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Genetics of skeletal muscle strength and its determinants in healthy, untrained males

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Contents

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Stebbings, GK, Morse, CI, Williams, AG, Day, SH. (2013). Variability and distribution of muscle strength and its determinants in humans. BASES Annual Conference 2013, 141-142 (Preston, UK).

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Abbreviation	Definition
θ	Pennation angle
ACE	Angiotensin I-converting enzyme
ACSA	Anatomical cross-sectional area
$ACSA_{VL}$	Anatomical cross-sectional area of the vastus lateralis
ACTN3	Alpha-actinin-3
ALM	Arm lean mass
AVI	Audio video interleave
BMC	Bone mineral content
BMI	Body mass index
CcT	Co-activation torque
CNTF	Ciliary neurotrophic factor
Col I	Collagen type I
Col III	Collagen type III
COL5A1	Collagen type V alpha 1
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
d_{PT}	Patellar tendon moment arm length
DXA	Dual energy x-ray absorptiometry
ECM	Extracellular matrix
EMG	Electromyography
FAC	Favourable allele count
FF_{VL}	Fascicle force of the vastus lateralis
F_{PT}	Patellar tendon force
HWE	Hardy-Weinberg equilibrium
ICC	Intraclass correlation coefficient
kDa	Kilodalton
KE	Knee extension
$kg \cdot m^{-2}$	Kilograms per square metre
kVp	Peak kilovoltage
L_f	Fascicle length
LLM	Lean leg mass
LoA	Limits of agreement
L_{VL}	Muscle length of the vastus lateralis
MF_{VL}	Muscle force of the vastus lateralis
MVC	Maximal voluntary contraction
MVC_{KE}	Maximal voluntary isometric knee extension contraction
MVC_{KF}	Maximal voluntary isometric knee flexion contraction
ng	Nanogram
PCR	Polymerase chain reaction
PCSA	Physiological cross-sectional area
$PCSA_{VL}$	Physiological cross-sectional area of the vastus lateralis
PTK2	Protein tyrosine kinase 2
rCNTF	Recombinant ciliary neurotrophic factor
RMS	Root mean square
SNP	Single nucleotide polymorphism

STT	Superimposed twitch torque
TALM	Total appendicular lean mass
TGS	Total genotype score
TTN	Titin
VI	Vastus intermedius
VL	Vastus lateralis
μg	Micrograms
μl	Microlitres
μSv	Micro sieverts

Abstract

It is well accepted that inter-individual variability exists in muscle strength (more specifically maximal voluntary contraction torque), and many of its determinants. The extent of this inter-individual variability, however, has yet to be quantified despite many researchers suggesting genetic factors contribute. The aims of the present thesis were to first quantify the inter-individual variability within skeletal muscle phenotypes in a homogenous population, and secondly to investigate the contribution of multiple genetic polymorphisms to the inter-individual variability within these phenotypes. Genotype and phenotype data was collected from 120 untrained Caucasian males (aged 18-39 yr). Considerable inter-individual variability in muscle strength phenotypes and many of its determinants was observed. Subsequently, polymorphisms in the *CNTF*, *TTN*, *PTK2*, *TRHR* and *ACTN3* genes demonstrated significant associations with one or more of the skeletal muscle phenotypes, but neither *ACE* nor *COL5A1* were found to associate with any of the measured phenotypes. Adopting a polygenic approach that incorporated all of these genetic polymorphisms did not account for the inter-individual variability observed within VL muscle size (inter-individual variability = 13-20%; $P \geq 0.166$) or strength (14-19%; $P \geq 0.220$). The results identified novel genetic associations between *TTN*, *CNTF* and skeletal muscle architecture, in addition to providing the first independent replications of associations between *PTK2* and specific force, and *TRHR* and lean mass. In conclusion, there appears to be a genetic influence on skeletal muscle phenotypes, however, further research is necessary to replicate the associations observed within the current thesis in comparable and different populations. Nonetheless, the work presented here has applications for improving physical performance, in addition to enhancing our understanding of skeletal muscle disorders, which may have implications for how

individuals exercise and how skeletal muscle disorders are treated and/or prevented in future.

Chapter 1

Literature review

1.1 Introduction

Skeletal muscle strength is a key determinant of an individual's ability to perform activities of daily living and achieve peak physical performance (Thompson et al., 2004; Beunen & Thomis, 2004). Muscle weakness has been suggested as an independent predictor of long-term mortality risk (Rantanen et al., 2000), in addition to being associated with other predictors of mortality such as low body weight (Era et al., 1994) and chronic diseases (Bernard et al., 1998; Häkkinen et al., 1995). Improvements in quality of life, on the other hand, have been associated with increased muscle mass and strength (Guralnik et al., 1995). Muscle strength is multifactorial and is influenced by both intrinsic and extrinsic factors. For instance, reductions in muscle strength are known to occur as a consequence of the ageing process and have also been observed in certain disease states secondary to a reduction in skeletal muscle mass (Lindle et al., 1997; Larsson et al., 1979; Park et al., 2006; Tisdale, 2002; Mitch et al., 1994). Gains in both muscle size and strength are known to occur with growth and in response to training (Larsson et al., 1979; Atha, 1981; Jones & Rutherford, 1987). It is unsurprising therefore, that differences in muscle strength have been extensively reported between young and elderly individuals (Clarkson et al., 1981; Frontera et al., 1991; Lindle et al., 1997; D'Antona et al., 2003; Morse et al., 2005a), males and females (Danneskiold-Samsøe et al., 1984; Miller et al., 1993; Leyk et al., 2007), and as a consequence of training (Maughan et al., 1983). In well-matched, homogenous groups however, where apparent stimuli for gains or losses in muscle strength are lacking, inter-individual variability in strength persists (Maughan et al., 1983; Kanehisa et al., 1994; Erskine et al., 2009). It is likely that genetic factors may partially explain this persistent inter-individual variability, as muscle strength phenotypes demonstrate a reasonable degree of heritability, although the true extent of this heritability remains unclear (Thomis

et al., 1997; Thomis et al., 1998; Beunen & Thomis, 2004; Tiainen et al., 2004). Heritability refers to the proportion of total phenotypic variation in a population that is attributable to genetic variation. More specifically, broad sense heritability is the ratio of total genetic variance to phenotypic variance and represents the extent to which a phenotype is genetically determined, whereas narrow sense heritability is the ratio of additive genetic variance to total phenotypic variance and represents the extent to which a phenotype is passed from parents to offspring (Visscher et al., 2008). Furthermore, a phenotype is the physical manifestation of the underlying genotype for a particular trait. For example in humans, blue eyes would be one possible phenotype for the eye colour trait. However, there exist more complex traits, such as muscle strength, that are quantitatively measured on a continuous scale and are influenced by multiple genotypes. Consequently the work presented in this thesis is concerned with investigating the genetic influence on muscle strength and its determinants in a homogenous population, where muscle strength nominally represents the varying terms used to denote the measurement of contractile force under different experimental set ups (e.g. isometric maximal voluntary torque, force and also broader measures of maximal concentric load). The findings of which may have implications for facilitating the development of new treatments or interventions for ageing, disuse or certain disease states, where reductions in muscle strength and size are readily observed, by identifying novel gene targets.

1.2 Inter-individual variability

Muscle strength, or rather the measurement of torque/force during an isometric maximal voluntary contraction (MVC) is influenced by a descending cascade of factors ranging from the degree of recruitment from depolarisation of the motor neurone, to the final actin-

myosin cross-bridge formation, and all the propagation and contractile elements in between. It is unsurprising therefore given the neural, muscular and structural components involved in this process that a degree of inter-individual variability persists in otherwise homogenous population groups. The inter-individual variability in muscle strength represents the spread of values produced by individuals within a sample population, and is dependent on experimental error, environmental factors and an individual's genetic profile (Tiainen et al., 2005; Beunen & Thomis, 2004; Silventoinen et al., 2008; Thomis et al., 1998; Simoneau & Bouchard, 1995). The environmental contribution to inter-individual variability in muscle strength and its determinants ranges from ~20-60% (Thomis et al., 1997; Tiainen et al., 2004; Carmelli & Reed, 2000), and is likely to be the consequence of factors such as habitual physical activity levels and diet, which can often prove difficult to control when using strength as an outcome measure in human populations. Using well-matched, homogenous samples however, could minimise the variability observed within the values produced, and therefore demonstrates the need for careful consideration of these factors in aspects of research design and statistical analysis. Contrastingly, experimental error in the measurement of muscle strength and its determinants is generally under greater control, depending on what variable is being measured and the measurement method being used, and thus contributes the least to overall estimates of inter-individual variability (Simoneau & Bouchard, 1995). The remaining variability may be accounted for by the genetic profile of an individual, however the magnitude of this genetic contribution remains inconclusive (Beunen & Thomis, 2004).

1.2.1 Genetic contribution

Attempts to elucidate the genetic contribution to the inter-individual variability in muscle strength and its determinants have been undertaken using heritability estimates (Visscher et al., 2008), typically by completing twin and/or family studies. Twin studies draw comparisons between monozygotic (identical) twin pairs, who have identical genotypes; and dizygotic (non-identical) twin pairs, who share approximately 50% of their genotype. It is assumed that as twin pairs are likely to share the same environment, the environmental contribution to variability in a given phenotype will be reduced. Therefore, for a phenotype under strong genetic control, it is expected that monozygotic twin pairs would demonstrate a higher intra-pair correlation coefficient than dizygotic twin pairs (Figure 1.1) (Beunen & Thomis, 2004).

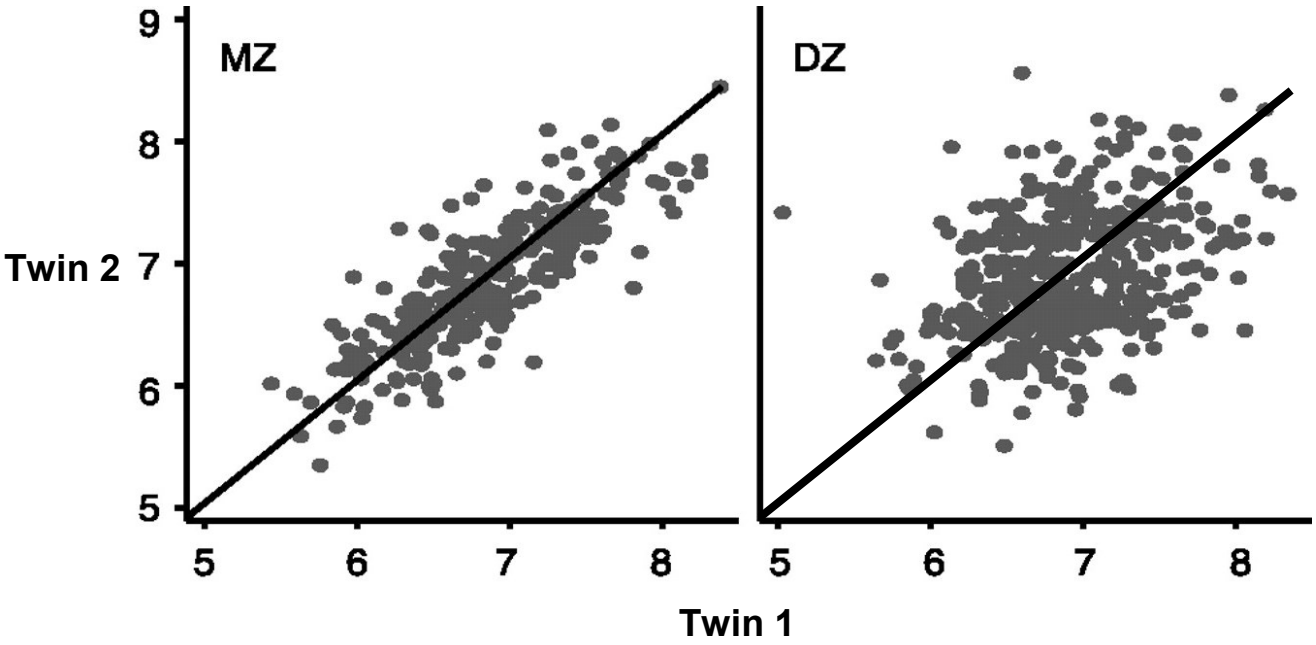

Figure 1.1. An example of a higher intra-pair correlation coefficient for monozygotic (MZ) twin pairs (r = 0.82) in comparison to dizygotic (DZ) twin pairs (r = 0.42) for serum surfactant protein D levels. Figure modified from Sorensen et al. (2006).

Family studies draw comparisons between more combinations of family members than twin studies and are therefore more representative of the population (Bouchard, 1997). Furthermore, flexible statistical analyses allow for the discovery of genomic regions of

interest in relation to the phenotype, making family studies a desirable approach. To date, heritability estimates have been calculated for a variety of somatic and performance-related phenotypes in humans (Beunen & Thomis, 2000; Arden & Spector, 1997; Silventoinen et al., 2003; Silventoinen et al., 2008), and it is probable that many of these estimates have led to the identification of new associations between genetic polymorphisms and observed phenotypes. With particular reference to the human muscle strength phenotype, reports of heritability are extensive, however estimates range from ~40-80% (Table 1.1), thus the extent of the genetic contribution to inter-individual variability in muscle strength remains uncertain (Thomis et al., 1997; Thomis et al., 1998; Beunen & Thomis, 2004; Tiainen et al., 2004). Nonetheless, it is accepted that the multifactorial muscle strength phenotype is under strong genetic control, with a number of studies reporting significant associations between muscle strength and various genetic polymorphisms (Bray et al., 2009).

Table 1.1. Heritability estimates for various muscle strength phenotypes in different muscle groups.

Strength Phenotype	Muscle Group	Heritability [Mean (confidence intervals)]	Article
Isometric MVC	Knee extension	0.82 – 0.96	Huygens et al. (2004)
		0.66 – 0.70	Thomis et al. (1997)
	Forearm flexion	0.36 (0.03 – 0.69)	Arden and Spector (1997)
	Elbow flexion	0.66 – 0.78	Thomis et al. (1998)
Isokinetic MVC	Knee extension	0.63 – 0.87	Huygens et al. (2004)
1 RM	Elbow flexion	0.65 – 0.75	Thomis et al. (1998)

1.3 Skeletal muscle properties

The work presented in this thesis aims to identify associations between gene variants and the structural and functional properties of human skeletal muscle, which may impact on an individual's ability to generate muscle strength. An overview of skeletal muscle structure and function is presented in the following sections of this thesis, and provides a foundation upon which potential genetic associations can be made in subsequent sections.

1.3.1 Skeletal muscle structure and composition

The regular arrangement of human skeletal muscle reflects its function to produce movement by generating force. Skeletal muscle is comprised of fascicular bundles that each contains numerous smaller bundles of muscle fibres surrounded by the sarcolemma (Figure 1.2). These individual muscle fibres contain an abundance of long, thin myofibrils that in turn house the muscle contractile proteins and an array of structural proteins; together known as the myofilament (Figure 1.2). The serial arrangement of myofilaments into repeated segments, known as sarcomeres, run along the length of the myofibril and it is the contraction and co-ordination of these sub-units that result in muscle contraction and thus force generation. This classical description of skeletal muscle structure, however, is not a true reflection of the structure of pennate fibred muscles in which muscle fibres do not run parallel to the tendon (Narici, 1999), or each other (Scott et al., 1993).

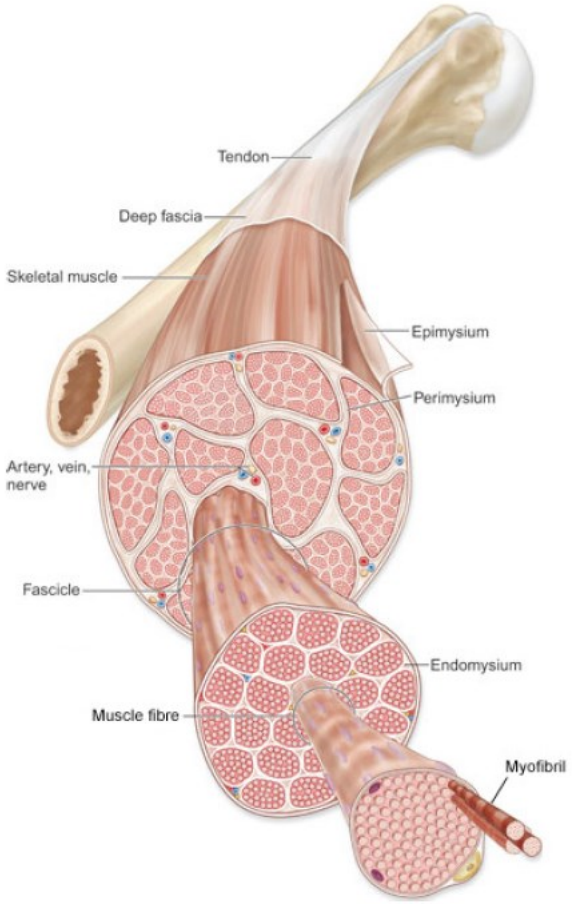

Figure 1.2. Overview of the regular arrangement of skeletal muscle structures from whole muscle level down to the myofilaments. Figure taken from (Jones et al., 2004).

Furthermore, the presence of numerous additional proteins within skeletal muscle, such as those involved in myofilament cross-linking (Patel & Lieber, 1997), inevitably increases the potential sources of inter-individual variability within the production of muscle strength and its determinants. Thus, identification of the roles of a number of these proteins to elucidate sources of inter-individual variability within them seems prudent at this stage in the review.

1.3.2 Sarcomere

The sarcomere is the basic contractile unit of the myofibril and has a striated appearance that demonstrates the regular arrangement of the sarcomere's principle components, actin and myosin (Figure 1.3). At rest a degree of overlap between interdigitating actin and myosin filaments exists, which is evidenced by the darker, anisotropic 'A'-band under light microscopy. Lying within the A-band, the H-zone is characterised by a relatively paler appearance due to a lack of myofilament overlap in this region. Central to the H-

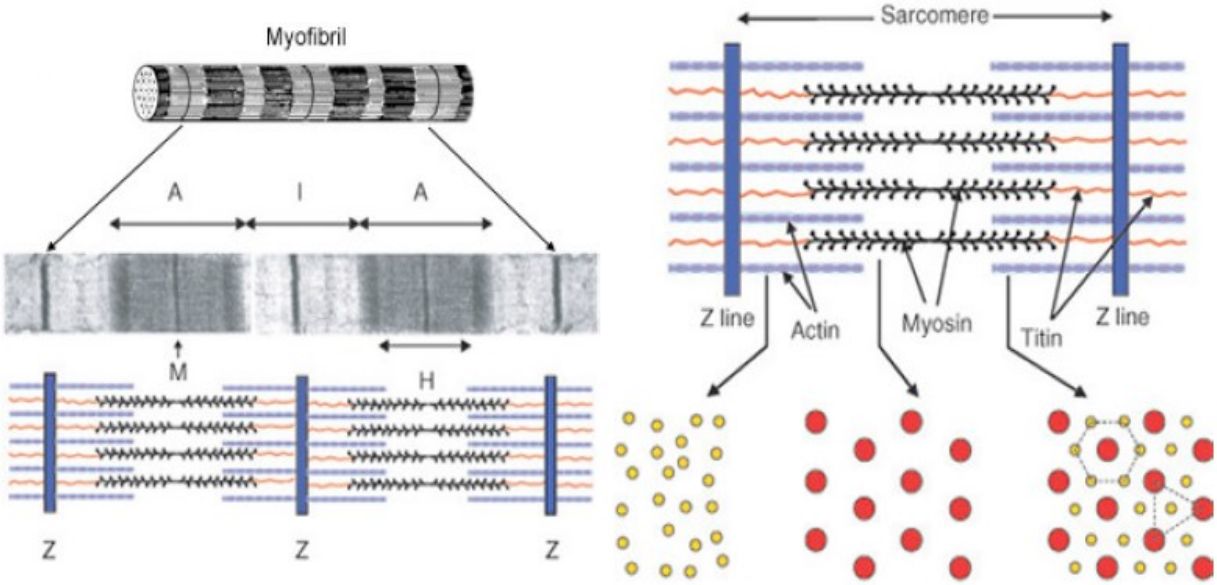

Figure 1.3. Overview of sarcomere structure including contractile and structural components, and the characteristic regions as observed under a microscope. Figure modified from (Jones et al., 2004).

zone, A-band and sarcomere in general is the M-line, which is indicative of the proteins involved in myosin filament cross-linking to form M-bridges, namely myomesin (Ehler et al., 1999) and M-proteins (Masaki & Takaiti, 1974). The isotropic band (I-band), identified as the palest region using light microscopy, is the sarcomeric region where only thin filaments are present, and these are anchored at the lateral boundary of the sarcomere, identified by the Z-disc (Clark et al., 2002). In addition, numerous accessory proteins are

involved in maintaining myofilament uniformity throughout the sarcomere. The functions of many of these sarcomeric components, protein interactions and links to the cytoskeleton are discussed in more detail below, however to comment on all currently identified sarcomeric and cytoskeletal components is beyond the scope of this review, although existing reviews are available (Clark et al., 2002; Bottinelli & Reggiani, 2000).

1.3.3 Thin filament – Actin, tropomyosin and troponin

Actin is the most abundant protein expressed in skeletal muscle cells, and is the major component of the thin filaments alongside tropomyosin and troponin, at a molar ratio of 7:1:1 respectively (Ebashi et al., 1968). Approximately, 200 G-actin monomers comprise each F-actin filament, and two F-actin filaments form a twisted α-helix that is ~1000 nm in length and ~8 nm in diameter (Hanson & Lowy, 1963). Cross-linking of actin filaments by α-actinin homodimers forms mechanical 'Z-links', and helps stabilize and maintain the parallel arrangement of the filaments. The location of actin filaments, in either a predominantly slow-oxidative or fast-glycolytic muscle generally dictates the number of Z-links formed. Typically, more Z-links are present in slow muscle types such as the soleus than in fast muscle types such as the extensor digitorum longus (Gautel, 2011). Consequently, slow muscle fibres typically possess Z-discs of approximately ~100-130 nm wide in comparison to the narrower Z-discs (~50 nm) observed in fast muscle fibres (Luther et al., 2003). An increase in the lateral mechanical strength of myofibrils has been associated with increased Z-disc width (Gautel, 2011). Therefore individual differences in the fibre type composition of heterogeneous muscles, such as the vastus lateralis (Lexell et al., 1983; Lexell et al., 1988), could contribute to the inter-individual variability in muscle

strength by enhancing or reducing transmission of contractile force to the tendon, possibly by altering the arrangement of actin filaments within the sarcomere.

Along the length of the actin filament in the groove between the F-actin filaments, end-to-end binding of individual tropomyosin molecules forms an elongated regulatory unit that is involved in modulating actin and myosin filament interaction (Lehman et al., 2000). Individual tropomyosin molecules are ~40 nm in length and span 7 G-actin monomers on each F-actin filament (Lehman et al., 2000). In human striated muscles, nine different tropomyosin isoforms are expressed and depending on which tropomyosin and actin isoforms are expressed will determine the position of tropomyosin along the actin filament (Lehman et al., 2000; Chandy et al., 1999). The continuous strand of neighbouring tropomyosin molecules along the actin filament is involved in the regulation of sarcomere shortening (and thus muscle contraction) through troponin-linked positional changes to effectively block or reveal the myosin-binding site on F-actin (Poole et al., 1995). It is suggested, however, that these positional changes differ slightly depending on the original position of tropomyosin, and may therefore contribute to the varied contractile properties of different muscle and fibre types (Clark et al., 2002).

Troponin is a complex of three sub-units that attaches to tropomyosin and actin at regular 38 nm intervals (Ohtsuki & Shiraishi, 2002) via its tropomyosin-binding site (TnT) and adenosine triphosphatase (ATPase) inhibitor (TnI) sub-units (Greaser & Gergely, 1973). A third calcium-binding (TnC) sub-unit initiates myofibril contraction in the presence of calcium by instigating conformational change within the troponin-tropomyosin complex (Greaser & Gergely, 1973). Mutations in the genes encoding the sub-units of cardiac troponin present as various types of cardiomyopathy, most notably hypertrophic

cardiomyopathy (Hofmann et al., 2001), and have also been associated with an increased risk of sudden death (Watkins et al., 1995). Despite the evident significance of these mutations within cardiac muscle, the impact of such mutations within skeletal muscle has not been extensively investigated. Nonetheless, it is clear that troponin is essential for the regulation of skeletal muscle contraction.

1.3.4 Thick filament – Myosin

Myosin II is the primary component of myofibril thick filaments, and is a two-headed molecule consisting of two pairs of light chains and two identical heavy chains. On each myosin heavy chain, the N-terminal forms the globular myosin heads, known as S1, and the C-terminal forms an α-helical tail by interweaving with the tail of the identical heavy chain to form a dimer. The staggered arrangement of hundreds of myosin II molecules ultimately forms the thick myofilament, each containing approximately 300 globular heads (Alberts et al., 2008). Furthermore, antiparallel myosin tail interactions in the H-zone of the sarcomere are the consequence of the bipolar myosin filament arrangement, such that their relative polarity is the same on adjacent sides of the M-line and allows for interaction with successive actin filaments (Luther, 2009). Within human skeletal muscle, three distinct myosin isoforms exist; myosin heavy chain-I (MHC-I), MHC-IIa and MHC-IIx, which correspond to type I, type IIa and type IIx muscle fibres, respectively (Bottinelli & Reggiani, 2000). Previous research has demonstrated type II fibres produce more force than type I fibres (Bottinelli et al., 1996; D'Antona et al., 2006; Degens & Larsson, 2007), and a difference in fibre cross-sectional area is apparent depending on the myosin heavy chain isoform present (Staron et al., 2000; Kofotolis et al., 2005). Thus, it is probable that some of the inter-individual variability in the measurements of muscle strength and/or muscle

size originates at the molecular level of skeletal muscle and can be attributed to differences in the myosin heavy chain content, and thus fibre type.

1.3.5 Structural filaments – Titin and nebulin

In addition to actin and myosin, striated muscle cells also contain the two giant proteins, titin and nebulin. Titin is estimated to have a molecular mass of ~3 MDa (Maruyama et al., 1984), is ~1 μm in length (Nave et al., 1989), and behind actin and myosin is the third most abundant protein in skeletal muscle, comprising 8-10% of skeletal muscle content (Trinick et al., 1984). Titin filaments extend from the Z-disc to the M-line, thus spanning half-sarcomere length (Maruyama et al., 1985; Wang et al., 1984). Within the I-band region of the sarcomere, titin demonstrates elastic properties thought to impact on myofibrillar stiffness (Clark et al., 2002), whereas in the A-band region of the sarcomere titin interacts closely with myosin filaments and is reported to have an organisational and structural role as a template for thick filament assembly (Granzier & Labeit, 2002). Moreover, titin interacts with myofibrillar proteins such as α-actinin at the Z-disc and further demonstrates its role in maintaining the structural integrity of the myofilament. A mutation in the Z-disc region of titin filaments has been known to reduce its affinity for α-actinin and is observed in some dilated cardiomyopathy patients, who typically experience cardiac muscle stretch and weakness (Itoh-Satoh et al., 2002). Furthermore, the presentation of a number of muscular dystrophies has been associated to mutations in the protein encoding titin (Hackman et al., 2002; Liu et al., 2008; Garvey et al., 2002). Similarly to titin, nebulin filaments anchor at the Z-disc of the sarcomere, however dissimilarly, nebulin associates with only the thin filaments in striated muscle cells (Keller III, 1995). Suggestions of nebulin as a regulator of thin filament length arose partly due to its inextensible structural nature

(Clark et al., 2002), and further evidence demonstrated that thin filament length was proportional to nebulin isoform size (Kruger et al., 1991). Therefore, whilst the primary phenotype of interest in the work of the current thesis is strength, it is possible that variability in the titin and nebulin proteins may indirectly increase the inter-individual variability observed in measurements of muscle strength by altering skeletal muscle architecture.

It is pertinent at this stage to briefly introduce the potential genetic role in influencing muscle structure and function. Many of the aforementioned proteins of the sarcomere and cytoskeleton are known to respond to mechanical stimuli, which in turn impacts on gene expression, regulation and control of gene products at a molecular level. Thus, an understanding of the pathways of force transmission is essential to investigate the genetic influences of muscle strength production.

1.3.6 Skeletal muscle contraction

Within a resting muscle, the concentration of calcium ions (Ca^{2+}) remains low and tropomyosin effectively blocks the myosin-binding site preventing the formation of cross-bridges. Initiation of skeletal muscle contraction by the release of Ca^{2+} from the sarcoplasmic reticulum increases the concentration of intracellular free Ca^{2+}, which binds to the TnC sub-unit of troponin and instigates conformational change. This change results in tropomyosin movement via action at the TnT sub-unit from its position in the centre of the actin filament to reveal the myosin-binding site, thus allowing the myosin heads to bind to the actin filament and form cross-bridges. Once attached, partial hydrolysis of ATP by

ATPase results in a change in the angle of the myosin head attachment, which then exerts a small force on the thin filaments causing them to slide past the thick filament. In the presence of sufficient ATP supply, the myosin head detaches, returns to its resting orientation and can reattach to a different actin monomer further along the thin filament. During rapid contractions, each myosin head can cycle up to five times a second, which equates to sarcomere shortening rates of up to 15 μm per second (Alberts et al., 2008). This well accepted model of muscle contraction, by which serial force transmission between adjacent sarcomeres continues along the length of the muscle to the muscle-tendon unit, is known as the sliding filament theory (Huxley, 1957). Evidence of an additional model of force transmission, however, suggests contractile force is also transmitted laterally between parallel myofibrils towards the sarcolemma and extracellular matrix (ECM) (Bloch & Gonzalez-Serratos, 2003).

1.3.7 Extracellular matrix

The ECM is a network of interstitial connective tissue located between individual myofibrils, and is composed primarily of collagens, tenascins and fibronectin. The basement membrane of the ECM comprises laminins, collagen IV and proteoglycans, and associates closely with the myofibril cell surface via regular connections called costameres; which are integral in the lateral transmission of force between superficial myofibrils, the sarcolemma and ECM (Danowski et al., 1992).

1.3.8 Costameres

Costameres were first identified as transverse, rib-like structures overlying the Z-line of myofibrils (Pardo et al., 1983), but this term also describes similar structures over the M-

line (Porter et al., 1992) and lying longitudinally over the long axis of the myofibril (Bloch & Gonzalez-Serratos, 2003). In addition to having a major role in effective lateral force transmission, costameres help maintain the structural integrity of the sarcolemma during muscle contraction/relaxation cycles. This is achieved through the concurrent actions of three cytoskeletal networks; integrin/focal adhesion complex, dystroglycan-sarcoglycan complex and spectrin-based complex (Figure 1.4) (Clark et al., 2002).

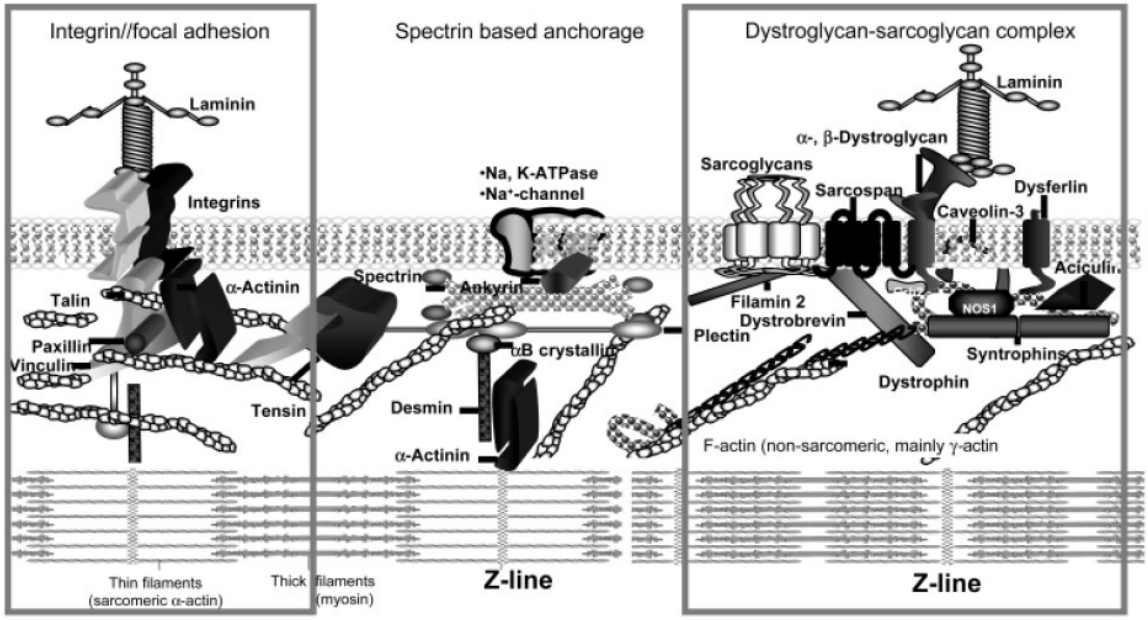

Figure 1.4. Cytoskeletal costamere networks involved in lateral force transmission to the ECM and maintaining membrane stability. Figure taken from Grounds et al. (2005).

Increased expression of some proteins located within these networks (e.g. focal adhesion kinase) has been observed in hypertrophied skeletal muscle and unsurprisingly, is associated with increased muscle force per unit cross-sectional area (Flück et al., 1999). Contrastingly, mutations in several of these costameric proteins (e.g. integrins and dystrophin) are associated with muscle weakness, and in some instances muscular dystrophy (Blake et al., 2002), thus emphasising the role of costameres in maintaining the structural integrity of the muscle and in the lateral transmission of contractile force.

1.4 *In vivo* assessment of skeletal muscle strength and its determinants

It is probable that some of the inter-individual variability observed in measurements of muscle strength and its determinants are artefacts of methodological differences, with a variety of techniques previously employed to investigate muscle anatomical cross-sectional area, muscle volume, voluntary activation capacity and tendon moment arm length. Furthermore, assessments of muscle function *in vivo* are susceptible to the inherent error associated with equipment and the measurement techniques employed.

Interest in the genetic contribution to the functional and morphological characteristics of muscle has increased considerably in the past 10 years but remains limited to predominantly measurements of strength and size. Furthermore, the majority of studies investigating the genetic associations with muscle strength and size use simple measurements such as one repetition maximum (1 RM), maximum voluntary contraction (MVC) and anatomical cross-sectional area (ACSA) instead of, for example, specific force and physiological cross-sectional area (PCSA). These simple measurements are known to underestimate the intrinsic strength of a muscle as they include both agonist and antagonist muscle efforts and do not account for factors known to affect the intrinsic strength of a muscle (Maganaris et al., 2001; Erskine et al., 2009). Collecting data using more controlled assessments of the functional and morphological characteristics of a muscle, even if in a relatively smaller population, may be beneficial in determining the genetic contribution to such factors.

A range of muscles have been the focus of investigations in human muscle function, although muscles of the appendices appear favourable, perhaps due to their accessibility and the superficial location of the muscles within them. Differences in function, loading patterns and fibre type composition between different muscles exist, (Bottinelli & Reggiani, 2000), which may impact on assessments of muscle size and strength, specifically ACSA, maximal joint torque and specific force. As previously stated, human skeletal muscle fibres can be separated into three different categories; type I, type IIa and type IIx. Of these categories, type II fibres are able to produce more force than type I fibres regardless of contraction velocity (Bottinelli et al., 1996; D'Antona et al., 2006; Degens & Larsson, 2007), and in male populations fibre cross-sectional area is greater for type IIa fibres and smallest for type IIx fibres (Staron et al., 2000; Kofotolis et al., 2005). It remains unclear, however, if these single fibre-type differences are observed in vivo or at the level of whole muscle (Miller et al., 2015; Schantz et al., 1983; Tesch & Karlsson, 1978; Harridge et al., 1998). Nonetheless, an awareness of these fibre type differences is therefore necessary when comparing data from previous studies, especially those investigating athletes or clinical patients for whom fibre type transitions due to chronic loading/unloading patterns are a likely possibility (D'Antona et al., 2006; Gallagher et al., 2005). With this in mind, studies investigating the morphological and functional characteristics of the quadriceps femoris muscle (or one or more of its constituent muscles) will be the main focus of the review from this point on, thus allowing for more appropriate comparisons between the methods used.

1.4.1 Muscle mass and architecture

Muscle size is generally accepted as being the greatest determinant of muscle strength and a significant relationship ($r \geq 0.51$, $P \leq 0.01$) exists between them (Maughan et al., 1983), although a variety of measurements to assess size have been reported in the literature (Maughan et al., 1983; Narici et al., 1988; Bamman et al., 2000; Miyatani et al., 2002; Reeves et al., 2004b; Wakahara et al., 2009). A number of early studies investigating the relationship between muscle size and strength reported a direct proportionality between maximal voluntary muscle force and muscle size by measuring ACSA (Ikai & Fukunaga, 1968; Young et al., 1984; Maughan et al., 1984). Anatomical CSA is defined as the area of muscle perpendicular to the longitudinal axis of the limb and can be measured non-invasively using imaging techniques such as magnetic resonance imaging (MRI), computed tomography (CT) and ultrasonography (Maughan et al., 1983; Narici et al., 1988; Reeves et al., 2004b). Using measurements of ACSA as a measure of contractile area in investigations of pennate muscle function is limited because differences in muscle architecture are not accounted for. Physiological CSA on the other hand, is defined as the area of muscle perpendicular to the muscle fibres and accounts for inter-individual differences in muscle architecture, more specifically fibre length and fibre pennation angle (Figure 1.5). Consequently, PCSA offers a more accurate measurement of muscle contractile area than ACSA, which represents only an estimate of true contractile area in pennate-fibred muscles (Alexander & Vernon, 1975). It remains unclear however, if the inter-individual variability in measurements of PCSA and ACSA is similar or different in magnitude.

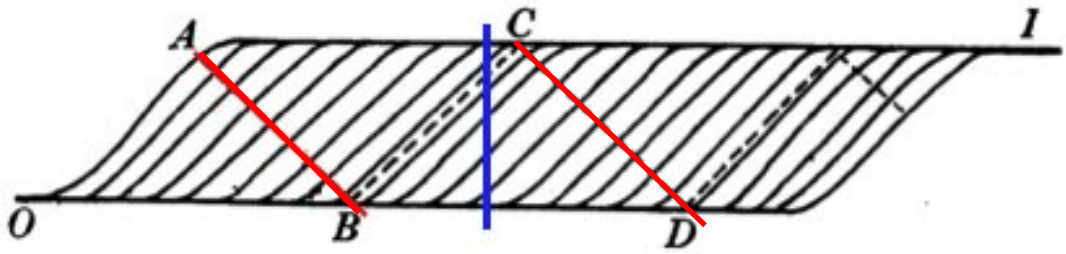

Figure 1.5. Schematic diagram adapted from Haxton (1944) demonstrating the difference between measurement of PCSA (red line) and ACSA (blue line) in pennate-fibred muscles.

Muscle fibre length is determined by the number of sarcomeres arranged in series, whereas the number of sarcomeres arranged in parallel determines muscle PCSA, and the latter can be affected by fibre pennation angle. Pennation angle refers to the angle of muscle fibre insertion into the aponeurosis and an increased angle of pennation allows for a greater proportion of muscle contractile material to attach to the tendon, subsequently increasing the force generating capacity of the muscle (Aagaard et al., 2001). Antithetically, greater pennation angles reduce the ability of the individual muscle fibres to effectively transmit force to the associated tendon, therefore decreasing maximal muscle force production (Aagaard et al., 2001). Consequently, the trade-off between these two opposing factors culminates in an increase in maximal muscle force production with an increasing pennation angle up to 45° (Alexander & Vernon, 1975). Thus, assessment of muscle architecture is pertinent to accurately estimate muscle fascicle force production from measurements of joint torque, especially when considering the changes in fibre length and pennation angle as a function of joint angle (Fukunaga et al., 1997). Production of muscle force is dependent on the amount of overlap between the muscle contractile proteins actin and myosin within the sarcomere (Gordon et al., 1966). If joint angle is manipulated to lengthen or shorten the muscle, sarcomere length will change accordingly.

In human skeletal muscles, the optimal sarcomere length for force production is ~2.5 μm, therefore in instances where sarcomere length is too long (i.e. the overlap between actin and myosin is limited), or too short (i.e. the degree of overlap exceeds the optimum length) production of force decreases (Gordon et al., 1966).

Inter-individual variability in muscle architecture and differences in optimum joint angle for force production exist within the literature, and are often the consequence of training adaptations (Aagaard et al., 2001; Reeves et al., 2004c; Reeves et al., 2004a). Reports in untrained individuals alone, however, have also demonstrated inter-individual variability in muscle architecture and optimum joint angle. In particular, several authors have demonstrated maximal torque production at the knee joint occurs between 60-90° (Houtz et al., 1957; Lindahl et al., 1969; Scudder, 1980; Marginson & Eston, 2001; Reeves et al., 2004a; Knapik et al., 1983; Erskine et al., 2009), although when focussing specifically on a population of untrained, young males, this range is reduced to between 70-90° knee flexion (Marginson & Eston, 2001; Knapik et al., 1983; Erskine et al., 2009). Consequently, this evidence emphasises the importance of identifying optimum joint angle and measuring muscle architecture when assessing maximal muscle force.

Measurement of muscle architecture *in vivo* is possible from images obtained using ultrasonography to identify individual muscle fascicular paths between the deep and superficial aponeuroses, and the angle of fascicular insertion into these aponeuroses (Henriksson-Larsen et al., 1992; Rutherford & Jones, 1992) (Figure 1.6).

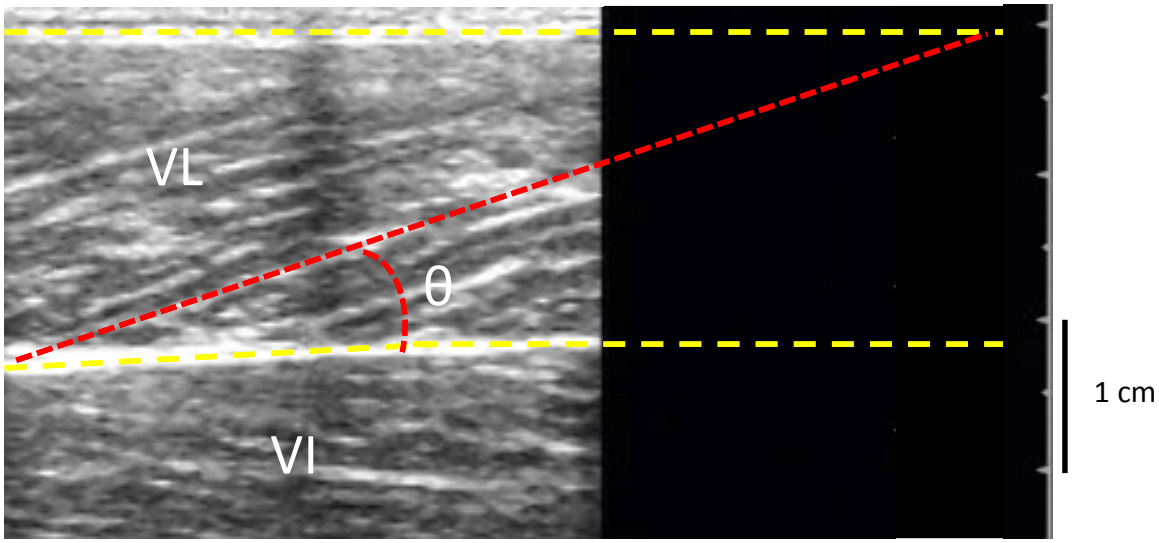

Figure 1.6. Sagittal ultrasonography scan of the right vastus lateralis (VL) demonstrating identification of muscle fascicular path (red dashed line) and pennation angle (θ) from the fascicle insertion into the deep and superficial aponeurosis (yellow dashed lines). VI = vastus intermedius. Scan was obtained from a participant of the work described in the current thesis.

1.4.2 Agonist muscle activation and antagonist muscle co-activation

To accurately assess maximum skeletal muscle force it is imperative that the agonist muscle is maximally activated, that is, all available motor units are stimulated at their optimal firing rate. Reports of voluntary activation capacity in untrained individuals are somewhat contradictory (Moritani & deVries, 1979; Häkkinen et al., 1998; Thorstensson et al., 1976; Komi & Buskirk, 1972), however sensitive use of the interpolated twitch technique suggests that untrained individuals probably cannot activate 100% of their motor units (Folland & Williams, 2007b). Failure to account for voluntary activation capacity is likely to lead to inaccurate measurements of maximum muscle force and could result in greater inter-individual variability in maximal muscle strength measurements. Additionally, during agonist muscle contractions, antagonist muscle co-activation occurs to provide stability to the joint about which the movement is occurring (Baratta et al., 1988). The level of this

antagonist muscle co-activation has also been found to vary considerably between similarly-aged, untrained individuals (Carolan & Cafarelli, 1992; De Vito et al., 2003; Macaluso et al., 2002). A consequence of not accounting for co-activation could lead to an underestimation of maximal muscle force and overestimation of inter-individual variability in the calculation of net joint torque and subsequently muscle force.

1.4.3 Tendon moment arm length

In addition to accounting for the level of agonist and antagonist muscle activation, *in vivo* assessments of muscle force should also account for variation in tendon moment arm length. During knee joint extension, the patellar tendon moment arm functions as the lever of effective force transmission to the tibia during contractions of the quadriceps femoris (Tsaopoulos et al., 2006), and can be identified by measuring the perpendicular distance from the tibio-femoral contact point to the patellar tendon line of action (Nisell et al., 1986) (Figure 1.7). Patellar tendon moment arm length can be measured *in vivo* using video fluoroscopy, sagittal MRI scans and more recently using dual energy x-ray absorptiometry (DXA) (Wretenberg et al., 1996; Kellis & Baltzopoulos, 1999; Maganaris, 2004; Erskine et al., 2014). It has been suggested that bone geometry is the primary determinant of moment arm length (Krevolin et al., 2004), and as this is known to differ inter-individually, it is probable that this is also the main source of inter-individual variability in tendon moment arm length (Maganaris et al., 2001; Tsaopoulos et al., 2006; Tsaopoulos et al., 2007a). Furthermore, as tendon moment arm length has been found to differ with changing joint angle (Baltzopoulos, 1995), it is necessary to obtain contraction-specific measurements of tendon moment arm when calculating maximal muscle force (Maganaris

et al., 2001). Therefore, imaging of patellar tendon moment arm length, for example, should occur at the knee joint angle that coincides with the production of peak MVC.

Figure 1.7. A sagittal DXA scan of the right knee joint from which patellar tendon moment arm length can be estimated as the perpendicular distance from the tibio-femoral contact point (TFCP) and the patellar tendon. Scan was obtained from a participant of the work described in the current thesis.

1.4.4 Specific force

Accounting for all of the aforementioned determinants of strength is necessary for accurate assessment of muscle force. Measurement of muscle specific force takes into account all of these determinants to estimate the intrinsic strength of the muscle. Furthermore, by accounting for the inter-individual variability in the determinants of strength, it could be expected that reported values of specific force would be homogenous. Measurements of *in vivo* human muscle specific force, however, range from 6-86 $N \cdot cm^{-2}$ (Table 1.2) (Maughan et al., 1983; Erskine et al., 2009; Reeves et al., 2004c; Narici et al., 1992; Chow et al., 1999; Gorgey et al., 2006; Claassen et al., 1989).

Table 1.2. Summary of knee extension specific force values reported in humans.

Muscle(s)	Participant Number	Age (years)	Training Status	Specific Force ($N \cdot cm^{-2}$)	Reference
Vastus lateralis Vastus intermedius Vastus medialis Rectus femoris	27 males	18 – 39	Untrained	30.3 ± 4.9 29.1 ± 4.8 (calculated using L*f* of VL to represent L*f* of whole QF)	Erskine et al. (2009)*
Knee extensors	1 female 6 male	28 ± 4		20 ± 3	Gorgey et al. (2006)
Vastus lateralis	*Training group*: 5 females and 4 males *Control group:* 5 females and 4 males	74.3 ± 3.5 67.1 ± 2		27 ± 6.3* 23.6 ± 6.1*	Reeves et al. (2004c)*
Quadriceps femoris	1 female	24		21.4 – 30.5	Chow et al. (1999)
Vastus lateralis Vastus intermedius Vastus medialis Rectus femoris	6 male	34 ± 4.7		23.7 ± 13.0 24.1 ± 16.0 27.9 ± 20.0 24.3 ± 22.0	Narici et al. (1992)*
Knee extensors	12 participants 7 male and 5 female	18 – 40	4 trained and 6 sedentary	7.6 ± 1.3	Rutherford and Jones (1992)
Knee extensors	6 males	27.8 ± 3.5		80.1 ± 15.5	Narici et al. (1988)
Knee extensors	12 participants 4 females and 8 males	20 – 38	Untrained	42.2	Wickiewicz et al. (1984)
Vastus lateralis Vastus intermedius Vastus medialis Rectus femoris	25 males	20 – 39	Recreationally active or sedentary	7.1 – 12.6	Maughan et al. (1983)
Knee extensors	25 females	20 – 36		6.6 – 11.1	McCullagh et al. (1983)

NB. * = baseline data presented; L*f*, fascicle length; VL, vastus lateralis; QF, quadriceps femoris.

Evident inconsistencies in sample populations and measurements are likely to contribute to such widespread values, and this range was reduced considerably (20-30 $N \cdot cm^{-2}$) when only those studies accounting for all of the necessary factors were considered (Erskine et al., 2009; Reeves et al., 2004c; Narici et al., 1992; Chow et al., 1999). Despite this, inter-individual variability of approximately 16% in muscle specific force has been reported within the literature, suggesting differences in the force-generating capacity of the individual fibres is contributing to the observed variability in specific force (Erskine et al., 2009). Individual variability in fibre type composition has been documented among untrained, young adults (Glenmark et al., 1992; Simoneau & Bouchard, 1989; Staron et al., 2000) and variability in fibre type composition of different muscles is evident (Harridge et al., 1996; Schantz et al., 1983). Furthermore, it is possible that the presence of intramuscular fat and connective tissue could also contribute to the reported variability in specific force (Kent-Braun et al., 2000; Evans et al., 1995; Macaluso et al., 2002; Frontera et al., 1991).

Failure to account for the presence of intra-muscular non-contractile material would result in an underestimation of muscle specific force by overestimating PCSA (Frontera et al., 1991; Erskine et al., 2009). Whereas, the consequence of increased connective tissue content could be an increase in muscle specific force through improvements in lateral force transmission via costameres to the tendon (Jones et al., 1989). Finally, a potential genetic contribution to the inter-individual variability in specific force should not be overlooked. Recently, two single nucleotide polymorphisms (SNPs) in the protein tyrosine kinase 2 gene, responsible for encoding focal adhesion kinase, were found to explain ~10% of the

inter-individual variability in the muscle specific force of untrained individuals (Erskine et al., 2012).

1.5 Genetics of skeletal muscle

In excess of 200 genetic polymorphisms have been associated with health and fitness related phenotypes within the literature over the past 50 years (Bray et al., 2009). Of these, ~30 polymorphisms have specifically been associated with a muscle-strength or power-related phenotype (Hughes et al., 2011; Garatachea & Lucía, 2013; Leońska-Duniec, 2013), but few have had a significant association replicated independently. This suggests, therefore, that more research is needed to confirm or refute these reported significant associations independently and strengthen the body of literature.

