculation of results

By plotting the concentration of each of the six standards against the absorbance, a standard curve was constructed (Figure 20). The mean absorbance value of each sample and control was determined concurrently with the corresponding concentration from the standard curve, using Gen5 data analysis software (Biotek Instruments Inc, Winooski, USA). The concentration of the samples was read directly from the standard curve.

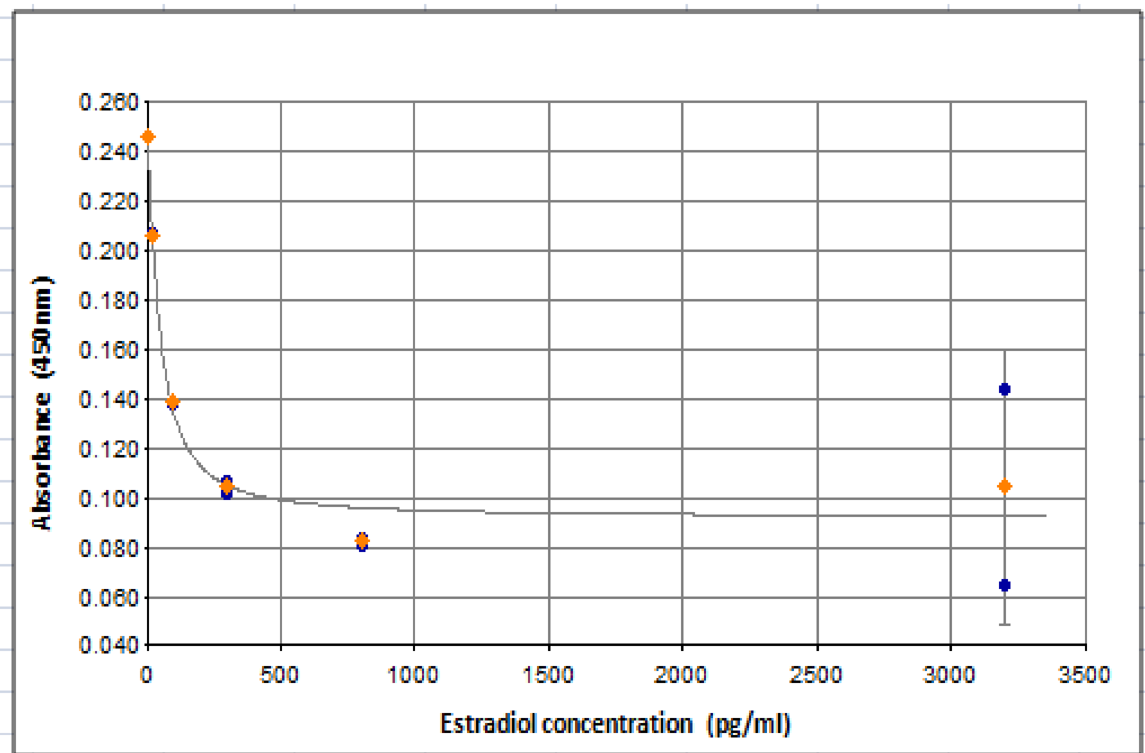


Figure 20. A standard curve constructed from our own laboratory data based on absorbance at 450nm vs. standard E2 concentrations. ◇ represents the mean absorbance values of the standard E2 concentrations. ○ represents both absorbance values read at each E2 concentration

In order to standardise the E2 concentrations for all females to the same day of the menstrual cycle (day 1), further calculations were made with reference to range data for normal cycling women with similar characteristics to the females within this study (i.e. age range 20-36 years, no use of contraceptives) (Stricker et al., 2006). Table 6 displays the results of the oestradiol analyses for the female participants in this study.

Table 6. Standardised oestradiol levels for day one of the menstrual cycle for all female participants in this study.

	FEMALES (N=39)
	Mean (SD)
Oestradiol (pg/mL) on day 1	34.70 (27.95)

Values are expressed as mean (standard deviation)

2.5 Statistical analyses

All data were analysed with SPSS version 19.0.0. Before commencing any statistical analyses on the data obtained, parametricity tests checks were performed to determine whether the population was normally distributed and there was equal variance (homogeneity) in the variables of interest (Young's Modulus and volume). The homogeneity of variance was assessed using Levene's statistic. If the data set did not meet the assumptions of a parametric measurement scale, i.e. the data were not normally distributed, and the variance of the data sets was not equal (Williams and Wragg, 2004), then the appropriate non-parametric statistical tests were conducted. The Kruskal-Wallis test was performed instead of the ANOVA and the Mann-Whitney U test was performed instead of independent t-tests.

Hardy-Weinberg Equilibrium

Each SNP was tested for Hardy-Weinberg Equilibrium (HWE) (genotype and allelic associations) within each population (male and female), using a freely available software package (Rodriguez et al., 2009). This test was conducted before selecting individuals for the phenotype tests, based on a higher degree of homozygosity, in order to establish whether the genotype and allele frequencies were constant between this sample and the general population. All SNPs for each population were in HWE ($P > 0.05$ with 1df (one degree of freedom)).

Statistical power to detect genotype-phenotype associations

Once the participant subgroup had been identified based on a higher degree of homozygosity, for prospective correlations with the phenotype measurements, it was prudent to perform a-priori, statistical procedures to estimate the extent to which trait variation (i.e. tendon properties) is explained by particular polymorphisms. So, based on power calculations with alpha set at 0.05 and beta set at 0.80 and using mean and standard deviation data of tendon properties obtained in our lab, it was estimated that approximately 40 of the original approximate 100 participants in both sexes would be required to complete the tests of tendon properties, in order to detect differences in tendon properties in the order of ~1-2% for tendon volume, and ~10-15% for tendon modulus. G*Power 3.1.6 (Franz Faul, Universitat Kiel, Germany) was used to calculate sample size.

Differences between genotypes for the SNPs, *COL5A1* rs12722 and *MMP3* rs650108, and the continuous data, namely the Young' Modulus (*E*) and the volume (mm^3) of the patellar tendon, were determined by one-way analysis of variance (ANOVA). Where appropriate, and hypothesis-driven, the TT genotype group was compared to the combined TC and CC genotype groups of the *COL5A1* SNP. Similarly, the GG genotype group of the *MMP3* rs650108 SNP was compared with the AG and AA genotypes combined, on the premise that the AA genotype has a low allele frequency in the population and sample. In these instances, independent t-tests were used to compare the two groups.

In the case of the male genotype data, only two genotype groups were present for the *MMP3* rs591058 and rs679620 SNPs, in accordance with one of the main hypothesis-driven aims to 'stress the genotype', by only retaining homozygote genotype groups. Additionally, these two SNPs are in perfect linkage disequilibrium (LD) so ultimately they were analysed in combination using independent t-tests. Where data were non-parametric, appropriate equivalent tests were carried out (Kruskal-Wallis test for ANOVA, and Mann-Whitney U test for independent t-tests), with values presented as median and interquartile range.

For genotype effects on Young's Modulus and volume, both unadjusted *P* values and *P* values adjusted for BMI, age, and oestradiol (in females only) were calculated where appropriate. Correlation analyses were used to determine any relationships between the variables of interest (Young's Modulus and volume) and the aforementioned numerical characteristics (such as BMI, age, oestradiol). Where a significant correlation was determined, an ANCOVA was run with these characteristics acting as covariates on the data. Significance was set to $P \leq 0.05$ with all data being presented as mean and standard deviation (SD).

Z-score analyses

To provide more stable measures of the overall impact of genotype on measures of tendon properties, composites were formed with unit-weighted z-scores of constituent tests (Ackerman and Cianciolo, 2000), i.e. Young's Modulus and volume measures. Z-scores are used to standardize scores from different groups of data such as Young's Modulus and volume measures. So, in this instance, the structural (volume) and functional (Young's Modulus) properties of tendon could be scaled and analysed together. Thus, the raw test

scores of Young's Modulus in GigaPascals (GPa) and volume in cubic millimetres (mm^3) were converted to z-scores using equation 6:

Equation 6: z-score = (variable score-mean) / standard deviation

Essentially, the z-score represents the number of standard deviations the raw score being converted, is from the mean. A value of '0' is equal to the group mean of a particular variable so z-scores can be positive or negative depending on whether the unit weighted standard deviations are above the mean or below the mean (95% of the data should have a z-score between '-2' and '+2', if normally distributed).The complexity of data can be simplified and a more efficient explanation of the analyses can be presented by adding up the z-scores of each variable (Young's Modulus and volume, combined).

Reliability of measurements

Reliability statistics for measures of tendon properties were computed as intraclass correlation coefficients (ICCs) using a two-way mixed, absolute agreement model (SPSS version 19.0.0). The intra-reliability (or test-retest reliability) for measuring constituent properties of tendon was assessed in one investigator between sessions; the repeated session was within 1-2 weeks of the first session for each participant. The ICC of the structural (volume) and functional (Young's Modulus) properties of tendon were carried out on a total of five participants. However, the use of ICC to quantify reliability has been criticised (Bland and Altman, 1990) because the strength of the correlation is dependent on the range of values in the sample, and also because it is not entirely clear what value of R indicates 'excellent', 'good' or 'poor' reliability. Therefore, together with the ICC measurement, the ratio limits of agreement (Nevill and Atkinson, 1997) was also used to detect systematic bias in the measures of tendon properties. Specifically, the absolute reliability or 'agreement' can be better detected between participants and provide a magnitude of disagreement between measurements on separate occasions, based on taking natural logarithms (Nevill and Atkinson, 1997).

Table 7. Reliability results showing the Intraclass Correlation (ICC) and ratio levels of agreement between volume and Young's Modulus measures

	Volume	**Young's Modulus**
ICC	0.99	0.98
Mean bias ratio	0.998* (*P* = 0.62)	1.059* (*P* = 0.36)
Random error component	×/÷1.019	×/÷ 1.144
Upper ratio limits	1.017	1.211
Lower ratio limits	0.980	0.926

*A significant correlation between test and re-test indicated by *P*-values (parentheses)

When referring to Table 7, the ICC of both measurement parameters is excellent (volume- 0.99; Young's Modulus- 0.98); a general rule is that an ICC over 0.75 is considered good (Landis and Koch, 1977, Nussbaumer et al., 2010). In addition, the ratio levels of agreement measurement indicate that the volume re-test reliability show little bias (0.998, *P* = 0.62) and an excellent agreement ratio (×/÷ 1.019) - that is, 95% of ratios are constrained between approximately 1.9% of the mean bias ratio, either in a positive or negative direction. The Young's Modulus re-test reliability shows little bias also, although not as good as the volume measure (1.059, *P* = 0.36) and good agreement ratio (×/÷ 1.144) with 95% of ratios constrained between approximately 14% of the mean bias ratio, either in a positive or negative direction .

Chapter 3.

Human *COL5A1* rs12722 gene polymorphism and tendon properties in males

3.1 Introduction

The primary role of tendons is to transmit contractile forces from muscle to bone enabling movement to occur (Butler et al., 1978). Tendons are well known to operate as spring-like structures exhibiting elastic and force dependent properties, which provide important functional characteristics for the muscle-tendon complex as a whole. The interaction between muscle and tendon not only influences force transmission (Burgess et al., 2007, Reeves et al., 2003a), but also energy storage and return for locomotion (Alexander, 1991, Voigt et al., 1995, Fukunaga et al., 2001), joint positional control (Loram et al., 2004, Loram et al., 2005a, Loram et al., 2005b), and to protect from muscle fibre damage (Griffiths, 1991, Lieber and Friden, 2000). Therefore, the tendon mechanical properties play a pivotal role in determining the function of the overall muscle-tendon complex, The tendon mechanical property most commonly associated with *in vivo* function is the 'modulus,' i.e. the relation between stress and strain. Modulus represents the material properties of tendon independent of its structural size, making it possible to compare tendon mechanical properties between individuals with different tendon dimensions. Essentially, a high tendon modulus represents a relatively stiff tissue.

Recently, it has been reported that the *COL5A1* gene is associated with tendon pathologies (Mokone et al., 2006, September et al., 2008), range of motion (ROM) (Collins et al., 2009, Brown et al., 2011b), and endurance running performance (Brown et al., 2011a, Posthumus et al., 2011). The *COL5A1* gene encodes the pro α1 chain of type V collagen (Col V), a quantitatively minor fibrillar collagen that through its heterotypic interactions with type I collagen (Col I), may have regulatory roles in controlling fibril diameter within connective tissues such as tendon (Birk et al., 1990, Wenstrup et al., 2011). In particular, the CC genotype of the *COL5A1* rs12722 single nucleotide polymorphism (SNP) was significantly over-represented in asymptomatic participants compared with chronic Achilles tendinopathy (AT) in two independent Caucasian populations ((South Africa, (Mokone et al., 2006); Australia, (September et al., 2008)). Similarly, the CC genotype was significantly associated with increased sit and reach ROM (Brown et al., 2011b, Brown et al., 2011a), although Collins et al. (2009) reported that the CT genotype had significantly lower sit and reach, and standing leg raise ROM, than the homozygous individuals. In contrast, the TT genotype of the *COL5A1* rs12722 SNP has been reported to be associated with enhanced endurance running performance (Posthumus et al., 2011, Brown et al., 2011a).

The multifactorial nature of tendon pathologies (Riley, 2004), ROM (Gleim and McHugh, 1997), and endurance running performance (Joyner and Coyle, 2008), reduces the ability to identify the main causative factors that contribute to the phenotype, although tendon stiffness may be one such intermediate phenotype linking genetic variation to risk of injury, ROM and endurance running performance. Independent of genetics, there are relationships that appear to exist between these phenotypes, in that ROM has been associated with tendon injuries (Witvrouw et al., 2004, Witvrouw et al., 2007). For instance, a more compliant tendon (low stiffness) is able to absorb more energy for a given mechanical load, and thus, reduce the risk of strain overload (Witvrouw et al., 2004). Tendon stiffness has been associated with running economy in that an inverse relationship between running economy and tendon stiffness has been reported to exist (Arampatzis et al., 2006, Fletcher et al., 2010). It may be that the *COL5A1* rs12722 SNP is associated with structural and morphological changes to the collagen fibrils, which may directly or indirectly modify the mechanical properties of tendon.

The mechanical properties of tendon itself can be assessed *in vivo* in humans with high accuracy and reliability, as detailed by Pearson and Onambele (2006), This *in vivo* assessment of tendon properties provides a more direct association with the role of *COL5A1* rs12722 SNP, in contrast to the previous surrogate measures of tendon properties obtained from sit and reach, and standing leg raise tests (Collins et al., 2009, Brown et al., 2011b). These tests are not precise measures of the phenotype under investigation, and hence, the assessment methods adopted in this study to measure tendon properties are in line with the objectives of associating the genetic contribution to the interindividual variability in mechanical properties of human tendon. The aim of this study was therefore to investigate whether the *COL5A1* rs12722 gene variant influences the modulus of the patellar tendon, in an asymptomatic male population, using an accurate, reproducible and non-invasive assessment of tendon properties *in vivo*. In addition, the aim encompasses whether the structural volume of the tendon, and the composite effect of both modulus and volume, is influenced by this gene variant.

3.2 Method

Participants: Forty-five male participants (age 22.9 (3.3) years; BMI 24.6 (2.6) kg/m²) were recruited for this study (as described in section 2.1). Physical characteristics of all participants are presented in Table 4 (section 2.1). Participants gave written informed consent (section 2.1). A detailed description of the methods for measures of tendon properties, and genotyping the *COL5A1* rs12722 SNP is given elsewhere (section 2.2 and 2.3, respectively).

Tendon properties: Briefly, Young's Modulus represented the mechanical properties of tendon and was calculated by multiplying the tendon stiffness (derived from the force-elongation curve), by the ratio of patellar tendon length (PTL) to patellar tendon cross-sectional area (PTCSA). The volume represented the structural extent of the patellar tendon and was calculated by geometric principles assuming a uniformly tapering truncated cone between measurement positions (i.e. the product of PTCSA at the three sections of the tendon, 25, 50, 75%, and PTL). The z-scores represented the scaling together of the structural (volume) and functional (Young's Modulus) properties of the tendon, and was calculated from the combination of the scores derived from the means of each tendon measure (Young's Modulus and volume), divided by their respective standard deviations.

Genotyping: The *COL5A1* rs12722 genotypes were determined using fluorescence-based TaqMan technique of polymerase chain reaction (PCR), based on the amplification of a fragment of genomic DNA overlapping the *COL5A1* rs12722 polymorphism, within the 3' untranslated region (UTR) of the *COL5A1* gene.

