Are ACE I/D and ACTN3 R577X polymorphisms associated with the muscle function of young and older men, and frequent fallers?

by

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Abstract

Angiotensin Converting Enzyme (ACE) I/D, and α actinin 3 (ACTN3) R577X polymorphisms have been linked to the strength and power performance of elite athletes and suggested to influence skeletal muscle function in the general population. This research investigated the association of these two candidate gene polymorphisms with the muscle function of young and older men, and the distribution of these genotypes in frequent fallers compared to controls. Muscle function measurements of young and older men included isometric strength, absolute and relative isokinetic strength at high velocity (ratio of torque at $240°{\cdot}s^{-1}$: torque at $30°{\cdot}s^{-1}$) and the time course of an evoked twitch. Additionally body composition was measured by skinfold thickness (young men) and DXA scanning (old men) to estimate fat-free mass, an index of muscularity, and fat mass. ACE and ACTN3 genotypes were determined from whole blood samples using polymerase chain reaction, and serum ACE activity using spectrophotometry. The genotypes of frequent fallers referred to a Falls Clinic were compared to a control group of healthy men. ACE genotype was not associated with any measure of muscle function, including the time course of an evoked twitch or absolute and relative high velocity torque, or body composition in these populations (ANOVA, $0.12<P<0.97$). Serum ACE activity appeared to be weakly associated with knee extensor ($R = 0.19$, $P = 0.07$) and elbow flexor ($R = 0.20$, $P = 0.06$) isometric strength in older men, and was negatively correlated with the relative torque at high velocity ($R = -0.23$, $P = 0.03$). ACTN3 genotype was associated with fat mass in older men ($P = 0.04$), but was not associated with any measure of muscle function or muscularity (Kruskal-Wallis, $0.26<P<0.95$). Finally there was no apparent difference in the distribution of ACE I/D ($\chi^2 = 0.54$, $P = 0.77$) and ACTN3 R/X ($\chi^2 = 0.76$, $P = 0.68$) genotypes between frequent fallers and controls. Any influence of these individual polymorphisms seems unlikely to be of sufficient magnitude to produce genotype related differences in muscle function in young or older free living UK Caucasian men. Serum ACE activity may have a small association with the isometric and dynamic strength of older men. However, ACTN3 genotype was associated with increased fat mass in XX individuals, that suggests this polymorphism may have an association with the accumulation of body fat over the life span of older men.

Key words: ACE, ACTN3, age, contractile properties, frequent fallers, muscle strength, muscle function.

Conference Proceedings

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CHAPTER 1

INTRODUCTION

1 Introduction

The ability to perform movement is a fundamental feature of life that is an essential component to the well-being and survival of man. A requirement for human movement is the generation of force by skeletal muscles, producing torque and movement of the skeleton (Fitts *et al.*, 1991). The maximum force or torque generated by a muscle or muscle group is defined as skeletal muscle strength (Lieber, 2002). The physiological factors that determine muscular strength have been extensively researched and encompass a range of morphological, neurological and task specific factors (Maughan *et al.*, 1984; Ikai *et al.*, 1967; Marginson and Eston, 2001)

An individual's muscular strength reaches its peak during the third decade of life and is well maintained until 50 years old (Deschenes, 2004). Beyond the fifth decade of life, strength tends to decrease at a pace of ~15% per decade (Larsson *et al.*, 1979). A reduction in physical function due to lower strength and power in old age can have a profound effect on a person's ability to live independently, as carrying out daily tasks such as rising from a chair, or climbing stairs becomes increasingly difficult. Poor physical function in old age leads to an increased risk of older adults developing disabilities (Roubenoff, 2000) therefore, understanding the basis of dysfunction of muscle in old age has important clinical and social implications (Frontera *et al.*, 2000).

A primary mechanism for the decrease in strength with ageing is sarcopenia - a progressive loss of muscle mass. Muscle power output and the ability to produce force quickly appears to decline more rapidly than strength (Metter *et al.*, 1997; Skelton *et al.*, 1994), which could be due to a preferential atrophy of type II muscle fibres (Lexell, 1995). The evoked contractile (twitch) response of the quadriceps has been found to be ~10% slower in older compared to younger persons (Roos *et al.*, 1992) and may reflect a slowing of the contractile properties that contributes to the decline in power. From a functional perspective dynamic strength and power are considered to be more relevant than isometric strength (Wilson & Murphy, 1996).

This loss of strength and power of the lower limb muscles is a primary risk factor for falling (American Geriatrics Society, 2001). Falls are a major health issue for the ageing population as they increase morbidity, contribute to decreased quality of life (Robbins *et al.*, 1989) and create a high financial burden. Falls also lead to an increased fear of falling, reduced activity and can cause a downward spiral of inactivity-related disability (Simey *et al.*, 1999).

Genetic factors appear to account for a substantial proportion of the phenotypic variability of muscle strength and function. In young men, the genetic contribution to strength and power appears to be 30-80%, depending on the nature of the functional test employed (Thomis *et al.*, 1998; Calvo *et al.*, 2002). The heritability of strength, power and walking speed has been found to be 34-52% in older men (Carmelli *et al.*, 2000; Tiainen *et al.*, 2007). Finding the specific genetic variants responsible for this heritability in muscle function will enhance our understanding of the mechanisms regulating muscle strength.

The annual publication of the human gene map for performance and health related fitness phenotypes (Rankinen *et al.*, 2006) list more than 200 genes or genetic regions associated with athletic performance and physical fitness traits. The angiotensin converting enzyme (ACE) I/D and α actinin 3 (ACTN3) R577X polymorphisms have recently been identified as potential influences contributing to variations in skeletal muscle composition, function and performance (Williams *et al.*, 2005; Mills *et al.*, 2001).

A functional polymorphism of the ACE gene is defined as the presence (insertion, I allele) or absence (deletion, D allele) of a 287 amino acid base pair Alu repeat sequence within intron 16 of the ACE gene on chromosome 17 (Rigat *et al.*, 1990). This polymorphism results in the genotypes II, ID and DD with representation among Caucasian Europeans of ~24, 50 and 26% respectively (Myerson *et al.*, 1999). Case control studies have reported a higher frequency of the DD genotype in sprint athletes compared to endurance athletes and controls (Myerson *et al.*, 1999; Woods *et al.*, 2001;

Nazarov *et al.*, 2001), indicating that this genotype may influence skeletal muscle function. The D allele variant of the ACE gene is associated with higher concentrations of serum ACE activity (Rigat *et al.*, 1990). ACE catalyses the production of Angiotensin II (ANG II), which appears to enhance skeletal muscle hypertrophy (Gordon *et al.*, 2001; Westercamp and Gordon, 2005) and degrades kinins (Brown *et al.*, 1998), which have growth inhibitory properties (Ishigai *et al.*, 1997). There is recent evidence that ACE genotype influences two important determinants of muscle strength and power; muscle size (Charbonneau *et al.*, 2008) and fibre type composition (Zhang *et al.*, 2003). The D allele has been associated with greater isometric strength in COPD patients (Hopkinson *et al.*, 2004) and the isometric and isokinetic strength of healthy young men (Williams *et al.*, 2005). However several investigations have found no association of ACE genotype with isometric and dynamic strength in young and older adults (Folland *et al.*, 2000; Fredericksen *et al.*, 2003; Thomis *et al.*, 2004; Pescatello *et al.*, 2006; Woods *et al.*, 2001b).

A common genetic variation in the ACTN3 gene results in the replacement of an arginine (R), the normal functional allele, with a stop codon (X) at amino acid 577 (R577X) that negates the production of the functional ACTN3 protein (North *et al.*, 1999). This polymorphism results in the genotypes RR, RX and XX with representation among healthy Caucasian Australian adults of 30, 52 and 18% respectively (Yang *et al.*, 2003).

ACTN3 expression is restricted to the fast glycolytic (type IIX) muscle fibres (Mills *et al.*, 2001) and the absence of ACTN3 could inhibit the performance of these fibres that are important for rapid powerful contractions (MacArthur and North, 2004). The mechanistic actions of ACTN3 deficiency are currently being explored. Muscle from ACTN3 knockout (KO) mice was found to have a slower contractile response and lower force generating capacity than those with ACTN3 (Chan *et al.*, 2008). Furthermore KO mice show a reduced fast twitch fibre diameter, lean mass and total body mass compared with wild type mice (MacArthur *et al.*, 2008) suggesting a clear effect of ACTN3 genotype on skeletal muscle growth and muscularity. Case control studies have reported

a higher frequency of the RR genotype in sprint, strength and power athletes compared with endurance athletes and controls (Niemi *et al.*, 2005; Yang *et al.*, 2003 Roth *et al.*, 2008; Druzhevskaya *et al.*, 2008; Papadimitriou *et al.*, 2008), indicating a beneficial effect on skeletal muscle function. Moreover ACTN3 genotype has been found to influence fibre type composition (Vincent *et al.*, 2007). However the evidence from cross sectional association studies remains equivocal with some reports of ACTN3 genotype influencing muscle strength of females but not males (Clarkson *et al.*, 2005; Delmonico *et al.*, 2007), but others have found no influence on muscle strength in either sex (Delmonico *et al.*, 2008; Moran *et al.*, 2007).

Many of the previous reports studying the associations of ACE I/D and ACTN3 R/X polymorphisms with muscle strength and function have studied populations with a mixed ethnic origin (Pescatello *et al.*, 2006), a wide age range (Clarkson *et al.*, 2005; Walsh *et al.*, 2008) or variable physical activity status (Woods *et al.*, 2001; Hopkinson *et al.*, 2004), which could confound results. For example skeletal muscle phenotypes are known to be influenced by ethnic origin (Newman *et al.*, 2003). The majority of these studies have assessed isometric strength as the only muscle function measure (Frederikson *et al.*, 2003; Hopkinson *et al.*, 2004; Moran *et al.*, 2006; Moran *et al.*, 2007), which may not be as functionally relevant as dynamic strength. To date no studies have examined the associations of ACE and ACTN3 genotype with high velocity muscle strength and the contractile properties of younger and older males. The vast majority of the previous studies have examined the association of only single gene polymorphisms with muscle function. Considering that the influence of a single gene variant is probably quite small (Williams and Folland, 2008) any association of candidate genes should be investigated together in order to determine broader genetic effects on a given phenotype. Therefore studies of multiple candidate genes are required.

The aim of this research was to investigate the association of two candidate gene (ACE I/D and ACTN 3 R/X) polymorphisms with the muscle function of young and older men, and the distribution of these genotypes in frequent fallers compared to controls. The first experiment (Chapter 3) examined knee extensor muscle function of healthy

young Caucasian men. A novel aspect of this study and Chapter 5 was the measurement of high velocity strength and contractile properties. Considering the importance of muscle strength and power to healthy ageing the second and third studies examined the association of ACE I/D and ACTN3 R/X polymorphisms with muscle function and body composition phenotypes of older Caucasian men. The first of these studies (Chapter 4) was in part a preliminary trial, but the second (Chapter 5) was a comprehensive investigation that included strength measures from a range of upper and lower body muscle groups and assessment of non-skeletal lean mass by dual energy x-ray absorptiometry (DXA). The fourth and final study (Chapter 6) investigated the frequency of ACE and ACTN3 genotypes in frequent fallers compared to a control population.

CHAPTER 2

LITERATURE REVIEW

2.1 Muscle Structure and Contraction

The ability to perform movement is a fundamental feature of life that is essential to the well being and survival of man. A requirement for human movement is the generation of force by skeletal muscles, producing a torque and movement of the skeleton (Fitts *et al.*, 1991).

2.1.1 *The structure of skeletal muscle*

Skeletal muscle comprises approximately 40% of total body weight. A skeletal muscle is a collection of muscle fibres (cells) that are bound with connective tissue and arranged with their long axis in parallel (Figure 2.1). The connective tissue surrounding the fibres is contiguous with the tendon that attaches the muscle to adjacent or in series bones. Groups of muscle fibres that function together and the motor neuron that innervate them are called a motor unit. Larger muscles such as the quadriceps femoris of adult humans contain several hundred thousand fibres organised as many motor units. Each skeletal muscle fibre is long and cylindrical in shape, and multinucleated with several hundred nuclei at the periphery of the fibre (Figure 2.1). Collagen, elastic fibres, nerves, capillaries and blood vessels are found between the bundles of muscle fibres (Martini, 2001).

The sarcolema or cell membrane surrounds the cytoplasm of the muscle fibre that contains ~2,000 myofibrils in human adult fibres (Martini, 2001). Each myofibril is surrounded by a webbing structure known as the sarcoplasmic reticulum, which releases Ca^{2+} when depolarisation spreads along the sarcolemma and into the fibre via the T-tubules. Myofibrils consist of a longitudinally repeating structure, known as the sarcomere, that contains the contractile filaments (proteins) actin and myosin. These filaments are responsible for force generation (Martini, 2001) and are arranged in a very precise hexagonal array within a scaffold of structural proteins (e.g. titin).

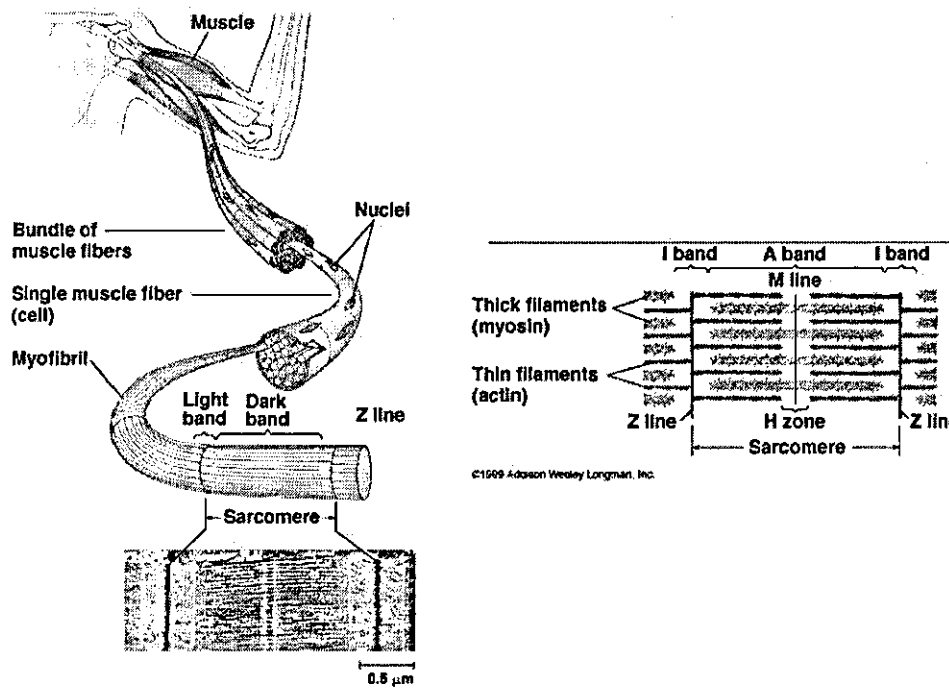

Figure 2.1 Basic structure of skeletal muscle (themedicalbiochemistrypage.org).

2.1.2 *The sliding filament theory*

The sliding filament theory proposes that muscle fibres shorten or lengthen because thick and thin filaments slide past each other without the filaments themselves changing length. When Ca^{2+} is present within a myofibril the myosin cross bridges cyclically attach, rotate, and detach from the actin thin filaments (with energy provided by ATP hydrolysis), driving the shortening process. Essentially during concentric activity the actin filaments are pulled passed the myosin filaments shortening the I band and reducing the length of the sarcomere. As all the sarcomeres in a muscle fibre and indeed within a motor unit are also shortening simultaneously this applies tension to the tendon and ultimately the skeleton generating torque about the joints. An isometric muscle contraction generates force while the fibres length remain relatively unchanged. In this situation the relative spacing of I and A bands remains constant as there is minimal movement of the actin filaments passed myosin. In contrast the A band widens when a muscle generates force while lengthening in an eccentric contraction (McArdle *et al.*, 2000).

2.2 Factors affecting skeletal muscle strength

The strength of skeletal muscle is defined as its capacity to produce active tension and thus force or torque (Hislop and Perrine, 1967). It is thought to depend on a number of morphological, neurological and task specific factors. The main factor influencing strength is the cross sectional area of the muscle (Maganaris *et al.*, 2001; Holmback *et al.*, 2002). Additional factors include, muscle architecture (Lieber, 2002), the muscle's length tension relationship (Marginson and Eston, 2001), fibre type composition (Aagaard and Andersen, 1998), the velocity at which the movement takes place (Gaines and Talbot, 1999) and the extent to which the muscle can be activated by voluntary effort (Gandevia and McKenzie, 1988).

2.2.1 *Measurements of skeletal muscle strength*

Muscular strength can be assessed with isometric, isokinetic and isoinertial measurements. Isometric assessment involves a muscle contraction with no noticeable muscle lengthening or shortening i.e. the body segment remains stationary and displacement is zero. Isometric tests are considered highly reliable, easily administered, require little skill involvement and are relatively inexpensive (Mital and Kumar, 1998). However isometric measurements are not considered to be a good representation of the capacity for functional movements, which are dynamic and occur throughout a range of movement rather than at just one joint angle (Wilson & Murphy, 1996).

Isokinetic assessment involves movement at a constant velocity, with the resistive torque accommodating to the individual's net joint torque which changes throughout the range of motion (Adams, 1998). Accommodating resistance enables the active musculature to contract at its maximum ability at all points through its range of motion (Armundsen, 1990). Isokinetic measurements possess high internal validity (Abernethy *et al.*, 1995).

Isoinertial measurement involves the generation of force or torque against a resistance of constant inertia or mass. In practice a participant's maximal lifting capacity with free weights provides an assessment of their isoinertial strength for a given exercise e.g. bench press. As free weights are widely available this is a practical and relatively quick assessment of strength.

2.2.2 *Morphological Factors influencing Strength*

2.2.2.1 *Muscle size, Cross sectional area (CSA) and Muscularity*

The maximum voluntary isometric force which can be produced by human skeletal muscle is partly determined by the muscle cross sectional area (Lieber, 2002). Theoretically the force generated by the contractile elements depends on the number of actin and myosin filaments arranged in parallel, rather than in series (Jones, 1992). The geometric measurement that captures the number of cross-bridges in parallel is CSA. Therefore a larger muscle with a greater CSA has a greater number of cross bridges in parallel and would be expected to produce more force.

Anatomical cross sectional area (ACSA) is a convenient measurement of muscle size that is made perpendicular to the long axis of the muscle, which can be obtained by a variety of scanning techniques including magnetic resonance imaging (MRI). Several studies have demonstrated a moderate to high correlation between ACSA of a muscle and maximum voluntary force (Maughan *et al.*, 1984; Narici *et al.* 1989). Maughan *et al.* (1984) examined muscle strength and cross sectional area and reported that the cross sectional area of a muscle accounts for approximately 50% of the difference in maximum voluntary knee extension force between individuals.

However in pennate muscles, (as is the case for most locomotor muscles) the muscle fibres run at an angle to the axis of traction of the muscle. This geometrical arrangement of fibres can directly influence the amount of force generated through voluntary contraction, and the ACSA does not represent the cross section perpendicular to the fibres in the muscle. (Lieber, 2002). In this case the physiological cross sectional area

(PCSA) measured perpendicular to the direction of the muscle fibres, is thought to better represent the contractile ability of the fibres and their collective cross sectional areas (Maughan *et al.*, 1984).

Furthermore Bamman *et al.* (2000) examined plantar flexor isometric strength in a cohort of 39 women. CSA was measured with magnetic resonance imaging (MRI). Isometric strength showed significant correlations with ACSA and PCSA (54 and 51% of strength explained by ACSA and PCSA, respectively). Additionally using MRI, Akima *et al.* (2001) tested isometric knee extensor strength in a group of men. Results demonstrated a significant correlation between PCSA of the quadriceps femoris and peak force in men demonstrating 34% of strength explained by CSA. These studies have shown that muscle size, assessed by ACSA or PCSA, appears to be the most important single determinant of strength.

Whole body muscularity is normally inferred from body composition measurement of fat-free mass (FFM). Methods of determining FFM include skinfold thickness, bioelectrical impedance analysis (BIA) and dual energy X-ray absorptiometry (DXA). The principle behind the technique of skinfold thickness is that the amount of subcutaneous fat is proportional to the total amount of body fat. It is assumed that close to one third of the total fat is located subcutaneously. Regression equations are used to convert the sum of skinfolds to percent body fat. Body composition determined from skinfolds correlates well (0.70-0.90) with body composition determined by hydrodensitometry (the standard method for measuring body composition). The accuracy of predicting percent fat from skinfolds is approximately ± 3.5% assuming that appropriate techniques and equations have been used (ACSM, 2010). BIA determines the electrical impedance, or opposition to the flow of an electric current through body tissues which can then be used to calculate an estimate of total body water (TBW). TBW can be used to estimate fat-free body mass and, by difference with body mass, body fat (Eston and Reilly, 1996). The accuracy of BIA is similar to skinfolds as long as stringent protocol is followed and the equations programmed into the analyser are valid and accurate for the population tested (ACSM, 2010). DXA can be used to assess total bone

mass as well as regional estimates of bone, fat and lean tissue. It uses a 3 components model to predict body fatness and is a reliable and accurate measure of body composition (ACSM, 2010).