1.5.1 Identifying candidate genes

Muscle strength is a quantitative phenotype measured on a continuous scale, and thus a range of obtainable strength values is possible within a population. Traditionally, an unmeasured genotype, or top-down, approach has been used to identify the heritability of the muscle-strength phenotype. Whilst heritability estimates are useful for identifying phenotypes under strong genetic control, they are unable to provide information on the specific gene, or combination of genes that contribute to these phenotypes. Much of the recent research into muscle strength and muscle-related phenotypes, therefore, has focussed on identifying associations with a single candidate gene through case-control, cross-sectional or longitudinal association studies (MacArthur & North, 2005), collectively known as the measured genotype or bottom-up approach (Sing & Boerwinkle, 1987). When investigating the genetic contribution to muscle strength and muscle-related

phenotypes, much of the challenge is identifying the specific gene variants that contribute to the variability within the phenotype, ultimately to determine how the polymorphism(s) influences a given phenotype. For example, a polymorphism within the angiotensin I-converting enzyme (*ACE*) gene is known to influence concentrations of the associated enzyme product, whereas a functional polymorphism in the alpha-actinin-3 (*ACTN3*) gene results in an inability to produce the protein product. Furthermore, alterations to gene transcription and translation, protein function and rates of protein degradation are other possible outcomes associated with genetic polymorphisms.

Identification of candidate genes is possible by first establishing a complete theoretical understanding of a particular protein and how it may contribute towards the phenotype of interest. Subsequent screening of the gene encoding for the protein may reveal polymorphisms that, if functionally significant, could be used to determine an association with the phenotype. Despite this approach working successfully in the past, demonstrated by the association between a polymorphism in the *ACE* gene and cardiac hypertrophy (Montgomery et al., 1997; Montgomery et al., 1998), recent estimates suggests there are ~19,000 genes in the human genome (Ezkurdia et al., 2014) meaning this process can be time-consuming and expensive.

Genome wide association studies (GWAS) are an increasingly popular alternative approach to identifying candidate genes now possible as a result of technological advances and decreasing costs, albeit these remain high in contrast to candidate gene approaches. GWAS identify genomic loci associated with a phenotype in a large population of individuals by completing a comprehensive scan of the genome to simultaneously genotype as many

as 4 million gene variants. Often the variants identified within adjacent loci are correlated, and are said to be in linkage disequilibrium (Wall & Pritchard, 2003). Linkage disequilibrium refers to the non-random association of alleles from neighbouring loci, occurring as a consequence of allele crossover in haplotype blocks during meiotic recombination (Daly et al., 2001). It is therefore possible for two neighbouring loci in linkage disequilibrium to both demonstrate significant genotype-phenotype associations, but for only one of these loci to have functional relevance for the phenotype. Due to the simultaneous analysis of thousands or millions of gene variants for associations with one or more phenotypes, the risk of identifying false positives is high (Cantor et al., 2010). Consequently, current practice in GWAS is to adopt a significance threshold of $P \leq 5 \times 10^{-8}$ (Panagiotou & Ioannidis, 2012), although some earlier GWAS used a lower threshold of $P \leq 5 \times 10^{-7}$ (Panagiotou & Ioannidis, 2012). Thus the sample size required for such studies increases substantially, an increase that would likely be unfeasible in studies performing detailed assessments of multiple phenotypes such as those of the subsequent chapters. The work presented in this thesis investigated the genetic contribution to the inter-individual variability in human skeletal muscle strength and its determinants. As completion of GWAS or sequence analyses was not possible, a candidate gene approach was taken. Ideally, genotyping of as many candidate genes as possible would occur, however due to the logistics surrounding time, expense and equipment available this was not feasible. Consequently, seven individual candidate genes were identified (Table 1.3), from an initial group of 11 candidate genes (inclusive of insulin-like growth factor, interleukin-6, vitamin D receptor and myostatin), for investigations relating to the aforementioned skeletal muscle phenotypes.

Table 1.3. Candidate genes, candidate proteins and their respective abbreviations.

Candidate Protein	Candidate Protein Abbreviation	Candidate Gene(s)	Candidate Gene Abbreviation
Angiotensin I-converting enzyme	ACE	Angiotensin I-converting enzyme	*ACE*
Alpha-actinin-3	ACTN3	Alpha-actinin-3	*ACTN3*
Ciliary neurotrophic factor	CNTF	Ciliary neurotrophic factor	*CNTF*
Type V collagen	Col V	Collagen type V alpha 1	*COL5A1*
Focal adhesion kinase	FAK	Protein tyrosine kinase 2	*PTK2 intron* *PTK2 UTR*
Titin	TTN	Titin	*TTN*
Thyrotropin-releasing hormone receptor	TRHR	Thyrotropin-releasing hormone receptor	*TRHR*

The selection of these seven specific candidate gene polymorphisms was based on 1) the greater existing frequency of associations with skeletal muscle phenotypes for some polymorphisms, particularly *ACE* and *ACTN3*; 2) existing gene transcriptional analyses demonstrating the likelihood of polymorphisms being functional (*ACE*, *COL5A1*, *CNTF*, *PTK2*); 3) the necessity for independent replications with particular skeletal muscle phenotypes (*PTK2* and *TRHR*); and 4) the potential for novel genotype associations with skeletal muscle phenotypes (particularly *COL5A1*, *CNTF* and *TTN*). Specific reviews of the selected candidate genes are detailed in the subsequent section of this review.

1.6 Candidate genes of interest

1.6.1 Angiotensin I-converting enzyme

In relation to human physical performance phenotypes, the *ACE* gene has been studied more extensively than any other. ACE is a zinc metallopeptidase, and is recognised as being integral to the renin-angiotensin system (RAS) (Rigat et al., 1990). Production of

angiotensin II (Ang II), thought to enhance skeletal muscle growth, is catalysed by ACE (Gordon et al., 2001) and the actions of Ang II are predominantly mediated by the angiotensin type-1 (AT_1R) and type-2 (AT_2R) receptors (Payne & Montgomery, 2003). ACE is also involved in the degradation of bradykinin, a potent vasodilator due to its actions on the bradykinin type-1 (BK_1R) and type-2 (BK_2R) receptors (Dendorfer et al., 2001; Regoli et al., 1998). Thus, ACE activity has a key role in the regulation of blood pressure in humans (Kem & Brown, 1990). In addition to endocrine RAS, local RAS are known to exist in skeletal muscle (Dragović et al., 1996), cardiac muscle (Dzau, 1988) and adipose tissue (Jonsson et al., 1994), which are involved in the regulation of tissue growth (Figure 1.8) (Ishigai et al., 1997; Nazarov et al., 2001; Dzau, 1988).

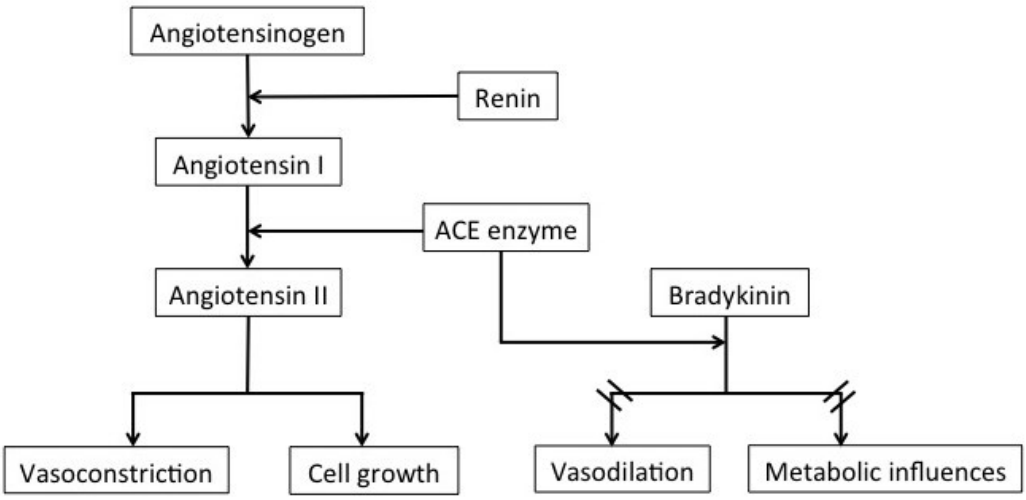

Figure 1.8. Angiotensin I-converting enzyme (ACE) activity within the renin-angiotensin system. Increased ACE production elevates angiotensin II-dependent vasoconstriction and enhances cell growth but decreases bradykinin-dependent vasodilation and metabolic influences. Figure modified from Roth (2007).

A functional polymorphism of *ACE* has been identified within intron 16 of the gene on chromosome 17 (Rigat et al., 1990). The absence (deletion, D) rather than presence (insertion, I) of a 287 amino acid base pair Alu repeat sequence is associated with increased

concentrations of tissue and serum ACE activity (Rigat et al., 1990; Danser et al., 1995). Accordingly, this polymorphism results in the II, ID and DD genotypes with the respective frequencies among Caucasian adults approximately 25%, 50% and 25% (Myerson et al., 1999). In humans, *ACE* genotype has been associated with cardiac and skeletal muscle hypertrophy in response to exercise training (Montgomery et al., 1997; Folland et al., 2000). The D-allele in particular has been repeatedly associated with increased left ventricular mass following training, with this being observed in Caucasian military recruits (Montgomery et al., 1997), endurance athletes (Di Mauro et al., 2010) and elite footballers (Fatini et al., 2000). Similarly, in skeletal muscle, D-allele carriers have been associated with greater increases in isometric and dynamic quadriceps muscle strength following 9-week of training (Folland et al., 2000), and in strength and power oriented athletes there exist an abundance of studies reporting an overrepresentation of the D-allele and/or underrepresentation of the I-allele (Nazarov et al., 2001; Woods et al., 2001; Costa et al., 2009; Tsianos et al., 2004). When considering untrained muscle strength, the D-allele has also been associated with baseline isometric quadriceps muscle strength in patients with chronic obstructive pulmonary disorder (Hopkinson et al., 2004). In age-matched healthy controls, however, Hopkinson et al. (2004) observed no association between *ACE* genotype and quadriceps muscle strength. Contrastingly, Williams et al. (2005) identified a significant linear trend between *ACE* genotype and baseline isometric quadriceps strength, although there have since been numerous reports on healthy, untrained individuals reporting no association between *ACE* genotype and muscle strength (Thomis et al., 2004; Erskine et al., 2013; McCauley et al., 2008), which might indicate untrained muscle strength is independent of *ACE* genotype.

Considering Ang II (production of which may be elevated with the presence of the D-allele) is a potent growth factor, stimulates the release of systemic growth hormone and is known to alter steroid metabolism (Messerli et al., 1977), a link between muscle size and/or mass and *ACE* genotype may be expected. To date, however, few studies have investigated this potential association and inconsistencies in the assessment of muscle size or mass, and the muscle of interest are evident between these studies (Frederiksen et al., 2003; Thomis et al., 2004; Erskine et al., 2013). Nonetheless, no association between *ACE* genotype and muscle size and mass has been identified, although independent replications of these initial reports are warranted.

1.6.2 Alpha-actinin-3

Alpha-actinins constitute a family of actin-binding proteins necessary to anchor actin filaments to the sarcomeric Z-line to stabilise the muscle contractile components (MacArthur & North, 2004; Blanchard et al., 1989). Alpha-actinin-3 (*ACTN3*) is the gene that codes for the myofibrillar α-actinin protein expressed only in type II muscle fibres (Beggs et al., 1992). A functional polymorphism of *ACTN3* has been located in humans at position 1,747 in exon 16 where a C > T transition results in the conversion of an arginine (R) to a premature stop codon (X) at amino acid 577 (R577X) (North & Beggs, 1996). Therefore, RR homozygotes have the fully functioning gene variant, whereas individuals homozygous for the 577X allele are unable to produce the ACTN3 protein in their muscle (Clarkson et al., 2005; Mills et al., 2001). The frequency of the 577X allele has been reported to be 42% in a Caucasian population, 52% in Asian Americans and 16% in Africans (Mills et al., 2001). Furthermore, approximately 18% of Europeans are estimated to be homozygous for the 577X allele (Yang et al., 2003).

Similar to *ACE*, extensive reports of *ACTN3* genotype and associations with muscle strength/power and endurance in athletic populations exist. In such populations, the R-allele appears to be overrepresented and the X-allele underrepresented in strength and/or power oriented athletes (Yang et al., 2003; Druzhevskaya et al., 2008; Roth et al., 2008), whereas the X-allele appears to be overrepresented and R-allele underrepresented in endurance athletes, although this latter observation remains contentious (Ahmetov et al., 2010; Niemi & Majamaa, 2005). These observations may be explained to some extent by genotype-dependent differences in fibre type composition; with RR homozygotes demonstrating significantly more type IIx fibres than XX homozygotes (Vincent et al., 2007). Therefore, as type II fibres are known to produce greater force than type I fibres, RR homozygotes may be expected to produce greater muscle force, and consequently may be more suited to competing in strength and/or power oriented sports. Additionally, as type I fibres contain greater concentrations of mitochondria than type II fibres and respire aerobically, they are more resistant to fatigue and XX homozygotes may therefore be advantaged when completing endurance exercise.

In non-athlete populations, reports of associations between *ACTN3* genotype and skeletal muscle-related phenotypes are less conclusive, thus it is unclear if *ACTN3* influences untrained muscles in the same way as is observed in athletes. In support of a comparable influence of *ACTN3* genotype on skeletal muscle phenotypes in untrained populations, as is observed in athletes, Moran et al. (2007) reported improved 40 m sprint time in R-allele carrying adolescent males compared to XX homozygotes. However, no associations between *ACTN3* and a number of other skeletal muscle phenotypes were observed, nor

were any genotype-phenotype associations observed in female adolescents. Contrastingly, isometric elbow flexion MVC and 1 RM (Clarkson et al., 2005), and concentric and eccentric knee extension peak torque (Walsh et al., 2008) were lower in females homozygous for the X-allele compared to R-allele carriers, whereas no associations were observed in males. Thus these initial reports, although inconsistent, may suggest the influence of *ACTN3* on skeletal muscle differs according to age and sex. However, there exist a number of studies that have found no association between *ACTN3* genotype and skeletal muscle strength or power phenotypes in young males (McCauley et al., 2009), young females (Gavin & Williams, 2010) or young adults (Santiago et al., 2010), which may suggest muscle strength and/or power is independent of *ACTN3* genotype in young non-athletes. Furthermore, in contrast with the aforementioned hypothesis that the R-allele is beneficial for strength and/or power and the X-allele perhaps more suited for endurance performance, Delmonico et al. (2007) reported the XX genotype was associated with increased muscle peak power in older females but not older males. Consequently, these often contrasting reports demonstrate the lack of parity within the existing literature and highlights the requirement for further research to be undertaken.

1.6.3 Ciliary neurotrophic factor

Ciliary neurotrophic factor (CNTF) is a cytokine belonging to the interleukin-6 family and has a molecular mass of approximately 22 kDa (Sleeman et al., 2000). The binding of CNTF to its receptor activates signal transduction pathways involved in cell survival, proliferation, differentiation, activation and death (Sleeman et al., 2000; Vergara & Ramirez, 2004). CNTF is pleiotropic, meaning it can elicit different responses in different tissues, and although it was originally identified as a factor supporting the survival of parasympathetic neurons of

the chick ciliary ganglion *in vitro* (Adler et al., 1979), subsequent reports have demonstrated CNTF also influences sympathetic neurons, sensory neurons, motor neurons and skeletal muscle in addition to other tissues (Sleeman et al., 2000).

The *CNTF* gene is localised to chromosome 11q.12 and contains a single 1 kB intron within the coding domain (Lam et al., 1991). A G-6A transition identified in intron 1 of the *CNTF* gene results in three *CNTF* genotypes; AA, GA, GG with corresponding frequencies of 2.2%, 21.3% and 76.5% in a Caucasian population (Roth et al., 2001). The rare AA genotype results in the production of a non-functional protein (Takahashi et al., 1994), and as lower levels of CNTF have been associated with lower muscle strength in rats (Guillet et al., 1999), Roth et al. (2001) hypothesized that individuals homozygous or heterozygous for the rare A-allele would produce lower muscle strength than GG homozygotes. Despite AA homozygotes exhibiting the lowest values of knee extension and flexion concentric torque, it was the heterozygous individuals who demonstrated the greater concentric torque values in their investigation (Roth et al., 2001). In agreement with these initial findings, a subsequent investigation of bilateral handgrip strength in older females (aged 70-79 years) demonstrated those females homozygous for the rs1800169 A-allele produced, on average, 3.8 kg less handgrip strength than G-allele carriers (Arking et al., 2006). Furthermore, De Mars et al. (2007) reported middle-aged females homozygous for the rare A-allele produced the lowest knee flexion concentric torque (at $180^{\circ} \cdot sec^{-1}$) in comparison to G-allele carriers, thus providing further evidence to suggest the *CNTF* rs180169 A-allele is associated with lower muscle strength. However, De Mars et al. (2007) did not find any associations between *CNTF* genotype and muscle strength in their male participants, which may suggest this association is sex specific. Additionally, in response to resistance training

of the elbow flexors, it appears that females carrying the A-allele exhibit significantly lower gains in muscle strength compared to females homozygous for the G-allele, though again no significant associations were observed in males (Walsh et al., 2009).

Despite the *CNTF* rs1800169 AA genotype appearing unfavourable for muscle strength production, it remains unclear which genotype (GG or GA) is preferential for muscle strength production, and if the associations observed to date are sex-specific or not (Roth et al., 2001; De Mars et al., 2007; Arking et al., 2006; Walsh et al., 2009). Furthermore, evident in the methodologies of the aforementioned investigations is the repeated use of gross measurements of muscle strength, such as 1 RM and MVC (De Mars et al., 2007; Arking et al., 2006; Roth et al., 2001; Walsh et al., 2009). When considering the inter-individual variability present within these measurements, the use of them in these investigations might demonstrate a lack of sensitivity by which genotype-phenotype associations in muscle strength can be detected, and may explain the inconsistent associations between muscle strength and *CNTF* G-allele carriers, and between *CNTF* genotype and male participants. Thus, there is a requirement for more research to investigate this polymorphism for associations with muscle strength, and its determinants, by using more sensitive assessments of the phenotype, such as muscle specific force.

1.6.4 Collagen type V alpha 1

Collagen is the primary structural protein of the ECM, and although types III and I are predominantly expressed, collagen type V is known to associate with both types (Gillies & Lieber, 2011). Together, collagen type V and I fibrils co-polymerise to form heterotypic fibres, an interaction which is reported as a regulatory mechanism to control fibril structure

and diameter (Birk et al., 1990). The major collagen type V isoform comprises two α1 (V) chains, encoded by the *COL5A1* gene and one α2 (V) chain encoded by the *COL5A2* gene (Wenstrup et al., 2004; Malfait et al., 2010). The *COL5A1* gene is located in region 9q.34.3 and comprises 66 exons, whereas *COL5A2* is located in region 2q.32.2 and comprises 54 exons (Birney et al., 2004).

Mutations within *COL5A1* have been identified in patients of Ehlers-Danlos syndrome (EDS), a disease characterised by joint hypermobility, joint laxity and muscle hypotonia (Beighton et al., 1998). Haploinsufficiency is reportedly common among EDS patients (Wenstrup et al., 2006; Malfait et al., 2010), resulting in irregular collagen fibrils of a larger size located within connective tissue (Vogel et al., 1979), which is attributed to a reduced synthesis of collagen type V (Malfait & De Paepe, 2005). In addition to the association between *COL5A1* and this rare skeletal muscle disease, the common *COL5A1* rs12722 polymorphism has also been previously associated with flexibility (Collins et al., 2009), anterior cruciate ligament injury in females (Posthumus et al., 2009) and Achilles tendinopathy (Mokone et al., 2006; September et al., 2009), but not patellar tendon properties (Foster et al., 2014).

Differences in *COL5A1* mRNA stability, associated with increased α1 (V) chain protein production, have been reported between *COL5A1* genotypes (Laguette et al., 2011). More specifically, the rs12722 T-allele was associated with increased mRNA stability and by implication, increased collagen type V production in a Caucasian population (Laguette et al., 2011; Abrahams et al., 2013). Furthermore, considering that collagen type V is ubiquitous in human tissues and that both collagens type III and I associate with collagen

type V, differences in collagen type V production as a consequence of the rs12722 polymorphism might have implications for skeletal muscle morphological and/or functional phenotypes. For instance, as the ECM plays a major role in the transmission of force during muscle contraction, differences in collagen type V production may impact on the structure and/or diameter of heterotypic collagen fibrils, thus enhancing or impairing force transmission to the skeleton. In addition, several animal models have observed a greater collagen content, most likely due to larger collagen fibrils, in muscles with a greater composition of slow twitch muscle fibres in comparison to those with less slow twitch fibres (Nakamura et al., 2003), although causality between collagen content and fibre type composition has not been confirmed. Additionally, it is not clear if the findings from these earlier animal models can be extrapolated to humans, greater muscle strength production might be expected from those individuals producing less collagen type V, as this might indicate a greater quantity of the more powerful fast twitch fibres (Nakamura et al., 2003) within the muscle and warrants further research. Furthermore, increased collagen type V production as a consequence of *COL5A1* rs12722 could influence fibril diameter and structure within the connective tissue of skeletal muscle. Thus, differences in the arrangement of the muscle connective tissue may influence the normally precise parallel arrangement of muscle fibres they surround. Subsequently, this may affect the muscle architecture of individuals with altered collagen type V production, such that genotype differences in pennation angle and/or fascicle length might be observed and should be investigated further.

1.6.5 Protein tyrosine kinase 2

Focal adhesion kinase (FAK) is an integrin-associated protein tyrosine kinase localised at focal adhesion complexes via interactions between its focal adhesion targeting domain and other integrin-associated proteins (Hildebrand et al., 1993; Schaller, 2001). Focal adhesion complexes are important components of cell costameres, which in skeletal muscle provide an integral link between the ECM, cytoskeleton and muscle fibres (Flück et al., 1999; Patel & Lieber, 1997) and have a major role in the effective lateral transmission of force as well as in the maintenance of sarcomeric structural integrity during muscle contraction. Autophosphorylation of FAK is critical in the initiation of intracellular integrin signalling pathways (Wolfson et al., 2009), involving both mitogen-activated protein kinase signalling and cytoskeletal remodelling (Mitra et al., 2005). Ultimately, this intracellular integrin-signalling cascade is responsible for the regulation of such processes as cell growth, migration and differentiation (Quach & Rando, 2006). Furthermore FAK plays an integral role in costamere formation and turnover, and it has been suggested that the observed increases in muscle force per unit CSA (Flück et al., 1999) following skeletal muscle hypertrophy, may be the consequence of improved lateral force transmission due to enhanced costamere density (Erskine et al., 2012).

FAK is encoded by protein tyrosine kinase 2 (*PTK2*), and two polymorphisms within this gene have been investigated for associations with human phenotypes (Garatachea et al., 2014; Erskine et al., 2012). Most recently the rs7843014 and rs7460 polymorphisms were investigated for associations with exceptional longevity in several European cohorts and a Japanese cohort (Garatachea et al., 2014). Garatachea et al. (2014) reported a possible association between the rs7843014 CC and rs7460 TT genotypes, which are in linkage disequilibrium (Erskine et al., 2012), and lower gene expression as potentially increasing

the likelihood of reaching exceptional longevity in a Spanish population only. The first study involving these two polymorphisms demonstrated a significant association between the rs7843014 AA and rs7460 TT genotypes and higher baseline specific force production, which was suggested to be the consequence of improved lateral force transmission due to an increased costamere density in the muscles of rs7843014 AA and rs7460 TT homozygotes compared to C-allele and A-allele carriers respectively (Erskine et al., 2012). This initial investigation, however, was only a preliminary report on a relatively small population (n = 51), therefore independent replications are required to confirm these observations. Furthermore, when taken together, the results from Garatachea et al. (2014) and Erskine et al. (2012) indicate a potential complex relationship between *PTK2* gene expression and subsequent influence on FAK and thus costamere formation and turnover that also warrants further investigation to elucidate.

1.6.6 Titin

Titin (*TTN*) encodes the largest described protein to date, which behind actin and myosin is the third most abundant protein within the myofilament of human cardiac and skeletal muscle (Vikhlyantsev & Podlubnaya, 2012; Fürst et al., 1988). Within the sarcomere, TTN is important for both myocyte development and function (Rankinen et al., 2003). Due to its size, TTN provides a molecular blueprint for the assembly and organisation of the thin and thick filaments during myofibrillogenesis (Chauveau et al., 2014; Gregorio et al., 1999), in addition to providing an attachment site for several myofibrillar proteins (Figure 1.8) (Trinick, 1994). During contraction, TTN functions to maintain the structural integrity of the sarcomere (Freiburg & Gautel, 1996), and is also involved in intracellular signalling (Mayans et al., 1998). The TTN protein is characterised by four regions; Z-disc, I-band, A-

band and M-line (Figure 1.8). The I-band region is highly elastic and plays an important role in the development of muscle passive tension, primarily due to the PEVK region [named according to its composition of proline (P), glutamate (G), valine (V) and lysine (K)] and immunoglobulin (Ig)-like domain (Herzog et al., 2012).

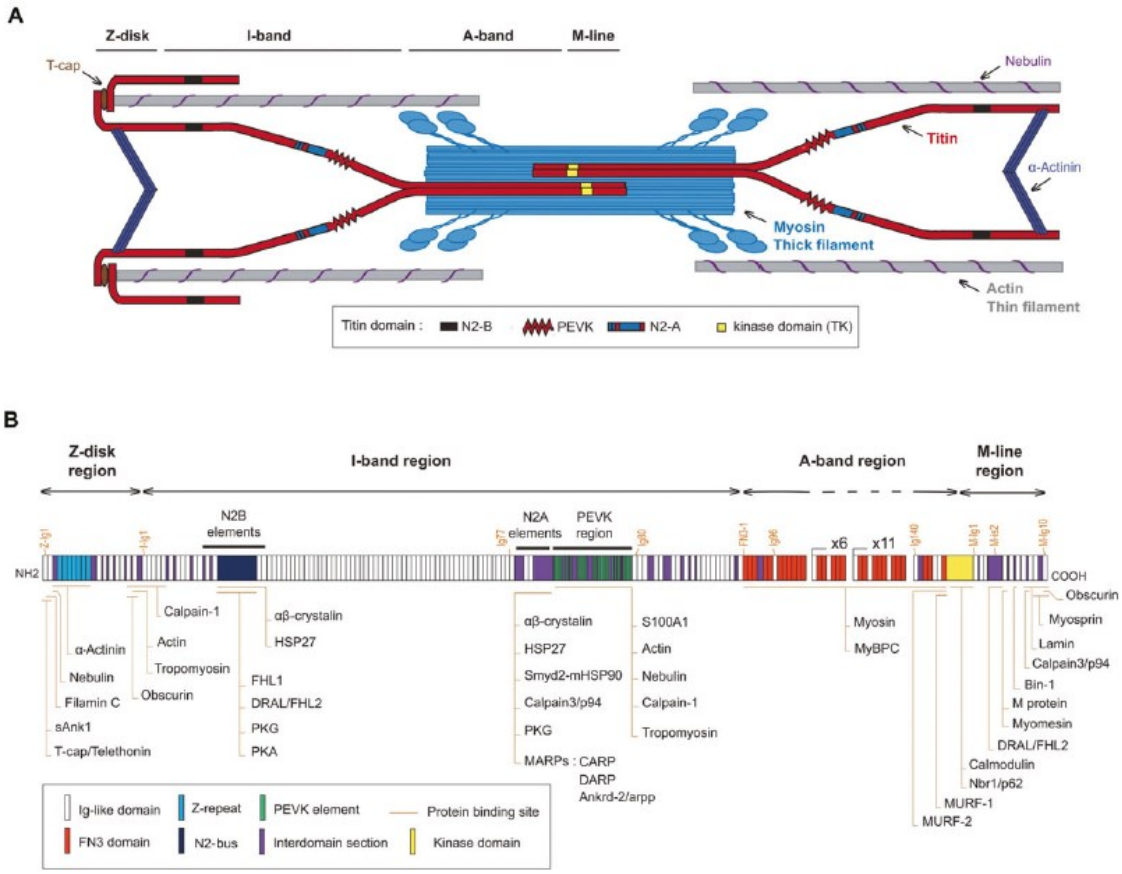

Figure 1.9. (A) Schematic diagram of TTN structure and associated myofibrillar proteins; **(B)** schematic diagram of a TTN isoform demonstrating the four distinct regions of the protein. Figure taken from Chauveau et al. (2014).

Seven splice isoform variants of TTN exist within human striated muscle, and three of these isoforms are known to differ according to the length of their PEVK region and Ig-like domains (Chauveau et al., 2014). Within human skeletal muscle, the predominant TTN

isoform is N2A, although both the novex-2 and novex-3 isoforms are also expressed (Bang et al., 2001; Vikhlyantsev & Podlubnaya, 2012; Freiburg et al., 2000).

To date several skeletal muscle diseases have been associated with mutations in *TTN*, with 39 known mutations reported to contribute to four known purely skeletal muscle diseases, and a further nine mutations associated with combined skeletal and cardiac muscle diseases (Chauveau et al., 2014). A commonality evident across all of these known skeletal muscle diseases is muscle atrophy and/or muscle weakness (Udd, 2012; Edström et al., 1990; Romero, 2010). Although much of the research surrounding *TTN* has tended to focus upon mutations associated with skeletal muscle diseases, other non-pathological polymorphisms have been identified (Herman et al., 2012), and one in particular has demonstrated an association with an exercise-related phenotype in healthy, untrained Caucasians (Timmons et al., 2010; Rankinen et al., 2003).

A missense C > T transition identified within *TTN* has been reported to contribute to the variability in the training response of maximal oxygen consumption (Timmons et al., 2010) and stroke volume (Rankinen et al., 2003). Within cardiac muscle, TTN is suggested to be a key regulator of the Frank-Starling mechanism (Fukuda et al., 2001), and considering substantial differences in the elasticity of cardiac TTN isoforms, this C > T transition, an exon splicing enhancer (ESE), is likely to contribute to the variability within TTN isoform expression. In turn, differences in the TTN isoforms expressed may explain the training-related increases in stroke volume (Rankinen et al., 2003) and consequently the maximal oxygen consumption (Timmons et al., 2010). Furthermore, if this *TTN* polymorphism influences TTN isoform expression as suggested, there exists a distinct possibility that a

similar influence is occurring within skeletal muscle tissues. To the author's knowledge there exist no investigations on potential associations between the *TTN* rs10497520 polymorphism and human skeletal muscle functional or morphological phenotypes. Differences in TTN isoform expression within skeletal muscle, such as increased expression of the larger N2A isoform and decreased expression of the smaller novex-2 and -3 isoforms, or vice versa, may have implications for muscle architecture and/or strength phenotypes. For instance, a recent investigation on TTN isoform size in rat skeletal muscle demonstrated an association between isoform size and sarcomere length, which could impact on muscle fascicle length (Greaser & Pleitner, 2014). Subsequently, if differences in skeletal muscle fascicle length are apparent, then variability in the length-tension relationship of muscle contraction might also be observed. Accordingly, individuals with longer fascicles would in theory experience a rightward shift in their length-tension relationship, which would result in a concurrent change in the optimal joint angle for maximal torque production in the direction of full flexion. Therefore, *TTN* is a suitable candidate gene for investigations between the aforementioned C > T transition and skeletal muscle properties in humans.

1.6.7 Thyrotropin-releasing hormone receptor

Belonging to the G protein-coupled receptor-1 family, thyrotropin-releasing hormone receptor (TRHR) is integral in the activation of the phospholipid-calcium-protein kinase C transduction pathway (Liu et al., 2009; Matre et al., 1999). This complex signalling cascade is primarily initiated by the binding of thyrotropin-releasing hormone (TRH) to its receptor, both of which are found throughout the central and peripheral nervous systems and extraneural tissues (Sharif, 1989; Satoh et al., 1993). Ultimately, this transduction pathway is responsible for controlling the synthesis and secretion of thyroid stimulating hormone

(TSH) and prolactin from the anterior pituitary gland (Matre et al., 1999). In turn, the secretion of TSH results in the release of thyroxin (T_4), a hormone that has an important role in skeletal muscle development (Norenberg et al., 1996; Larsson et al., 1994). *TRHR*, located in the region 8q.23.1, is the gene encoding TRHR and mutations in this gene have been reported to alter the binding capacity of TRH to TRHR, and can result in central hypothyroidism (Collu et al., 1997).

A link between central hypothyroidism and MHC isoform expression is well established, with several authors identifying a significant increase in MHC-I transcription following induction of hypothyroidism compared with euthyroidism (Norenberg et al., 1996; Montgomery, 1992; Vadaszova et al., 2006). Additionally, concomitant reductions in the CSA of both type I and type II fibres, attributed to a disproportionate increase in protein degradation (Brown & Millward, 1983) following the induction of hypothyroidism, have also been observed (Norenberg et al., 1996). Consequently, the generation of absolute tension is significantly lower in the soleus muscle fibres of hypothyroid rats than euthyroid rats (Norenberg et al., 1996). Interestingly, however, due to simultaneous reductions in cell size and myofibrillar protein content, no differences in the specific force generating capacity of these fibres were observed (Norenberg et al., 1996).

Although many of these aforementioned observations were from hypothyroid rats, a more recent investigation has identified two single nucleotide polymorphisms within *TRHR* as important for lean body mass in humans (Liu et al., 2009). These two SNP were identified following an initial GWAS and are in strong linkage disequilibrium (r^2 = 0.98). Lean body mass of individuals homozygous for the rs16892496 G-allele was, on average, 2.7 kg higher

than T-allele homozygotes; similarly, the lean body mass of individuals homozygous for the rs7832552 T-allele was, on average, 2.5 kg higher than heterozygotes and C-allele homozygotes (Liu et al., 2009). A subsequent investigation was able to replicate the association between *TRHR* and lean body mass for the rs16892496 polymorphism but not the rs7832552 polymorphism (Lunardi et al., 2013). Furthermore, as total body lean mass correlates with muscle strength (r = 0.365) (Bamman et al., 2000), Lunardi et al. (2013) also investigated a potential association between *TRHR* and muscle strength. The authors demonstrated a tendency for *TRHR* rs16892496 genotype to differ according to isokinetic strength, such that those individuals with significantly lower appendicular lean mass were more likely to produce lower values of peak torque, however, as this is the only study to date to investigate this genetic polymorphism for associations with a muscle strength phenotype, independent replications are warranted. In addition, differences in total body or appendicular lean mass might reflect differences in individual muscle size, thus potential associations between *TRHR* genotype and various indices of muscle size might also exist and should be investigated further.

1.7 Aims and objectives

Consequently, the overall aim of the current thesis was to investigate some of the genetic contribution to the inter-individual variability within skeletal muscle strength and some of its determinants. More specifically, the objectives were:

1. To investigate the extent of inter-individual variability within skeletal muscle strength and some of its determinants in healthy, untrained males.

2. To determine whether eight gene polymorphisms in seven separate genes (*ACE*, *ACTN3*, *CNTF*, *COL5A1*, *PTK2*, *TRHR* and *TTN*) are associated with skeletal muscle strength and/or some of its determinants in healthy, untrained males.
3. To investigate whether polygenic profiles comprising these eight gene polymorphisms are associated with skeletal muscle strength and/or some of its determinants in healthy, untrained males.

1.8 Overview of thesis

Chapters 2 and 3 of this thesis are both concerned with the research methodology and design adopted to investigate the skeletal muscle phenotypes necessary for Chapters 4, 5, 6 and 7. More specifically, Chapter 2 describes the measurement, calculation or estimation of each of the skeletal muscle phenotypes under investigation in subsequent chapters, in addition to outlining the procedures necessary for DNA collection, extraction and genotyping. In Chapter 3 the reliability of the measurements necessary for the calculation of muscle specific force was investigated to confirm that these measurements would be appropriate for use in subsequent chapters.

The main aim of Chapter 4 was to investigate the inter-individual variability within the measurements of muscle strength and its determinants. It was hypothesized that the inter-individual variability within the more stringent measurements of muscle strength and size, such as muscle specific force and PCSA would be less than that identified within the less stringent measurements, such as maximal voluntary contraction torque and ACSA.

Chapters 5, 6 and 7 all followed a similar research design, in which associations between polymorphisms in the candidate genes identified in the current chapter and skeletal muscle functional and morphological phenotypes were investigated. More specifically, in Chapter 5 associations between polymorphisms in five of these candidate genes (*ACTN3*, *CNTF*, *COL5A1*, *PTK2* and *TTN*) and vastus lateralis muscle fascicle length and pennation angle were investigated. In Chapter 6, polymorphisms in *ACE, ACTN3*, *CNTF*, *PTK2* and *TRHR* were investigated for associations with several skeletal muscle size phenotypes (vastus lateralis ASCA, PCSA, volume, thickness, total body lean mass and measures of appendicular lean mass); and in Chapter 7, polymorphisms in all seven candidate genes (*ACE, ACTN3*, *CNTF*, *COL5A1*, *PTK2*, *TRHR* and *TTN*) were investigated for associations with maximal voluntary knee extension isometric torque, maximal voluntary knee extension net torque and vastus lateralis specific force. It was hypothesized that associations would exist between the genetic polymorphisms and skeletal muscle phenotypes.

Subsequently in Chapter 8, separate polygenic profiles were completed for the size and strength phenotypes investigated in Chapters 6 and 7 respectively. These polygenic profiles and their associations with the respective muscle size and strength phenotypes were assessed. It was expected that by involving all of the genetic polymorphisms investigated in the preceding chapters in a polygenic profile, a greater proportion of the observed inter-individual variability described in these phenotypes in Chapter 4, would be accounted for than could be achieved when investigating associations with single candidate polymorphisms.

Finally, Chapter 9 took a retrospective view of the results observed within each chapter and attempts to combine these to, in particular, identify how each of the candidate genes under investigation influences both skeletal muscle function and morphology. In combining the findings of the preceding chapters, this chapter also considers the implications of these for future research in addition to outlining possible future directions.

Chapter 2

General methodology

2.1 Participants

Participants were healthy, untrained and unrelated Caucasian males [*n* = 120, age 20.6 (2.3) yr, height 1.79 (0.06) m and mass 75.1 (10.1) kg; mean (SD)], and all gave written consent to participate prior to involvement. Participants self-reported as not having a known musculoskeletal or neurological disorder, were aged between 18 yr and 39 yr, had a body mass index (BMI) between 18.5 $kg \cdot m^{-2}$ and 30 $kg \cdot m^{-2}$, and had not undertaken any structured resistance training in the preceding 12 months. Additionally, a questionnaire designed to assess habitual activity levels (Baecke et al., 1982) (Appendix 1) was used to ensure that only untrained participants, those undertaking less than 3 hours of low-to-moderate intensity habitual work-based and leisure time physical activity per week, took part in the study. All experimental procedures were conducted in accordance with the guidelines in the Declaration of Helsinki (World Medical Association, 2013) and approved by the Ethics Committee of Manchester Metropolitan University.

2.2 Measurement of muscle morphological and functional properties

2.2.1 Knee extension and flexion torque

Maximum voluntary isometric knee extension (MVC_{KE}) and flexion (MVC_{KF}) torque was measured using an isokinetic dynamometer (Cybex Norm, Cybex International Inc., NY, USA) with participants seated at 85° hip flexion (Figure 2.1). A minimum of 3 maximal voluntary contractions (MVC) were performed at knee joint angles of 70°, 80° and 90° of flexion on the right leg only. If the third MVC was the highest, additional MVC attempts were performed until no further increases in MVC were apparent. This range of knee joint angles has been shown previously to include the optimum angle for a comparable sample population (Erskine et al., 2009). Alignment of the dynamometer rotational axis with the

participant's knee joint centre of rotation took place prior to attaching the cuff of the lever arm to the lower leg proximal to the lateral malleolus. Participants were secured in the dynamometer via inextensible straps across the shoulders, waist and right thigh to prevent any extraneous movement during maximal contraction efforts (Figure 2.1). Following a series of sub-maximal knee extension and flexion contractions to warm-up, participants were instructed to maintain each MVC for approximately 3 s until they received a verbal signal to relax. Maximal isometric knee extension and flexion torques were assessed at all knee joint angles in a randomised order, and a 2 min rest period was given between contractions (Parcell et al., 2002). The knee joint angle at which peak MVC torque occurred was considered the optimal angle and was used for subsequent measurements.

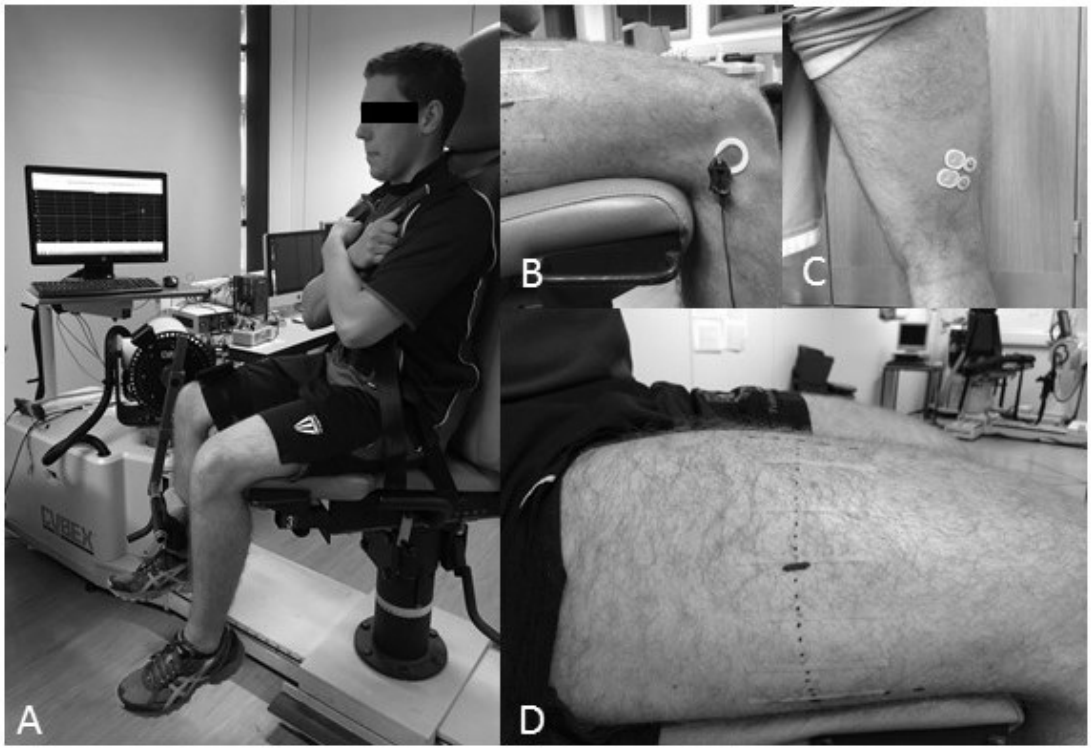

Figure 2.1. Example of participant set-up for measurement of knee extension and flexion MVC torque (**A**); reference (**B**) and active (**C**) EMG electrode placement; and vastus lateralis mark-up and external reference marker placement (**D**).

2.2.2 Muscle activation and co-activation

Two self-adhesive electrodes (7.5 x 12.5 cm; Tyco Galvanic Pad, Uni-Patch, MN, USA) were positioned over the muscle belly of the vastus medialis, rectus femoris and vastus lateralis and connected to an external stimulation device (DS7, Digitimer stimulator, Welwyn, Garden City, UK) such that the anode was placed 5-10 cm proximal to the superior border of the patella and the cathode was located 5-10 cm distal to the inguinal crease (Place et al., 2010). With the participant at rest, the maximal twitch torque stimulation intensity was identified by administering single twitches of increasing current intensity until no further increases in twitch torque were observed. The current intensity at which no further increases in twitch torque were observed was defined as the supramaximal stimulation intensity and was used to deliver two doublets to the participant during MVC_{KE} and one doublet during relaxation (each separated by 1.5 s) to assess voluntary activation capacity as (Behm et al., 1996):

$$\text{Activation (\%)} = (1 - t/T) \times 100 \quad \text{(Eq 1)}$$

where t is the higher of the two interpolated doublet amplitudes and T is the potentiated doublet amplitude (Behm et al., 2001). Doublets were preferred to singlets as these have been associated with improved signal-to-noise ratio (Behm et al., 1996); reduced variability (Suter & Herzog, 2001), and multiple stimuli are more sensitive in the detection of muscle activation than single stimulations (Kent‐Braun & Le Blanc, 1996). Furthermore, to overcome potential reductions in MVC_{KE} torque as a consequence of twitch anticipation (Button & Behm, 2008) doublets were only superimposed on those MVC_{KE} efforts eliciting comparable torque values to those previously assessed (see section 2.2.1). Antagonist muscle co-activation during MVC_{KE} was determined through electromyographic (EMG)

assessment of the biceps femoris, as this muscle is reportedly representative of the hamstrings (Kellis & Baltzopoulos, 1999). Two pre-gelled Ag-AgCl electrodes (Ambu, Neuroline 720, Denmark) were positioned with an inter-electrode distance of 20 mm over the long head of the biceps femoris. Prior to electrode placement the skin was shaved, abraded and cleansed with an alcohol wipe to reduce skin impedance (Hermens et al., 2000). To minimise muscle cross talk from adjacent muscles, electrodes were placed over the distal third of the muscle in the mid-sagittal plane and a reference electrode was positioned over the lateral tibial condyle (Figure 2.1). Pre-amplified raw EMG activity was filtered using low (10 Hz) and high (500 Hz) band pass filters. The integral of the root mean square (RMS) EMG activity corresponding to peak MVC_{KE} torque at the optimal knee joint angle was calculated and averaged over 0.5 s either side of the instantaneous peak. EMG activity of the biceps femoris during MVC_{KF} was measured and, assuming a linear relationship between torque and EMG activity, was used to estimate co-activation torque (Reeves et al., 2004c). Subsequently, net MVC_{KE} torque was calculated as:

$$\text{Net } MVC_{KE} \text{ torque} = CcT + (MVC_{KE} + STT) \quad \text{(Eq 2)}$$

where CcT is the co-activation torque and STT is the superimposed twitch torque. All signals of torque, electrical stimuli and EMG activity were displayed on a computer screen (Macintosh, iMac, Apple Computer, Cupertino, USA) interfaced with an acquisition system (AcqKnowledge, Biopac Systems, Santa Barbara, USA) to enable analogue-to-digital conversion at a sampling frequency of 2000 Hz.

2.2.3 Muscle architecture

Muscle architecture of the vastus lateralis (VL) muscle was assessed *in vivo* during MVC_{KE} at the pre-determined optimum joint angle using B-mode ultrasonography (AU5, Esaota, Italy). With the participant seated on the dynamometer as described previously, the origin and insertion and the medial and lateral borders of the VL muscle were identified at rest. VL muscle length was measured, and an external echo-absorptive reference marker was placed at 50% of muscle length (Figure 2.1). Scans were obtained using a 40 mm, 7.5 MHz linear-array probe coated in water-soluble transmission gel to increase acoustic contact. The probe was positioned perpendicular to the skin surface over the echo-absorptive marker in the mid-sagittal plane of the VL muscle. The external reference marker was visible on the scanned image; thus, any movement of the probe in relation to the marker during each MVC trial would be identified. If movement of the probe was apparent, the trial was omitted and an additional trial would take place. An external voltage trigger enabled synchronization of the ultrasound scans with the acquisition system to allow for the ultrasound image corresponding to peak MVC_{KE} torque to be exported for subsequent analysis. All ultrasound scans were recorded in audio video interleave (AVI) format at a sampling frequency of 25 Hz, and single images were captured using frame-capture software (Adobe Premiere Elements version 10, Adobe Systems). Measurement of VL muscle fibre pennation angle and fascicle length was completed on single images using digitising software (NIH ImageJ, version 1.44o, National Institutes of Health, Bethesda, USA) (Reeves et al., 2004c). Pennation angle was measured as the angle of fascicular insertion into the deep aponeurosis. Identification of fascicle length was achieved by measuring the distance from fascicular origin to insertion on the aponeuroses (Narici et al., 1996). Often the VL muscle fascicles extended beyond the scanning window; therefore estimation of fascicle length was necessary by extrapolating the deep and superficial aponeuroses and

fascicle (Figure 2.2) (Reeves & Narici, 2003). Pennation angle and fascicle length were measured on a minimum of three fascicles for every ultrasound image, and an average of these measurements was taken as the pennation angle and fascicle length.

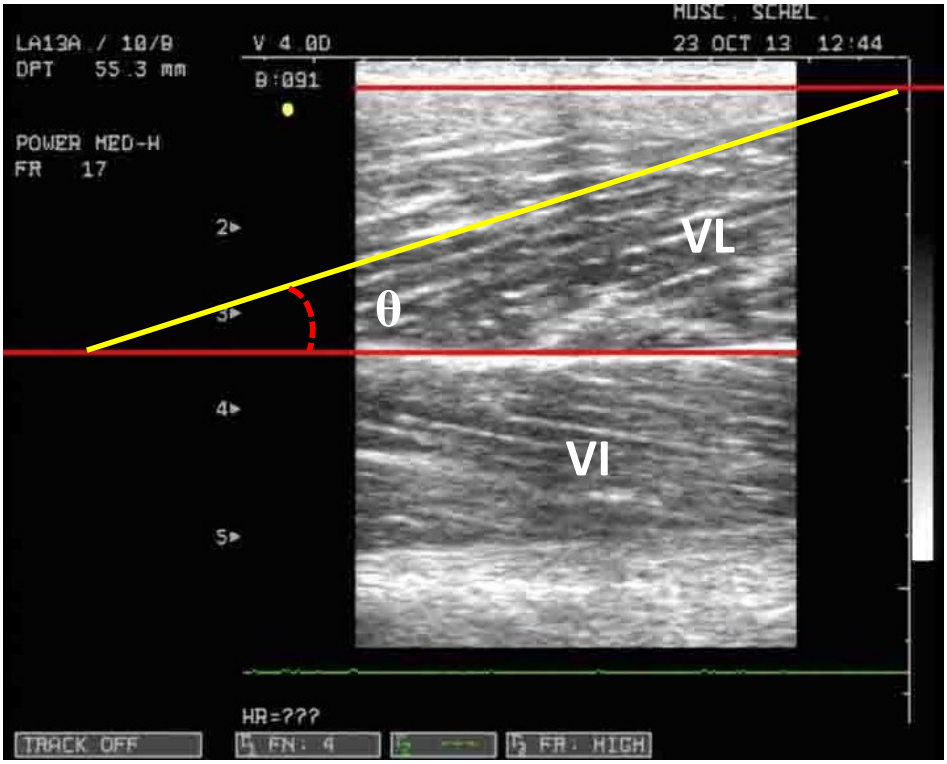

Figure 2.2. Extrapolation of VL fascicle length and aponeuroses beyond the ultrasound-scanning window. VL = vastus lateralis, VI = vastus intermedius, θ = pennation angle.

2.2.4 Muscle volume

VL muscle anatomical cross-sectional area (ACSA) was measured using previously validated methods with B-mode ultrasonography (Reeves et al., 2004b). A series of transverse plane scans were taken at the level of 50% of VL muscle length with the use of external reference markers to identify sections from the medial to lateral edge of the VL (Figure 2.3). Care was taken to ensure minimal pressure was applied to the VL during scanning to avoid compression of the muscle. A recording of the scans was saved in AVI format, and single scans were captured using frame-capture software (Adobe Premiere Elements version 10, Adobe Systems) and used for subsequent analysis. Single scans were fitted using contour

matching (Figure 2.3), and ACSA was measured using digitising software (ImageJ 1.44o, National Institutes of Health, Bethesda, USA).