Statistical analyses: A one-way analysis of variance (ANOVA) was used to determine any significant difference between the characteristics of the genotype groups and where appropriate, independent t-tests were conducted when combining genotype groups, as well as non-parametric equivalents if the data set did not meet certain assumptions (section 2.5). As only age (z-scores, $r = 0.308$, $P = 0.04$) and BMI (Young's Modulus, $r = 0.348$, $P = 0.019$) showed a significant correlation with any of the phenotypic measures, age and BMI were used as covariates. Statistical significance was accepted when $P \leq 0.05$. All measurements showed high reliability (section 2.5).

3.3 Results

An ANOVA was performed on all three genotype groups and the measures of patellar tendon volume and z-scores. The Kruskal-Wallis non-parametric equivalent statistical test was performed on Young's Modulus and its association with the three genotype groups, as the data was not normally distributed. Independent t-tests were performed on the volume and z-score measures when combining two of the genotype groups. The Mann-Whitney U test was assigned to the Young's Modulus measure, and its association with the two groups.

There were no significant differences in patellar tendon volume and Young Modulus measurements and z-scores, between the genotype groups (Figure 21 A) (volume, P = 0.936; Young's Modulus, P = 0.897; z-scores, P = 0.820). The z-scores were co-varied for age but remained not significant (z-score, P = 0.635) (Table 8). In addition, there were no significant differences in volume, Young's Modulus and z-scores when comparing the TT genotype group to the combined TC and CC genotype groups (Figure 21 B) (volume, P = 0.765; Young's Modulus, P = 0.768; z-scores, P = 0.744). The z-scores were also adjusted for age in the combined genotype groups (P = 0.694) (Table 9). In addition, there were no *COL5A1* gene variant genotype effects on any of the participant characteristics (Table 8 and 9).

Table 8. General characteristics with measures of patellar tendon properties for the TT, TC or CC *COL5A1* rs12722 genotype groups

N = 45	***COL5A1* rs12722 genotype groups**			
	TT (n=12)	**TC (n=28)**	**CC (n=5)**	***P*-value**
Age (years)	22.5 (20-27)	22.5 (19-32)	23 (20-30)	0.651
Height (cm)	177.1 (157-191)	180 (167-190)	183.5 (160-187)	0.366
Weight (kg)	75.5 (66-95)	79.2 (58-99)	78.6 (60-88)	0.784
BMI (kg/cm^2)	24.3 (22-29)	23.8 (20-30)	23.4 (21-29)	0.728
Volume (mm^3)	2315 (429)	2275 (246)	2280 (366)	0.936
Young's Modulus (GPa)	0.48 (0.64)	0.41 (1.23)	0.37 (0.39)	0.897
Z-scores	0.128 (1.681)	0.031 (1.244)	-0.332 (1.347)	0.820 (0.635)

General characteristics are expressed as median (range)

Volume and Z-scores are expressed as mean (standard deviation)

Young's Modulus is expressed as median (range)

The *P*-value for Z-scores are reported with age as a covariant in parenthesis

Table 9. General characteristics with measures of patellar tendon properties for the TT, or TC and CC *COL5A1* rs12722 genotype groups

N = 45	***COL5A1* rs12722 genotype groups combined**		
	TT (n=12)	**TC & CC (n=33)**	***P*-value**
Age (years)	22.5 (20-27)	23 (19-32)	0.806
Height (cm)	177.1 (157-191)	180.5 (160-190)	0.248
Weight (kg)	75.5 (66-95)	78.6 (58-99)	0.712
BMI (kg/cm^2)	24.3 (22-29)	23.4 (20-30)	0.438
Volume (mm^3)	2315 (429)	2276 (261)	0.715
Young's Modulus (GPa)	0.48 (0.64)	0.41 (1.23)	0.768
Z-scores	0.128 (1.681)	-0.024 (1.245)	0.744 (0.694)

General characteristics are expressed as median (range)

Volume and Z-scores are expressed as mean (standard deviation)

Young's Modulus is expressed as median (range)

The *P*-value for Z-scores are reported with age as a covariant in parenthesis

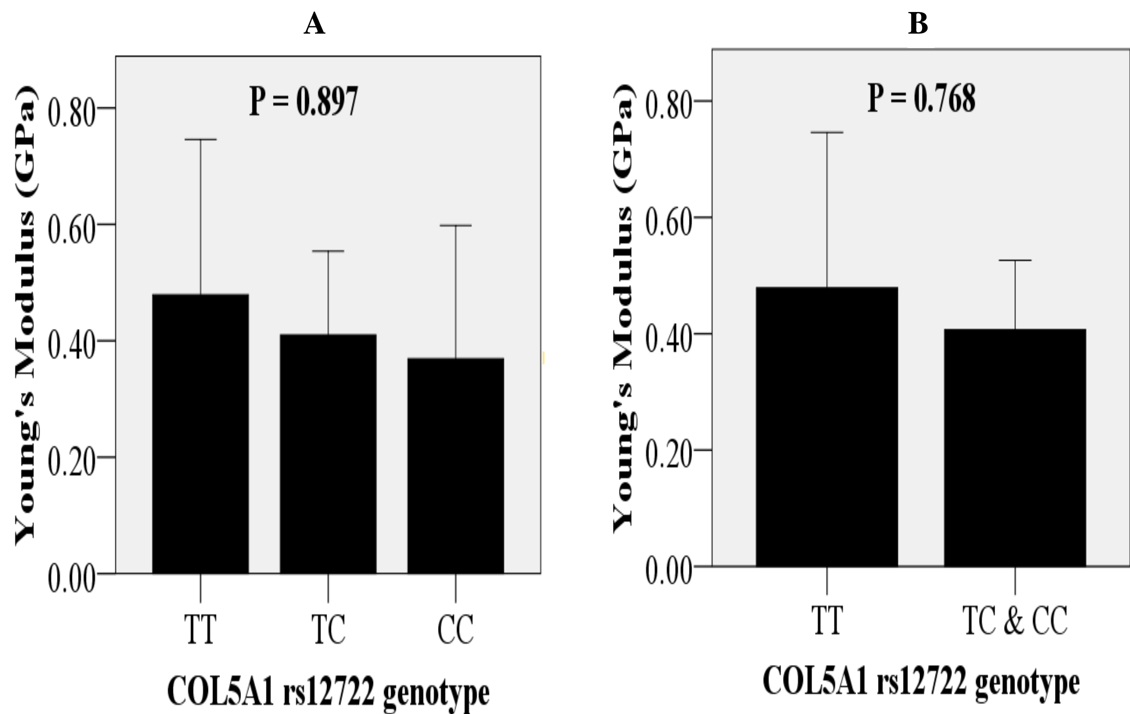


Figure 21. Young's Modulus values for the genotype groups of the *COL5A1* rs12722 gene variant. **A**: TT vs. TC vs. CC. **B**: TT vs. TC & CC. Between genotype group comparisons did not reach statistical significance as indicated by the *P*- values. Error bars represent the upper quartile range.

3.4 Discussion

This study shows no significant association between the *COL5A1* rs12722 variant and any of the measures of tendon properties. These data suggest that the *COL5A1* rs12722 variant does not associate with structural (volume) and functional (modulus) properties of the patellar tendon, or indeed as a composite (z-scores) (Tables 7 and 8), in an asymptomatic male population. The hypothesis of an association between *COL5A1* rs12722 SNP and structural and mechanical properties of the patellar tendon was based on the role of the *COL5A1* gene, as it encodes the pro α1 chain of type V collagen (Col V), which through its heterotypic interactions with type I collagen (Col I), may have regulatory roles in controlling fibril diameter within connective tissues such as tendon (Birk et al., 1990, Wenstrup et al., 2011). Indeed, previous published reports have shown that the *COL5A1* rs12722 variant associates with phenotypes such as Achilles tendon pathologies (Mokone et al., 2006, September et al., 2008), ROM (Collins et al., 2009, Brown et al., 2011b), and endurance running performance (Brown et al., 2011a, Posthumus et al., 2011); phenotypes that are likely to be influenced by tendon modulus. However, in the present investigation

we have observed no link between the *COL5A1* gene variant and tendon modulus (Figure 21 A and B). This would suggest that where the *COL5A1* gene variant has previously been associated with the tendon through some other surrogate of tendon modulus, other factors should be considered.

Collins and Posthumus (2011) have proposed an association between injury, ROM and endurance running ability phenotypes, and the *COL5A1* rs12722 variant, with the mechanical properties of musculoskeletal soft tissue being a possible intermediate phenotype. In this investigation, we have identified tendon modulus under isometric conditions, and although it is an established method for determining the material properties *in vivo,* it may be limited in defining the consequence of the muscle-tendon complex as a whole. For example, although endurance running performance is linked to the *COL5A1* rs12722 gene variant and here we report no relation, it is perhaps relevant to note that tendon hysteresis (viscoelastic property relating to energy economy) as opposed to modulus is likely to influence the performance of endurance running (Sano et al., 2012). Indeed, in regards to injury there remains no link between tendon properties and tendon or muscle damage. If anything, this data is consistent with what has previously been demonstrated, that flexibility or ROM (associated with the *COL5A1* gene) has no association with tendon modulus (no association with *COL5A1* gene). In addition, the patellar tendon may play a different functional role than the Achilles tendon, which is known to contribute significantly to ROM (Morse et al., 2008), and due to its greater length, contributes significantly to attenuating length changes in the muscle during eccentric loading of the lower limb (Spanjaard et al., 2008).

The lack of association reported in this study between measures of patellar tendon properties and the *COL5A1* rs12722 variant may indirectly suggest that tendon structural and functional properties do not associate with phenotypes previously reported to associate with this gene variant. The multifactorial nature of such phenotypes due to multiple factors (e.g. non-genetic) being implicated in their aetiology, such as different functional roles of tendon based on anatomical positions, makes observations of interindividual genetic variation in phenotypes such as tendon properties more complex. Compounding this complexity is the fact that non-genetic factors can be considered multifactorial phenotypes in their own right (Collins and Raleigh, 2009).

Within this study an attempt was made to maximise the ability to detect genotype-phenotype associations by controlling for non-genetic factors and variables known to contribute to the variability on tendon structure and function, by adopting strict exclusion criteria (see methods, 2.1). For instance, an asymptomatic group of participants with a pre-selected age range was recruited, because it has been reported that there is an age-dependent increase in the distribution of the CC genotype of the *COL5A1* rs12722 variant (Collins and Posthumus, 2011).

In conclusion, there was no association between the *COL5A1* rs12722 gene variant and measures of patellar tendon properties in an asymptomatic male cohort. Nevertheless, future identification of sequence variants within genes with structural and regulatory roles in the tendon extracellular matrix should be investigated for their potential influences on tendon properties, in coming years. Specifically, those of the lower limbs including the patellar and Achilles tendon would be of precedence. This could potentially enhance the efficacy of multifactorial models, developed to understand the molecular mechanisms that contribute to physical performance and cause tendon-specific injuries.

Chapter 4.

Human *COL5A1* rs12722 gene polymorphism and tendon properties in females

4.1 Introduction

A higher incidence of tendon injuries among women suggests that the structure and/or metabolism of tendon may be different between the sexes (Trappe, 2007). Indeed, cross-sectional studies highlight such sex-specific differences, in that it has been reported that sex hormones specific to women such as oestradiol have deleterious effects on tendon tissue quality (Hansen et al., 2009b, Hansen et al., 2008). In addition, collagen synthetic rates at rest and following an acute bout of exercise are lower in women than in men (Miller et al., 2007), suggesting an inferior responsiveness to adaptation. At a molecular level, structural and regulatory mRNA expression levels relating to collagen and matrix metalloproteinase-3 (MMP3) protein levels differ between the sexes, with women being more susceptible to tendon pathologies as a result (Sullivan et al., 2009).

From a structural and functional perspective, women are less responsive to increases in tendon size or hypertrophy and exhibit a tendon elastic modulus (relation between stress and strain of material) less than half of that of men, as deduced from tendon biopsies (Magnusson et al., 2007). Indeed, in a cross-sectional study investigating the structural and mechanical properties of tendon *in vivo* between young men and women, has reported that females have a ~21% smaller CSA and a 53% lower Young Modulus than males (Onambele et al., 2007). These sex-specific differences may be explained in some part by the effects of higher levels of circulating oestradiol in females. In an elderly cohort where oestradiol is not likely to have an effect on sex-specific differences in tendon properties, no differences in the mechanical properties of tendon between sexes *in vivo* were evident (Carroll et al., 2008, Carroll et al., 2011, Burgess et al., 2009b). It may also be that sex-specific differences in metabolism, structure, and function reflect the levels of absolute loading, related to inferior force output in women.

Variation in genes that code for proteins that have structural roles within tendon may associate with the mechanical properties of tendon. Indeed, the *COL5A1* rs12722 gene variant has shown to be associated with Achilles tendinopathies (Mokone et al., 2006, September et al., 2008), range of motion measures (ROM) (Brown et al., 2011b, Collins et al., 2009), and endurance running performance (Brown et al., 2011a, Posthumus et al., 2011). Recently, the biological function of the region of genomic DNA in which the *COL5A1* gene is found (3' UTR) has been investigated (Laguette et al., 2011). An increase in mRNA stability associated with the 'T' allele variant has been reported to produce more pro α1 (V) chain protein, and thus, increased collagen type V (Col V) production.

Phenotypically, it has been proposed that this particular genetic variation may result in structural and architectural changes within the collagen fibril, which could further result in altered mechanical properties of musculoskeletal soft tissues, such as tendon (Collins and Posthumus, 2011).However, we have shown previously that there is no significant association between the *COL5A1* rs12722 gene variant and measures of structure and mechanical properties of the patellar tendon, in an asymptomatic male population (see chapter 3). The reported differences in tendon structure, function and metabolism between the sexes, as explained above, may be a result of gene-hormone interactions specific to women, and so the genotype-phenotypes association may differ as a result.

The aim of this study was therefore to investigate whether the *COL5A1* rs12722 gene variant within the 3' UTR of the *COL5A1* gene was partly responsible for the inter-individual variation in structural size and modulus of the patellar tendon, independently, and in combination, in an asymptomatic female population.

4.2 Method

Participants: Thirty-nine female participants (22.4 (4.8) years; BMI 23.2 (2.8) kg/m^2) were recruited for this study (as described in section 2.1). Physical characteristics of all participants are presented in Table 4 (section 2.1). Participants gave written informed consent (section 2.1). A detailed description of the methods for measures of tendon properties, and genotyping the *COL5A1* rs12722 SNP is given elsewhere (section 2.2 and 2.3, respectively).

Tendon properties: Briefly, Young's Modulus represented the mechanical properties of tendon and was calculated by multiplying the tendon stiffness (derived from the force-elongation curve), by the ratio of patellar tendon length (PTL) to patellar tendon cross-sectional area (PTCSA). The volume represented the structural extent of the patellar tendon and was calculated by geometric principles assuming a uniformly tapering truncated cone between measurement positions (i.e. the product of PTCSA at the three sections of the tendon, 25, 50, 75%, and PTL). The z-scores represented the scaling together of the structural (volume) and functional (Young's Modulus) properties of the tendon, and was calculated from the combination of the scores derived from the means of each tendon measure (Young's Modulus and volume), divided by their respective standard deviations.

Genotyping: The *COL5A1* rs12722 genotypes were determined using fluorescence-based TaqMan technique of polymerase chain reaction (PCR), based on the amplification of a fragment of genomic DNA overlapping the *COL5A1* rs12722 polymorphism, within the 3' untranslated region (UTR) of the *COL5A1* gene.

Oestradiol: Oestradiol levels were analysed using standard ELISA procedures from serum content taken on the day of measures of tendon properties (see section 2.4 for more detail on this procedure), to assess the contribution of this ligand to measures of tendon properties. Serum levels of oestradiol have been associated with tendon stiffness in young females (Burgess et al., 2009c). Oestradiol was therefore factored into statistical analyses as a potential covariate.

Statistical analyses: A one-way analysis of variance (ANOVA) was used to determine any significant difference between the characteristics of the genotype groups and values of volume and z-scores. Independent t-tests were conducted when combining genotype groups and its relation with volume and z-scores. The Kruskal-Wallis non-parametric statistical test was used for assessing differences between all three genotype groups and Young's Modulus, with the Mann-Whitney U test being used when assessing differences between the combined genotype groups and Young's Modulus. As only BMI showed a significant correlation with any of the phenotypic measures (volume, $r = 0.334$, $P = 0.038$; Young's Modulus, $r = 0.440$, $P = 0.005$; z-scores, $r = 0.473$, $P = 0.002$) BMI was used as a covariate. Oestradiol showed no significant correlation with tendon properties (volume, $r = 0.010$, $P = 0.950$; Young's Modulus, $r = 0.146$, $P = 0.375$; z-scores, $r = 0.055$, $P = 0.740$) and so was not used as a covariate. Statistical significance was accepted when $P \leq 0.05$. All measurements showed high reliability (section 2.5).