2.2.2.2 *Muscle architecture*

The force developed by pennate muscle fibres acts at an angle to the long axis of a muscle. The component of force, which acts along the length of a muscle, is equal to the cosine of the angle of pennation. The PCSA represents the maximal number of acto-myosin cross bridges that can be activated in parallel during contraction (Fukunaga, 2001). Based on the parallelogram models of bipennate muscle, total physiological cross sectional area increases in proportion to sin ($\varnothing p$), where $\varnothing p$ is the muscle fibre pennation angle. Greater pennation angles would result in more contractile material attached to a larger area of the tendon and therefore greater total force produced by the fibres (Kawakami *et al.*, 1993). However, the contractile force transmitted from the fibres to the tendon decreases as the angle of pennation increases according to cos ($\varnothing p$). From mathematical modelling of these opposing effects, maximum muscle force is expected to increase with fibre pennation angle up to an optimum of 45° (Rutherford and Jones, 1992). As a consequence, all other factors being equal, pennate muscles are stronger than non-pennate muscles (Lieber, 2002).

2.2.2.3 *Specific tension*

The intrinsic force generating capacity of the muscle fibres is known as specific tension and it is expressed as the force that a muscle fibre can exert per unit of CSA ($N \cdot cm^{-2}$) (Fitts *et al.*, 1991). It has been suggested that specific tension differs according to muscle fibre types and that this could influence muscle function and strength. If this were the case then a muscle rich in type II fibres would produce more force than a muscle high in type I fibres (Burke, 1967).

Fitts *et al.* (1991) reported a 24% difference in specific tension between type I and II fibres (1.36 and 1.69 $kg \cdot cm^{-2}$, respectively). In vivo specific tension of the soleus and

tibialis anterior muscles in man were calculated to be 150 and 155 $kN \cdot m^{-2}$, respectively (Maganaris *et al.*, 2001). Further more Larsson and Moss (1993) reported on human skinned and freeze dried muscle fibres in which specific tension of type I and type II fibres was ~120 and 180 kN/m^2, respectively, indicating that specific tension may be fibre type dependent.

Recent findings suggest greater specific tension of human fibres expressing myosin heavy chains (MHC) IIX isoform than fibres expressing only MHC I (+50%, Stienen *et al.*, 1996; +20%, Bottinelli *et al.*, 1999; +32%, Widrick *et al.*, 2002). Consequently fibre type composition does appear to influence specific tension and likely exerts a small influence on the isometric strength of human skeletal muscle. The influence of fibre type composition on muscle function during dynamic contractions is addressed.

2.2.3 *Task Specific Factors*

2.2.3.1 Angle-torque relationship

The change in joint torque as a result of changes in muscle length is well documented both *in-vivo* and *in-vitro*. As the angle of a joint alters, the length of the muscle(s) acting over that joint also changes. This leads to the arrangement of the contractile filaments in the sarcomere to change, in particular the overlap of actin and myosin filaments, which affects force generation (Jones, 1992). Gordon *et al.* (1966) first described the length-tension properties of skeletal muscle sarcomeres. They reported that peak active tension was constant across a sarcomere length of 2.0-2.2 μm. At this length, optimal overlap exists between the myosin and actin filaments, which is turn produces the maximal number of cross-bridges and therefore peak force. At progressively longer sarcomere lengths, active tension decreases linearly, reaching zero at 3.6 μm. Below sarcomere lengths of 2.0 μm force during an isometric contraction falls due to overlap of the thin filaments from opposite ends of the sarcomere and to an increase in the filament lattice spacing (Allen and Moss, 1987). This relationship can be plotted on a graph and demonstrates a length-tension curve.

It has been demonstrated that the length-tension relationship *in-vivo* does not entirely reflect the same characteristics as an isolated muscle due to *in-situ* biomechanical factors such as the tendon and joint mechanics (Kawakami and Lieber, 2000). *In-vivo* the relationship is referred to as the angle-torque relationship (Figure 2.2). Marginson and Eston (2001) investigated the relationship between torque and joint angle during knee extension in males. These authors documented a characteristic torque-joint angle curve in which torque increased until an optimal joint angle of 80°, after which it began to decrease.

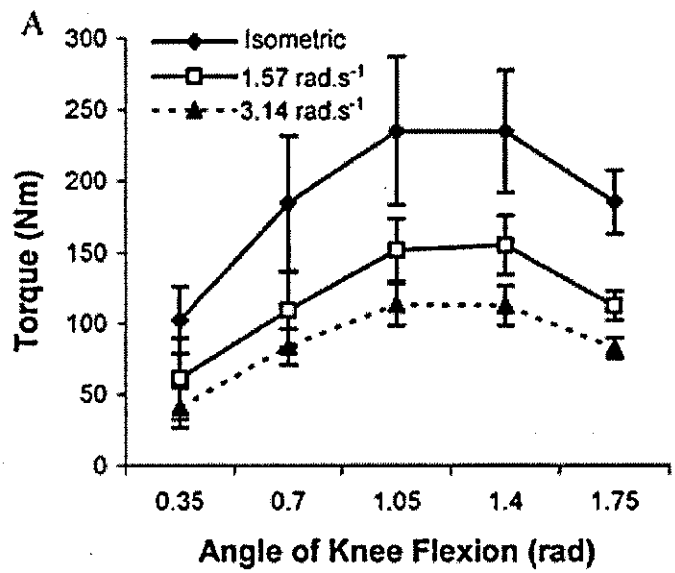

Figure 2.2 *In-vivo* angle-torque relationship at 3 velocities (Reproduced from Folland and Morris, 2008).

2.2.3.2 Torque-angular velocity relationship

This is the relationship between the maximum torque and the corresponding velocity of muscle lengthening and shortening. The maximal torque a muscle can produce decreases with an increase in the velocity of shortening (Herzog, 2000). Since the early work of A.V. Hill (1938), this relationship for isolated skeletal muscle has been known to be

hyperbolic. As the velocity at which the actin and myosin slide past one another increases, a resulting lower number of attachments occur and so force decreases (Lieber, 2002). In humans the torque-angular velocity relationship is markedly less hyperbolic, but does show a clear reduction in torque with an increase in the velocity of shortening (Figure 2.3).

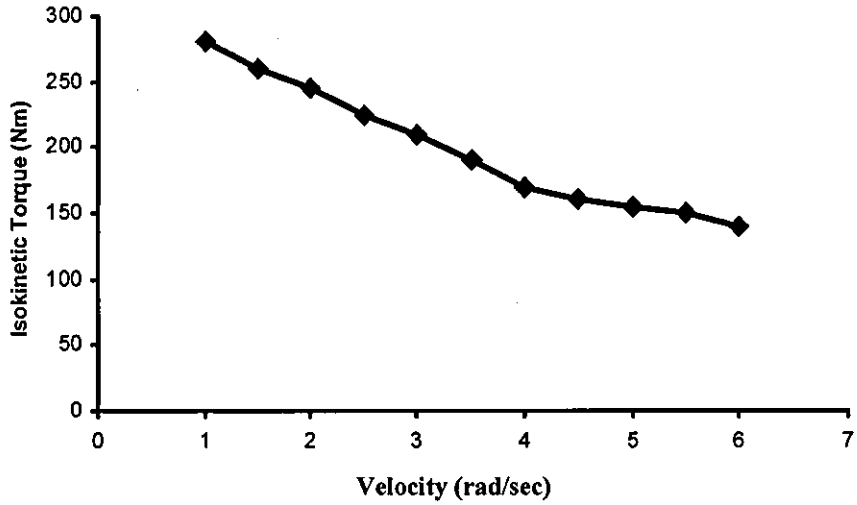

Figure 2.3 *In-vivo* Torque-angular velocity relationship (Reproduced from Racz *et al.*, 2002).

2.2.4 *Fibre type composition and the function of skeletal muscle*

Human skeletal muscle fibres can be divided into the following groups I, I-IIA, IIA-IIX and IIX by using myosin heavy chain isoforms (Bottinelli *et al.*, 1999). Fast twitch fibres (type II) show considerably higher maximal shortening speeds and power outputs than slow twitch fibres (type I). In rat skeletal muscle fibres the maximal shortening velocity is 5-6 fold higher in the type IIX than type I, while the type IIA is 3-4 fold higher than the type I (Fitts *et al.*, 1989). Bottinelli *et al.* (1999) measured the maximum velocity shortening and power output of different types of human skeletal muscle fibres. Maximum power output was ~ 9 times higher in type IIX fibres compared to type I; the velocity at which maximum power was achieved showed large and significant variations (about 4 times) between fibre types (Figure 2.4). Additionally the difference in velocity of shortening was approx. 4 times between the fastest and the slowest group of fibres. In

human skeletal muscle Faulkner *et al.* (1986) showed the peak power output of the type II fibres was fourfold that of the type I fibres. Additionally it was shown that the muscle with 50% type II and 50% type I fibres had a peak power that was 55% of the peak power for a muscle composed exclusively of type II fibres. Consequently muscles composed of type I fibres are at a disadvantage in performing tasks requiring substantial power development (Faulkner *et al.*, 1986).

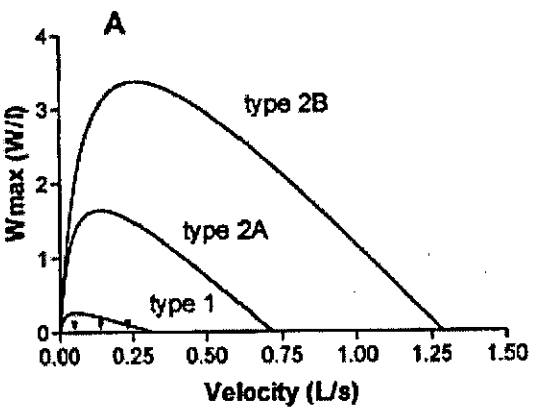

Figure 2.4 Power–velocity curves for each fibre type (Reproduced from Bottinelli *et al.*, 1999).

Force production depends on the binding of the myosin head to actin. The force-velocity relationship is likely to reflect the kinetic properties of the cross bridges and attempts have been made to evaluate the various steps in the cross bridge cycle using the information provided by the force-velocity curve. There is reason to believe that the maximum speed of shortening expresses the maximum cycling rate of the cross bridges. In support of this the maximum speed of shortening had been found to correlate with the splitting of ATP within the contractile system. This was first demonstrated in studies of the whole muscle (Barany, 1967) and later in a more quantitative way (Edman *et al.*, 1988) by comparing the maximum speed of shortening and myofibrillar ATPase activity in isolated single muscle fibres and showed an increase in ATPase activity as velocity of unloaded shortening increased. Furthermore the speed of relaxation of the muscle is

related to the activity of the myosin ATPase and the re-uptake of calcium by the sarcoplasmic reticulum, these properties being different in the different fibre types (Rutherford *et al.* 1995).

Aagaard and Andersen (1998) investigated the correlation between contraction strength and myosin heavy chain composition in human skeletal muscle. The percentage of myosin heavy chain II (MHC) in the quadriceps muscle was positively correlated to maximum concentric quadriceps strength obtained at medium to high knee angular velocity ($120°\cdot s^{-1}$ and $240°\cdot s^{-1}$). It was suggested that the relationship between muscular MHC composition and maximal contractile strength appears as a consequence of the MHC-related difference in contractile force-velocity characteristics. A growing body of evidence suggests that the ratio of muscle torque produced at fast versus slow speeds is highly correlated to the area and isoform profile of muscle fibres (Gur *et al.* 2003; Ivy *et al.* 1981; Suter *et al.* 1993). In particular Gur *et al.* (2003) found the ratio of maximum isokinetic torque at $240°\cdot s^{-1}$ to that generated at $30°\cdot s^{-1}$, correlated ($r = 0.74$; $p < 0.001$) with the relative area of type II fibres. On the basis of this evidence, isokinetic dynamometry may have the sensitivity to detect substantial individual differences in fibre type composition or even subtle differences between groups.

Furthermore the concept of examining the speed of relaxation of a muscle from a contraction in order to infer fibre type has already been applied to studies of tendon reflexes and brief electrically stimulated contractions (Lambert *et al.* 1951). Time to peak tension (TPT) and half relaxation time (HRT) have been previously used to infer fibre type composition (Hamada *et al.* 2000), suggesting that short TPT and HRT reflects a high percentage of type II fibres. This suggestion concurs with an early study by Burke *et al.* (1973) in which the physiological properties of single motor units were studied in the gastrocnemius muscle (primarily in the medial head) of cats. Results showed that the units studied could be classified into one of three major fibre types, 2 of which exhibited relatively short twitch contraction times (types IIA and IIX), and one group with relatively long contraction times (type I).

2.2.5 *Neurological Factors*

2.2.5.1 *Agonist Activation*

In order to function a muscle must have an intact nerve supply and force generation is determined by the number of motor units recruited as well as their firing frequency (Jones, 1992). When the greatest number of motor units is recruited in combination with the highest firing frequency, then maximal force is obtained. Early evidence from Merton (1954) demonstrated the ability to maximally activate a muscle during a voluntary effort. It has also been put forward that during maximal efforts most major muscle groups can be fully activated without training (Jones, 1992; Kent-Braun and LeBlanc, 1996).

However the majority of recent evidence suggests that skeletal muscle cannot be fully activated by voluntary effort, (Ikai, 1967; Hakkinen *et al.*, 1986; Herbert and Gandevia, 1999) and there are many muscles that the average person is unable to control; trivial examples include the muscles involved in moving ears and eyebrows but it is possible that major muscle groups may not be fully used. In support of this idea, Ikai (1967) documented that during maximum contractions force produced by the thumb adductor muscle in response to tetanic stimulation was 30% greater than that which could be produced by voluntary effort. More recent studies concur with this suggestion (Hakkinen *et al.*, 1986; Herbert and Gandevia, 1999). Additionally evidence indicates that voluntary activation declines as a consequence of inactivity (Kawakami *et al.*, 2001), in which 20 days bed rest resulted in a 6% reduction in the extent to which knee extensors could be activated. This suggests that inactive individuals are less likely than active individuals to be able to maximally activate skeletal muscle.

The maximality of neurological activation may be muscle and joint position / muscle length specific. For example the elbow flexors appear to be more completely activated than the quadriceps femoris during maximum isometric efforts (Belanger and McComas, 1981). Kubo *et al.* (2004) also reported that the activation level of quadriceps femoris was highest at the knee flexed positions (80-110°) than at extended positions (40-70°)

suggesting that the activation level of an agonist muscle is higher at longer than shorter lengths.

The inability to fully activate muscle during an MVC may be a consequence of a lack of neural drive from supraspinal centres or inhibitory input from sensory afferents including muscle mechanoreceptors, golgi tendon organs or joint receptors. This protective mechanism may inhibit individuals from completely activating all their motor units within a specific muscle group (Kendall *et al.*, 2006) consequently reducing the capacity to create maximal force.

2.2.5.2 *Antagonist activation*

It is known that muscle activation is not confined to the agonist, but is generally seen in the antagonist as well, appearing as a burst of antagonist activity if the movement is quite rapid (Karst and Hasan, 1987). Kubo *et al.* (2004) reported that the co-activation of an antagonist to knee extension, the biceps femoris at 90-110° was significantly higher than at the other knee angles, suggesting that co-activation of an antagonist muscle is higher at longer lengths. Furthermore Baratta *et al.* (1988) showed co-activation of the knee flexors during knee extension tasks at various joint angles. Co-contraction of antagonists reduces torque output, but may also impair, by reciprocal inhibition, the ability to fully activate the agonists (Folland and Williams, 2007). Greater co-activation can reduce the performance of agonist muscle and therefore the torque produced (Carolan and Cafarelli 1992).

2.3 Strength and Power in Old Age

2.3.1 *Loss of strength as a consequence of ageing*

Frontera *et al.* (1991) assessed the isokinetic strength of the elbow and knee extensors and flexors in 200 healthy 45 to 78 year old men and women to examine the relationship between muscle strength, age, and body composition. Peak torque was measured at 60 and 240°·s^{-1} in the knee and at 60 and 180°·s^{-1} in the elbow with an isokinetic dynamometer. Strength of all muscle groups at both velocities was significantly lower (range 16-27%) in the 65-78 than the 45-54 yr old men and women. When strength was adjusted for fat free mass (FFM) and muscle mass (MM) the age-related differences were not significant in all muscle groups except the knee extensors tested at 240°·s^{-1} suggesting that reduced muscle mass accounts for a high proportion of the decline in strength with age. Based mostly on cross sectional studies the mean annual loss of strength among healthy people from 30-70 years of age has been estimated to be 0.5-1 % when comparing the isokinetic torque of young and older persons (Akima *et al.*, 2001; Zu *et al.*, 2007). The loss of strength over time is larger in longitudinal studies (Bassey *et al.*, 1993; Frontera *et al.*, 2000) than in cross sectional studies indicating that cross sectional studies may underestimate the age related changes in muscle function.

Bassey *et al.* (1993) investigated grip strength over a four year period in a large cohort of over 65 year old men and women and found a 2% per year decline. In a more recent longitudinal study the overall rate of loss of strength in the quadriceps and hamstrings has been reported to be 2 and 2.4% per year and hand grip strength decreased by ~3% per year (Frontera *et al.*, 2000) in a cohort initially aged 65.4 ± 4.2 yrs. Additionally isokinetic muscle strength of the knee extensors and flexors and elbow flexors showed losses ranging from 1.7-2.5% per year at slow and fast angular velocities (Frontera *et al.*, 2000). Furthermore Frederiksen *et al.* (2006) carried out a longitudinal study of Danes aged 45 to 102 years and reported an annual decline in grip strength with increasing age of 1.4% in men and 1% in women over 4 years.

2.3.2 *Loss of muscle Power*

Skelton *et al.* (1994) measured isometric knee extensor, isometric elbow flexor and handgrip strength and leg extensor power in 50 men and 50 women (65-89 yrs). In men and women the rates of loss of leg extension power were 3.7% and 3.2% per year respectively. The rate of decline over this age range was 1-2% year for isometric strength and 3.5% per year for leg extensor power. Additionally over a 25-year period Metter *et al.* (1997) measured strength and power every 2 years and found the decline to begin by age 40 in both women and men. After which power declined about 10% more than strength in men. Confirming that muscle power output and the ability to produce force quickly appears to decline more rapidly than strength. This affects the torque power–velocity relationship and data from young and elderly men shows the estimated maximum shortening velocity (V_{max}) of the plantar flexor muscles of the elderly is 16% lower that that of the young (Narici and Maganaris, 2006). The graph below demonstrates the reduction in power and torque at various velocities between young and old men as a consequence of ageing (Figure 2.5).

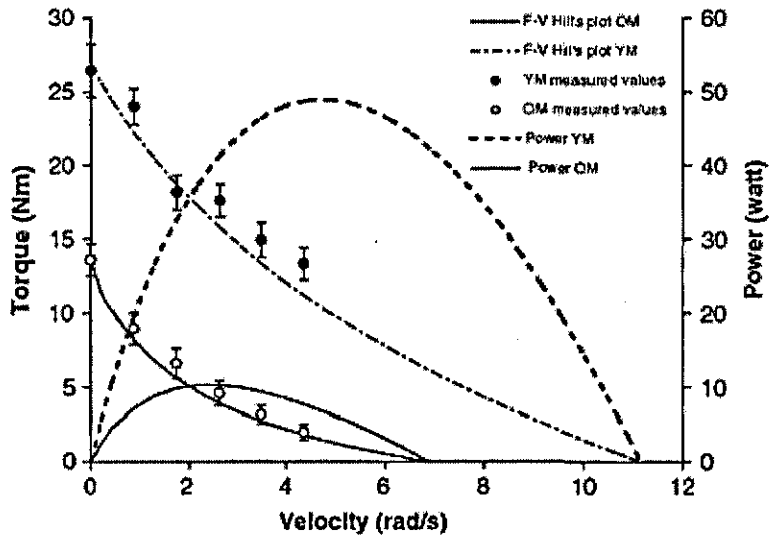

Figure 2.5 The torque and power–velocity relationships for young (YM) and older (OM) men. At all velocities, the younger men were significantly stronger than the older men ($P < 0.001$). (Reproduced from Thom *et al.*, 2007).

2.3.3 *Sarcopenia*

A primary mechanism for this reported decrease in strength and power is sarcopenia - a progressive loss of muscle mass (Frontera *et al.*, 1991). In a cross sectional study Janssen *et al.* (2000) examined the influence of age on skeletal muscle mass with whole body magnetic resonance imaging of 468 men and women. They observed a reduction in skeletal muscle mass starting in the third decade primarily attributed to a decrease in lower body skeletal muscle. Skeletal muscle mass declined by 17.1 % in females and 26.3% in males between the youngest age group (18-20 yrs) and the oldest group (+70 yrs).