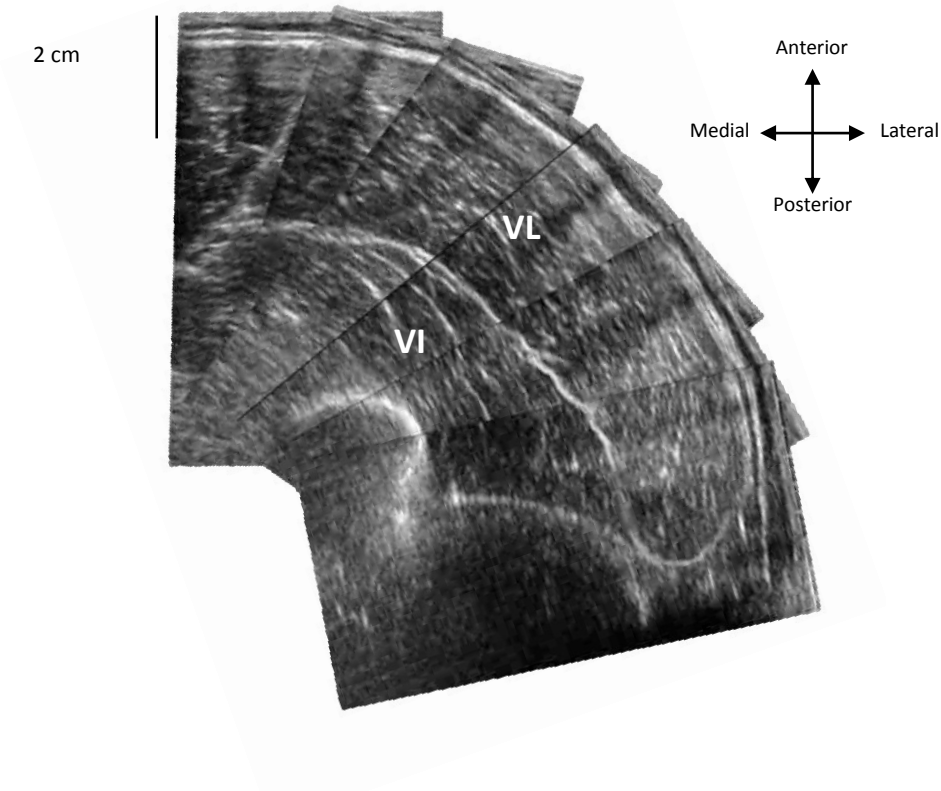

Figure 2.3. VL muscle ACSA measured using single transverse plane B-mode ultrasound scans and contour matched. VI = vastus intermedius.

The mean of 3 measurements was taken and used to estimate VL muscle volume using previously applied methods based on a series of regression derived constants (Morse et al., 2007) along with VL muscle length.

$$\text{Equation 3: Muscle Volume} = L_{VL} \times ACSA_{VL} \times (a/4 + b/3 + c/2 + d) \quad \text{(Eq 3)}$$

where L_{VL} equates to VL muscle length, $ACSA_{VL}$ is the mean of three ACSA measurements taken at 50% muscle length and a (-2.9244), b (0.74), c (2.2178) and d (0.0244) are the

regression-derived constants (Morse et al., 2007). Subsequently, calculation of VL muscle physiological cross-sectional area (PCSA) was achieved as:

$$\text{Equation 4: } PCSA_{VL} = \text{Muscle volume} / L_f \quad \text{(Eq 4)}$$

where, L_f is the fascicle length obtained under contraction at the optimal knee joint angle.

2.2.5 Tendon moment arm length

Moment arm length of the patellar tendon was measured to calculate patellar tendon forces using a dual energy X-ray absorptiometry (DXA) scanner (Hologic Discovery W, Vertec Scientific Ltd, UK), consistent with Erskine et al. (2014). A single, low-energy (0.9 μSv) sagittal plane scan was obtained using a 22.6 x 13.7 cm field of view, which lasted approximately 11 s. During scanning, each participant lay on their side with the hip flexed at 85° and the right knee joint positioned at the previously determined optimum angle using a goniometer (Figure 2.4).

Scans were exported to a DICOM file viewer (OsiriX 5.0.2, Pixmeo Sarl, Geneva, Switzerland), and the perpendicular distance between the tibiofemoral contact point and the axis of the patellar tendon was measured as the patellar tendon moment arm length (Figure 2.3)(Tsaopoulos et al., 2006). Measurement of patellar tendon moment arm length using DXA imaging is highly reproducible [ICC = 0.97, ratio limits of agreement (LoA) (Atkinson & Nevill, 1998) = 1.01(x/÷ 1.07)], but has been shown to consistently overestimate moment arm length by 9.7% in comparison with MRI obtained data (Erskine et al., 2014).

2.2.6 Patellar tendon, VL fascicle and specific force

Patellar tendon force was calculated by dividing net MVC_{KE} torque at the optimum knee joint angle by patellar tendon moment arm length.

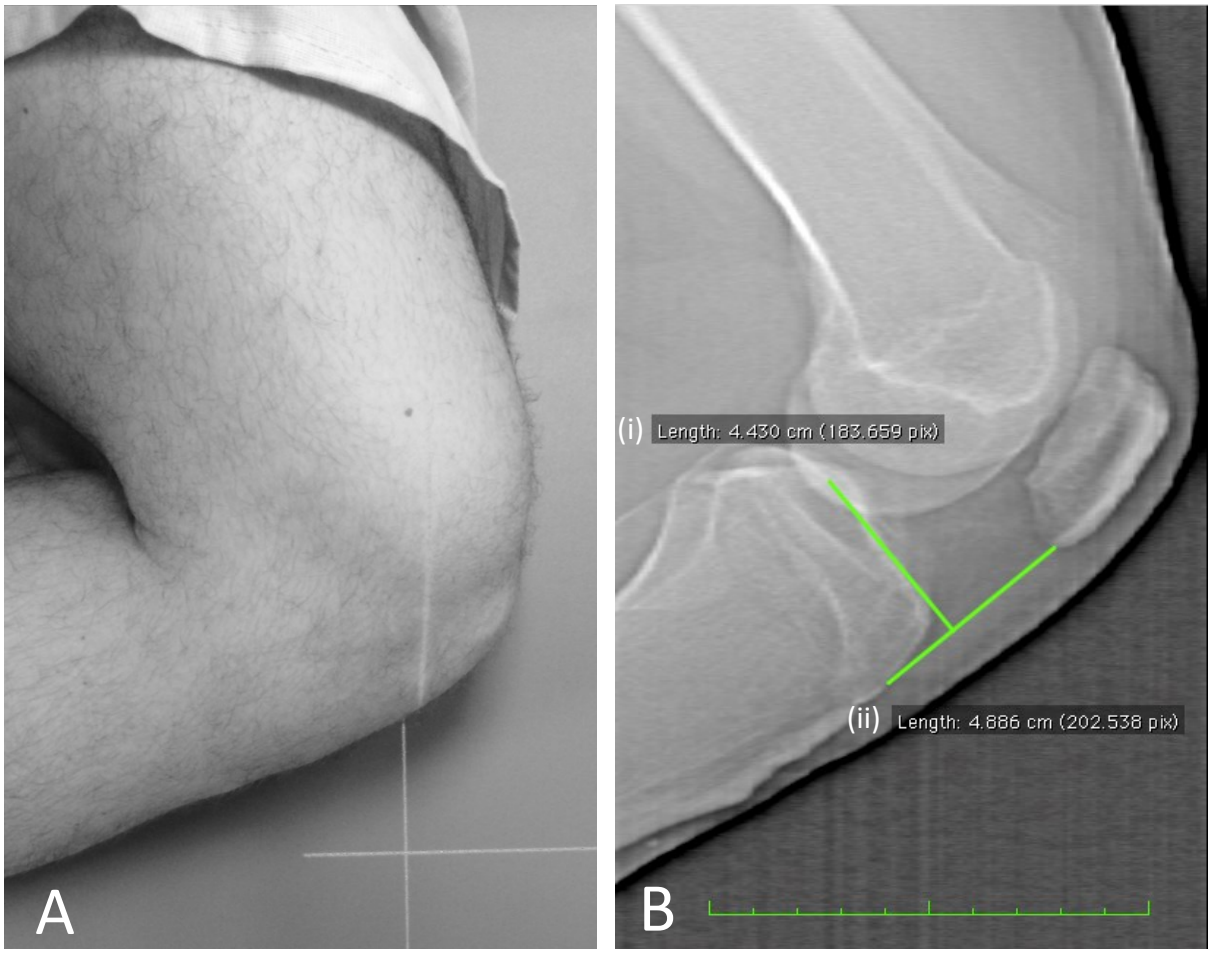

Figure 2.4. (**A**) Participant positioning during single-energy DXA scan of the patellar tendon moment arm. (**B**) DXA scan image showing the patellar tendon moment arm length (i), and patellar tendon length (ii). Both images taken from a participant involved in the work described in the current thesis.

Equation 5: F_{PT} = Net MVC_{KE} torque / d_{PT}

(Eq 5)

where F_{PT} is the patellar tendon force and d_{PT} is patellar tendon moment arm length. The contribution of the VL muscle to patellar tendon force was calculated by estimating the

relative PCSA of the VL in relation to the quadriceps femoris muscle as approximately 21% using previously reported data (Narici et al., 1992). Subsequently, fascicle force of the VL was estimated by dividing VL muscle force by the cosine of the pennation angle.

$$\text{Equation 6: } FF_{VL} = MF_{VL} / \cos\theta \quad \text{(Eq 6)}$$

where, FF_{VL} is VL fascicle force, MF_{VL} is VL muscle force and $\cos\theta$ is the cosine of the pennation angle obtained at the optimum joint angle during MVC. Finally, VL muscle specific force was calculated by dividing VL fascicle force by VL PCSA (Reeves et al., 2004c).

$$\text{Equation 7: Specific force} = FF_{VL} / PCSA_{VL} \quad \text{(Eq 7)}$$

2.3 Body composition

Quantification of fat mass, lean mass and percentage body fat was completed using DXA, following a period of overnight fasting for 12 hours by all participants (Tomlinson et al., 2014). All participants wore a cotton wraparound examination gown and were instructed to remove all metal items prior to the scan. Participants lay supine in the centre of the table with arms by their side and legs outstretched and internally rotated (Figure 2.5). Care was taken to ensure enough space was left between the arms and torso, and between legs to maximise the accuracy of the subsequent analysis. To aid participant comfort and minimise movement during the scan, medical tape (Transpore™ Medical Tape, 3M™, USA) was placed around the outside of the feet. The default whole-body scan mode was selected which emits dual energy (140/100 kVp) fan-beam x-rays to estimate body

composition. The scanning region was 195 cm x 65 cm with 1.3 cm line spacing and 0.2 cm point resolution. Scan duration was approximately 7 minutes and the effective radiation dose to each participant was 8.4 μSv (Blake et al., 2006). All images were analysed by the same trained investigator using Physician's Viewer v6.1 software.

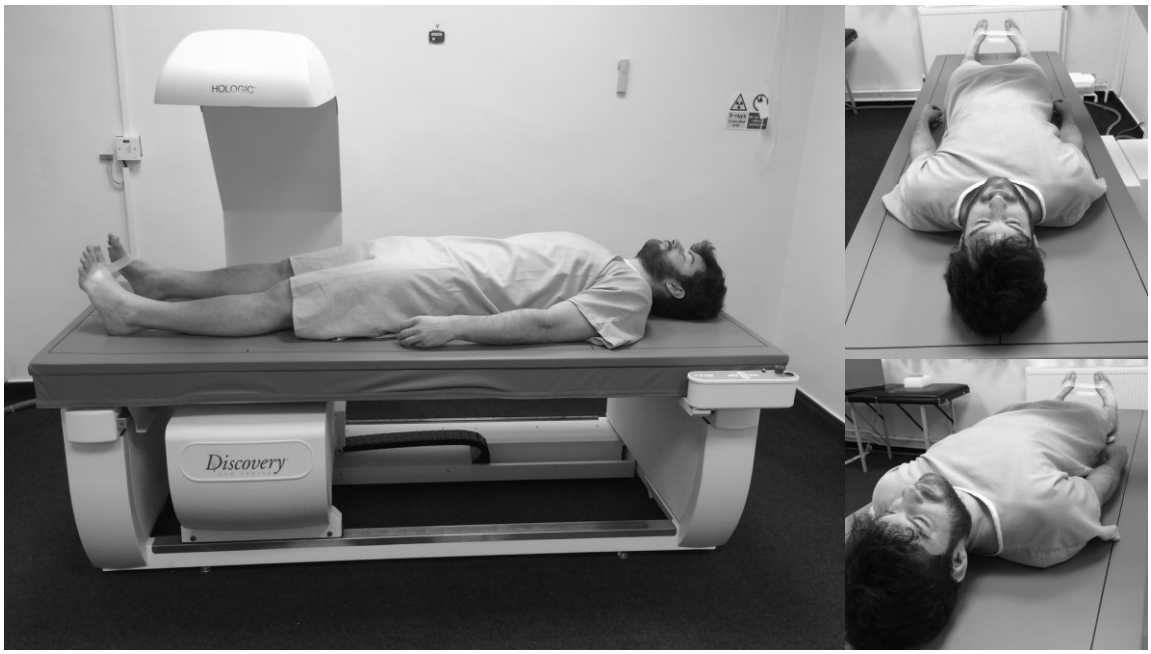

Figure 2.5. Participant positioning for whole-body DXA scanning.

2.4 Genetic analysis

2.4.1 DNA sample collection

A 5 ml blood sample was collected by a trained phlebotomist (the principal investigator in all cases) from a superficial forearm vein into EDTA tubes (BD Vacutainer Systems, Plymouth, UK) before being aliquotted into 2 mL microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) and stored at -20°C. Although whole-blood sampling is preferable for collection of large amounts of genomic DNA, buccal cell sampling provides a less invasive alternative and is therefore preferable for some participants (Feigelson et al., 2001). For

24 participants, buccal cell samples were obtained in duplicate (Whatman Sterile OmniSwab, GE Healthcare, USA) following a minimum 1-hour abstinence from food and drink. Participants were instructed to brush one OmniSwab collection tip firmly against the inside of the cheek for approximately 30 s and repeat with a second swab on the opposite cheek. Each collection tip was ejected into a 2 mL microcentrifuge tube and stored at -20°C. All collection tubes were coded and labelled to ensure participant anonymity in accordance with the Human Tissue Act (2004).

2.4.2 DNA extraction

The Qiagen QIAcube spin protocol (Qiagen, Crawley, UK) was used for the extraction of genomic DNA from both whole blood and buccal samples. The protocol was completed in accordance with the manufacturer's guidelines and used the buffers contained in the Qiagen DNA Blood Mini kit (Qiagen, Crawley, UK). Briefly, DNA extraction from whole blood required cell lysing with protease and AL buffer during incubation at 56°C for 10 mins. Following brief centrifugation and the addition of ethanol, the resultant lysate was centrifuged at 8000 rpm for 60 s to allow silica gel membrane binding to occur. Removal of proteins, nucleases and other impurities was achieved following additional buffer-centrifugation cycles before elution of the remaining solution with 200 μL of AE buffer into a 1.5 mL microcentrifuge tube. DNA extraction from buccal swabs followed the same process as detailed for whole blood, however an additional stage of transferring the lysate into clean 2 mL microcentrifuge tubes prior to the DNA purification phase was necessary. The automated Qiagen QIAcube was used to standardise these procedures and could process a maximum of 12 samples at a time. Although genomic DNA yield is sample-dependent and affected by the number of cells contained in each sample, typical yields

from 200 μL of whole blood (5-11 μg) and one buccal swab (1.3-2.9 μg) in the current samples using this protocol are deemed good quality with A_{260}/A_{280} ratios of 1.2-1.7 (Glasel, 1995). Furthermore, during the subsequent genotyping process for blood and buccal samples, mean (±SD) DNA content of each 10 μl reaction volume was 9.9±1.1 ng and 18.6±4.6 ng, respectively. These data were taken from a sub-set of 30 samples to negate superfluous use of ~10 μl of participant DNA.

2.4.3 Genotyping

Each participant was genotyped for eight polymorphisms (Table 2.1) using the fluorophore-based detection technique of TaqMan® real-time polymerase chain reaction (PCR). This technique requires the amplification of a segment of genomic DNA overlapping the specific polymorphism being genotyped. To achieve amplification, forward primers were used to identify the starting point of the genomic DNA segment and reverse primers to identify the end-point (Applied Biosystems®, UK). Allele-specific probes, identified by either VIC® or FAM® (Table 2.1; Applied Biosystems®, UK) attached to their respective complementary sequences and emitted a fluorescent dye that was detected by the PCR machine.

Real-time PCR was carried out in 96-well plates with each well containing a reaction volume of 10 μL. The reaction volume for genotyping of *ACE* using DNA obtained from whole blood samples contained 0.5 μL of participant DNA, 5 μL of TaqMan® genotyping master mix (Applied Biosystems®, UK), 1.55 μL of nuclease-free H_2O (Qiagen, Crawley, UK), 0.9 μL of I and D allele-specific probes and 0.38 μL of *ACE* primer 111, 112 and 113 (Koch et al., 2005)(refer to Table 2.1 for specific sequences).

Table 2.1. Polymorphisms used in genotyping, identification of allele-specific probes and when known the flanking primers and probes used for DNA amplification.

Polymorphism	VIC®	FAM®	Primers (5'-3')
ACE I/D (rs4341)	I-allele (5'-AGGCGTGA-TACAGTCA-3')	D-allele (5'-TGCTGCCT-TATACAGTCA-3')	***ACE*111** CCCATCCTTTCTCCCATTTCTC ***ACE*112** AGCTGGAATAAAATTGGCGAAAC ***ACE*113** CCTCCCAAAGTGCTGGGATTA
ACTN3 (rs1815739)	R-allele	X-allele	
COL5A1 (rs12722)	C-allele	T-allele	
CTNF (rs1800169)	A-allele	G-allele	
PTK2 (rs7843014)	A-allele	C-allele	
PTK2 3'UTR (rs7460)	A-allele	T-allele	
TRHR (rs7832552)	C-allele	T-allele	
TTN (rs10497520	C-allele	T-allele	

For DNA samples obtained from buccal cells, volumes of the TaqMan® genotyping master mix (Applied Biosystems®, UK), probes and primers remained the same, instead 0.05 μL of nuclease-free H_2O (Qiagen, Crawley, UK) and 2 μL of participant DNA were used. For genotyping of all other polymorphisms using DNA obtained from whole blood samples, the reaction volume contained 0.2 μL of participant DNA, 5 μL of TaqMan® genotyping master

mix (Applied Biosystems®, UK), 4.3 μL of nuclease-free H_2O (Qiagen, Crawley, UK) and 0.5 μL of TaqMan® genotyping assay mix (Applied Biosystems®, UK). For DNA samples obtained from buccal cells, the 10 μL reaction volume contained 1 μL of participant DNA, 5 μL of TaqMan® genotyping master mix, 3.5 μL of nuclease-free H_2O and 0.5 μL of TaqMan® genotyping assay mix. Control wells were included on each 96-well plate, in which nuclease-free H_2O replaced the DNA sample.

Real-time PCR was performed on two different PCR machines such that the 96-well plate (LightCycler® 480 Multiwell Plate 96, Roche Diagnostics Ltd, UK) used for the genotyping of *ACTN3* and *CNTF* was sealed (Microseal 'B' Adhesive Seal, BioRad Laboratories, Hercules, USA) and DNA amplification completed on the LightCycler® 96 Real-Time PCR System (Roche Diagnostics Ltd, UK). For the remaining polymorphisms, the PCR plate (MicroAmp® EnduraPlate™ Optical 96-Well Clear Reaction Plate, Applied Biosystems®, Crawley, UK) was sealed (MicroAmp® Optical Adhesive Film, Applied Biosystems®, Crawley, UK) and run on a StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Crawley, UK). To identify the agreement between the two different PCR machines, a sub-set of participant samples were analysed on both machines and 100% agreement was achieved in all samples. DNA amplification of each polymorphism (except *ACE* I/D) was completed using the following PCR protocol: denaturation for 10-min at 95° followed by 40 cycles of incubation for 15 s at 92°C, primer annealing and extension for 1-min at 60°C and plate read. The DNA amplification protocol for *ACE* I/D was 50 cycles of denaturation for 15 s at 92°C, primer annealing and extension for 1-min at 57°C and plate read. All samples were analysed in duplicate to minimise the occurrence of genotyping errors known to negatively affect the statistical power of genetic association studies (Tintle

et al., 2009) and 100% agreement between all duplicate samples was achieved. Genotypes were determined by measurement of the end-point fluorescence of VIC® and FAM® detected by the PCR machine. Results were subsequently analysed using a computer interfaced with software supplied by the respective manufacturers of each PCR machine. An example of the results from both PCR machines is displayed in Figure 2.6.

2.5 Statistical analysis

Statistical analysis was completed using the Statistical Package for Social Sciences (SPSS) version 19.0. Prior to completing any statistical analyses the data was tested for parametricity. Normal distribution of the population was identified using Kolmogorov Smirnov and the homogeneity of variance of each phenotype was assessed using Levene's statistic. The frequency of each SNP was assessed for compliance with Hardy-Weinberg equilibrium (HWE) using X^2 tests and all SNPs were in HWE ($P > 0.05$; Table 2.2). To determine the statistical power to detect genotype-phenotype associations, power calculations, using an alpha of 0.05 and beta of 0.80 in addition to mean and standard deviation data on muscle morphology obtained in our laboratory, were computed using G*Power 3.1.9 (Franz Faul, Universitat Kiel, Germany). Consequently, it was estimated that approximately 100 participants would be required to complete assessments of muscle morphology to detect differences of ~3-5% for muscle strength (MVC_{KE} torque, net MVC_{KE} torque and specific force), ~6% for muscle architecture (fascicle length and pennation angle) and ~3-5% for muscle size (muscle thickness, ACSA, PCSA and muscle volume) and mass.

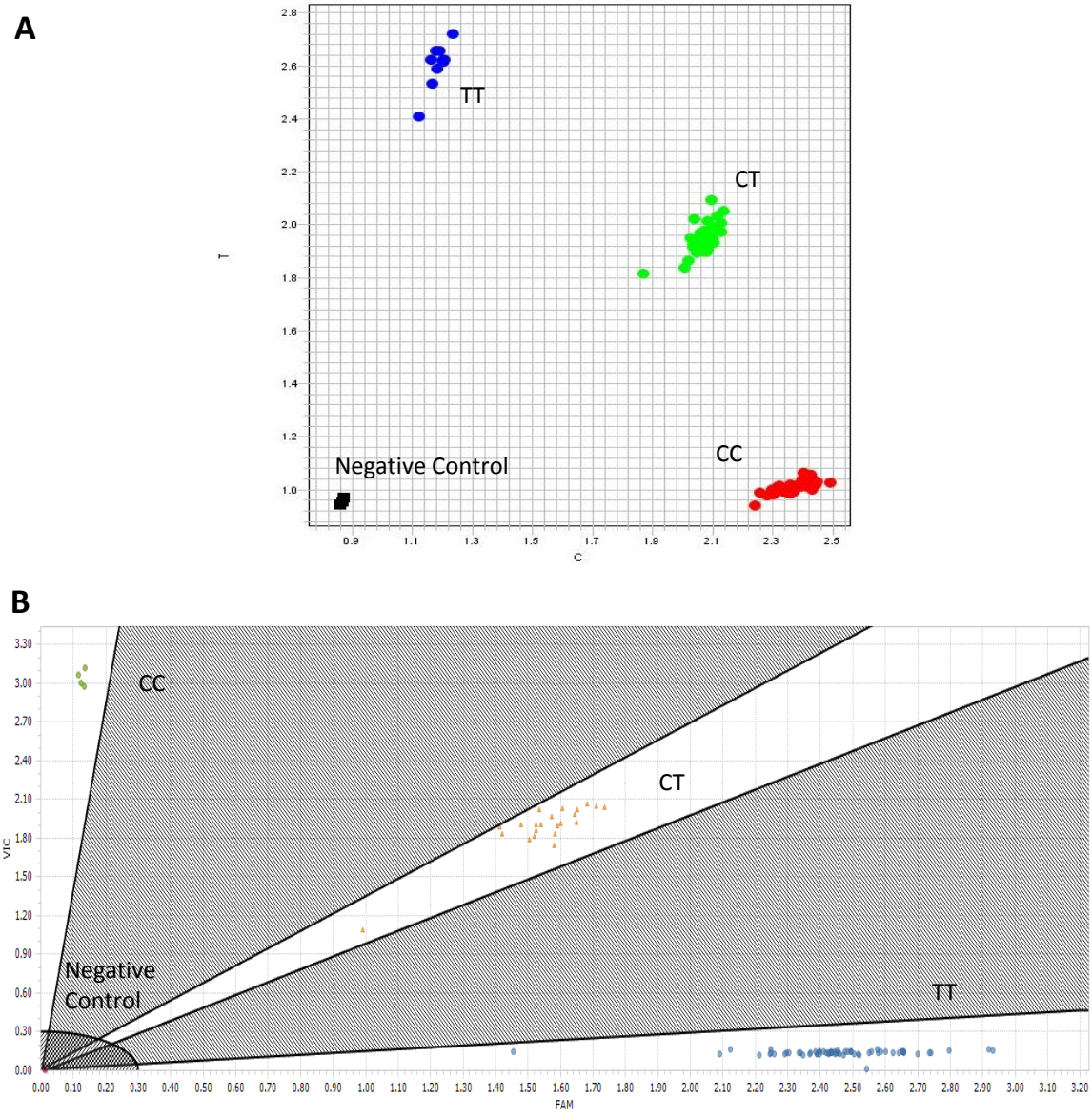

Figure 2.6. Example allelic discrimination plots for *TRHR* rs7832552 obtained using the StepOnePlus™ Real-Time PCR System (**A**), and *PTK2* rs7460 obtained using the LightCycler® 96 Real-Time PCR System (**B**).

A one-way analysis of variance (ANOVA) was conducted to determine any significant differences in physical characteristics (stature, mass, BMI and age) between genotype groups (Table 2.2). When genotype groups were combined into a dominant or recessive model, an independent samples t-test was used to identify any differences in physical

characteristics. Pearson's correlation coefficient were used to identify the variables that made a meaningful contribution to the variability within the phenotype under investigation, and those identified were included as confounding variables in subsequent analyses of covariance (ANCOVA). ANOVA, and where appropriate ANCOVA, was conducted to identify any genotype differences in muscle phenotypes. Furthermore, the effects of each genotype on the muscle phenotypes of interest were assessed for linear trend using ANOVA or ANCOVA. All significant associations identified in the main ANOVA or ANCOVA analyses were subject to post-hoc pairwise comparisons using the Benjamini-Hochberg correction (Benjamini & Hochberg, 1995). Independent samples t-tests were used to complete two group analyses on genotype groups following combination into a dominant or recessive model. Only in those instances were a tendency between genotype groups was observed, such that $P > 0.05$ but < 0.15 (Danilovic et al., 2007; Fischer et al., 2004), were the two genotype groups with the closest means combined and a two group analysis completed. Statistical significance was accepted when $P \leq 0.05$ and data are presented as means (SD).

Table 2.2. Genotype frequency and participant physical characteristics according to genotype for all candidate gene polymorphisms.

SNP		Frequency (%)	Hardy-Weinberg		Stature (m)	Mass (kg)	BMI	Age (yr)	*P*
			X^2	*P*					
ACE	II (*n* = 32)	26.7			1.80 (0.07)	77.1 (10.1)	23.9 (3.0)	20.8 (2.5)	
	ID (*n* = 54)	45.0	1.194	0.550	1.79 (0.07)	75.1 (10.2)	23.5 (2.5)	20.5 (2.2)	≥ 0.140
	DD (*n* = 34)	28.3			1.79 (0.05)	72.8 (9.4)	22.7 (2.7)	20.6 (2.2)	
ACTN3	RR (*n* = 44)	36.6			1.78 (0.05)	75.3 (11.1)	23.8 (3.2)	20.3 (2.2)	
	RX (*n* = 57)	47.5	0.006	0.997	1.80 (0.07)	74.7 (9.6)	23.2 (2.4)	20.9 (2.4)	≥ 0.282
	XX (*n* = 19)	15.9			1.80 (0.07)	75.1 (9.2)	23.2 (2.6)	20.5 (2.2)	
COL5A1	CC (*n* = 19)	15.8			1.81 (0.08)	79.4 (12.4)	24.2 (2.8)	21.0 (1.7)	
	CT (*n* = 61)	50.8	0.286	0.867	1.78 (0.06)	73.1 (9.0)	22.9 (2.4)	20.3 (1.8)	≥ 0.135
	TT (*n* = 40)	33.4			1.79 (0.05)	76.1 (10.0)	23. 8 (3.1)	20.9 (3.0)	
CNTF	AA (*n* = 2)	1.7			1.75 (0.01)	72.3 (10.3)	23.7 (3.3)	21.5 (2.1)	
	GA (*n* = 33)	27.5	0.356	0.837	1.80 (0.05)	77.4 (9.9)	23.8 (3.0)	20.9 (2.6)	≥ 0.211
	GG (*n* = 85)	70.8			1.79 (0.07)	74.1 (10.0)	23.2 (2.6)	20.5 (2.2)	
PTK2 (rs7843014)	AA (*n* = 42)	35.0			1.78 (0.07)	72.9 (9.3)	23.0 (2.5)	20.4 (2.3)	
	AC (*n* = 52)	43.3	1.661	0.436	1.79 (0.07)	75.4 (10.4)	23.4 (2.7)	20.7 (2.3)	≥ 0.177
	CC (*n* = 26)	21.7			1.79 (0.06)	77.4 (10.0)	24.1 (3.1)	20.8 (2.4)	
PTK2 (rs7460)	AA (*n* = 34)	28.3			1.79 (0.07)	74.9 (11.0)	23.5 (3.0)	20.4 (2.3)	
	AT (*n* = 54)	45.0	1.194	0.550	1.80 (0.05)	76.8 (9.3)	23.7 (2.7)	20.8 (2.4)	≥ 0.091
	TT (*n* = 32)	26.7			1.77 (0.07)	72.0 (9.7)	22.8 (2.5)	20.4 (2.2)	
TTN	CC (*n* = 95)	79.2			1.79 (0.06)	74.8 (9.8)	23.4 (2.7)	20.7 (2.3)	
	CT (*n* = 25)	20.8	1.622	0.444	1.80 (0.07)	76.2 (11.4)	23.5 (3.0)	20.3 (2.2)	≥ 0.441
	TT (*n* = 0)	0			-	-	-	-	
TRHR	CC (*n* = 53)	44.2			1.78 (0.06)	75.0 (11.1)	23.6 (3.1)	21.1 (2.8)	
	CT (*n* = 58)	48.3	1.638	0.441	1.80 (0.07)	75.0 (9.5)	23.2 (2.5)	20.2 (1.7)	≥ 0.094
	TT (*n* = 9)	7.50			1.79 (0.04)	74.5 (7.7)	23.1 (1.8)	20.0 (1.7)	

Chapter 3

Reliability of the *in vivo* assessment of vastus lateralis specific force.

3.1 Introduction

Human muscle strength is essential for locomotion and other activities of daily living, and is known to change in response to training, ageing and disease (Reeves et al., 2004c; Morse et al., 2005a; Park et al., 2007). In addition to environmental influences, muscle strength is strongly influenced by genetics and heritability estimates range from ~40-80% for a number of strength phenotypes (Thomis & Aerssens, 2012; Huygens et al., 2004). Measurement of muscle strength is useful for both athlete populations, to assess the effectiveness of training, and patient populations to determine functional ability to complete activities of daily living. Typical assessments of muscle strength use simple measurements such as maximal voluntary contraction (MVC) or one repetition maximum (Reeves et al., 2004c). These gross measurements of muscle strength do not account for the physiological determinants of strength and are therefore susceptible to inter-individual variability in said determinants, and could over- or underestimate the influence of genetic or environmental factors on muscle strength (see Chapter 4).

Calculating the intrinsic strength of muscle, or specific force, takes into account the neural and structural determinants of strength and provides a more stringent measure of the contractile capacity of the muscle. Despite this, calculating muscle specific force is a complex process and requires the accurate measurement of isometric MVC torque, muscle activation and co-activation, muscle architecture (fascicle length and pennation angle), muscle size [anatomical cross-sectional area (ACSA), physiological cross-sectional area (PCSA) and muscle volume], and tendon moment arm length, ideally using gold standard techniques (Reeves et al., 2004b). Although it is well established that MRI represents the gold standard for *in vivo* measures of muscle mass, due to the clear contrast between

hyperechoic and hypoechoic tissues and ability to complete contiguous scans along the length of a muscle (Reeves et al., 2004b; Morse et al., 2007), alternative techniques such as ultrasound alleviate some of the limitations associated with using MRI (Reeves et al., 2004c). For example in populations with impaired mobility, or in instances where an MRI is not available, ultrasound provides a more mobile and cheaper alternative to assess individuals that may not have access to MRI, or are not able to attain supine postures. Further application of ultrasound imaging is in the measurement of muscle architecture necessary for the calculation of specific force, during isometric MVC. Kwah et al. (2013) recently demonstrated moderate to high reliability of using ultrasound to measure VL fascicle length, both at rest and during MVC (ICC = 0.62-0.99). Additionally, due to the small ultrasound-scanning window, it is often necessary to extrapolate the fascicles beyond the field of view during analysis. Despite this, the linear extrapolation method has been associated with only 2.4% error when used to measure fascicle length under contraction, thus suggesting high validity of applying this technique (Reeves et al., 2003). However, using ultrasound to measure the muscle architecture of several muscles such as in the quadriceps femoris (QF) can be a timely process, especially when large sample sizes are required as in studies of inter-individual variability or genetic associations.

To save on participant time and resources in the measurement of specific force, previous research has estimated QF muscle volume using a single QF ACSA measurement and this alternative method correlates highly with measurements of QF muscle volume using the contiguous MRI method (Erskine et al., 2009). Furthermore, measurement of VL muscle ACSA using ultrasound in comparison to MRI-obtained measurements has previously been associated with ~2% error, therefore demonstrating that ultrasound is a suitable

alternative to MRI (Reeves et al., 2004c). Despite this, the reliability of using ultrasound to measure VL ACSA for use in the estimation of VL muscle volume currently remains unreported. Furthermore, as the VL muscle is known to be the predominant constituent of the QF (Alexander & Vernon, 1975; Wickiewicz et al., 1983; Scott et al., 1993) it would be useful to accurately and reliably measure VL muscle architecture in the calculation of specific force using alternative methods to MRI, such as ultrasound and dual energy x-ray absorptiometry (DXA) respectively. Therefore the aim of the current study was to identify the reliability of using an alternative method of assessing VL muscle volume, which may benefit studies requiring large sample sizes in future. Additionally, the study aimed to assess the reliability of measuring other determinants of muscle strength that are used in the calculation of muscle specific force *in vivo*.

3.2 Method

3.2.1 Participants

Eight Caucasian males [age 22.1 (2.2) yrs, stature 1.74 (0.10) m, mass 79.4 (11.1) kg; mean (SD)] gave written informed consent to participate in this study. All participants were identified as recreationally active, defined here as undertaking less than 3 hours of low-to-moderate intensity exercise per week. Participants were excluded if they had taken part in resistance exercise during the preceding 12 months, had a BMI outside the normal range (18.5-24.0 $kg \cdot m^{-2}$) or reported a history of lower limb injury. The study was in agreement with the Declaration of Helsinki and approval was obtained from the local Ethics Committee of Manchester Metropolitan University.

3.2.2 Experimental procedure

Participants were required to attend the laboratory on two occasions separated by at least 24-hrs at the same time of day on each occasion (Coldwells et al., 1994). All participants completed a series of sub-maximal knee extension and flexion contractions as a warm-up prior to collection of the following measurements on day 1 and day 2.

3.2.3 Knee extension and flexion

Participants sat in an isokinetic dynamometer with hips flexed at 85° (Cybex Norm, Cybex International Inc., NY, USA). The dynamometer axis of rotation was visually aligned with the knee joint centre of rotation and participants were secured in the dynamometer using inextensible straps positioned over the thigh, hips and shoulders to prevent any extraneous movement during contraction. Maximum isometric voluntary contraction torque was measured for knee extension (MVC_{KE}) and flexion (MVC_{KF}) of the right limb. Three MVCs were completed at knee joint angles of 60°, 70°, 80°, 90° and 100° of flexion in a randomised order to counterbalance order effects, and participants were given a 2-min rest period between contractions. Visual feedback and verbal encouragement were given throughout each trial and participants were asked to maintain each MVC for ~3 s until receiving a signal to relax.

3.2.4 Activation capacity and co-activation torque

Voluntary activation capacity was measured using the interpolated twitch technique (Behm et al., 2001). Two self-adhesive electrodes (7.5 x 12.5 cm; Tyco Galvanic Pad, Uni-Patch, MN, USA) were used to administer a supramaximal doublet (DS7, Digitimer stimulator, Welwyn, Garden City, UK) with a 50 μs pulse width and 50 ms interstimulus gap to the QF (Behm et al., 1996). Maximal twitch torque stimulation intensity was identified with the

participant at rest by administering repeated single twitches of increasing current intensity until no further increases in twitch torque were measured (Behm et al., 2001). Voluntary activation capacity of the QF was calculated as:

Equation 1: Activation (%) = (1 – *t*/T) x 100

(Eq 1)

where, *t* is the interpolated doublet amplitude and T is the potentiated doublet amplitude (Behm et al., 2001). Co-activation torque was calculated by measuring the electromyographic (EMG) activity of the biceps femoris (BF) during MVC_{KE}, and a linear relationship between EMG activity and torque when measuring MVC_{KF} torque was assumed (Kellis & Baltzopoulos, 1997). EMG activity was measured using two pre-gelled percutaneous electrodes positioned in the mid-sagittal plane over the distal third of the BF long head using a constant 20 mm inter-electrode distance (Ambu, Neuroline 720, Denmark). A third electrode was placed on the lateral tibial condyle as a reference. Recording of torque, electrical stimuli and EMG activity was via a multi-channel analogue-to-digital converter at 2 kHz, with pre-amplified raw EMG data filtered using low (10 Hz) and high (500 Hz) band pass filters (AcqKnowledge, Biopac Systems, Santa Barbara, USA). The integral of the root mean square of the EMG activity was calculated over 0.5 s either side of peak MVC_{KE} torque for the knee joint angle at which peak torque was recorded. Net knee extension torque was calculated as:

Net torque = co-activation torque + (MVC_{KE} torque + superimposed stimulation torque)

Patellar tendon moment arm length was measured using DXA with participants at rest (Hologic Discovery, Vertec Scientific Ltd, UK) consistent with Erskine et al (2014). Sagittal plane DXA scans were taken of the knee joint positioned at the angle corresponding to that of peak torque production. The perpendicular distance between the patellar tendon and

tibiofemoral contact point was measured as the tendon moment arm length using an offline DICOM viewer (OsiriX 5.0.2, Pixmeo Sarl, Geneva, Switzerland). Subsequently, patellar tendon force was calculated by dividing net torque by patellar tendon moment arm length.

3.2.5 Muscle architecture

In vivo measurements of VL muscle architecture were completed during contraction using ultrasound at the knee joint angle corresponding to peak knee extension torque (AU5, Esaota, Italy). VL muscle length was measured as the distance between the muscle origin and insertion using a 40 mm, 7.5 MHz linear-array probe. An external reference marker was positioned over the skin at 50% of muscle length, and measurements of VL muscle architecture were obtained during MVC_{KE} with the probe held perpendicular to the external reference marker in the mid-sagittal plane. MVC_{KE} trials during which movement of the probe in relation to the external reference marker was observed were omitted and an additional trial was completed. Synchronisation of the ultrasound scans via an external trigger and data acquisition system enabled offline analysis of the ultrasound image corresponding to peak MVC_{KE} torque using digitising software (NIH ImageJ, version 1.44o, National Institutes of Health, Bethesda, USA). Scans were recorded using a 25 Hz sampling frequency in audio video interleave format. The angle of fascicle insertion into the deep aponeurosis was measured as pennation angle, and the distance between fascicle insertion into the superficial and deep aponeuroses was measured as fascicle length. When necessary, estimates of fascicle length were made by extrapolating the aponeuroses beyond the ultrasound field of view (Reeves et al., 2003c). The mean of three measurements for pennation angle and fascicle length was taken for each participant.

3.2.6 Muscle size

VL muscle ACSA was measured at 50% of VL muscle length using ultrasound (Reeves et al., 2004c). External reference markers were placed along the line of 50% muscle length from the medial to lateral border of the VL. A series of ultrasound scans taken in the transverse plane were recorded and single scans were isolated using frame-capture software (Adobe Premiere Elements v10, Adobe Systems) and used for offline analysis. Individual ultrasound scans were contour matched to allow for the measurement of ACSA using digitising software (NIH ImageJ, version 1.44o, National Institutes of Health, Bethesda, USA). VL muscle volume was estimated by multiplying the mean of three VL ACSA measurements by VL muscle length and a series of regression equation constants (Morse et al., 2007). VL PCSA was calculated by dividing muscle volume by the mean fascicle length during contraction at the knee joint angle at which peak MVC_{KE} torque was recorded.

3.2.7 Specific force

Calculation of VL muscle force was achieved by estimating the contribution of the VL muscle to patellar tendon force based on the relative PCSA of the VL accounting for 21.3% within the QF (Narici et al., 1992). VL muscle force was then divided by the cosine of the pennation angle during contraction at the optimum knee joint angle to estimate VL fascicle force. VL specific force was subsequently calculated by dividing VL fascicle force by PCSA (Reeves et al., 2004c).

3.2.8 Statistical analysis

Two-tailed paired t-tests were used to identify any differences in the measurements of strength and its determinants between day 1 and day 2 (Statistical Package for Social Sciences 19.0, SPSS Inc., Chicago, Il, USA). To determine the level of agreement between tests (day 1 vs. day 2), the reliability of the measurements of muscle strength and its determinants was calculated using coefficients of variation (CV) as (SD*1.96)/mean*100) (Reeves et al., 2004c), one-way random effects intra-class correlation coefficients (ICC) and ratio limits of agreement (Atkinson & Nevill, 1998). Statistical significance was accepted at $P \leq 0.05$.

3.3 Results

Descriptive data on the functional and morphological characteristics of the VL are displayed in Table 3.1. Measurements of muscle size between day 1 and day 2 demonstrated significant ICCs (muscle volume = 0.969, ACSA = 0.967 and PCSA = 0.958, all $P < 0.0005$). Ratio limits of agreement revealed no systematic bias on the estimation of muscle volume (0.994 ×/÷ 1.055, $t = -0.619$, $P = 0.555$), ACSA (0.995 ×/÷ 1.056, $t = -0.494$, $P = 0.637$) and PCSA (0.999 ×/÷ 1.068, $t = -0.856$, $P = 0.421$). The bias ratio (0.994) for muscle volume demonstrates <1.0% difference between measurements taken on day 1 and day 2 which is in agreement with the mean inter-day difference (Table 3.1). Based on the agreement ratio, 95% of measurements taken on day 2 were within 5.5% above or below the measurement taken on day 1.

Table 3.1. Reliability of the functional and morphological characteristics of the VL.

Variable	Day 1	Day 2	CV (%)	ICC
Isometric MVC_{KE} torque (N·m)	249 (7.4)	250 (8.2)	4.2	0.989
Net knee extension torque (N·m)	274 (13.5)	277 (9.3)	5.6	0.986
Muscle volume (cm^3)	622 (6.2)	626 (12.0)	2.5	0.969
ACSA (cm^2)	18.7 (0.2)	18.8 (0.4)	2.7	0.967
Fascicle length (cm)	8.5 (0.2)	8.5 (0.2)	2.5	0.978
Pennation angle (°)	14.6 (0.3)	14.6 (0.4)	2.1	0.998
PCSA (cm^2)	66.2 (1.1)	66.9 (2.5)	3.6	0.958
Moment arm (cm)	4.51 (0.1)	4.50 (0.1)	1.8	0.974
Patella tendon force (N)	6112 (313)	6190 (249)	5.6	0.989
VL fascicle force (N)	1345 (68.5)	1362 (53.7)	5.5	0.990
Specific force ($N \cdot cm^{-2}$)	24.2 (1.1)	24.3 (1.4)	5.7	0.979

ACSA, anatomical cross-sectional area; CV, coefficient of variation MVC_{KE}, maximal voluntary knee extension contraction; PCSA, physiological cross-sectional area; VL, vastus lateralis.

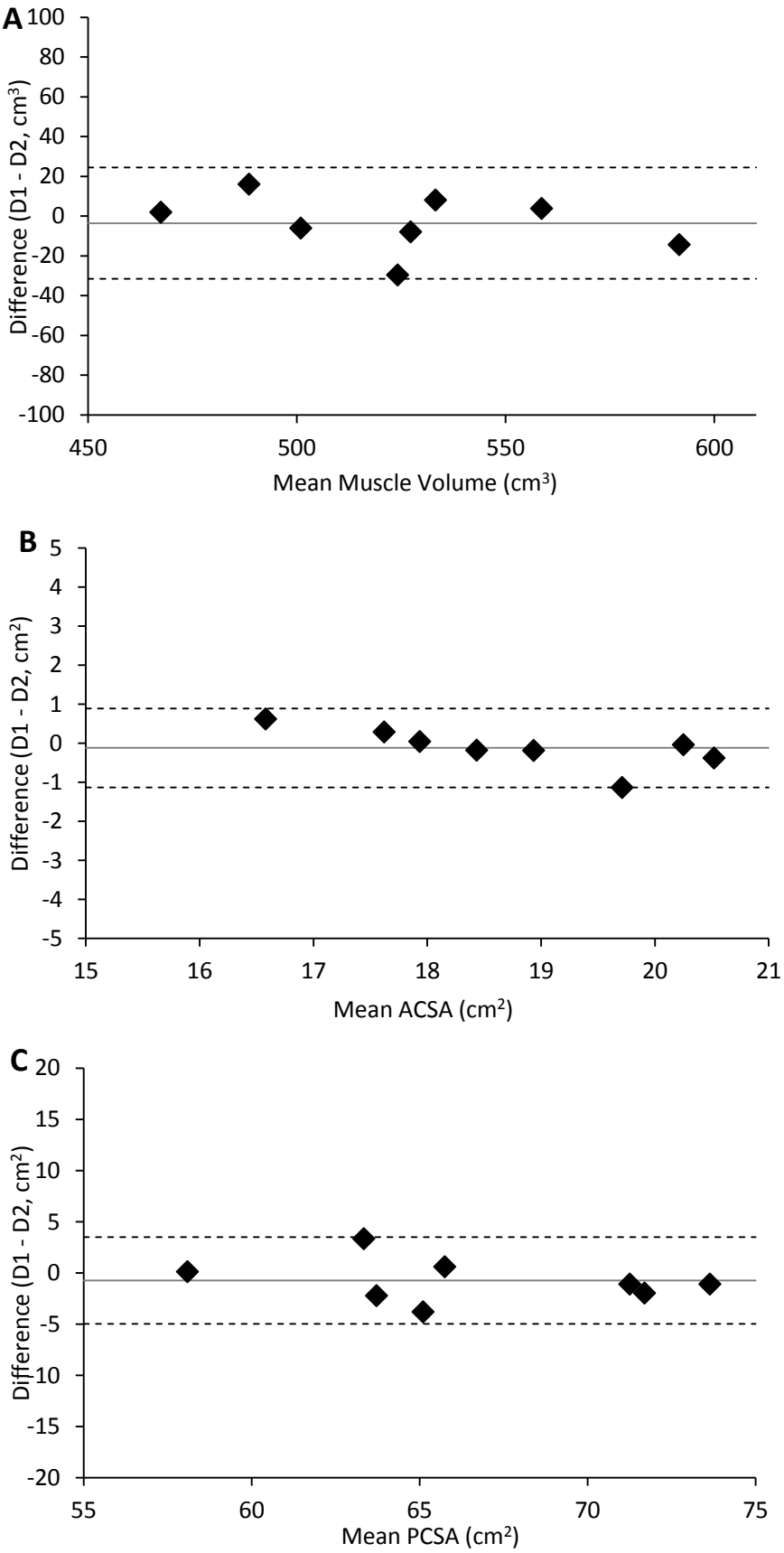

Figure 3.1. Bland-Altman plots identifying the bias (solid line) and the 95% limits of agreement (dashed line) between day 1 and day 2 for the measurement of muscle volume (**A**) and ACSA (**B**) and PCSA (**C**).

Calculation of VL fascicle force and specific force identified significant inter-day ICCs of 0.990 and 0.979 respectively ($P < 0.0005$). There was no systematic bias for VL fascicle force (0.988 ×/÷ 1.084, $t = -0.857$, $P = 0.420$) or specific force (0.998 ×/÷ 1.107, $t = -0.128$, $P = 0.902$), and the ratio limits of agreement indicate 95% of the mean inter-day ratios should be within 8.4% and 10.7% of the bias ratio respectively.

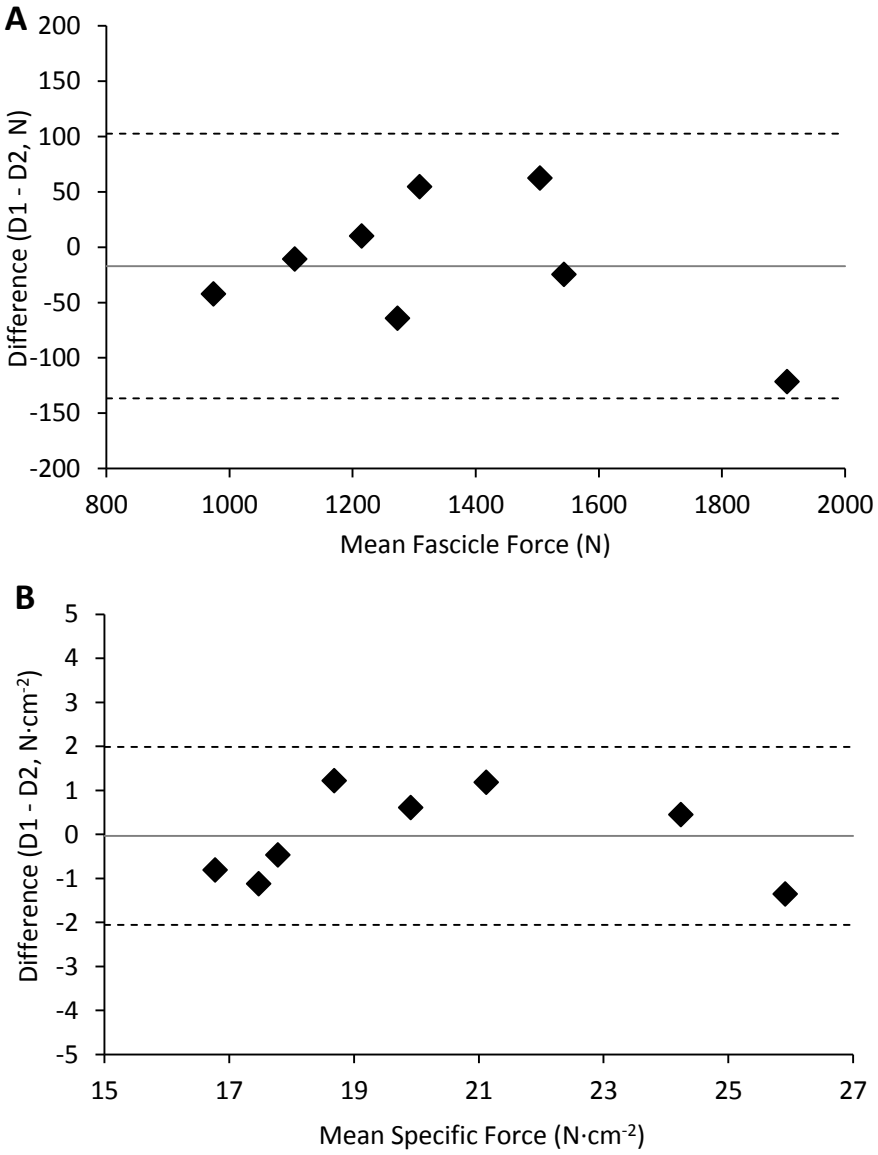

Figure 3.2. Bland-Altman plots identifying the bias (solid line) and the 95% limits of agreement (dashed line) between day 1 and day 2 for the measurement of VL fascicle force (**A**) and specific force (**B**).

Measurements of isometric MVC_{KE} torque and net knee extension torque on day 1 were significantly correlated with the measurements taken on day 2 (ICC = 0.989, $P < 0.0005$ and

ICC = 0.986, $P < 0.0005$, respectively). Analysis of the inter-day agreement revealed no systematic bias in the measurement of isometric MVC_{KE} (0.998 ×/÷ 1.073, $t = -0.126$, $P = 0.903$) and net knee extension torque (0.990 ×/÷ 1.085, $t = -0.662$, $P = 0.529$), with 95% of the mean inter-day ratios expected between 7.3% and 8.5% of the bias ratio respectively.