4.3 Results

There were no significant differences in the patellar tendon volume and Young's Modulus measurements and z-scores, between the genotype groups (Figure 22 A) (volume, $P = 0.667$, Young's Modulus, $P = 0.227$; z-scores, $P = 0.398$). *P*-values were adjusted for BMI where appropriate (volume, $P = 0.938$; z-scores, $P = 0.896$) (Table 10). In addition, there were no significant differences in volume, Young's Modulus and z-scores when comparing the TT genotype group to the combined TC and CC genotype groups (Figure 22 B) (volume, $P = 0.627$; Young's Modulus, $P = 0.053$; z-scores, $P = 0.437$). Again, the volume and z-scores were adjusted for BMI ($P = 0.385$; $P = 0.720$, respectively) (Table 11). There

were no *COL5A1* gene variant genotype effects on any of the participant characteristics (Table 10 and 11)

Table 10. General characteristics and measures of patellar tendon properties for the TT, TC or CC *COL5A1* rs12722 genotype groups

N = 39	*COL5A1* rs12722 genotype groups			
	TT (n=14)	TC (n=17)	CC (n=8)	*P*-value
Age (years)	21 (19-23)	21 (18-39)	21.5 (19-24)	0.808
Height (cm)	166.8 (154-179)	165.5 (153-183)	166.75 (159-175)	0.604
Weight (kg)	63.5 (50-80)	63.2 (47-77)	68 (48-79)	0.655
BMI (kg/cm^2)	23.8(19-30)	24.1 (21-27)	21.3 (19-24)	0.075
Oestradiol (pg/ml)	24.6 (9-90)	27.1 (7-116)	26 (6-46)	0.839
Volume (mm³)	1505 (208)	1543 (195)	1457 (305)	0.667 (0.938)
Young's Modulus (GPa)	0.64 (1.02)	0.49 (1.01)	0.42 (0.84)	0.227
Z-scores	0.209 (1.731)	0.160 (1.385)	-0.711 (1.972)	0.398 (0.896)

General characteristics are expressed as median (range)

Volume and Z-scores are expressed as mean (standard deviation)

Young's Modulus is expressed as median (range)

The *P*-value for Volume and Z-scores are reported with BMI as a covariant in parenthesis

Table 11. General characteristics and measures of patellar tendon properties for the TT, or TC and CC *COL5A1* rs12722 genotype groups

N = 39	*COL5A1* rs12722 genotype groups combined		
	TT (n=14)	TC & CC (n=25)	*P*-value
Age (years)	21 (19-23)	21 (18-39)	0.534
Height (cm)	166.8 (154-179)	165.5 (153-183)	0.623
Weight (kg)	63.5 (50-80)	64 (47-79)	0.685
BMI (kg/cm^2)	23.8 (19-30)	22.9 (19-28)	0.134
Oestrodiol (pg/ml)	24.6 (9-90)	27.1 (6-116)	0.598
Volume (mm³)	1505 (208)	1515 (233)	0.893 (0.755)
Young's Modulus (GPa)	0.636 (1.02)	0.488 (1.13)	0.151
Z-scores	0.209 (1.731)	-0.119 (1.608)	0.556 (0.689)

General characteristics are expressed as median (range)

Volume and Z-scores are expressed as mean (standard deviation)

Young's Modulus is expressed as median (range)

The *P*-value for Volume and Z-scores are reported with BMI as a covariant in parenthesis

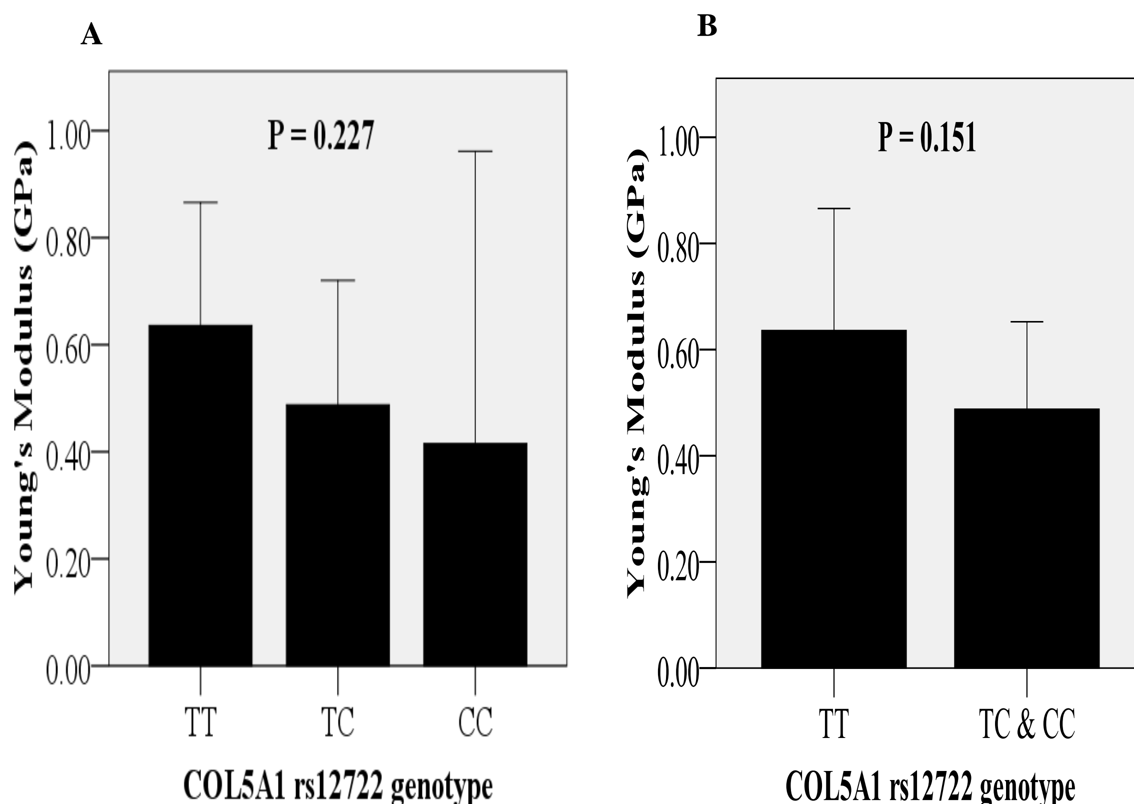


Figure 22. Young's Modulus values for the genotype groups of the *COL5A1* rs12722 gene variant. **A**: TT vs. TC vs. CC. **B**: TT vs. TC & CC. Between genotype group comparisons did not reach statistical significance as indicated by the *P*- values. Errors bars represent the upper quartile range.

4.4 Discussion

This study shows no significant association between the *COL5A1* rs12722 variant and measures of patellar tendon properties. Specifically, these data suggest that structural (volume) and functional (modulus) properties of the tendon, or the interaction of both (z-scores), do not associate with this gene variant in an asymptomatic female population. Previous published reports associate the *COL5A1* rs12722 variant with Achilles tendinopathies (Mokone et al., 2006, September et al., 2008), as well as tendon phenotypes that partly rely on the functional ability of tendon, such as range of motion (ROM) (Brown et al., 2011b, Collins et al., 2009), and endurance running performance (Brown et al., 2011a, Posthumus et al., 2011).

The *COL5A1* gene encodes for the pro α1 (V) chain of type V collagen (Col V), a quantitatively minor fibrillar collagen which is thought to initiate fibril assembly and regulate lateral fibril growth within tendon (Birk et al., 1990, Wenstrup et al., 2004). A reduced Col V content has been reported to compromise the diameter of the collagen fibril on *in vitro* cultures (Birk, 2001, Wenstrup et al., 2004), which may reduce the linear modulus (Young's Modulus), i.e. the material stiffness of tendon (Dressler et al., 2002). It is possible that variations within the *COL5A1* gene may influence a tendon's material and mechanical properties, and indeed from a functional perspective, the 'T' allele variant has been reported to produce more pro α1(V) chain protein and thus, increased Col V production (Laguette et al., 2011). As a mechanical consequence, this may translate into an increased tendon modulus when considering the above rational. However, no association was evident between the genotypes, individually and combined, with Young's Modulus ($P = 0.227$; $P = 0.151$; Tables 7 and 8), although a linear trend does exist across the genotypes, such that Young's Modulus is higher in the TT genotype groups compared to the CC genotype groups (0.64 GPa vs. 0.42 GPa) (Figure 22). It may be that the 'T' allele associates with higher tendon modulus and that a positive relationship exists between Young's Modulus and the risk of tendon pathologies, as the 'T' allele has been generally identified in Achilles tendinopathic patients (Laguette et al., 2011).

Various intrinsic risk factors have been proposed to be associated with female tendon injuries (Kannus, 1997), the exact mechanisms by which these factors contribute to an increased risk of tendon pathologies in women remains unknown. It may be that a gene-hormone interaction exists, as sex hormones such as oestrogen are known to exert their biologic effects indirectly on collagen-rich tissues, through regulating gene expression of MMPs (matrix metalloproteinases) (Moalli et al., 2002, Sato et al., 1991); *MMP3* gene expression has been reported to be higher in women compared with men (Slauterbeck et al., 2006). Although, no previous research has investigated the effect of female sex hormones such as oestrogen on the regulation of *COL5A1* gene expression, Col V has been reported to be a substrate for MMP3 proteolytic activity (Birkedal-Hansen et al., 1993), so it is possible that the *COL5A1* rs12722 gene variant interacts with oestrogen to modify material and mechanical properties of tendon, which makes this genetic association specific to females. Higher levels of oestrogen have however been linked to smaller tendon sizes, but this was suggested to be independent of genetic factors (Finni et al., 2009).

Within this study, oestradiol levels had no association with any of the measures of tendon properties, including volume, Young's Modulus and z-scores, and as no association was observed between genotypes and these same measures of tendon properties, it is unlikely that there were gene-hormone interactions specific to females that influence the structural, material and mechanical properties of tendon in *vivo,* at least not in this study.

To conclude, this study found no association between the *COL5A1* rs12722 gene variant and measures of patellar tendon properties, although using genetic association studies still remains an important prelude in identifying genetic variants, which predispose individuals to altered risk of tendon injuries, as well as the potential for enhanced physical performance. Additional investigations into the effect of female sex hormones on the expression of genes which code for collagen structures, such as *COL5A1*, specifically relating to tendon, would enhance our understanding of sex-specific differences in the metabolism, structure and functional properties of tendon.

Chapter 5.

Variants within the *MMP3* gene and tendon properties in males

5.1 Introduction

Internal structures such as tendon undergo deformation when resisting external loads that act on the body (McGinnis, 2005). The degree of deformation or strain experienced by the tendon structures is related to the stress caused by these external loads and the integrity of the material. Knowledge of the mechanical properties of musculoskeletal tissues including tendon, can assist in understanding the aetiologies of injury as well as physical performance potential. The primary parameters describing tendon material and mechanical properties is tendon stiffness, which describes the change in length in relation to the force applied (force-displacement relation) and is dependent on the volume of the tendon, and tendon modulus, which describes the relation between tendon stress and strain. Therefore, the mechanical properties of tendon are influenced by the volume of tendon as well as the tissue material properties.

Recently, a genetic component has been associated with tendon phenotypes, particularly that of the *COL5A1* rs12722 gene variant (Collins and Posthumus, 2011). In addition, gene variants within the *MMP3* gene which encodes for matrix metalloproteinase-3 protein , a key regulatory enzyme of the extracellular matrix (ECM) capable of degrading multiple structural components of the ECM such as the collagens (Matrisian, 1990), has also been associated with tendinopathies (Raleigh et al., 2009). Specifically, three gene variants within the *MMP3* gene (rs679620, rs591058, rs650108) have independently been associated with chronic Achilles tendinopathies. Furthermore, the rs679620 gene variant is non-synonymous, in that it causes a change in the amino acid sequence, and consequently an altered protein function.

Even though no research literature exists associating these gene variants with tendon structural and mechanical properties *per se,* it is however important to state that all genes that contain sequence variants shown to be associated with tendon tissue injury to date, encode for proteins that serve essential structural and functional roles within tendon (Collins and Raleigh, 2009). Therefore, it could be postulated that relatively small changes in MMP3 expression within non-pathological ranges, as a result of the function of these gene variants, could result in interindividual variation in the degree of fibrillar collagen degradation, and ultimately the mechanical properties of tendon.

The aim of this study was therefore to investigate whether the *MMP3* rs679620, rs591058 and rs650108 gene variants influence structural and functional roles within the patellar

tendon, through volume and modulus measures *in vivo,* in an asymptomatic male population.

5.2 Method

Participants: Forty-five male participants (age 22.9 (3.3) years; BMI 24.6 (2.6) kg/m²) were recruited for this study (as described in section 2.1). Physical characteristics of all participants are presented in Table 4 (section 2.1). Participants gave written informed consent (section 2.1). A detailed description of the methods for measures of tendon properties, and genotyping the *MMP3* rs650108, rs591058 and rs679620 gene variants, are given elsewhere (section 2.2 and 2.3, respectively).

Tendon properties: Briefly, Young's Modulus represented the mechanical properties of tendon and was calculated by multiplying the tendon stiffness (derived from the force-elongation curve), by the ratio of patellar tendon length (PTL) to patellar tendon cross-sectional area (PTCSA). The volume represented the structural extent of the patellar tendon and was calculated by geometric principles assuming a uniformly tapering truncated cone between measurement positions (i.e. the product of PTCSA at the three sections of the tendon, 25, 50, 75%, and PTL). The z-scores represented the scaling together of the structural (volume) and functional (Young's Modulus) properties of the tendon, and was calculated from the combination of the scores derived from the means of each tendon measure (Young's Modulus and volume), divided by their respective standard deviations.

Genotyping: The genotypes of the *MMP3* gene variants were determined using fluorescence-based TaqMan technique of polymerase chain reaction (PCR), based on the amplification of a fragment of genomic DNA overlapping the each of the *MMP3* polymorphisms, within its non-coding regions (rs591058, rs650108) and coding regions (rs679620).

Statistical analyses: A one-way analysis of variance (ANOVA) was used to determine any significant difference between the characteristics of the genotype groups of the *MMP3* rs650108 variant, and volume and z-scores. Independent t-tests were conducted when combining genotype groups and assessing an association with volume and z-scores, as well as when analysing the *MMP3* rs591058 and rs679620 variants, due to only including homozygote genotype groups. Non-parametric statistical equivalent tests were conducted if

the data set did not meet certain assumptions (section 2.5). This was true of the Young's modulus values and the independent variables (Kruskal-Wallis test for three genotype groups; Mann-Whitney U test for combined genotype groups). As only age (z-scores, r = 0.308, *P* = 0.04) and BMI (Young's Modulus, r = 0.348, *P* = 0.019) showed a significant correlation with any of the phenotypic measures, age and BMI were used as a covariates. Statistical significance was accepted when $P \leq 0.05$. All measurements showed high reliability (section 2.5).

5.3 Results

There were no significant differences in patellar tendon volume and Young's Modulus measurements and z-scores, between the genotype groups for *MMP3* rs650108 (volume, *P* = 0.952; Young's Modulus, *P* = 0.170; z-scores, *P* = 0.585), as well as when comparing the GG genotype group to the combined GA and AA genotype groups (volume, *P* = 0.825; Young's Modulus, *P* = 0.103; z-scores, *P* = 0.470). In addition, when factoring in the covariate of age for the z-scores, there were no significant differences between all genotype groups (*P* = 0.681) and when combined (*P* = 0.629) (Table 12). Because the *MMP3* gene variants rs591058 and rs679620 were in perfect disequilibrium, the results were identical with no significant differences between genotype groups (volume, *P* = 0.796; Young's Modulus, *P* = 0.238; z-scores, *P* = 0.450). When adjusting the z-scores statistic for age, there was still no significant difference (*P* = 0.346), (Table 13). There was a genotype effect evident from the *MMP3* rs679620 and rs591058 gene variants on BMI, such that the GG and CC genotypes, respectively, presented a higher BMI than the AA and TT genotypes, respectively (*P* = 0.034) (Table 13), yet BMI did not correlate with any of the tendon properties.