In a 12 year longitudinal study Frontera *et al.* (2000) examined the age related changes in skeletal muscle size and function in older men and women (initial age 65 years). A significant reduction in muscle CSA in leg muscles over the 12-year period was reported, ranging form 12.5% to 16.1%. It was estimated from this study that the reduction in muscle CSA after 50 years of age was 1.4% per year and suggested that this is a major contributor to the decrease in muscle strength seen with advancing age.

2.3.4 *Fibre type, number and size*

Sarcopenia and the decline in the size of whole muscles with age could theoretically be caused by a reduction in the number of muscle fibres, a reduction in the size of muscle fibres (atrophy) or a combination of the two. In humans muscle fibre size reaches its peak in the third or fourth decade of life and thereafter gradually decreases (Larsson, 1978). Early investigations reported fibre type distribution changed towards a decrease in the percentage of type II fibres due to ageing (Larsson *et al.*, 1978; Jakobson *et al.*, 1988). In a study by Larsson *et al.* (1978) biopsies were taken from the vastus lateralis muscle of 55 untrained, healthy male subjects from 22 to 65 years of age. Fibre type distribution changed towards a decrease in the percentage of type II fibres, both in type IIA and type IIX fibres, whereas type IIX/IIA fibre ratio did not change with increasing age. It was found that the type IIX/IIA fibre ratio was inversely related to type I fibres, i.e. subjects rich in type I fibres had a relatively smaller proportion of type IIX fibres. Fibre area determinations revealed a selective decrease in type II fibre area.

Consequently, the type II/I fibre area ratio and relative type II fibre area decreased. Lexell *et al.* (1988) carried out a human autopsy on whole vastus lateralis (VL) muscle from 43 men between 15 and 83 years old. Results indicated that ageing begins at 25 years and progressively accelerates, mainly caused by a quantitative loss of fibre number. In the most thorough study of this topic to date Lexell and Taylor (1991) investigated cross sections of whole VL from 20 men, 19-84 year olds. Type I and type II fibres were measured in 5 different regions throughout each muscle. Older individuals had 6% and 35% smaller mean CSA of type I and II fibres, respectively, than young subjects.

Lexell *et al.* (1986) described in detail the age-induced alteration in muscle size and showed type II fibre size decreased by 20%. Additionally Andersen (2003) found 80-year-old men had much greater atrophy of the type II fibres than type I fibres of young men (25 yrs). Therefore greater atrophy of type II fibres occurs with ageing, and this may contribute to a greater decline in muscle power than strength (Lexell, 1995). And considering fast twitch fibres show considerably higher maximal shortening speeds and power outputs then this reduction in fast twitch fibre size would have negative consequence for tasks involving power.

Muscle fibre atrophy in the elderly may be associated with a specific reduction in the number of satellite cells in type II fibres (Verdijk *et al.*, 2007). Satellite cells or "muscle stem cells" are the sole source for the generation of new myonuclei in vivo (Allen *et al.*, 1999) and are essential for the maintenance of skeletal muscle mass (Shefer *et al.*, 2006). Fibre type specific satellite cell content from the vastus lateralis in eight elderly (76 yrs) and eight young (20 yrs) healthy males was assessed via muscle biopsies (Verdijk *et al.*, 2007). The number of satellite cells per type II fibre was substantially lower in the elderly compared to the young (0.044 ± 0.003 vs. 0.080 ± 0.007). Additionally in the type II fibres, the number of satellite cells relative to the total number of nuclei and the number of satellite cells per fibre area were also significantly lower in the elderly group. This study was the first to show type II fibre atrophy in the elderly to be associated with a fibre type-specific decline in satellite cell content. This decline in content might be an

important factor in the etiology of type II muscle fibre atrophy, which accompanies the loss of skeletal muscle with aging.

2.3.5 *Evoked twitch response with ageing*

The slowing of the contractile properties (through electrical stimulation) has been demonstrated in investigations of the lower limbs (Roos *et al.*, 1999) and in the adductor pollicis muscle (Narici *et al.*, 1991). The evoked contractile (twitch) response of the quadriceps has been found ~10% slower in older compared to younger persons (Roos *et al.*, 1999). The effect of ageing on the electrically evoked contractile properties of the human adductor pollicis muscle was investigated in 70 healthy male subjects aged 20-91 yr, (10 subjects for each decade) (Narici *et al.*, 1991). Maximum relaxation rate from tetanic stimulation dropped by 49% from the second to the eighth decade. Leg muscles are 10% slower in individuals over 73 yrs compared to young people in their twenties (Vandervoort and McComas 1986; Roos *et al.*, 1999) as demonstrated by the longer time taken to reach peak tension and to relax after the twitch. The reason for this slower relaxation rate (or longer twitch contraction duration) is probably related to selective atrophy of type II fibres (Macaluso and De Vito, 2004). The muscles relax at a lower rate as a result of the predominance of type I fibres, which are slower.

2.3.6 *Fibre angle of pennation*

The effect of ageing on human gastrocnemius medialis (GM) pennation angle was evaluated in older individuals (aged 70–81 yrs) and was significantly smaller by 10 and 13% (Narici *et al.*, 2003; Morse *et al.*, 2004) than those in younger adults. Findings were based on ultrasound scanning, a method that has been validated by comparisons with direct anatomical measurements on cadaveric muscles (Narici *et al.*, 1991; Kawakami *et al.*, 1993). Modifications in muscle architecture are unlikely to be associated with disuse, given that in one study Narici *et al.* (2003) purposely matched the level of physical activity of young and elderly individuals. Therefore the differences between age groups appear to have been due to the process of aging *per se.*

2.3.7 *Specific tension*

Larsson and colleagues (1997) determined specific tension in 400 single quadriceps muscle cells of young and old men and observed age related differences in specific tension in type I and IIA fibres. The specific tension of chemically skinned type IIA fibres in a young group was 0.25 N/mm^2 compared to 0.18 N/mm^2 in the older group. In a more recent study biopsy samples were taken from the vastus lateralis muscle of young (30.2 +/- 2.2 yrs), and elderly (72.7 +/- 2.3 yrs) persons in order to evaluate the specific tension of single skinned muscle fibres (D'Antona *et al.*, 2003). The specific tension of type I and IIA fibres was significantly lower in the older group than in the young group with a larger decrease in type I (-22 %) than in type IIA (-16 %). Furthermore at the single muscle fibre level Yu *et al.* (2007) reported a 20-28% decline in specific tension of the vastus lateralis muscle fibres expressing type I MHC isoform in young compared to older men and women.

Contrastingly Kent-Braun and Ng (1999) reported that dorsiflexor specific tension was unaffected by ageing and suggested that the decline in specific tension reported in other studies may actually be due to a reduction in physical activity and not ageing *per se.* However, the investigation by Larsson *et al.* (1997) and D'Antona *et al.* (2003) do not support this suggestion as both studies utilised immobilised elderly as well as physically active elderly participants and still reported a decline in specific tension for both groups compared to young adults. In contrast to Kent-Braun and Ng (1999), Macaluso *et al.* (2002) reported lower specific tension at the whole muscle level in seventy year old women compared to women in their twenties. The change in specific tension provides evidence of quantitative changes in contractile properties of human skeletal muscle with ageing, which likely plays an important role in the age-related impairment of skeletal muscle function by reducing power and speed of movement (Larsson *et al.*, 1997).

2.3.8 *Neurological factors*

2.3.8.1 *Agonist activation*

Neural alterations could contribute to muscle weakness by reducing central drive to the agonist muscles or increasing coactivation of the antagonist muscles (Klass *et al.*, 2007). Morse *et al.* (2004) reported the maximum activation of the plantar flexor muscles was lower in elderly males compared to young males (86% and 98% respectively) suggesting that the loss in muscle strength may partly be due to a reduced voluntary drive to the muscles. Muscles such as the tibialis anterior (Kent-Braun and Ng, 1999) and quadriceps (Roos *et al.*, 1999) have shown no impairment of muscle activation with age. Roos *et al.* (1999) utilised the twitch interpolation technique and found there was no difference in the ability of young and old groups to activate their quadriceps muscle (93.6% and 95.5% respectively). In contrast a clear deficit in muscle activation has been reported in very elderly inactive adults (69-93% of full activation) (Harridge *et al.*, 1999).

2.3.8.2 *Antagonist activation*

Greater co-activation can reduce the performance of agonist muscles both through the opposing action of the antagonist muscle and also by reciprocal inhibition (Crone and Nielsen, 1989). Several studies have found higher levels of co-activation of older adults during maximal voluntary efforts of various muscle groups: isometric knee extension (Izquierdo *et al.*, 1999), elbow flexion and extension (Klein *et al.*, 2001) and concentric knee extension (Izquierdo *et al.*, 1999). Contrastingly Klass *et al.* (2005) did not observe differences in co-activation during isometric contraction of the ankle dorsiflexors when comparing young and older groups.

No definitive answer can be drawn from existing literature as to whether voluntary activation and co-activation during MVC is modified with ageing due to discrepancies ascribed to differences in physical condition, the muscle group that is tested and the type of contraction performed (Klass *et al.*, 2007).

2.4 Falls

The continual decline in muscle strength and power restricts locomotion and contributes to a loss of mobility and is a primary risk factor for falling (American Geriatrics Society, 2001). Perry *et al.* (2007) found a falls group had 85% of the strength and 79% of the power of non-fallers for a range of lower limb muscle groups. Pijnappels *et al.* (2008) assessed how muscle strength influenced individuals' ability to deal with a gait perturbation that led to either falling or recovery of balance. Results showed that ankle and knee voluntary function measures (both maximum and rate of moment development), maximum leg press force, jump height, and grip strength were significantly lower for fallers compared to non-fallers. Therefore individuals who fall repeatedly (frequent fallers) or are less able to cope with gait perturbations have lower muscle function (strength and power) than non-fallers (Perry *et al.*, 2007; Pijnappels *et al.*, 2008).

Falls are a major health issue for the ageing population. It is estimated that 30-50% of people over the age of 65 years of age experience at least one fall per year (Campbell *et al.*, 1981), and about half of these will suffer recurrent falls (Masud and Morris, 2001). Falls frequently result in trauma, musculo-skeletal injury and disability. Approximately 20% of falls require medical attention; 15% result in joint dislocations, soft tissue bruises, and concussions (Kannus *et al.*, 1999). With ageing the skeleton becomes progressively weaker, therefore falling when bones are in a weakened state increases the likelihood of a fracture with 5% of falls resulting in fracture (Kannus *et al.*, 1999). As well as a high financial burden and morbidity falls typically contribute to decreased quality of life (Robbins *et al.*, 1989) and increased fear of falling, that leads to a reduction in activity and can cause a downward spiral of inactivity-related disability (Simey *et al.*, 1999).

Clearly there is a great need to investigate further how the factors influencing skeletal muscle strength and power impact upon the risk of falling. Genetic differences could via

their influence on factors such as sarcopenia and muscle fibre composition effect the predisposition of an individual to falling.

2.5 Inheritance

Heritability is the relative contribution of genetic and non-genetic factors to the total phenotypic variance in a population. Genetics is the study of genes, heredity, and the variation of organisms (World book encyclopedia, 1991). Heredity forms the basis of genetics. Modern molecular biology techniques have provided tools for investigating the function of specific genetic variants, and how they influence phenotype. Within organisms, genetic information is generally carried on chromosomes, where it is represented in the chemical structure of particular DNA molecules (Roth, 2007). Genes encode the information necessary for synthesizing the amino-acid sequences in proteins, which in turn play a large role in determining the final phenotype of the organism. Basically a gene is a segment of DNA which contains the information for making a protein; it codes for the nucleotide sequence in mRNA and rRNA that provides the information necessary to build a specific protein. Twenty to twenty five thousand genes exist within the human genome; although the complete list is still to be determined. The thousands of genes are found across all of the 22 autosomes, the X and Y chromosomes, and the mitochondrial genome, in locations specific to the gene. Nucleotide differences within the DNA sequence of a gene influences the function of that gene and therefore the protein synthesised, which results in an individual's specific phenotype or trait (Roth, 2007). Phenotype can be defined as any observable or measureable characteristic or trait, and is typically determined by a combination of genetic and environmental factors. Genotype is the combination of 2 alleles, at a particular locus, present within a single individual, corresponding to the 2 alleles present on the paired chromosomes. Genotype also refers more generally to the genetic profile or genetic make-up of an individual.

Many traits, including health outcomes and behaviours, have a tendency to run in families, which share a similar environment but also genetic factors (Frederickson and Christeensen, 2003). During the past decade numerous studies have assessed the relative contribution of genetic factors to traits involved in physical functioning. Physical function and performance encompasses a number of phenotypes e.g. muscular strength and power, flexibility, balance, VO_2 max, blood pressure, body composition etc. Twin studies have provided evidence of a genetic contribution to a number of measures reflecting physical function. The heritability of muscle strength and power, the primary phenotypes of interest in this research, appears to be 30-80%, depending on the nature of the functional test employed (Thomis *et al.*, 1998; Calvo *et al.*, 2002).

Thomis *et al.*, (1998) assessed the quantification of genetic and environmental factors in arm strength in male monozygotic (MZ, N = 25) and dizygotic (DZ, N = 16) twins (22.4 ± 3.7 yrs). Variation in muscle phenotypes indicated a high contribution of genetic factors (Heritability) for isoinertial strength (0.77), isometric strength (0.69), and muscle cross-sectional area (0.85). Calvo *et al.* (2002) tested the heritability of anaerobic power and capacity in a group of 16 pairs (8 MZ and 8 DZ) of Caucasian male twins. Results revealed a high index of heritability (HI) for maximal 5 s power (HI = 0.74), mean 30 s power (HI = 0.84) and maximal lactate concentration (HI = 0.82) with the Wingate test.

Carmelli *et al.* (2000) assessed the contribution of genetic influences to measures of lower-extremity function in 95 monozygotic (MZ) and 92 dizygotic (DZ), white male twins aged 68 to 79 years. The heritability of the lower-extremity performance was 57%. Tiainen *et al.* (2007) found genetic factors accounted for 52% of the variance in strength, 36% in power, and 34% in walking speed of MZ and DZ female twins aged 63-76 years.

Recently the focus has moved toward the molecular level with studies of specific genetic factors using various approaches. The Human Gene Map for Performance and Health-Related Fitness Phenotypes reports on advances in research of the relationship between gene variants and these phenotypes. The fitness and performance map includes 214

autosomal gene entries and quantitative trait loci, seven variants on the X chromosome, and 18 mitochondrial genes that have been quantitatively linked to fitness and performance phenotypes. The most up to date version of the map can be found in the review by Bray *et al.* (2009). Two candidate genes that have been suggested to have associations with a variety of physical performance phenotypes including muscle strength and power, and the physiological determinants of these phenotypes (e.g. muscle size and composition) are Angiotensin Converting Enzyme (ACE) and α Actinin-3 (ACTN).

2.6 Angiotensin Converting Enzyme (ACE) Gene Polymorphism

A polymorphism within intron 16 of the ACE gene on chromosome 17q23 concerns the insertion (I allele) or deletion (D allele) of a 287 base alu repeat segment. Alu repeats are short segments of DNA interspersed throughout the genome, and is a member of the SINE (short interspersed nuclear element) family of repeat elements (reviewed by Jasinska and Krzyzosiac, 2004). The alu repeat in the ACE gene is thought to have integrated into the ancestral human genome in the recent evolutionary past and has been found to be present at some frequency in almost every ethnic population that has been studied (see ALFRED: the ALelle FREquency Database for allele frequency data). The polymorphism results in the genotypes II, ID, DD with representation among young healthy Caucasian persons ~ 24%, 50%, 26% respectively (Myserson *et al.*, 1999). Intronic polymorphisms can have a phenotypic effect cumulative data show that introns are helpful symbionts of eukaryotic genomes that carry out various gene regulatory functions (Fedorova and Fedorov, 2003). These functions involve sources of non-coding RNA, carriers of transcription regulatory elements, actors in alternative and trans-splicing, substrates for exon shuffling and signals for mRNA export from the nucleus (Fedorova and Fedorov, 2003). However it is possible that the ACE I/D alleles are not causal but are in linkage disequilibrium (that is not randomly segregating during intergenerational transmission) with other causal variants elsewhere or near ACE.

The entire functions of carrying the intron 16 *alu* on ACE expression is not known however studies have shown a greater effect of the D allele resulting in higher levels of serum ACE and tissue ACE activity (Rigat *et al.*, 1990; Danser *et al.*, 1995). Rigat *et al.* (1990) reported the I/D polymorphism accounted for 47% of the total phenotype variance of serum ACE (II = 299.3 ± 49 μg/L, I/D = 392.6 ± 66.8 μg/L, DD = 494.1± 88.3 μg/L). Danser *et al.* (1995) reported cardiac ACE activity was higher in subjects with the ACE DD genotype (12.7 +/- 1.9 mU/g wet wt) compared with subjects with the ID (8.7 +/- 0.8 mU/g) and the II (9.1 +/- 1.0 mU/g) genotypes.

2.6.1 *Role of ACE within the Renin–Angiotensin-Aldosterone System (RAAS) Pathway*

The RAAS is a principal regulator of blood pressure and fluid homeostasis (Wang and Scherer, 2008). The major components are angiotensinogen, renin, Angiotensin I (ANG I) and Angiotensin II (ANG II).

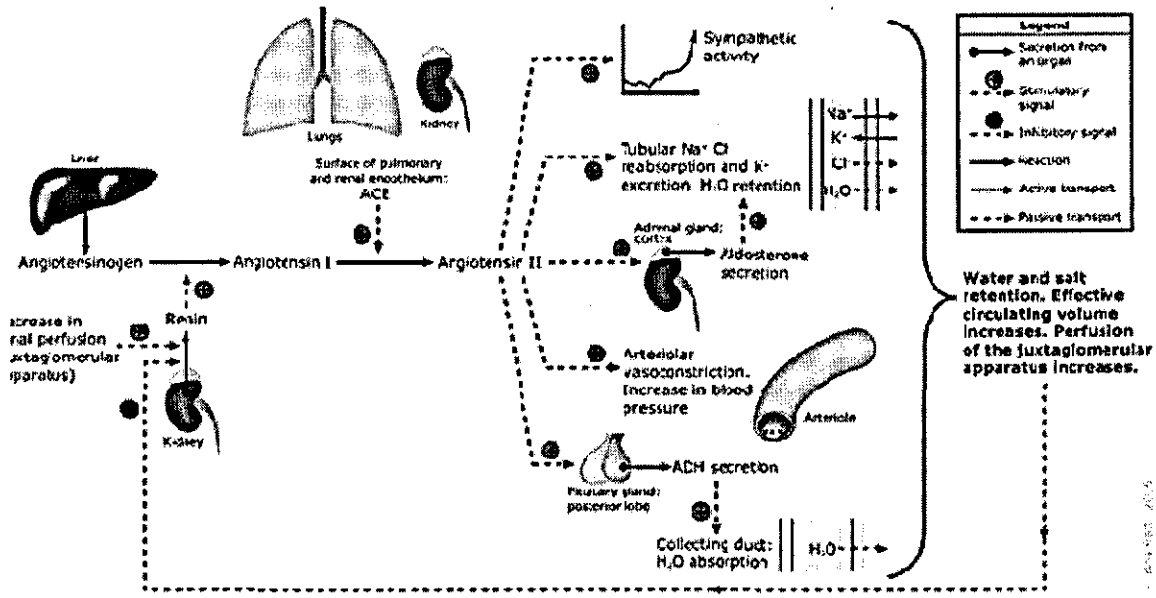

Figure 2.6 The Renin-angiotensin- aldosterone system (Author: A. Rad Date: February 2nd, 2006 License: GFDL-self Created using XaraX[1])

The RAAS pathway begins in the kidney when the juxtaglomerular cells in the afferent arterioles of nephrons secrete an enzyme called renin. Renin converts an inactive plasma

protein angiotensinogen into ANG I. Angiotensin Converting Enzyme (ACE), a proteolytic enzyme, converts ANG I to the octapeptide ANG II and also inactivates bradykinin, an endothelium-dependent vasodilator that increases vascular permeability (Silverthorn, 2004). Three roles of ANG II are: it is a potent vasoconstrictor that is important in the regulation of mean arterial blood pressure; it stimulates aldosterone release from the adrenal cortex; and the release of ADH from the hypothalamus and therefore triggers the sensation of thirst (Germann and Stanfield, 2005).

Aldosterone is a steroid hormone released from the adrenal cortex that regulates both the reabsorption of Na^+ and secretion of K^+. It increases the number of sodium channels and potassium channels in the apical membrane by causing existing channels to open and by stimulating the synthesis of new channels (Germann and Stanfield, 2005). It also increases the synthesis of Na^+/K^+ pumps which increase the concentration of Na^+/K^+. Through these actions aldosterone increases sodium reabsorption and potassium secretion simultaneously (Germann and Stanfield, 2005).