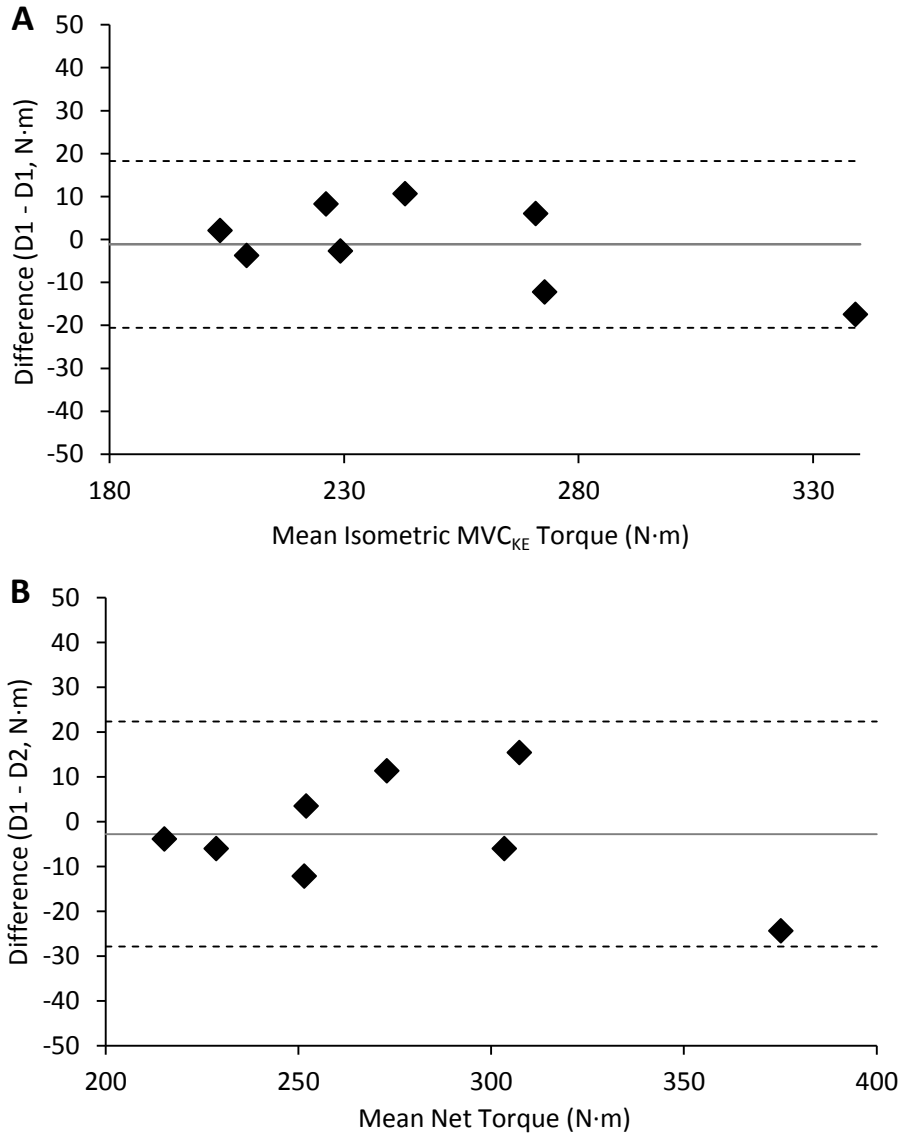

Figure 3.3. Bland-Altman plots identifying the bias (solid line) and the 95% limits of agreement (dashed line) between day 1 and day 2 for the measurement of isometric MVC_{KE} torque (**A**) and net knee extension torque (**B**).

Inter-day ICC assessments of VL fascicle length (ICC = 0.978) and pennation angle (ICC = 0.998) were significant ($P < 0.0005$). Analysis of ratio limits of agreement identified no systematic bias for VL fascicle length ($t = 0.530$, $P = 0.613$) and the 95% limits of agreement

were between 0.96 and 1.04. No systematic bias was identified for VL pennation angle (*t* = -0.141, *P* = 0.892) and the 95% limits of agreement were between 0.97 and 1.03.

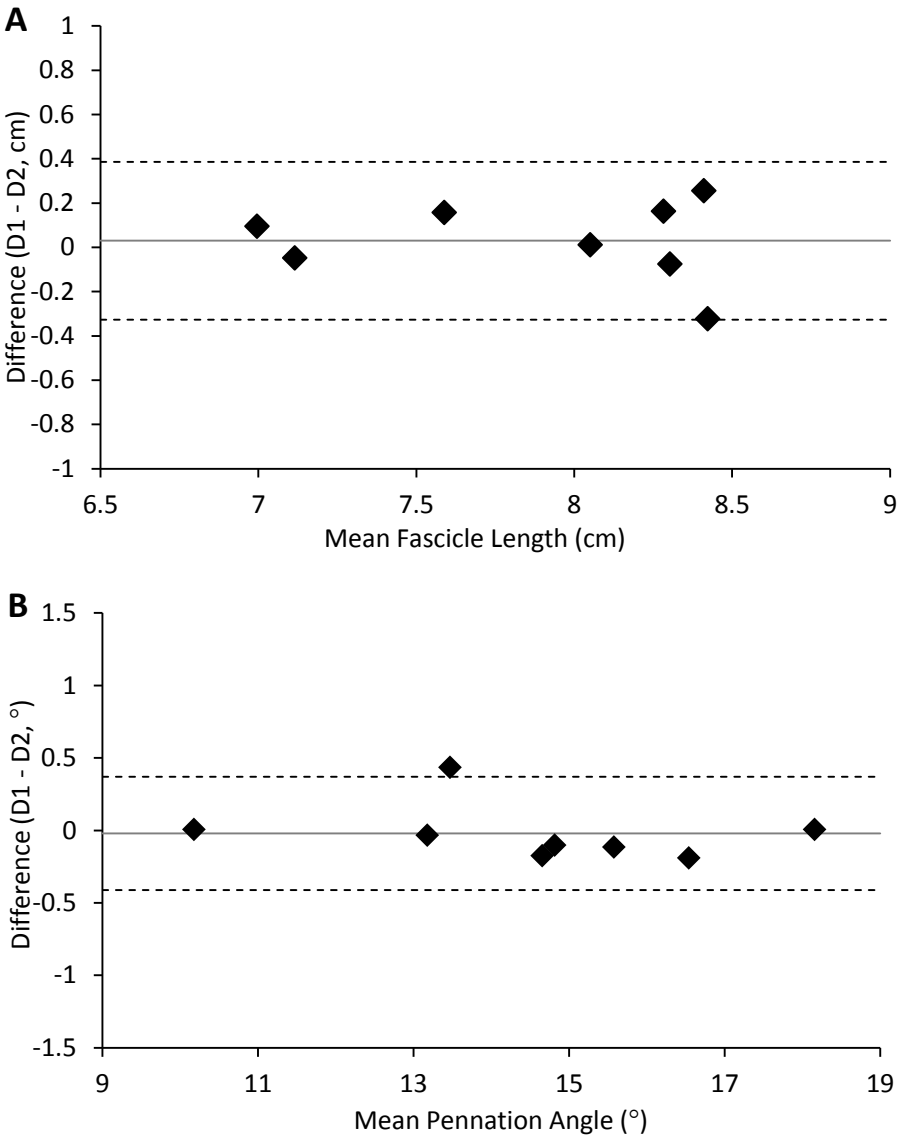

Figure 3.4. Bland-Altman plots identifying the bias (solid line) and the 95% limits of agreement (dashed line) between day 1 and day 2 for the measurement of fascicle length (**A**) and pennation angle (**B**).

3.4 Discussion

The aim of the current study was to ascertain the reliability of an alternative method for measuring VL muscle volume, and to identify the reliability of the methods used to measure muscle MVC_{KE} torque, specific force and its determinants. The results demonstrate that the method used to estimate VL muscle volume was reliable, as were the methods used in

the measurement of VL muscle specific force and its constituent elements. These methods could be used to reliably identify inter-individual variability in the functional and morphological characteristics of the VL in studies requiring large sample sizes.

Triangulation of multiple statistical tests when assessing test re-test reliability is recommended to obtain a more complete understanding of measurement error (Atkinson & Nevill, 1998). This is particularly salient when considering the limitations of employing some popular statistical tests that can be affected by sample variance (Atkinson & Nevill, 1998). By using triangulation in the current study, it is evident that the measurement of ACSA, and subsequent estimation of muscle volume and PCSA using ultrasound are highly repeatable and reliable methods (Atkinson & Nevill, 1998). Furthermore, analysis of ratio limits of agreement indicates no significant systematic bias and strong measurement agreement between day 1 and day 2. Based on this data, it is possible that for an individual whose VL muscle volume is estimated at 620 cm^3 using the current method, a repeated measurement could yield values as low as 596 cm^3 or as high as 645 cm^3. Together with the mean data presented in Table 3.1 these values would translate into inter-day measurement differences of 2.98 cm^2 for PCSA and 0.77 $N \cdot cm^{-2}$ for specific force, which according to previous data would suggest appropriate measurement sensitivity (Erskine et al., 2013). Furthermore, the values of VL muscle volume (624 cm^3), ACSA (18.8 cm^2) and PCSA (66.6 cm^2) in the current study are comparable to those obtained in previous in assessments of muscle size (674 cm^3, 18.3 cm^2 and 75.1 cm^2 respectively) using gold standard techniques on a similar population (Erskine et al., 2009). Therefore suggesting that the reported methods of measuring VL muscle size are sensitive enough to allow for

the detection of genetic associations and accurately quantify inter-individual variability in muscle volume, ACSA and PCSA.

Accurate calculation of VL specific force requires the measurement of maximal knee extension torque and muscle architecture during contraction (Chow et al., 1999; Maganaris et al., 2001). It is reasonable to assume that for the calculation of VL fascicle force and specific force to be reliable, the measurement of the contributing functional and morphological characteristics of the VL would also need to be reliable. According to all statistical analyses, measurements of isometric MVC_{KE} torque, fascicle length and pennation angle were deemed reliable. In particular, ratio limits of agreement indicate small inter-day differences in measurements of isometric MVC_{KE} torque (7.3%), fascicle length (<1.0%) and pennation angle (2.8%), which would suggest adequate repeatability of the current measurement techniques. Comparisons with previous literature demonstrated the mean isometric MVC_{KE} torque in the current study was similar to those obtained (~200-270 N·m) using equivalent methods in other studies (Erskine et al., 2009; Reeves et al., 2004c). Similarly, the measurements of VL fascicle length and pennation angle in the current study (8.5 cm and 14.6° respectively) were comparable to those obtained previously using ultrasound, which ranged from 8.4 cm to 9.1 cm and 12.5° to 16.0°, respectively (Erskine et al., 2009; Reeves et al., 2004c). It is evident from the close ratio limits of agreement (<10.7%), high ICCs (>0.979) and the relatively small CVs (<5.6%) that the calculation of VL fascicle force and specific force based on the aforementioned methods was reliable. Furthermore, VL specific force has previously been reported between 23.6-27.0 $N \cdot cm^{-2}$ in studies employing a variety of techniques to assess muscle volume (Reeves

et al., 2004c; Narici et al., 1992), suggesting the current method of estimating muscle volume does not negatively affect the reliability of subsequent measurements.

Measurement reliability can be compromised as a result of investigator, procedural and equipment error (Dvir, 2004). For example, when using ultrasound to image muscle properties, Esformes (2002) stated the importance of applying minimal pressure to the probe to prevent muscle tissue compression, which could impact on the reliability of measurements of muscle architecture and ACSA. Furthermore, high reliability of the Cybex isokinetic dynamometer system used to assess isometric MVC has been reported previously, with ICCs between 0.92-0.98 (Impellizzeri et al., 2008; Bandy & McLaughlin, 1993). However, visual alignment of the knee joint centre of rotation to the dynamometer axis could be a potential source of error if not recorded in studies requiring one or more re-tests (Sole et al., 2007). In an attempt to increase the measurement reliability of the current study, procedural and investigator errors were minimised by having one investigator following a standardised procedure for all participants. Furthermore, the inclusion of a familiarisation session prior to day 1 may have reduced any learning effects from repeated trials and thus reduced the measurement errors associated with participant variability (Sole et al., 2007).

3.5 Conclusion

For studies requiring large (>50), homogenous samples such as for performing genetic associations between muscle-related phenotypes and single nucleotide polymorphisms, it is essential to use reliable measurements of muscle properties to minimise the likelihood of type II error. The very good agreement observed for the repeated measurement of VL

muscle volume, specific force and a range of other measurements of functional and morphological muscle characteristics demonstrated in the current study suggest the aforementioned method is suitable when gold standard equipment is inaccessible or time may be limited.

Chapter 4

Variability and distribution of muscle strength and its determinants in humans

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4.1 Introduction

Human skeletal muscle is a highly adaptive tissue that responds to changes in functional loading, and consequently muscle strength (here defined as maximal isometric joint torque) is known to vary between individuals. Much of the variability among untrained, asymptomatic individuals of a similar age has been attributed to differences in the structural and neural determinants of muscle strength (Maughan et al., 1983; Erskine et al., 2009).

Muscle size is generally considered to be the greatest determinant of muscle strength (Maughan et al., 1983; Knuttgen, 1976), and measurements of muscle thickness have revealed inter-individual variability in appendicular muscle size ranging from 9-18% (Wakahara et al., 2009). Assessments of muscle thickness, however, are relatively simple and may actually underestimate the true contractile area of the muscle that contributes to force production (Alexander & Vernon, 1975; Wickiewicz et al., 1983; Reeves et al., 2004b). Physiological cross-sectional area (PCSA) on the other hand, provides a more accurate assessment of muscle contractile area than measurements of muscle thickness or anatomical cross-sectional area (ACSA) by accounting for inter-individual differences in muscle architecture and muscle length. Taking account of such differences by using PCSA could reduce the inter-individual variability compared with ACSA, which represents only an estimate of true contractile area in pennate muscles (Alexander & Vernon, 1975). However, as PCSA is affected directly by muscle length while ASCA is not, it could alternatively be that PCSA may demonstrate greater inter-individual variability than ACSA. Thus, the relationships between PCSA, ACSA, muscle length, and muscle architecture are

complex, and it is difficult to predict realistically whether the inter-individual variability in PCSA and ACSA in a population will be similar or different in magnitude.

The importance of measuring agonist and antagonist muscle activation during assessments of *in vivo* maximal isometric strength has been highlighted previously (Maganaris et al., 2001; Reeves et al., 2004c). While reports of voluntary activation capacity in untrained individuals are somewhat contradictory (Moritani & deVries, 1979; Häkkinen et al., 1998; Thorstensson et al., 1976; Komi & Buskirk, 1972), sensitive use of the interpolated twitch technique suggests that untrained individuals probably cannot activate 100% of their motor units (Folland & Williams, 2007b). All else being equal, individuals with greater observed maximal voluntary contraction (MVC) torque are likely to have greater voluntary activation capacity. Therefore, accounting for inter-individual differences in voluntary activation capacity is likely to increase the calculated joint torque relatively more in weaker muscles, bringing the values closer to the mean in a population. This should have the net effect of reducing inter-individual variability in calculated maximal joint torque compared to that in observed MVC torque. Furthermore, antagonist muscle co-activation of the hamstrings during knee extension MVCs has been reported to range between 15-30% in healthy, untrained, adults (Carolan & Cafarelli, 1992; De Vito et al., 2003; Macaluso et al., 2002). Once again, all else being equal, individuals with greater observed MVC torque are likely to have lower antagonist co-activation, whereas those with lower observed MVC torque are likely to have higher antagonist co-activation. Consequently, accounting for inter-individual differences in antagonist co-activation should increase the calculated

maximal joint torque relatively more in the weaker muscles, thus bringing those values closer to the mean in a population and reducing inter-individual variability.

A tendon moment arm functions as a lever of effective force transmission during muscle contraction (Tsaopoulos et al., 2006) and is therefore central to accurate measurement of muscle force from torque. Bone geometry has been suggested as the primary determinant of tendon moment arm length and, as this is known to differ between individuals, is probably also the main source of inter-individual variability in moment arm length (Maganaris et al., 2001; Tsaopoulos et al., 2006; Tsaopoulos et al., 2007b). Therefore, assuming all else is equal, individuals with longer tendon moment arms would produce greater isometric MVC torque than those with shorter tendon moment arms. One would also expect individuals with larger bone geometry and longer moment arms to generally possess larger, stronger muscles. Therefore, individuals capable of producing values of MVC torque above the observed mean value in a population are likely to have MVC torque further inflated by a longer moment arm, and those below the mean further reduced by the shorter moment arm, which would exaggerate the deviation of observed MVC torque values from the mean in a population. Consequently, controlling for moment arm length when calculating tendon force (and subsequently muscle force) should result in reduction in the distribution of observed force values and thus reduction in inter-individual variability in muscle force compared with observed isometric MVC torque.

Muscle specific force reflects the intrinsic strength of a muscle and is estimated by accounting for all of the aforementioned determinants of strength. As such, it could be

expected that reports of specific force within the literature would be homogenous. However, measurements of human muscle specific force *in vivo* are widespread, ranging from 6-86 $N \cdot cm^{-2}$ (Maughan et al., 1983; Erskine et al., 2009; Reeves et al., 2004c; Narici et al., 1992; Chow et al., 1999; Gorgey et al., 2006; Claassen et al., 1989). Differences in sample selection and/or inconsistencies in measurements could contribute to such widespread values. Unsurprisingly, when only those studies that accounted for all of the necessary factors were considered, the range of values for *in vivo* specific force of the vastus lateralis (VL) was noticeably reduced to 20-30 $N \cdot cm^{-2}$ (Erskine et al., 2009; Reeves et al., 2004c; Narici et al., 1992; Chow et al., 1999).

Consequently, the aim of the study was three-fold; firstly to develop a normative set of data on the inter-individual variability in measurements of muscle strength and its determinants in a relatively large, homogenous sample. Secondly, to demonstrate the extent of the differences in the inter-individual variability between the less stringent measurements of strength and its determinants (isometric MVC torque and ACSA) compared with the more stringent measurements (specific force, fascicle force, and PCSA). The final aim was to ascertain the strength of the relationship between the different measurements of muscle strength and size. We hypothesized that a stronger relationship would exist between PCSA and VL fascicle force than between ACSA and isometric MVC torque, because greater physiological variability is accounted for in the more stringent measurements of muscle size and strength.

4.2 Methods

Detailed descriptions of participant recruitment and the assessment of skeletal muscle properties are included in Chapter 2 (section 2.1 and 2.2 respectively), thus only a brief description of these methods is detailed below.

4.2.1 Participants

A sample of 73 untrained Caucasian males [age 20.6 (2.5) yr, stature 1.78 (0.07) m and mass 76.0 (9.8) kg; mean (SD)] volunteered to participate in this study. All participants met the inclusion criteria (described in Chapter 2.1) and provided written informed consent prior to involvement.

4.2.2 Skeletal muscle properties

Maximal voluntary knee extension (MVC_{KE}) and flexion (MVC_{KF}) torque was measured at three knee joint angles to identify the angle of peak torque production. Agonist muscle activation (Eq 1) and antagonist muscle co-activation during MVC_{KE} was determined to enable the calculation of net MVC_{KE} torque (Eq 2). Measurement of VL ACSA, by contour matching a series of transverse plane scans, was used in the estimation of VL muscle volume (Eq 3). VL fascicle length, pennation angle and patellar tendon moment arm length were measured and used in the calculation of PCSA (Eq 4), fascicle force (Eq 6) and tendon force (Eq 5), respectively. Ultimately, VL specific force was calculated by dividing VL fascicle force by VL PCSA (Eq 7).

4.2.3 Statistical analysis

Coefficients of variation (CV) were calculated to identify the extent of inter-individual variability in all functional and morphological characteristics of the VL using Microsoft Excel. To determine any differences in inter-individual variability between isometric MVC_{KE} torque, VL fascicle force, VL ACSA, and VL PCSA, a Friedman ANOVA was conducted using corrected percentage distribution data, which breached the parametric assumption of normal distribution following correction (Statistical Package for Social Sciences 19.0, SPSS Inc., Chicago, Il, USA). The Wilcoxon signed-rank test was used to perform appropriate *post-hoc* analyses where necessary. Regression analyses were conducted to determine the relation between isometric MVC_{KE} torque and VL ACSA, and VL fascicle force and VL PCSA. The use of the Fisher Z-transformation enabled the difference between these correlations to be analysed. Reliability of the architectural measurements was determined by calculation of ratio limits of agreement (LoA)(Atkinson & Nevill, 1998) and CVs on data collected during pilot testing on 2 separate occasions separated by 1 day for 8 participants (Chapter 3). Data are displayed as means (SD), and statistical significance was set at $P \leq 0.05$.

4.3 Results

Descriptive data on the functional and morphological characteristics of the VL are presented with CVs in Table 4.1. Notably, the CVs of VL specific force and VL ACSA were lower than those for isometric MVC_{KE} torque and VL PCSA, respectively. The Shapiro-Wilk test revealed that the data were distributed normally (P = 0.063-0.706). Calculation of ratio LoA and CVs were used to determine the repeatability of architectural measurements on two occasions by the same investigator on eight participants (Table 4.2). There was no

significant difference between day 1 and day 2; all limits of agreement were less than 10%, and most were less than 6%, which when taken in the context of the measurement showed very good reliability. For example, based on the reliability data presented in Chapter 3, together with the mean data presented in Table 4.1, differences in the measurements of VL specific force (1.2 $N \cdot cm^{-2}$) and PCSA (4.2 cm^2) could be expected for an individual estimated to have a VL muscle volume of 561 cm^3, which according to previous data would suggest appropriate measurement sensitivity (Erskine et al., 2013).

Table 4.1. Functional and morphological characteristics of the vastus lateralis.

Variable	Mean (SD)	Range	CV (%)
Isometric MVC_{KE} torque (N·m)	259 (49)	168-363	18.9
Activation capacity (%)	89.5 (5.1)	80.1-98.5	5.7
Antagonist co-activation (%)	13.9 (1.0)	8.0-24.8	6.8
Net KE torque (N·m)	282 (50)	146-339	17.9
Muscle volume (cm^3)	561 (115)	424-816	20.2
ACSA (cm^2)	21.3 (2.8)	14.1-28.6	13.0
Fascicle length (cm)	8.0 (1.3)	6.3-11.5	16.6
Pennation angle (°)	14.6 (2.4)	9.0-21.4	16.7
PCSA (cm^2)	65.7 (11.0)	43.3-114.5	17.2
Moment arm (cm)	4.4 (0.4)	3.5-5.3	8.8
Patellar tendon force (N)	6430 (1113)	4624-8270	17.3
VL fascicle force (N)	1458 (213)	1079-1812	14.6
Specific force ($N \cdot cm^{-2}$)	23.8 (3.5)	17.7-27.9	13.5

ACSA, anatomical cross-sectional area; CV, coefficient of variation MVC_{KE}, maximal voluntary knee extension contraction; PCSA, physiological cross-sectional area; VL, vastus lateralis.

Table 4.2. Inter-day measurement reliability.

Variable	CV (%)	LoA (%)	Mean (SD)
Muscle volume (cm^3)	2.5	5.3	523 (14)
Pennation angle (°)	2.1	2.6	14.5 (2.4)
Fascicle length (cm)	2.5	4.5	7.8 (0.6)
Moment arm length (cm)	1.8	2.5	4.5 (0.2)
Specific force ($N \cdot cm^{-2}$)	5.6	9.9	20.2 (3.3)

The mean knee joint angle at which maximal MVC_{KE} torque was determined was 80°. Histograms showing the percentage deviation from the mean value for isometric MVC_{KE} torque, VL fascicle force, VL ACSA, and VL PCSA can be seen in Figure 4.1. Output from a Friedman ANOVA revealed a significant difference ($P < 0.0005$) in corrected distribution data. *Post-hoc* analyses identified a significant difference in the percentage distribution between isometric MVC_{KE} torque and VL fascicle force ($P = 0.025$) and between VL ACSA and VL PCSA ($P < 0.0005$).

A regression analysis revealed a significant relationship between VL ACSA and isometric MVC_{KE} torque ($r^2 = 0.57$; $P < 0.0005$, Figure 4.2 A). Additionally, there was a significant relationship between VL PCSA and VL fascicle force ($r^2 = 0.68$; $P < 0.0005$, Figure 4.2 B). However, the relationship between VL PCSA and VL fascicle force was not significantly different from the relationship between VL ASCA and isometric MVC_{KE} torque ($P = 0.359$). The relation between stature and both PCSA and ACSA was assessed using regression

analyses and a stronger significant relationship between PCSA and stature (r^2 = 0.674, P < 0.0005) when compared with ACSA and stature (r^2 = 0.217, P = 0.001) was apparent.

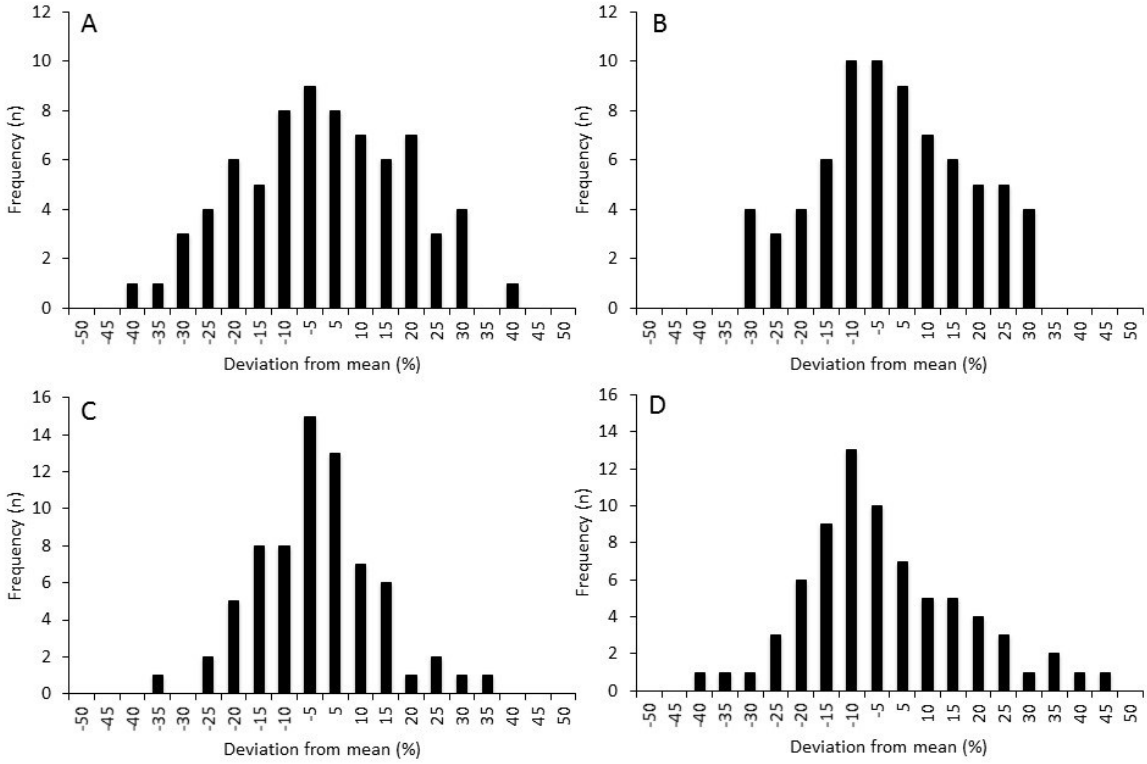


Figure 4.1. Frequency distributions around the mean (displayed as %) for: **A**) MVC_{KE} torque, **B**) VL fascicle force, **C**) VL ACSA, and **D**) VL PCSA. Significant differences were apparent between MVC_{KE} torque and VL fascicle force (P = 0.025) and ASCA and PCSA (P < 0.0005).

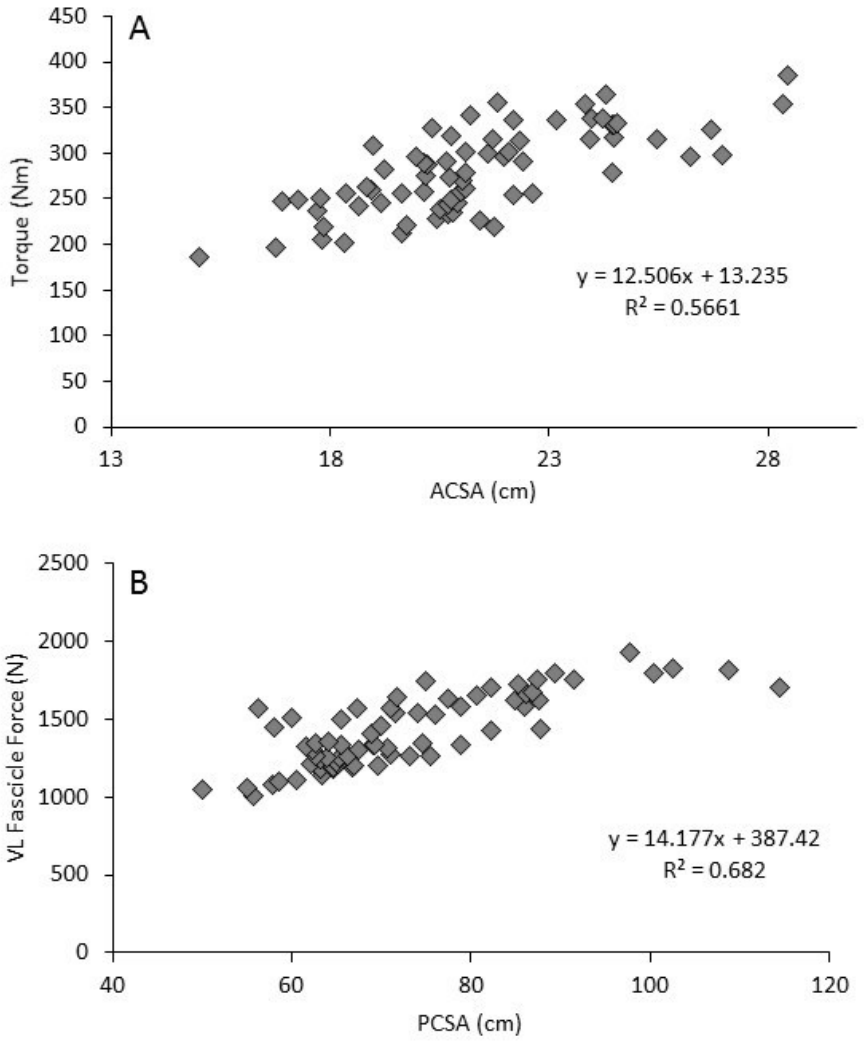


Figure 4.2. A) The relationship between VL ACSA and isometric MVC_{KE} torque (P < 0.0005); **B**) The relationship between VL PCSA and VL fascicle force (P < 0.0005).

4.4 Discussion

One of the aims of this study was to develop a normative set of data on the inter-individual variability in measurements of muscle strength and its determinants in a relatively large, homogenous sample. This was achieved for 73 asymptomatic young men using a range of measurements related to muscle size and strength. This not only applies to mean values,

but in particular to inter-individual variability. Table 4.1 presents those data and includes CVs ranging from 5.7% (agonist activation capacity) to 20.2% (muscle volume). These data are useful for researchers who investigate the causes of inter-individual variability in these parameters, such as various genetic and environmental factors.

Inter-individual variability in the measurement of isometric MVC_{KE} torque is associated with the variability of its determinants. It was hypothesized that accounting for these determinants in the calculation of specific force would result in a reduction in inter-individual variability compared to that present in the measurement of isometric MVC_{KE} torque. Inter-individual variability in specific force (13.5%) is comparable with previous reports in healthy, untrained adults (16.2%)(Erskine et al., 2009; Degens et al., 1995). Despite this, the hypothesis is only partially accepted, as the inter-individual variability in specific force was only 4% less than the inter-individual variability in isometric MVC_{KE} torque. Nevertheless, the difference in inter-individual variability between specific force and isometric MVC_{KE} torque is slightly more than that reported previously (3%) in the only other study to the author's knowledge that investigated inter-individual variability in specific force *in vivo* in a smaller (n = 27), but comparable sample (Erskine et al., 2009).

Differences in the inter-individual variability of isometric MVC_{KE} torque, tendon force and specific force are dependent on the differing extent to which the physiological determinants are accounted for. For example, gross measurements of strength such as isometric MVC_{KE} torque are likely to be more susceptible to this inherent variation, because they are influenced by the inter-individual variability in agonist and antagonist muscle

activity (Reeves et al., 2009). In contrast, specific force may provide a more accurate representation of the contractile properties of the muscle while accounting for inter-individual variability in neural properties, tendon moment arm length, and muscle architecture. Unsurprisingly, in the current study the inter-individual variation in isometric MVC_{KE} torque was greater than that for specific force and all of its determinants (with the exception of muscle volume). However, the inter-individual variation observed in agonist activation and antagonist co-activation was relatively small (5.7% and 6.8%, respectively). It is evident that muscle activation in untrained, young males is relatively complete and likely contributes to a lower degree of inter-individual variability in the measurement of isometric MVC_{KE} torque than in some other populations. It could be expected that within a more heterogeneous population where activation levels show greater variability greater inter-individual variability in isometric MVC_{KE} torque would also be observed.

A significant relationship was observed between isometric MVC_{KE} torque and ACSA (r^2 = 0.57), which is comparable to that reported previously in the plantar flexors and dorsiflexors (r^2 = 0.59-0.62)(Fukunaga et al., 1996). As expected, this relationship was weaker than that between VL fascicle force and PCSA (r^2 = 0.68), although this difference was not significant statistically. The high inter-individual variability in isometric MVC_{KE} torque and ACSA is likely to contribute to this tendency for a difference. Inter-individual variability in VL ACSA was found to be 13%, which is comparable to previous reports of approximately 14% in muscle thickness of the rectus femoris and vastus intermedius in untrained men (Wakahara et al., 2009). In comparison, the observed inter-individual variability in VL PCSA was greater than for VL ACSA. A plausible explanation for the greater

inter-individual variability in VL PCSA compared with VL ACSA could be the consequence of the inter-individual variability observed in pennation angle and fascicle length. The source of inter-individual variation in these measures of muscle size and architecture could be differences in body size (Gallagher et al., 1997; Janssen et al., 2000). Both PCSA and ACSA are related to body mass, whereas only PCSA is related to stature, given that fascicle length is proportional to femur length. This is substantiated in this study, which revealed a stronger relationship between VL PCSA and stature than between VL ACSA and stature.

The lower inter-individual variability observed in VL fascicle force compared to that in isometric MVC_{KE} torque could be attributed to architectural and structural factors. By accounting for inter-individual variability in tendon moment arm length it is possible to account for differences in bone geometry (and hence body size) within a population; this has been suggested previously to be the key determinant of tendon moment arm length (Krevolin et al., 2004). For example, for any given VL fascicle force, the 8.8% inter-individual variability in moment arm length observed in the current chapter would result in an isometric MVC_{KE} torque difference of 23 N·m. It should be noted that much of the reported variation in bone geometry, however, has been observed between different ethnic populations (Seeman, 1997) and genders (Krevolin et al., 2004), whereas the current chapter sampled only Caucasian men. Nonetheless, the inter-individual variation in tendon moment arm length made a contribution to the inter-individual variability observed in the estimation of VL specific force.

Despite the obvious sources of variation in the measurement of isometric MVC_{KE} torque and its determinants, the calculation of specific force failed to reduce this to the extent envisaged initially. It is possible that differences in the intrinsic force-generating capacity of individual fibres exist which contribute to the observed inter-individual variability (Erskine et al., 2009). One plausible explanation is inter-individual differences in fibre type composition of the VL (Glenmark et al., 1992; Simoneau & Bouchard, 1989; Staron et al., 2000), as type I fibres are reported to have lower specific tension than type II fibres (Bottinelli et al., 1996; Harridge et al., 1996). Additionally, inter-individual variability in VL specific force may be explained by the presence of intramuscular fat and connective tissue (Macaluso et al., 2002; Kent-Braun et al., 2000; Frontera et al., 1991). Variation in intra-muscular non-contractile material has been observed previously in a sample of young adults (Macaluso et al., 2002), and although this was not measured in the current chapter, it could contribute to some of the unexplained inter-individual variability in specific force. Failure to account for the presence of intra-muscular non-contractile material would result in an overestimation of muscle PCSA and thus underestimate muscle specific force (Erskine et al., 2009; Frontera et al., 1991). On the other hand, it has been suggested that increased connective tissue content may be associated with improved lateral force transmission from the muscle fibre to the tendon, the consequence of which would be an increase in muscle specific force (Jones et al., 1989). Furthermore, myofilament-packing density is known to influence cross-bridge interaction of actin and myosin filaments and consequently may also contribute to the observed inter-individual variability in specific force (Alway, 1980). Data on inter-individual variability in human skeletal myofilament-packing density is lacking, although reports from training studies have found no change in pre- and post-training

packing densities (MacDougall et al., 1980; McCullagh et al., 1983). Nonetheless, more research is needed to establish if myofilament packing density varies in untrained adults. One way in which fibre type composition and intramuscular values of collagen and adipose tissue could be accounted for is through biopsy, however, the estimate of whole muscle properties based on biopsies may be limited (Clarkson et al., 2005).

The assessment of muscle specific force includes a number of assumptions or surrogate measures where direct measurement is not possible. For example, previous studies have adopted estimates of fascicle length based on previously published values of muscle length (Kawakami et al., 1994). Similarly, where MRI is not available to measure muscle volume directly, estimates have been made based on single measures of ACSA multiplied by limb length. Indeed the measurement of moment arm during MVC requires X-ray fluoroscopy to account for deformation and extension of the moment arm through contraction; in contrast, moment arm is often estimated based on external anthropometric measures (Morse et al., 2005a). In the current body of work, direct measurement of moment arm length during MVC was not possible, and force was estimated from resting measures of moment arm length. Furthermore, muscle volume was estimated based on a single measure of ACSA. Although both of these methods have been demonstrated to be valid surrogates in the calculation of specific force (Morse et al., 2007; Cotofana et al., 2010; Wretenberg et al., 1996; Reeves et al., 2004b), direct measurement may have improved the validity of the measures.

One of the applications of these data is in research into genetic factors that may be associated with specific force, by seeking to minimise unexplained inter-individual variability in associated strength measurements. By 2007, 22 genetic polymorphisms associated specifically with a muscle strength-related phenotype had been reported, however this number will have increased in subsequent years (Hughes et al., 2011). Using more stringent measurements of muscle strength and size that reduce confounding variability, as demonstrated here, would increase the likelihood of identifying small associations between individual genetic polymorphisms and strength-related phenotypes.

4.5 Conclusion

In conclusion, the current chapter confirmed the extent of the inter-individual variability previously reported in human muscle specific force and isometric MVC torque. Furthermore, establishing the inter-individual variability in the factors involved in the determination of muscle strength provides normative data on a relatively large sample of apparently healthy, untrained men that had previously remained unreported. These results substantiate previous findings that calculation of inter-individual variability in human knee extension specific force explained little of the inter-individual variability observed in MVC torque. Thus, factors other than muscle fibre architecture, moment arm length, and agonist muscle activation and antagonist muscle co-activation appear to contribute to the observed variation.

Chapter 5

Individual influence of *ACTN3, CNTF, COL5A1, PTK2* and *TTN* polymorphisms on muscle architectural phenotypes

5.1 Introduction

Skeletal muscle architecture, here defined as fascicle length and pennation angle, is an important determinant of muscle function. Maximal muscle fibre shortening velocity is proportional to the number of sarcomeres arranged in series, or fascicle length; whilst the production of maximal muscle force is proportional to the number of sarcomeres arranged in parallel [physiological cross-sectional area (PCSA)], which for a given muscle volume is largely determined by fibre pennation angle (Narici, 1999).

Muscle shortening velocity is one determinant of power output (Josephson, 1993), and previous research into elite power athletes has identified a significant correlation (r = -0.43 – -0.57) between muscle fascicle length and sprint performance (Kumagai et al., 2000; Abe et al., 2000). Conversely, reductions in power output (-81% and -27%) have been observed alongside decreases in muscle fascicle length (-19%) in ageing populations (Thom et al., 2007) and following disuse (Rittweger et al., 2007; de Boer et al., 2008). Whilst it is probable that environmental factors such as training and detraining adaptations contribute to the differences in fascicle length in these populations, the work presented in Chapter 4 demonstrates that in homogenous untrained populations, where the contribution of such environmental influences is likely to be reduced, inter-individual variability in fascicle length persists.

The ability to generate maximal muscle force is the product of two opposing factors culminating in an increase in pennation angle of up to 45°, beyond which point the ability

of the muscle to transmit force effectively to the tendon is diminished and force production decreases accordingly (Alexander & Vernon, 1975; Rutherford & Jones, 1992). Changes in pennation angle are commonly reported following muscle hypertrophy and atrophy, and greater pennation angles have been observed for resistance trained compared to untrained individuals (+8-36%) (Aagaard et al., 2001; Reeves et al., 2004c; Seynnes et al., 2007). Again, although it is probable that training and detraining adaptations contribute substantially to the observed differences in fibre pennation angle within these populations, it is also possible that these individuals are completing the training most suited to them as a consequence of their muscle architecture. Furthermore, in untrained populations where the contribution of environmental factors to the observed variability is likely to be reduced, inter-individual variability in pennation angle of the vastus lateralis (VL) is approximately 17% (Chapter 4). Thus, it is possible that differences in both pennation angle and fascicle length are influenced to some extent by genetic factors.

To date there has been a considerable amount of research into skeletal muscle fascicle length and pennation angle in relation to different aspects of muscle function, which together with the work presented in Chapter 4, demonstrates the persistent inter-individual variability apparent within these phenotypes (Abe et al., 2000; Erskine et al., 2009; Fukunaga et al., 1997; Reeves et al., 2004a). Although many of the authors within this area have speculated about a potential genetic contribution to the inter-individual variability in fascicle length and pennation angle, research is lacking and thus appropriate candidate genes need identifying.

A plethora of proteins exist within the sarcomere, thus polymorphisms in any number of the genes encoding these proteins could contribute to the variability observed in muscle architecture. Due to the close association between muscle architecture and contractile force, a reasonable starting point to identify suitable candidate genes would be to investigate those polymorphisms within sarcomeric proteins that have already demonstrated associations with strength and/or power-related phenotypes. For example, a common polymorphism within α-actinin-3 (*ACTN3*), the gene encoding the ACTN3 protein involved in anchoring actin filaments within type II muscle fibres, has been identified (North & Beggs, 1996). Individuals homozygous for the 'R' allele are able to produce the fully functioning ACTN3 protein whereas homozygous XX individuals are not (Clarkson et al., 2005). Overexpression of the R-allele and under-expression of the X-allele has been reported in strength and power trained athletes (Eynon et al., 2013). Although an association between *ACTN3* and muscle architecture has yet to be investigated, it is possible that the presence of the protein may influence fascicle length and/or pennation angle and thus explain some of the previous associations between *ACTN3* and increased muscle strength and/or power.

Furthermore, two polymorphisms within protein tyrosine kinase 2 (*PTK2)*, a gene encoding the phosphoprotein focal adhesion kinase (FAK), have previously been associated with increased muscle specific force (Erskine et al., 2012). An association believed to exist as a result of an improved capacity to transmit muscle contractile force laterally to the extracellular matrix and tendon (Erskine et al., 2012). It is reasonable to speculate therefore, that improvements in lateral force transmission may be facilitated by increased

muscle fibre pennation angles, as this would allow for the arrangement of a greater number of smaller parallel muscle fibres, subsequently increasing muscle costamere density.

Titin is a major sarcomeric protein commonly referred to as a 'ruler' for myofilament length, therefore polymorphisms within the gene encoding this protein (*TTN*) could potentially contribute to the inter-individual variability observed in muscle fascicle length. To the author's knowledge, however, no reports of this single nucleotide polymorphism (SNP) and associations with skeletal muscle phenotypes currently exist. Interestingly, a C > T transition identified within *TTN* has previously been reported as one of 11 SNPs explaining a proportion of the variability in the training response of maximal oxygen consumption (Timmons et al., 2010). This is most likely due to differences in a cardiac titin isoform influencing the training-related increase in stroke volume due to more/less effective use of the Frank-Starling mechanism, depending on the genotype present (Rankinen et al., 2003). Therefore, if *TTN* genotype is responsible for regulating these cardiac-specific changes to the myofilament, it stands to reason that *TTN* genotype may influence skeletal muscle similarly.

In addition to identifying genes encoding sarcomeric proteins, the possible influence of proteins within the extracellular matrix (ECM) should not be overlooked. The primary structural protein within skeletal muscle ECM is collagen, of which type I and type III are predominantly expressed in the perimysium (type I), endomysium and epimysium of skeletal muscle (both type III) (Gillies & Lieber, 2011). Collagen type V associates with both type I and type III collagen, and mutations in type V collagen have been associated with

joint range of motion (Collins et al., 2009) and are also responsible for the development of Ehlers-Danlos syndrome, a connective tissue disorder characterised by joint instability, chronic myalgia and muscle hypotonia (Voermans et al., 2008). Mutations identified in the collagen type V alpha 1 (*COL5A1*) gene (encoding the α1 chain of collagen type V) disrupt collagen fibril formation and results in irregular fibril packing and diameter (Beighton et al., 1998). Thus, it is possible that potential differences in collagen fibres of the ECM may impact on the parallel arrangement of the muscle fibres they surround, and should be investigated.

Another potential candidate gene is ciliary neurotrophic factor (*CNTF*), a member of the interleukin-6 cytokine family with known myotrophic effects (Guillet et al., 1999), which has also previously been associated with muscle strength and size both at baseline and in response to resistance training in humans (Roth et al., 2001; Walsh et al., 2009). However, as neither of the aforementioned studies measured muscle architecture, it is unclear whether the observed genotype differences in muscle strength and size could be explained to some extent by differences in muscle fascicle length and/or pennation angle. Thus, *CNTF* is an ideal candidate gene to investigate the potential genetic influence on muscle architecture.

The aim of this chapter, therefore, was to investigate the influence of polymorphisms within *ACTN3*, *CNTF*, *COL5A1*, *PTK2* and *TTN* on muscle architectural phenotypes (fascicle length and pennation angle) in an untrained, apparently healthy population.

5.2 Methods

Detailed descriptions of participant recruitment, assessment of skeletal muscle properties, and the genotyping of the *ACTN3* (rs1815739), *CNTF* (rs1800169), *COL5A1* (rs12722), *PTK2* (rs7843014 and rs7460) and *TTN* (rs10497520) polymorphisms is included in Chapter 2 (section 2.1, 2.2 and 2.4, respectively), thus only a brief description of these methods is detailed below.

5.2.1 Participants

A sample of 120 untrained Caucasian males [age 20.6 (2.3) yr, stature 1.79 (0.06) m and mass 75.1 (10.1) kg; mean (SD)] volunteered to participate in this study. All participants met the inclusion criteria (described in Chapter 2.1) and gave written informed consent prior to involvement.

5.2.2 Skeletal muscle architecture

Muscle architecture of the VL was assessed *in vivo* using ultrasound at 50% of muscle length. Single ultrasound images were used for the measurement of VL fascicle length and pennation angle. Identification of fascicle length was achieved by measuring the distance from fascicular origin to insertion on the aponeuroses. In instances where muscles extended beyond the ultrasound field of view, fascicle length was estimated by extrapolating the deep and superficial aponeuroses and fascicle. Pennation angle was measured as the angle of fascicular insertion into the deep aponeurosis. A minimum of

three fascicles were measured per image and an average was taken as VL fascicle length and pennation angle.

5.2.3 Genotyping

Genotyping was completed using the fluorophore-based detection technique of TaqMan® real-time PCR. Genomic DNA amplification of fragments overlapping polymorphisms in the *ACTN3*, *CNTF, COL5A1*, *PTK2* and *TTN* genes was completed for all participants.

5.2.4 Statistical analysis

The frequency of each SNP was assessed for compliance with Hardy-Weinberg equilibrium using X^2 tests. A one-way analysis of variance (ANOVA) was conducted to determine any significant differences in physical characteristics (stature, mass, BMI and age) between genotype. When genotype groups were combined, an independent samples *t*-test was used to identify any differences in physical characteristics. Pearson's correlation coefficient was used to identify the variables that made a meaningful contribution to the variability within muscle fascicle length and pennation angle, and were then included as confounding variables. ANOVA, and where appropriate analysis of covariance (ANCOVA), was conducted to identify any genotype differences in muscle architectural phenotypes. Additionally, any genotype effects on muscle architectural phenotypes were also assessed for linear trend using ANOVA or ANCOVA. In instances when too few participants represented one genotype group, this group was combined with the heterozygous group and an independent samples *t*-test was used to identify any differences between muscle architectural phenotypes. All significant associations identified in the main ANOVA or

ANCOVA analyses were subject to *post-hoc* pairwise comparisons using the Benjamini-Hochberg correction. In instances where a tendency between genotype groups was observed, such that $P > 0.05$ but < 0.15, the two groups with similar means were combined and the analysis re-run using independent samples *t*-tests. All statistical analyses were performed using SPSS version 19.0 and statistical significance was accepted when $P \leq 0.05$. Data are presented as mean (SD).

5.3 Results

Genotype frequencies for SNPs in the *ACTN3*, *COL5A1*, *CNTF*, *PTK2* and *TTN* genes are presented in Table 5.1 and were all in Hardy-Weinberg equilibrium ($P \geq 0.436$).

No differences between any SNP genotypes were observed for stature ($P \geq 0.196$), mass ($P \geq 0.091$), BMI ($P \geq 0.130$) or age ($P \geq 0.094$; Chapter 2, Table 2.2). Pearson's correlation coefficients revealed significant weak correlations between VL muscle architecture (both pennation angle and fascicle length), and body mass ($r \geq 0.190$, $P \leq 0.039$), BMI ($r \geq 0.200$, $P \leq 0.03$), muscle thickness ($r \geq 0.225$, $P \leq 0.014$) and muscle length ($r \geq 0.231$, $P \leq 0.012$). Consequently, body mass, BMI, muscle thickness and muscle length were all included as covariates where appropriate in subsequent analyses.

Table 5.1. Means (SD) for fascicle length and pennation angle according to genotype for SNPs in the *ACTN3*, *COL5A1*, *CNTF*, *PTK2* and *TTN* genes.

SNP	Genotype Number	Frequency (%) Genotype	Frequency (%) Minor Allele	Fascicle Length (cm)	Pennation Angle (°)	Hardy-Weinberg
ACTN3 (rs1815739)	RR (*n* = 44)	36.7	0.396	7.1 (1.6)	19.3 (3.7)	0.997
	RX (*n* = 57)	47.5		7.2 (1.8)	19.7 (3.9)	
	XX (*n* = 19)	15.8		7.1 (1.1)	19.2 (4.2)	
COL5A1 (rs12722)	CC (*n* = 19)	15.8	0.413	7.4 (1.8)	20.3 (3.3)	0.867
	CT (*n* = 61)	50.8		7.0 (1.4)	19.2 (4.0)	
	TT (*n* = 40)	33.4		7.3 (1.7)	19.4 (4.0)	
CNTF (rs1800169)	AA (*n* = 2)	1.7	0.154	6.5 (1.6)	22.2 (0.6)	0.837
	GA (*n* = 33)	27.5		7.0 (1.5)	21.0 (3.6)	
	GG (*n* = 85)	70.8		7.2 (1.6)	18.8 (3.8)	
PTK2 (rs7843014)	AA (*n* = 42)	35.0	0.433	7.2 (1.8)	19.2 (3.8)	0.436
	AC (*n* = 52)	43.3		7.0 (1.4)	20.1 (4.0)	
	CC (*n* = 26)	21.7		7.5 (1.4)	18.5 (3.6)	
PTK2 (rs7460)	AA (*n* = 34)	28.3	0.492	7.3 (1.4)	18.8 (3.2)	0.550
	AT (*n* = 54)	45.0		7.1 (1.7)	20.2 (4.6)	
	TT (*n* = 32)	26.7		7.2 (1.7)	18.8 (3.0)	
TTN (rs10497520)	CC (*n* = 95)	79.2	0.104	7.3 (1.6)	19.3 (3.8)	0.444
	CT (*n* = 25)	20.8		6.6 (1.1)	20.1 (3.9)	
	TT (*n* = 0)	0.0		-	-	

Due to low numbers of *CNTF* AA homozygotes (*n* = 2) present in the sample population, these data were combined with those of the heterozygotes and a two-group analysis completed. VL pennation angle was significantly greater for A-allele carriers than GG homozygotes for *CNTF* (11.1%, *t* = 3.148, *P* = 0.002; Figure 5.1). No differences between *CNTF* A-allele carriers and GG homozygotes for VL fascicle length (*t* = 0.924, *P* = 0.357) were observed.