Table 12. General characteristics and measures of patellar tendon properties for the GG, GA or AA *MMP3* rs650108 genotype groups. Combined GA and AA genotype groups are also presented, together with the GG genotype group for this gene variant

N = 45	***MMP3* rs650108 genotype groups**				***MMP3* rs650108 genotype groups combined**		
	GG (n=38)	**GA (n=3)**	**AA (n=4)**	***P*-value**	**GG (n=38)**	**GA & AA (n=7)**	***P*-value**
Age (years)	22.5 (19-32)	23 (21-27)	24 (21-27)	0.466	22.5 (19-32)	22.5 (19-32)	0.218
Height (cm)	179.8 (157-190)	180.5 (177-191)	179.2 (172-184)	0.715	179.8 (157-190)	180.5 (172-191)	0.730
Weight (kg)	76.5 (58-99)	87.8 (66-95)	74.7 (64-82)	0.550	76.5 (58-99)	81.7 (64-95)	0.865
BMI (kg/cm^2)	23.5 (20-30)	26 (24-27)	27.5 (21-30)	0.270	23.5 (20-30)	26 (21-30)	0.113
Volume (mm³)	2291 (294)	2293 (492)	2239 (413)	0.952	2291 (294)	2262 (409)	0.825
Young's Modulus (GPa)	0.376 (1.23)	0.452 (0.14)	0.647 (0.51)	0.170	0.376 (1.23)	0.454 (0.58)	0.103
Z-scores	-0.047 (1.305)	-0.087 (1.639)	0.698 (1.865)	0.585 (0.681)	-0.047 (1.305)	0.361 (1.677)	0.470 (0.629)

General characteristics are expressed as median (range)

Volume and Z-scores are expressed as mean (standard deviation)

Young's Modulus is expressed as median (range)

The *P*-value for Z-scores are reported with age as a covariant in parenthesis

Table 13. General characteristics and measures of patellar tendon properties for the CC and GG genotype groups of the *MMP3* rs591058 and rs679620 gene variants, respectively, together with the TT and AA genotype groups of the same two variants, respectively

N = 45	*MMP3* rs591058 and rs679620 genotype groups		
	591058 (CC) 679620 (GG) (n=12)	591058 (TT) 679620 (AA) (n=33)	*P*-value
Age (years)	21.5 (19-27)	23 (21-27)	0.806
Height (cm)	180.5 (172-191)	179 (157-187)	0.355
Weight (kg)	81.8 (64-99)	75.4 (58-99)	0.404
BMI (kg/cm^2)	25.9 (21-30)	23.4 (20-30)	0.034*
Volume (mm^3)	2306 (304)	2279 (336)	0.796
Young's Modulus (GPa)	0.45 (0.75)	0.37 (1.23)	0.238
Z-scores	0.273 (1.320)	-0.077 (1.376)	0.450 (0.346)

General characteristics are expressed as median (range)

*Genotype effect on BMI significant ($P = 0.034$)

Volume and Z-scores are expressed as mean and standard deviation (parentheses)

Young's Modulus is expressed as median and range (parentheses)

The *P*-value for Z-scores are reported with age as a covariant in parenthesis

5.4 Discussion

In this study there were no significant associations between the *MMP3* gene variants, rs679620, rs591058 and rs650108, and measures of patellar tendon properties, related to structure (volume), function (modulus), and both (z-scores). Although none of the variants investigated in this study were found to be associated with tendon properties (Tables 11 and 12), it does not exclude the possibility that other variants within genes clustered in close proximity to the *MMP3* gene on chromosome 11q22, such as *MMP10*, *MMP1*, and *MMP12,* associates with tendon properties. Like *MMP3*, the gene products of these genes are capable of degrading a diverse array of extracellular matrix (ECM) proteins (Pasternak and Aspenberg, 2009, Somerville et al., 2003), so it is prudent to assume that because *MMP3* variants have been associated with tendinopathies (Raleigh et al., 2009), similar associations may be observed in tendon properties with these other *MMP* gene variants, by

virtue of the fact that the intrinsic regulatory proteins associated with tendon pathologies, are also directly involved in maintenance processes within tendon.

The lack of associations between the three gene variants and tendon properties is as expected if one variant does not associate, given they are in high linkage disequilibrium with one another. In particular, the rs591058 and rs679620 variants are in perfect linkage disequilibrium, allele ‘C’ in the former corresponds to allele ‘G’ in the latter. In addition, the rs650108 variant displays high linkage disequilibrium with the rs679620 and rs591058 variants ($r = 0.673$, $P = 0.01$). This non-random association suggests that one gene variant acts as a marker for the other, and so knowing if one variant is associated with tendon properties, provides information as to the likelihood of the other also being associated.

A promoter polymorphism within the *MMP3* gene (rs3025058) is also believed to be tightly linked to the rs679620 variant (Chen et al., 2012, Beyzade et al., 2003, Raleigh et al., 2009). Either marker is associated with MMP3 expression levels, notably the rs3025058 5A allele, and the rs679620 ‘A’ allele are associated with relatively lower levels of MMP3, and the highest level of pathological activity related to rheumatoid arthritis. In addition, lower MMP3 protein expression levels have been reported in human tendon displaying pathological characteristics (Ireland et al., 2001, Parkinson et al., 2010, de Mos et al., 2007, Jones et al., 2006, Riley et al., 2002). These observations may represent a failure of the normal matrix remodelling process (Riley et al., 2002). However within a non-pathological range, lower MMP3 levels may favour a state of imbalance with greater synthesis relative to degradation, thus, substrates involved in cross-linking and stabilisation of intact fibrillar collagen may be relatively less affected by degradation processes. Ultimately, the AA genotype of the rs679620 variant may associate with a higher matrix stiffness and higher Young’s Modulus, when considering the above rational.

Although clearly not approaching statistical significance ($P = 0.238$), the AA genotype shows a weak tendency to associate with higher Young’s Modulus values, compared with the GG genotype (0.37 GPa vs. 0.45 GPa) (Table 12), and hence, the TT genotype of the rs591058 variant may also tend to associate with higher tendon modulus, compared with the CC genotype. However, the functional significance of the rs650108 variant has yet to be determined, most likely due to the fact that it resides in an intron, which makes it difficult to explain the association relationship, yet as part of a haplotype it associates with pathological states (Koch et al., 2010, Raleigh et al., 2009).

Future research should attempt to determine whether there is a differential expression of *MMP3* genes in tendon displaying relatively high and low tendon modulus, to assist in determining whether these genes are causally implicated in modifying mechanical properties of tendon, through mechanisms which remodel its microstructure. Additionally, relationships can be established between protein expression levels and the genotypes of the three *MMP3* sequence variants.

In conclusion, the data suggests no evidence of an association between the variants of the *MMP3* gene and structural and functional characteristics of the patellar tendon. Possible links between pathological states within tendon and tendon mechanical parameters should be investigated to aid in injury prevention models.

Chapter 6.

Variants within the *MMP3* gene and tendon properties in females

6.1 Introduction

It has been identified that female hormones may negatively affect collagen protein synthetic rates in connective tissue such as tendon (Hansen et al., 2009b, Hansen et al., 2008, Miller et al., 2007), which may be why there are sex-specific differences in injury rates and adaptational responses associated with healing related to physical activity (Gray et al., 1985, Kannus et al., 1987, Geary et al., 2002). In tendon, there are oestrogen receptors that are responsive to female sex hormones (Hart et al., 1998, Wentorf et al., 2006), and it has been demonstrated in animal models that oestrogen may have an inhibiting effect on collagen synthesis (Fischer, 1973, Irie et al., 2010, Liu et al., 1997a, Yu et al., 1999).

At a molecular level, a lower resting gene expression of *MMP3* in women compared to men has been reported, which also has implications concerning tendon pathologies (Sullivan et al., 2009). Indeed, down-regulated expression levels of MMP3 mRNA and its associated proteins, have been found in injured or ruptured tendons (de Mos et al., 2007, Ireland et al., 2001, Jones et al., 2006, Riley et al., 2002, Lo et al., 2004). MMP3 (matrix metalloproteinase-3) is an essential and functionally diverse regulating enzyme, capable of degrading a wide range of extracellular matrix (ECM) components such as collagens (Matrisian, 1990). Ultimately, its function is thought to be crucial for the maintenance of a healthy ECM in tendon (Riley et al., 2002), so lower mRNA expression levels of this gene in women may indicate an impaired ECM state and thus, an increased susceptibility to tendon injuries (Sullivan et al., 2009).

Sequence variants within the *MMP3* gene (rs679620, rs591058, rs650108) have been associated with tendinopathies in a predominantly male cohort (Raleigh et al., 2009), however, there was no physiological evidence to explain as to why there was an increased/decreased risk of incurring a tendinopathy. The mechanical properties of tendon may contribute to the aetiology of the tendon injury model, namely stiffness (force-displacement relationship dependent on structural dimensions), and modulus (stress and strain relation of tendon material). However, the role of stiffness in soft-tissue injury risk is far from conclusive (Witvrouw et al., 2004). In theory, an increase in stiffness of the tendon structure may lead to increased injury risk through lower force dissipation ability, and a smaller distance over which to absorb external or internal forces.

There was no evidence of an association between the variants of the *MMP3* gene with structural and functional characteristics of the patellar tendon in males (chapter 5). However, it is unknown whether the same gene variants can be associated with tendon properties in females. Therefore, the aim of this study was to investigate whether the gene variants, rs679620, rs591058 and rs650108, influence structural and functional measures of patellar tendon properties in an asymptomatic female population.

6.2 Method

Participants: Thirty-nine female participants (22.4 (4.8) years; BMI 23.2 (2.8) kg/m^2) were recruited for this study (as described in section 2.1). Physical characteristics of all participants are presented in Table 4 (section 2.1). Participants gave written informed consent (section 2.1). A detailed description of the methods for measures of tendon properties, and genotyping the *MMP3* rs650108, rs591058 and rs679620 gene variants, are given elsewhere (section 2.2 and 2.3, respectively).

Tendon properties: Briefly, Young's Modulus represented the mechanical properties of tendon and was calculated by multiplying the tendon stiffness (derived from the force-elongation curve), by the ratio of patellar tendon length (PTL) to patellar tendon cross-sectional area (PTCSA). The volume represented the structural extent of the patellar tendon and was calculated by geometric principles assuming a uniformly tapering truncated cone between measurement positions (i.e. the product of PTCSA at the three sections of the tendon, 25, 50, 75%, and PTL). The z-scores represented the scaling together of the structural (volume) and functional (Young's Modulus) properties of the tendon, and was calculated from the combination of the scores derived from the means of each tendon measure (Young's Modulus and volume), divided by their respective standard deviations.

Genotyping: The genotypes of the *MMP3* gene variants were determined using fluorescence-based TaqMan technique of polymerase chain reaction (PCR), based on the amplification of a fragment of genomic DNA overlapping the each of the *MMP3* polymorphisms, within its non-coding regions (rs591058, rs650108) and coding regions (rs679620).

Oestradiol: Oestradiol levels were analysed using standard ELISA procedures from serum content taken on the day of measures of tendon properties (see section 2.4 for more detail

on this procedure), to assess the contribution of this ligand to measures of tendon properties. Serum levels of oestradiol has been associated with tendon stiffness in young females (Burgess et al., 2009c). Oestradiol was therefore factored into statistical analyses as a potential covariate.

Statistical analyses: A one-way analysis of variance (ANOVA) was used to determine any significant differences between the characteristics of the *MMP3* rs650108 genotype groups, and volume and z-scores. Independent t-tests were conducted when combining genotype groups for these same two measures of tendon properties. Non-parametric equivalents of the ANOVA (Kruskal-Wallis test) and independent t-tests (Mann-Whitney U test) were conducted on Young's Modulus values, for all three genotype groups and combined genotype groups, respectively. These same tests were performed on the *MMP3* rs591058 and rs679620 genotype groups, although only volume met the assumptions of a parametric test, with Young's Modulus and z-scores being assessed using non-parametric tests. As only BMI showed a significant correlation with any of the phenotypic measures (volume, r =0.334, P = 0.038; Young's Modulus, r = 0.440, P = 0.005; z-scores, r =0.473, P = 0.002) BMI was used as a covariate. Statistical significance was accepted when $P \leq$ 0.05. All measurements showed high reliability (section 2.5).

6.3 Results

There were no significant differences in patellar tendon volume and Young's Modulus measurements as well as the z-scores, between the genotype groups for *MMP3* rs650108 (volume, P = 0.828; Young's Modulus, P = 0.557; z-scores, P = 0.719). This outcome holds true when comparing the GG genotype group to the combined GA and AA genotype groups (volume, P = 0.627; Young's Modulus, P = 0.797; z-scores, P = 0.708). Furthermore, when factoring in the covariate of BMI, volume and z-scores remain insignificant between all genotype groups (volume, P = 0.676; z-scores, P = 0.531), and when combined (volume, P = 0.399; z-scores, P = 0.358) (Table 14). Results were combined between the *MMP3* gene variants, rs591058 and rs679620, as they were in perfect disequilibrium with one each other. However, no significant differences were evident between genotype groups for measures of volume and Young's Modulus and z-scores (volume, P = 0.835; Young's Modulus, P = 0.680; z-scores, P = 0.862). As volume was the only parametric measure, BMI was included as a covariate, but no significant difference between genotype groups were evident (P = 0.532) (Table 15). Oestradiol showed no significant correlation with volume (volume, r =0.010, P = 0.950) and so was

not used as a covariate. There were no *MMP3* gene variant genotype effects on any of the subject characteristics (Table 14 and 15).

Table 14. General characteristics and measures of patellar tendon properties for the GG, GA or AA *MMP3* rs650108 genotype groups. Combined GA and AA genotype groups are also presented, together with the GG genotype group for this gene variant

N = 39	***MMP3* rs650108 genotype groups**				***MMP3* rs650108 genotype groups combined**		
	GG (n=23)	**GA (n=12)**	**AA (n=4)**	***P*-value**	**GG (n=23)**	**GA & AA (n=16)**	***P*-value**
Age (years)	21 (18-39)	20 (18-24)	22 (20-24)	0.130	21 (18-39)	20 (18-24)	0.153
Height (cm)	166.5 (153-179)	165.8 (155-183)	167.8 (158-175)	0.927	166.5 (153-179)	165.8 (155-183)	0.830
Weight (kg)	63.2 (47-80)	62.8 (48-75)	69.5 (52-79)	0.669	63.2 (47-80)	64.7 (48-79)	0.971
BMI (kg/cm^2)	23.9 (19-30)	22.1 (19-27)	23.3 (19-27)	0.630	23.9 (19-30)	22.6 (19-27)	0.356
Oestradiol (pg/ml)	24.9 (6-116)	18.8 (8-70)	35.7 (28-90)	0.197	24.9 (6-116)	27.5 (8-90)	0.797
Volume (mm^3)	1497 (211)	1521 (257)	1570 (217)	0.828 (0.676)	1497 (211)	1533 (241)	0.627 (0.399)
Young's Modulus (GPa)	0.499 (1.19)	0.547 (0.90)	0.712 (0.49)	0.557	0.499 (1.19)	0.607 (0.90)	0.797
Z-scores	-0.085 (1.640)	-0.056 (1.729)	0.643 (1.636)	0.719 (0.531)	-0.085 (1.640)	0.119 (1.680)	0.708 (0.358)

General characteristics are expressed as median (range)

Volume and Z-scores are expressed as mean (standard deviation)

Young's Modulus is expressed as median (range)

The *P*-value for Volume and Z-scores are reported with BMI as a covariant in parenthesis

Table 15. General characteristics and measures of patellar tendon properties for the CC and GG, CT and GA, and TT and AA genotype groups of the *MMP3* rs591058 and rs679620 genotype groups, respectively

N = 39	*MMP3* rs591058 and rs679620 genotype groups			
	591058 (CC) 679620 (GG) (n=17)	591058 (CT) 679620 (GA) (n=6)	591058 (TT) 679620 (AA) (n=16)	*P*-value
Age (years)	20 (18-24)	21.5 (21-23)	21.5 (18-39)	0.241
Height (cm)	165.5 (154-183)	164.8 (163-179)	166.9 (153-175)	0.771
Weight (kg)	64 (48-79)	61.9 (50-78)	67.7 (47-80)	0.868
BMI (kg/cm^2)	22.7 (19-27)	23.8 (20-30)	23.7 (19-27)	0.343
Oestradiol (pg/ml)	28.3 (8-90)	20.5 (9-46)	28 (6-116)	0.544
Volume (mm^3)	1534 (234)	1474 (314)	1502 (179)	0.835 (0.532)
Young's Modulus (GPa)	0.558 (0.90)	0.715 (1.19)	0.493 (1.01)	0.680
Z-scores	-0.190 (5.69)	0.260 (7.93)	-0.465 (3.72)	0.862

General characteristics are expressed as median (range)

Volume is expressed as mean (standard deviation)

Young's Modulus and Z-scores are expressed as median (range)

The *P*-value for Volume is reported with BMI as a covariant in parenthesis

6.4 Discussion

In this study no significant associations were found between the three *MMP3* gene variants, rs679620, rs591058 and rs650108, and patellar tendon properties in an asymptomatic female population. These data suggest that the *MMP3* gene does not associate with structural and mechanical characteristics of the patellar tendon, which is in agreement with data in a male population (chapter 5). No sex-specific differences were evident between these genotype-phenotype proposed associations, in that the results are similar between the male (chapter 5) and female cohorts.