As previously mentioned studies have shown a greater effect of the D allele on serum ACE activity and tissue ACE with lower levels of the enzyme associated with the I allele. Theoretically higher serum ACE activity could enhance production of ANG II, and it has been demonstrated that with ANG I infusion, plasma ANG II concentrations increase in DD compared to II subjects (Brown *et al.*, 1998). Bradykinin is a nonapeptide that causes blood vessels to enlarge (dilate), and therefore causes blood pressure to lower. Bradykinin works on blood vessels through the release of prostacyclin, nitric oxide, and endothelial-derived hyperpolarizing factor (Germann and Stanfield, 2005). Kinins have shown to have growth inhibitory properties (McDonald *et al.*, 1995). McDonald *et al.* (1995) showed that bradykinin antagonism inhibits the antigrowth effect of angiotensin converting enzyme inhibition in the dog myocardium. ACE degrades kinins and the extent of degradation is dependent on ACE activity (Campbell, 1994). Myerson *et al.* (2001) discovered that subjects with the ACE DD genotype experienced greater left ventricular hypertrophy after physical training compared with II genotypes that they attributed to possible lower kinin levels.

2.6.2 *The physiology of ACE and ACE genotype within muscle tissues: potential influence on skeletal muscle phenotype?*

Apart from blood pressure regulation ACE may exert influences upon skeletal muscle through actions of ANG II and bradykinin as a consequence of higher ACE levels associated with the DD genotype. By using immunohistochemistry, ACE was detected in endothelial cells of capillaries in skeletal muscle (Schaufelberger *et al.*, 1998). The number of ACE mRNA transcripts was related to muscle fibre area and an inverse relationship between the number of ACE transcripts and capillary density was also found. The close relationship between gene expression and capillary density in this study may suggest a role for the RAAS in modulating capillary growth in the skeletal muscle in humans. ANG II has also been found to be synthesised in skeletal muscle (Danser *et al.*, 1995) and localized to the endothelial cells (Schaufelberger *et al.*, 1998). ANG II appears to optimise skeletal muscle hypertrophy in response to overload (Gordon *et al.*, 2001). Gordon and colleagues showed that inhibition of endogenous ANG II production with an oral ACE inhibitor decreased rat plantaris and soleus muscle hypertrophy by 57 and 96% respectively.

Danser *et al.* (1995) assessed ACE activity in cardiac muscle which was significantly higher in subjects with ACE DD genotype (II = 9.1 ± 1.0 mu/g, ID = 8.7 ± 0.8 mu/g, DD = 12.7 ± 1.9 mu/g). Additionally Reneland and Lithell (1994) reported higher ACE activity within the vastus lateralis muscle associated with the D allele in 50 essential hypertensive subjects. Danser *et al.* (1999) investigated whether ACE genotype influenced production of ANG II in human leg muscle with infusion of ANG I. Production of ANG II within human leg muscle did not parallel with the D allele-related differences in ACE concentration. Additionally Lachurie *et al.* (1995) infused exogenous ANG I into muscle and despite the ACE genotype effect on serum ACE activity, genotype did not influence ANG II or plasma aldosterone production. Therefore it appears that ACE has no limiting influence on ANG II generation, so higher ACE may not necessarily result in higher concentrations of ANG II.

Ahmad *et al.* (2006) reported that ACE is the major cardiac enzyme involved in the degradation of bradykinin during a single passage through the coronary vascular bed of the healthy rat heart. Brown *et al.* (1998) tested the hypothesis that the ACE deletion allele is associated with enhanced degradation of bradykinin. ACE levels were increased in DD compared with II subjects. During ANG I infusion, plasma ANG II concentrations were increased in DD compared with II subjects which, is contrary to the evidence presented by Lachuire *et al.* (1995) The half-life of bradykinin was significantly decreased in sera obtained from ACE DD compared with II subjects. Additionally there was a relationship between the half-life of bradykinin and serum ACE activity and between the half-life of bradykinin and the conversion of ANG I to ANG II confirming that ACE genotype determines bradykinin degradation.

Ishigai *et al.* (1997) found that bradykinin significantly attenuated protein DNA content (which is an index of hypertrophy in heart cells) and it can work as an antihypertrophic substance in cardiomyocytes. Since the DD genotype is associated with higher serum ACE activity, individuals with the DD genotype may have lower kinins levels. It is therefore possible that DD individuals experience less protein degradation that could contribute to a greater net protein balance and synthesis.

To date two published papers have considered the influence of ACE genotype on skeletal muscle fibre type composition. The possibility that the DD genotype maybe associated with a greater proportion of fast twitch fibres (Zhang *et al.*, 2003) could explain any association of the ACE D allele with strength/power, particularly at high velocities. Zhang *et al.* (2003) investigated the association between fibre type and ACE genotype. The DD genotype was associated with a greater proportion of fast twitch fibres, which supports the notion that the ACE D allele maybe associated with skeletal muscle function. Contrastingly Akhmetov *et al.* (2006) reported an increased frequency of the D allele (68.8% vs 34.4%) in 48 healthy young men with the highest proportion of slow-twitch fibers (56-70%) compared to the group with the lowest proportion (25-43%). However, this article is written in Russian and only an Abstract is available in English. Finally, there is recent evidence that ACE genotype is associated with the muscle volume of healthy older men and women (Charbonneau *et al.*, 2008) a key

determinant of muscle strength and power. Quadriceps muscle volume (MV) was measured by computed tomography (CT) in 86 men and 139 women. Higher MV was reported in male and female DD individuals compared to II individuals.

2.6.3 *ACE genotypes and athletic performance*

Over ten years ago Montgomery *et al.* (1998) described a genetic association that demonstrated an over representation of the I variant of the ACE gene in high altitude mountaineers. Their paper entitled the Human Gene for Physical Performance provided the impetus for many other groups to test the association of this polymorphism with various performance related phenotypes.

Initially studies investigated the association of the I allele with endurance performance reporting that the frequency of the I allele was higher amongst endurance athletes compared to controls (Alvarez *et al.*, 1999; Gayagay *et al.*, 1998; Lucia *et al.*, 2005). Myerson *et al.* (1999) studied 79 Olympic standard runners ranging from ≤200 m sprinters to ≥5000 m endurance runners who were assessed for ACE genotype. Results showed a linear trend of increasing I allele frequency with distance run. A significant excess and skew toward the I allele was found in the ≥5000 m group, with a skew in favour of the D allele amongst sprinters (≤200 m). Similarly Nazarov *et al.* (2001) showed an excess of the D allele in short distance athletes in their study of 217 athletes (swimmers, skiers, triathletes and track-and-field participants) compared to 449 controls. An excess of the D allele amongst sprinters led Woods *et al.* (2001) to examine whether the ratio of I and D alleles in swimmers competing over different distances would vary. One hundred and twenty swimmers from the European and Commonwealth championships had their ACE genotype determined and their gene and allele frequencies compared with control groups. Of the 103 Caucasians, there was a significant excess of the D allele in elite swimmers compared to the control group. This association remained in those competing over shorter distances (400 m and below) but not in the longer events.

2.6.4 *ACE genotype and skeletal muscle strength and function*

To date two reports have positively associated the ACE DD genotype with muscular strength (Table 2.1). Hopkinson *et al.* (2004) demonstrated that the D allele variant was associated with greater isometric quadriceps strength (P = 0.04) in a cohort of 103 patients suffering from chronic obstructive pulmonary disease (II = 31.4 kg; ID = 34.1; DD = 38.3). Williams *et al.* (2005) extended the findings of Hopkinson *et al.* (2004) to a young and healthy population. ACE genotype had a significant association with isometric strength (P = 0.026), with DD genotype individuals significantly stronger than ID or II genotype individuals. Furthermore, ACE activity was positively correlated with both isometric and dynamic muscle strength.

However the majority of work conducted is in contrast to the findings of Hopkinson *et al.* (2004) and Williams *et al.* (2005). Woods *et al.* (2001b) reported no differences between ACE genotypes in the maximal voluntary force (MVF) of adductor pollicis (AP) in a cohort of 45 women. Furthermore two early studies found no association of ACE genotype with elbow flexor (Thomis *et al.*, 2004) or knee extensor (Folland *et al.*, 2000) isometric strength of healthy young men. However these studies involved small cohorts of <60 participants.

In a much larger cohort (n=631) Pescatello *et al.* (2006) reported no ACE genotype association with isometric elbow flexor muscle strength of men and women. The large population was a strength of their study and could provide confidence in results, however the cohort had mixed ethnicity (Caucasian 80%, mixed origin 20%) and sex, which may have confounded the results. Likewise no genotype association with physical functioning strength in older persons was reported by Giaccaglia *et al.* (2008). The potentially stronger study by Frederickson *et al.* (2003b) comprising 684 older Caucasian males and females assessed isomeric hand grip strength. This study did not identify any genotype influence upon muscle strength. Moran *et al.* (2006) measured the influence of ACE genotype on hand grip strength in male and female Greek teenagers and again found no association in males and contrastingly reported greater hand grip strength in II genotype females. Additionally in the largest cohort (n=2966) involving

older persons no genotype association with isokinetic knee extensor strength was reported by Kritchevsky *et al.* (2005). Conversely their population was quite heterogeneous comprising both males and females as well as mixed race and involved active and non active participants. The lack of a genotypic effect may have been confounded due to such heterogeneity.

It is apparent that controversy surrounds the possible associations of ACE genotype with skeletal muscle strength and body composition. The mechanisms by which ACE genotype could be associated with skeletal muscle function seems plausible and case control studies suggest that there is a genotype connection with muscle function (Myerson *et al.*, 1999; Nazarov *et al.*, 2001). A number of studies have recognised the affect of the ACE D allele upon muscle phenotypes (Zhang *et al.*, 2003; Charbonneau *et al.*, 2008) as well as muscle strength (Williams *et al.*, 2005; Hopkinson *et al.*, 2004) however, the evidence to date provides no consensus.

Table 2.1 Association studies of ACE I/D polymorphism with muscle strength

Reference	Strength	Ethnicity Sex/Age (yrs)	N	Difference between genotypes
Folland *et al.*, 2000	Isometric & isokinetic KE	European Caucasian (UK) M 18-30	33	NS
Woods *et al.*, 2001	MVC of AP	UK Caucasian F 60	83	NS
Frederikson *et al.*, 2003	Hand grip	Danes Caucasian M&F 73+	684	NS
Thomis *et al.*, 2004	Isometric & concentric EF torque	European Caucasian M 22.4 ± 3.7	57	NS
Hopkinson *et al.*, 2004	Isometric KE	European Caucasian (UK) M&F 64.1±9.1 P M&F 61.8±8.6 C	103 P 101 C	D allele associated with > isometric strength in P NS in C
Kritchevsky *et al.*, 2005	Isokinetic KE ($60°\cdot s^{-1}$)	USA Caucasian & Black M&F 70-79	2966 Active & non active	NS
Williams *et al.*, 2005	Isometric & isokinetic (1.05 $rads\cdot s^{-1}$) KE	European Caucasian (UK) M 22.2±3.9	81	ACE DD was associated with > isometric strength
Pescatello *et al.*, 2006	EF 1RM and isometric	USA 80% Caucasian M &F 24.2±0.2	631	NS
Moran *et al.*, 2006	Hand grip	Greek M&F 11-18	1027	NS in males II females > handgrip strength
Giaccaglia *et al.*, 2008	Physical functioning muscle strength	USA M&F 60+	231	NS
Charbonneau *et al.*, 2008	KE 1RM	USA Caucasian& Black M&F 50-85	86 139	NS

KE, knee extensors. EF, elbow flexors. AP, adductor pollicis. NS, non significant. P, patient C, control. MVC, maximum voluntary contraction.

2.7 α Actinin-3 (ACTN3) R577X Polymorphism

The ACTN 3 R577X polymorphism has been widely suggested to have an association with skeletal muscle composition, function and performance. The α actinins are a family of actin binding proteins related to dystrophin and the spectrins (Blanchard *et al.*, 1989): ACTN1, ACTN2, ACTN3 and ACTN4. ACTN1 and ACTN4 are non-muscle proteins (Kaplan *et al.*, 2000), ACTN2 and ACTN3 are myofibrillar proteins located at the Z disk where they form a lattice structure.

The ACTN3 gene situated on chromosome 11q14, encodes a protein constituent of the Z disk of myofibrils, and a polymorphism of this gene results in a loss of the ACTN3 constituent. ACTN3 deficiency is due to homozygosity for a premature stop codon in ACTN3: C→T transversion at position 1,747 in exon 16, converting an arginine to a stop codon at residue 577 (R577X) resulting in the replacement of an arginine (R), the normal functional allele, with a stop codon (X) at amino acid 577 (R577X) that negates the production of the functional ACTN3 protein (North *et al.*, 1999). This polymorphism results in the genotypes RR, RX, XX with representation among healthy Caucasian Australian adults of 30, 52 and 18% respectively (Zhang *et al.*, 2003). The frequency of the 577X null allele differs between human groups: it is ~10% in African and approaches 50% in Eurasian populations (Mills *et al.*, 2001).

2.7.1 *ACTN3 genotypes and athletic performance*

Numerous investigators have associated the ACTN3 polymorphism with elite athletic performance (Table 2.2). The first of these studies obtained DNA samples from a large group of elite Australian athletes and non-athletic controls (Yang *et al.*, 2003). The frequency of the XX genotype was lower in power athletes than in controls and higher in female endurance athletes than controls. Additionally the power Olympians and female power athlete groups included no XX genotype individuals. It was suggested that the presence of the R allele of ACTN3 has a beneficial effect on skeletal muscle in generating forceful contractions at high velocities as ACTN3 protein could confer a

greater capacity for the absorption or transmission of force at the Z line during rapid contraction (Yang *et al*, 2003). A similar study in Finnish athletes and controls also showed a marked decrease in the frequency of ACTN3 deficiency in sprint athletes and a slight increase in the frequency of the XX genotype in endurance athletes that was most apparent at elite level (Niemi and Majamma, 2005). Furthermore recent studies have reported that the frequency of the RR genotype has been found to be higher amongst elite strength (Roth *et al.*, 2008) and power athletes (Druzhevskaya *et al.*, 2008; Papadimitiou *et al.*, 2008) compared to endurance runners or controls, indicating a beneficial effect on skeletal muscle function and in particular the ability to generate powerful contractions. These findings of an ACTN3 genotype association with athletic performance are consistent with the proposed mechanisms of ACTN 3 outlined below.

Table 2.2 Case control studies of ACTN 3 polymorphism frequencies in elite athletes and controls

Reference	Discipline	Ethnicity Sex/Age (Yrs)	N	Difference in distribution
Yang *et al.*, 2003	Elite from 14 sports	Australian Caucasian M & F	107 sprint & power 192 endurance 441controls	> freq. of the R allele in sprinters than controls.
Niemi & Majamaa, 2005	Elite track & field	Finnish	52 endurance 89 sprinters	XX genotype > and RR < in endurance athletes.
Roth *et al.*, 2008	Elite level body builders & strength	USA Caucasian Black M & F	75 athletes 876controls	< XX genotype freq. in athlete's vs. controls.
Druzhevskaya *et al.*, 2008	Power oriented	Russian Caucasian M & F 17-24	486 Athletes Controls 1197	XX genotype < freq. in power athletes vs. controls.
Papadimitiou *et al.*, 2008	Elite track & field	Greek Caucasian	Athletes Controls	> freq. of RR genotype in power athletes.
Ahmetov *et al.*, 2009	Endurance	Russian &Tartars Caucasian M & F 17-24	Athletes 456 Controls 1211	Freq. of the XX genotype < endurance athletes None of the highly elite athletes had the XX genotype

Freq., frequency.

2.7.2 *ACTN3 genotypes and skeletal muscle strength and function*

Cross sectional association studies for the association of ACTN3 with muscle function are more equivocal, however (Table 2.3). Clarkson *et al.* (2005) studied the association between ACTN3 genotype and isometric and dynamic strength of men (n=247) and women (n=355). No genotype-muscle phenotype association was reported in men. However women homozygous for the ACTN3 X allele (XX) had lower isometric strength compared with heterozygotes (RR). It was concluded that 2.2% of the variability in isometric strength was attributable to ACTN3 genotype, and thus one of many genes contributing to the variation in muscle strength. A recent report investigated the association of ACTN3 R/X polymorphism with muscle fibre type and fast velocity knee extensor strength in a group of ninety healthy young men (18-29) (Vincent *et al.*, 2007). Homozygotes for the R allele showed higher relative dynamic quadriceps torque at $300^{\circ}\cdot s^{-1}$ compared to XX carriers; additionally the number of type IIX fibres was greater in the RR than the XX genotype groups.

In a more recent study Delmonico *et al.* (2007) reported XX genotype women had greater absolute peak power than RR and RX genotypes; this was not observed in the male population. An additional study by the same group reported no differences between genotypes when testing for knee extensor torque at $60^{\circ}\cdot s^{-1}$ in men and women (Delmonico *et al.*, 2008). Delmonico *et al.* (2008) recruited a mixed race cohort and although analysis was performed for each race their results contradicts their previous study. A large group of adolescent Greek male and females were genotyped and measured for grip strength and similar to the work of Delmonico *et al.* (2008) no genotype association with strength was observed (Moran *et al.*, 2007). However, RR and RX genotype males had lower (faster) 40 m sprint times compared with XX genotype males and was not observed in the female cohort.

Table 2.3 Cross sectional studies of muscle strength according to ACTN3 genotype

Reference	Phenotype	Population Sex Age (Yrs)	N	Difference between genotypes
Vincent *et al.*, 2007	Isometric & isokinetic 300°·s^{-1} KE strength	Belgium M 18-29	90	RR genotype individuals > relative dynamic quadriceps torque vs. with XX genotype.
Clarkson *et al.*, 2005	EF isometric (MVC) and dynamic 1-RM	USA Mixed ethnicity M & F 25	602	XX genotype women < MVC vs. RR genotypes.
Delmonico *et al.*, 2007	KE concentric PP	USA M & F 65 ± 8	157	XX genotype women > absolute PP than RR & RX NS in men
Delmonico *et al.*, 2008	KE torque 60°·s^{-1}	USA Caucasian&Black M & F 79	1367	NS
Moran *et al.*, 2007	Hand grip strength 40m sprint	Greek M & F 11-18	992	NS of handgrip strength RR & RX males faster 40m sprint times vs. XX. NS in females
Walsh *et al.*, 2008	KE torque 30 & 80°·s^{-1}	USA M & F 22-90	848	XX females < torque vs. RR &RX NS in men

KE, knee extensors. EF, elbow flexors. PP, peak power. NS, non significant. 1-Rm, 1 repetition max. MVC, maximum voluntary contraction.

2.7.3 *Physiological effects of ACTN3 genotype in skeletal muscle*

Studies of ACTN3 were originally motivated by the possibility that mutations in the ACTN3 gene might underlie muscle disease in humans (MacArthur and North, 2007). The complete absence had no obvious phenotypic effect and as outlined above the presence may have a more subtle association with muscle function. ACTN3 is found almost exclusively in the primarily anaerobic, glycolytic type IIX fibres (Mills *et al.*, 2001). The specialised expression pattern and strong sequence conservation of ACTN3 over evolutionary time (North *et al.*, 1999) suggests that this protein may possess some advantageous role in type II fibres. If this is the case then the absence of ACTN3 in the skeletal muscle may inhibit the performance of the type IIX fast glycolytic muscle fibres important for rapid powerful contractions (MacArthur and North, 2007) that anchors together actin-containing thin filaments and stabilises the contractile apparatus (Squire, 1992). ACTN3 was also suggested to be functionally redundant (Mills *et al.*, 2001) meaning that the function of one gene completely overlaps, but extends beyond that of another gene, as long as the gene whose function is completely overlapped performs the common function of the two genes more effectively (Krakauer and Nowak, 1999). This may be the case for ACTN2 and ACTN3 but ACTN3 performs the role in fast twitch fibres more effectively.

The mechanistic actions of ACTN3 deficiency are currently being explored, and current knowledge is evolving rapidly. It is known that expression is restricted to type IIX muscle fibres and its presence is somehow beneficial for power and sprint athletes at an elite level (Chan *et al.*, 2008). A number of hypotheses have been put forward. First, it may stabilize the sarcomere when muscles are exercised to their maximum capacity, as in sprinting, and without ACTN3 the sarcomere may be weakened. This role is suggested due to the protein being located in the Z-disc and its actin binding properties. Second, ACTN3 may influence fibre type differentiation toward a fast twitch, glycolytic profile that is beneficial for sprint performance, with its absence leading to differentiation toward a slower oxidative profile that is beneficial for endurance performance (Chan *et al.*, 2008).