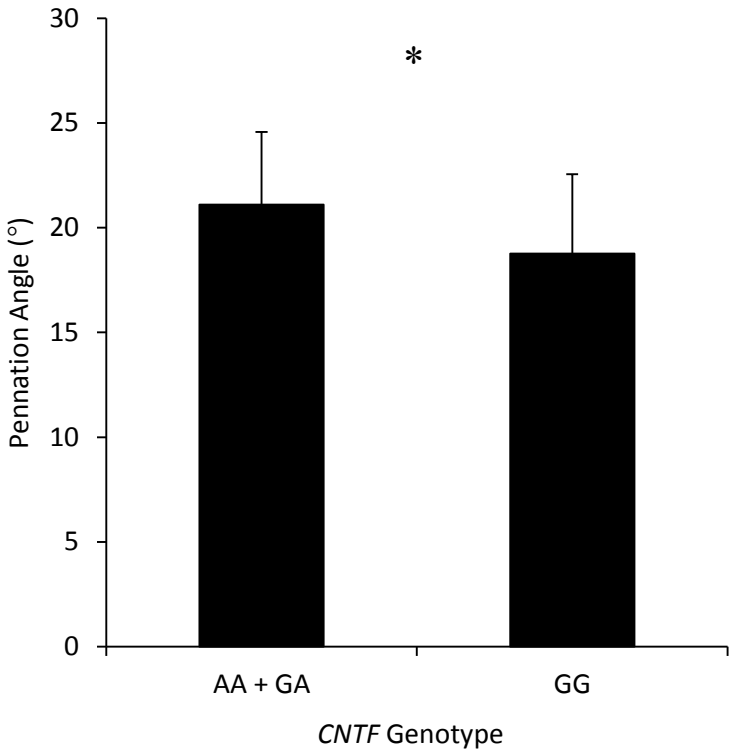


Figure 5.1. Comparison of VL pennation angle by the combined preferential (*n* = 35) and non-preferential (*n* = 85) *CNTF* genotype (**P* = 0.002). Data presented are means (SD).

Analysis of *TTN* genotype revealed VL fascicle length was longer in CC homozygotes in comparison to CT heterozygotes when controlling for covariates (10.1%, $F = 4.780$, $P = 0.031$; Figure 5.2). No TT homozygotes were identified so further comparisons were not completed. No differences between *TTN* genotype for VL pennation angle ($t = 0.929$, $P = 0.337$) were observed.

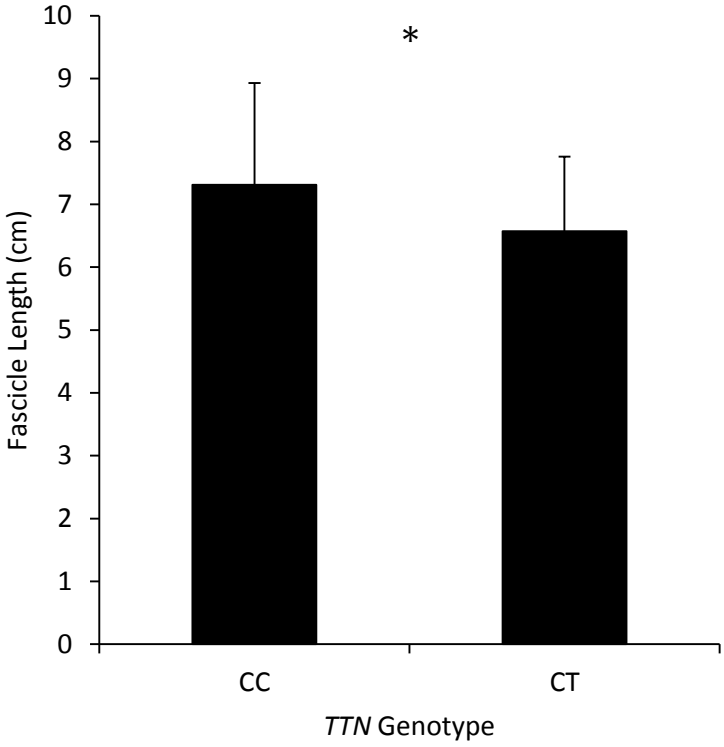


Figure 5.2. Comparison of VL fascicle length by *TTN* CC (*n* = 95) and CT (*n* = 25) genotype (**P* < 0.05). Data presented are means (SD).

No significant differences in fascicle length or pennation angle were observed between genotype groups for *ACTN3* ($F \leq 1.830$, $P \geq 0.165$), *COL5A1* ($F \leq 0.898$, $P \geq 0.410$), *PTK2* rs7843014 ($F \leq 1.721$, $P \geq 0.183$) or *PTK2* rs7460 ($F \leq 2.051$, $P \geq 0.133$). Similarly, there were no significant linear trend effects on fascicle length or pennation angle for *ACTN3* ($P \geq 0.562$, $\eta_p^2 \leq 0.01$), *COL5A1* ($P \geq 0.410$, $\eta_p^2 \leq 0.025$), *PTK2* rs7843014 ($P \geq 0.431$, $\eta_p^2 \leq 0.029$) or *PTK2* rs7460 ($P \geq 0.585$, $\eta_p^2 \leq 0.034$).

5.4 Discussion

The genetic contribution to inter-individual variability in the muscle architecture of untrained, apparently healthy individuals is presently unknown. In the current chapter

polymorphisms in *ACTN3*, *CNTF*, *COL5A1*, *PTK2* and *TTN* were investigated for associations with VL muscle fascicle length and pennation angle in untrained and apparently healthy young men. Significant genotype-phenotype associations were identified between *CNTF* and resting VL pennation angle, and between *TTN* and resting VL fascicle length, thus highlighting novel genetic associations with skeletal muscle architecture. No other significant associations were observed.

Genotype and allele frequencies for SNPs in the current chapter (Table 5.1) were similar to previous reports in Caucasian populations (North et al., 1999; Posthumus et al., 2011; De Mars et al., 2007; Erskine et al., 2012)(allele frequencies based on European [CEU] HapMap data). Mean VL fascicle length in the current sample (7.2 ± 1.6 cm) was comparable to some previous reports of VL fascicle length (~7 cm)(Fukunaga et al., 1997; Abe et al., 2000), but less than others (~8 cm and ~9 cm)(Reeves et al., 2004c; Erskine et al., 2009). Mean VL pennation angle for the current sample (19.4 ± 3.8°) was in accordance with some (~20°; (Fukunaga et al., 1997; Abe et al., 2000), but greater than other reports of VL pennation angle (~13° and ~16°)(Reeves et al., 2004c; Erskine et al., 2009). Differences in participant positioning [knee at 0° flexion in the current chapter compared to 60°- 90° flexion in Reeves et al. (2004c) and Erskine et al. (2009)] during measurement of muscle architecture may explain the reported differences between VL fascicle length and pennation angle in the current chapter and reports elsewhere (Fukunaga et al., 1997).

The *CNTF* G6-A gene polymorphism was associated with VL pennation angle but not fascicle length in untrained, apparently healthy males. Individuals carrying the mutant A-allele had

the biggest pennation angle, while GG homozygotes had the smallest pennation angle (Figure 5.1). Greater pennation angles allow for more parallel muscle fibres to attach along the aponeurosis and tendon, and are associated with bigger muscle PCSAs and the ability to generate increased muscle strength (Narici, 1999; Blazevich, 2006). Therefore, in addition to the bigger pennation angles observed, A-allele carriers in the current chapter could also be expected to have increased muscle CSA and knee extension muscle voluntary contraction (MVC_{KE}) torque.

Previous studies, however, have associated the AA genotype with lower concentric muscle strength at $180°\cdot s^{-1}$ in a predominantly (but not exclusively) Caucasian, mixed-gender cohort (Roth et al., 2001), and a middle-aged Caucasian female cohort (De Mars et al., 2007), both of which were apparently healthy and untrained (with the exception of <1% of the cohort from Roth et al.). The low frequency of AA homozygotes (n = 2) in the current sample, although reflective of other studies on Caucasian populations (De Mars et al., 2007), was insufficient to perform statistical analyses on independently. Consequently combination of the AA and GA genotype groups demonstrated that pennation angle was significantly greater for A-allele carriers compared to GG homozygotes (Figure 5.1), therefore suggesting that the A-allele may confer an advantage for greater pennation angles. It is possible that the greater pennation angles observed in the A-allele carriers of the current chapter created a mechanical disadvantage for force transmission during contraction, which may be reflected by a decreased MVC_{KE} torque production (see Chapter 7). Since loss of contractile force transmission is proportional to $1 - \cos\theta$ (where θ = pennation angle), when current pennation angle data is considered and all else is assumed

equal, A-allele carriers could expect to experience up to a 6.8% loss in force transmission during MVC_{KE} in comparison to only 5.3% by GG homozygotes. This may explain why previous studies have observed lower muscle strength in AA homozygotes (Roth et al., 2001; De Mars et al., 2007). If, however, current differences in VL fascicle length are taken into account, in addition to potential differences in muscle activation and co-activation, moment arm length and muscle fibre type composition it is unlikely that the greater pennation angles observed for A-allele carriers would translate into lower MVC_{KE} torques. Furthermore, previous research has identified significantly greater concentric torque at $180^{\circ}\cdot s^{-1}$ for heterozygotes of the G6-A polymorphism, which may identify a role for *CNTF* genotype in the development of muscle power (De Mars et al., 2007).

As *CNTF* is a pleiotropic cytokine, it is possible that through a combination of neurological factors and influences on skeletal muscle, such as altering pennation angle to accommodate the fascicle length necessary to maintain sarcomere contractile force during a high velocity of shortening (Blazevich, 2006), *CNTF* genotype could be influential for muscle power production (Vergara & Ramirez, 2004). It should be noted, however, that the association observed by De Mars et al. between GA heterozygotes and concentric torque was in females only, and no associations between *CNTF* genotype and muscle torque was observed in males. Thus, the lack of association in the current chapter between *CNTF* genotype and fascicle length in males may not reflect the genotype-phenotype associations within a female population. Indeed, Forger (2006) reported evidence to suggest the interactions between trophic factors and their receptors mediate sex differences in motor unit development, and this is substantiated by reports of sex

differences in neural cell number and cell death in rats (Sumida et al., 1993). Consequently, there is a requirement to further investigate muscle architectural phenotypes for associations with *CNTF* G6-A genotype in a female cohort to further understand this polymorphism.

Alternatively, previous research has demonstrated increases in muscle fibre number (+300%) without increasing muscle fibre size following exogenous administration of CNTF in developing rat muscle (Peroulakis & Forger, 2000). It is likely such a substantial increase in fibre number was possible due to a concurrent increase in pennation angle (although this was not measured), however as AA homozygotes express the non-functional protein, and thus lack CNTF, this does not explain why GG homozygotes in the current chapter had the smallest fibre pennation angles. Consequently, it remains unclear what mechanism is responsible for the observed differences in pennation angle in the mature adult muscles of participants in the current chapter. Although it is apparent that CNTF may influence developing muscle and different muscle types in different ways (Guillet et al., 1999), it should also be noted that previous observations in rat models may not be applicable to humans (Rennie et al., 2010). More specifically, differences in muscle fibre type distribution (Schiaffino & Reggiani, 2011) and myosin heavy chain isoform expression (Pellegrino et al., 2003) have been observed between humans and rats, which may respond differently to the same *CNTF* genotype. Thus more research in human populations is required to elucidate the mechanism underpinning the observed *CNTF* genotype group differences in pennation angle in the current chapter.

TTN acts as a template for myofibrillar protein assembly during sarcomere formation and provides an attachment site for a plethora of myofibrillar proteins to maintain the structural integrity of the sarcomere (Chauveau et al., 2014). In the current chapter, the *TTN* C > T gene polymorphism was associated with VL fascicle length but not pennation angle in untrained, apparently healthy males. Individuals homozygous for the wild type C-allele had longer VL fascicles than heterozygotes but as no individuals homozygous for the rare T-allele were present, it is unclear if the VL fascicles of TT homozygotes would have been smaller still. This missense gene variant is an exon splicing enhancer (ESE) involved in facilitating the assembly of the spliceosome during mRNA transcription (Lam & Hertel, 2002). Although multiple ESEs are involved in the activation of regulated exons (Lam & Hertel, 2002), it is possible that presence of the T-allele affects *TTN* splicing thus increasing expression of a different, smaller TTN isoform within the muscle fascicles of heterozygotes. To date, 7 different TTN isoforms have been identified within human striated muscle that differ in size (Vikhlyantsev & Podlubnaya, 2012). In skeletal muscle, the predominant TTN isoform is N2A, of which multiple isovariants exist ranging from 3400 kDa to 3700 kDa, although the smaller novex-2 (~3000 kDa) and novex-3 (616 kDa) isoforms are also expressed in smaller quantities (Freiburg et al., 2000; Bang et al., 2001; Vikhlyantsev & Podlubnaya, 2012). Thus, it is possible that altered *TTN* splicing as a result of the C > T polymorphism may influence the expression of one of more of these TTN isoforms and might explain the observations of the current chapter. Evidence to support this idea arises from observations of a *TTN* mutation that alters splicing in rat cardiac muscle and results in the expression of a large (3900 kDa) cardiac isoform (Greaser et al., 2005). Furthermore, the same rat *TTN* mutation was recently associated with both cardiac and skeletal muscle

sarcomere length, with resting sarcomere lengths corresponding to the larger mutant TTN isoforms significantly longer than those of wild type counterparts (Greaser et al., 2008; Greaser & Pleitner, 2014), suggesting that TTN isoform size directly influences sarcomere length in striated muscles. Consequently, it stands to reason that for individuals with an equal number of serial sarcomeres, fascicles would be longer in those expressing more of the larger TTN isoforms, and thus exhibiting longer resting sarcomeres. It is important to note, however, that sarcomere length (like TTN isoform expression) is not homogeneous within skeletal muscle (Wickiewicz et al., 1983; Greaser et al., 2005) and it remains unclear if the findings of the aforementioned studies using rat models can be extrapolated to human populations, hence more research is necessary to confirm or refute the current interpretations.

Regardless of the mechanism(s) responsible for the association between *TTN* and VL fascicle length observed in the current chapter, there are a number of possible implications of this association. Firstly, when considering the length-tension relationship of muscle contraction, individuals with longer fascicles would in theory experience a rightward shift in their length-tension relationship resulting in a concurrent change in the optimal joint angle for maximal torque production in the direction of full flexion. Such a shift in the length-tension relationship has been linked to a reduction in injury occurrence, as a longer optimum muscle length would ensure that less of the muscle's functional range would be along the more unstable descending limb of the length-tension curve (Brughelli & Cronin, 2007). Secondly, shorter TTN isoforms are reportedly less compliant than longer isoforms (Freiburg et al., 2000), therefore at any given joint angle, individuals expressing more

shorter N2A, novex-2 and/or novex-3 isoforms could experience increased passive tension compared to individuals expressing more of the longer TTN isoforms, although this has yet to be shown experimentally. If future research is able to confirm these early speculations, the findings of the current chapter may be beneficial for improving performance in athletes, and improving the ability to complete certain activities of daily living in ageing or diseased populations. Until further research is conducted to investigate the impact of *TTN* on muscle architecture and muscle functional phenotypes however, the interpretations of the current chapter regarding this genetic polymorphism should be taken with caution.

Despite extensive reports of *ACTN3* influencing muscle strength and/or power phenotypes in the literature, the findings of the current chapter suggest this is independent of any genotype-phenotype interactions between *ACTN3* and muscle architecture. A complex network of cytoskeletal protein interactions closely regulates the precise structured arrangement of muscle fibres. ACTN3 is expressed exclusively in type II muscle fibres (North & Beggs, 1996), and is one such protein involved in maintaining the structural organisation of the sarcomere, which having been associated with muscle strength and power phenotypes elsewhere (Eynon et al., 2013), was an ideal candidate polymorphism to examine the possibility of a potential influence on muscle architecture. Inter-individual variability in fascicle length and/or pennation angle, for example, could be expected as a consequence of differences in sarcomere Z-disc width, known to occur according to the quantity of α-actinin proteins present (Luther et al., 2003; Luther, 2009). However, since there is no obvious detrimental consequence on muscle structure for individuals lacking the ACTN3 protein (North et al., 1999), perhaps due to a compensatory upregulation of the

α-actinin-2 isoform (Mills et al., 2001), there appears to be no influence of *ACTN3* on either muscle fascicle length or pennation angle in untrained, apparently healthy males. Despite this, there is a necessity to confirm this observation in other skeletal muscles as the fibre type composition of the human VL muscle in untrained individuals is ~45/55% in favour of type II fibres, although considerable inter-individual variability in this ratio is known to exist in this population (Simoneau & Bouchard, 1989). Thus, in muscles composed of predominantly type II muscle fibres, where the consequence of lacking the ACTN3 protein may be more pronounced considering ACTN3 deficiency is associated with a shift in fibre-type towards a slow oxidative phenotype (Seto et al., 2013), *ACTN3* genotype may well be associated with muscle architecture.

No associations between the *COL5A1 3'-UTR* C > T polymorphism and either VL fascicle length or pennation angle were observed in the untrained and apparently healthy male population of this chapter. *COL5A1* is the gene encoding the α1 chain of collagen type V, a minor fibrillar collagen known to intercalate with collagen types I and III in the skeletal muscle ECM (Collins & Posthumus, 2011). Collagen type V is primarily involved in the regulation of collagen fibrillogenesis (Wenstrup et al., 2004), and previous research has demonstrated that soft tissues relatively abundant in type V collagen are characterised by small collagen fibril diameters (Birk et al., 1990). Thus, as muscle fibres are embedded within a matrix of collagen-enriched ECM, it is reasonable to assume that even minor changes to collagen fibril diameter may affect the precise arrangement of the muscle fibres between them, which, in turn could be reflected by changes in muscle pennation angle. This has potential implications for force transmission, as ultimately, following contraction

of the sarcomere, the contractile force produced is transmitted via the ECM to the tendon (Grounds, 2008). It could be assumed therefore, that individuals possessing larger collagen fibrils would experience faster force transmission and/or a greater torque would emanate at the joint. However, as no significant genotype group differences in fibre pennation angle for *COL5A1* rs12722 were observed, it is unlikely that this polymorphism is influencing VL muscle architecture in such a way. Nonetheless increases in collagen fibril diameter as a consequence of *COL5A1* genotype might confer an advantage during maximal voluntary contraction efforts and a potential association with this phenotype should be investigated (Chapter 7). Furthermore, over 79 SNPs have been identified within the *COL5A1 3'-UTR* gene (www.ncbi.nlm.nih.gov, accessed on 8 August 2014), some having a more profound impact on muscle function than others, thus potential associations between muscle architecture and one or more of these additional polymorphisms should be investigated to confirm the current finding that *COL5A1* does not influence skeletal muscle architecture.

Focal adhesion kinase (FAK), a major integrin effector encoded by *PTK2*, is influential in costamere formation and turnover (Flück et al., 1999). In the current chapter, no significant associations between *PTK2* rs7843014 and rs7460 polymorphisms and VL muscle architecture were observed, suggesting that a previous link between these SNPs and muscle specific force was independent of any architectural influences (Erskine et al., 2012). This recent study reported a baseline difference in specific force between genotype groups for both *PTK2* SNPs, which was attributed to differences in costamere density (Erskine et al., 2012). It remains unclear, however, if the proposed increase in costamere density is due to a greater number of costameres per muscle fibre, or a greater number of smaller

fibres with more costameres relative to fibre area. If the latter were correct, it is possible that fibre pennation angle would remain relatively low, and thus fascicle length may increase alongside a concomitant decrease in PCSA to accommodate the greater number of smaller fibres. Although it would appear this is not the case as no association between polymorphisms of *PTK2* and VL muscle architecture were observed in the current chapter. Furthermore, without measurements of VL PCSA to accompany these architectural measurements, the confidence of these findings is somewhat limited. Nonetheless, regardless of the mechanism underpinning the association between *PTK2* and muscle specific force, more research to attempt to replicate this finding in a larger sample is required.

5.6 Conclusion

This chapter has identified novel associations between polymorphisms in *CNTF* and *TTN* and VL muscle architecture and has also found that no association between polymorphisms in the *ACTN3*, *COL5A1* and *PTK2* genes and muscle architecture exist. Consequently, there is reason to assume an architectural mechanism may underlie the genetic association previously reported between *CNTF* and muscle strength; and could also suggest a potential influence of *TTN* genotype on muscle strength that should be investigated in future. Furthermore, the findings of this chapter have applications for a variety of individuals, including athletic, ageing and clinical populations, for whom completion of activities of daily living or sporting performance may be improved as a consequence of better understanding their individual-specific muscle mechanics.

Chapter 6

Influence of *ACE*, *ACTN3*, *CNTF*, *PTK2* and *TRHR* polymorphisms on muscle size phenotypes

6.1 Introduction

Inter-individual variability in muscle size is considerable (Chapter 4) (Seeman et al., 1996), and can be attributed to differences in fibre cross-sectional area (CSA), and/or differences in fibre number (Sale et al., 1987). Bigger fibre CSA and/or more fibres correspond to a greater number of muscle sarcomeres in parallel, which during muscle contraction translates into the formation of a greater number of cross-bridges and thus increased force production. Accordingly, muscle size is generally considered to be the main determinant of muscle strength ($r \geq 0.51$, $P \leq 0.01$) (Maughan et al., 1983), and increases in both are commonly observed following functional overload (Jones & Rutherford, 1987). For many athletic populations, a bigger muscle mass and thus ability to produce greater force is essential, and athletes from strength/power-oriented sports are often reported as having larger muscle thickness, CSA and/or volume than their endurance and sedentary counterparts (Abe et al., 2000; Hakkinen & Keskinen, 1989; Fukunaga et al., 2001). Additionally, for populations in which sarcopenia and cachexia are prevalent, an individual's ability to complete activities of daily living is diminished (Hyatt et al., 1990), whilst mortality risk is increased (Wannamethee et al., 2007). Therefore, individuals with a smaller muscle mass predisposed by their genetic profile may be less likely to compete at the top level athletically, or may be at a greater risk of early mortality following the onset of sarcopenia or cachexia.

The role of muscle size and mass in health and disease is evident, so it is unsurprising that a number of genetic polymorphisms have already been investigated for associations with this phenotype (Thompson et al., 2004). Perhaps the most extensively studied of these genes is myostatin (*MSTN*), although as with many of these polymorphisms, this has

predominantly been in livestock where muscle size has been linked to meat tenderness (Koohmaraie et al., 2002). The *MSTN* gene is a known negative regulator of skeletal muscle mass and a mutation in this gene, resulting in MSTN knockout, has been observed in the Belgian Blue and Piedmontese breeds of cattle, which typically exhibit a 'double-muscled' appearance (McPherron & Lee, 1997). In humans, however, there has been only one reported homozygote with this mutation, a German infant who also demonstrated a similar 'double-muscled' appearance from birth (Schuelke et al., 2004). Although the impact of *MSTN* on muscle mass cannot be disputed, with only one reported case in humans to date, it is unlikely that another case would be identified in the apparently healthy population recruited in the current chapter. Furthermore, as considerable inter-individual variability in muscle mass is known to exist in homogenous populations (Chapter 4) (Arden & Spector, 1997; Seeman et al., 1996), it is reasonable to assume that control of muscle mass is polygenic and thus under the influence of a number of genetic polymorphisms, six of which are discussed below as candidate genes for investigation in the current chapter.

Angiotensin I-converting enzyme (*ACE*) is central to the renin-angiotensin system (RAS), where it is essential for the conversion of angiotensin I to angiotensin II (Reid, 1998). The absence (D) rather than the presence (I) of a 287 amino acid base pair is associated with increased concentrations of tissue and serum ACE activity (Rigat et al., 1990; Danser et al., 1995). Although *ACE* and the gene encoding growth hormone (GH) are not related (McKenzie et al., 1995), they are closely located and angiotensin II is known to stimulate the release of GH systemically (Messerli et al., 1977), in addition to being an effective growth factor in smooth and striated muscle tissues (Geisterfer et al., 1988). Furthermore, *ACE* is associated with cardiac and skeletal muscle hypertrophy in response to exercise

training (Montgomery et al., 1997; Folland et al., 2000), as well as degrading growth-inhibitory kinins (Ishigai et al., 1997). Subsequently there appears to be a role for *ACE* in the regulation of muscle size, however, investigations into a potential association between *ACE* and muscle size in an apparently healthy, untrained population to date are limited (Erskine et al., 2013; Thomis et al., 2004; Frederiksen et al., 2003), thus there is a requirement to further investigate this potential link in a relatively large homogenous population.

Alpha-actinin-3 (ACTN3) is a protein expressed exclusively in type II muscle fibres (North & Beggs, 1996; Mills et al., 2001), however, individuals homozygous for the X-allele of the R577X polymorphism within the *ACTN3* gene are unable to produce the fully functioning protein (Clarkson et al., 2005). In addition to anchoring actin filaments to the sarcomeric Z-line, ACTN3 also interacts with cell-signalling proteins and may have a role in fibre type differentiation (Mills et al., 2001; Seto et al., 2013). Observations of smaller muscle fibre diameters and an increased number of oxidative enzymes in type II fibres of XX mice compared to RR mice (Chan et al., 2008; MacArthur et al., 2008; Seto et al., 2013), suggests fibre type differentiation from type II to type I muscle fibres in the absence of ACTN3 may contribute to inter-individual variability in muscle mass, especially in muscles composed of approximately similar quantities of type I and type II fibres, such as the vastus lateralis (VL). Furthermore, type II fibres are known to have larger fibre CSA than type I fibres (Bottinelli et al., 1996), therefore it could be expected that RR individuals able to produce the fully functioning ACTN3 protein, may have a greater percentage of type II fibres and thus an increased muscle mass (Vincent et al., 2007). Recently, a significantly larger quadriceps femoris muscle volume was observed in RR homozygotes compared to XX homozygotes

(Erskine et al., 2013), however because this study used only a small sample (*n* = 51), attempts to replicate these findings in a larger sample are required.

Ciliary neurotrophic factor (CNTF) is a cytokine with myotrophic factors known to influence muscle fibre number and muscle volume (Peroulakis & Forger, 2000; Bengston et al., 1996; Forger et al., 1995). Previously, exogenous administration of CNTF to developing rat muscle resulted in a ~300% increase in muscle fibre number, which led to an increase in muscle CSA and volume (Peroulakis & Forger, 2000). In addition, a mutation in the *CNTF* receptor gene has been associated with fat-free mass (FFM), potentially via alterations in how the receptor interacts with CNTF, although the exact mechanism by which this occurs is unclear (Roth et al., 2003). Secondary to an increase in muscle fibre number, is likely to be an increase in muscle strength due to a rise in the number of sarcomeres increasing cross-bridge formation during muscle contractions. In humans, polymorphisms in *CNTF* have been associated with muscle strength in some Caucasian populations (Roth et al., 2001; De Mars et al., 2007), although no association between the *CNTF* G6-A polymorphism and FFM was observed in these studies. However, as accurate assessments of muscle size, such as ultrasound assessments of anatomical and physiological CSA (ACSA and PCSA respectively) were not carried out in these studies, an association of this polymorphism with muscle size cannot be discounted and should be investigated.

Focal adhesion kinase (FAK) is a phosphoprotein located within integrin/focal adhesion complexes of muscle costameres (Quach & Rando, 2006). In hypertrophied rooster skeletal muscle, increased expression of FAK has been observed (Flück et al., 1999) and although this has yet to be investigated in humans, may demonstrate a link between FAK and muscle

size. Protein tyrosine kinase 2 (*PTK2*) is the gene encoding FAK, and two polymorphisms in this gene have demonstrated an association with muscle specific force (Erskine et al., 2012). Although Erskine et al. (2012) did not measure FAK density, inherent differences in FAK density may explain their findings, as greater FAK densities (and thus costamere densities) in skeletal muscle are believed to occur through one of two mechanisms, 1) a greater number of costameres per muscle fibre; or 2) a greater number of smaller fibres per costamere (Erskine et al., 2012), however the exact mechanism remains unclear at this stage. Nonetheless, as *PTK2* genotype influences FAK expression within skeletal muscle (Flück et al., 1999), genotype differences in muscle size could be expected as a consequence of one, or both of the aforementioned mechanisms and a potential association should be investigated in a homogenous group of untrained individuals.

Thyroid hormone signalling is important for skeletal muscle development (Norenberg et al., 1996) and muscle weakness is a notable symptom of some thyroid-related conditions such as hypothyroidism and thyrotoxicosis (Salvatore et al., 2014). Production of triiodothyronine (T_3) and thyroxine (T_4) thyroid hormones is regulated by thyroid-stimulating hormone (TSH), secretion of which is determined by the binding of thyrotropic-releasing hormone (TRH) to the thyrotropin-releasing hormone receptor (TRHR) (Salvatore et al., 2014). A mutation in the *TRHR* gene which alters the TRH:TRHR binding capacity, has been identified as the primary cause of central hypothyroidism in some instances (Collu et al., 1997). Although this mutation was associated with a serious disease condition, two different polymorphisms in the *TRHR* gene have since been identified as having less severe consequences, following a genome-wide association study (GWAS) linking this gene to FFM (Liu et al., 2009). More recently, Lunardi et al. (2013) replicated these initial findings with

limited success. Whilst both of these studies used dual energy x-ray absorptiometry (DXA), the gold-standard, to assess body composition, neither study attempted to isolate individual muscles by identifying ACSA, PCSA or volume to identify if these polymorphisms were also linked to individual muscle size. Considering sarcopenia affects the muscles of the lower body to a greater extent than the upper body (Lynch et al., 1999), investigating the potential association between *TRHR* and individual muscle size and muscle mass may be more beneficial in targeting the development of preventions and treatments against muscle wasting.

The aim of this chapter, therefore, was to investigate the influence of polymorphisms within *ACE*, *ACTN3*, *CNTF*, *PTK2* and *TRHR* genes on muscle size phenotypes (total body, appendicular, arm, and leg lean mass, VL muscle thickness, ACSA, PCSA and volume) in an untrained, apparently healthy population.

6.2 Methods

Detailed descriptions of participant recruitment, assessment of skeletal muscle size and mass, and the genotyping of the *ACE* (rs4341), *ACTN3* (rs1815739), *CNTF* (rs1800169), *PTK2* (rs7843014 and rs7460) and *TRHR* (rs7832552) polymorphisms is included in Chapter 2 (section 2.1, 2.2 and 2.4, respectively), thus only a brief description of these methods is detailed below.

6.2.1 Participants

One hundred and twenty untrained active Caucasian males [age 20.6 (2.3) yr, stature 1.79 (0.06) m and mass 75.1 (10.1) kg; mean (SD)] volunteered to participate in this study. All

participants met the inclusion criteria (described in Chapter 2.1) and gave written informed consent prior to involvement.

6.2.2 Skeletal muscle properties

VL muscle thickness was identified from sagittal plane ultrasound scans at 50% of muscle length. Measurement of VL ACSA, by contour matching a series of transverse plane ultrasound scans, was used in the estimation of VL muscle volume (Eq 3). VL fascicle length was measured from a single ultrasound image and used in the calculation of PCSA (Eq 4). Finally, DXA was used to quantify total body, appendicular, arm and leg lean mass, all of which were analysed excluding bone mineral content (BMC).

6.2.3 Genotyping

Genotyping was completed using the fluorophore-based detection technique of TaqMan® real-time PCR. Genomic DNA amplification of fragments overlapping polymorphisms in the *ACE* (rs4341), *ACTN3* (rs1815739), *CNTF* (rs1800169), *PTK2* (rs7843014 and rs7460) and *TRHR* (rs7832552) genes was completed for all participants.

6.2.4 Statistical analysis

The frequency of each polymorphism was assessed for compliance with Hardy-Weinberg equilibrium using X^2 tests. A one-way analysis of variance (ANOVA) was conducted to determine any significant differences in physical characteristics (stature, mass, BMI and age) between genotype. When genotype groups were combined, an independent samples *t*-test was used to identify any differences in physical characteristics. Pearson's correlation coefficient was used to identify any covariates with muscle size phenotypes (VL ACSA,

PCSA, muscle volume, muscle thickness, and total body, appendicular, leg and arm lean mass). ANOVA, and where appropriate additional analysis using analysis of covariance (ANCOVA), was conducted to identify any genotype differences in muscle size phenotypes. Additionally, any genotype effects on muscle size phenotypes were also assessed for linear trend using ANOVA or ANCOVA. In instances when too few participants represented one genotype group, this group was combined with the heterozygous group and an independent samples *t*-test was used to identify any differences between muscle size phenotypes. All significant associations identified in the main ANOVA or ANCOVA analyses were subject to *post-hoc* pairwise comparisons using the Benjamini-Hochberg correction. In instances where a tendency between genotype groups was observed, such that $P > 0.05$ but < 0.15, the two groups with similar means were combined and the analysis re-run using an independent samples *t*-tests. All statistical analyses were performed using SPSS version 19.0 and statistical significance was accepted when $P \leq 0.05$. Data are presented as mean (SD).

6.3 Results

Genotype frequencies for polymorphisms in the *ACE*, *ACTN3*, *CNTF*, *PTK2* and *TRHR* genes are presented in Table 6.1 and all were in Hardy-Weinberg equilibrium ($P \geq 0.436$). No differences between genotypes for any polymorphism were observed for stature ($P \geq 0.217$), mass ($P \geq 0.106$), BMI ($P \geq 0.140$) or age ($P \geq 0.094$; Chapter 2: Table 2.2). Pearson's correlation coefficients revealed significant weak correlations between pennation angle ($r \geq 0.231$, $P \leq 0.012$) and VL muscle length and muscle thickness; and moderate positive correlations between ACSA ($r \geq 0.422$, $P \leq 0.0005$) and VL muscle volume, PCSA and thickness; and VL muscle length ($r \geq 0.424$, $P \leq 0.0005$) and VL muscle volume and thickness.

Consequently, pennation angle, ACSA and VL muscle length were included as covariates where appropriate in subsequent analyses. Additionally, stature ($r \geq 0.235$, $P \leq 0.01$), mass ($r \geq 0.328$, $P \leq 0.0005$) and BMI ($r \geq 0.227$, $P \leq 0.013$) were moderately correlated with all muscle size phenotypes (with the exception of muscle thickness for BMI and stature) and were included as covariates in subsequent analyses.

Analysis of limb lean mass revealed significant differences between *TRHR* genotypes for total appendicular lean mass ($F = 4.629$, $P = 0.012$), leg lean mass ($F = 4.004$, $P = 0.021$) and arm lean mass ($F = 3.841$, $P = 0.024$). Similarly, significant linear trend effects of *TRHR* on total appendicular lean mass ($P = 0.004$, $\eta_p^2 = 0.073$) and leg lean mass ($P = 0.006$, $\eta_p^2 = 0.062$) were observed, whilst a linear trend effect of *TRHR* on arm lean mass approached significance ($P = 0.055$, $\eta_p^2 = 0.064$). *Post-hoc* comparisons revealed that TT homozygotes had significantly greater appendicular, leg and arm lean mass than their CT (all $P = 0.025$) and CC counterparts ($P = 0.025$, $P = 0.05$ and $P = 0.025$ respectively; Table 6.1). Following combination of the CC and CT genotypes, TT homozygotes demonstrated significantly greater appendicular lean mass (9.6%, $t = 3.005$, $P = 0.003$), arm lean mass (11.4%, $t = 2.417$, $P = 0.017$) and leg lean mass (8.6%, $t = 2.676$, $P = 0.008$; Figure 6.1). There was a tendency for VL muscle thickness of *TRHR* TT individuals to be greater than that of both CT and CC genotype groups when mass was included as a covariate (TT, 2.61 cm; CT, 2.34 cm; CC, 2.42 cm; $F = 2.527$, $P = 0.084$; Table 6.1), however, when CT and CC groups were pooled, this difference remained non-significant ($t = 1.671$, $P = 0.097$). No significant linear trend effect of *TRHR* on VL muscle thickness was observed ($P = 0.197$, $\eta_p^2 = 0.034$). Furthermore, no significant *TRHR* genotype group differences existed for total body lean mass ($F = 0.746$, $P = 0.477$), VL muscle ACSA ($F = 0.056$, $P = 0.945$), PCSA ($F = 0.334$, $P = 0.717$) or volume ($F =$

0.035, $P = 0.966$), nor did any significant linear trend effects on these phenotypes exist ($P \geq 0.513$, $\eta_p^2 \leq 0.013$)

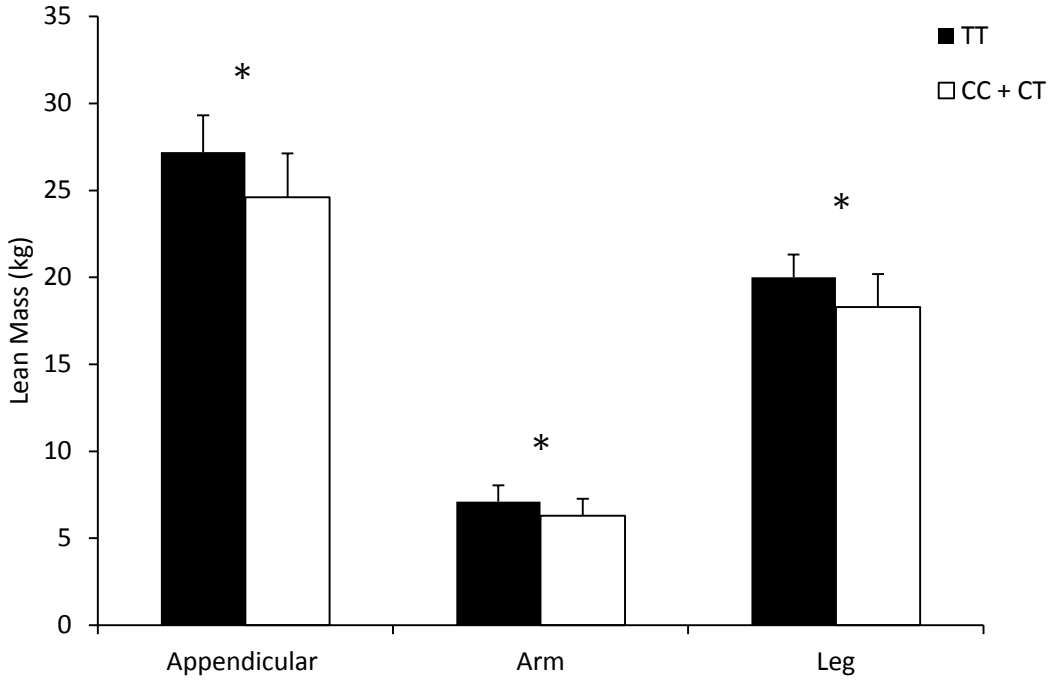


Figure 6.1. Limb lean mass according to *TRHR* genotype. Preferential (black bars) TT (n = 9) homozygotes had significantly greater lean mass (kg) than non-preferential (white bars) C-allele carriers (n = 111; *$P < 0.05$). Data presented are means (SD).

Due to low numbers of *CNTF* AA homozygotes (n = 2) in the current sample, a two-group analysis was completed between GG homozygotes and A-allele carriers. VL muscle thickness was significantly smaller for GG homozygotes than A-allele carriers for *CNTF* (7.9%, $t = 2.657$, $P = 0.009$; Figure 6.2). No *CNTF* genotype group differences were apparent for limb lean mass ($F \leq 1.027$, $P \geq 0.361$), total body lean mass ($F = 0.165$, $P = 0.848$), VL ACSA ($F = 0.557$, $P = 0.574$), PCSA ($F = 0.379$, $P = 0.686$) or volume ($F = 1.058$, $P = 0.350$).

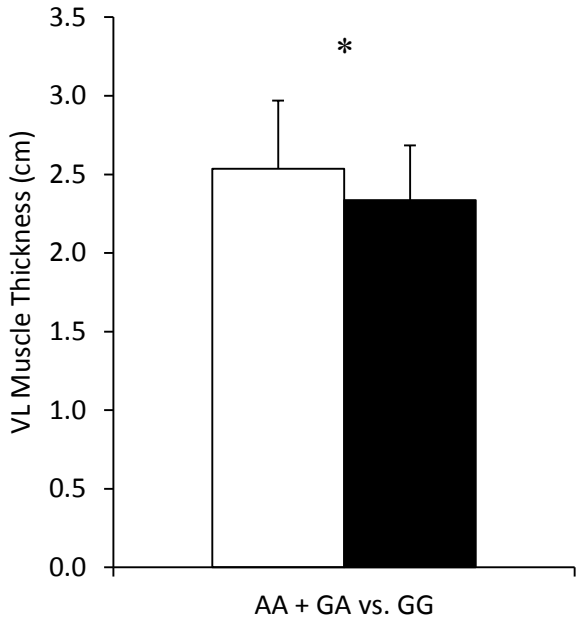


Figure 6.2. Comparison of VL muscle thickness by the combined preferential (*n* = 35) and non-preferential (*n* = 85) *CNTF* genotype (*$P < 0.01$). Data presented are means (SD).

VL PCSA of *ACTN3* heterozygotes was significantly greater than that of both homozygote groups when mass and BMI were included as covariates ($F = 3.085$, $P = 0.049$, Figure 6.3). Consequently, no significant linear trend was observed between *ACTN3* genotype and VL PCSA ($P = 0.460$; $\eta_p^2 = 0.042$). Following combination of RR and RX, however, no significant difference in VL PCSA between XX homozygotes and R-allele carriers was apparent ($F = 2.950$, $P = 0.089$). Furthermore, no significant *ACTN3* genotype group differences existed for limb lean mass ($F \leq 0.540$, $P \geq 0.584$), total body lean mass ($F = 0.188$, $P = 0.829$), or VL ACSA ($F = 1.957$, $P = 0.146$), thickness ($F = 0.447$, $P = 0.641$) or volume ($F = 1.315$, $P = 0.272$). Similarly, there were no significant linear trend effects of *ACTN3* genotype on limb lean mass ($P \geq 0.544$, $\eta_p^2 \leq 0.009$), total body lean mass ($P = 0.638$, $\eta_p^2 = 0.003$) or muscle size phenotypes ($P \geq 0.859$, $\eta_p^2 \leq 0.032$).

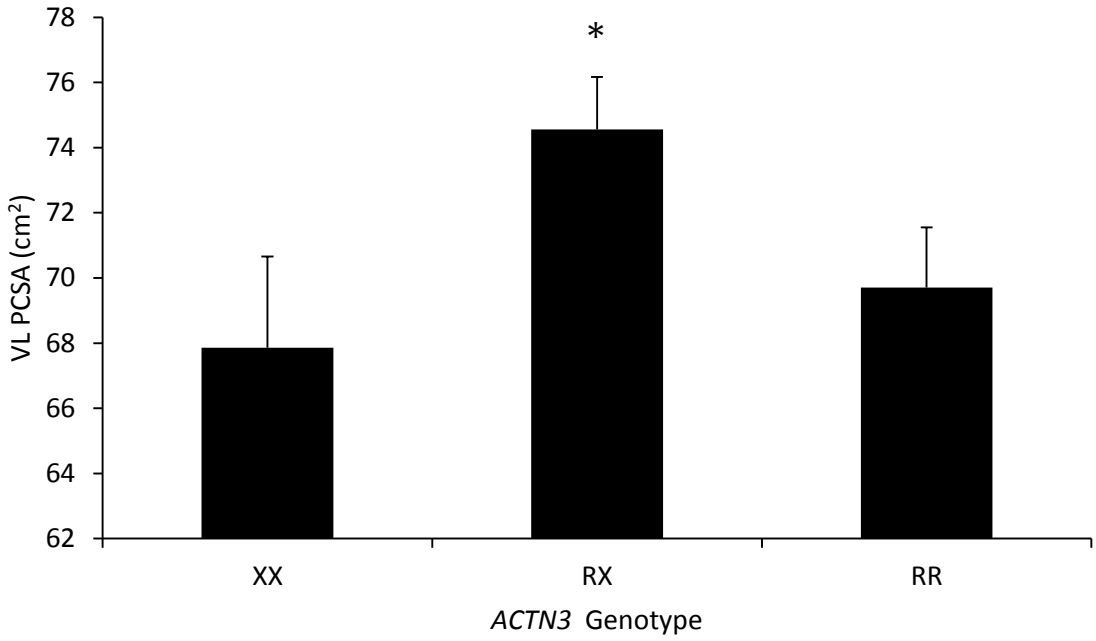


Figure 6.3. *ACTN3* genotype group differences for VL PCSA (*$P < 0.05$). Data presented are means (SD).

No significant differences in any of the measured phenotypes were observed between genotype groups for *ACE* ($F \leq 1.448$, $P \geq 0.239$) and *PTK2* rs7843014 ($F \leq 2.615$, $P \geq 0.077$) or rs7460 ($F \leq 2.730$, $P \geq 0.069$). Furthermore, no linear trend effects were observed for *ACE* ($P \geq 0.155$, $\eta_p^2 \leq 0.024$), *PTK2* rs7843014 ($P \geq 0.073$, $\eta_p^2 \leq 0.034$) or rs7460 ($P \geq 0.128$, $\eta_p^2 \leq 0.035$) and any of the measured phenotypes.

Table 6.1. Means (SD) for muscle size phenotypes according to genotype for SNPs in the *ACE*, *ACTN3*, *CNTF*, *PTK2* and *TRHR* genes.

SNP	Genotype Number	Genotype Frequency (%)	ACSA (cm^2)	PCSA (cm^2)	Muscle Volume (cm^3)	Muscle Thickness (cm)	Lean Mass (kg)			
							Total Body	Total Appendicular	Arm	Leg
ACE (rs4341)	II (*n* = 32)	26.7	21.6 (2.4)	71.1 (12.2)	670 (87)	2.4 (0.3)	55.2 (4.6)	25.0 (2.4)	6.4 (0.9)	18.6 (1.8)
	ID (*n* = 54)	45.0	21.5 (2.4)	71.6 (14.1)	671 (89)	2.4 (0.4)	54.9 (5.7)	24.8 (2.6)	6.4 (1.0)	18.4 (2.0)
	DD (*n* = 34)	28.3	21.4 (2.8)	72.5 (10.9)	655 (80)	2.3 (0.3)	54.0 (6.1)	24.5 (2.8)	6.2 (1.1)	18.3 (1.8)
ACTN3 (rs1815739)	RR (*n* = 44)	36.6	21.0 (1.9)	70.2 (9.6)	653 (60)	2.4 (0.4)	54.7 (5.2)	24.7 (2.3)	6.4 (1.0)	18.3 (1.6)
	RX (*n* = 57)	47.5	21.9 (2.7)	74.3 (13.9)§	679 (93)	2.4 (0.4)	54.5 (5.8)	24.7 (2.7)	6.3 (1.0)	18.5 (2.0)
	XX (*n* = 19)	15.9	21.1 (3.0)	67.6 (14.3)	656 (109)	2.4 (0.3)	55.4 (5.5)	25.1 (3.0)	6.5 (1.0)	18.6 (2.2)
CNTF (rs1800169)	AA (*n* = 2)	1.7	19. 7 (1.0)	68.4 (17.2)	592 (25)	2.7 (0.7)	52.6 (5.2)	22.6 (0.1)	5.9 (0.4)	16.6 (0.5)
	GA (*n* = 33)	27.5	21.3 (2.4)	70.3 (12.2)	657 (100)	2.5 (0.4)	54.6 (4.5)	24.5 (2.1)	6.3 (0.9)	18.3 (1.8)
	GG (*n* = 85)	70.8	21.5 (2.6)	72.3 (12.9)	671 (80)	2.3 (0.4) ‡	54.8 (5.9)	24.9 (2.8)	6.4 (1.0)	18.5 (2.0)
PTK2 (rs7843014)	AA (*n* = 42)	35.0	21.0 (2.4)	71.3 (10.9)	651 (82)	2.4 (0.4)	53.5 (5.6)	24.1 (2.8)	6.3 (1.1)	17.9 (2.1)
	AC (*n* = 52)	43.3	21.6 (2.7)	71.6 (13.6)	672 (81)	2.4 (0.4)	54.9 (5.9)	25.0 (2.7)	6.3 (1.1)	18.6 (1.9)
	CC (*n* = 26)	21.7	21.9 (2.2)	72.6 (13.8)	675 (101)	2.5 (0.3)	56.3 (4.1)	25.3 (1.8)	6.6 (0.6)	18.8 (1.5)
PTK2 (rs7460)	AA (*n* = 34)	28.3	21.6 (2.3)	70.3 (15.0)	666 (100)	2.4 (0.4)	54.9 (5.6)	24.8 (2.5)	6.4 (0.9)	18.5 (1.9)
	AT (*n* = 54)	45.0	21.5 (2.6)	72.3 (11.5)	670 (75)	2.4 (0.4)	55.7 (5.2)	25.2 (2.5)	6.4 (0.1)	18.7 (1.8)
	TT (*n* = 32)	26.7	21.1 (2.7)	72.2 (12.3)	659 (88)	2.4 (0.4)	52.8 (5.7)	24.0 (2.7)	6.2 (0.1)	17.8 (2.0)
TRHR (rs7832552)	CC (*n* = 53)	44.2	21.5 (2.4)	72.8 (14.3)	665 (86)	2.4 (0.4)	55.0 (5.9)	24.6 (2.5)	6.2 (1.0)	18.2 (1.9)
	CT (*n* = 58)	48.3	21.4 (2.8)	70.9 (11.4)	667 (89)	2.3 (0.3)	54.1 (5.3)	24.6 (2.6)	6.4 (1.0)	18.4 (1.8)
	TT (*n* = 9)	7.5	21.3 (1.6)	70.9 (10.9)	659 (60)	2.6 (0.4)	56.3 (5.0)	27.2 (2.1)*	7.1 (1.0)*	20.0 (1.3)*

Grey shading denotes significance; §Significant difference between RX and both homozygote genotype groups for *ACTN3* ($P < 0.05$); ‡Significant difference between GG and GA genotype groups for *CNTF* ($P < 0.05$); * Significant difference between TT and both CC and CT genotype groups for *TRHR* ($P \leq 0.05$).

6.4 Discussion

The current chapter aimed to identify associations between polymorphisms in *ACE*, *ACTN3*, *CNTF*, *PTK2*, *TRHR* and muscle size phenotypes in untrained and apparently healthy young men. Significant genotype-phenotype associations were identified between *TRHR* and measures of appendicular lean mass; *CNTF* and VL muscle thickness; and *ACTN3* and VL PCSA, thus highlighting novel associations with skeletal muscle size, in addition to independently replicating the findings of some previous reports. No other significant associations were observed.

Genotype frequencies for polymorphisms in the current chapter (Table 6.1) were similar to previous reports in Caucasian populations (Williams et al., 2005; North et al., 1999; De Mars et al., 2007; Erskine et al., 2012). Mean lean mass for the legs (18.4 kg), arms (6.3 kg), total appendicular (24.8 kg) and total body (54.7 kg) in the current sample were comparable to previous reports (Roth et al., 2003). Similarly, VL muscle volume (666 cm^3), ACSA (21.4 cm^2), PCSA (71.7 cm^2) and muscle thickness (2.4 cm) were all in accordance with values (674 cm^3, 18.3 cm^2, 75.1 cm^2 and 2.3 cm, respectively) reported previously in comparable adult male samples (Erskine et al., 2009; Abe et al., 2000).