MMP3 is a potent degrading enzyme capable of degrading multiple ECM and non-ECM protein components (Visse and Nagase, 2003, Matrisian, 1990). MMP3 also indirectly affects the degradation of the ECM by activating the proteolytic activity of other MMPs. Both highly stressed (Maeda et al., 2009, Asundi and Rempel, 2008, Birch, 2007) and

load-deprived (Asundi and Rempel, 2008, Thornton et al., 2010, Leigh et al., 2008) tendon display elevated MMP3 protein expression levels compared to 'normal' tendon, determined in animal models and *in vitro* cell cultures. Conversely, relatively lower levels of MMP3 expression have been reported in human tendon displaying pathological characteristics (de Mos et al., 2007, Ireland et al., 2001, Jones et al., 2006, Riley et al., 2002, Lo et al., 2004). This differential expression of MMP3 highlights the importance of negating non-genetic factors, when investigating the association of genetic factors with phenotypes. Controlling for non-genetic factors such as the level of activity and only recruiting individuals who have no history of tendon injury or pathology (see section 2.1), was a notable strength of this study design. In addition, a standardised measure of oestradiol was a necessary requirement in order to maximise the detection of measurable associations with genetic variation, as oestradiol may be a non-genetic variable contributing to tendon metabolism, structure and function of tendon in females (Burgess et al., 2009d, Miller et al., 2007). This consideration is of particular importance as the expression of MMP3 has reported to be suppressed in the presence of oestradiol, through a possible gene-hormone interaction (Moalli et al., 2002, Sato et al., 1991). However, within this study, oestradiol was found not to associate with any of the tendon measurement parameters (volume, $r = -0.087$; Young's Modulus, $r = 0.146$; z-scores, $r = -0.020$). Oestrogen may not have an effect on the expression of MMP3 within normal physiological limits, and so is not likely to be a confounding variable in genotype-phenotype associations proposed in this study at least.

Sex differences in the mechanical properties of tendon have been explored on numerous occasions previously (Onambele-Pearson and Pearson, 2012, Kubo et al., 2003a, Magnusson et al., 2007, Onambele et al., 2007), however in this study the aim was to better understand the role of genetic variation and in particular, *MMP3* gene variants (rs679620, rs591058, rs650108) on the structural and functional characteristics of the patellar tendon, in an asymptomatic female population. The high *P*-values observed clearly indicate no significant association for all three gene variants, with measures of tendon properties (Tables 13 and 14).

Previous research has associated these gene variants independently with chronic Achilles tendinopathies, in a predominantly male population (Raleigh et al., 2009), but it is not possible to elucidate between sex-specific associations from this data. It has also been reported that females have half the amount of resting mRNA expression levels of MMP3 in

patellar tendon, compared to men (Sullivan et al., 2009), which may indicate an impaired ability to maintain a healthy ECM, and thus, females may be more susceptible to tendon injury. Therefore, it could be postulated that the turnover of the ECM is relatively less than that of men, so the matrix stiffness is likely to be relatively higher, and hence, this may translate into a higher tendon modulus *in vivo*. Nevertheless, tendon modulus is evenly matched across all genotypes for all gene variants, which ultimately suggests that oestradiol has no influence on the *MMP3* gene in females within normal physiological levels.

In conclusion, the sequence variants rs679620, rs591058 and rs650108 within the *MMP3* gene, do not associate with patellar tendon properties in females. Other *MMP* gene variants displaying high heterozygous frequencies could be potentially informative, and should be considered as candidates for association studies, given their importance as regulatory components at a protein level, within human tendon.

Interindividual variability in tendon properties in humans and polygenic profiling

7.1 Introduction

It has been demonstrated that the *MMP3* rs679620 single nucleotide polymorphism (SNP) interacts with the *COL5A1* rs12722 SNP, in modifying the risk of chronic Achilles tendinopathies (AT) (Raleigh et al., 2009). Raleigh et al. (2009) report that individuals with the CC genotype of the *COL5A1* rs12722 SNP, and the AA genotype of the *MMP3* rs679620 SNP, may be at less risk of developing AT compared to individuals with the TT genotype of the *COL5A1* rs12722 SNP and the GG genotype of the *MMP3* rs679620 SNP. They found that when combining the alleles of both gene variants (pseudo-haplotypes), a significant association was established between the controls (asymptomatic) and cases (tendinopathies), in that the frequency of the 'A' and 'C' alleles (*MMP3* and *COL5A1* SNPs, respectively) were greater in the control group compared to the case group (P = 0.002), whereas, the frequency of the 'G' and 'T' alleles were greater in the case group compared to the control group (P = 0.006).

It is plausible that tendon injury can be linked to tendon mechanical stiffness in that both ends of the stiffness continuum can potentially lead to injury, as well as being protective against injury. A relatively stiffer tendon would decrease the stress and strain for a given magnitude of force per unit area, in direct contrast to a more compliant tendon, thus, dissipating the stress imposed on the structures, and as a consequence a reduced injury risk (Ker et al., 1988, Couppe et al., 2008, Seynnes et al., 2009). Conversely, a stiffer tendon would induce a relative increase in elongation of the contractile apparatus in response to mechanical loading, and hence, the myotendinous junction may be at greater risk of strain overload, whereas, a compliant tendon has a greater energy absorbing capacity (Witvrouw et al., 2004).

Even in the absence of any significant associations between the genotypes of the previously mentioned *COL5A1* and *MMP3* SNPs independently, with volume, Young's Modulus, and both in combination (z-scores), it is possible that the alleles of both gene variants interact to modify patellar tendon structural volume and modulus. The rationale supporting such an interaction derives from the premise that collagen type V (Col V) expression levels appear critical in determining a tendon's microstructure, through diameter and cross-linking (Ottani et al., 2001, Wenstrup et al., 2004, Niyibizi and Eyre, 1993), and as Col V is a possible substrate for the proteolytic activities of MMP3 protein (Sternlicht and Werb, 2001), the cross-linking and stabilisation of fibrillar collagen may be

degraded. As a result, there may be a reduction in fibril diameter and matrix stiffness (Eliasson et al., 2007, Reddy, 2004).

Investigating the cumulative effect of several polymorphisms in genes related to tendon structural and regulatory processes will account for a larger proportion of the variability in the phenotypes, which could ever be possible by studying one gene variant at a time (Akey et al., 2001, Zollner and von Haeseler, 2000). By attempting to accumulate small tendencies (clearly no significant) for association between the genotypes and phenotypes reported in the previous chapters (chapters 3, 4, 5, 6), may provide a greater potential for something approaching statistical significance, so long as these associations are analysed appropriately (Akey et al., 2001).

By applying a mathematical and statistical model, as well as using current knowledge and analysed data from our own research regarding polymorphic associations with related phenotypes, the assessment of a polygenic profile was made possible. Therefore, the aim of this investigation was to determine whether gene-gene interactions between the *COL5A1* and *MMP3* SNPs were involved in modifying patellar tendon structural and functional properties, in asymptomatic males and females.

7.2 Method

Participants: Forty-five males (age 22.9 (3.3) years; BMI 24.6 (2.6) kg/m^2) and thirty-nine females (22.4 (4.8) years; BMI 23.2 (2.8) kg/m^2) took part in the full range of tests for tendon properties. Physical characteristics of all participants are presented in Table 4 (section 2.1). Participants gave written informed consent (section 2.1). A detailed description of the methods for measures of tendon properties, and genotyping the *COL5A1* rs12722, *MMP3* rs650108, rs591058 and rs679620 gene variants, are given elsewhere (section 2.2 and 2.3, respectively).

Tendon properties: Briefly, Young's Modulus represented the mechanical properties of tendon and was calculated by multiplying the tendon stiffness (derived from the force-elongation curve), by the ratio of patellar tendon length (PTL) to patellar tendon cross-sectional area (PTCSA). The volume represented the structural extent of the patellar tendon and was calculated by geometric principles assuming a uniformly tapering truncated cone between measurement positions (i.e. the product of PTCSA at the three sections of the tendon, 25, 50, 75%, and PTL). The z-scores represented the scaling

together of the structural (volume) and functional (Young's Modulus) properties of the tendon, and was calculated from the combination of the scores derived from the means of each tendon measure (Young's Modulus and volume), divided by their respective standard deviations.

Genotyping: The genotypes of the *COL5A1* and *MMP3* gene variants were determined using fluorescence-based TaqMan technique of polymerase chain reaction (PCR), based on the amplification of a fragment of genomic DNA overlapping the each of the gene polymorphisms, within the 3' untranslated region of the *COL5A1* rs12722 gene variant, and the non-coding regions (rs591058, rs650108) and coding regions (rs679620) of the *MMP3* gene.

Oestradiol: In females only, oestradiol levels were analysed using standard ELISA procedures from serum content taken on the day of measures of tendon properties (see section 2.4 for more detail on this procedure), to assess the contribution of this ligand to measures of tendon properties. Serum levels of oestradiol has been associated with tendon stiffness in young females (Burgess et al., 2009c). Oestradiol was therefore factored into statistical analyses as a potential covariate.

Data analyses:

Allele combinations

A simple additive model of continuous data was applied such that the allele combinations consisting of markers in two different genes were modelled together, and their association with measures of tendon properties was tested (volume, Young's Modulus, z-scores).
A detailed explanation of how the alleles were combined, and the model used for the purposes of analysing the combination of alleles are displayed in appendix 2 and 3, respectively.

Ultimately, four independent groups of data with distribution of the weighted variables ('Allele combination count'), relating to the four possible combination of alleles were entered into SPSS version 19.0.0. Because none of the datasets including volume, Young's Modulus, and z-scores for both sexes met the assumptions of parametricity, the non-parametric Kruskal-Wallis statistical test was used in these instances.

Total genotype score

To examine the combined influence of the four gene variants on measures of tendon properties (volume, Young's Modulus and z-scores), scores were allocated to each genotype within each of the polymorphisms. All four polymorphisms are bi-allelic, providing three possible genotypes. The homozygote genotype associated with the highest volume and Young's Modulus values as well as z-scores were allocated a 'score' of 2, with a linear trend applied such that the heterozygotes scored 1 and the other homozygotes scored 0. Where only homozygotes were used in the case of the male group for *MMP3* rs591058 and rs679620 variants, heterozygotes were allocated an intermediate score of 1.

Criteria for allocation of scores: As no previous research has associated genetics with tendon properties, scores were allocated based on the following rationale; Brown et al. (2011b) report a significant linear trend for the *COL5A1* rs12722 variant and measures of flexibility or range of motion (ROM) ((sit and reach test, (SR)), in a mixed sex population. A greater SR ROM was associated with the CC genotype compared to the TT genotype (321 mm vs. 225 mm, respectively, $P = 0.017$), which indirectly suggests that individuals homozygote for the TT genotype are more 'stiff'/inflexible than individuals homozygote for the CC genotype. Although SR ROM cannot be directly associated with mechanical properties of the patellar tendon, it does intuitively imply an association. SR ROM is partly determined by the function of the muscle-tendon unit, and therefore a relatively stiff tendon would contribute to the overall stiffness and inflexibility of the ROM phenotype. Therefore, for Young's Modulus (male and female), individuals with the TT genotype were allocated a score of 2, and individuals with a CC genotype were allocated a 0 score. Heterozygotes were given a score of 1.

As no significant findings exist in the research literature linking genetics with muscle and/or tendon stiffness, or ROM parameters, for the *MMP3* gene variants (rs679620, rs591058 and rs650108), allocation of scores were based on our own Young's Modulus results for these gene variants. Even though there were no significant associations between any of these gene variants and Young's Modulus, a linear trend exists between the genotypes of the *MMP3* rs650108 variant, so that the AA genotype was allocated a score of 2 and the GG genotype a score of 0. The AG genotype was allocated a score of 1. As for the gene variants *MMP3* rs679620 and rs591058, the genotypes GG and CC, respectively, were allocated a score of 2, and the AA and TT genotypes, respectively, a score of 0.

For volume and z-scores, scores were allocated based on tendencies in our own data for all four gene variants. The homozygote genotype displaying the highest values for these measures of tendon properties were given a score of 2, with the corresponding homozygote given a score of 0. The heterozygote genotype was allocated a score of 1. It was not possible to assign scores to the z-score data in the male cohort, as the highest volume values for the *MMP3* rs650108 gene variant were reversed in relation to the highest Young's Modulus scores (volume – 2 for GG genotype, Young's Modulus – 2 for AA genotype). Therefore in this instance, the z-scores could not represent the functional significance of the volume and Young's Modulus values together.

To quantify the combined influence of all four gene variants on tendon modulus, an algorithm was used to combine all four genotype scores (GS) for any given participant, in a simple additive model (Williams and Folland, 2008). The total score was then transformed mathematically to lie within the range of 0-100 (Equation 7), to allow a more meaningful interpretation of the results, and defined as the 'total genotype score' (TGS). Taking into account the four genotype scores and a maximum score of 8 before transformation for all GS combined, the TGS was calculated as:

$$\text{Equation 7: } TGS = (100 / 8) \times (GS_1 + GS_2 + GS_3 + GS_4)$$

A TGS of 100 represents a polygenic profile which demonstrates the highest volume, tendon modulus, and z-scores, and a TGS of 0 represents a profile which demonstrates the lowest volume, tendon modulus, and z-scores in this particular model.

In order to test whether an association exists between the TGS and volume, Young's Modulus, and z-scores, the Spearman's rho correlation co-efficient was conducted on all the variables, as the TGS did not meet the assumptions of a parametric statistical test. In addition, a linear regression model was applied to this association to predict the degree of variance in the outcome measures (volume, Young's Modulus, z-scores), determined by the polygenic profile (TGS). Furthermore, to determine which predictive variables (gene variants that constitute the TGS) contribute to the variance in volume, Young's Modulus and z-scores, a forward and backward stepwise regression model were used. Briefly, by adding each predictive variable one after another to the model, a test was made to check if some variables could be deleted without decreasing the predictive power of the model, and its effect on volume, Young's Modulus and z-scores. All data were analysed with SPSS

version 19.0.0 software, with all data presented as mean (SD) unless otherwise stated. Statistical significance was accepted when $P \leq 0.05$.

7.3 Results

Allele combinations

Raleigh et al. (2009) have previously shown that the *MMP3* gene variant rs679620 interacts with the *COL5A1* rs12722 gene variant to increase the risk of chronic Achilles tendinopathy. The four possible combinations of the alleles of these two gene variants and their respective values of volume, Young's Modulus and z-scores, for both sexes, are represented in Table 16. No significant differences were observed in patellar volume and Young's Modulus measurements as well as z-scores, between all four allele combinations, in either sex (males - volume, $P = 0.359$; Young's Modulus, $P = 0.073$; z-scores, $P = 0.110$. females - volume, $P = 0.949$; Young's Modulus, $P = 0.067$; z-scores, $P = 0.579$). Even though no significant differences were evident in any of the measures, differences in Young's Modulus values did however approach significance, in both sexes (males, $P = 0.073$; females, $P = 0.067$). Figures 23 and 24 show the distributions between the weighted allele combinations for Young's Modulus values, for males and females, respectively. All values are expressed as the median and range, as the data sets did not meet the assumptions of a parametric statistical test. The allele combinations for both sexes had an effect on BMI (Male/Female – P = < 0.001/0.028) (Table 16), yet BMI had no effect on tendon phenotypic measures.