Sarcomeric α actinins are known to interact with cell signaling proteins that are involved in fibre differentiation and with enzymes involved in metabolic pathways. It is therefore possible that ACTN3 could influence fibre type differentiation toward a fast glycolytic profile. MacArthur *et al.* (2007) reported that ACTN3 deficient muscle fibres mice had higher levels of oxidative enzymes that would be expected in a fast glycolytic fibre. MacArthur *et al.* (2008) revealed than the activity of oxidative enzymes was higher, and anaerobic enzymes were lower in KO mice than in wild types. Therefore ACTN3 may facilitate anaerobic metabolism, and its absence may promote more oxidative pathways. To determine if this alteration in skeletal metabolism affects performance MacArthur and North (2007) subjected ACTN3 deficient and ACTN3 sufficient mice to an endurance run and the XX mice performed significantly better that the RR mice running ~33% further; suggesting that the shift toward oxidative metabolism increases intrinsic performance consistent with the over representation of the 577XX null genotype in endurance athletes (discussed above). Chan *et al.* (2008) reported features consistent with this hypothesis such as smaller cross sectional areas, lower twitch to tetanus ratios and better recovery from fatigue in ACTN3 deficient fibres in the mouse model. In the same study Chan *et al.* (2008) reported a 2.6 ms increase in the twitch half relaxation time of knockout mice compared with wild types which is consistent with the observation that elite sprinters have a very low incidence of ACTN3 deficiency. Lack of ACTN3 would appear to prolong the time taken for muscles to relax and therefore would be disadvantageous to activities requiring repeated rapid contractions such as sprinting (Allen *et al.*, 1995).

Additionally it is suggested that the α actinins form a hub at the intersection of several important functional pathways in skeletal muscle, helping to maintain the integrity of both the contractile apparatus and the crucial link between the sarcomere and plasma membrane, and potentially playing important roles in the communication between the sarcomeric machinery and other cellular pathways and the regulation of skeletal muscle metabolism (MacArthur and North, 2004).

Moreover, there is also recent evidence that ACTN3 genotype may have an association with fibre type composition in human skeletal muscle (Vincent *et al.*, 2007). Muscle biopsies of the right vastus lateralis were obtained from 22 XX and 22 RR young men, analysis revealed the number of type IIX fibres was greater in RR than XX individuals and ACTN3 protein content was systematically higher in type IIX compared to type IIA fibres. These authors suggested that the mechanism by which ACTN3 has its effect on muscle power may partly rely on the regulation of fibre type proportions. Norman *et al.* (2009) obtained muscle biopsies from the vastus lateralis muscle of 21 men to determine fibre type composition and ACTN3 mRNA levels. In contrast to Vincent *et al.* (2007) fibre type composition across the 3 genotypes did not differ suggesting that ACTN3 do not play a significant role in determining muscle fibre type composition. Norman *et al.* (2009) additionally showed that ACTN2 expression is affected by the content of α actinin-3, which implies that α actinin-2 may compensate for the lack of α actinin-3 and hence counteract the phenotypic consequences of the deficiency.

ACTN3 has also been proposed as a candidate metabolically 'thrifty gene' (Neel, 1962), with the absence of ACTN3 hypothesised to improve energetic efficiency (Mc Arthur and North, 2004) that could promote energy storage and body fat accumulation. However there has been little support for this in young people (Vincent *et al.*, 2007). Vincent *et al.* (2007) did not observe differences between genotypes for body fat percentage, fat mass and fat free mass (FFM) in ninety young Belgium males using bioelectrical impedance. Moran *et al.* (2000) reported similar results of no genotype influence on BMI and skinfold measures in their group of young athletes. In mouse models however Chan *et al.* (2008) reported ACTN3 deficient mice displayed lower lean mass and total body mass. It is possible that an effect may only accrue over the human lifespan and a subtle genetic association with energy balance may only become apparent over a prolonged period of several decades.

Considering the evidence that ACTN3 may perform important functions in type II skeletal muscle, it seems reasonable to assume that there may be subtle differences in skeletal muscle function between humans with different ACTN3 genotypes. The

apparent benefit for the presence of functional ACTN3 in elite sprint/power performance is consistent with the localisation of ACTN3 to skeletal muscle type II fibres and the overrepresentation of the functional allele among elite sprinters.

It has been suggested that the presence or absence of ACTN3 in humans has functional implications only in certain environments or under extreme conditions, which could be why the frequency of functional ACTN3 is shown to be abundant in elite level sprint and power athletes compared to the normal population (Neimi and Majamaa, 2005; Yang *et al*; 2003).

CHAPTER 3

ACE I/D and ACTN3 R577X Polymorphisms and Human Muscle Functional and Contractile Properties in Young Caucasian Men.

3.1 Introduction

Genetic factors are thought to determine 20-80% of the variation in a number of traits important to athletic performance (MacArthur & North, 2007) for example the relative proportion of fast and slow twitch skeletal muscle fibres (Simoneau and Bouchard, 1995; ~ 45% due to inherited factors). The annual publication of the human gene map for performance and health related fitness phenotypes (Rankinen *et al.*, 2006) lists more than 150 genes or genetic regions associated with athletic performance and physical fitness traits. The angiotensin converting enzyme (ACE) I/D polymorphism (Williams *et al.*, 2005) and α actinin 3 (ACTN3) R577X polymorphism (Mills *et al.*, 2001) have recently been identified as potentially contributing to variations in skeletal muscle composition, function and performance.

The ACE gene has been the most extensively studied gene in the area of human performance phenotypes. A functional polymorphism of the ACE gene is defined as the presence (insertion, I allele) or absence (deletion, D allele) of a 287 amino acid base pair Alu repeat sequence within intron 16 of the ACE gene on chromosome 17 (Rigat *et al.*, 1990). The polymorphism results in the genotypes II, ID, DD with representation among white European adults of approximately 24%, 50% and 26% respectively (Myerson *et al.*, 1999). A high proportion of the DD genotype has been reported in elite sprint athletes compared with endurance athletes and controls (Myerson *et al.*, 1999; Woods *et al.*, 2001; Nazarov *et al.*, 2001), indicating that this genotype may be associated with skeletal muscle function.

ACE is recognised as a key enzyme in the renin-angiotensin system and it has been shown that the D allele variant of the ACE gene is associated with higher concentrations of serum ACE activity (Rigat *et al.*, 1990). ACE catalyses the production of ANG II, which appears to enhance skeletal muscle hypertrophy (Gordon *et al.*, 2001; Westerkamp and Gordon, 2005). Additionally ACE degrades kinins (Brown *et al.*, 1998), which have been shown to have growth inhibitory properties (Ishigai *et al.*, 1997). There is recent evidence that ACE genotype is associated with the muscle volume

of healthy older men and women (Charbonneau *et al.*, 2008) a key determinant of muscle strength and power. Fascinatingly Zhang *et al.* (2003) reported that the DD genotype was associated with a greater proportion of fast twitch fibres in a Japanese population. This evidence supports the notion that the ACE D allele may be associated with skeletal muscle function particularly strength/power at high velocities. However, in contrast Akhmetov *et al.* (2006) reported that the DD genotype was associated with a greater proportion of slow twitch fibres in a Russian population.

Several investigations have found no association of ACE genotype with isometric and dynamic strength (Folland *et al.*, 2000; Thomis *et al.*, 2004; Woods *et al.*, 2001; Pescatello, *et al.*, 2006). Although the D allele has been associated with greater isometric strength in COPD patients (Hopkinson *et al.*, 2004) and isometric and isokinetic strength in healthy young men (Williams *et al.*, 2005). To date no studies have examined the association of ACE genotype with high velocity strength and the contractile properties of human skeletal muscle which is of great interest given the potential association of ACE genotype with fibre type composition.

α actinins are a family of actin-binding proteins found in humans (Blanchard *et al.*, 1989): ACTN1 and ACTN4 are non muscle proteins (Kaplan *et al.*, 2000) whereas ACTN2 and ACTN3 are myofibrillar proteins located at the Z disk where they form a lattice structure that anchors together actin containing thin filaments and stabilises the contractile apparatus (Squire, 1992). The functions of ACTN3 are likely to involve a static function in maintaining the ordered myofibrillar array (Mills *et al.*, 2001). A common genetic variation in the ACTN3 gene results in the replacement of an arginine (R) with a stop codon (X) at amino acid 577 (R577X). The 577R allele is the normal functional allele of the gene, whereas 577X allele contains a sequence change that negates the production of the functional ACTN3 protein (North *et al.*, 1999). This polymorphism results in the genotypes RR, RX, XX with representation among healthy white Australian adults of 30%, 52% and 18% respectively (Yang *et al.*, 2003).

ACTN3 is found almost exclusively in the primarily anaerobic, type II fibres (North and Beggs, 1996). The specialised expression pattern and strong sequence conservation of ACTN3 over evolutionary time (North *et al.*, 1999) suggests that this protein may possess some advantageous role in type II fibres. The absence of ACTN3 in the skeletal muscle has been suggested to inhibit the performance of the type II muscle fibres that are important for rapid powerful contractions (MacArthur and North, 2007). Moreover, there is also recent evidence that ACTN3 genotype may be associated with fibre type composition (Vincent *et al.*, 2007).

It has been demonstrated that the frequency of the ACTN3 RR genotype was higher amongst elite sprinters compared to endurance runners (Niemi and Majamaa, 2005) or controls (Yang *et al.*, 2003) while none of the top sprinters harboured the XX (ACTN3 deficient) genotype. This suggests that the presence of ACTN3 may have a beneficial effect on skeletal muscle function and in particular the ability to generate powerful contractions at high velocity.

Interesting reports have highlighted an apparent association of ACE and ACTN3 genotypes with sprint performance. The association of these polymorphisms with the contractile properties of human muscle and with strength throughout the torque velocity relationship has not been investigated. The purpose of this study was to carry out a comprehensive assessment of knee extensor muscle function and contractile properties in relation to the ACE I/D and ACTN3 R/X polymorphisms in healthy young men, and in particular their association with muscular strength at high velocities.

3.2 Methodology

3.2.1 *Participants and anthropometric assessment*

Ethical approval for this study was provided by the Loughborough University ethical advisory committee. The study was performed according to the *Declaration of Helsinki.* Seventy-nine UK Caucasian males, aged 18-30 years, volunteered to participate in this study and provided written informed consent. Participants were recruited from amongst University students, had no history of strength training, moderate-low overall physical activity (<4 sessions of exercise per week) and were free from locomotor or neuromuscular disease.

Participants' height was measured with a wall-mounted stadiometer (Holtain Ltd, Crymych, Wales) and body mass with a beam balance scale (Avery Ltd, Fairmont, MN) whilst participants wore shorts and a T-shirt. Skinfold thickness was measured at four sites (biceps, triceps, suprailiac and subscapular) using Harpenden skinfold calipers (British Indicators Ltd, Wolverhampton, UK). The measures were taken on the right side of the body and repeated 3 times, in order to calculate an average value. From the sum of four skinfolds body density was calculated using the formula of Durnin and Womersley (1974) and percentage body fat estimated using the Siri equation (Siri, 1956). Fat mass and fat-free mass (FFM) were derived from the percentage body fat and body mass values.

3.2.2 *Protocol overview*

Participants were tested between the hours of 1400 and 1800 and were asked to refrain from heavy physical exertion in the 36 hours prior to measurement. Prior to strength assessment participants performed a warm up of 5 minutes on a cycle ergometer (Monark 818e) at ~50 W. A range of muscle function tests were performed on the knee extensors. All tests were performed on both legs and were completed on two separate occasions with at least 72 hours separating each laboratory visit. Values from both legs

measured on each occasion were averaged to provide representative values for each individual. Participants were given verbal encouragement on all tests.

3.2.3 *Knee extensor isometric strength and contractile properties*

Both voluntary and electrically stimulated isometric contractions of the knee extensors were assessed using a conventional isometric strength testing chair (Parker *et al.*, 1990) where participants were seated in an upright position with the back supported, and knee and hip joint angles at 90° (1.57 rad) and 80° (1.41 rad), respectively. Participants were firmly secured with straps, which fastened at hips and shoulders. Participants lower leg was tightly strapped, approximately 2 cm above the ankle, to a calibrated U-shaped aluminum strain gauge (Jones and Parker, 1989) that was always in the direct line of action of the applied force. The strain gauge signal was sampled at 2,000 Hz with an analogue to digital (A/D) converter (Micro 1401, Cambridge Electronic Design Ltd, Cambridge, UK) and logged and displayed by PC with Spike 2 software (Cambridge Electric Design Ltd, Cambridge, UK).

Following some progressive sub-maximal contractions maximum isometric strength was determined by having participants perform 3-4 maximum voluntary contractions of the knee extensors, each of ~3 s duration and with 30 s rest between each contraction. The highest value was recorded as the participants' maximum strength. The coefficient of variation (CV) for maximum isometric strength over repeated test occasions was 3.3%.

The contractile properties of the knee extensors were measured using electrically stimulated contractions. The participants remained seated in the chair and 2 large (13 x 10 cm) flexible carbon rubber electrodes (Electro Medical Supplies Ltd, Wantage, UK), coated with electrode gel (Camcare Gels, Ely, UK) were placed over the anterior surface of the muscle. Percutaneous electrical stimulation with unidirectional square wave pulses of 50 μs duration was applied through a constant current Digitimer stimulator (DS 7AH, Welwyn, Garden City, UK). The stimulation current began at 5 mA and gradually increased until the twitch response reached a plateau of maximal twitch force. The

twitch response was filtered by applying a digital filter with a low pass frequency of 30 Hz and a transition gap of 16 Hz. Semi automated cursors within the spike 2 software were used to identify discrete events within each twitch. Time to peak tension (TPT) was taken as the time between force onset and peak twitch force, with force onset defined as 3% of peak gradient. Half relaxation time (HRT) was the time for the muscle to relax from peak twitch force to half peak force. The peak rate of force development (PeakRFD) during the twitch was assessed as the maximum gradient of the force-time graph. Three twitches were analysed, the average values were recorded for each leg. The coefficient of variation (CV) for TPT, HRT and RFD over repeated test occasions was 6.8, 13.8 and 8.7 % respectively.

3.2.4 *Torque-velocity relationship*

For torque measurements participants were seated in a Cybex Norm Isokinetic Dynamometer (Lumex Inc, NY, USA) with the axis of the knee joint aligned with the centre of rotation of the dynamometer arm, and the lower leg strapped to the lever arm just proximal to the ankle. Participants were restrained at the waist, shoulders and the distal part of the thigh, and the backrest was set at 100° (1.73 rad) from the horizontal base of the seat. Peak torque of the knee extensors was assessed isometrically at 73° (1.27 rad) of knee flexion (identified by pilot work as the angle of peak torque) and isokinetically at 30, 90 and 240°·s^{-1} (0.52, 1.57, 4.19 rad·s^{-1}). The relative torque at high velocity (ratio of strength at 240°·s^{-1} to 30°·s^{-1} (4.19:0.52 rad·s^{-1})) was calculated as a percentage. For isometric measurements there were 3 practice contractions at 50% effort followed by 3 maximum voluntary contractions. For the isokinetic measurements participants performed a set of 3 practice trials of sub-maximal knee extension at each angular velocity, prior to 2 sets of 2 maximum contractions at 30°·s^{-1} and 2 sets of 3 maximum contractions at 90°·s^{-1} and 240°·s^{-1}. There was at least 30 s rest between each set of contractions, and ~60 s between velocities. The range of motion for the isokinetic measurements was from 120° - 15° (2.09 - 0.26 rad) of knee flexion. Knee extensor torque values were corrected for gravitational torque, and contractions at the highest velocity 240°·s^{-1} (4.19 rad·s^{-1}) were typically isovelocity for at least the range 90° - 30°

(1.57-0.52 rad). The CV for the different torque measurements over repeated test occasions was from 5.3-6.3%.

3.2.5 *Blood collection and genotyping*

A trained phlebotomist performed venous puncture. Participants lay in a semi - supine position whilst a 10 ml blood sample was obtained from a superficial forearm vein following a 12 hour fast to avoid lipemic sera. Blood samples were collected in a serum tube and applied onto filter paper in preparation for genomic deoxyribonucleic acid (DNA) isolation. All blood samples were left at room temperature for 2-3 hours to allow blood to clot. The samples were then spun at 2300 revs·min^{-1} (rpm) for 10 minutes at 4°C in a refrigerated centrifuge (Beckman Instruments, Palo Alto, CA) and stored at -20°C enabling the determination of serum ACE activity on a separate occasion.

Genomic DNA was obtained from previously bloodstained filter paper using Chelex extraction and genotypes for the polymorpisms of ACE I/D and ACTN3 R/X were determined. ACE I/D polymorphism was typed using polymerase chain reaction (PCR) based amplification. The final reaction mixture of 15 µl for ACE contained: ACE Forward and Reverse Primers at 0.3 µl each, 5 µl chelex extraction DNA, 7.5 µl 2xReddy mix (ABgene) which contained 1.25 units Thermoprime Plus DNA Polymerase, 75 mM Tris-HCl, 20 mM$(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.01 % (v/v) Tween® 20 and 0.2 mM each of dATP, dCTP, dGTP and dTTP and precipitant and red dye for electrophoresis and 1.9 µl H_2O. Mistyping of ID heterozygotes' has been found in some studies to occur due to preferential amplification of the D allele, and inefficiency in the amplification of the I allele. To increase the specificity of DD genotyping, PCR amplifications were also performed with an I-specific primer pair (5'TGGGACCACAGCGCGCCCGCCACTAC3'and 5'TCGCCAGCCCTCCCATGCCCATAA3') in all the samples that were found to be DD after amplification with the flanking primers. Briefly I specific amplification was performed using 25 µl reaction with 1 min denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at 67°C (annealing), and 2 min at 72°C (extension). The 335bp

fragment was identified on 2% agarose gel containing ethidium bromide. Under these conditions only the I allele produces a 335bp amplicon. The reaction yielded no products in a sample of all the DD genotypes.

ACTN 3 polymorphism was typed using PCR. The final reaction mixture of 20 µl for ACTN 3 contained: ACTN3 Forward and Reverse Primers at 1 µl each, 10 µl of 2x Readdy Mix, (5 mM $MgCl_2$) (ABgene), 5 µl chelex extracted DNA and 3 µl H_2O. The PCR reaction conditions protocol (Table 3.1) was programmed onto and run on Mastercycler PCR machine (Eppendorf, Hamburg, Germany).

Table 3.1 Forward and reverse primers and PCR conditions for ACE and ACTN3

	Forward Primer (5'-3')	Reverse Primer (5'-3')	PCR reaction conditions		
			Initial Denaturation	Denaturation (D) Annealing (A) Extension (E)	Final Extension
ACE	CTGGAGACCACT CCCATCCTTTCT	GATGTGGCCATC ACATTCGTCAGAT	95°Cfor5min	30cyclesof 95°Cfor1min (D), 58°Cfor1min (A), 72°Cfor2 min (E)	72°Cfor10min
ACTN3-16	CTGTTGCCTGTG GTAAGTGGG	TGGTCACAGTATG CAGGAGGG	95°Cfor5mins	35cyclesof 95°Cfor30sec (D), 60°Cfor30sec (A), 72°Cfor30sec (E)	72°Cfor10min

PCR product (15 µl) for ACE was then analysed by electrophoresis at 140 V for 45 minutes through a 2% agarose gel containing 0.5 µg/ml ethidium bromide and 1xTris-borate, ethylenediaminetetraacetic acid (EDTA) electrophoresis buffer pH 8.3 (TBE). Additionally, 5 µl of 100bp ladder (Abgene) was loaded to identify 190 and 490bp fragments for the ACE I/D polymorphism. A UV transilluminator (UVP, Cambridge, UK) was then used to visualize and score DNA fragments.

Following genomic DNA amplification, ACTN3 PCR product was digested using *Dde*I restriction enzyme (New England, Biolabs Inc., Ipswich, MA) at 37°C for 4 h (Mastercycle, Eppendorf). The reaction mixture of 20µl contained: 0.4µl Enzyme *Dde*I, 2 µl of 10x NEBuffer 4 (pH 7.9 at 25°C) constituting of 50 mM potassium acetate, 20

mM Tris-acetate, 10 mM magnesium acetate and 1mM dithothreitol, 0.2 µl 100x bovine serum albumin (BSA), 7.6 µl H_2O and 10 µl ACTN3 PCR product. The recognition site for the *Dde*I restriction enzyme was CTNAG (5'-3').

PCR product (15 µl) for ACTN3 was then analysed by electrophoresis at 120 V for 90 minutes through a 3% agarose gel containing 0.5 µg/ml ethidium bromide and 1xTris-borate, ethylenediaminetetraacetic acid (EDTA) electrophoresis buffer pH 8.3 (TBE). Additionally, 5 µl of 100bp ladder (Abgene) was loaded to identify 86, 97, 108 and 205bp fragments distinctive of the ACTN3 polymorphism. A UV transilluminator (UVP, Cambridge, UK) was then used to visualize and score DNA fragments.

3.2.6 *Serum ACE activity*

Diagnostic determination of ACE activity in serum was carried out by an enzymatic assay using an automated spectrophotometer (Cobas Mira Plus, Roche, Switzerland). The enzyme mediates the cleavage of a synthetic substrate and the kinetics of this cleavage reaction are measured following the decrease in absorbance at 340 nm. This was measured twice with the Bühlmann ACE kinetic kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland). The intra-assay coefficient of variation for the serum ACE measurements was 4.9%.