The *TRHR* rs7832552 gene polymorphism was associated with appendicular lean mass but not total body lean mass or other measures of muscle size in untrained, apparently healthy males. Individuals homozygous for the T-allele had the greatest leg, arm and total appendicular lean mass, whilst for CC homozygotes leg (-1.8 kg compared to TT),

arm (-0.94 kg compared to TT) and total appendicular lean mass (-2.6 kg compared to TT) was smallest (Figure 6.1). Furthermore, 6-7% of the observed variation in appendicular lean mass could be attributed to *TRHR* genotype. As muscle fibre number and CSA largely determine muscle size (Sale et al., 1987), individuals with greater appendicular lean mass might also be expected to produce greater maximal voluntary contraction (MVC) torques. Although data on muscle strength is addressed in the subsequent chapter (Chapter 7), a recent study has reported an association between a different polymorphism in *TRHR* and total body FFM, appendicular FFM and muscle strength (Lunardi et al., 2013). Presumably, therefore, the greater muscle strength in these individuals could be largely attributed to their greater FFM, which in turn may reflect an increased number of sarcomeres per muscle, and therefore improve the force-generating capacity of the muscle. It is important to note, however, that in the same study no association between appendicular FFM and the *TRHR* rs732552 gene polymorphism was observed (Lunardi et al., 2013), which is in contrast to the findings of the current study. Selection of an older female population in the former compared to the young male population recruited in the latter may explain this discrepancy, as females are known to have fewer muscle fibres and smaller fibre CSA than males (Henriksson-Larsen et al., 1985; Sale et al., 1987).

Despite observations of an association between *TRHR* rs7832552 and appendicular lean mass in the current chapter, no association between this genetic polymorphism and total body lean mass was observed. In contrast, however, a recent GWAS identified an association between *TRHR* rs7832552 and total body lean mass, which demonstrated a 2.6 kg larger total body lean mass of TT homozygotes compared to C-allele carriers (Liu et al.,

2009). GWAS allow the simultaneous genotyping of as many as 4 million gene variants across the genome of thousands of participants, and the greater sample sizes increase the power to detect associations, which may explain the discrepancy between these studies. Alternatively, whilst in the current chapter BMC was excluded from the measurement of total body lean mass, it is unclear if total body lean mass in the previous study was inclusive of BMC or not. It is possible that a greater discrimination of whole body lean mass in the current study enabled a closer approximation of muscle mass and could explain the discrepancies between the findings of the current chapter and those of Liu et al. (2009), although without confirmation this remains speculative.

The current chapter is the first study to the author's knowledge to investigate a potential association of *TRHR* with VL muscle size, as determined by muscle thickness, ACSA, PCSA and volume. However, no significant association of the *TRHR* gene polymorphism with any of these measures of muscle size was observed, despite leg lean mass demonstrating a significant association. A plausible explanation for this could be that *TRHR* genotype confers only a modest influence on individual muscle size phenotypes. Therefore, when individual muscles are investigated alone, such as the current analyses of VL muscle size, this genotype influence is undetected but when multiple muscles are considered together, such as in the current analysis of leg lean mass, a detectable genotype association becomes apparent. It would be useful, therefore, for future research to measure muscle volume, ACSA, PCSA and thickness in several muscles to ascertain if *TRHR* is associated with muscle size phenotypes.

Nonetheless, the association of *TRHR* rs7823552 with appendicular lean mass observed in the current study has implications for a number of populations. With ageing, for example, sarcopenia is associated with a reduction in functional capacity (Baumgartner et al., 1998), increased risk of falls (Whipple et al., 1987) and is an independent predictor of mortality risk (Wannamethee et al., 2007). Furthermore, in athletes muscle mass is important for the production of strength, speed and power (Arden & Spector, 1997), and those predisposed to have a lower lean muscle mass may be weaker, slower, less powerful and subsequently less likely to succeed in certain playing positions (Sutton et al., 2009) or at the top level of elite sporting competition (Olds, 2001). Although previous research has demonstrated altered levels of thyroid hormone following exercise bouts (Schmid et al., 1982; Galbo et al., 1977), the exact effect of exercise on thyroid hormone remains a controversial topic (Huang et al., 2004). It stands to reason, however, that if baseline thyroid levels respond to prolonged exercise training as can be expected for athletes, a reversal of this response could be expected following a period of detraining or disuse. Thus, it is evident that the potential impacts of *TRHR* genotype for lean muscle mass are extensive, and this may well be a worthwhile target for researchers hoping to treat and/or prevent muscle-wasting diseases in the future.

CNTF is a member of the interleukin-6 family of cytokines and is known to influence muscle fibre number and volume (Peroulakis & Forger, 2000; Bengston et al., 1996; Forger et al., 1995). In the current chapter, the *CNTF* G6-A polymorphism was associated with VL muscle thickness only. Individuals homozygous for the G-allele had significantly smaller VL muscle thickness than A-allele carriers (Figure 6.2). This is the first study to the author's knowledge

to report an association between *CNTF* G6-A and muscle thickness in humans. Previously, the presence of CNTF, either through production of the functioning protein *in vivo* or exogenous administration of recombinant CNTF has been linked to a reduction in fibre degeneration (Peroulakis & Forger, 2000), increased fibre number (Peroulakis & Forger, 2000) and increased fibre CSA (Guillet et al., 1999). Although these earlier studies were conducted in rats, and the applicability of the findings to a human population are unclear, it appears that CNTF affects muscle differently according to the stage of its development. For example, in developing muscle, changes in fibre number appeared to be the primary effect (Peroulakis & Forger, 2000), whereas in mature muscle, increases in fibre CSA were more likely (Guillet et al., 1999). Accordingly, evidence exists to suggest, that although *CNTF* genotype remains stable throughout an individual's lifetime, muscle fibre number in humans is determined before birth (Alberts et al., 2008; Rehfeldt et al., 1999), and that hyperplasia does not occur thereafter (MacDougall et al., 1984; McCall et al., 1996). Muscle fibre hypertrophy, however, is extensively reported in response to functional overload in humans (McCall et al., 1996; MacDougall et al., 1980; Widrick et al., 2002), thus, it is unclear which of these mechanisms underlies the association between *CNTF* and VL muscle thickness currently observed. Despite this, it is reasonable to assume that those individuals able to produce the functioning CNTF protein would exhibit the greatest muscle thickness. However, in the current study it is the GG homozygotes, predicted to produce the CNTF protein, who exhibit the smallest VL muscles which contradicts these earlier reports (Peroulakis & Forger, 2000; Guillet et al., 1999). Differences in the potency of human and rat recombinant CNTF have been noted (Helgren et al., 1994), and as the majority of this previous research focussed on rats rather than humans, it is possible that CNTF affects

skeletal muscle differently in the two species and explains this discrepancy. Contrastingly, exogenous administration of recombinant human CNTF in certain quantities, actually results in muscle atrophy and cachexia in rodents (Martin et al., 1996; Henderson et al., 1994), and is not associated with reduced fibre degeneration, increased fibre number or CSA. A more plausible explanation for the current findings, therefore, is that assuming G-allele carriers were producing normal circulatory levels of CNTF such that the atrophic effects of CNTF would be milder than those experienced previously in rodents, GG individuals may be predisposed to smaller muscles fibres, which is subsequently reflected by their reduced muscle thickness. Nonetheless, when considering the results from Chapter 5 together with the current findings, both report genotype differences in the order of GG < GA < AA for VL fibre pennation angle and muscle thickness. This suggests that the mechanism underlying the association of *CNTF* and VL muscle thickness, may also be contributing to the observed association between *CNTF* and VL fibre pennation angle, although future research should be undertaken to confirm this.

As both VL muscle thickness and pennation angle are associated with *CNTF* G6-A, it is somewhat surprising that this association is not continuous with VL ACSA, PCSA or volume. Whilst it is probable that inter-individual variability in VL muscle and fascicle length may explain the lack of association between *CNTF,* VL PCSA and volume, the reason underlying the lack of association between VL ACSA and *CNTF* is less clear. Nonetheless, muscle fascicle gearing is the process by which muscle fibre shortening and lengthening velocities are limited to maintain the functional capacity of the muscle through a range of joint angles, contraction intensities and speeds, and is largely determined by fibre pennation

angle and muscle thickness (Wakeling et al., 2011). Considering the findings of the current chapter and those of Chapter 5, it is evident that GG homozygotes have smaller VL pennation angles and muscle thickness, which may subsequently confer an advantage during muscle shortening contractions for force production as a consequence of allowing for a greater gearing ratio (Wakeling et al., 2011). This is consistent with previous research that reported lower concentric MVC torque for AA homozygotes in comparison to G-allele carriers (Roth et al., 2001; De Mars et al., 2007), and might identify the *CNTF* G6-A G-allele or GG genotype as influential for muscle power production. However, whilst this cannot be confirmed using the data from the current chapter, subsequent chapters of this thesis investigate genotype associations with muscle strength and simultaneous measures of muscle morphology (Chapter 7 and Chapter 8). Furthermore, due to the low frequency of AA homozygotes within Caucasian populations, and the relatively low sample sizes of studies investigating an association of *CNTF* with human skeletal muscle to date, a general consensus on the impact of *CNTF* G6-A polymorphism has yet to be reached and researchers are therefore encouraged to complete future studies on thousands, rather than hundreds of participants where possible.

ACTN3 is expressed in type II muscle fibres only (North & Beggs, 1996), and as these fibres have a greater CSA than type I fibres (Bottinelli et al., 1996) the common R577X polymorphism of the *ACTN3* gene was investigated for associations with muscle size phenotypes. The data indicate that *ACTN3* was associated with VL PCSA only, with R-allele carriers exhibiting the largest values (Figure 6.3). Considering VL PCSA is dependent on muscle volume and fascicle length, genotype differences in either one or both of these

phenotypes could underlie, or contribute to the observed association. In the previous chapter, however, no significant association between VL fascicle length and *ACTN3* was observed, despite the values demonstrating the same trend as those observed for VL PCSA (Chapter 5). Furthermore, in the current chapter VL muscle volume and *ACTN3* genotype were not associated, although again the same trend in values (RX > RR > XX) was observed (Table 6.1). Contrastingly, a recent study reported that quadriceps femoris volume was greater for R-allele carriers compared to XX homozygotes (Erskine et al., 2013). Differences in the muscles investigated (VL compared to quadriceps femoris) may explain these contrasting results. It is probable that the influence of *ACTN3* genotype on individual muscle volume, such as the VL of the current study, is only modest and could be attributed to a greater proportion of type II fibres in the muscles of R-allele carriers (Vincent et al., 2007). However, when multiple phenotypes, each experiencing a modest influence of *ACTN3,* are investigated in combination, such as quadriceps femoris muscle volume or in the calculation of muscle PCSA, a detectable genotype association may be apparent. Although the size of the current sample (n = 120) is larger than that of some previous studies investigating *ACTN3* in relation to skeletal muscle phenotypes (Erskine et al., 2013; Gavin & Williams, 2010; Garatachea et al., 2012), and *a priori* statistical power was ≥80% for all of the phenotypes studied (Chapter 2), it is possible that the influence of *ACTN3* on muscle volume (and/or fascicle length) is so modest that the current chapter was underpowered to detect such an association. Adopting a polygenic approach to investigations of muscle size would account for a greater proportion of the inter-individual variability in muscle size than is possible when investigating individual polymorphisms, and may therefore be more appropriate in such instances (see Chapter 8).

ACE is responsible for the conversion of angiotensin I to angiotensin II, which is a known muscle growth factor (Geisterfer et al., 1988) and stimulant for growth hormone release (Messerli et al., 1977). Extensive research on the *ACE* I/D polymorphism to date has identified the overrepresentation of the D-allele among power/strength athletes in comparison to endurance athletes (Nazarov et al., 2001). As muscle size is one of the main determinants of muscle strength, *ACE* genotype was analysed in what the author believes is the first study to investigate this polymorphism for potential associations with a plethora of muscle size and mass measurements. In the current chapter, however no associations between *ACE* genotype and any of the muscle size phenotypes measured were observed, which is comparable to previous findings from studies investigating just single measures of muscle size (Erskine et al., 2013; Thomis et al., 2004; Frederiksen et al., 2003). Crucially, like the participants sampled in the current chapter, the aforementioned studies all recruited untrained participants. Together these findings indicate that *ACE* I/D genotype does not influence muscle size in this population, however the influence of *ACE* genotype on muscle size in response to strength training in this population remains unclear (Erskine et al., 2013; Thomis et al., 2004; Frederiksen et al., 2003). Knowledge of *ACE* genotype in response to training may benefit future practitioners prescribing exercise training for the treatment and prevention of muscle wasting disorders. Similarly, athletes following structured resistance training programmes are likely to benefit from programmes tailored to their individual genotype, although more research on the influence of *ACE* in response to training is required if the application of such information is to be successful. Additionally, there are substantial ethical concerns surrounding the application of sensitive genetic

information and these should be carefully considered prior to using the data in such a way (Wackerhage et al., 2009).

No associations between the *PTK2* rs7843014 or rs7460 polymorphisms and any of the muscle size or mass phenotypes were observed in the untrained and apparently healthy male population of this chapter. *PTK2* is the gene encoding FAK, which is primarily involved in the formation and turnover of muscle costameres (Quach & Rando, 2006). Costameres overlie the Z-lines and M-lines of skeletal muscle sarcomeres and are responsible for the lateral transmission of force from the muscle contractile proteins to the extracellular matrix during muscle contraction (Bloch & Gonzalez-Serratos, 2003), and previous research has demonstrated an increase in FAK expression following muscle hypertrophy (Flück et al., 1999). It is unclear if a causal relationship between increased FAK expression and muscle hypertrophy exists, as the current chapter did not investigate the association between *PTK2* and muscle size following exercise training. Furthermore, as *PTK2* genotype is associated with specific force in untrained individuals, it is probable that individuals able to produce greater specific force have a greater number of costameres per fibre rather than a greater number of fibres or increased fibre CSA. However, as there exists only one study to date that has investigated the association between *PTK2* genotype and muscle specific force, the subsequent chapter will investigate a potential association between the *PTK2* genotype and this phenotype.

6.5 Conclusion

This study has identified novel associations between polymorphisms in *ACTN3* and VL PCSA; *CNTF* and VL muscle thickness; and *TRHR* and appendicular lean mass. Furthermore, the lack of association between *ACE* I/D genotype and any of the muscle size phenotypes is in agreement with previous research and serves to strengthen the case that muscle size is independent of *ACE* genotype in untrained populations. Finally, the observation of no association between two polymorphisms in the *PTK2* gene demonstrates that in untrained males, *PTK2* does not influence VL muscle size or measures of lean mass. Consequently, there exists a clear genetic influence on some of these skeletal muscle size and mass phenotypes, providing evidence therefore, to suggest variability in muscle size may underlie some of the aforementioned genotype associations with muscle functional phenotypes.

Chapter 7

Influence of *ACE*, *ACTN3*, *CNTF, COL5A1*, *PTK2*, *TRHR* and *TTN* polymorphisms on muscle strength phenotypes

7.1 Introduction

Muscle strength, here defined as muscle voluntary contraction (MVC) torque, is the term used to express the ability of a muscle or groups of muscles to overcome an external load. Muscle strength is essential to complete activities of daily living (Beunen & Thomis, 2004; Thompson et al., 2004), and enhanced muscle strength is associated with strength and power performance (Abe et al., 2000; Hakkinen & Keskinen, 1989; Fukunaga et al., 2001). Muscle tissue is extremely plastic and adapts to different loading patterns accordingly, consequently, increases in muscle strength are commonly associated with muscle hypertrophy following exercise training and vice versa following detraining and disuse (Larsson et al., 1979; Atha, 1981; Jones & Rutherford, 1987; Gallagher et al., 2005; de Boer et al., 2008; MacDougall et al., 1980).

Measurement of muscle strength is typically undertaken using isokinetic dynamometry to ascertain isometric, concentric or eccentric strength, however a number of surrogate measures of strength exist. Reports of one repetition maximum, for example, are often used to represent muscle strength (McCall et al., 1996; McBride et al., 2003) and whilst such gross measurements of strength are accepted within the literature; unlike the measurement of specific force (fascicle force/physiological cross-sectional area), they do not take into account the previously identified inter-individual variability in the determinants of muscle strength (Chapter 4). Nonetheless, it is evident that inter-individual variability persists in the measurement of specific force and it is probable that some of the observed inter-individual variability in these muscle strength phenotypes may be explained by genetic differences. Accordingly, previous research has demonstrated the heritability of maximal isometric and isokinetic knee extension torque ranges from 31% to

75% (Tiainen et al., 2004; Thomis et al., 1997; Huygens et al., 2004). Furthermore, when investigating the genetic influence on muscle strength it would be useful to obtain both gross measurements of strength such as MVC torque, and more stringent measurements of specific force, to isolate the potential influence of genetic polymorphisms on the determinants of strength, which may also direct future research in this field.

To date, a number of genes have been investigated for associations with skeletal muscle strength phenotypes (Hughes et al., 2011; Bray et al., 2009), including polymorphisms in the angiotensin I-converting enzyme (*ACE*)(Williams et al., 2005; Thomis et al., 2004), alpha-actinin-3 (*ACTN3*)(Clarkson et al., 2005; Walsh et al., 2008; Ahmetov et al., 2013), ciliary neurotrophic factor (*CNTF*)(Roth et al., 2001; De Mars et al., 2007; Walsh et al., 2009) and protein tyrosine kinase 2 (*PTK2*)(Erskine et al., 2012) genes. However, before a consensus on the genotype associations with muscle strength phenotypes in an untrained population can be reached, it is evident that further research is necessary. Furthermore, polymorphisms in the collagen type V alpha 1 (*COL5A1*)(Collins & Posthumus, 2011), thyrotropin-releasing hormone (*TRHR*)(Chapter 6)(Liu et al., 2009; Lunardi et al., 2013) and titin (*TTN*)(Chapter 5) genes have been associated with one or more of the determinants of muscle strength, thus it is reasonable to assume that they might also associate with a muscle strength phenotype.

The aim of this chapter, therefore, was to investigate the individual influence of polymorphisms in the *ACE*, *ACTN3*, *CNTF*, *COL5A1*, *PTK2*, *TRHR* and *TTN* genes on the muscle strength phenotypes in an untrained population.

7.2 Methods

Detailed descriptions of participant recruitment, assessment of skeletal muscle properties, and the genotyping of *ACE* (rs4341), *ACTN3* (rs1815739), *CNTF* (rs1800169), *COL5A1* (rs12722), *PTK2* (rs7843014 and rs7460), *TRHR* (rs7832552) and *TTN* (rs10497520) polymorphisms are included in Chapter 2 (section 2.1, 2.2 and 2.4, respectively), thus only a brief description of these methods is detailed below.

7.2.1 Participants

Untrained Caucasian males [*n* = 120, age 20.6 (2.3) yr, stature 1.79 (0.06) m and mass 75.1 (10.1) kg; mean (SD)] volunteered to participate in this study. All participants met the inclusion criteria (described in Chapter 2.1) and provided written informed consent prior to involvement.

7.2.2 Skeletal muscle properties

Isometric maximal voluntary knee extension (MVC_{KE}) and flexion (MVC_{KF}) torque was measured at three knee joint angles to identify the optimal angle of peak torque production. Agonist muscle activation (Eq 1) and antagonist muscle co-activation during MVC_{KE} was determined to enable the calculation of net MVC_{KE} torque (Eq 2). Measurement of vastus lateralis (VL) anatomical cross-sectional area (ACSA), by contour matching a series of transverse plane scans, was used in the estimation of VL muscle volume (Eq 3). VL fascicle length, pennation angle (B-mode ultrasonography) and patellar tendon moment arm length (single energy DXA scan) were measured and used in the calculation of physiological cross-sectional area (PCSA; Eq 4), fascicle force (Eq 6) and tendon force (Eq

5), respectively. Ultimately, VL specific force was calculated by dividing VL fascicle force by VL PCSA (Eq 7).

7.2.3 Genotyping

Genotyping was completed using the fluorophore-based detection technique of TaqMan® real-time PCR. Genomic DNA amplification of fragments overlapping polymorphisms in the *ACE* (rs4341), *ACTN3* (rs1815739), *CNTF* (rs1800169), *COL5A1* (rs12722), *PTK2* (rs7843014 and rs7460), *TRHR* (rs7832552) and *TTN* (rs10497520) genes was completed for all participants.

7.2.4 Statistical analysis

The frequency of each polymorphism was assessed for compliance with Hardy-Weinberg equilibrium using X^2 tests. A one-way analysis of variance (ANOVA) was conducted to determine any significant differences in physical characteristics (stature, mass, BMI and age) between genotype. When genotype groups were combined, an independent samples *t*-test was used to identify any differences in physical characteristics. Pearson's correlation coefficient was used to identify the variables that made a meaningful contribution to the variability within muscle strength phenotypes (MVC_{KE} torque, net MVC_{KE} torque and VL specific force). ANOVA, and where appropriate analysis of covariance (ANCOVA), were conducted to identify any genotype differences in muscle strength phenotypes. Additionally, any genotype effects on muscle strength phenotypes were also assessed for linear trend using ANOVA or ANCOVA. In instances when too few participants represented one genotype group, this group was combined with the heterozygous group and an independent samples *t*-test was used to identify any differences between muscle strength

phenotypes. All significant associations identified in the main ANOVA or ANCOVA analyses were subject to *post-hoc* pairwise comparisons using the Benjamini-Hochberg correction. In instances where a tendency between genotype groups was observed, such that $P > 0.05$ but < 0.15, the two groups with similar means were combined and the analysis re-run using an independent samples *t*-test. To identify any *TTN* genotype differences in optimal angle for MVC_{KE} torque production an independent samples *t*-test was completed between the CC and CT individuals as no TT homozygotes were present in the current sample. Furthermore, the extent of linkage disequilibrium between *PTK2* rs7843014 and rs7460 was determined using CubeX online software (http://www.oege.org/software/cubex) to estimate haplotype frequencies, and in the calculation of *D'* and R^2 as the difference between the observed and expected haplotype frequencies (Gaunt et al., 2007). All statistical analyses were performed using SPSS version 19.0 and statistical significance was accepted when $P \leq 0.05$. Data are presented as means (SD).

7.3 Results

Genotype frequencies for polymorphisms in the *ACE*, *ACTN3*, *CNTF*, *COL5A1*, *PTK2*, *TRHR* and *TTN* genes are presented in Table 7.1 and were all in Hardy-Weinberg equilibrium ($P \geq 0.436$). No differences between any genotypes for any polymorphism were observed for stature ($P \geq 0.196$), mass ($P \geq 0.091$), BMI ($P \geq 0.140$) or age ($P \geq 0.455$; Table 7.1). Pearson's correlation coefficients revealed significant moderate correlations between muscle volume ($r \geq 0.343$, $P = 0.0005$), PCSA ($r \geq 0.417$, $P \leq 0.0005$) and all muscle strength phenotypes. Consequently, muscle volume and PCSA were included as covariates in analyses of MVC_{KE} torque and net MVC_{KE} torque but not specific force, as both factors are already accounted for during the calculation of muscle specific force. Additionally, stature ($r \geq 0.265$, $P \leq 0.04$),

mass ($r \geq 0.412$, $P = 0.0005$) and BMI ($r \geq 0.312$, $P \leq 0.001$) were moderately correlated with MVC_{KE} torque and net MVC_{KE} torque and were included as covariates in subsequent analyses.

Table 7.1. Genotype and allele frequencies, and muscle strength characteristics for single nucleotide polymorphisms (SNPs) in the *ACE*, *ACTN3*, *CNTF*, *COL5A1*, *PTK2*, *TRHR* and *TTN* genes. *Denotes significant difference between AA homozygotes and C-allele carriers for *PTK2* rs7843014, and between TT homozygotes and A-allele carriers for *PTK2* rs7460 ($P < 0.05$).

SNP	Genotype Number	Frequency (%) Genotype	Minor Allele	MVC_{KE} torque (N·m)	Net MVC_{KE} torque (N·m)	Specific Force ($N \cdot cm^{-2}$)
ACE rs4341	II (*n* = 32)	26.7		252 (42.7)	266 (61.3)	22.0 (2.8)
	ID (*n* = 54)	45.0	0.492	251 (49.4)	275 (53.3)	21.7 (2.6)
	DD (*n* = 34)	28.3		248 (43.9)	273 (46.0)	20.8 (2.5)
ACTN3 rs1815739	RR (*n* = 44)	36.6		252 (42.0)	273 (43.6)	21.5 (2.5)
	RX (*n* = 57)	47.5	0.396	250 (51.2)	269 (64.0)	21.7 (2.6)
	XX (*n* = 19)	15.9		249 (39.2)	278 (38.7)	21.4 (2.8)
CNTF rs1800169	AA (*n* = 2)	1.7		281 (18.6)	301 (9.47)	20.3 (0.7)
	AG (*n* = 33)	27.5	0.154	249 (32.0)	269 (39.1)	21.8 (2.6)
	GG (*n* = 85)	70.8		250 (50.6)	272 (58.6)	21.5 (2.6)
COL5A1 rs12722	CC (*n* = 19)	15.8		250 (39.5)	276 (48.9)	20.3 (3.1)
	CT (*n* = 61)	50.8	0.413	243 (40.5)	262 (51.9)	19.9 (2.6)
	TT (*n* = 40)	33.4		263 (53.9)	286 (55.5)	20.7 (4.7)
PTK2 rs7843014	AA (*n* = 42)	35.0		260 (50.9)	286 (53.0)	22.3 (2.6)*
	AC (*n* = 52)	43.3	0.433	250 (43.0)	272 (56.1)	21.1 (2.6)
	CC (*n* = 26)	21.7		243 (44.7)	257 (46.2)	21.1 (2.6)
PTK2 rs7460	AA (*n* = 34)	28.3		243 (45.9)	259 (46.9)	21.1 (2.6)
	AT (*n* = 54)	45.0	0.492	250 (42.0)	267 (53.8)	21.3 (2.5)
	TT (*n* = 32)	26.7		253 (51.7)	276 (57.8)	22.4 (2.8)*
TRHR rs7832552	CC (*n* = 53)	44.2		255 (48.1)	277 (50.8)	20.6 (4.4)
	CT (*n* = 58)	48.3	0.317	247 (43.9)	268 (57.6)	20.0 (2.7)
	TT (*n* = 9)	7.5		246 (47.0)	263 (40.2)	19.3 (1.6)
TTN rs10497520	CC (*n* = 95)	79.2		252 (46.7)	272 (55.4)	20.3 (3.6)
	CT (*n* = 25)	20.8	0.104	246 (43.1)	272 (45.9)	20.0 (3.2)
	TT (*n* = 0)	0.0		-	-	-

Analysis of VL specific force identified non-significant tendencies between genotype groups for *PTK2* rs7843014 (F = 2.881, P = 0.060) and rs7460 (F = 2.466, P = 0.089). Following combination of AC and CC *PTK2* rs7843014 genotypes, the VL specific force produced by AA homozygotes was significantly higher than that of the C-allele carriers (8.3%, t = 2.410, P = 0.017; Figure 7.1). Similarly, when the AA and AT *PTK2* rs7460 genotypes were pooled, TT homozygotes produced significantly higher VL specific force compared to A-allele carriers (5.4%, t = 2.205, P = 0.029; Figure 7.1). Due to the significant associations of both *PTK2* SNPs with VL specific force, linkage disequilibrium between them was calculated as D' = 0.894 and R^2 = 0.591. Furthermore, the individual contributions of the *PTK2* rs7843014 and rs7460 SNPs to the inter-individual variability in VL specific force were 3.5% (R^2 = 0.035, P = 0.041) and 3.3% (R^2 = 0.033, P = 0.048) respectively. The combined influence of these *PTK2* SNPs on the inter-individual variability in specific force was 3.8%, but this was non-significant (R^2 = 0.038, P = 0.107).

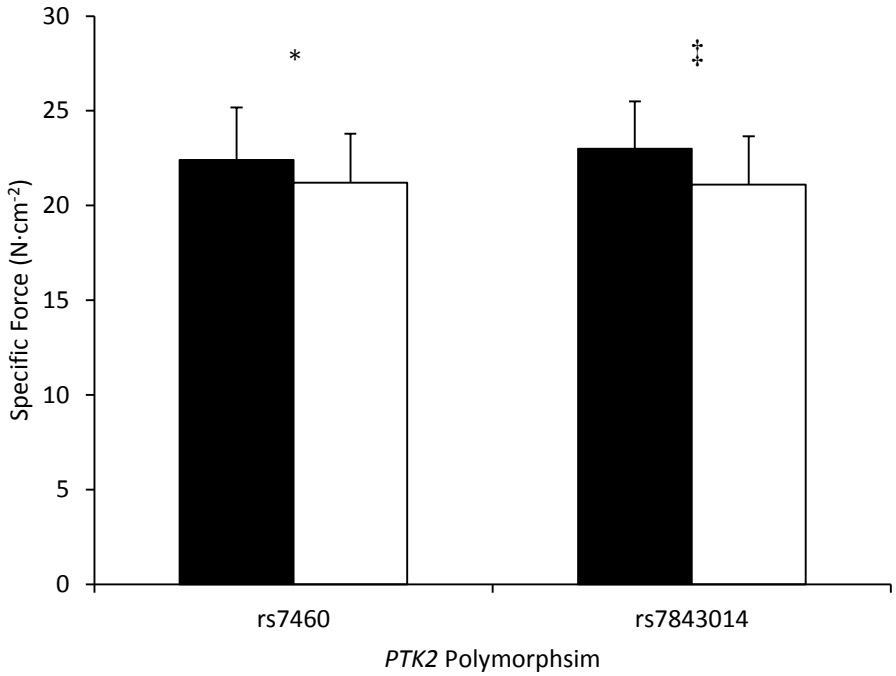


Figure 7.1. VL specific force according to genotype of the *PTK2* gene rs7843014 and rs7460 SNPs. Preferential (black bars) rs7460 TT homozygotes were significantly different from non-preferential (white bars) A-allele carriers (**P* = 0.029). Preferential rs7843014 AA homozygotes were significantly different to non-preferential C-allele carriers (‡*P* = 0.017). Data presented are means (SD).

A non-significant tendency between genotype groups of *PTK2* rs7843014 (*F* = -2.871, *P* = 0.094) was observed for net MVC_{KE} torque. Furthermore, linear trend analysis revealed a significant effect of *PTK2* rs7843014 (*P* = 0.041, η_p^2 = 0.042) on net MVC_{KE} torque, but not rs7460. Subsequently, the analysis was re-run with the rs7843014 CC and AC genotype groups pooled, however, no significant differences in net MVC_{KE} torque were observed between genotype groups (*t* = 1.531, *P* = 0.128). No significant differences in net MVC_{KE} torque were observed between genotype groups for *PTK2* rs7460 (*F* = 1.305, *P* = 0.275), nor were any significant linear trend effects of rs7460 on net MVC_{KE} torque apparent (*P* = 0.120, η_p^2 = 0.022). No significant differences in MVC_{KE} torque were observed between genotype groups for either *PTK2* polymorphism ($F \leq 2.276$, $P \geq 0.107$).

No significant differences in MVC_{KE} torque, net MVC_{KE} torque or VL specific force were observed between genotype groups for *ACE* ($F \leq 2.13$, $P \geq 0.123$), *ACTN3* ($F \leq 0.21$, $P \geq 0.815$), *CNTF* ($F \leq 0.46$, $P \geq 0.631$), *COL5A1* ($F \leq 2.643$, $P \geq 0.075$), *TRHR* ($F \leq 0.768$, $P \geq 0.466$) or *TTN* ($F \leq 0.312$, $P \geq 0.578$). Furthermore, no differences in optimal angle of peak MVC_{KE} torque production were observed between *TTN* genotype groups ($P \geq 0.762$). There were no significant linear trend effects on MVC_{KE} torque, net MVC_{KE} torque or VL specific force for *ACE* ($P \geq 0.451$, $\eta_p^2 \geq 0.009$), *ACTN3* ($P \geq 0.641$, $\eta_p^2 \geq 0.021$), *COL5A1* ($P \geq 0.323$, $\eta_p^2 \leq 0.043$) or *TRHR* ($P \geq 0.219$, $\eta_p^2 \geq 0.009$).

7.4 Discussion

The current chapter aimed to identify associations between polymorphisms in the *ACE*, *ACTN3*, *CNTF*, *COL5A1*, *PTK2*, *TRHR* and *TTN* genes and muscle strength phenotypes in an untrained, apparently healthy population. A significant genotype-phenotype association was identified between both *PTK2* SNPs and VL specific force only, no other significant associations were observed. Genotype and allele frequencies for SNPs in the current chapter (Table 7.1) were similar to previous reports in Caucasian populations (Williams et al., 2005; North et al., 1999; De Mars et al., 2007; Posthumus et al., 2011; Erskine et al., 2012)(allele frequency data compared to CEU HapMap where available). Values of mean MVC_{KE} torque, net MVC_{KE} torque and VL specific force in the current sample (250 N·m, 274 N·m and 21.4 $N \cdot cm^{-2}$, respectively) were in accordance with previous reports (245-269 N·m, 285 N·m, 20.0-23.9 $N \cdot cm^{-2}$, respectively) in comparable samples (Erskine et al., 2009; Erskine et al., 2012; Kellis & Baltzopoulos, 1997; Gorgey et al., 2006; Narici et al., 1992).

PTK2 is the gene encoding focal adhesion kinase (FAK), a protein integral for the formation and turnover of muscle costameres (Quach & Rando, 2006). During muscle contraction, muscle costamere complexes are involved in transmitting force laterally from the muscle contractile elements to the extracellular matrix (ECM)(Bloch & Gonzalez-Serratos, 2003). Altering the ability of the muscle to transmit force laterally, therefore, may translate into an increase or decrease in the production of maximal joint torque and/or muscle specific force. In the current study, polymorphisms in the *PTK2* gene were associated with VL specific force but not MVC_{KE} torque or net MVC_{KE} torque in untrained, apparently healthy males. Individuals homozygous for the rs7843014 A-allele had greater VL specific force than their C-allele counterparts, and individuals homozygous for the rs7460 T-allele demonstrated greater VL specific force than A-allele carriers (Figure 7.1). Similarly, a previous report on the influence of *PTK2* rs7843104 and rs7460 on muscle strength also reported a significant association between quadriceps femoris muscle specific force and A-allele (rs7843104) and T-allele (rs7460) homozygotes in a smaller Caucasian cohort (Erskine et al., 2012). Together these findings demonstrate a probable influence of *PTK2* on muscle specific force, although as both of these studies have investigated knee extensor muscles, future research should attempt to replicate these findings in other muscle groups. A plausible explanation for the findings of the current chapter may be that AA (rs7843104) and TT (rs7460) homozygotes experience altered *PTK2* expression compared to their C-allele (rs7843104) and A-allele (rs7460) carrying counterparts respectively. Interestingly, a recent study reported a possible association between the CC (rs7843104) and TT (rs7460) genotype and lower gene expression in Spanish Caucasians (Garatachea et al., 2014). Furthermore, FAK-null cells have previously been shown to form stronger adhesions, possess enhanced contractile properties and migrate slower than their wild-type

counterparts (Ilic et al., 1995; Chen et al., 2002; Ren et al., 2000). Although it is unlikely that either the rs7843104 or rs7460 polymorphisms would result in changes to the amino acid sequence of FAK that would elicit comparable effects to those of FAK-null cells. However, potential alterations to the magnitude, location and timing of gene expression, or mRNA stability as a consequence of one or both of these SNPs cannot be discounted (Erskine et al., 2012; Tabor et al., 2002).

Although speculative at this stage, the association between the TT (rs7460) genotype and enhanced VL specific force in the current study may be attributed to an increased muscle costamere density as a consequence of lower gene expression as observed by Garatachea et al. (2014). Understanding the association between the AA (rs7843014) genotype and enhanced specific force in the current study, however, appears more complex as it was the CC (rs7843014) genotype that was previously associated with a lower gene expression and might therefore be expected to have increased costamere density and thus muscle specific force. It is possible that because these two SNPs are in linkage disequilibrium, only one locus is functionally important for VL muscle specific force. In the current study, the individual and combined contributions of these two SNPs to the inter-individual variability in VL specific force was ~3.5%. Of the rs7843014 and rs7460 polymorphisms, it is the A-allele of the former that corresponds to the T-allele of the latter, and the non-random association of these SNPs might suggest that rs7843014 merely acts as a marker for the functional rs7460 SNP.

Although it remains unclear how an increased costamere density corresponds to an increase in specific force, it could be achieved by 1) having a greater number of costameres

per muscle fibre and/or, 2) having a greater number of smaller fibres per muscle and thus a higher fibre perimeter-to-area ratio (Erskine et al., 2012). Considering smaller muscle fibres, such as type I fibres, typically produce a lower fibre specific force than their larger, type II counterparts (Bottinelli et al., 1996; Widrick et al., 2002), it seems unlikely that having a greater number of smaller fibres would correspond to a greater muscle specific force without considerable genotype differences in muscle size or architecture. The findings of Chapters 5 and 6, however, revealed no significant *PTK2* genotype associations with any muscle architectural or size phenotypes, thus suggesting that an increased costamere density is not achieved by a greater number of smaller muscle fibres, rather by a greater number of costameres per fibre. Researchers are encouraged, however, to investigate *PTK2* genotype differences in muscle costamere density following direct measurement to confirm or refute this hypothesis.

For the remaining polymorphisms investigated in the current study, no genotype associations were observed with any of the muscle strength phenotypes. The lack of association between *COL5A1* and muscle strength was somewhat unsurprising considering no associations between this SNP and muscle architecture were observed in Chapter 5. Previous research has reported a link between *COL5A1* rs12722 and collagen fibril formation and diameter (Beighton et al., 1998), and as collagen is the primary structural protein in muscle connective tissue, the underlying muscle architecture may be influenced by differences in collagen fibril diameter, which could ultimately impact on the production of muscle strength and/or specific force. However, when considering the findings of the current thesis, it is possible that the consequences of *COL5A1* rs12722 on muscle architecture and strength are negligible. Future research may instead consider

investigating alternative collagen type V polymorphisms, such as collagen type V alpha 2 (*COL5A2*), and/or mutations in the more abundant collagen type I, such as collagen type I alpha 1 (*COL1A1*) to ascertain any influence on phenotypes linked with strength and its determinants.

Similarly to *COL5A1*, the lack of associations between *ACE* I/D, *ACTN3* R577X and the muscle strength phenotypes were also somewhat unsurprising considering *ACE* did not significantly associate with muscle size in previous chapters (Chapters 5 and 6), and *ACTN3* was significantly associated with only VL PCSA in Chapter 6. These findings are consistent with previous reports in untrained populations spanning ~20-90 yr and incorporating a range of muscle strength measurements (Garatachea et al., 2012; McCauley et al., 2009; Gavin & Williams, 2010; Erskine et al., 2013; Thomis et al., 2004; Delmonico et al., 2007; Pereira et al., 2013), although a number of contrasting reports do exist (Erskine et al., 2013; Walsh et al., 2008; Williams et al., 2005). Nonetheless, the findings of the current study add to the growing body of literature suggesting the inter-individual variability in muscle strength of untrained individuals is independent of *ACE* I/D and *ACTN3* R577X genotype when each polymorphism is considered individually. Recently, however, three studies have investigated the combined influence of *ACE* and *ACTN3* on muscle strength phenotypes and reported contrasting findings (Erskine et al., 2013; Garatachea et al., 2012; Pereira et al., 2013). Whilst Erskine et al. (2013) observed significant differences between the optimal and suboptimal combined *ACE* and *ACTN3* genotype for maximal leg strength and power, no significant associations between the combined *ACE* and *ACTN3* genotype and baseline muscle strength and strength endurance were reported by either Garatachea et al. (2012) or Pereira et al. (2013). Although all three studies were in agreement over the 'optimal'

combined *ACE* and *ACTN3* profile (DD and RR/RX, respectively), considerable differences in the mean age of the sample populations and the muscle strength phenotypes being assessed in each of these studies may explain this discrepancy, especially considering significant differences in the muscle specific force of recreationally active elderly (~74 yr) and young (~25 yr) males has previously been reported (Morse et al., 2005b). Consequently, there remains a requirement to conduct further polygenic investigations of muscle strength to elucidate the combined influence of these polymorphisms on such phenotypes (see Chapter 8).

CNTF is part of the interleukin-6 cytokine family and has previously been associated with MVC concentric torque in untrained populations (Roth et al., 2001; De Mars et al., 2007). Although only isometric torque and specific force were measured in the current study, no differences in these phenotypes according to *CNTF* genotype were observed. Nonetheless, it is possible that through previously observed associations with muscle architecture (Chapter 5) and pennation angle (Chapter 6), that *CNTF* is influential for concentric contractions, but negligible during isometric contractions. This hypothesis is consistent with De Mars et al. (2007), who found no significant effects of *CNTF* genotype on isometric MVC_{KE} strength in untrained Caucasian males. It is possible that genotype-dependent effects on both muscle thickness and fascicle length contribute to maintaining the functional capacity, and thus force production, of a muscle throughout a joint range of motion and at varying intensities as a consequence of fascicle gearing (Wakeling et al., 2011). Further research is, however, necessary to investigate muscle isokinetic strength, size and architecture simultaneously to confirm these speculations. Additionally, due to inter-muscular differences in pennation angle and joint articulation, future research should

investigate these phenotypes in a variety of muscles to ascertain the extent, if any, of *CNTF* genotype on muscle concentric strength and its determinants.

TRHR is involved in the secretion of thyroid-stimulating hormone (TSH), via the binding of thyrotropin-releasing hormone (TRH) to the receptor (Salvatore et al., 2014). A mutation in this gene causes hypothyroidism, a condition associated with muscle weakness (Salvatore et al., 2014). Additionally, the *TRHR* rs7832552 polymorphism has previously been significantly associated with various indices of lean mass in four independent samples (Liu et al., 2009; Lunardi et al., 2013)(Chapter 6). Furthermore, as lean mass correlates (r = 0.30-0.79) with muscle strength (Maughan et al., 1983; Reed et al., 1991), *TRHR* rs7832552 was an ideal candidate for investigating associations with muscle strength. No significant associations, however, between *TRHR* and muscle strength (isometric MVC_{KE} torque, net MVC_{KE} torque or VL specific force) were observed in the current chapter despite the *TRHR* genotype explaining ~6% of the inter-individual variability in leg lean mass of the current sample population (Chapter 6). Similarly, Lunardi et al. (2013) did not find any significant associations between isokinetic MVC_{KE} torque and *TRHR* in their older female cohort. Thus, it is possible that the influence of *TRHR* on muscle strength is so modest that it remained undetected during the current assessment of the VL muscle, and previous assessment of the quadriceps femoris (Lunardi et al., 2013). Accordingly, no associations between *TRHR* genotype and VL muscle size (ACSA, PCSA or volume) were observed in the previous chapter (Chapter 6). Assessments of muscle strength using multiple muscle groups, such as a maximal squat, may be necessary to ascertain if *TRHR* genotype contributes to muscle strength in an untrained population.

TTN is a template for myofibrillar protein assembly in the muscle sarcomere and is associated with resting sarcomere length in rat cardiac and skeletal muscle (Greaser et al., 2008; Greaser & Pleitner, 2014) and fascicle length in the human VL muscle (Chapter 5). Those individuals with shorter fascicles, therefore, may be expected to have larger pennation angles and thus produce greater force (Van Eijden et al., 1997; Blazevich, 2006). In the current chapter, however, there was no association between *TTN* genotype and any of the muscle strength phenotypes. Furthermore, no *TTN* genotype group differences in pennation angle were observed in Chapter 5. Despite the lack of association with muscle strength phenotypes, genotype differences in fascicle length may influence the length-tension relationship of the muscle, such that those with longer fascicles (CC) would generate peak torque at greater knee joint angles (i.e. in the direction of full flexion), whereas those individuals with shorter fascicle lengths would generate peak torque at smaller knee joint angles (i.e. in the direction of full extension). No differences in the optimal angle of peak MVC_{KE} torque production, however, were observed between CC and CT genotype groups, although as the current sample did not contain any TT homozygotes, future research is warranted to confirm the current observations.

7.5 Conclusions

This chapter has identified a significant association between polymorphisms in the *PTK2* gene and VL muscle specific force, which is consistent with previous research conducted on the quadriceps femoris muscle group (Erskine et al., 2012). The lack of association between muscle strength phenotypes and *COL5A1* genotype suggests that this polymorphism does not influence muscle strength, and researchers are encouraged to perform similar investigations on more abundant skeletal muscle collagens such as,

collagen type I and collagen type III, in the future. Additionally, no association between *ACE* I/D and *ACTN3* R577X and any muscle strength phenotypes were observed, which adds to the growing body of research suggesting muscle strength in untrained populations is independent of *ACE* I/D or *ACTN3* R577X genotype (Garatachea et al., 2012; McCauley et al., 2009; Gavin & Williams, 2010; Erskine et al., 2013; Thomis et al., 2004; Delmonico et al., 2007; Pereira et al., 2013). Furthermore, it would appear that despite polymorphisms within *CNTF*, *TRHR* and *TTN* contributing to the inter-individual variability in some of the determinants of muscle strength and specific force, they do not significantly influence muscle strength. Consequently, the extent and source of the genetic contribution to muscle strength remains unclear and it seems appropriate to adopt a polygenic approach when investigating this area in future.

Chapter 8

An assessment of the polygenic influence of *ACE*, *ACTN3*, *CNTF, COL5A1*, *PTK2*, *TRHR* and *TTN* polymorphisms on muscle strength and size phenotypes

8.1 Introduction

Muscle strength is a polygenic trait dependent on many genetic variants, each making a small contribution to the observed inter-individual variability. Previous research has highlighted that elite strength/power-trained athletes carry more of the favourable genetic variants for successful strength/power production than elite endurance athletes and non-athletes (Ruiz et al., 2010). In addition, a recent study explained between 3-9% of the inter-individual variability in untrained isokinetic knee extensor strength, endurance and rectus femoris diameter by investigating the combined influence of multiple genetic polymorphisms (Thomaes et al., 2013). Specifically, Thomaes et al (2013) reported the polygenic contribution to muscle strength and size in an untrained population, however, it should be noted that the authors selected the number of polymorphisms that were included in each phenotype-specific polygenic investigation. Consequently, it is likely that this manipulation inflated the probability of identifying significant polygenic-phenotype associations than if all selected polymorphisms had been included for each phenotype.

The multifactorial muscle strength phenotype is determined by a number of factors such as muscle architecture (Aagaard et al., 2001), size (Maughan et al., 1983), activation and co-activation capacity (Reeves et al., 2004a), tendon moment arm length (Tsaopoulos et al., 2006) and fibre type composition (Bottinelli et al., 1996). Each of these factors is independently influenced to some extent by genetic factors (Abe et al., 2000; Chan et al., 2008; Erskine et al., 2013; MacArthur et al., 2008; Karasik & Kiel, 2008; Thomis et al., 1997), therefore it is reasonable to assume that those genetic variants influencing muscle architecture or size, for example, may also confer a slight advantage/disadvantage for

muscle strength production. Despite the absence of many significant associations between a number of genetic polymorphisms and muscle architectural, size and strength phenotypes in the earlier chapters of the current thesis (Chapter 5, 6 and 7), it is possible that by quantifying the cumulative effect of these genetic polymorphisms on skeletal muscle phenotypes, a greater proportion of the inter-individual variability in these phenotypes will be accounted for than when each polymorphism was investigated independently (Akey et al., 2001). By adopting a polygenic approach, in which the cumulative effect of several non-significant tendencies (and some significant associations) between the genotypes and phenotypes of the previous chapters (Chapter 5, 6 and 7), the statistical power of the analysis is likely to increase, which may increase the potential for associations to approach statistical significance.

Furthermore, when investigating individual genotype-phenotype associations independently, potential gene-gene interactions are not accounted for. For example, Erskine et al. (2013) recently reported a significant combined effect of the 'optimal' angiotensin I-converting enzyme (*ACE;* DD/ID) and α-actinin-3 (*ACTN3*; RR/RX) genotypes on knee extensor strength and power in untrained males. This suggests that the influence of *ACE* genotype on untrained muscle strength and power is only significant when considered alongside the optimal *ACTN3* genotype. Contrastingly, there exist a number of other studies that report no significant combined effect of the 'optimal' *ACE* and *ACTN3* genotypes for muscle strength/power in untrained populations (Garatachea et al., 2012; McCauley et al., 2009). Thus, further research into the combined effect of multiple genetic polymorphisms is warranted, but there exists a possibility that the

Table 8.1. Genetic polymorphisms included in the polygenic analysis, their functions and interactions.

Gene Symbol	Gene	Polymorphism	Function
ACE	Angiotensin I-converting enzyme	I/D (rs4341)	Synthesises the vasoconstrictor angiotensin II and degrades growth-inhibitory kinins (Ishigai et al., 1997). The I/D polymorphism is involved in cardiovascular and skeletal muscle function and hypertrophy (Montgomery et al., 1997; Folland et al., 2000).
ACTN3	Alpha-actinin-3	R/X (rs1815739)	Encodes the actin-binding protein α-actinin-3, which is expressed exclusively in type II muscle fibres (North & Beggs, 1996). The R-allele of the R/X polymorphism is consistently overrepresented in elite strength/power training athletes (MacArthur & North, 2005).
CNTF	Ciliary neurotrophic factor	A/G (rs12722)	A pleiotropic cytokine involved in cell survival and differentiation (Sleeman et al., 2000). Involved in regulating muscle fibre number (Peroulakis & Forger, 2000) and the A/G polymorphism has been associated with muscle size and strength (Roth et al., 2001).
COL5A1	Collagen type V alpha 1	C/T (rs1800169)	Associates with both collagens type I and type III, and is involved in collagen fibril formation and diameter (Beighton et al., 1998). Polymorphisms linked to joint range of motion and connective tissue disorders (Collins et al., 2009; Voermans et al., 2008).
PTK2	Protein tyrosine kinase 2	A/C (rs7843014) C/T (rs7460)	Encodes focal adhesion kinase, a phosphoprotein involved in the formation and turnover of muscle costameres (Quach & Rando, 2006). Both polymorphisms linked to specific force production (Erskine et al., 2012) and exceptional longevity (Garatachea et al., 2014).
TRHR	Thyrotropin-releasing hormone receptor	C/T (rs7832552)	Regulates the secretion of thyroid-stimulating hormone via binding with thyrotropic-releasing hormone (Salvatore et al., 2014). Polymorphisms in *TRHR* have been associated with fat-free mass (Liu et al., 2009).
TTN	Titin	C/T (rs10497520)	Template for myofibrillar protein assembly during sarcomere formation (Chauveau et al., 2014). C/T polymorphism associated with cardiac and skeletal muscle sarcomere length (Greaser & Pleitner, 2014).

optimal genotypes of two or more of the genetic polymorphisms under investigation in the previous chapters (Chapter 5, 6 and 7) interact to alter muscle strength or size phenotypes to a greater extent than when investigated individually. The aim of the current chapter, therefore, was to investigate the combined influence of eight common polymorphisms, located within genes previously associated with skeletal muscle phenotypes (Table 8.1), on skeletal muscle size and strength phenotypes.

8.2 Methods

Detailed descriptions of participant recruitment, assessment of skeletal muscle properties, and the genotyping of *ACE* (rs4341), *ACTN3* (rs1815739), *CNTF* (rs1800169), *COL5A1* (rs12722), *PTK2* (rs7843014 and rs7460), *TRHR* (rs7832552) and *TTN* (rs10497520) polymorphisms are included in Chapter 2 (section 2.1, 2.2 and 2.4, respectively), thus only a brief description of these methods is detailed below.

8.2.1 Participants

Untrained Caucasian males [*n* = 120, age 20.6 (2.3) yr, stature 1.79 (0.06) m and mass 75.1 (10.1) kg; mean (SD)] volunteered to participate in this study. All participants met the inclusion criteria (described in Chapter 2.1) and provided written informed consent prior to involvement.