Table 16. Four allele combinations constructed from the *MMP3* rs679620 and *COL5A1* rs12722 SNPs, and their respective values for general characteristics and measures of patellar tendon properties in both sexes. First allele of each combination corresponds to the *MMP3* SNP, and the second allele, the *COL5A1* SNP.

	Males					**Females**				
	A + C	**G + C**	**A + T**	**G + T**	***P*-value**	**A + C**	**G + C**	**A + T**	**G + T**	***P*-value**
Age (years)	23 (19-32)	21.5 (19-27)	23 (19-32)	21.5 (20-27)	0.900	21.5 (18-39	20 (18-24)	22 (20-24)	20 (18-25)	0.928
BMI (kg/cm^2)	23.4 (20-30)	27 (21-30)	23.5 (20-30)	25.5 (21-30)	< 0.001*	23.7 (19-27)	22.5 (19-27)	23.8 (19-30	24.4 (19-30)	0.028*
Oestradiol (pg/ml)						27 (6-116)	27.1 (6-90)	25.9 (6-116)	26.5 (8-90)	0.876
Volume (mm³)	2311 (264)	2243 (990)	2300 (1592)	2365 (1179)	0.359	1489 (839)	1585 (1021)	1489 (642)	1557 (684)	0.949
Young's Modulus (GPa)	0.35 (1.23)	0.45 (0.64)	0.39 (1.23)	0.45 (0.75)	0.073	0.50 (1.13)	0.44 (0.96)	0.56 (1.07)	0.66 (1.04)	0.067
Z-scores	-0.290 (6.02)	0.320 (4.26)	-0.285 (6.72)	0.675 (4.26)	0.110	-0.545 (6.01)	-0.190 (7.17)	-0.370 (5.64)	-0.170 (6.41)	0.579

General characteristics are expressed as median (range)

*Allele combinations have significant effects on BMI

Values are expressed as median and range is presented in parenthesis

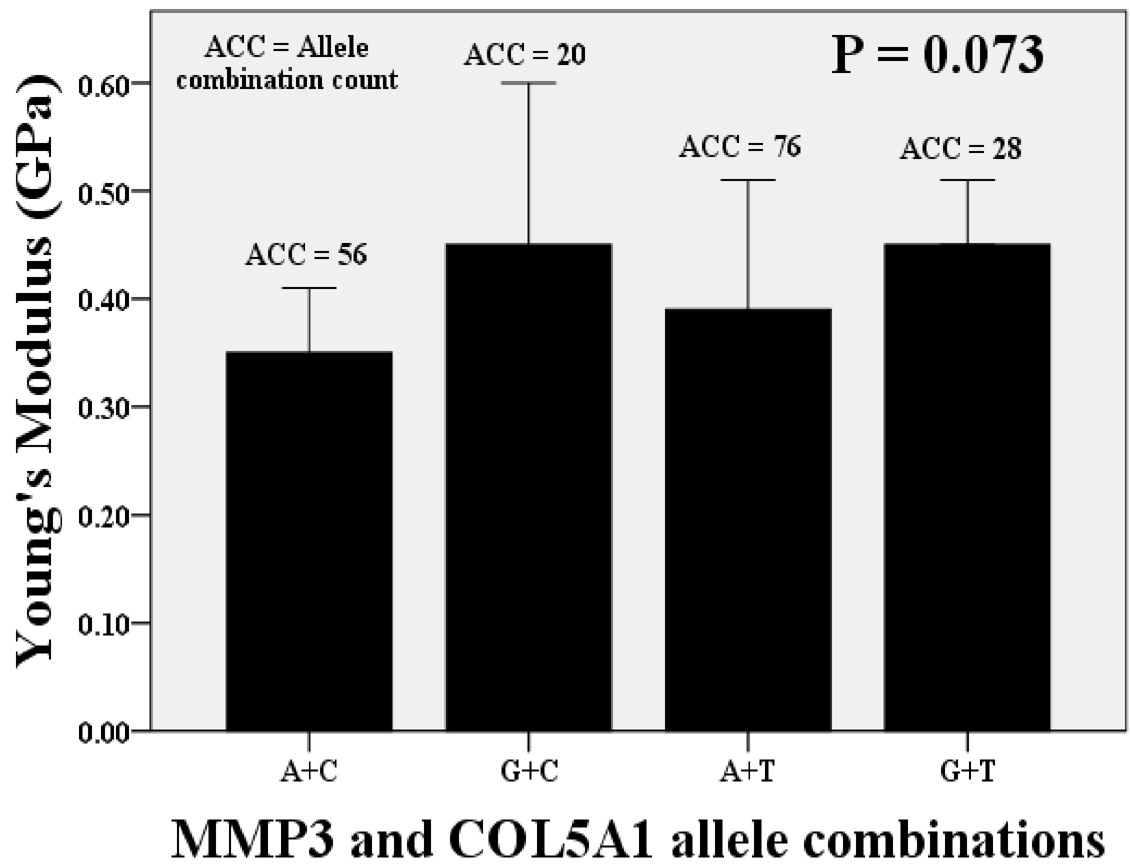


Figure 23. Allele combinations of *MMP3* SNP rs679620 (A/G) and *COL5A1* SNP rs12722 (C/T) and median Young's Modulus values for males. Error bars represent the upper quartile range. Annotations above each error bar indicate the total number of allele combinations derived from the model (termed 'Allele combination count')

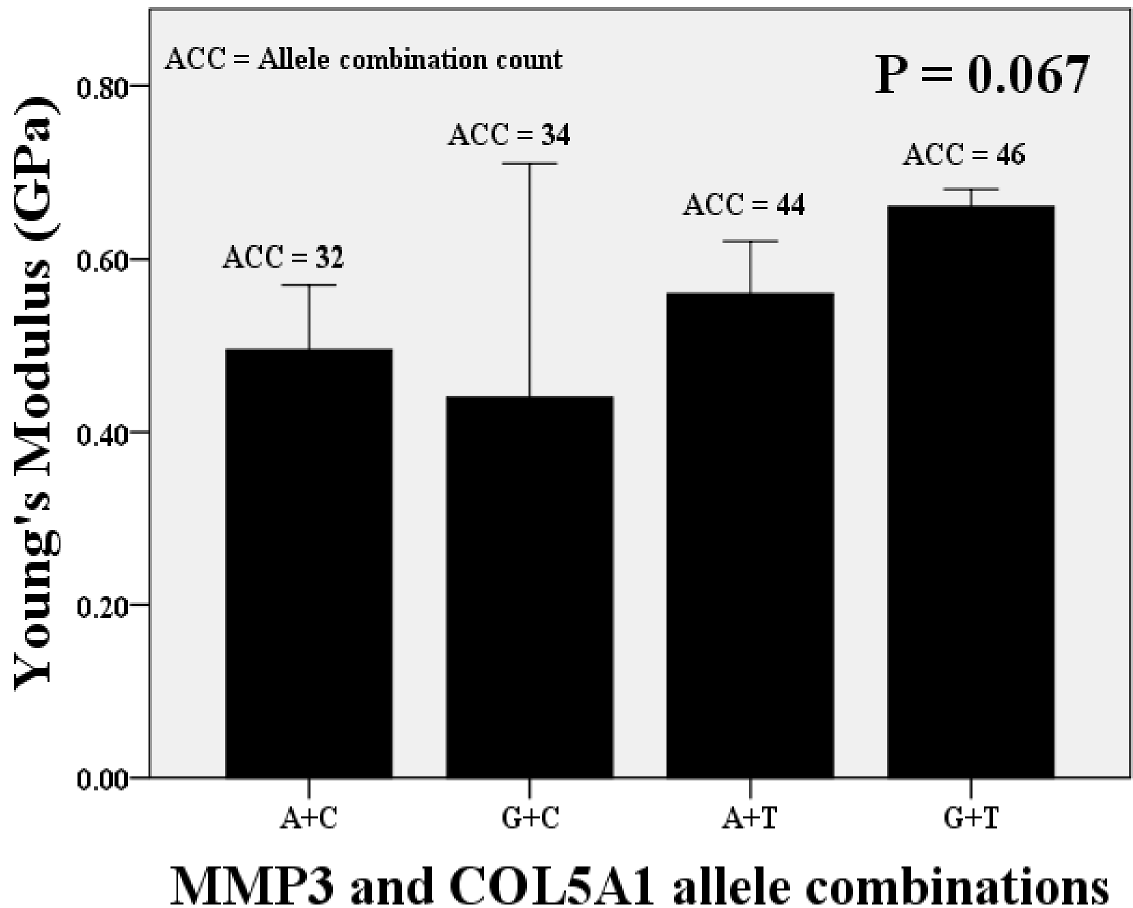


Figure 24. Allele combinations of *MMP3* SNP rs679620 (A/G) and *COL5A1* SNP rs12722 (C/T) and median Young's Modulus values for females. Error bars represent the upper quartile range. Annotations above each error bar indicate the total number of allele combinations derived from the model (termed 'Allele combination count')

Total genotype score

The total genotype score (TGS) attempts to quantify the combined influence of all four gene variants (*COL5A1* rs12722, *MMP3* rs679620, rs591058 and rs650108) on the measures of patellar tendon properties (volume, Young's Modulus, z-scores). There were very weak correlations with no significant associations with the TGS and each of the measures of tendon properties, both in males (volume, r = 0.067, $P = 0.663$; Young's Modulus, r = 0.227, $P = 0.134$) and females (volume, r = 0.012, $P = 0.942$; Young's Modulus, r = 0.125, $P = 0.449$; z-scores, r = 0.076, $P = 0.645$). In addition, a forward and backward stepwise regression model was used to assess the effect of adding and deleting, respectively, each independent variable (the gene variants) to the model at each step, on the volume and Young's Modulus values as well as z-scores. None of the gene variants

included in this stepwise regression model, nor any combination of them in a regression equation, could predict an effect on volume, Young's Modulus or z-scores.

7.4 Discussion

The main findings of this study were that the allele combinations constructed from the *MMP3* rs679620 and *COL5A1* rs12722 gene variants were not significantly associated with patellar tendon structural (volume) and functional (modulus) measures, independently and when combined (z-scores), and that a polygenic profile including the four tested polymorphisms (*COL5A1* rs12722, *MMP3*, rs679620, rs591058, rs650108) does not significantly associate with patellar tendon volume, modulus, and z-scores in either sex.

Akey et al. (2001) report that two and four marker locus haplotypes (combination of neighbouring alleles) offer higher power to detect associations than single marker tests, as was investigated in this study. In addition, even though in the previous chapters no associations were evident with single genetic markers and tendon properties, it is still possible to identify allele combination/phenotype associations (Fallin et al., 2001). However, none of the mathematical and statistical models investigated in this study were found to be associated with patellar tendon properties. Despite these findings, it does not exclude the possibility that additional variants and therefore gene-gene interactions within the *MMP3* and *COL5A1* genes associates with tendon properties. Although there was no significant association with the allele combinations and Young's Modulus for both sexes, the association did however approach significance (male, $P = 0.073$; female, $P = 0.067$). There was a tendency toward higher Young's Modulus values (0.45 GPa) for males with 'G' and 'T' allele combinations of the *MMP3* and *COL5A1* gene variants, and a tendency toward lower Young's Modulus values (0.35 GPa) for the same cohort of males, with an 'A' and 'C' allele combination for these same gene variants (Figure 23). This represents ~22% difference in material stiffness between male individuals for the allele combinations. In females, a similar trend was apparent (Figure 24), with higher Young's Modulus values (0.66 GPa) evident with the 'G' and 'T' allele combination, and lower Young's Modulus values (0.50 GPa) evident with the 'A' and 'C' allele combination, signifying an approximate difference of 24% in material stiffness between these two allele combinations. Interestingly, Raleigh et al. (2009) reported in the only other study to combine the alleles of these two gene variants and their association with a tendon phenotype (Achilles tendinopathies), that the 'G' and 'T' allele combination was significantly overrepresented in the tendinopathy group compared to the asymptomatic control group ($P = 0.006$), and

the ‘A’ and ‘C’ allele combination was significantly underrepresented in the tendinopathy group compared to the control group ($P = 0.002$). Therefore, it could be speculated that higher Young’s Modulus values are associated with increased risk of tendon pathologies. Conversely, lower Young’s Modulus values may be associated with being protective against such pathologies, regardless of sex. This assumption makes intuitive sense in that a relatively stiffer tendon is less able to conform to elongation and thus, dissipate the force developed within the muscular system overtime, leading to an increased risk of strain overload to the tendon, particularly at the myotendinous junction (Witvrouw et al., 2007). However, the proposed relationship between tendon material stiffness and risk of injury remains far from conclusive.

Further research is required to decipher whether significant associations exist between these allele combinations and patellar tendon modulus. By only assessing the Young’s Modulus of individuals with the AA genotype of the *MMP3* rs679620 variant and the CC genotype of the *COL5A1* rs12722 variant, and comparing it with individuals with the GG genotype and TT genotype of the same gene variants, respectively, the expected difference between groups (and thus statistical power) could be maximised, and thus the ability to detect an association between allele combinations.

For the polygenic profile analyses which sought to identify the combined influence of the four gene variants, *COL5A1* rs12722, *MMP3* rs679620, rs591058 and rs650108, all of which have been shown to associate with tendinopathies, independently (*COL5A1* rs12722 (Mokone et al., 2006, September et al., 2008); *MMP3* gene variants (Raleigh et al., 2009), there were no significant correlations with volume, Young’s Modulus in both sexes, and z-scores in females (males - volume, $r = 0.067$, $P = 0.663$; Young’s Modulus, $r = 0.227$, $P = 0.134$; females - volume, $r = 0.012$, $P = 0.942$; Young’s Modulus, $r = 0.125$, $P = 0.449$; z-scores, $r = 0.076$, $P = 0.645$). Furthermore, a stepwise regression model could not predict an effect on volume, Young’s Modulus, and z-scores from any possible combination of these gene variants. Consequently, the polygenic profiling has no influence on patellar tendon structural and mechanical properties *in vivo*. The genes in which these four gene variants derive (*COL5A1*, *MMP3*), encode for proteins directly involved in biological processes within tendon (Collins and Raleigh, 2009), so a potential influence of these gene variants on tendon properties is rational.

A decrease in tendon size or CSA as well as tendon tissue stiffness in a murine model, can be explained by a decrease in Col V concentrations, and thus, a reduction in the assembly of fewer fibrils (Wenstrup et al., 2011). Even though these findings have been reported in *COL5A1* gene knockout models in mice, it can be speculated that common gene variants such as *COL5A1* rs12722 could be partly responsible for the normal interindividual variation in the tendon size and modulus at maturation. The functional significance of this gene and its 3' UTR in which the rs12722 gene variant resides has been reported previously (Laguette et al., 2011). These findings suggest that the mRNA stability of the 'T' alleles equates to a greater amount of Col V being produced. Ultimately, a relative increase in Col V compared to the amount produced by the 'C' alleles, would result in an increased fibril diameter and increased material stiffness (Wenstrup et al., 2011, Wenstrup et al., 2006). However, no differences were evident between the homozygotes of this gene variant and tendon volume and modulus, in the human cohorts studied in this thesis (see chapters 3 and 4). By factoring in the influence of the *MMP3* gene variants with the *COL5A1* gene variant on patellar tendon properties, there was a greater possibility that an association would be evident, than would be possible with these gene variants independently. From a physiological standpoint, the proposed interactions of these gene variants' respective proteins could influence tendon structural and mechanical properties, in that Col V may influence the synthesis and activation of the MMP3 protein during catalysis, leading to an altered rate of degradation and change in the stiffness of the matrix. However, no significant differences were found between the TGS and all measures of tendon properties.

Researchers should be encouraged to explore the influences of additional sequence variants within genes which assemble and regulate the microstructure of tendon, on tendon mechanical properties including those within collagens I and XI. These proteins have been reported to interact with Col V to regulate tendon fibril diameter and assembly (Birk, 2001, Segev et al., 2006). Ultimately, if these gene variants were to associate with tendon modulus for example, they could then be factored into a polygenic model, which would account for a greater proportion of the interindividual variability, than would be possible with only single marker genetic variants.

A notable limitation of this method to categorise genotypes with scores, was the complete lack of data in the research literature associating the *MMP3* gene variants with phenotypes related to tendon mechanical properties, owing to the novel associations proposed.

Genotype scoring was therefore based on non-significant tendencies in our own observations, which is inherently limited.