3.2.7 *Statistical analysis*

A Chi squared test was used to confirm that the observed frequencies for ACE and ACTN3 were in Hardy–Weinberg Equilibrium. All data was confirmed as normally distributed using SPSS for windows 12.0 (SPSS, Chicago, IL). A one way analysis of variance (ANOVA) was used to test the differences in strength, contractile properties and serum ACE activity between ACE I/D and ACTN3 R/X genotypes. A Pearson's product moment correlation was used to examine the relationship between serum ACE activity and the functional and contractile properties of the knee extensors. Statistical analyses were performed using SPSS for windows 12.0 (SPSS, Chicago, IL) and Microsoft Excel 2000 for windows (Microsoft, Redmond, WA). Data are presented as

mean and standard deviation of the mean (SD) with statistical significance defined as P values of < 0.05.

3.3 Results

3.3.1 *Physical characteristics*

A total of 79 participants were studied, whose characteristics were (mean ± SD): age, 20.1 ± 2.2 yr; body mass, 75.3 ± 11.1 kg; height, 1.79 ± 0.06 m; body mass index, 23.2 ± 3.9 kg·m^{-2}; FFM 57.1 ± 8.4 kg. The distribution of ACE (II = 17, ID = 40, DD = 22) and ACTN3 (XX = 15, RX = 37, RR = 27) genotypes were in Hardy-Weinberg equilibrium. Physical characteristics were independent of ACE and ACTN3 genotypes.

3.3.2 *ACE I/D polymorphism, serum ACE activity and knee extensor functional and contractile properties*

No differences existed between ACE genotypes for knee extensor isokinetic torque at any velocity from 0°·s^{-1} – 240°·s^{-1} (ANOVA, 0.12 < P < 0.23; Figure 3.1a) or the relative torque at high velocity (240:30°·s^{-1}; II 59 ± 5, ID 59 ± 6, DD 59 ± 5 %; ANOVA, P = 0.971; Figure 3.2). No differences existed between ACE genotypes for the time course of the twitch (TPT, ANOVA, P = 0.135; HRT, ANOVA, P = 0.312; Figure 3.3a) or peak RFD (II 4678 ± 1654; ID 4535 ± 1189; DD 4611 ± 1226 N·s^{-1}; ANOVA, P = 0.86). There was no significant difference in isometric strength between ACE genotypes (II 600 ± 88; ID 632 ± 89; DD 577 ± 86 N; ANOVA, P = 0.07; Figure 3.4). Serum ACE activity for the whole group was 53 ± 22 U/L, and this was significantly influenced by ACE genotype (II 30 ± 11; ID 55 ± 22; DD 66 ± 16 U/L; ANOVA, P < 0.01). Serum ACE activity was not associated with any functional measurement (isometric strength R = 0.11, P = 0.33; isokinetic strength at 30°·s^{-1} R = 0.16, P = 0.18; isokinetic strength 30°·s^{-1} R = 0.06, P = 0.62).

3.3.3 *ACTN3 R577X polymorphism and knee extensor functional and contractile properties*

No differences existed between ACTN3 genotypes for knee extensors isokinetic torque at any velocity from 0°·s^{-1}– 240°·s^{-1} (ANOVA, 0.64 < P < 0.99; Figure 3.1b) or the

relative torque at high velocity (240:30°·s^{-1}; RR 60 ± 6, RX 58 ± 4, XX 60 ± 6 %; ANOVA, P = 0.19; Figure 3.2). No significant differences existed between ACTN3 genotypes for the time course of the twitch (Figure 3.3b) or peak RFD (RR 4493 ± 1241; RX 4748 ± 1344; XX 4611 ± 1226 N·s^{-1}; ANOVA, P = 0.65). Isometric strength was independent of ACTN3 genotype (RR, 599 ± 81; RX, 619 ± 92; XX, 606 ± 103 N; ANOVA, P = 0.66; Figure 3.4).

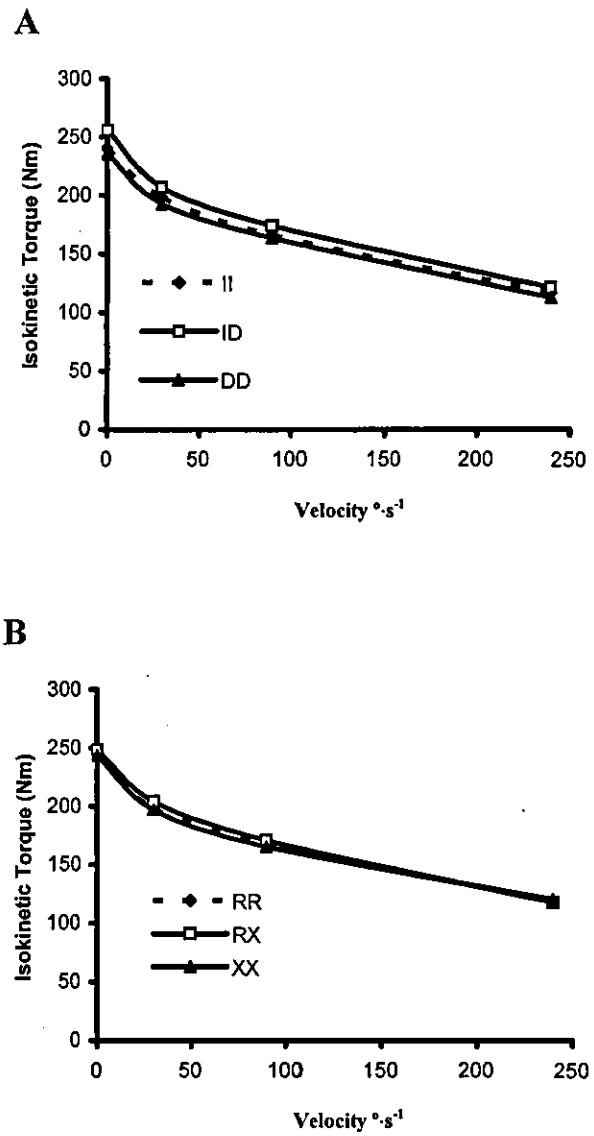

Figure 3.1 ACE (A) and ACTN3 (B) genotype were not significantly associated with isokinetic torque. Data presented as mean values (n = 79).

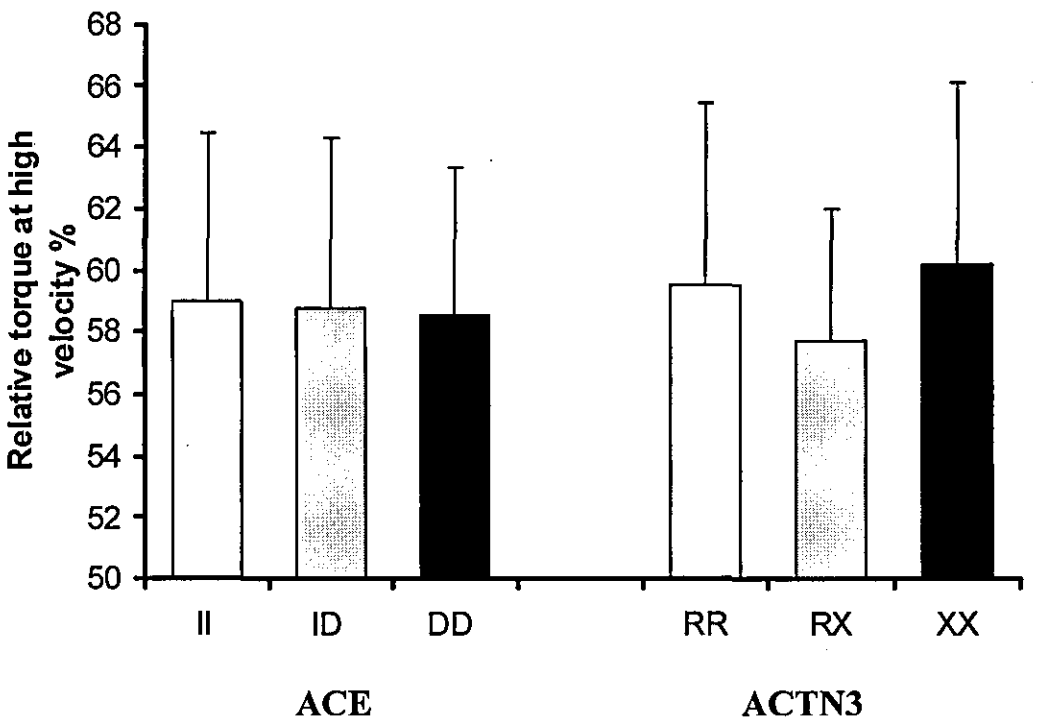

Figure 3.2 ACE and ACTN3 genotype were not associated with the relative torque at high velocities. Values are presented as mean and SD ($n = 79$).

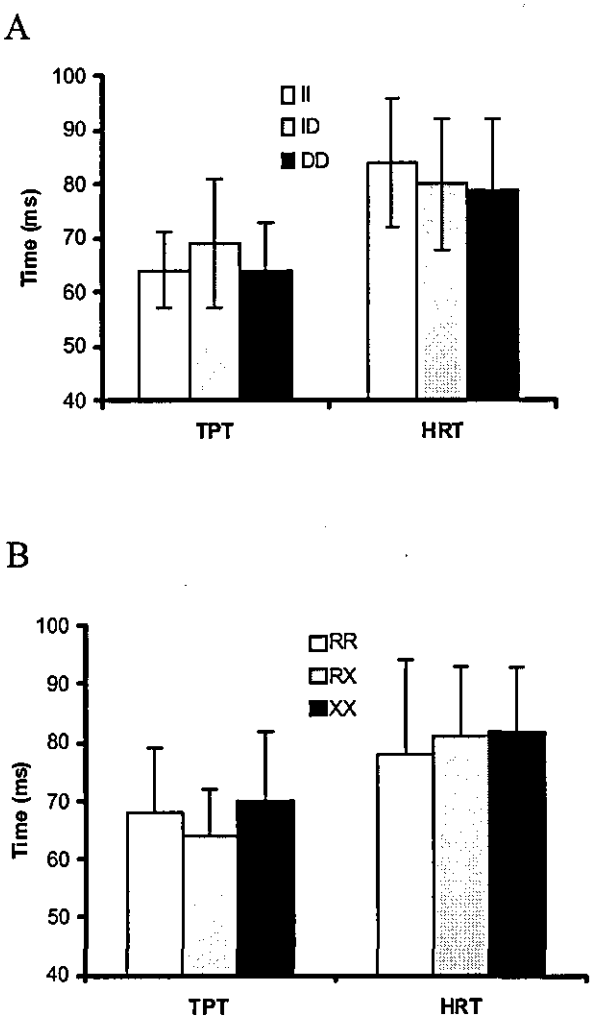

Figure 3.3 Contractile properties were not significantly associated with ACE (A) and ACTN3 (B) genotype. Values are presented as mean and SD (n =79).

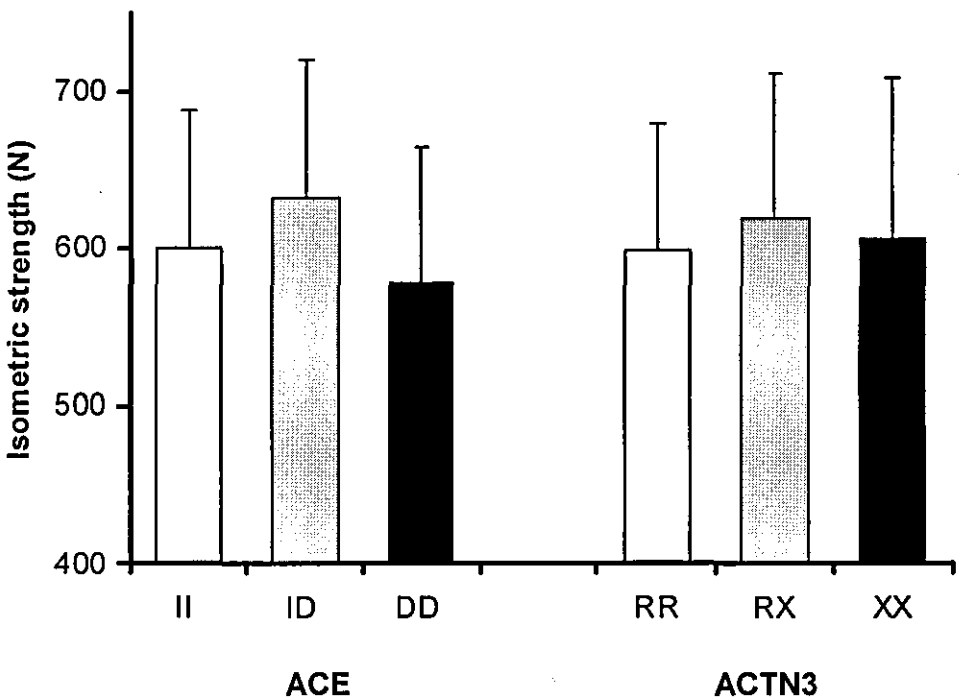

Figure 3.4 Isometric strength according to ACE and ACTN3 genotypes. Data are mean and SD (n=79).

3.4 Discussion

The aim of this study was to evaluate the association of ACE I/D and ACTN3 R/X polymorphisms on knee extensor muscle function and contractile properties of healthy young men. Specifically it examined the association of two candidate gene polymorphisms with muscle strength at high velocities as well as the time course of an electrically stimulated twitch. ACE I/D and ACTN3 R/X polymorphisms were not associated with muscular strength at high velocities or the time course of the twitch response.

The strength values recorded in this experiment were typical of untrained healthy young men (Folland *et al.*, 2000; Folland *et al.*, 2005). Every effort was made to ensure that the participants made genuinely maximal contractions, and the reliability of the measurements was generally high (CV: 3.3% for isometric force; 5.3-6.3% for torque measurements; 5.8% relative torque at high velocity; 6.8% TPT; 13.8% HRT; 8.7% peak RFD). We endeavored to avoid the potentially confounding effects of chronic physical training and ethnic diversity in order to highlight the association of genes with strength. Therefore we recruited volunteers with moderate to low levels of physical activity, which likely explains the wide variation in body composition, as well as ensuring participants were UK Caucasian.

Dynamic strength and power are critical aspects of muscle function that have greater validity for sports performance and health (general mobility and locomotion) than isometric strength measurements. Hence the importance of identifying genetic variations which influence dynamic strength and power. The current study was the first to examine the association of ACE genotype with the torque-velocity relationship and the time course of a twitch response. It was thought that if the DD genotype is associated with a greater proportion of fast twitch fibres then this could be associated with muscle function, particularly torque at high velocity and the time course of the twitch response. However ACE genotype was not associated with the absolute or relative torque at high

velocities or the timecourse or RFD of a maximal twitch response, and in the current study had no association with muscle function. The relative torque at high velocity ($240:30^{o}\cdot s^{-1}$) that we have calculated is also thought to be a functional indicator of fibre type ($r = 0.74$ with the relative area of type II fibres; Gur *et al.*, 2003). Whilst it is clearly not a direct measure of isoform profile a growing body of evidence suggests that the ratio of muscle torque produced at fast versus slow speeds is highly correlated to the area and isoform profile of muscle fibres (Gur *et al.*, 2003; Ivy *et al.*, 1981; Suter *et al.*, 1993). The TPT has also been found to reflect the percentage of type II fibres (Hamada *et al.*, 2000; Edwards *et al.*, 1976).

Zhang *et al.* (2003) reported individuals with DD genotype had a greater proportion of fast twitch fibres in a Japanese population. This evidence supports the notion that the ACE D allele may be associated with skeletal muscle function particularly strength/power at high velocities. However, a recent contrasting study of Russian men found ACE D allele carriers to have a higher proportion of slow twitch fibers (Akhmetov *et al.*, 2006). Our results demonstrate that there is either no association of ACE genotype with fibre type composition or the effect is too small to show association with the muscle function of UK Caucasian men.

In keeping with the literature, ACE genotype was associated with circulating ACE activity (Rigat *et al.*, 1990). Whilst it has previously been reported that circulating ACE activity is associated with muscular strength (Williams *et al.*, 2005), in the current study, there was no association of ACE activity with any measure of muscle function. Furthermore, we found no association of ACE genotype and muscle function. A similar recent study by Williams *et al.* (2005) reported a significant effect of ACE genotype on isometric and isokinetic strength at 1.05 $rads\cdot s^{-1}$ in 81 Caucasian men. The current investigation and that of Williams *et al.* (2005) studied very similar subject populations and functional measurements. Therefore it is difficult to account for the contrasting findings. The lack of an ACE genotype association with isometric strength in the current study is similar to previous findings (Folland *et al.*, 2000; Frederiksen *et al.*, 2003; Thomis *et al.*, 2004; Pescatello *et al.*, 2006; Hopkinson *et al.*, 2004). It is increasingly

recognized, with evidence from a number of studies, that an individual gene has, at best, a small association with physical function (Williams and Folland, 2008). This may explain the contrasting findings from single gene studies, and in part motivated this attempt to quantify the simultaneous influence of two candidate genes upon a specific phenotype (muscle function). As ~20-80% of the phenotypic variation appears to be due to inherited factors it is likely that numerous as yet unidentified genes collectively influence muscle function (Williams and Folland, 2008).

The other candidate gene considered in this study was the ACTN3 R/X polymorphism which, was not associated with the absolute or relative torque at high velocities or the twitch response. This is surprising giving the apparent role that ACTN3 may play in fast twitch fibers and the suggested advantageous effect on skeletal muscle function particularly the ability to generate powerful contractions at high velocity (Yang *et al.*, 2003). Although, in older women (64 yrs) knee extensor concentric peak power was found to be higher in X allele homozygotes compared to RR genotype individuals (Delmonico *et al.*, 2007). In contrast to the current study and Delmonico *et al.* (2007), another recent study reported higher relative dynamic torque at $300°\cdot s^{-1}$ for RR genotype individuals compared to XX carriers (Vincent *et al.*, 2007). Fiber type differed significantly between the homozygote genotypes of Vincent *et al.* (2007), with the percentage surface area and number of type IIX fibres being greater in the RR than the XX genotype group. The velocity used in the current study was $240°\cdot s^{-1}$ compared to the $300°\cdot s^{-1}$ used by Vincent *et al.* (2007). A higher velocity of torque measurement may more effectively discriminate for fibre type differences between genotypes.

Isometric strength of the knee extensors was not associated with ACTN3 genotype in our cohort. Similarly Clarkson *et al.* (2005) reported no association between ACTN3 R577X genotype and muscle phenotype in men when investigating isometric elbow flexor strength although these authors reported that women homozygous for the ACTN3 577X allele (XX) had lower isometric strength compared with heterozygotes (RX). Whilst the current study found no association of the two candidate genes considered with muscle function phenotypes of young men, it is feasible that they may be associated

with the muscle function of other populations e.g. women, patient groups or older individuals. For example a gene-environment interaction could occur over several decades, and accelerate or delay the decline in function with age.

To conclude, in the present study ACE I/D and ACTN3 R/X polymorphisms were not associated with muscular strength at high velocities or the time course of the twitch response. Any association of these individual polymorphisms does not appear to be of sufficient magnitude to influence muscle function in young free living UK Caucasian men. It remains to be determined if these gene variants are associated with the muscle function of older individuals.

CHAPTER 4

The Association of ACE I/D and ACTN3 R577X Polymorphisms with Hand Grip Strength and Muscularity in Older Caucasian men.

4.1 Introduction

An individual's muscular strength reaches its peak around the age of 30 and is well maintained until 50 years old (Deschenes, 2004). Beyond the fifth decade of life, strength tends to decrease at a pace of ~15% per decade (Larsson *et al.*, 1979). Thus ageing is a strong environmental influence on muscle function. Age associated losses in muscle strength and power can have a profound effect on a person's ability to live independently and contributes to an increased risk of falls. The decline in functional capacity has detrimental effects not only rates of injury but also morbidity and mortality (Rantanen *et al.*, 1999; Rantanen *et al.*, 2000).

Grip strength has been found to be a strong predictor of physical functioning, disability, morbidity and mortality (Giampaoli *et al.*, 1999) hence it is a suitable phenotype for identifying genetic variants of importance in mid and late life. In young men, the genetic contribution to strength measures appears to be 30-80%, depending on the angle, type and velocity of contraction (Thomis *et al.*, 1998). Estimates of the grip strength heritability of older twins range from 30-65% (Arden and Spector, 1997; Frederiksen and Christensen, 2003; Carmelli and Reed, 2000; Frederiksen *et al.*, 2003a). As genetic factors appear to account for a substantial proportion of the phenotypic variability of muscle function, further research is required to find the specific genetic variants that are responsible for this inheritance.