8.2.2 Skeletal muscle properties

Maximal voluntary knee extension (MVC_{KE}) and flexion (MVC_{KF}) torque was measured at three knee joint angles to identify the optimal angle of peak torque production. Agonist muscle activation (Eq 1) and antagonist muscle co-activation during MVC_{KE} was determined

to enable the calculation of net MVC_{KE} torque (Eq 2). Measurement of vastus lateralis (VL) anatomical cross-sectional area (ACSA), by contour matching a series of transverse plane scans, was used in the estimation of VL muscle volume (Eq 3). VL fascicle length, pennation angle and patellar tendon moment arm length were measured and used in the calculation of physiological cross-sectional area (PCSA; Eq 4), fascicle force (Eq 6) and tendon force (Eq 5), respectively. Ultimately, VL specific force was calculated by dividing VL fascicle force by VL PCSA (Eq 7). Finally, dual energy x-ray absorptiometry (DXA) was used to quantify appendicular (TALM), arm (ALM) and leg lean mass (LLM), all of which were analysed excluding bone mineral content.

8.2.3 Genotyping

Genotyping was completed using the fluorophore-based detection technique of TaqMan® real-time PCR. Genomic DNA amplification of fragments overlapping polymorphisms in the *ACE*, *ACTN3*, *CNTF*, *COL5A1*, *PTK2*, *TRHR* and *TTN* genes was completed for all participants.

8.2.4 Data analysis

The combined impact of all eight polymorphisms on muscle strength (MVC_{KE} torque, net MVC_{KE} torque and specific force) and size (VL ACSA, PCSA, volume, TALM, ALM, LLM and all measurements relative to body mass) phenotypes was assessed using a 'favourable' allele count (FAC) and total genotype score (TGS) for both strength ($FAC_{STRENGTH}$ and $TGS_{STRENGTH}$) and size (FAC_{SIZE} and TGS_{SIZE}). For each approach, the 'favourable' allele and genotype for muscle strength and size phenotypes was identified using previous literature and data from the current thesis (Table 8.2 and 8.3).

8.2.4.1'Favourable' allele count

Following identification of the 'favourable' allele for each polymorphism, participants were categorised into one of five groups (0-3, 4-6, 7-10, 11-13, 14-16) based on the number of 'favourable' alleles they possessed. A one-way analysis of variance (ANOVA) was used to assess linear trend effects between $FAC_{STRENGTH}$ and muscle strength phenotypes, and FAC_{SIZE} and muscle size phenotypes. Additionally, ANOVA was used to identify any group differences in muscle strength and size phenotypes for $FAC_{STRENGTH}$ and FAC_{SIZE}, respectively.

8.2.4.2 Total genotype score

To determine TGS, each genotype within each polymorphism was allocated a 'genotype score' (GS) of 0, 1 or 2. Allocation of genotype scores was based on the assumption that allele effects were co-dominant, and homozygotes deemed to have the favourable genotype for the phenotypes of interest (muscle strength or size) were allocated a GS of 2, heterozygotes scored 1 and the non-favourable homozygotes scored 0. Combination of each GS and transformation of the total score (Eq 8) allowed the combined influence of all eight gene variants on both muscle strength and size phenotypes to be quantified (Williams & Folland, 2008).

Equation 8: $TGS = (100/16) * (GS_{ACE} + GS_{ACTN3} + GS_{COL5A1} + GS_{CNTF} + GS_{PTK2a} + GS_{PTK2b} + GS_{TRHR} + GS_{TTN})$ (Eq 8)

In this instance, GS_{PTK2a} equates to the rs7843014 polymorphism and GS_{PTK2b} equates to the rs7460 polymorphism. Two TGS were calculated in the current chapter, one to represent the optimal profile for muscle strength phenotypes ($TGS_{STRENGTH}$) and another to reflect the optimal profile for muscle size phenotypes (TGS_{SIZE}). A $TGS_{STRENGTH}$ of 100 is

representative of a polygenic profile hypothesized to demonstrate the highest MVC_{KE} torque, net MVC_{KE} torque and specific force, whereas a $TGS_{STRENGTH}$ of 0 reflects a polygenic profile hypothesized to demonstrate the lowest MVC_{KE} torque, net MVC_{KE} torque and specific force. Similarly, a TGS_{SIZE} of 100 represents a polygenic profile which is hypothesized to demonstrate the greatest VL ACSA, PCSA, volume, TALM, ALM and LLM, and vice versa for a TGS_{SIZE} of 0.

8.2.4.3 Statistical analysis

The frequency of each polymorphism was assessed for compliance with Hardy-Weinberg equilibrium using X^2 tests. Pearson's correlation coefficients were conducted to ascertain whether an association existed between $TGS_{STRENGTH}$ and MVC_{KE} torque, net MVC_{KE} torque and specific force, and between TGS_{SIZE} and VL ACSA, PCSA, volume, TALM, ALM, LLM and all size measurements relative to body mass. As the $FAC_{STRENGTH}$ and FAC_{SIZE} data did not meet the assumptions of parametricity, the non-parametric Kruskal-Wallis test was conducted to identify any differences between $FAC_{STRENGTH}$ and MVC_{KE} torque, net MVC_{KE} torque and specific force, and between FAC_{SIZE} and VL ACSA, PCSA, volume, TALM, ALM and LLM, and all size phenotypes relative to body mass. Spearman's rank correlation coefficient was used to identify any linear associations between $FAC_{STRENGTH}$, FAC_{SIZE} and their respective phenotypes. All statistical analyses were performed using SPSS version 19.0 and statistical significance was accepted when $P \leq 0.05$.

Table 8.2. Genotype score for each polymorphism in relation to muscle strength phenotypes and rationale for score allocation.

Gene	Genotype score (2 = optimal)	Rationale	References (if available)
ACE	0 = II, 1 = ID, 2 = DD	D-allele and DD genotype consistently overrepresented in elite strength and power-trained athletes.	Nazarov et al. (2001); Costa et al. (2009); Tsianos et al. (2004); Woods et al. (2001)
ACTN3	0 = XX, 1 = RX, 2 = RR	R-allele and RR genotype consistently overrepresented and X-allele and XX genotype consistently underrepresented in elite strength and power-trained athletes.	Druzhevskaya et al. (2008); Yang et al. (2003); Niemi and Majamaa (2005); (Roth et al., 2008); Eynon et al. (2009)
CNTF	0 = GG, 1 = GA, 2 = AA	A-allele previously identified as the optimal allele for muscle strength production; A-allele was identified as the optimal allele for strength and architectural phenotypes in our data.	Roth et al. (2001); Chapter 5; Chapter 7
COL5A1	0 = CC, 1 = CT, 2 = TT	Tendency for the TT genotype to produce greater MVC_{KE} torque, net MVC_{KE} torque and specific force in Chapter 7, therefore genotype scoring was based on our own data.	Chapter 7
PTK2	0 = CC, 1 = AC, 2 = AA	AA previously identified as the optimal genotype for specific force in an untrained male population; scoring also reflects our own data.	Erskine et al. (2012); Chapter 7
	0 = CC, 1 = CT, 2 = TT	TT previously identified as the optimal genotype for specific force in an untrained male population; scoring also reflects our own data.	Erskine et al. (2012); Chapter 7
TRHR	0 = TT, 1 = AT, 2 = AA	Tendency for the CC genotype to produce greater MVC_{KE} torque, net MVC_{KE} torque and specific force than T-allele carriers, therefore scoring was based on our own data.	Chapter 7
TTN	0 = CC, 1 = CT, 2 = TT	Shorter muscle fascicles allow for greater pennation angles, and thus number of parallel force-generating sarcomeres, therefore genotype scores were allocated according to our own fascicle length data.	Chapter 5; Narici (1999); Blazevich (2006)

Table 8.3. Genotype score for each polymorphism in relation to muscle size phenotypes and rationale for score allocation.

Gene	Genotype score (2 = optimal)	Rationale	References (if available)
ACE	0 = II, 1 = ID, 2 = DD	D-allele and DD genotype consistently overrepresented in elite strength and power-trained athletes.	Nazarov et al. (2001); Costa et al. (2009); Tsianos et al. (2004); Woods et al. (2001)
ACTN3	0 = XX, 1 = RX, 2 = RR	R-allele and RR genotype consistently overrepresented and X-allele and XX genotype consistently underrepresented in elite strength and power-trained athletes.	Druzhevskaya et al. (2008); Yang et al. (2003); Niemi and Majamaa (2005); ; (Roth et al., 2008); Eynon et al. (2009)
CNTF	0 = GG, 1 = GA, 2 = AA	A-allele previously identified as the optimal allele for muscle strength production; A-allele was identified as the optimal allele for strength and architectural phenotypes in our data.	Roth et al. (2001); Chapter 5; Chapter 6; Chapter 7
COL5A1	0 = TT, 1 = CT, 2 = CC	Greater pennation angles are associated with increased muscle size, therefore scoring based upon our own muscle architectural data.	Chapter 5; Chapter 7; Narici (1999); Blazevich (2006)
PTK2	0 = AA, 1 = AC, 2 = CC 0 = TT, 1 = AT, 2 = AA	No existing research reporting significant associations between *PTK2* and muscle size phenotypes, therefore genotype scoring was based on our own muscle size phenotype data.	Chapter 6
TRHR	0 = CC, 1 = CT, 2 = TT	T-allele previously identified as the optimal allele for increased lean mass; TT genotype was significantly associated with measures of appendicular lean mass in Chapter 6.	Chapter 6; Liu et al. (2009)
TTN	0= CC, 1 = CT, 2 = TT	Shorter muscle fascicles associated with greater pennation angles and thus, increased muscle size, therefore genotype scores were allocated according to our own fascicle length data.	Chapter 5; Chapter 7; Narici (1999); Blazevich (2006)

8.3 Results

Genotype frequencies for polymorphisms in *ACE*, *ACTN3*, *CNTF*, *COL5A1*, *PTK2*, *TRHR* and *TTN* were all in Hardy-Weinberg equilibrium ($P \geq 0.436$).

8.3.1 Favourable allele count

No significant differences between $FAC_{STRENGTH}$ groups were observed for MVC_{KE} torque ($P = 0.715$), net MVC_{KE} torque ($P = 0.843$) or specific force ($P = 0.468$; Figure 8.1). Similarly, no significant linear trends were evident between $FAC_{STRENGTH}$ groups for any of the muscle strength phenotypes ($r \leq 0.087$, $P \geq 0.345$).

Furthermore, no significant differences were observed between FAC_{SIZE} groups and VL ACSA ($P = 0.292$), PCSA ($P = 0.727$), volume ($P = 0.572$), TALM ($P = 0.596$), ALM ($P = 0.556$) or LLM ($P = 0.642$; Figures 8.2). Additionally, no linear trend effects were observed between FAC_{SIZE} groups for any of the muscle size phenotypes ($r \leq 0.101$, $P \geq 0.271$). No significant differences were observed between FAC_{SIZE} groups and any muscle size relative to body mass phenotypes ($P \geq 0.162$), nor were any linear trend effects observed ($r \leq 0.073$, $P \geq 0.430$).

8.3.2. Total genotype score

None of the participants in the current chapter had the minimal (0) or maximal (100) $TGS_{STRENGTH}$ or TGS_{SIZE}. Scores ranged from 13-81 for $TGS_{STRENGTH}$ and 6-81 for TGS_{SIZE}. No significant correlations between the $TGS_{STRENGTH}$ and MVC_{KE} torque ($r = 0.040$, $P = 0.331$), net MVC_{KE} torque ($r = 0.055$, $P = 0.274$), or specific force ($r = 0.071$, $P = 0.220$) were observed. Similarly, no significant correlations were identified between the TGS_{SIZE} and

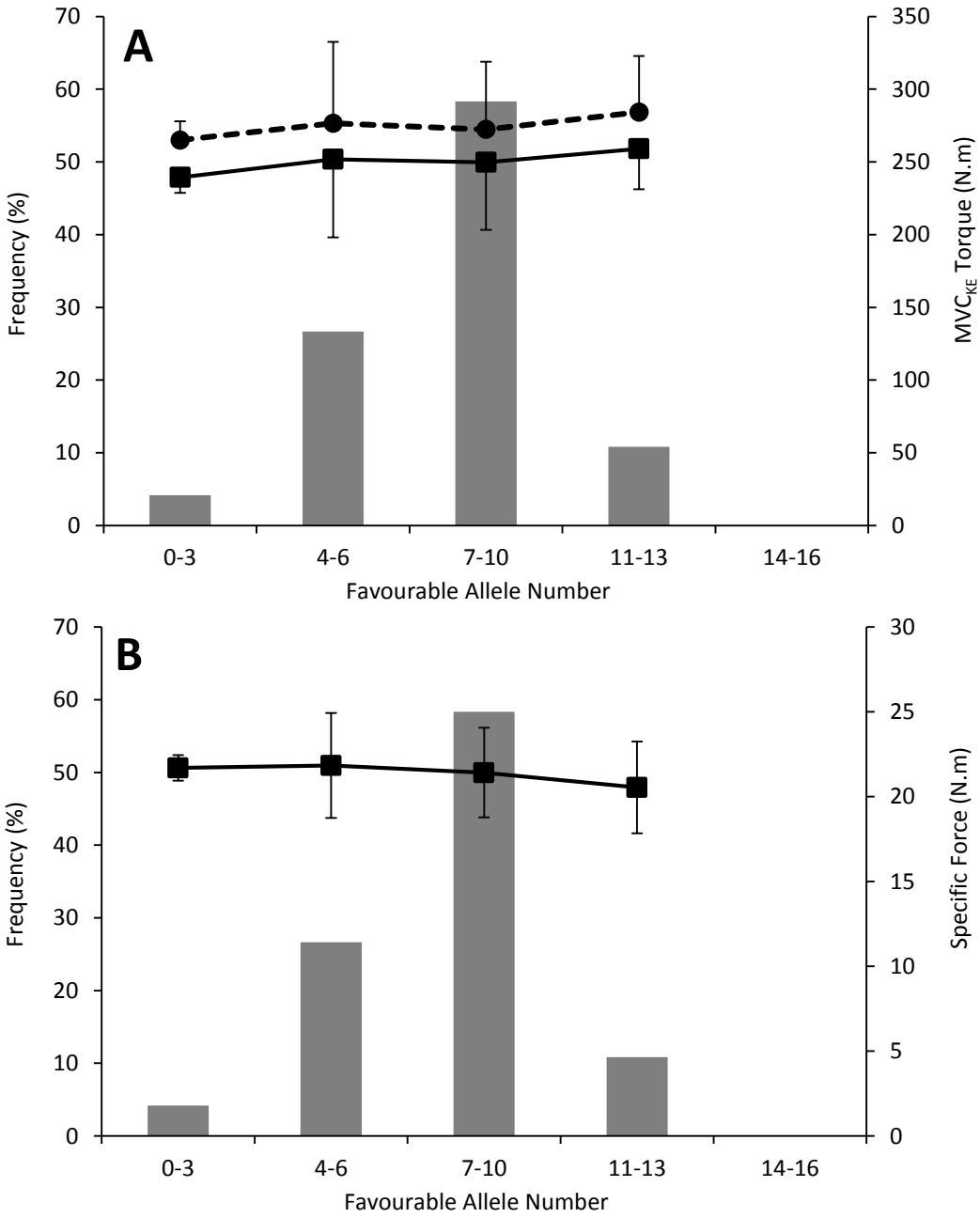


Figure 8.1. Favourable allele count ($FAC_{STRENGTH}$; grey bars), MVC_{KE} torque (black squares with solid line) and net MVC_{KE} torque (black circles with dashed line) (**A**); $FAC_{STRENGTH}$ (grey bars) and vastus lateralis specific force (**B**). Data are presented as means (SD).

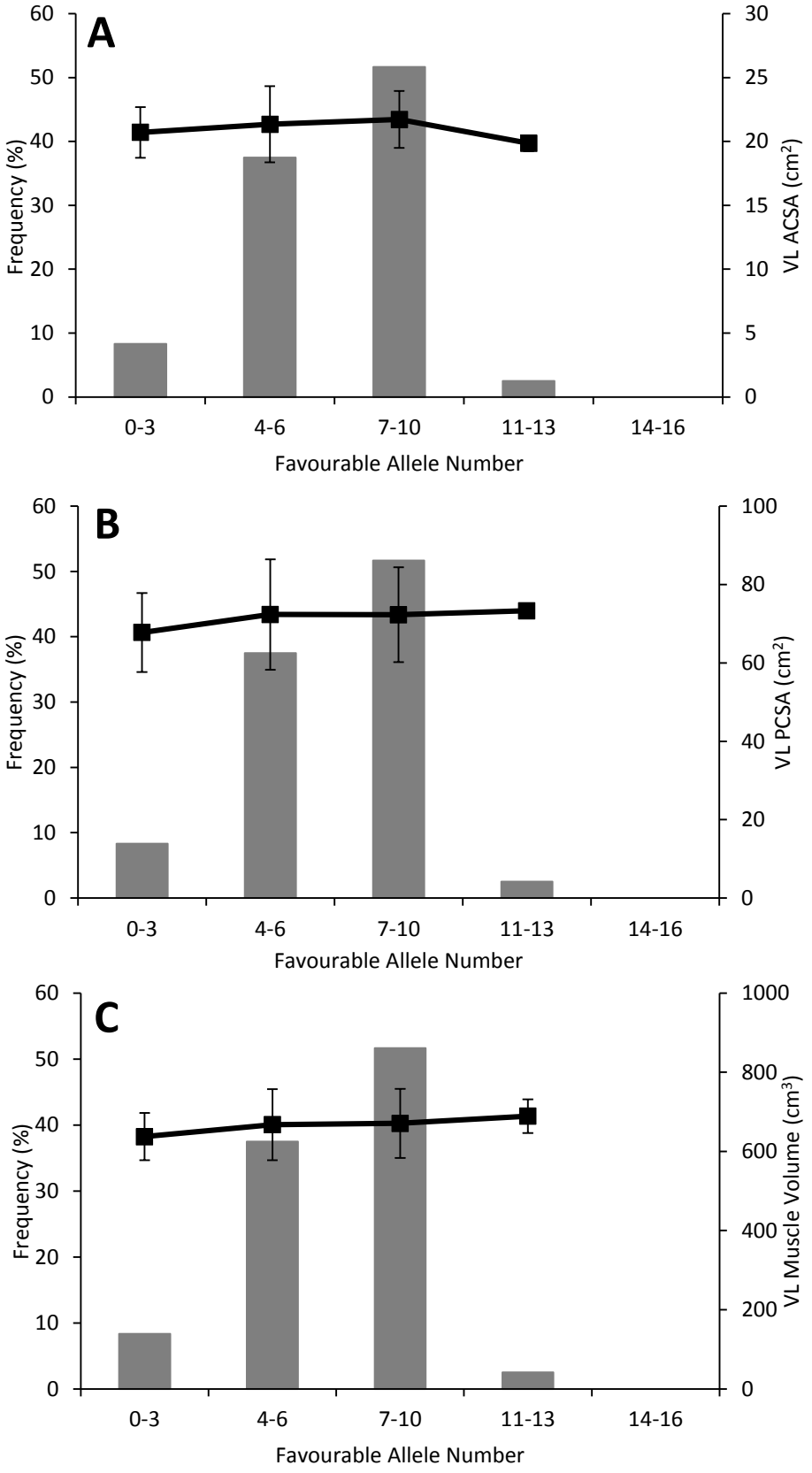


Figure 8.2. Favourable allele count (FAC_{SIZE}; grey bars) and, vastus lateralis (VL) anatomical cross-sectional area (**A**); FAC_{SIZE} and VL physiological cross-sectional area (**B**); and FAC_{SIZE} and VL muscle volume (**C**). Data are presented as means (SD).

VL ACSA (r = 0.059, P = 0.263), PCSA (r = 0.020, P = 0.415), muscle volume (r = 0.033, P = 0.360), or any measure of lean mass ($r \leq 0.089$, $P \geq 0.166$). Furthermore, there was a tendency for a weak correlation between TGS_{SIZE} and VL PCSA relative to body mass (r = 0.150, P = 0.051), although no significant correlations were observed between the TGS_{SIZE} and any of the other muscle size relative to body mass phenotypes ($r \leq 0.085$, $P \geq 0.178$).

8.4 Discussion

The current chapter aimed to identify the combined influence of polymorphisms within *ACE*, *ACTN3*, *CNTF*, *COL5A1*, *PTK2*, *TRHR* and *TTN* for associations with skeletal muscle strength and size phenotypes in an untrained, apparently healthy population. No significant associations were apparent between either of the calculated favourable allele counts ($FAC_{STRENGTH}$ or FAC_{SIZE}) and their respective muscle strength and size phenotypes. Similarly neither of the polygenic profiles ($TGS_{STRENGTH}$ or TGS_{SIZE}), including all eight of the aforementioned polymorphisms, were significantly associated with any of the muscle strength or size phenotypes.

Interestingly none of the participants in the current chapter possessed as few as one or none of the favourable alleles, or as many as 14, 15 or all of the favourable alleles. The average number of favourable alleles in possession was eight for muscle strength and seven for muscle size phenotypes. It is possible that those individuals possessing 14 or more favourable alleles are predisposed to athletic success, whereas those individuals in possession of one or fewer favourable alleles may be predisposed to skeletal muscle disorders, and consequently it is possible that those individuals at the extremes of this range did not meet the criteria of being untrained and healthy for inclusion within the

current research. For instance, *CNTF* deficiency has been observed in individuals with early onset multiple sclerosis (Giess et al., 2002), whereas mutations in *COL5A1* have been linked to the development of Ehlers-Danlos syndrome (Voermans et al., 2008). Furthermore, an overrepresentation of *ACE* 'D' and *ACTN3* 'R' alleles in elite strength/power trained athletes has been observed independently in a number of different athlete groups (Druzhevskaya et al., 2008; Nazarov et al., 2001; Roth et al., 2008). This suggests, therefore, that the FAC and TGS observed in the current chapter may be accurate representations of an untrained and apparently healthy population.

Although no significant associations between the polygenic profile and muscle strength phenotypes were observed in the current study, these findings are in agreement with a recent study on a population of untrained, elderly males (Thomaes et al., 2013). This recent study reported no significant associations between isometric MVC_{KE} strength and FAC based on four different polymorphisms to the eight used to calculate $FAC_{STRENGTH}$ in the current chapter. Together these findings might reflect the highly polygenic nature of isometric MVC_{KE} strength, which in the current investigation and that of Thomaes et al. (2013) may have been inadequately captured by including too few contributory polymorphisms in the FAC and TGS models. Despite observing few significant associations between muscle strength phenotypes and the polymorphisms in the previous chapter independently (Chapter 7), all eight of these polymorphisms exist within genes encoding proteins involved in skeletal muscle function (Table 8.1). A potential polygenic influence of these polymorphisms on skeletal muscle strength phenotypes, therefore, would seem reasonable. Furthermore, it appears probable that as new polymorphisms influencing muscle strength are discovered, and more are included within polygenic profiles, the

cumulative effect of these insignificant associations may begin to approach statistical significance, however, as this happens the probability of an individual possessing the optimal TGS (i.e. 100) will decrease accordingly (Williams & Folland, 2008). An alternative explanation for the lack of associations between the polygenic profiles and muscle strength phenotypes is that other yet undiscovered polymorphisms contribute substantially to skeletal muscle strength in an untrained population. To ascertain which of these hypotheses is correct, future research is warranted to continue identifying new polymorphisms using genome-wide association studies and candidate gene approaches before more extensive polygenic profiles can be investigated.

In contrast with previous literature, the current chapter observed no associations between either of the polygenic profiles and any muscle size phenotypes, although a tendency between TGS_{SIZE} and VL PCSA relative to body mass was evident. A recent study reported a significant positive correlation between rectus femoris diameter and FAC based on five different polymorphisms to those included in the current chapter (Thomaes et al., 2013). Evident methodological differences exist between the current chapter and that of the earlier study, particularly in the assessment of muscle size and the muscle of interest, which may explain this discrepancy. However, it is possible that the polymorphisms included in the polygenic profile of the earlier study are more influential for muscle size than those included in the current chapter. Nonetheless, this does not exclude the possibility of potential gene-gene interactions between these two sets of polymorphisms, and additional polymorphisms, from associations with muscle size phenotypes. Consequently, it is evident that there is a requirement for future research to investigate the polygenic influence on skeletal muscle phenotypes, in particular muscle size.

It is important to note that polygenic profiling using the models chosen in the current chapter is not without its limitations. Perhaps most notably is the identification of the favourable allele, and therefore the allocations of scores to each genotype. Due to the paucity of research available on some polymorphisms, scores were sometimes based on the associations and non-significant tendencies apparent within the data of the previous chapters (Chapter 5, 6 and 7). However, in instances where an extensive body of literature existed and multiple independent replications had occurred, or when the findings of the current thesis were comparable to those of previous research, identification of the favourable allele, and thus allocation of genotype scores was perhaps more justified. Allocation of genotype scores was based on the assumption that allele effects were co-dominant and the difference between each possible genotype score was therefore equal. Several authors, however, have suggested weighting the genotype scores, and polymorphisms, according to their influence on the phenotype (Williams & Folland, 2008; Hughes et al., 2011). Although a recent attempt to use this approach has been documented within the literature (Massidda et al., 2013), research on the discrete contribution of each polymorphism to the phenotype is lacking. Until more research identifying the deterministic power of polymorphisms is undertaken, adopting this approach would be premature.

8.5 Conclusion

In conclusion, there was no significant polygenic influence of *ACE*, *ACTN3*, *CNTF*, *COL5A1*, *PTK2*, *TRHR* and *TTN* on muscle strength and size phenotypes in an untrained, apparently healthy population when applying the mathematical and statistical models used in the

current chapter. However, previous studies using the same or similar models have identified significant polygenic profiles, and have included some of the same polymorphisms to those of the current chapter (Thomaes et al., 2013; Ben-Zaken et al., 2013). Future research including greater combinations of polymorphisms is therefore necessary to identify potential gene-gene interactions relevant for muscle strength and size. Furthermore, although there are several limitations of polygenic profiling, researchers should be encouraged to continue developing the existing polygenic approaches and models to eventually incorporate accurate genotype and polymorphism weightings that will allow more valid estimations of the polygenic influence on skeletal muscle phenotypes.

Chapter 9

General discussion

9.1 Overview

Skeletal muscle strength is a key determinant of an individual's ability to perform activities of daily living and achieve peak physical performance (Thompson et al., 2004; Beunen & Thomis, 2004). Following a review of the existing literature in Chapter 1, it is evident that muscle strength is multifactorial and is influenced by both intrinsic and extrinsic factors. An interesting recurrent observation is the considerable differences that exist between individuals in their muscle strength, and in their responses to such extrinsic factors. Furthermore, previous research investigating phenotypes related to skeletal muscle structure and function has identified a genetic influence on muscle strength and some of its determinants, which may partly explain the inter-individual variability evident in muscle strength. Consequently, the overall aim of the work presented in the current thesis was to investigate some of the genetic contribution to the inter-individual variability within skeletal muscle strength and some of its determinants, which may have identified novel genes or gene variants as targets for new treatments or interventions for sarcopenia and/or cachexia, for example, in future. More specifically, the objectives were:

1. To investigate the extent of inter-individual variability within skeletal muscle strength and some of its determinants in healthy, untrained males.
2. To determine whether eight gene polymorphisms in seven separate genes (*ACE*, *ACTN3*, *CNTF*, *COL5A1*, *PTK2*, *TRHR* and *TTN*) are associated with skeletal muscle strength and/or some of its determinants in healthy, untrained males.
3. To investigate whether polygenic profiles comprising these eight gene polymorphisms are associated with skeletal muscle strength and/or some of its determinants in healthy, untrained males.

9.2 Main findings and implications for future research

The main findings of the current thesis are discussed in the subsequent sections of this chapter. Briefly, the data confirm the extent of inter-individual variability previously observed in human muscle specific force and isometric maximal voluntary contraction (MVC) torque; and thereby demonstrate that the calculation of vastus lateralis (VL) specific force explains little of the inter-individual variability observed in isometric MVC torque (Chapter 4). Additionally, by establishing normative data inclusive of the inter-individual variability in some of the determinants of muscle strength, the first study objective was achieved (Chapter 4). Completion of the second study objective revealed a number of associations between some of the polymorphisms and skeletal muscle phenotypes of interest. Most notably, A-allele carriers of the ciliary neurotrophic factor (*CNTF*) rs1800169 polymorphism demonstrated greater VL pennation angle (Chapter 5) and muscle thickness (Chapter 6) than GG homozygotes, despite *CNTF* genotype not associating with other VL muscle size phenotypes (Chapter 6), VL fascicle length (Chapter 5), isometric MVC_{KE} torque or VL specific force (Chapter 7). The significance of titin (*TTN*) genotype for VL fascicle length was established for the first time in human skeletal muscle in Chapter 5, although this association did not appear to influence the optimal angle for peak torque production (Chapter 7). Within the protein tyrosine kinase 2 (*PTK2*) gene, both the rs7843014 and rs7460 polymorphisms were associated with VL specific force, thus confirming the observations of a previous smaller cohort study (Erskine et al., 2012), and provides the first independent replication of an association between *PTK2* and muscle specific force (Chapter 7). Similarly, the association between thyrotropin-releasing hormone receptor (*TRHR*) genotype and measures of appendicular lean mass indicates the first independent

replication of these data in a young, Caucasian male population (Chapter 6). Furthermore, despite *TRHR* associating with appendicular lean mass, no genotype associations with measures of VL muscle size were evident (Chapter 6). Finally, incorporating all eight of the aforementioned polymorphisms into a polygenic profile revealed no significant polygenic influence on muscle strength or size phenotypes in the untrained Caucasian population of the current thesis (Chapter 8).

9.2.1 Inter-individual variability

Prior to investigating the genetic influence on muscle strength and/or its determinants it was important to identify the extent of inter-individual variability within each of these phenotypes for a relatively large, homogeneous sample using the methods of the current study. Although Erskine et al. (2009) have previously reported the inter-individual variability of isometric MVC_{KE} torque [coefficient of variation (CV) = 17.8%] and quadriceps femoris specific force (CV = 16.2%), this was conducted using a slightly different method, using gold standard equipment (specifically MRI to determine VL muscle volume), and a smaller cohort than that of the current study (*n* = 27 vs. 73 in Chapter 4). Throughout the current thesis, MRI was not available for assessing VL muscle volume or patellar tendon moment arm length in all individuals. Thus, Chapter 3 was conducted to assess the reliability of an alternative method of measuring muscle specific force, more specifically to identify the reliability of using ultrasound to assess VL anatomical cross-sectional area (ACSA) for subsequent use in the estimation of VL muscle volume. Triangulation of multiple statistical tests (coefficient of variation, intra-class correlation coefficient and ratio limits of agreement) demonstrated very good agreement between the repeated measurements of VL muscle volume and specific force, amongst other measurements of muscle function

and morphology. Furthermore, the values of muscle function and morphology, specifically muscle size, obtained in Chapter 3 were comparable with those data obtained by previous research utilising gold standard techniques to measure VL muscle volume (Erskine et al., 2009), VL muscle architecture (Erskine et al., 2009; Reeves et al., 2004b) and quadriceps femoris muscle function (Erskine et al., 2009; Reeves et al., 2009). This suggests therefore, that the methods used to measure VL muscle size reported in Chapter 3 are reliable enough to allow for the accurate quantification of inter-individual variability in VL ACSA, physiological cross-sectional area (PCSA) and muscle volume. Consequently, these methods were utilised throughout the subsequent chapters (Chapters 4-8).

Muscle size is generally considered to be the greatest determinant of muscle strength (Maughan et al., 1983; Knuttgen, 1976), with approximately 50% of the inter-individual variability in MVC_{KE} torque previously attributed to differences in quadriceps femoris ACSA (Maughan et al., 1983; Maughan et al., 1984; Kanehisa et al., 1994). In pennate-fibred muscles, such as the constituents of the quadriceps femoris, however, ACSA underestimates the PCSA, and it is the PCSA that is understood to be the primary determinant of maximal muscle force production (Powell et al., 1984). Measurements of muscle architecture, activation capacity and co-activation during maximal voluntary contractions, and tendon moment arm length at the optimum joint angle allows for the calculation of muscle specific force, as described in Chapter 4. Despite accounting for potential differences within each of these determinants when calculating muscle specific force, inter-individual variability in specific force is evident (Maughan et al., 1983; Erskine et al., 2009; Narici et al., 1992; Chow et al., 1999; Gorgey et al., 2006; Reeves et al., 2004c). However, due to a variety of methodological inconsistencies between previous reports of

VL or quadriceps femoris specific force, there was a requirement to establish a normative set of data on the inter-individual variability in specific force, which was achieved in Chapter 4 on a relatively large, homogeneous sample. Additionally, because some of the measurements necessary for the calculation of muscle specific force could be identified as more or less stringent than other measurements, for example PCSA compared to ACSA, it was also appropriate to establish normative data on each of the determinants of VL specific force. By establishing a normative set of data on the inter-individual variability in the determinants of muscle specific force, it was apparent that the use of more stringent measures of muscle strength and size did not consistently equate to reduced inter-individual variability within these measurements. For instance, the inter-individual variability in the more stringent measurement of VL fascicle force was approximately 5% less than that identified in isometric MVC_{KE} torque, whereas the inter-individual variability in the more stringent measurement of VL PCSA was approximately 3% greater than that in ACSA. Despite this, a somewhat stronger correlation was observed between the more stringent measurements of VL fascicle force and VL PCSA (r^2 = 0.68) than that between isometric MVC_{KE} torque and VL ACSA (r^2 = 0.57), albeit these correlation coefficients were not significantly different. As specific force is ultimately calculated using the more stringent measurements (VL fascicle force/ VL PCSA; Eq 7), it was expected that the inter-individual variability in VL specific force would be lower than that in isometric MVC_{KE} torque. Although the inter-individual variability in VL specific force (13.5%) was lower than that in isometric MVC_{KE} torque (18.9%), accounting for all of the aforementioned determinants did not appreciably reduce this variability. Thus, it is probable that differences in the intrinsic force-generating capacity of individual fibres exist which contribute to the observed inter-individual variability in VL specific force. For example, differences in fibre

type composition are influenced to some extent by genetic factors (Simoneau & Bouchard, 1995) and may contribute to the observed inter-individual variability in VL specific force. Only one previous study investigating the genetic influence on muscle specific force exists (Erskine et al., 2012), and there are currently no studies reporting the polygenic influence on muscle specific force to the author's knowledge. Subsequently, the aim of Chapters 5-8 was to identify if any associations between eight common gene polymorphisms and skeletal muscle architectural, size and strength phenotypes were apparent.

9.2.2 Ciliary neurotrophic factor

The rationale for including each of the eight gene polymorphisms was established in Chapter 1 following a review of existing literature; there was evidence to suggest that each of the selected polymorphisms may be associated with skeletal muscle strength and/or some of its determinants. For instance, an association between muscle fibre number and/or volume (Peroulakis & Forger, 2000; Bengston et al., 1996; Forger et al., 1995) and *CNTF* has been demonstrated previously, which may indicate a link between *CNTF* and both muscle architecture and size. Accordingly, an association between *CNTF* and VL fibre pennation angle was observed in Chapter 5, with A-allele carriers demonstrating larger pennation angles than GG homozygotes. Similarly, A-allele carriers were also identified as having thicker VL muscles than their GG counterparts, a complementary finding considering the muscle size dependence of pennation angle in pennate muscles such as the VL (Kawakami et al., 2006).

Previous research has demonstrated a link between *CNTF* and a reduction in fibre degeneration, an increased fibre number (Peroulakis & Forger, 2000) and an increased fibre

cross-sectional area (CSA) (Guillet et al., 1999). It is reasonable to assume, therefore that those individuals able to produce the functioning *CNTF* protein, GG homozygotes, would exhibit the greatest muscle thickness and thus larger pennation angles, whereas in the current investigation it is the A-allele carriers who exhibit the greatest muscle thickness (Chapter 6) and larger pennation angles (Chapter 5). Furthermore, as both VL muscle thickness and pennation angle were associated with *CNTF*, it is somewhat surprising that this association was not continuous with other measures of muscle size (VL ACSA, PCSA and volume). Whilst it is probable that the inter-individual variability in VL muscle and fascicle length (Chapter 4) may explain the lack of association between *CNTF*, VL PCSA and volume, the reason underlying the lack of association between *CNTF* and VL ACSA is less clear.

Increases in fibre number (hyperplasia) and/or fibre CSA (hypertrophy) offer potential explanations as to how *CNTF* influences VL muscle thickness and pennation angle. However, as VL muscle fibre number and fibre CSA were not assessed in the present work, the mechanism by which *CNTF* influences muscle thickness and pennation angle remains unclear. Evidence exists to suggest that, although *CNTF* genotype remains stable throughout an individual's lifetime, muscle fibre number is determined before birth (Alberts et al., 2008; Rehfeldt et al., 1999) and hyperplasia does not occur thereafter (MacDougall et al., 1984; McCall et al., 1996). Furthermore as muscle fibre hypertrophy is extensively reported in response to functional overload (McCall et al., 1996; MacDougall et al., 1980; Widrick et al., 2002), it appears reasonable to assume that the association observed between *CNTF* genotype and VL muscle thickness in Chapter 6 was more likely due to fibre hypertrophy rather than hyperplasia, however, future research measuring the differences in fibre CSA is necessary to confirm this hypothesis.

As stated previously, the association between muscle size and muscle strength is well established (Maughan et al., 1983; Knuttgen, 1976). Furthermore, moderate pennation angles (<25°)(Blazevich, 2006), reportedly increase force production by allowing a greater quantity of contractile material to attach to the tendon or aponeurosis, therefore, increasing PCSA for any given muscle volume; and secondly by allowing fibres to function closer to their optimum length (Blazevich, 2006). Consequently, due to the aforementioned associations with VL muscle thickness and pennation angle, an association between *CNTF* and muscle strength phenotypes was investigated in Chapter 7. Consistent with some previous research, no significant association between *CNTF* and isometric MVC_{KE} torque was observed (De Mars et al., 2007). Contrastingly, however, other literature has demonstrated that *CNTF* is associated with knee extension concentric torque (Roth et al., 2001; De Mars et al., 2007), which considering the data from Chapter 5 and 6, could be the consequence of allowing for a greater fascicle gearing ratio (Wakeling et al., 2011).

Muscle fascicle gearing is the process by which muscle fibre shortening and lengthening velocities are limited to maintain sarcomere length close to its optimum throughout the joint range of motion, and different contraction intensities and speeds (Wakeling et al., 2011). Greater fascicle gearing ratios, determined by smaller pennation angles and muscle thickness, are therefore likely to confer an advantage for force production during muscle shortening contractions. Accordingly, lower concentric MVC_{KE} torques have been observed by AA homozygotes compared to G-allele carriers (Roth et al., 2001; De Mars et al., 2007), which might identify the *CNTF* G-allele or GG genotype as influential for muscle power

production, although this remains speculative at this stage and warrants further investigation.

9.2.3 Titin

TTN, a template for myofibrillar protein assembly in the muscle sarcomere, was associated with VL fascicle length but not pennation angle (Chapter 5), or any muscle strength phenotypes (Chapter 7). The mechanism(s) responsible for the association between *TTN* and VL fascicle length observed in Chapter 5 is unclear, however, the author has speculated that the presence of the T-allele, associated with shorter VL fascicles, may affect *TTN* splicing which may increase the expression of a smaller TTN isoform within the fascicles of heterozygotes. Earlier studies in rat cardiac muscle have demonstrated a link between *TTN* and alternative isoform splicing (Greaser et al., 2005), and more recently, *TTN* has been associated with both cardiac and skeletal muscle sarcomere length (Greaser et al., 2008; Greaser & Pleitner, 2014). Furthermore, considering the observed association between *TTN* genotype and VL fascicle length, it is possible that other skeletal muscle phenotypes, such as joint range of motion and/or maximum velocity of shortening, may be influenced by *TTN* genotype and more research is warranted to investigate these potential associations.

Evidence exists to suggest that within pennate muscles, those individuals with shorter muscle fascicles have correspondingly larger pennation angles and are more suited to producing higher forces and/or working over a smaller range of motion (Blazevich, 2006; Van Eijden et al., 1997). Therefore, the lack of association between *TTN* and VL fascicle pennation angle might explain the lack of association between *TTN* and muscle strength

observed in Chapter 7. It is pertinent to state that the current sample comprised no TT homozygotes, and although this is reflective of the low frequency of the T-allele within a Caucasian population (CEU HapMap), may indicate that an association between *TTN* and either VL fascicle pennation angle or muscle strength cannot be ruled out. Future research is warranted to investigate these potential associations on larger sample sizes comprising meaningful genotype group sizes. More specifically, based on the T-allele frequency observed in the current thesis, future studies would require ~1800 participants to obtain a TT homozygous group of ~20 individuals. Despite this, the genotype differences observed in VL fascicle length may influence the length-tension relationship of the VL muscle. For instance, CC homozygotes, which in Chapter 5 possessed longer VL fascicles, would in theory experience a rightward shift in their length-tension relationship and would therefore generate peak torque at greater knee joint angles than T-allele carriers with shorter fascicles. In Chapter 7, however, no significant differences in the optimal angle of peak MVC_{KE} torque production were observed between CC and CT genotype groups, although again as the current sample did not contain any TT homozygotes it is necessary for further research to replicate these findings independently.

9.2.4 Alpha-actinin-3

No associations between *ACTN3* genotype and VL muscle fascicle length or pennation angle (Chapter 5) were apparent in the current thesis. This is the first study to the author's knowledge to investigate *ACTN3* in relation to muscle architecture, however, previous research has identified differences in sarcomere Z-disc width according to the quantity of α-actinin proteins present within the sarcomere (Luther et al., 2003; Luther, 2009). Thus, *ACTN3* was selected as an appropriate candidate gene for such an investigation. Since

there are no substantial deleterious consequences of lacking the ACTN3 protein on muscle structure (North et al., 1999), probably due to a compensatory upregulation of the α-actinin-2 isoform (Mills et al., 2001), there appears to be no influence of *ACTN3* on VL muscle architecture in untrained, healthy males.

Additionally, associations between *ACTN3* and VL muscle volume, PCSA, ACSA, thickness and measures of lean body mass were investigated in Chapter 6. As type II fibres are known to have larger fibre CSAs than type I fibres (Bottinelli et al., 1996) and RR homozygotes, who are able to produce the fully-functioning ACTN3 protein, have been reported to possess a greater percentage of type II fibres, R-allele carriers were expected to have a greater muscle size than XX homozygotes. The findings of Chapter 6, however, demonstrated a significant association between *ACTN3* genotype and VL PCSA only, and this was regardless of the lack of genotype group differences observed in VL fascicle length and muscle volume, the determinants of VL PCSA. Previous research, has demonstrated significant associations between *ACTN3* and quadriceps femoris muscle volume (Erskine et al., 2013) and thigh muscle CSA (Zempo et al., 2010). Together, these findings might suggest the influence of *ACTN3* on measures of individual muscle size, such as the VL of the current thesis, is only modest. However when multiple phenotypes, each experiencing a modest influence of *ACTN3*, are investigated in combination, such as the quadriceps femoris muscle volume, thigh muscle CSA or in the calculation of muscle PCSA, a detectable genotype association is apparent.

Considering PCSA is understood to be the primary determinant of maximal muscle force production in pennate muscles, such as the VL (Powell et al., 1984), an association between

ACTN3 and VL muscle strength could be expected. However, no associations between *ACTN3* genotype groups and any VL muscle strength phenotypes were observed in the current thesis, which is consistent with previous reports in untrained populations (Gavin & Williams, 2010; McCauley et al., 2009; Santiago et al., 2010), although a number of contradictory reports do exist (Garatachea et al., 2012; Pereira et al., 2013). It is possible that other factors, such as tendon moment arm length, voluntary activation capacity and antagonist co-activation, which contribute to the inter-individual variability in isometric MVC_{KE} torque and net torque (Reeves et al., 2004c), may explain the lack of association between *ACTN3* genotype and these muscle strength phenotypes. However, when all of these additional variables were accounted for in the calculation of VL muscle specific force, no discernible contribution of *ACTN3* genotype to the phenotype was observed. Following these independent replications in untrained populations, each reporting no influence of *ACTN3* on muscle strength, it appears that muscle strength within this population may be independent of *ACTN3* genotype. This is unlike in athletic populations, where extensive reports have associated the *ACTN3* R-allele with increased muscle strength and/or power (Eynon et al., 2013), and may indicate a genotype interaction with exercise training (Clarkson et al., 2005), which should be investigated further.

9.2.5 Thyrotropin-releasing hormone receptor

Thyrotropin-releasing hormone (*TRHR*) was investigated for associations with skeletal muscle mass, size (both Chapter 6) and strength (Chapter 7), having previously been associated with measures of lean mass and strength following the use of genome-wide association studies (GWAS)(Liu et al., 2009). In Chapter 6, *TRHR* rs7832552 was significantly associated with measures of appendicular lean mass but not total body lean mass or any

muscle size phenotypes. Individuals homozygous for the T-allele possessed the greatest leg, arm and total appendicular lean mass, with a linear trend observed such that CC homozygotes possessed the smallest leg, arm and total appendicular lean mass. Whilst it is well established that the binding of thyrotropin-releasing hormone to *TRHR* regulates the secretion of thyroid-stimulating hormone, the underlying mechanism(s) responsible for the observed association between *TRHR* and appendicular lean mass remains unclear and warrants further research.

Liu et al. (2009) suggested the rs7832552 polymorphism as being influential for lean body mass in their GWAS, and identified the T-allele as being associated with increased lean mass, thus demonstrating the findings of Chapter 6 are in agreement with Liu et al. (2009). Furthermore, a recent independent replication of this earlier investigation observed no significant associations between *TRHR* rs7832552 and any measure of appendicular lean mass (Lunardi et al., 2013). This discrepancy may be explained by differences in sample population, with young males being recruited in the current thesis compared to older females in the recent investigation (Lunardi et al., 2013), as females are known to have fewer muscle fibres and smaller fibre CSAs than males (Henriksson-Larsen et al., 1985; Sale et al., 1987). Nonetheless, these contrasting findings highlight the necessity for future research to attempt independent replications to identify the extent of the influence of *TRHR* on indices of lean mass, specifically in a female population and in individuals of all ages.

Lean mass is known to correlate with muscle strength (r = 0.30-0.79)(Maughan et al., 1983; Reed et al., 1991), therefore an association between *TRHR* and the muscle strength

phenotypes measured in Chapter 7 was expected. No association, however, was observed between *TRHR* and isometric MVC_{KE} torque, net MVC_{KE} torque or VL specific force, which is comparable with a previous report investigating *TRHR* rs7832552 genotype and isokinetic MVC_{KE} torque (Lunardi et al., 2013). It is possible that the influence of *TRHR* on muscle strength is so modest that it remained undetected during the current assessment of the knee extensors. Similarly, a modest influence of *TRHR* on muscle size might also explain the lack of associations observed between *TRHR* and VL muscle volume, PCSA, ACSA and thickness in Chapter 6. Therefore, assessing multiple muscles when investigating genotype-associations with muscle size, such as the entire quadriceps femoris, and strength, such as during a maximal back squat, may be necessary to ascertain if *TRHR* contributes to muscle size and strength in an untrained male population.

9.2.6 Protein tyrosine kinase 2

PTK2 was investigated for associations with VL muscle architecture (Chapter 5), VL muscle size, lean mass (both Chapter 6), and muscle strength (Chapter 7). No significant associations between *PTK2* and VL muscle architecture, muscle size or lean mass were observed, despite both rs7843014 and rs7460 being significantly associated with VL muscle specific force in Chapter 7 and in a previous report (Erskine et al., 2012). As *PTK2* encodes focal adhesion kinase (FAK), a protein integral for the formation and turnover of muscle costameres (Quach & Rando, 2006), which have a major role in effective lateral force transmission, expecting an association between polymorphisms within this gene and VL specific force was justified.

In Chapter 7, it was the A-allele (rs7843014) and T-allele (rs7460) homozygotes that demonstrated greater VL specific force than their C-allele and A-allele carrying counterparts respectively. Although the mechanism underlying this significant association between *PTK2* and VL specific force is unclear, FAK-null cells demonstrate enhanced contractile properties, form stronger adhesions and migrate at a slower rate than their wild-type counterparts (Ilic et al., 1995; Chen et al., 2002; Ren et al., 2000). Although it is unlikely that either the rs7843014 or rs7460 polymorphisms would elicit comparable effects to the FAK-null cells, the AA and TT genotypes might alter the magnitude, location and timing of gene expression and subsequent differences in FAK expression might contribute to this observed association. A recent study reported a possible association between the CC (rs7843014) and TT (rs7460) genotype and lower gene expression (Garatachea et al., 2014). Therefore, the association between the TT (rs7460) genotype and increased VL specific force observed in Chapter 7 could be attributed to an increased costamere density as a consequence of a lower gene expression. The association between the AA (rs7843014) genotype, associated with higher gene expression according to Garatachea et al. (2014), and increased VL specific force, however, is more complex. A plausible explanation is the rs7843014 polymorphism is non-functional and is acting as a marker for the functional rs7460 polymorphism due to linkage disequilibrium.

Nonetheless the data presented in Chapter 7 are in agreement with those of Erskine et al. (2012) who also reported increased muscle specific force production by AA and TT homozygotes, therefore Chapter 7 provides the first independent replication of the findings from this previous smaller cohort study and together these suggest that *PTK2* genotype does influence muscle specific force. There is a requirement, however, for future

research to extend these observations to different muscles and population samples to ascertain if the findings of Chapter 7 and Erskine et al. (2012) are specific to the quadriceps of untrained Caucasian males or if these findings can be replicated, for example, in the upper limb, females and/or other ethnic groups.

9.2.7 Angiotensin I-converting enzyme

Investigations of angiotensin I-converting enzyme (*ACE*) genotype are reported in Chapter 6 and 7. *ACE* genotype has previously been associated with strength (DD genotype) and endurance (II genotype) performance (Nazarov et al., 2001; Woods et al., 2001), and cardiac and skeletal muscle hypertrophy (Montgomery et al., 1997; Folland et al., 2000), due to its role in catalysing the production of angiotensin II within the renin-angiotensin system (Rigat et al., 1990), and was therefore considered an appropriate candidate gene. The data indicated there were no significant associations between *ACE* genotype and any of the measured muscle size or strength phenotypes in untrained, healthy males. These data are comparable to previous research that reported no association between *ACE* genotype and measures of handgrip and elbow flexion strength (Garatachea et al., 2012; Thomis et al., 2004) and lower limb muscle power (Pereira et al., 2013; Erskine et al., 2013) in untrained populations. Additionally, the current data offer an independent replication in a relatively large cohort, which confirms two earlier investigations also reporting that knee extensor muscle strength and size is independent of *ACE* genotype (Erskine et al., 2013; McCauley et al., 2009). Thus, suggesting that the influence of *ACE* genotype on untrained muscle strength and size is minimal, however, the influence of *ACE* genotype on these phenotypes in response to strength training in an untrained population is less clear

and should be investigated further (Erskine et al., 2013; Thomis et al., 2004; Frederiksen et al., 2003).

9.2.8 Collagen type V alpha 1

The results from investigations into the influence of collagen type V alpha 1 (*COL5A1*) on muscle architectural and strength phenotypes are presented in Chapters 5 and 7. *COL5A1* encodes the α1 chain of collagen type V, which associates with collagen types I and III in skeletal muscle ECM (Collins & Posthumus, 2011); and tissues relatively abundant in collagen type V have been characterised by small collagen fibril diameters (Birk et al., 1990). Thus, any differences in collagen fibril diameter could have implications for the precise arrangement of muscle fibres, and subsequently force transmission during contraction. No significant associations between *COL5A1* genotype and VL fascicle length or pennation angle were observed in the untrained, healthy male population of the current thesis (Chapter 5). Consequently, it was somewhat unsurprising that *COL5A1* genotype did not associate with any of the muscle strength phenotypes of Chapter 7. These results suggest that *COL5A1* genotype does not contribute to the inter-individual variability observed in the muscle architectural and strength phenotypes (Chapter 4). Despite this, there have been over 79 polymorphisms identified within the *COL5A1 3'-UTR* gene and further research should investigate potential associations between these polymorphisms and skeletal muscle properties to elucidate if *COL5A1* makes a discernible contribution to the variability within skeletal muscle properties.