In conclusion, the main finding of this study was that the mathematical and statistical approaches used to assess the associations of the gene variants with allele combinations and polygenic constructs, with measures of patellar tendon properties, yielded no significant findings. Previous findings have shown that allele combinations constructed from the *COL5A1* rs12722 and *MMP3* rs679620 gene variants, associates with discrete datasets of chronic Achilles tendinopathy and asymptomatic control groups. The findings of the current study do not support the notion that these two gene variants alter the structural and mechanical properties of patellar tendon, however, a tendency exists that may link the mechanical properties of tendon to risk of incurring tendon pathologies. Therefore, further research is required to investigate the effect of these associations and their clinical applications to injury prevention models.

Chapter 8.

General discussion

8.1 Overview of rational for conducting genetic association studies on tendon properties

The definitive aetiology of variability in structural and mechanical properties of tendon is not yet fully understood, in spite of a large volume of published research in this area (Mokone et al., 2006, September et al., 2008, Mokone et al., 2005, Posthumus et al., 2010a, Raleigh et al., 2009), Brown et al., 2011a, Posthumus et al., 2011, Collins et al., 2009, Brown et al., 2011b). Nonetheless, in reviewing the existing literature (chapter 1), it is clear that tendon properties are defined by a multitude of factors for which diverse extrinsic and intrinsic factors have been identified, for instance, mechanical loading (Hansen et al., 2003, Kubo et al., 2002, Kubo et al., 2004, Reeves et al., 2005a), sex (Kubo et al., 2003a, Magnusson et al., 2007, Onambele et al., 2007, Onambele-Pearson and Pearson, 2012) and ageing (Reeves et al., 2003a, Reeves et al., 2005b, Magnusson et al., 2003a, Kubo et al., 2003b, Karamanidis and Arampatzis, 2006, Karamanidis and Arampatzis, 2005, Narici et al., 2005, Maganaris et al., 2006, Morse et al., 2005, Onambele et al., 2006, Mian et al., 2007, Mademli et al., 2008, Baudry et al., 2012).

Previous research examining physical performance phenotypes related to tendon structure and function has been performed for which a genetic basis can be suspected. Specifically, these phenotypes include Achilles tendon pathologies (Mokone et al., 2006, September et al., 2008, Mokone et al., 2005, Posthumus et al., 2010a, Raleigh et al., 2009), range of motion measures of the lower limbs (Collins et al., 2009, Brown et al., 2011b), and endurance running performance (Brown et al., 2011a, Posthumus et al., 2011). In the latter two phenotypes, tendon function can be considered an essential contributing factor to the overall phenotype.

Variations within genes that encode for protein components expressed and involved in structural and regulatory processes within tendon, are potential areas for investigation in genetic association studies.

8.2 Limitations of genetic association studies

Although genetic association studies such as the above, have achieved great successes in identifying a genetic component to complex exercise and health-related phenotypes, these type of studies only investigate variation at one loci, which only explains a limited proportion of heritability of the tendon phenotype, leaving the vast majority of heritability unexplained (Eichler et al., 2010). Hence, on the basis of current evidence, it is difficult to

assume that there is only a single causative gene variant or gene involved in tendon phenotypes, and it is more likely that a multitude of genes are involved (polygenics) (Spurway, 2006, Bray et al., 2009). This concurs with the expectation that tens or even hundreds of genes, their associated proteins, and their heterogeneous interactions, are required to maintain normal tendon structure, regulation and ultimately function, thus, the literature documenting genotype-phenotype associations is often ambiguous. Compounding the ambiguity with these associations is the lack of a systematic process, particularly when recruiting subjects, in that the vast majority of the genetic association studies on tendon phenotypes addressed above, do not maximise the effects of the genetic portion of the associations. Generally these studies are retrospective in nature, in that the phenotype was evident before genetic variables were considered, which invariably pertains to non-genetic factors being highly influential on the phenotype (Kavvoura and Ioannidis, 2008). Age, body mass, and physical activity levels were notable non-genetic factors not controlled for in these studies. In addition, sample size is a reoccurring limitation in that the power to detect genotype-phenotype associations is limited when dealing with very small effects from the gene variant (s) under investigation (Hong and Park, 2012).

With regards the phenotype in these association studies, there appears to be a clear lack of definition, in that they are too generalised to highlight the significance of genetic factors. The problem lies in the fact that the endpoint assessment may be too ubiquitous to detect the modifying effects of the gene variants on the phenotype under investigation. For instance, flexibility tests such as the sit and reach test were used in associating the *COL5A1* rs12722 variant and range of motion measures (Collins et al., 2009, Brown et al., 2011b), but flexibility is a broad ill-defined phenotype encompassing various tissues, such as tendon, ligament, joint capsules, aponeuroses and fascia sheaths, of which differential expression of the protein coded for by the *COL5A1* gene between the tissues is likely. Hence, a precise relation between this gene variant and range of motion cannot be identified. To this end, it is essential that a direct and precise assessment of the phenotype is available to minimise noise, which may otherwise contribute to the variability between the measures (Newton-Cheh and Hirschhorn, 2005). This accuracy becomes particularly important when a specific intermediate phenotype is assessed. Therefore, when investigating the candidate gene variants and their association with tendon structural and mechanical properties, it was necessary to adopt a comprehensive *in vivo* assessment of the patellar tendon, whilst minimising the effect of confounding variables (non-genetic),

reported in the research literature to influence the structure, metabolism and function of tendon.

8.3 Aim of the thesis

The overall aim of the work described in this thesis was to examine some of the genetic factors that contribute to independent parameters describing the structural and mechanical properties of the patellar tendon. Specific aims were:

1. To determine whether the *COL5A1* rs12722 gene variant and *MMP3* rs679620, rs591058 and rs650108 gene variants associate with patellar tendon properties in asymptomatic male and female populations

2. To determine whether allele combinations deriving from these gene variants associate with patellar tendon properties

8.4 Main findings and implications

No variability was evident between the genotype groups of the four gene variants and any of the measures of patellar tendon properties investigated in this thesis. Common single nucleotide polymorphisms in two separate genes, *COL5A1* rs12722 and *MMP3* rs679620, rs591058 and rs650108, were investigated.

The *COL5A1* rs12722 gene variant has been associated with tendon pathologies, range of motion, and endurance running performance, through mechanisms proposed to be related to the structural and mechanical properties of soft-tissue, including that of tendon, so it was considered a rational candidate gene to examine. The *MMP3* gene variants have also been associated with tendon pathologies, although there is a lack of replication in affirming this association, and there is no clear consensus over possible mechanisms through which any effect is mediated. Tendon structural and mechanical properties are therefore possible intermediate phenotypes, through which the effect of the gene variants on the phenotypes stated above, are related.

The examination of the *COL5A1* rs12722 genotypes are described in chapter 3 and 4. No significant associations were found between *COL5A1* rs12722 genotypes and volume of tendon, modulus of tendon, or indeed both in combination, in either sex. Thus, the data suggests that the *COL5A1* rs12722 gene variant does not make a discernible contribution to

the variability on the patellar tendon properties measured, and that there is no influence of sex on this genotype-phenotype association, at least not in the asymptomatic male and female cohorts studied here.

The results from the examination of the *MMP3* rs679620, rs591058, and rs650108 genotypes are reported in chapters 5 and 6. Again, no significant associations were evident between the genotypes of the three *MMP3* gene variants independently, with volume and modulus of tendon, as well as both in combination (z-scores), in either sex. Hence, the data suggests that the three *MMP3* gene variants do not make discernible contributions to the interindividual variability of patellar tendon properties measured, and that there are no sex-specific effects on the genotype-phenotype associations, in these asymptomatic cohorts.

The results from the polygenic profile including the four tested gene variants (*COL5A1* rs12722, *MMP3* rs679620, rs591058, rs650108) on structural (volume) and functional (modulus) measures of the patellar tendon are reported in chapter 7. No significant associations were evident between the allele combinations of the *COL5A1* rs12722 and *MMP3* 679620 gene variants, as well as the total genotype scores (TGS), which included all four gene variants, on patellar tendon properties. In addition, no significant associations were evident when factoring in the influence of sex.

The association between the allele combinations and Young's Modulus, albeit not significant in either sex, did approach significance (male, $P = 0.073$; female, $P = 0.067$). What was intriguing about these findings was that there was a tendency for the allele combination counts to concur with a previous case-control study investigating the same combination of alleles, and risk of incurring a tendinopathy. Higher tendon modulus values were associated with increased risk of tendon pathologies, and conversely, lower tendon modulus values were associated with being protective against such pathologies, in relation to their respective combination of alleles. There was no influence of sex on these tendencies, as similar findings were evident in both male and female cohorts.

Even though the link between tendon material stiffness and risk of injury remains a contentious issue (Witvrouw et al., 2004, Witvrouw et al., 2007), the findings documented in chapter 7 together with the previous association with risk of tendinopathies, suggests that the mechanical properties of tendon may be a possible causative factor and prelude to tendon injuries, through common genetic variation in proteins expressed in tendon.

Accordingly, these proposed relationships highlight the importance of investigating associations between sequence variants in DNA with more 'immediate' phenotypes (closer to the genotype), which are involved in complex phenotypes such as tendon injury. The advancements in technologies have made possible the *in vivo* assessments of soft-tissue structures and mechanics, associated with these 'immediate' phenotypes. It could therefore be assumed that there is a direct chain of influence on the interindividual variability from the genotype to the more easily measurable phenotypes.

Ultimately, by establishing the intermediate phenotypes (*in vivo* structural and mechanical properties) that are influenced by the same genetic variants as tendon injury will enhance our understanding of the individual components that contribute to the complexities of tendon injury.

8.5 Directions for future research

When investigating a gene variant and its influence on tendon properties, it is crucially important to negate confounding variables that are likely to influence tendon phenotypes, specific to its anatomical region and physiological heterogeneity. Also, minimising experimental error associated with phenotype measurement techniques is paramount, in order to establish a valid and reliable association between gene variants, and tendon structural and mechanical properties. Previous genetic association studies on soft tissue-related phenotypes have not adopted precise measurement techniques; hence, attempting to verify a specific gene-phenotype relationship is limited. Recent advances in technologies have allowed for the accurate, reproducible and non-invasive assessment of soft tissue properties *in vivo,* such as tendon, as utilised in chapters 3-6. In addition, such techniques enhance the power to detect a genetic influence on the interindividual variability of these types of phenotypes. Future efforts should be concentrated on associating gene variants with intermediate phenotypes, ordered more closely to the genotype, to further our understanding of the molecular mechanisms involved in the more outward phenotypes (whole body level). In addition, our understanding of these relationships may be accelerated by continuing to construct polygenic profiles through appropriate statistical techniques (such as those utilised in chapter 7). These profiles can be potentially more informative than single variant analyses, when associations are being proposed with tendon modulus.

A limitation of the studies documented in chapters 3, 4, 5, 6, and 7, were the relatively small sample sizes in the region of 40 participants for genotype-phenotype associations, for both sexes. However, the results from power calculations performed prior to conducting the experimental chapters (see section 2.1), suggests that the sample sizes were sufficient to detect true associations between the genotype groups and measures of tendon properties, albeit the sample sizes were at the lower end of the power continuum. In addition, even though by combining male and female data sets would inevitably enhance the statistical power analyses in detecting these genotype-phenotype associations, the hypothesis-driven research question relating to sex differences in tendon properties (see section 1.10-Aims of thesis), underlined the need to maintain separate subgroups. Accordingly, a larger sample size is encouraged in future research investigating these genotype-phenotype associations in order to increase the power, and thus, the ability to detect these proposed associations, particularly when investigating the contribution of a single genetic marker. Given that this thesis presents the first investigations into associations between the gene variants and patellar tendon properties, until a larger sample size is used, it remains a possibility that weak associations do exist.

The approach undertaken in each of the experimental chapters (chapters 3 ,4 ,5 ,6) to maximise the detection of phenotypic variability, was to recruit a high proportion of participants from the original cohort (see section 2.1) who were homozygote for the gene variants under investigation, hence, the genotype was being ‘stressed’ (Montgomery et al., 2002). This approach is pragmatic in that it represents an efficient model for future research in sport and exercise genetics. Therefore, future researchers investigating gene variants and their association with tendon properties should be encouraged to replicate this approach in synergy with a larger sample size, in order to maximise the ability to detect such associations.

From a purely genetic standpoint, a more powerful approach is required to find significant DNA polymorphisms associated with a higher proportion of the variability in tendon phenotypes, especially those that display continuous or quantitative traits (i.e. tendon modulus), due to the likelihood of many polymorphisms on several loci on the genome, being influential. Genotyping arrays (SNP chips) can now assay upward of 2 millions variants simultaneously, which due to linkage disequilibrium captures a substantial proportion of total genomic variability (The International HapMap, 2005), so by cataloguing key genetic variants involved in tendon structure and function, genome-wide

association studies (GWAS) could pinpoint key genes/gene variants and shed light on underlying mechanisms with extensive scans of DNA. However, GWAS are considered to be non-candidate-driven approaches in contrast to candidate gene-specific studies, such as the genetic association studies within this thesis, and as with these candidate gene association studies cannot specify on their own which genes are causal in genotype-phenotype associations (Manolio, 2010, Pearson and Manolio, 2008). Therefore, over the next decade to improve the strength of these associations, it would be beneficial to 1) conduct twin/family studies to provide estimated interindividual variability of specific tendon properties that is inherited, which has not yet been done; 2) conduct genetic association studies of thousands of asymptomatic individuals with measures of specific tendon properties; 3) use appropriate combinations of GWAS and sequencing; 4) conduct intensive functional studies to characterise the genes and pathways; 5) construct animal models that represent human tendon physiology. This would assist in eventually asserting a cause and effect relationship between the gene variants and tendinopathies as well as physical performance potential.

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Appendices

Appendix 1. Algorithmic model used to predict the number of participants from a hypothetical 100 individuals, optimised for the phenotypic tests

Participant		MMP3 rs679620		MMP3 rs591058		MMP3 rs650108		COL5A1 rs12722	No. '0' homozygotes	No. '2' homozygotes	Total No. homozygotes
		A/G (FreqG = 0.43)		T/C (FreqC = 0.46)		G/A (FreqA = 0.23)		T/C (FreqC = 0.43)			
		0.32 and 0.82		0.29 and 0.79		0.59 and 0.95		0.32 and 0.82			
1	0.83	2	0.77	1	0.85	1	0.00	0	1	1	2
92	0.11	0	0.77	1	0.33	0	0.43	1	2	0	2
93	0.13	0	0.59	1	0.92	1	0.48	1	1	0	1
94	0.29	0	0.61	1	0.47	0	0.13	0	3	0	3
95	0.05	0	0.02	0	0.45	0	0.98	2	3	1	4
96	0.87	2	0.59	1	0.34	0	0.13	0	2	1	3
97	0.78	1	0.35	1	0.78	1	0.64	1	0	0	0
98	0.20	0	0.90	2	0.27	0	0.87	2	2	2	4
99	0.83	2	0.25	0	0.30	0	0.13	0	3	1	4
100	0.21	0	0.97	2	0.03	0	0.47	1	2	1	3

No. 0	4
No. 1	23
No. 2	33
No. 3	28
No. 4	12

Appendix 2. Allele combination

Four possible allele combinations were constructed from the two gene variants, *MMP3* rs679620 and *COL5A1* rs12722, for both sexes independently, such that the A and C, G and C, A and T, and G and T alleles were assembled for the aforementioned gene variants, respectively.

For each participant a total score of 4 was allocated, although the distribution of this score was determined by their genotypes for both gene variants. For example, a score of **4** was allocated to one combination of alleles if the participant was homozygote for both gene variants. If the participant was heterozygote for one gene variant and homozygote for the other, a score of **2** was allocated to each of the two possible allele combinations. Finally, if the participant displayed heterozygosity for both gene variants, a score of **1** was given to each of the four possible allele combinations.

The variable or measure of tendon properties (volume, Young's Modulus, z-scores) was then weighted per allele combination for each participant, such that if the participant scored a **4** for one particular allele combination, the numerical value of the variable would appear four times for that allele combination. The variable would appear twice for two different allele combinations if a score of **2** was allocated for the two relevant allele combinations.