The angiotensin converting enzyme (ACE) I/D and α actinin 3 (ACTN3) R577X polymorphisms have received attention for their potential association with human performance phenotypes. Case controlled studies have found good evidence that the ACE DD genotype and ACTN3 RR genotype are over represented in elite sprint and power athletes than controls (Myerson *et al.*, 1999; Woods *et al.*, 2001; Nazarov *et al.*, 2001; Niemi and Majamaa, 2005; Yang *et al.*, 2003; Druzhevskaya *et al.*, 2008; Papadimitriou *et al.*, 2008), indicating that these genotypes may be associated with muscle function. ACE is recognised as a key enzyme in the renin-angiotensin system and the D allele

variant of the ACE gene is associated with higher concentrations of serum ACE activity (Rigat *et al.*, 1990). ACE catalyses the production of ANG II, which appears to enhance skeletal muscle hypertrophy (Gordon *et al.*, 2001; Westerkamp and Gordon, 2005), and degrades kinins (Brown *et al.* 1998), which have been shown to have growth inhibitory properties (Ishigai *et al.*, 1997). α actinins are a family of actin-binding proteins found in humans (Blanchard *et al.*, 1989). ACTN2 and ACTN3 are myofibrillar proteins located at the Z disk and stabilise the thin filaments and contractile apparatus (Squire, 1992). ACTN3 expression is restricted to type II muscle fibres (Mills *et al.*, 2001) and the absence of ACTN3 could inhibit the performance of these fibres.

The findings from cross-sectional association studies of these candidate genes are more equivocal than the case-control studies with elite athletic groups. Several investigations have found no association of ACE genotype with isometric strength in young (Folland *et al.*, 2000; Thomis *et al.*, 2004; Woods *et al.*, 2001b; Chapter 3) and older adults (Frederikson *et al.*, 2003b). Although the D allele has been associated with the muscle volume of healthy older men and women (Charbonneau *et al.*, 2008) a key determinant of strength and power. Other functional studies have found the D allele to be associated with greater isometric strength in COPD patients (Hopkinson *et al.*, 2004) and in healthy young men (Williams *et al.*, 2005). Two studies reported an association between ACTN3 R577X genotype and muscle phenotypes in women (lower elbow flexor (Clarkson *et al.*, 2005) and knee extensor (Walsh *et al.*, 2008) strength (for the XX genotype)), but not in men.

In the previous study (Chapter 3) there was no association of ACE I/D or ACTN3 R/X polymorphisms with the muscle function of young men. However ageing and the typical decline in muscle function that occurs represents a potent environmental influence. It is possible that the two candidate genes under consideration are associated with the muscle function phenotypes of older men by the interaction of ageing muscle with the environment. The possible genetic association with muscle function in older age should be considered due to the importance of muscle function to healthy ageing.

The objectives of this study was to demonstrate: (i) the feasibility of recruiting a substantial cohort of healthy older Caucasian men with a reasonably homogenous age range and physical activity status (ii) examine the association of ACE I/D and ACTN3 R/X with grip strength and muscularity of this cohort.

4.2 Methodology

4.2.1 *Screening*

The Loughborough University ethical advisory committee approved the study. One hundred and twenty three UK Caucasian men, aged 60-70 years, volunteered to participate in this study and provided written informed consent. Participants had no history of strength training, moderate-low overall physical activity (<4 sessions of exercise per week) and were free from locomotor or neuromuscular disease.

4.2.2 *Anthropometric assessment*

Participants' height was measured with a wall-mounted stadiometer (Holtain Ltd, Crymych, Wales) and body mass with a beam balance scale (Avery Ltd, Fairmont, MN) whilst participants wore shorts and a T-shirt. Skinfold thicknesses were measured at four sites (biceps, triceps, suprailiac and subscapular) using Harpenden skinfold calipers (British Indicators Ltd, Wolverhampton, UK). The measures were taken on the right side of the body and repeated 3 times, in order to calculate an average value. From the sum of four skinfolds body density was calculated using the formula of Durnin and Womersley (1974) and percentage body fat estimated using the Siri equation (Siri, 1956). Fat mass and fat-free mass (FFM) were derived from the percentage body fat and body mass values. FFM was used as an indicator of whole body muscularity.

Right and left forearm circumferences were measured using an anthropometric tape (Harpenden, British Indicators Ltd, London, UK). Maximum circumference of the proximal forearm, perpendicular to the long axis of the segment, was recorded while participants stood in the anatomical position. Skinfold thickness was determined at two sites on the forearm, lateralis and volaris (Eston and Reilly, 2001), using skinfold calipers (Harpenden, British Indicators Ltd, London, UK). Measurements were taken at least twice, if readings were discrepant by more that 0.2 mm a third measurement was taken. Circumference and skinfold measurements were averaged and an estimate of forearm

muscle and bone cross sectional area (lean forearm CSA) was calculated (Eston and Reilly, 2001) as an index of forearm muscularity.

4.2.3 *Grip strength*

Isometric hand grip strength was assessed with a JAMAR dynamometer (Sammons Preston Rolyan, Illinois, USA). Participants were familiarised with the dynamometer and test procedure and were required to stand with feet shoulder width apart with their elbow flexed at an angle of 90° (1.57 rad), with the shoulder adducted. Verbal encouragement was given during the assessment. Participants performed three maximal efforts that were sustained for 3 s with 30 s rest between each contraction. If grip strength increased after the third maximal voluntary contraction (MVC) then a fourth contraction was performed. The highest value for each hand was recorded and the average of both hands gave one representative value for grip strength.

4.2.4 *Blood collection and genotype determination*

The participants' thumb was sterilised and capillary puncture at the tip of the thumb was performed with a sterile Accu-Chek lancet device (Softclix, Roche Diagnostics, Hergeshelt von, Germany). Blood was stained directly onto filter paper ready for genomic deoxyribonucleic acid (DNA) isolation. The blood stained filter paper was then left to dry at room temperature. Genomic DNA was later obtained from bloodstained filter paper using Chelex extraction and genotypes for the polymorphisms of ACE I/D and ACTN3 R/X were determined as described previously (Chapter 3).

4.2.5 *Statistical analysis*

A Chi squared test was used to confirm that the observed genotype frequencies for ACE and ACTN3 were in Hardy–Weinberg Equilibrium. All data were confirmed as normally distributed using SPSS for windows 12.0 (SPSS, Chicago, IL). A one way analysis of variance (ANOVA) was used to test the differences in strength between ACE I/D and ACTN3 R/X genotypes. Statistical analyses were performed using SPSS for windows 14.0 (SPSS, Chicago, IL) and Microsoft Excel 2000 for windows (Microsoft, Redmond,

WA). Data are presented as mean and standard deviation of the mean (SD) with statistical significance defined as P values of < 0.05.

4.3 Results

4.3.1 *Physical characteristics*

The recruitment of 123 men who fitted the inclusion criteria demonstrated the feasibility of studying this population. The participants physical characteristics were (mean ± SD): age, 64 ± 3 yr; body mass, 85.5 ± 13.2 kg; height, 1.75 ± 0.07 m; body mass index, 27.8 ± 3.7 $kg \cdot m^{-2}$; fat free mass, 60.5 ± 7.4 kg; body fat percentage, 28.9 ± 4.6 %; forearm muscularity, 51 ± 7 cm^2. The distribution of ACE (II 31, ID 59, DD 33) and ACTN3 (RR 48, RX 59, XX 16) genotypes were in Hardy-Weinberg equilibrium. Physical characteristics were independent of ACE and ACTN3 genotypes (Table 4.1 and 4.2). Isometric grip strength did not differ between ACE and ACTN 3 genotypes (Figure 4.1).

Table 4.1 Physical characteristics and grip strength in relation to ACE genotype

	II ($n = 31$)	**ID** ($n = 59$)	**DD** ($n = 33$)	**P**
Anthropometry:				
Height (m)	1.74 ± 0.06	1.75 ± 0.07	1.77 ± 0.07	0.32
Body mass (kg)	85.7 ± 15	85.3 ± 13.2	85.8 ± 11.3	0.98
Fat free mass (kg)	60.4 ± 8.2	60 ± 7.6	61.4 ± 6.3	0.68
Body fat (%)	28.9 ± 4.4	29.2 ± 4.6	28.4 ± 4.9	0.71
Lean forearm CSA (cm^2)	51 ± 7	50 ± 6	53 ± 9	0.40
Isometric grip strength (kg)	45 ± 6	45 ± 6	44 ± 6	0.73

Table 4.2 Physical characteristics and grip strength in relation to ACTN3 genotype

	RR (n = 48)	RX (n = 59)	XX (n = 16)	P
Anthropometry:				
Height (m)	1.75 ± 0.06	1.76 ± 0.08	1.78 ± 0.06	0.25
Body mass (kg)	85 ± 12.4	85 ± 14	88 ± 12.7	0.55
Fat free mass (kg)	60.5 ± 6.9	60.3 ± 8	61 ± 7.1	0.96
Body fat (%)	28.5 ± 4.1	28.7 ± 4.9	31.0 ± 4.4	0.14
Lean forearm CSA (cm^2)	51 ± 7	51 ± 7	52 ± 11	0.75
Isometric grip strength (kg)	45 ± 5	44 ± 6	45 ± 8	0.88

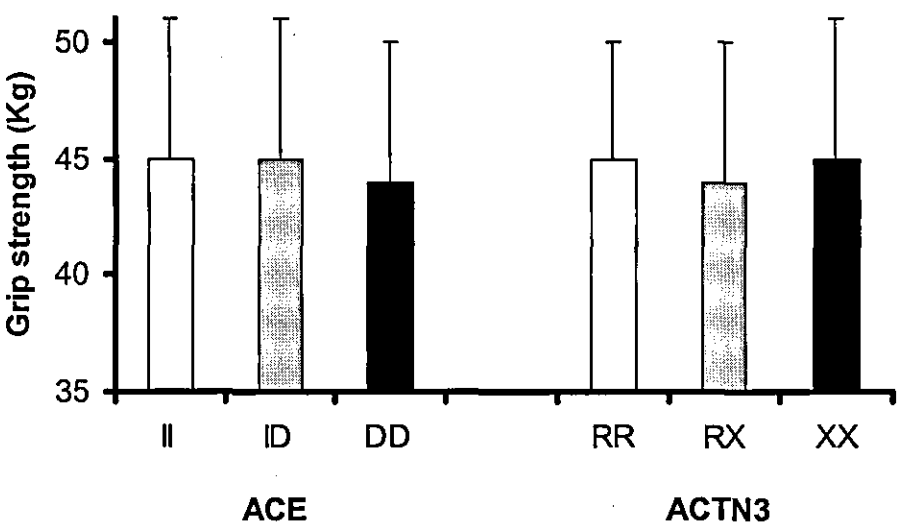

Figure 4.1 The association of ACE and ACTN3 genotype with isometric grip strength. There were no significant differences in strength for ACE (P = 0.73) or ACTN3 (P = 0.88) genotypes. Data are mean and SD (n = 123).

4.4 Discussion

The first aim of this study was to evaluate the feasibility of recruiting a substantial population of healthy older Caucasian men. With the inclusion of 123 suitable volunteers it was demonstrated that gene-phenotype association studies with this cohort were feasible with the available resources. The second aim of this study was to investigate the association of ACE I/D and ACTN3 R/X polymorphisms with muscle strength and indicators of muscularity in an older Caucasian male population. The two candidate gene polymorphisms considered were not associated with isometric grip strength, lean forearm CSA or whole body FFM.

We endeavored to avoid the potentially confounding effects of ethnic diversity and chronic physical training in order to highlight the association of genes with strength. Therefore we recruited UK Caucasian volunteers with moderate to low levels of physical activity, which likely explains the wide variation in body composition.

A high proportion of elite sprint athletes have been reported to have a DD genotype compared with endurance athletes and controls (Myerson *et al.*, 1999; Woods *et al.*, 2001; Nazarov *et al.*, 2001), indicating that this genotype may be associated with skeletal muscle function. The current study did not find an ACE genotype association with isometric grip strength. These findings are similar to previous investigations reporting no ACE genotype influence with strength in older individuals (Hopkinson *et al.*, 2004; Frederikson *et al.*, 2003a; Charbonneau *et al.*, 2008; Giaccaglia *et al.*, 2008) and young adults (Folland *et al.*, 2000; Thomis *et al.*, 2004; Pescatello *et al.*, 2006, chapter 3). Williams *et al.* (2005) is the only study to have reported a DD association with isometric strength, albeit in younger people.

As muscle size is a key determinant of muscle strength (Folland and Williams, 2007) the similar forearm muscularity of the three ACE genotypes is coherent with our finding of no ACE genotype association with strength. It is in contrast, however, with a recent report of an association of ACE genotype with the muscle volume of healthy older men and women (Charbonneau *et al.*, 2008). Furthermore in this study there was no

association of ACE or ACTN3 genotypes with any measure of body composition, although assessment of body composition using the sum of 4 skinfolds methods may lack validity. The sum of four skinfolds is considered a level III doubly indirect method of estimating body fat percentage, and thus also FFM, compared to a DXA scan which is categorised as a level II method (Eston and Reilly, 2001).

ACTN 3 genotype was also considered in the present investigation. It has been suggested that the sarcomeres within skeletal muscle fibres are inherently weaker when they lack ACTN3 and that this may be associated with the muscle function of free living humans (Chan *et al.*, 2008). The frequency of the RR genotype has been found to be higher amongst elite sprinters (Niemi and Majamaa 2005), strength (Roth *et al.*, 2008) and power athletes (Druzhevskaya *et al.*, 2008, Papadimitriou *et al.*, 2008) compared to endurance runners or controls. However, we found no association between ACTN3 genotype and muscle strength or muscularity. The lack of an association between ACTN3 genotype and isometric strength is similar to a number of other studies in young (Vincent *et al.*, 2007; chapter 3) and older men (Walsh *et al.*, 2008) but two studies have found lower strength in XX genotype women (Clarkson *et al.*, 2005, Walsh *et al.*, 2008), perhaps indicating a sex-genotype interaction. Given that ACTN3 is only present in type IIX fibres that are distinguished by their high velocity of shortening and hence capacity for producing power, functional tests at high velocities might be more likely to show an effect of this polymorphism (Clarkson *et al.*, 2005).

This study demonstrated the feasibility of recruiting a substantial population of healthy older Caucasian men for gene-muscle function phenotype investigation. In the present study, ACE I/D and ACTN3 R/X polymorphisms were not associated with muscular strength or muscularity. However, the muscle function and body composition measures included in this study were relatively simplistic measures taken on just one occasion, that were not as comprehensive or as thoroughly collected as those in first study (Chapter 3). Further studies investigating the association between these polymorphisms with more comprehensive measures of muscle function and body composition of older persons are required. Specifically, dynamic strength of the knee extensors.

CHAPTER 5

ACE I/D and ACTN3 R577X Polymorphisms, and the Muscle Function and Body Composition of Older Caucasian Men

5.1 Introduction

A reduction in physical function due to lower strength and power in old age can have a profound effect on a person's ability to live independently, as carrying out daily tasks such as rising from a chair, or climbing stairs become increasingly difficult. Poor physical function in old age leads to an increased risk of older adults developing disabilities (Roubenoff, 2000). Beyond the fifth decade of life strength tends to decrease at ~15% per decade (Larsson, 1979). A primary mechanism for the decrease in strength is sarcopenia - a progressive loss of muscle mass. Muscle power output and the ability to produce force quickly appears to decline more rapidly than strength (Metter *et al.*, 1997; Skelton *et al.*, 1994), which could be due to a preferential atrophy of type II muscle fibres (Lexell 1995). The evoked contractile (twitch) response of the quadriceps has been found ~10% slower in older compared to younger persons (Roos *et al.*, 1992) and this may reflect a slowing of the contractile properties that contributes to the decline in power.

Reductions in strength with age occur in both the upper limb and in the weight-bearing lower limb muscles (Izquirdo *et al.*, 1999), the latter are important for locomotion, posture and prevention of falls, and consequently quality of life and morbidity. The overall rate of loss of strength in the quadriceps and hamstrings has been reported to be 2 and 2.4% per year (Frontera *et al.*, 2000). Strength of the quadriceps is a predictor of dependency and survival (Rantanen *et al.*, 1999) therefore understanding the basis of dysfunction of this muscle group in old age has important clinical and social implications (Frontera *et al.*, 2000). The decline in upper body strength with age (hand grip strength decreases by ~3 % per year (Frontera *et al.*, 2000)) compromises the ability for manual tasks.

In young men, the genetic contribution to strength and power appears to be 30-80%, depending on the nature of the functional test employed (Thomis *et al.*, 1998; Calvo *et al.*, 2002). The heritability of lower extremity function (strength, power and walking speed) in older twins (>60 years) has been found to be 34-52% (Carmelli *et al.*, 2000;

Tiainen *et al.*, 2007). Finding the specific genetic variants responsible for this heritability in muscle function with ageing will enhance our understanding of the mechanisms regulating muscle weakness. Angiotensin converting enzyme (ACE) I/D and α actinin 3 R557X (ACTN3) polymorphisms have been suggested to have an association with the variations in skeletal muscle function.

A high proportion of the ACE DD genotype has been reported in elite sprint athletes compared with endurance athletes and controls (Myerson *et al.*, 1999; Woods *et al.*, 2001; Nazarov *et al.*, 2001), indicating that this genotype may be associated with skeletal muscle function. The D allele variant of the ACE gene is associated with higher concentrations of serum ACE activity (Rigat *et al.*, 1990). ACE catalyses the production of ANG II, which appears to enhance skeletal muscle hypertrophy (Gordon *et al.*, 2001; Westercamp and Gordon 2005) and degrades kinins (Brown *et al.*, 1998), which have growth inhibitory properties (Ishigai *et al.*, 1997). There is recent evidence that ACE genotype is associated with the muscle volume of healthy older men and women (Charbonneau *et al.*, 2008) a key determinant of muscle strength and power. The possibility that the DD genotype maybe associated with a greater proportion of fast twitch fibres (Zhang *et al.*, 2003) could explain any association of the ACE D allele with strength/power, particularly at high velocities, but this evidence remains equivocal (Akhmetov *et al.*, 2006). Several investigations have found no association of ACE genotype with isometric and dynamic strength in young and older adults (Folland *et al.*, 2000; Thomis *et al.*, 2004; Pescatello *et al.*, 2006; Woods *et al.*, 2001b). However the D allele has been associated with greater isometric strength in COPD patients (Hopkinson *et al.*, 2004) and isometric and isokinetic strength in healthy young men (Williams *et al.*, 2005). To date no studies have examined the association of ACE genotype with high velocity muscle strength and the contractile properties of older males.

The α actinin 3 (ACTN3) R/X polymorphism (Mills *et al.*, 2001) has also been identified as potentially associated with skeletal muscle composition, function and performance. ACTN3 expression is restricted to type IIX muscle fibres (Mills *et al.*, 2001) and the absence of ACTN3 could inhibit the performance of these fibres that are important for

rapid powerful contractions (MacArthur and North, 2004). Moreover, there is also recent evidence that ACTN3 genotype may be associated with fibre type composition (Vincent *et al.*, 2007). The frequency of the RR genotype has been found to be higher amongst elite sprinters (Niemi *et al.*, 2005; Yang *et al.*, 2003), strength (Roth *et al.*, 2008) and power athletes (Druzhevskaya *et al.*, 2008; Papadimitriou *et al.*, 2008) compared to endurance runners or controls, indicating a beneficial effect on skeletal muscle function and in particular the ability to generate powerful contractions at high velocity. The findings from cross-sectional association studies are more equivocal, however. We previously found ACTN3 genotype was not associated with the relative or absolute strength at high velocities, or the evoked contractile response, of young men (Chapter 3), but greater relative high velocity strength has been reported in young male R allele carriers (Vincent *et al.*, 2007). The mechanistic actions of ACTN3 deficiency are currently being explored, with muscle from ACTN3 knockout (KO) mice found to have a slower contractile response and lower force generating capacity than those with ACTN3 (Chan *et al.*, 2008). Furthermore, KO mice have a reduced fast fibre diameter, lean mass and total body mass (MacArthur *et al.*, 2008), which suggests a clear association of ACTN3 genotype with skeletal muscle growth and muscularity. ACTN3 has also been proposed as a candidate metabolically 'thrifty gene' (Neel, 1962) with the absence of ACTN3 hypothesised to improve energetic efficiency (MacArthur *et al.*, 2004) that could promote energy storage and body fat accumulation. Whilst there has been little support for this in young people (Vincent *et al.*, 2007; chapter 3) it is possible that an association may accrue over the lifespan.

Many previous reports have studied populations with a mixed ethnic origin (Pescatello *et al.*, 2006), wide age range (Clarkson *et al.*, 2005; Walsh *et al.*, 2008) or variable physical activity status (Woods *et al.*, 2001; Hopkinson *et al.*, 2004), which could confound and potentially explain some of the contrasting findings for both ACE and ACTN3 polymorphisms. For example skeletal muscle phenotypes are known to be influenced by ethnic origin (Newman *et al.*, 2003). Furthermore, the measurement of different muscle groups, e.g. upper vs lower body, which may be exposed to different levels of habitual loading and therefore more or less reflective of genetic factors could also account for the

equivocal findings. The vast majority of the previous studies have examined the association of only single gene polymorphisms with muscle function. Considering that the association of a single gene variant is probably quite small (Williams and Folland, 2008) any association of candidate genes should be investigated together in order to determine broader genetic effects on a given phenotype. Therefore studies of multiple candidate genes are required.