9.2.9 Polygenic Profiling

Polygenic profiling was conducted to ascertain the combined influence of all eight of the aforementioned gene polymorphisms on muscle size and strength phenotypes, however no significant associations between either of the muscle strength polygenic profiles and related phenotypes, or either of the muscle size polygenic profiles and related phenotypes were observed in Chapter 8. Few previous studies have investigated the polygenic influence on muscle strength and size phenotypes using profiles comparable to those used in Chapter 8. Thomaes et al. (2013) did, however, adopt a polygenic profile comparable to the favourable allele count used in Chapter 8, albeit using four different polymorphisms, and observed no significant polygenic influence on isometric MVC_{KE} torque. It is possible that the highly polygenic nature of muscle strength was inadequately captured by the polygenic profiles of both Chapter 8 and Thomaes et al. (2013) by including too few contributory polymorphisms. Contrastingly, Thomaes et al. (2013) did observe a significant polygenic influence on muscle size when incorporating five polymorphisms different to those included in Chapter 8 in their muscle size polygenic profile, which might suggest the polymorphisms selected by Thomaes et al. (2013) are more influential for muscle size than those included in the current thesis.

Despite observing few significant associations between muscle size and strength phenotypes in Chapters 6 and 7, it was appropriate to include all eight polymorphisms in each polygenic profile, as they all exist within genes encoding proteins involved in skeletal muscle function. However, it seems probable that as new polymorphisms are identified as influential for muscle size or strength, and are thus included in polygenic profiles in future, the cumulative effect of such modest independent associations observed in Chapters 6 and 7 may approach significance, but as this happens the likelihood of an individual possessing

the optimal polygenic profile will reduce accordingly. Furthermore, a potential limitation of the polygenic profiling in Chapter 8 was the allocation of genotype scores, as this was often based on the non-significant tendencies apparent in Chapters 5, 6 and 7, due to the lack of research concerning some of the polymorphisms being investigated. Additionally, genotype scores were allocated assuming allele effects were co-dominant, whereas some authors have suggested adopting a weighted scoring approach, in which genotypes and polymorphisms are given a weighted score according to their influence on the phenotype of interest. However, as research is lacking on the discrete contributions of the polymorphisms investigated in the current thesis, adopting such an approach in Chapter 8 would have been premature. Thus, it is evident that more research to uncover new polymorphisms for potential associations with the phenotypes investigated in Chapters 6, 7 and 8 is necessary. Perhaps more importantly, there is also a requirement for future studies to identify the deterministic power of newly discovered and existing polymorphisms associated with skeletal muscle strength and size phenotypes to allow existing polygenic profiles to continue to be used and developed that will allow for valid estimations of the polygenic influence on skeletal muscle phenotypes.

9.3 Methodological considerations and limitations

One of the implications of the findings presented in the current thesis is their potential association with the development of treatments for sarcopenia and/or cachexia, by identifying possible new target genes and thus generating new directions for future research. With this in mind, and considering that sarcopenia affects the lower body to a greater extent than the upper body (Lynch et al., 1999; Candow & Chilibeck, 2005), the quadriceps femoris was the muscle group chosen within the current thesis. This large anti-

gravity muscle group of the upper leg is essential for ambulation, jumping and squatting, functioning primarily as the knee extensor. Whilst it is relatively simple to assess whole quadriceps femoris function, via use of an isokinetic dynamometer as described in Chapter 2, detailed assessment of the morphological characteristics of each constituent is less simple (Blazevich, 2006). Consequently, the vastus lateralis was chosen as a representative of the quadriceps femoris for more detailed assessment, as is consistent with previous research (Alexander & Vernon, 1975; Wickiewicz et al., 1983; Scott et al., 1993; Trappe et al., 2001; Reeves et al., 2004b). Additionally, due the large CSA of the VL, its pennate fascicle arrangement and lateral location on the thigh, many gold-standard techniques (or acceptable alternatives) have been previously validated for completing assessments on this muscle (Erskine et al., 2009; Reeves et al., 2004b). However, due to differences in muscle CSA, fascicle arrangement and function between muscles of the upper and lower body, and indeed between muscles of the lower body, more research is required to ascertain if the potential implications of the current thesis in directing developments for sarcopenia and/or cachexia are comparable between muscle groups.

During assessments of quadriceps femoris function and morphology, the right leg of each participant was chosen for analysis, which is consistent with some previous research (Morse et al., 2007; Kent-Braun et al., 2000; Kellis & Baltzopoulos, 1997) but in contrast to others who opted to assess the dominant or non-dominant limbs of participants (Narici et al., 1996; De Vito et al., 2003; Folland & Williams, 2007a). Differences in MVC torque (Fousekis et al., 2010), muscle architecture (Kearns et al., 2001) and muscle size (McCreesh & Egan, 2011) have previously been observed between the dominant and non-dominant limb of trained participants and demonstrates the importance of accurately identifying the

dominant limb for assessments within this population. In untrained or sedentary participants, however, no significant bilateral difference in MVC torque (Lindle et al., 1997; Hageman et al., 1988; Häkkinen et al., 1998), architecture (Kearns et al., 2001), CSA and EMG (Häkkinen et al., 1996) have been observed, which suggests that the decision to assess only the right leg in the participants of the current thesis was not disadvantageous. Nonetheless, although no significant differences between limbs were observed in the aforementioned investigations, small non-significant differences were evident (4-9%), which may have influenced the results of Chapters 4-8. For instance, if small non-significant differences were evident between the limbs of participants in the current thesis, assuming that the dominant limb of each participant was marginally stronger and bigger, measurements of MVC torque and ACSA may have been underestimated in participants whose left leg was their dominant limb. Underestimating the MVC torque and ACSA in some participants may have subsequently increased the inter-individual variability within VL muscle volume and PCSA above what might otherwise be expected had the dominant limb of each participant been assessed. However, as differences in MVC torque and ACSA are accounted for in the calculation of specific force, this is unlikely to have been affected. Nonetheless, it is recommended that future research complete assessments of muscle function or morphology on the dominant limb.

It is acknowledged that variations in MVC torque occur according to circadian rhythm (Reilly & Waterhouse, 2009; Drust et al., 2005; Guette et al., 2005), and whilst it was possible to standardise the time of day at which participants were tested in Chapter 3, it was not logistically possible to standardise this for the 120 participants in the remaining chapters. Subsequently, an attempt to reduce the impact of time of day variations on the

measurements of muscle strength and its determinants was made by limiting data collection to between 0900-1600 hours for all participants. This timeframe has previously been associated with less than ~5% change in MVC torque production (Guette et al., 2005), which when considering previous data, is small enough to allow for the detection of any genotype differences in MVC torque (Erskine et al., 2013). Furthermore, evidence exists to suggest muscle activation capacity and co-activation do not vary according to the time of day (Guette et al., 2005). Moreover, as these latter measurements were both used in subsequent calculations of fascicle force and specific force in Chapters 7 and 8, the variability within them as a consequence of time of day is likely to be small.

Consistent with apparent common practice within the field of functional muscle physiology, one familiarisation session was completed for each participant prior to data collection in the current thesis (Maganaris et al., 1998; Bamman et al., 2000; Morse et al., 2007; Impellizzeri et al., 2008). The purpose of this session was to familiarise participants with the isokinetic dynamometer test protocol to reduce the effects of learning during subsequent data collection. Despite evidence demonstrating one familiarisation session is sufficient prior to assessing maximal muscle strength in previously untrained individuals, contrasting reports exist that suggest the completion of two (Oliveira et al., 2013) or three (Tracy et al., 1999; Tihanyi et al., 2007) familiarisation sessions is necessary to fully habituate an individual to maximal muscle strength testing protocols, and thus reduce the occurrence of systematic bias in the measurement. According to these latter studies, insufficient familiarisation may have occurred in the current thesis, which would likely underestimate – and increase the inter-individual variability within – the true MVC of participants, in addition to their VL specific force. However, when comparing both the MVC

torque and specific force values obtained in Chapters 4 and 7 to existing literature, this seems unlikely (Erskine et al., 2009; Kellis & Baltzopoulos, 1997; Gorgey et al., 2006; Narici et al., 1992). On the other hand, completing too many familiarisation sessions may overestimate true MVC torque as a consequence of participants experiencing a training effect (Sale, 1988). Thus, these complexities and the existing contrasting reports highlight the current lack of agreement surrounding the correct approach to familiarisation prior to assessments of maximal muscle strength, and warrants further investigation.

9.4 Conclusion

The current thesis extended the growing body of literature demonstrating there is a genetic influence on human skeletal muscle phenotypes, in particular muscle architecture, size and strength. Investigations into the genetic influence on muscle architecture revealed two novel associations. Firstly, VL muscle fascicle length was associated with *TTN*, which could be as a consequence of genotype-dependent TTN isoform splicing, although further research to elucidate this is required. Secondly, *CNTF* was associated with VL fascicle pennation angle, which, in addition to being associated with VL muscle thickness, might indicate a possible role of *CNTF* genotype in muscle fascicle gearing and thus power production, rather than maximal isometric torque that was measured in the current thesis.

Furthermore, the work presented in the current thesis included the first independent replications, to the author's knowledge, of associations between VL muscle specific force and *PTK2*, and appendicular lean mass and *TRHR* rs7832552. The former of these two associations provides independent confirmation that *PTK2* rs7843014 and rs7460 are influential for VL specific force production, possibly due to genotype-dependent

differences in gene expression affecting the adhesion strength and migration rate of FAK within the skeletal muscle costameres. The latter of these associations extends the findings of an initial GWAS by identifying the *TRHR* rs7832552 TT genotype as beneficial for appendicular lean mass. In addition, the non-associations between *COL5A1* and both VL muscle architectural and muscle strength phenotypes represent the first investigations of this polymorphism with such phenotypes, although no association was observed in the current thesis. Despite observing some individual candidate gene associations with skeletal muscle phenotypes, polygenic profiling revealed no significant polygenic influence on any muscle or strength phenotypes.

Nonetheless, the work presented here has applications for improving physical performance, in addition to enhancing our understanding of skeletal muscle disorders, which may have implications for how individuals exercise and how skeletal muscle disorders are treated and/or prevented in future.

9.5 Directions for future research

Throughout the current thesis specific areas for future research have already been highlighted; thus broader directions for future research are discussed here, which are based on both the findings of the current thesis and the movement of the field of sport and exercise genetics since the commencement of this thesis.

Within the studies documented in Chapters 5, 6, 7 and 8, eight polymorphisms from seven candidate genes (*ACE*, *ACTN3*, *CNTF*, *COL5A1*, *PTK2*, *TRHR* and *TTN*) were selected to try to capture the genetic variability within several functional and morphological skeletal muscle

phenotypes. Despite the findings of the current thesis identifying novel associations with muscle architecture and providing the first independent replications of two previously reported associations within untrained Caucasian males, a number of other genes have been identified as potential candidates for associations with skeletal muscle phenotypes (Hubal et al., 2010; Li et al., 2014; Zarebska et al., 2014). For example, 5,10-methylenetetrahydrofolate reductase (*MTHFR*) was recently indirectly associated with muscle strength in a case-control study of Russian and Polish elite athletes (Zarebska et al., 2014). A finding that has yet to be replicated independently or investigated further using direct measurements of skeletal muscle strength (and related phenotypes), such as those employed in the methods of the current thesis. Therefore, to improve the strength of new and existing associations in future, researchers are encouraged to 1) continue identifying new candidate genes for potential associations with skeletal muscle properties; 2) conduct genetic association studies with direct measurement of skeletal muscle phenotypes using thousands of individuals situated at all locations along the muscle-strength continuum, and; 3) perform independent replications of reported associations in similar and different populations to those previously investigated.

In the current thesis, the findings demonstrated the somewhat unpredictable nature of candidate gene associations. For instance, *ACTN3* genotype was associated with VL PCSA but not ACSA or muscle volume, and *PTK2* genotype was associated with VL specific force but no other measures of muscle strength. Thus, when investigating the genetic influence on these, and other skeletal muscle functional and morphological phenotypes, the findings of the current thesis demonstrate the necessity to include broad, and where possible, stringent measurements of the phenotype. Future candidate gene association studies are

therefore encouraged to incorporate multiple phenotype assessments in order to capture potential genotype-phenotype associations that may otherwise remain undetected if only one measurement was used.

It is well established that both skeletal muscle size and strength are polygenic phenotypes, and although the work presented in Chapter 8 did not reveal any significant polygenic associations with these phenotypes, it is pertinent to state that only eight polymorphisms were included in these polygenic profiles. Researchers are therefore encouraged to investigate the polygenic influence of these, and other phenotypes, using combinations of the polymorphisms reported in the current thesis and those reported elsewhere. Additionally, polygenic profiling in sport and exercise genetics remains a relatively new approach, being first reported less than 7 years ago (Williams & Folland, 2008), thus future research is warranted to continue to develop the application of polygenic profiling within sport and exercise genetics, but more specifically for skeletal muscle phenotypes. Furthermore, as more polymorphisms are identified as significant for skeletal muscle size and strength; extensive independent replications are conducted to support the genotype scoring process and improvements are made to existing polygenic profiling approaches, more valid estimations of the polygenic influence on these and other related phenotypes can be completed in future.

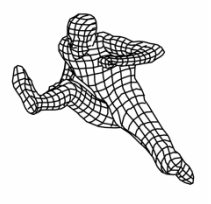
Appendix 1

Informed consent and questionnaire

Manchester Metropolitan University — Cheshire —

Department of Exercise and Sport Science

Sport and Exercise Science



Informed Consent Form

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

Name of Participant:

Principal Investigator: **Georgina Stebbings**

Project Title: **Genetic influence on skeletal muscle architecture and strength.**

Ethics Committee Approval Number: **01.06.11(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	

<u>Parental or guardian consent for research involving children.</u>

I confirm that the details of this study have been fully explained and described in writing to (insert name) and have been understood by him/her and I therefore consent to his/her participation in this study.

Signed :		Date :	

Pre-Test Medical Questionnaire & Physical Activity Assessment

Department of Exercise and Sport Science
MMU Cheshire

Name: ……

Date of Birth: ……/……/…… Age: …… Gender: ……

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. **Smoking Habits**
   Are you currently a smoker? Yes / No
   If yes, how many do you smoke …….. per day

   Are you a previous smoker? Yes / No
   If yes, how long is it since you stopped …….. years

3. **Do you drink alcohol?** Yes / No
   If you answered **Yes**, do you usually have?
   An occasional drink / a drink every day / more than one drink a day?

4. **Have you had to consult your doctor within the last 6 months?** Yes / No
   If you answered **Yes**, please give details……..……..
   …… ……..……

5. **Are you presently taking any form of medication?** Yes / No
   If you answered **Yes**, please give details……..
   …….. ……..……

6. **As far as you are aware, do you have or have you ever had:**

a. Diabetes	Yes / No	**b.** Asthma	Yes / No
c. Epilepsy	Yes / No	**d.** Bronchitis	Yes / No
e. Any form of heart	Yes / No	**f.** Raynaud's Disease	Yes / No
g. Marfan's Syndrome	Yes / No	**h.** Aneurysm/embolism	Yes / No
I. Anaemia	Yes / No		

   Any other medical condition or illness? Yes / No
   If you answered **Yes**, please give details ……
   …….

7. **Is there a history of heart disease in your family?** Yes / No

8. **Do you currently have any form of muscle or joint injury?** Yes / No
   If you answered **Yes**, please give details……
   ……

9. **Have you suspended your normal training in the last 2 weeks?** Yes / No
   If the answer is **Yes** please give details……
   ……

10. **Please read the following questions. As far as you are aware:**
    a) Do you have any known serious infection? Yes / No
    b) Have you had jaundice within the previous year? Yes / No
    c) Have you ever had any form of hepatitis? Yes / No
    d) Are you HIV antibody positive Yes / No
    e) Have you had unprotected sexual intercourse with any person from a HIV high-risk population? Yes / No
    f) Have you ever been involved in intravenous drug use? Yes / No
    g) Are you haemophiliac? Yes / No

11. **What is your main occupation?** ……

12. **At work I sit:**
    Never / Seldom / Sometimes / Often / Always

13. **At work I stand:**
    Never / Seldom / Sometimes / Often / Always

14. **At work I walk:**
    Never / Seldom / Sometimes / Often / Always

15. **At work I lift heady loads:**
    Never / Seldom / Sometimes / Often / Always

16. **After work I am tired:**
    Very Often / Often / Sometimes / Seldom / Never

17. **At work I sweat:**
    Very Often / Often / Sometimes / Seldom / Never

18. In comparison to others my own age I think my work is:
Much Heavier / Heavier / Same / Lighter / Much Lighter

19. Do you play sport or exercise? Yes / No

20. If *yes*, which sport do you play most frequently? ……

a. **How many hours per week?**
Less than 1 / 1-2 / 2-3 / 3-4 / More than 4

b. **How many months per year?**
Less than 1 / 1-3 / 4-6 / 7-9 / More than 9

21. If you play a second sport/exercise, which is it? …….

a. **How many hours per week?**
Less than 1 / 1-2 / 2-3 / 3-4 / More than 4

b. **How many months per year?**
Less than 1 / 1-3 / 4-6 / 7-9 / More than 9

22. In comparison with others of my own age I think my physical activity during leisure time is:
Much more / More / Same / Less / Much Less

23. During leisure time I sweat:
Very Often / Often / Sometimes / Seldom / Never

24. During leisure time I play sport:
Very Often / Often / Sometimes / Seldom / Never

25. During leisure time I watch TV:
Very Often / Often / Sometimes / Seldom / Never

26. During leisure time I walk:
Very Often / Often / Sometimes / Seldom / Never

27. During leisure time I cycle:
Very Often / Often / Sometimes / Seldom / Never

28. How many minutes do you walk and/or cycle per day to and from work, school and/or shopping?

Less than 5 / 5-15 / 15-30 / 30-45 / More than 45

29. Have you participated in any form of exercise testing before? Yes / No

If the answer is **Yes**, have you ever needed to terminate a test prior to completion, for health and safety reasons? Yes / No

If the answer is **Yes** please give details……
……
……

30. As far as you are aware, is there anything that might prevent you from successfully completing the tests that have been outlined to you?

Yes / No

……
……

Thank you for completing this questionnaire. All information will be kept confidential.

Appendix 2

Publications

VARIABILITY AND DISTRIBUTION OF MUSCLE STRENGTH AND ITS DETERMINANTS IN HUMANS

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Running title: Variability of muscle strength

VARIABILITY AND DISTRIBUTION OF MUSCLE STRENGTH AND ITS DETERMINANTS IN HUMANS

ABSTRACT

Introduction: Inter-individual variability in measurements of muscle strength and its determinants was identified to: 1) produce a normative data set describing the normal range and 2) determine whether some measurements are more informative than others when evaluating inter-individual differences.

Methods: Functional and morphological characteristics of the vastus lateralis were measured in 73 healthy, untrained adult men.

Results: Inter-individual variability (coefficient of variation) was greater for isometric maximal voluntary contraction (MVC) torque (18.9%) compared with fascicle force (14.6%, P = 0.025) and physiological cross-sectional area (PCSA, 17.2%) compared with anatomical cross-sectional area (ACSA, 13.0%, $P < 0.0005$). The relationship between ACSA and isometric MVC torque (r^2 = 0.56) was weaker than that between PCSA and fascicle force (r^2 = 0.68).

Conclusions: These results provide a normative data set on inter-individual variability in a variety of muscle strength-related measurements and illustrate the benefit of using more stringent measures of muscle properties.

Keywords: Inter-individual variation, vastus lateralis, muscle strength, muscle size, force

INTRODUCTION

Human skeletal muscle is a highly adaptive tissue that responds to changes in functional loading, and consequently muscle strength (here defined as maximal isometric joint torque) is known to vary between individuals. Much of the variability among untrained, asymptomatic individuals of a similar age has been attributed to differences in the structural and neural determinants of muscle strength.[1,2]

Muscle size is generally considered to be the greatest determinant of muscle strength,[1,3] and measurements of muscle thickness have revealed inter-individual variability in appendicular muscle size ranging from 9-18%.[1] Assessments of muscle thickness, however, are relatively simple and may actually underestimate the true contractile area of the muscle that contributes to force production.[2-4] Physiological cross-sectional area (PCSA) on the other hand, provides a more accurate assessment of muscle contractile area than measurements of muscle thickness or anatomical cross-sectional area (ACSA) by accounting for inter-individual differences in muscle architecture and muscle length. Taking account of such differences by using PCSA could reduce the inter-individual variability compared with ACSA, which represents only an estimate of true contractile area in pennate muscles.[2] However, as PCSA is affected directly by muscle length while ASCA is not, it could alternatively be that PCSA may demonstrate greater inter-individual variability than ACSA. Thus, the relationships between PCSA, ACSA, muscle length, and muscle architecture are complex, and it is difficult to predict realistically whether the inter-individual variability in PCSA and ACSA in a population will be similar or different in magnitude.

The importance of measuring agonist and antagonist muscle activation during assessments of *in vivo* maximal isometric strength has been highlighted previously.[5,6] While reports of

voluntary activation capacity in untrained individuals are somewhat contradictory,[7-10] sensitive use of the interpolated twitch technique suggests that untrained individuals probably cannot activate 100% of their motor units.[11] All else being equal, individuals with greater observed maximal voluntary contraction (MVC) torque are likely to have greater voluntary activation capacity. Therefore, accounting for inter-individual differences in voluntary activation capacity is likely to increase the calculated joint torque relatively more in weaker muscles, bringing the values closer to the mean in a population. This should have the net effect of reducing inter-individual variability in calculated maximal joint torque compared to that in observed MVC torque. Furthermore, antagonist muscle co-activation of the hamstrings during knee extension MVCs has been reported to range between 15-30% in healthy, untrained, adults.[12-14] Once again, all else being equal, individuals with greater observed MVC torque are likely to have lower antagonist co-activation, whereas those with lower observed MVC torque are likely to have higher antagonist co-activation. Consequently, accounting for inter-individual differences in antagonist co-activation should increase the calculated maximal joint torque relatively more in the weaker muscles, thus bringing those values closer to the mean in a population and reducing inter-individual variability.

A tendon moment arm functions as a lever of effective force transmission during muscle contraction[15] and is therefore central to accurate measurement of muscle force from torque. Bone geometry has been suggested as the primary determinant of tendon moment arm length and, as this is known to differ between individuals, is probably also the main source of inter-individual variability in moment arm length.[5,15,16] Therefore, assuming all else is equal, individuals with longer tendon moment arms would produce greater isometric MVC torque than those with shorter tendon moment arms. One would also expect individuals with larger

bone geometry and longer moment arms to generally possess larger, stronger muscles. Therefore, individuals capable of producing values of MVC torque above the observed mean value in a population are likely to have MVC torque further inflated by a longer moment arm, and those below the mean further reduced by the shorter moment arm, which would exaggerate the deviation of observed MVC torque values from the mean in a population. Consequently, controlling for moment arm length when calculating tendon force (and subsequently muscle force) should result in reduction in the distribution of observed force values and thus reduction in inter-individual variability in muscle force compared with observed isometric MVC torque.

Muscle specific force reflects the intrinsic strength of a muscle and is estimated by accounting for all of the aforementioned determinants of strength. As such, it could be expected that reports of specific force within the literature would be relatively homogenous. However, measurements of human muscle specific force *in vivo* are widespread, ranging from 6-86 $N \cdot cm^{-2}$.[6,17-22] Differences in sample selection and/or inconsistencies in measurements could contribute to such widespread values. Unsurprisingly, when only those studies that accounted for all of the necessary factors were considered, the range of values for *in vivo* specific force of the vastus lateralis (VL) was noticeably reduced to 20-30 $N \cdot cm^{-2}$.[6,18-20]

Consequently, the aim of the study was three-fold; firstly to develop a normative set of data on the inter-individual variability in measurements of muscle strength and its determinants in a relatively large, homogenous sample. Secondly, to demonstrate the extent of the differences in the inter-individual variability between the less stringent measurements of strength and its determinants (isometric MVC torque and ACSA) compared with the more

stringent measurements (specific force, fascicle force, and PCSA). The final aim was to ascertain the strength of the relationship between the different measurements of muscle strength and size. We hypothesized that a stronger relationship would exist between PCSA and VL fascicle force than between ACSA and isometric MVC torque, because greater physiological variability is accounted for in the more stringent measurements of muscle size and strength.

MATERIALS AND METHODS

Subjects. Participants were healthy, untrained, Caucasian men [n = 73, age 20.6 (2.5) yr, height 178.2 (6.7) cm, mass 76.0 (9.8) kg; mean (SD)], and all gave written consent to participate in this study prior to involvement. Participants were excluded from the study if they suffered from a known musculoskeletal or neurological disorder, were aged less than 18 years or more than 39 years, had a body mass index below 18.5 or above 30 $kg \cdot m^{-2}$, or if they had undertaken any structured resistance training in the preceding 12 months. Additionally, a questionnaire designed to assess habitual activity levels[23] was used to ensure that only untrained participants, those undertaking less than 3 hours of low-to-moderate habitual physical activity per week, took part in the study. All experimental procedures were conducted in accordance with the guidelines in the Declaration of Helsinki and approved by the Ethics Committee of Manchester Metropolitan University.

Knee extension and flexion strength. Maximum voluntary isometric knee extension (MVC_{KE}) and flexion torque was measured using an isokinetic dynamometer (Cybex Norm, Cybex International Inc., NY, USA) with participants seated at 85° hip flexion. A minimum of 2 MVCs were performed at knee joint angles of 70°, 80°, and 90° of flexion on the right leg only. A third MVC attempt was performed if the second MVC was more than 10%

higher than the first.[18] This range of knee joint angles has been shown previously to include the optimum angle for a comparable sample population.[18] Knee joint center of rotation was aligned visually with the dynamometer axis of rotation, and participants were strapped into the dynamometer across the hips, shoulders, and right thigh to limit any extraneous movement. Participants were instructed to maintain each MVC for approximately 3 s until they received a verbal signal to relax. Maximal isometric knee extension and flexion torque were assessed at all knee joint angles in a randomized order, and a 2-min rest period was given between contractions. The knee joint angle at which peak MVC torque occurred was considered the optimal angle and was used for subsequent measurements.

Co-activation. The level of antagonist muscle co-activation during knee extension MVC was determined through assessment of electromyographic (EMG) activity of the biceps femoris, as this muscle has previously been found to be representative of the entire knee flexor muscle group.[24] Two self-adhesive Ag-AgCl electrodes were placed over the long head of the BF in a bipolar configuration with an inter-electrode distance of 20 mm. In an attempt to reduce skin impedance to less than 5,000 Ω prior to electrode placement, the skin was shaved, abraded, and cleansed with an alcohol wipe. Electrodes were positioned in the mid-sagittal plane over the distal third of the muscle to minimize cross-talk from neighboring muscles, and a reference electrode was positioned over the lateral tibial condyle. Preamplified raw EMG activity was filtered using band pass filters set at 10 and 500 Hz. The root mean square of the EMG activity was calculated over 1 s, corresponding to peak knee extension MVC torque at each joint angle. Additionally, EMG activity of the biceps femoris was measured during knee flexion MVCs at the same joint angles.[25] Assuming a linear relationship between torque and EMG activity,[6] the torque produced by the knee flexors during knee extension MVC was estimated as co-activation torque.

Voluntary activation capacity. To assess voluntary activation capacity a supramaximal doublet (50-μs pulse width and 50-ms interstimulus gap) generated by an external stimulation device (DS7, Digitimer stimulator, Welwyn, Garden City, UK) was administered to the quadriceps femoris via 2 self-adhesive electrodes (7.5 x 12.5 cm; Tyco Galvanic Pad, Uni-Patch, MN, USA) positioned over distal (anode) and proximal (cathode) regions of the thigh. To ascertain maximal twitch torque stimulation intensity, single twitches were delivered to the participant at rest, and current intensity was increased using approximately 50 mA increments until no further increase in twitch torque was observed. This current intensity was defined as the supramaximal stimulation intensity. Consequently, quadriceps femoris voluntary activation capacity was calculated as:

$$\text{Activation (\%)} = (1 - t/\text{T}) \times 100$$

where, t is the interpolated doublet amplitude, and T is the potentiated doublet amplitude.[26] Additionally, net knee extension torque was calculated by adding co-activation torque to the sum of MVC_{KE} torque and superimposed stimulation torque. Signals of torque, electrical stimuli and EMG activity were displayed on a computer screen (Macintosh, iMac, Apple Computer, Cupertino, USA), interfaced with an acquisition system (AcqKnowledge, Biopac Systems, Santa Barbara, USA) to enable analogue-to-digital conversion at a 2,000 Hz sampling frequency.

Muscle architecture. Muscle architecture of the VL muscle was assessed *in vivo* during MVC_{KE} at the pre-determined optimum joint angle using B-mode ultrasonography (AU5, Esaota, Italy). With the participant seated on the dynamometer as described previously, the origin and insertion and the medial and lateral borders of the VL muscle were identified at rest. VL muscle length was measured, and an external echo-absorptive reference marker was

placed at 50% of muscle length. Scans were obtained using a 40-mm, 7.5-MHz linear-array probe coated in water-soluble transmission gel to increase acoustic contact. The probe was positioned perpendicular to the skin surface over the echo-absorptive marker in the mid-sagittal plane of the VL muscle. The external reference marker was visible on the scanned image; thus, any movement of the probe in relation to the marker during each MVC trial would be identified. If movement of the probe were apparent, the trial was omitted and an additional trial would take place. An external voltage trigger enabled synchronization of the ultrasound scans with the acquisition system to allow for the ultrasound image corresponding to peak MVC_{KE} torque to be exported for subsequent analysis. All ultrasound scans were recorded in audio video interleave (AVI) format at a sampling frequency of 25 Hz, and single images were captured using frame-capture software (Adobe Premiere Elements version 10, Adobe Systems). Measurement of VL muscle fiber pennation angle (θ) and fascicle length (L_f) was completed on single images using digitizing software (NIH ImageJ, version 1.44o, National Institutes of Health, Bethesda, USA).[6] θ was measured as the angle of fascicular insertion into the deep aponeurosis. Identification of L_f was achieved by measuring the distance from fascicular origin to insertion on the aponeuroses.[27] Often the VL muscle fascicles extended beyond the scanning window; therefore estimation of L_f was necessary by extrapolating the deep and superficial aponeuroses and fascicle. θ and L_f were measured on a minimum of 3 fascicles for every ultrasound image, and an average of these measurements was taken as the θ and L_f.

Muscle volume. VL muscle ACSA was measured using previously validated methods with B-mode ultrasonography.[4] A series of transverse plane scans were taken at the level of 50% of VL muscle length with the use of external reference markers to identify sections from the medial to lateral edge of the VL. Care was taken to ensure minimal pressure was

applied to the VL during scanning to avoid compression of the muscle. A recording of the scans was saved in AVI format, and single scans were captured using frame-capture software (Adobe Premiere Elements version 10, Adobe Systems) and used for subsequent analysis. Single scans were fitted using contour matching, and ACSA was measured using digitizing software (NIH ImageJ, version 1.44o, National Institutes of Health, Bethesda, USA). The mean of 3 measurements was taken and used to estimate VL muscle volume using previously applied methods based on a series of regression derived constants(Morse et al., 2007) along with VL muscle length. Muscle PCSA was calculated subsequently by dividing muscle volume by L_f obtained at the optimum knee joint angle.

Tendon moment arm length. Moment arm length of the patellar tendon was measured to calculate patellar tendon forces using a dual energy X-ray absorptiometry (DEXA) scanner (Hologic Discovery, Vertec Scientific Ltd, UK). Sagittal plane scans were obtained with the participant at rest and with the knee joint positioned at the previously determined optimum angle using a 22.3 x 13.7 cm field of view. Scans were exported to a dicom file viewer (OsiriX 5.0.2, Pixmeo Sarl, Geneva, Switzerland), and the perpendicular distance between the tibiofemoral contact point and the axis of the patellar tendon was measured as the patellar tendon moment arm length.[15] Patellar tendon force was calculated by dividing net torque at the optimum knee joint angle by patellar tendon moment arm length.

Specific force. The contribution of the VL muscle to patellar tendon force was calculated by estimating the relative PCSA of the VL in relation to the quadriceps femoris muscle using previously reported data.[19] Subsequently, fascicle force of the VL was estimated by dividing VL muscle force by the cosine of the θ obtained at the optimum joint angle during MVC.

Finally, VL muscle specific force was calculated by dividing VL fascicle force by VL PCSA.[6]

Statistics. Coefficients of variation (CV) were calculated to identify the extent of inter-individual variability in all functional and morphological characteristics of the VL using Microsoft Excel. To determine any differences in inter-individual variability between isometric MVC_{KE} torque, VL fascicle force, VL ACSA, and VL PCSA, a Friedman ANOVA was conducted using corrected percentage distribution data, which breached the parametric assumption of normal distribution following correction (Statistical Package for Social Sciences 19.0, SPSS Inc., Chicago, Il, USA). The Wilcoxon signed-rank test was used to perform appropriate *post-hoc* analyses where necessary. Regression analyses were conducted to determine the relation between isometric MVC_{KE} torque and VL ACSA and VL fascicle force and VL PCSA. The use of the Fisher Z-transformation enabled the difference between these correlations to be analyzed. Reliability of the architectural measurements was determined by calculation of ratio limits of agreement (LoA)[29] and CVs on data collected during pilot testing on 2 separate occasions separated by 1 day for 8 participants. Data are displayed as means (SD), and statistical significance was set at $P \leq 0.05$.

RESULTS

Descriptive data on the functional and morphological characteristics of the VL are presented with CVs in Table 1. Notably, the CVs of VL specific force and VL ACSA were lower than those for isometric MVC_{KE} torque and VL PCSA, respectively. The Shapiro-Wilk test revealed that the data were distributed normally ($P = 0.063$-0.706). Calculation of ratio LoA and CVs were used to determine the repeatability of architectural measurements on 2

occasions by the same investigator on 8 participants (Table 2). There was no significant difference between day 1 and day 2; all limits of agreement were less than 10%, and most were less than 6%, which showed very good reliability.

< INSERT TABLE 1 NEAR HERE >

<INSERT TABLE 2 NEAR HERE >

The mean knee joint angle at which maximal MVC_{KE} torque was determined was 80º. Histograms showing the percentage deviation from the mean value for isometric MVC_{KE} torque, VL fascicle force, VL ACSA, and VL PCSA can be seen in Figure 1. Output from a Friedman ANOVA revealed a significant difference ($P < 0.0005$) in corrected distribution data. *Post-hoc* analyses identified a significant difference in the percentage distribution between isometric MVC_{KE} torque and VL fascicle force ($P = 0.025$) and between VL ACSA and VL PCSA ($P < 0.0005$).

< INSERT FIGURE 1 NEAR HERE >

A regression analysis revealed a significant relationship between VL ACSA and isometric MVC_{KE} torque ($r^2 = 0.57$; $P < 0.0005$, Figure 2A). Additionally, there was a significant relationship between VL PCSA and VL fascicle force ($r^2 = 0.68$; $P < 0.0005$, Figure 2B). However, the relationship between VL PCSA and VL fascicle force was not significantly different from the relationship between VL ASCA and isometric MVC_{KE} torque ($P = 0.359$). The relation between stature and both PCSA and ACSA was assessed using regression

analyses and revealed a stronger significant relationship between PCSA and stature (r^2 = 0.674, $P < 0.0005$) when compared with ACSA and stature ($r^2 = 0.217$, $P = 0.001$).

< INSERT FIGURE 2 NEAR HERE >

DISCUSSION

One of the aims of this study was to develop a normative set of data on the inter-individual variability in measurements of muscle strength and its determinants in a relatively large, homogenous sample. This was achieved for 73 asymptomatic young men using a range of measurements related to muscle size and strength. This not only applies to mean values, but in particular to inter-individual variability. Table 1 presents those data and includes CVs ranging from 5.7% (agonist activation capacity) to 20.2% (muscle volume). These data are useful for researchers who investigate the causes of inter-individual variability in these parameters, such as various genetic and environmental factors.

Inter-individual variability in the measurement of isometric MVC_{KE} torque is associated with the variability of its determinants. We hypothesised that accounting for these determinants in the calculation of specific force would result in a reduction in inter-individual variability compared to that present in the measurement of isometric MVC_{KE} torque. Inter-individual variability in specific force (13.5%) is comparable with previous reports in healthy, untrained adults (16.2%).[18,30] Despite this, we can only accept partially the hypothesis, as the inter-individual variability in specific force was only 4% less than the inter-individual variability in isometric MVC_{KE} torque. Nevertheless, the difference in inter-individual variability between specific force and isometric MVC_{KE} torque is slightly more than that reported previously (3%) in the only other study to our knowledge that

investigated inter-individual variability in specific force *in vivo* in a smaller (n = 27), but comparable sample.[18]

Differences in the inter-individual variability of isometric MVC_{KE} torque, tendon force and specific force are dependent on the differing extent to which the physiological determinants are accounted for. For example, gross measurements of strength such as isometric MVC_{KE} torque are likely to be more susceptible to this inherent variation, because they are influenced by the inter-individual variability in agonist and antagonist muscle activity.[31] In contrast, specific force may provide a more accurate representation of the contractile properties of the muscle while accounting for inter-individual variability in neural properties, tendon moment arm length, and muscle architecture. Unsurprisingly, we found that the inter-individual variation in isometric MVC_{KE} torque was greater than that for specific force and all of its determinants (with the exception of muscle volume). However, the inter-individual variation observed in agonist activation and antagonist co-activation was relatively small (5.7% and 6.8%, respectively). We found that muscle activation in untrained, young males is relatively complete and likely contributes to a lower degree of inter-individual variability in the measurement of isometric MVC_{KE} torque than in some other populations. It could be expected that within a more heterogeneous population where activation levels show greater variability greater inter-individual variability in isometric MVC_{KE} torque would also be observed.

A significant relationship was observed between isometric MVC_{KE} torque and ACSA (r^2 = 0.57), which is comparable to that reported previously in the plantar flexors and dorsiflexors (r^2 = 0.59-0.62).[32] As expected, this relationship was weaker than that between VL fascicle force and PCSA (r^2 = 0.68), although this difference was not significant statistically. The

high inter-individual variability in isometric MVC_{KE} torque and ACSA is likely to contribute to this tendency for a difference. Inter-individual variability in VL ACSA was found to be 13%, which is comparable to previous reports of approximately 14% in muscle thickness of the rectus femoris and vastus intermedius in untrained men.[1] In comparison, the inter-individual variation of VL PCSA we found was greater than for VL ACSA. A plausible explanation for the greater inter-individual variability in VL PCSA compared with VL ACSA could be the consequence of the inter-individual variability observed in θ and L_f. The source of inter-individual variation in these measures of muscle size and architecture could be differences in body size.[33,34] Both PCSA and ACSA are related to body mass, whereas only PCSA is related to stature, given that L_f is proportional to femur length. This is substantiated in this study, which revealed a stronger relationship between VL PCSA and stature than between VL ACSA and stature.

The lower inter-individual variability observed in VL fascicle force compared to that in isometric MVC_{KE} torque could be attributed to architectural and structural factors. By accounting for inter-individual variability in tendon moment arm length it is possible to account for differences in bone geometry (and hence body size) within a population; this has been suggested previously to be the key determinant of tendon moment arm length.[35] For example, for any given VL fascicle force, the 8.8% inter-individual variability in moment arm length we observed would result in an isometric MVC_{KE} torque difference of 23 N·m. It should be noted that much of the reported variation in bone geometry, however, has been between different ethnic populations[36] and genders[35], whereas we sampled only Caucasian men. Nonetheless, the inter-individual variation in tendon moment arm length made a contribution to the inter-individual variability observed in the estimation of VL specific force.

Despite the obvious sources of variation in the measurement of isometric MVC_{KE} torque and its determinants, the calculation of specific force failed to reduce this to the extent envisaged initially. It is possible that differences in the intrinsic force-generating capacity of individual fibers exist which contribute to the observed inter-individual variability.[18] One plausible explanation is inter-individual differences in fiber-type composition of the VL,[37-39] as type I fibers are reported to have lower specific tension than type II fibers.[40-41] Additionally, inter-individual variability in VL specific force may be explained by the presence of intramuscular fat and connective tissue.[14,42,43] Variation in intra-muscular non-contractile material has been observed previously in a sample of young adults,[14] and although we did not measure this, it could contribute to some of the unexplained inter-individual variability in specific force. Failure to account for the presence of intra-muscular non-contractile material would result in an overestimation of muscle PCSA and thus underestimate muscle specific force.[18,43] On the other hand, it has been suggested that increased connective tissue content may be associated with improved lateral force transmission from the muscle fiber to the tendon, the consequence of which would be an increase in muscle specific force.[44] Furthermore, myofilament-packing density is known to influence cross-bridge interaction of actin and myosin filaments and consequently may also contribute to the inter-individual variability in specific force we observed.[45] Data on inter-individual variability in human skeletal myofilament-packing density is lacking, although reports from training studies have found no change in pre- and post-training packing densities.[46,47] Nonetheless, more research is needed to establish if myofilament packing density varies in untrained adults. One way in which fiber type composition and intramuscular values of collagen and adipose tissue could be accounted for is through biopsy, however, the estimate of whole muscle properties based on biopsies may be limited.[48]

The assessment of muscle specific force includes a number of assumptions or surrogate measures where direct measurement is not possible. For example, previous studies have adopted estimates of fascicle length based on previously published values of muscle length.[49] Similarly, where MRI is not available to measure muscle volume directly, estimates have been made based on single measures of ACSA multiplied by limb length.[29] Indeed the measurement of moment arm during MVC requires X-ray fluoroscopy to account for deformation and extension of the moment arm through contraction; in contrast, moment arm is often estimated based on external anthropometric measures.[50] In our study, direct measurement of moment arm length during MVC was not possible, and force was estimated from resting measures of moment arm length. Furthermore, muscle volume was estimated based on a single measure of ACSA. Although both of these methods have been demonstrated to be valid surrogates in the calculation of specific force,[28,51-53] direct measurement may have improved the validity of the measures.

One of the applications of these data is in research into genetic factors that may be associated with specific force, by seeking to minimize unexplained inter-individual variability in associated strength measurements. By 2007, 22 genetic polymorphisms associated specifically with a muscle strength-related phenotype had been reported, however this number will have increased in subsequent years.[54] Using more stringent measurements of muscle strength and size that reduce confounding variability, as demonstrated here, would increase the likelihood of identifying small associations between individual genetic polymorphisms and strength-related phenotypes.

In conclusion, we confirmed the extent of the inter-individual variability previously reported in human muscle specific force and isometric MVC torque. Furthermore, establishing the inter-individual variability in the factors involved in the determination of muscle strength provides normative data on a relatively large sample of healthy, untrained men that had previously remained unreported. These results substantiate previous findings that calculation of inter-individual variability in human knee extension specific force explained little of the inter-individual variability observed in MVC torque. Thus, factors other than muscle fiber architecture, moment arm length, and agonist muscle activation and antagonist muscle co-activation appear to contribute to the observed variation.

ABBREVIATIONS

ACSA, Anatomical cross-sectional area

AVI, audio video interleave

CV, Coefficients of variation

EMG, Electromyography

L_f, Fascicle length

LoA, Limits of agreement

MVC, Maximal voluntary contraction

MVC_{KE}, Maximal voluntary knee extension contraction torque

PCSA, Physiological cross-sectional area

VL, Vastus lateralis

θ, Pennation angle

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TABLES

Table 1. Functional and morphological characteristics of the vastus lateralis.

Variable	Mean (SD)	Range	CV (%)
Isometric MVC_{KE} torque (N·m)	259 (49)	168 – 363	18.9
Activation capacity (%)	89.5 (5.1)	80.1 – 98.5	5.7
Antagonist co-activation (%)	13.9 (0.96)	8.0 – 24.8	6.8
Net KE torque (N·m)	282 (50)	146 – 339	17.9
Muscle volume (cm^3)	561 (115)	424 – 816	20.2
ACSA (cm^2)	21.3 (2.8)	14.1 – 28.6	13.0
Fascicle length (cm)	8.0 (1.3)	6.3 – 11.5	16.6
Pennation angle (°)	14.6 (2.4)	9.0 – 21.4	16.7
PCSA (cm^2)	65.7 (11.0)	43.3 – 114.5	17.2
Moment arm (cm)	4.4 (0.38)	3.5 – 5.3	8.8
Patellar tendon force (N)	6,430 (1,113)	4624 – 8270	17.3
VL fascicle force (N)	1,458 (213)	1079 – 1812	14.6
Specific force ($N \cdot cm^{-2}$)	23.8 (3.5)	17.7 – 27.9	13.5

ACSA, anatomical cross-sectional area; CV, coefficient of variation MVC_{KE}, maximal voluntary knee extension contraction; PCSA, physiological cross-sectional area; VL, vastus lateralis.

Table 2. Inter-day measurement reliability.

Variable	CV (%)	LoA (%)	Mean (SD)
Muscle volume (cm^3)	2.5	5.3	523 (14)
Pennation angle (°)	2.1	2.6	14.5 (2.4)
Fascicle length (cm)	2.5	4.5	7.8 (0.6)
Moment arm length (cm)	1.8	2.5	4.5 (0.2)
Specific force ($N \cdot cm^{-2}$)	5.6	9.9	20.2 (3.3)

FIGURES

Figure 1. Frequency distributions around the mean (displayed as %) for: A) MVC_{KE} torque, B) VL fascicle force, C) VL ACSA, and D) VL PCSA. Significant differences were apparent between MVC_{KE} torque and VL fascicle force ($P = 0.025$) and ASCA and PCSA ($P < 0.0005$).

Figure 2. A) The relationship between VL ACSA and isometric MVC_{KE} torque ($P < 0.0005$); B) The relationship between VL PCSA and VL fascicle force ($P < 0.0005$).

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ABSTRACT SUBMISSION

Variability and distribution of muscle strength and its determinants in humans

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Background: Much of the variability in muscle strength among similarly aged, untrained, asymptomatic individuals has been attributed to differences in the structural and neural determinants of muscle strength (Maughan et al., 1983: Journal of Physiology, 338(1), 37-49; Erskine et al., 2009: European Journal of Applied Physiology, 106(6), 827-838). Measurements employed to investigate muscle strength and its determinants can range from simple measures, for example maximal voluntary contraction (MVC) torque and anatomical cross-sectional area (ACSA), to more stringent measurements of specific force and physiological cross sectional area (PCSA). However, it is unknown if some of these measurements are more informative than others when evaluating inter-individual differences.

Purpose: The purpose of this investigation was to identify inter-individual variability in measurements of muscle strength and its determinants to produce a normative set of data describing the normal range, and to establish which measurements – if any – are more informative than others in evaluations of inter-individual differences.

Methods: Healthy, untrained adult males (n = 73, age 20.6 ± 2.5 yr, height 178.2 ± 6.7 cm, mass 76.0 ± 9.8 kg; mean ± s) gave informed consent to participate in this investigation. Knee extension and flexion MVC torque was measured on an isokinetic dynamometer at 70°, 80° and 90° of knee flexion. The knee joint angle at which peak MVC torque occurred was taken as the optimum angle and used for subsequent measurements of *in vivo* muscle architecture. Pennation angle and fascicle length of the right vastus lateralis (VL) were measured during MVC using B-mode ultrasonography at 50% muscle length in the mid-sagittal plane. VL ACSA was measured *in vivo* at rest, using previously validated ultrasonography techniques (Reeves et al., 2004: European Journal of Applied Physiology, 91, 116-118) and used to estimate muscle volume with muscle length and a series of regression derived constants (Morse et al., 2007: European Journal of Applied Physiology, 100, 267-274). VL PCSA was calculated by dividing muscle volume by fascicle length. Dual energy X-ray absorptiometry was used to measure patella tendon moment arm length and was subsequently used in the calculation of patella tendon force, by dividing net joint torque by moment arm length. VL muscle force was calculated from tendon force by estimating VL PCSA relative to the entire quadriceps femoris, and VL muscle force was divided by the cosine of the pennation angle to estimate VL fascicle force. VL specific force was calculated by dividing VL fascicle force by PCSA. The Ethics committee of Manchester Metropolitan University approved all experimental procedures.

Results: Inter-individual variability was 4.3% greater in MVC torque compared with fascicle force (CVs = 18.9% and 14.6%, respectively, P = 0.025, ES = 0.624), and 4.2% greater in PSCA compared with ACSA (CVs = 17.2% and 13.0%, respectively, $P < 0.0005$, ES = 0.867). A stronger relationship was observed between PCSA and fascicle force (r^2 = 0.68, $P < 0.0005$) than between ACSA and MVC torque (r^2 = 0.57, $P < 0.0005$).

Discussion: These findings confirm the extent of the inter-individual variability previously reported in human muscle specific force and MVC torque (Erskine et al., 2009). Furthermore, establishing the inter-individual variability in the factors involved in the determination of muscle strength provides normative data on a relatively large sample of

untrained, asymptomatic males that had previously remained unreported, and illustrates the benefit of using more stringent measurements of muscle properties. These outcomes may be used to inform future investigations assessing the genetic contribution to inter-individual variability.

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ASSOCIATION OF ACTN3, CNTF AND PTK2 WITH SKELETAL MUSCLE PHENOTYPES IN UNTRAINED MALES

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Introduction

The ability to perform physical activities requires muscle strength, which is known to vary inter-individually (Stebbings et al., 2013). The genetic contribution to this inter-individual variation is yet to be confirmed, despite a number of associations between single-nucleotide polymorphisms (SNPs) and skeletal muscle phenotypes, most notably with elite athlete status. We aimed to establish if SNPs in the *ACTN3*, *CNTF* and *PTK2* genes were associated with maximal voluntary contraction (MVC) torque and specific force in untrained men.

Method

Vastus lateralis (VL) skeletal muscle phenotypes were measured in untrained Caucasian men (n = 100). Knee extension (KE) and flexion (KF) isometric MVC torque was measured in the right leg using isokinetic dynamometry, and VL muscle architecture, size and length were measured using B-mode ultrasonography. These data were used to calculate VL specific force. DNA was isolated from whole blood and participants were genotyped for *ACTN3* R577X (rs1815739), *CNTF* G-6A (rs1800169) and *PTK2* A/C (rs7843014) SNPs using real-time PCR.

Results

Genotype frequencies were all in Hardy-Weinberg equilibrium. There were no differences in specific force between the genotypes of *ACTN3*, *CNTF* ($P \geq 0.074$) or *PTK2* ($P = 0.051$) although this did approach significance. When combining *PTK2* AC and CC genotypes, VL specific force was 7.5% higher for homozygous AA individuals (20.9 ± 2.9 N·cm^{-2}) than C-allele carriers (19.4 ± 2.5 N·cm^{-2}; $P = 0.008$). No differences between *ACTN3, CNTF* or *PTK2* genotypes were observed for KE MVC ($P \geq 0.690$) or KF MVC ($P \geq 0.446$).

Discussion

The lack of association between isometric MVC and *ACTN3*, *CNTF* or *PTK2* genotype is similar to previous reports in untrained men (Erskine et al., 2012; De Mars et al., 2007; McCauley et al., 2009), suggesting minimal importance of these particular polymorphisms on MVC in an untrained population. The greater muscle specific force observed in homozygous A-allele individuals compared to C-allele carriers of the *PTK2* SNP is consistent with previous work from our lab and suggests that AA individuals are more effective at lateral force transmission through the impact of *PTK2* on the expression of focal adhesion kinase (Erskine et al., 2012). Specific force is a true reflection of intrinsic muscle strength in athletes and in disease conditions, so *PTK2* (rs7843014) should be investigated in relevant cohorts.

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