Appendix 3. The mathematical model used for the purposes of analysing the allele combinations

MMP3		COL5A1						YM
A/G		C/T		A+C	G+C	A+T	G+T	
G	G	T	T				4	0.20
A	A	T	T			4		0.32
A	A	T	T			4		0.78
G	G	T	T				4	0.45
G	G	C	T		2		2	0.67
A	A	C	C	4				0.21
A	A	C	T	2		2		0.35
A	A	T	T			4		0.39
A	A	C	T	2		2		0.30
A	A	C	T	2		2		0.31
A	A	C	T	2		2		0.26
A	A	C	T	2		2		0.10
A	A	C	C	4				0.46
A	A	T	T			4		0.23
G	G	C	C		4			0.37
A	A	C	T	2		2		0.27
A	A	T	T			4		0.56
A	A	C	T	2		2		0.41
A	A	C	C	4				0.34
G	G	T	T				4	0.51
A	A	T	T			4		0.51
A	A	C	T	2		2		0.31
G	G	C	T		2		2	0.45
A	A	C	T	2		2		0.36
A	A	C	T	2		2		0.61
A	A	C	T	2		2		0.35

A	A	C	T	2		2		0.41
A	A	C	T	2		2		0.59
A	A	C	T	2		2		0.20
G	G	C	T		2		2	0.95
A	A	C	T	2		2		0.55
G	G	C	T		2		2	0.43
A	A	C	T	2		2		0.34
G	G	C	T		2		2	0.45
A	A	C	T	2		2		0.53
A	A	C	T	2		2		0.78
A	A	C	T	2		2		0.31
G	G	C	C		4			0.60
G	G	C	T		2		2	0.31
G	G	T	T				4	0.84
A	A	T	T			4		0.20
A	A	T	T			4		0.75
A	A	C	T	2		2		1.33
A	A	C	T	2		2		0.73
A	A	C	T	2		2		0.90

Variable weighted per allele combination															
A+C				G+C				A+T				G+T			
												0.20	0.20	0.20	0.20
								0.32	0.32	0.32	0.32				
								0.78	0.78	0.78	0.78				
												0.45	0.45	0.45	0.45
				0.67	0.67							0.67	0.67		
0.21	0.21	0.21	0.21												
0.35	0.35							0.35	0.35						
								0.39	0.39	0.39	0.39				
0.30	0.30							0.30	0.30						
0.31	0.31							0.31	0.31						
0.26	0.26							0.26	0.26						
0.10	0.10							0.10	0.10						
0.46	0.46	0.46	0.46												
								0.23	0.23	0.23	0.23				
				0.37	0.37	0.37	0.37								
0.27	0.27							0.27	0.27						
								0.56	0.56	0.56	0.56				
0.41	0.41							0.41	0.41						
0.34	0.34	0.34	0.34												
												0.51	0.51	0.51	0.51
								0.51	0.51	0.51	0.51				
0.31	0.31							0.31	0.31						
				0.45	0.45							0.45	0.45		
0.36	0.36							0.36	0.36						
0.61	0.61							0.61	0.61						
0.35	0.35							0.35	0.35						

0.41	0.41							0.41	0.41						
0.59	0.59							0.59	0.59						
0.20	0.20							0.20	0.20						
				0.95	0.95							0.95	0.95		
0.55	0.55							0.55	0.55						
				0.43	0.43							0.43	0.43		
0.34	0.34							0.34	0.34						
				0.45	0.45							0.45	0.45		
0.53	0.53							0.53	0.53						
0.78	0.78							0.78	0.78						
0.31	0.31							0.31	0.31						
				0.60	0.60	0.60	0.60								
				0.31	0.31							0.31	0.31		
												0.84	0.84	0.84	0.84
								0.20	0.20	0.20	0.20				
								0.75	0.75	0.75	0.75				
1.33	1.33							1.33	1.33						
0.73	0.73							0.73	0.73						
0.90	0.90							0.90	0.90						

Appendix 4. Recruitment materials

EMAIL:

Dear all,

I am looking for some help for my PhD study I am conducting into the 'genetics of tendon properties'. Basically, I am trying to see how our DNA/genes affect our physical performance capabilities as well as our risk of incurring injuries. To do this, I'm studying how DNA influences how our tendons work. In total, 3 visits to the new Exercise and Sport Science laboratories would be required, so you get to see some cutting edge research in action. Note that no strenuous activity is involved!

Eligibility: Female, age 18-40, good health, no history of serious knee or ankle problems. You don't have to be very sporty – in fact, simply an average activity level would be perfect.

If you are interested in participating in this study, and helping with my PhD research and the exercise science research going on here at MMU Cheshire, please reply to me using the address below or phone number.

Thank You,

Brandon Foster (PhD student) b.foster@mmu.ac.uk /07847820666

POSTER:

YOUR HELP REQUIRED FOR WORLD-CLASS RESEARCH INTO GENETICS OF SPORT AND EXERCISE SCIENCE!

HI, MY NAME IS BRANDON FOSTER (BSC, MSC, CSCS) AND I AM A PHD STUDENT HERE AT CREWE CAMPUS AND I'M CONDUCTING A STUDY INTO HOW OUR GENETICS OR DNA/GENES AFFECT OUR PHYSICAL PERFORMANCE CAPABILITIES AS WELL AS OUR RISK OF INCURRING INJURIES. IN PARTICULAR, I'LL BE LOOKING AT HOW YOUR TENDONS WORK. I AM LOOKING FOR BOTH MALE AND FEMALE VOLUNTEERS TO HELP OUT WITH THIS STUDY.

SUITABILITY FOR STUDY

AGE- 18-40

GOOD HEALTH

NON-SMOKERS

NO HISTORY OF KNEE OR ANKLE PROBLEMS

YOU DON'T HAVE TO BE VERY SPORTY – IN FACT, AN AVERAGE ACTIVITY LEVEL WOULD BE PERFECT

WHAT'S IN IT FOR ME?

ABLE TO GAIN INCITE INTO WHAT GENES ARE POTENTIALLY AFFECTING YOUR EXERCISE PERFORMANCE AND RISK OF INCURRING INJURIES TO YOUR MUSCLES AND TENDONS

YOU HAVE THE OPPORTUNITY TO TAKE PART IN A STUDY THAT MAY PROVIDE ANOTHER IMPORTANT PROSPECTIVE ON HOW SPORTING AND EXERCISE PERFORMANCE MAY DIFFER DRAMATICALLY BETWEEN INDIVIDUALS, SOLELY BECAUSE OF OUR DNA/GENES

HAVE THE OPPORTUNITY TO GAIN EXPERIENCE OF THE PRACTICES/EXPERIMENTAL PROCEDURES INVOLVED IN A SPORT AND EXERCISE SETTING

For more details and if you are interested and are willing to help me please contact me by email/mobile,

(bpfoster@hotmail.co.uk/07847820666)

PRESENTATION:

'Cutting edge' research into the Genetics of Sport and Exercise Science!

- Does tendon/muscle flexibility differ between people due our unique DNA/gene profile? (genetic variation)

- Genetics potentially contributes around **50%** to our performance potential in any given task

- Applications
- Screen for individuals who are at an ↑ risk of injury or more suited to an activity
- Develop 'individualised' training/rehabilitation programs
- **Gene therapy.**

- Both male and female, Age 18-40, Good BMI, Good health, No history of serious knee and ankle problems.
- No strenuous exercise regimes or strenuous testing involved.
- Only 3 visits to lab ranging from only 10 -45 mins

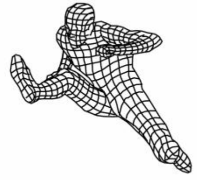
Thank you for your time!

Appendix 5. Ethical materials- participant ISP and ICF

Department of Exercise and Sport Science

Informed Consent Form

(Both the investigator and participant should retain a copy of this form)



Name of Participant:

Supervisor/Principal Investigator: **Brandon Paul Foster**

Project Title: **Genetics of Tendon Properties**

Ethics Committee Approval Number: **08.06.10(i)**

Participant Statement

I have read the participant information sheet for this study and understand what is involved in taking part. Any questions I have about the study, or my participation in it, have been answered to my satisfaction. I understand that I do not have to take part and that I may decide to withdraw from the study at any point without giving a reason. Any concerns I have raised regarding this study have been answered and I understand that any further concerns that arise during the time of the study will be addressed by the investigator. I therefore agree to participate in the study.

It has been made clear to me that, should I feel that my rights are being infringed or that my interests are otherwise being ignored, neglected or denied, I should inform the The University Secretary and Clerk to the Board of Governors, Manchester Metropolitan University, Ormond Building, Manchester, M15 6BX. Tel: 0161 247 3400 who will undertake to investigate my complaint.

Signed (Participant)   Date

Signed (Investigator)   Date

Prospective Participant Screening Questionnaire

MMU Cheshire

Department of Sport and Exercise Science

Name: ....

Date of birth:........................... Age: .... Gender:...................................

Height (m):... Weight (kg): ....

Email address:.. Mobile phone number: ....

Please answer the following questions by putting a circle round the appropriate response or filling in the blank.

1. **What is your ethnic group? (last 3 generations of your family history)**

White / Mixed heritage / Asian / Black / Chinese

2. **How would you describe your present level of activity?**

Less than 30 minutes of activity a day (excludes golf, gardening and fishing)

Over 30 minutes of activity a day (includes walking)

Structured physical exercise sessions more than 3 times a week (Give details please)

....

3. **Have you ever been diagnosed by a doctor with any muscle and/or tendon problems of the knee and/or ankle?** Yes / No

If you answered **Yes,** please give details..

As far as I am aware the information I have given is accurate.

Participant's Signature:..

Supervisor's Signature:...

Date:......../............/............

MANCHESTER METROPOLITAN UNIVERSITY

MMU Cheshire

Department of Exercise and Sport Science

Information Sheet for Participants (ISP Template)

Title of Study:

"Genetics of Tendon Properties"

Ethics Committee Reference Number:

08.06.10(i)

Participant Information Sheet

1) This is an invitation to take part in a piece of research.

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part.

2) What is the purpose of the research?

To investigate whether the flexibility of people's tendons is influenced by our genes.

3) Why is the study being performed?

Here at MMU we have track record of research into both the function of tendons, and the genetics of exercise performance. We are now combining these areas of our expertise to investigate the genetics of tendon function. This could help scientists understand why some people have more flexible tendons than others, why some people can perform better at certain sports than others, and even why some people are at greater risk of tendon injury than others.

4) Why am I being asked to take part?

To do our research, we need the help of quite a large number of volunteers who are willing to provide some DNA for genetic testing and who are also willing to take part in some tests of tendon function. We would like people who take part in normal amounts of physical activity to help us in this research – e.g. athletes in heavy regular training are not suitable for this research.

5) Do I have to take part?

You are under no obligation to take part in this study. If, after reading this information sheet and asking any additional questions, you do not feel comfortable taking part in the study you do not have to. If you do decide to take part you are free to withdraw from the study at any point, without having to give a reason. If you do withdraw from the study you are free to take any personal data with you and this will not be included when the research is reported. If you decide not to take part or withdraw from the study it will not affect the standard of care you receive in any way, nor will it affect your relationship with any of the staff at the Manchester Metropolitan University.

If you do decide to take part you will be asked to sign an informed consent form stating your agreement to take part and you will be given a copy together with this information sheet to keep.

6) What will happen to me if I agree to take part?

You will be asked to visit the MMU laboratories on four separate occasions. The first visit consists of a few physical measurements, which will include your height and weight, followed by providing a small blood sample. All this should take less than 30 minutes. At a later date the investigator will analyse your blood sample (if you are a female, additional analysis of oestrogen levels will be performed using half of your blood sample), and will subsequently let you know if you are required for a second visit, which will involve a practice session on a machine designed to measure muscle and tendon function, so you become familiar with the process. This should take no longer than an hour out of your time. The third visit involves measurements of tendon function, and could take 1-2 hours of your time. Specifically, this third visit will involve measurement of the size and length of your patellar tendon (the tendon just below the kneecap) using ultrasound, as well as measurements of your maximum leg strength. The fourth and final visit involves a trip to the MMU laboratory on Oxford Road, Manchester, for a further measure of your tendon length using a MRI scan. This could take 1 hour of your time.

The tests and procedures are given in more detail below:

1ST Visit, expected to take less than 30 minutes:

Questionnaire: We will ask you to fill in a medical questionnaire including questions about your lifestyle, to ensure you are eligible to take part in the study. The answers you give will be kept strictly confidential with only the principal investigators having access to them.

Body Composition: We will measure your height and weight and calculate your BMI (body mass index) from this information.

Blood sample: A small blood sample (about 10mL) will be taken from a vein in your forearm by qualified personnel. The procedure is usually not painful nor particularly uncomfortable and you will remain seated or be lying down. After leaving the laboratory, you can continue with your normal activities.

2nd Visit, expected to take up to 1 hour:

Familiarisation session: We will allow you to practice on a machine designed to measure your muscle and tendon function, so you will become more familiar with the process when you perform the test for real on the final visit. A description of this process is outlined below.

3rd Visit, expected to take between 1 and 2 hours:

Measurement of tendon size and stiffness: You will remain seated in a chair specially designed for testing muscle and tendon function. With your knee bent, you will be asked to try to straighten your leg and push against a pad strapped to the lower part of your shin, increasing the force gradually over 4-6 seconds. However, your leg will not actually move because the pad you push against will be in a fixed position. At the same time we will measure the amount your patellar tendon stretches using an ultrasound probe, which is completely painless. In a separate test, you will be asked to try to bend your leg as hard as possible so we can measure your strength during this action, but again your leg will not move because the pad will be in a fixed position.

Measurement of EMG activity: Small pads (EMG electrodes) will be stuck onto your skin near the front of the knee and on the back of your thigh. This will help us assess the contribution of the various muscles in your leg to the stretch of the tendon, during the measurements of tendon size and stiffness described above. The use of EMG electrodes in this way is completely painless.

4th Visit, expected to take up to 1 hour:

Measurement of tendon length: With your knee straight or bent, we will measure the length of your patellar tendon using an MRI scan

After leaving the laboratory, you can again continue with your normal activities.

7) Are there any disadvantages or risks in taking part?

All the procedures we will use are regularly used in our laboratories.

There is a small risk of a little muscle soreness for a day or two after the tests of tendon size and stiffness, but this would be minor and temporary.

There is a small risk of infection when taking blood but every precaution will be taken to minimise this risk, with the use of sterile equipment. For example, one of the researchers (Dr Alun Williams) has taken over 500 blood samples without a single case of infection. All blood samples will be drawn by qualified phlebotomists. In some cases there is a little bruising after the blood sample is taken, but this would be temporary.

8) What are the possible benefits of taking part?

There may be no direct benefits gained from the study by you, except for experiencing how research is carried out in a sport and exercise setting. However, the results in a broader sense will contribute to our knowledge of how certain genes affect the way tendons work, why some people can perform better than others in certain sports and activities, and even the risk of tendon injury. In addition, we would be happy to talk to you about your own results from the tendon tests and any implications these might have for your future exercise and training. We will not, however, be allowed to provide you with your own results from your genetic tests – this is standard practice in research of this kind.

9) Who are the members of the research team?

Principal investigators:

Brandon Paul Foster- PhD student involved in every aspect of study including genetic analysis and tendon function measures.

Dr Alun Williams- The main supervisor involved in the design of the whole study.

Collaborators:

Dr Gladys Pearson- Involved in design of tendon function measures and interpretation of data.

Dr Christopher Morse- Involved in design of tendon function measures and interpretation of data.

If you have any questions about the study now or at any time in the future, please contact Brandon Paul Foster (09985886@stu.mmu.ac.uk) or Dr Alun Williams (A.G.Williams@mmu.ac.uk).

10) Who is funding the research?

Myself (Brandon Foster) and the MMU Department of Exercise and Sport Science are funding this research study.

11) Who will have access to the data?

All information that is collected about you during the course of the study will be held securely in the Department of Exercise and Sport Science at Manchester Metropolitan University. Any information about you will have your name and details removed so that you cannot be recognised from it. Furthermore, to protect your privacy, confidentiality and security of the information you provide to the research team, a code number will be assigned to you. Only your code number will appear on your information generated for the study.

Information you provide will not be used or made available for any purpose other than for research, which is likely to be communicated at conferences or published in scientific journals at some point in the future.

As research into the genetics in exercise and health is in its infancy, the information we gain will be kept for future studies into establishing a link between genetics and physical performance capabilities. Only the members of the research team named in this document, who work in the Department of Exercise and Sport Science at MMU, will be permitted to access the data.

After the conclusion of the study, we will compile a short report for all participants to inform you about the contribution you will have made to the progression of knowledge in this field.

12) Who do I contact if I feel my rights have been violated?

If in the event you feel the wish to complain about the way you were treated during the study, please contact:

The University Secretary and Clerk to the Board of Governors, Manchester Metropolitan University, Ormond Building, Manchester, M15 6BX. Tel: 0161 247 3400.

13) Finally, a thank you!

Finally, thank you very much for your time, interest and help!