The aim of this study was to investigate the association of both ACE I/D and ACTN3 R/X polymorphisms with the body composition and muscle function of 60-70 year old Caucasian men with a low-moderate level of physical activity. Measurements included isometric strength of a range of upper and lower body muscle groups and assessment of non-skeletal lean mass and fat mass by Dual-energy X-ray Absorptiometry (DXA). In addition the evoked twitch response and isokinetic strength at a range of velocities were used to determine the contractile properties and dynamic strength of the knee extensors.

5.2 Methodology

5.2.1 *Protocol overview*

Participants visited the laboratory on three occasions. The first visit involved a range of muscle function measurements: hand grip, knee extensor and elbow flexor isometric strength of both limbs; the contractile properties of both knee extensors; and, isokinetic strength of the non dominant knee extensors at three velocities. On the second visit these functional measurements were repeated and a DXA scan assessed non-skeletal lean mass and fat mass. The third visit involved a fasted blood sample between 7.30 and 9.30 am. All functional measurements were performed between 9 am and 1 pm and repeated measurements were performed 1 week apart.

5.2.2 *Participants*

One hundred UK Caucasian males, aged 60-70 years, volunteered to participate in the study and provided written informed consent. Participants had no history of strength training, moderate-low overall physical activity (<4 sessions of activity per week), were free from locomotor or neuromuscular disease and conditions known to influence muscle function. Ethical approval for this study was provided by the Loughborough University ethical advisory committee. All tests were conducted in conformity with the principles of the declaration of Helsinki. Participants' height was measured with a wall-mounted stadiometer (Holtain Ltd, Crymych, Wales) and body mass with a beam balance scale (Avery Ltd, Fairmont, MN) whilst participants wore shorts and a T-shirt.

5.2.3 *Hand grip strength*

Isometric hand grip strength was assessed with a JAMAR dynamometer (Sammons Preston Rolyan, ILL). Participants were familiarised with the dynamometer and test procedure and were required to stand with feet shoulder width apart with their arm flexed at an elbow joint angle of 90°, with the shoulder abducted. Verbal encouragement was

given during the assessment. Participants performed three maximal voluntary contractions (MVCs) that were sustained for 3 seconds and with 30 seconds rest between each contraction. If the third MVC was the highest then a fourth contraction was performed. The coefficient of variation (CV) for isometric grip strength over repeated test occasions was 3.7%.

5.2.4 *Elbow flexor and knee extensor isometric strength, and the contractile properties of the knee extensors*

Elbow flexor isometric strength as well as voluntary and electrically evoked isometric contractions of the knee extensors were assessed using a conventional isometric strength testing chair (Parker *et al.*, 1990). Participants were seated in an upright position with the back supported and firmly secured with straps fastened at the hips and shoulders. Force was measured with a calibrated U-shaped aluminium strain gauge (Jones and Parker, 1989). The strain gauge signal was sampled at 2,000 Hz with an analogue to digital (A/D) converter (Micro 1401, Cambridge Electronic Design Ltd, Cambridge, UK) and logged and displayed by PC with Spike 2 software (Cambridge Electric Design Ltd, Cambridge, UK).

Participants performed a 5 min warm-up on a cycle ergometer (Monark 818e, Stockholm, Sweden) at ~50 W. For knee extensor measurements, knee and hip joint angles were 90° (1.57 rad) and 110° (1.41 rad), respectively (0 represents full extension). Their lower leg, ~2 cm above the ankle, was tightly strapped to a calibrated U-shaped aluminium strain gauge (Jones and Parker, 1989), that was always in the direct line of action of the applied force. Following some progressive sub-maximal contractions maximum isometric strength was determined by having participants perform 3-4 MVCs of the knee extensors, each of ~3 s duration and with 30 s rest in-between. The between sessions CV for knee extensor isometric strength was 2.6%.

Peak isometric elbow flexor strength was determined at a 90° (1.57 rad) elbow joint angle with the shoulder horizontally adducted, and the elbow resting on a rigid platform. A

wrist cuff was worn by participants with the strain gauge strapped to the participants' wrist. Following a series of sub-maximal contractions participants were instructed to perform three MVCs of the elbow flexors, each of ~3 s duration with 20 s between attempts. The isometric strength of each arm and leg was recorded as the single highest value from the three attempts. The between sessions CV for elbow flexor isometric strength was 3.9%.

The contractile properties of the knee extensors were measured using electrically stimulated contractions. The participants remained seated in the chair and 2 large (13 x 10 cm) flexible carbon rubber electrodes (Electro Medical Supplies Ltd, Wantage, UK), coated with electrode gel (Camcare Gels, Ely, UK) were placed over the anterior surface of the quadriceps. Percutaneous electrical stimulation with unidirectional square wave pulses of 50 μs duration was applied through a constant current Digitimer stimulator (DS 7AH, Welwyn, Garden City, UK). The stimulation current began at 5 mA and gradually increased until the twitch response reached a plateau of maximal twitch force. The twitch response was filtered by applying a digital filter with a low pass frequency of 30 Hz and a transition gap of 16 Hz. Semi automated cursors within the spike 2 software were used to identify discrete events within each twitch. Time to peak tension (TPT) was taken as the time between force onset and peak twitch force, with force onset defined as 3% of peak gradient. Half relaxation time (HRT) was the time for the muscle to relax from peak twitch force to half peak force. The peak rate of force development (Peak RFD) during the twitch was assessed as the maximum gradient of the force-time graph. Three twitches were analysed, the average values were recorded for each leg. The CV for TPT, HRT and RFD over repeated test occasions was 3.6, 4.7 and 9.7 % respectively.

5.2.5 *Torque-velocity relationship of the knee extensors*

Knee extensor torque of the non dominant leg was assessed at 3 velocities (0, 30 and 240°·s^{-1}). Participants were seated in a Cybex Norm Isokinetic Dynamometer (Lumex Inc, NY, USA) with the axis of the knee joint aligned with the centre of rotation of the dynamometer arm, and the lower leg strapped to the lever arm just proximal to the ankle. Participants were restrained at the waist, shoulders and the distal part of the thigh, and the

backrest was set at 100° (1.73 rad) from the base of the seat. Peak torque of the knee extensors was assessed isometrically at 73° (1.27 rad) of knee flexion (identified by pilot work as the angle of peak torque) and isokinetically at 30 and 240°·s^{-1} (0.52, 4.19 rad·s^{-1}). The relative torque at high velocity (ratio of strength at 240°·s^{-1} to 30°·s^{-1}) was calculated as a percentage. For isometric measurements there were 2 practice contractions at 50% effort followed by 3 MVCs. For the isokinetic measurements participants performed a set of practice trials of sub-maximal knee extension at each angular velocity, prior to 2 sets of 2 maximum contractions at 30°·s^{-1} and 2 sets of 3 maximum contractions at 240°·s^{-1}. There was at least 30 s rest between each set of contractions, and ~60 s between velocities. The range of motion for the isokinetic measurements was from 120° - 15° (2.09 - 0.26 rad) of knee flexion. Knee extensor torque values were corrected for gravitational torque, and contractions at the highest velocity 240°·s^{-1} (4.19 rad·s^{-1})) were typically isovelocity for at least the range 90° - 30° (1.57-0.52 rad). The CV for the torque measurements at 0, 30 and 240°·s^{-1} over repeated test occasions was 5.7, 7.8 and 8.3% respectively.

5.2.6 *Dual-energy X-ray Absorptiometry (DXA)*

On the 2nd visit a narrow angle fan beam DXA scan (Lunar Prodigy Advance DXA machine, GE Lunar, Madison, WI.) was performed whilst participants lay quietly on a couch fully clothed. The purpose of the whole body scan was to assess body composition, specifically non-skeletal lean mass, and fat mass. The non-skeletal lean mass component of body composition was the criterion measure of whole body muscularity. The scanner was calibrated daily, and an aluminium spine phantom was also scanned in order to confirm that there was no discernable drift during the course of the study.

5.2.7 *Blood collection and genotyping*

A trained phlebotomist performed venous puncture. Participants lay in a semi-supine position whilst a 10 ml blood sample was obtained from a superficial forearm vein following a 12 hour fast to avoid lipemic sera. Blood samples were collected in a serum tube and applied onto filter paper in preparation for genomic deoxyribonucleic acid (DNA) isolation. All blood samples were left at room temperature for 1-2 hours to allow blood to clot. The samples were then spun at 2300 revs·min^{-1} (rpm) for 10 minutes at 4°C in a refrigerated centrifuge (Beckman Instruments, Palo Alto, CA) and stored at -20°C enabling the determination of serum ACE activity on a separate occasion. Genomic DNA was obtained from previously bloodstained filter paper using Chelex extraction and genotypes for the polymorphisms of ACE I/D and ACTN3 R/X were determined as described previously (Chapter 3).

5.2.8 *Serum ACE activity*

Diagnostic determination of ACE activity in serum was carried out by an enzymatic assay using an automated spectrophotometer (Cobas Mira Plus, Roche, Switzerland). The enzyme mediates the cleavage of a synthetic substrate and the kinetics of this cleavage reaction are measured following the decrease in absorbance at 340nm. This was measured twice with the Bühlmann ACE kinetic kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland). The intra-assay CV for the serum ACE measurements was 4%.

5.2.9 *Statistical analysis*

A Chi squared test was used to confirm that the observed frequencies for ACE and ACTN3 genotypes were in Hardy–Weinberg Equilibrium. Data was confirmed as not normally distributed using SPSS for windows 12.0 (SPSS, Chicago, IL). A Kruskal-Wallis test (a non parametric version of ANOVA) was used to assess the differences in strength, contractile properties, body composition and serum ACE activity between ACE I/D and ACTN3 R/X genotypes. A Pearson's product moment correlation was used to examine the relationship between serum ACE activity and the other variables. Statistical

analyses were performed using SPSS v12.0 (SPSS, Chicago, IL) and Microsoft Excel 2000 (Microsoft, Redmond, WA). Data are presented as mean and standard deviation (SD) with statistical significance defined as $P < 0.05$.

5.3 Results

5.3.1 *Physical characteristics*

The participants physical characteristics were (mean ± SD): age, 65 ± 3 yr; body mass, 83.5 ± 11.4 kg; height, 1.76 ± 0.06 m; body mass index, 26.9 ± 3.3 $kg \cdot m^{-2}$; non-skeletal lean mass, 56.9 ± 5.5 kg; fat mass 22.9 ± 7.1 kg. The distribution of ACE (II 25, ID 48, DD 27) and ACTN3 (RR 43, RX 41, XX 16) genotypes were in Hardy-Weinberg equilibrium. Physical characteristics were independent of ACE and ACTN3 genotypes.

5.3.2 *ACE I/D polymorphism and serum ACE activity in relation to muscle phenotypes: strength, contractile properties and muscularity*

ACE genotypes had similar non-skeletal lean mass ($P = 0.41$; Table 5.1) and fat mass ($P = 0.22$; Table 5.1). No differences existed between ACE genotypes for knee extensor isokinetic torque at any velocity from 0–240° $\cdot s^{-1}$ ($0.27 < P < 0.67$; Figure 5.1A) or the relative torque at high velocity ($P = 0.60$; Table 5.1). The evoked twitch response was similar for the three ACE genotypes (TPT, $P = 0.79$; HRT, $P = 0.73$; Peak RFD, $P = 0.43$; Table 5.1). Isometric strength of the knee extensor ($P = 0.55$; Table 1), elbow flexor ($P = 0.60$; Table 5.1) and handgrip ($P = 0.38$; Table 5.1) muscles were independent of ACE genotype. Serum ACE activity for the whole group was 46.7 ± 20.2 U/L and this was influenced by ACE genotype (II 34.5 ± 14.6; ID 44.4 ± 17.7; DD 61.8 ± 20.4 U/L; $P < 0.001$).

Table 5.1 *Physical characteristics and muscle function of the different ACE genotypes. Data are mean ± SD*

	II ($n = 25$)	ID ($n = 48$)	DD ($n = 27$)	P
Anthropometry:				
Height (m)	1.74 ± 0.05	1.76 ± 0.06	1.77 ± 0.05	0.16
Body mass (kg)	80.9 ± 9.3	86.2 ± 12.2	81.3 ± 10.8	0.12
Lean mass* (kg)	55.7 ± 4.7	57.5 ± 5.2	56 ± 5.5	0.41
Fat mass (kg)	22.3 ± 5.8	24.3 ± 8.1	20.9 ± 5.9	0.22
Isometric Strength:				
Knee extensors (N)	474 ± 76	498 ± 84	487 ± 73	0.55
Elbow Flexors (N)	266 ± 34	272 ± 33	266 ± 31	0.60
Handgrip (kg)	44 ± 5	44 ± 5	43 ± 6	0.38
Relative Isokinetic Torque				
240:30°·s^{-1} (%)	55 ± 6	52 ± 7	53 ± 6	0.15
Contractile Properties:				
TPT (s)	71 ± 06	73 ± 09	71 ± 07	0.65
HRT (s)	94 ± 09	93 ± 13	95 ± 16	0.93
Peak RFD (N·s^{-1})	2075 ± 644	2150 ± 604	2011 ± 507	0.43

* *Non-skeletal lean mass*

Serum ACE activity was negatively correlated with the relative torque at high velocity (R = - 0.23, P = 0.03). There was a tendency for serum ACE activity to be correlated with quadriceps (R = 0.19; P = 0.07) and elbow flexor (R = 0.20; P = 0.06) isometric strength. Serum ACE activity was not correlated with any other functional, contractile or body composition measurement ($0.13<P<0.86$).

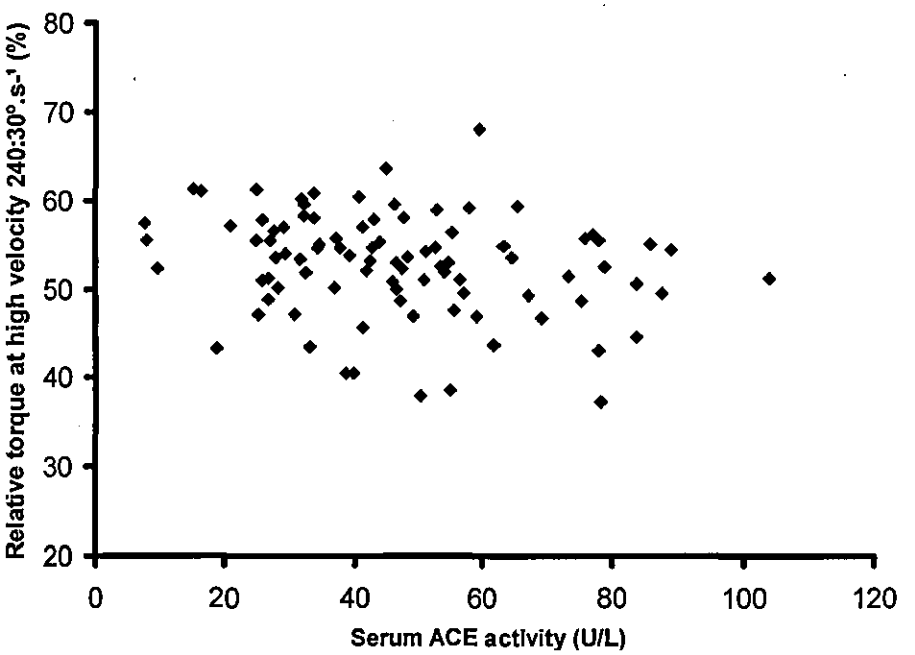

Figure 5.1 Relationship between serum ACE activity and relative torque at high velocities (r = - 0.23, P = 0.03, n=100).

5.3.3 *ACTN3 R577X polymorphism in relation to muscle phenotypes: strength, contractile properties and muscularity*

No differences existed between ACTN3 genotypes for non-skeletal lean mass (P = 0.72; Table 5.2). There was a significant difference in fat mass between genotypes (P = 0.04; Table 5.2; Mann Whitney corrected P = 0.045) with XX genotype individuals having higher fat mass than RR individuals. There was a tendency for differences in body mass (P = 0.09; Table 5.2) between ACTN3 genotypes. ACTN3 genotypes had similar knee extensor isokinetic torque at each velocity from 0–240°·s^{-1} ($0.53 < P < 0.99$; Figure 5.1B) and relative torque at high velocity (P = 0.44; Table 5.2). No significant differences

existed between ACTN3 genotypes for the evoked twitch response (TPT, $P = 0.89$; HRT, $P = 0.19$; Peak RFD $P = 0.39$; Table 5.2). Knee extensor isometric strength ($P = 0.77$; Table 5.2) elbow flexor strength ($P = 0.68$; Table 5.2) and handgrip strength ($P = 0.75$; Table 5.2) were independent of ACTN3 genotype.

Table 5.2 *Physical characteristics and muscle function of the different ACTN3 genotypes. Data are mean ± SD*

	RR ($n = 43$)	RX ($n = 41$)	XX ($n = 16$)	P
Anthropometry:				
Height (m)	1.75 ± .06	1.76 ± .06	1.79 ± .04	0.14
Body mass (kg)	80.8 ± 9.7	84.9 ± 12.6	87.3 ± 11.2	0.09
Lean mass* (kg)	56 ± 3.6	57.3 ± 6.3	58.3 ± 7.3	0.72
Fat mass (kg)	20.9 ± 5.9	23.7 ± 7.8	25.9 ± 6.7	0.04
Isometric Strength:				
Knee extensors (N)	483 ± 66	495 ± 86	477 ± 49	0.77
Elbow Flexors (N)	269 ± 32	270 ± 36	266 ± 26	0.68
Handgrip (kg)	43 ± 4	44 ± 6	45 ± 7	0.75
Relative Isokinetic Torque:				
240:30°·s^{-1} (%)	53 ± 6	52 ± 6	53 ± 6	0.65
Contractile Properties:				
TPT (ms)	72 ± 08	72 ± 09	72 ± 08	0.95
HRT (ms)	93 ± 14	97 ± 13	92 ± 15	0.26
Peak RFD (N·s^{-1})	2026 ± 555	2102 ± 641	2281 ± 499	0.39

* *Non-skeletal lean mass*

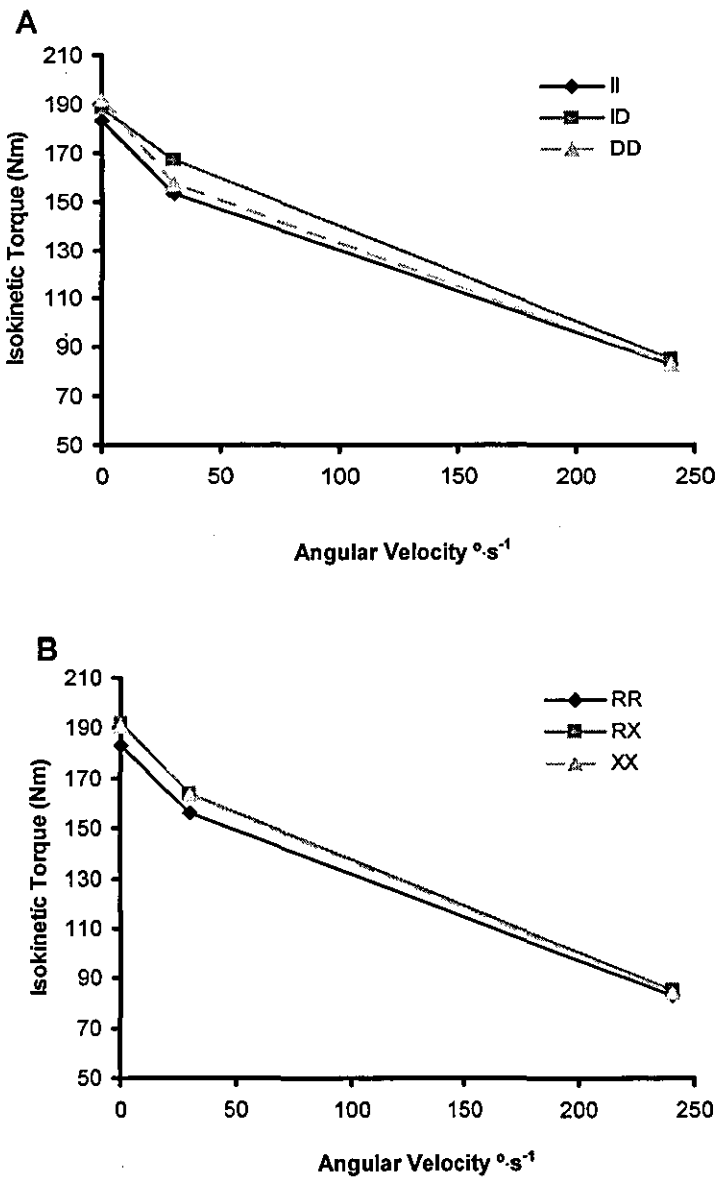

Figure 5.2 The association of ACE (A) and ACTN3 (B) genotypes with the knee extensor torque-velocity relationship. There were no significant differences in torque at any velocity for either genotype ($0.27<P<0.67$, $n=100$)

5.4 Discussion

The aim of this study was to evaluate the association of ACE I/D and ACTN3 R/X polymorphisms with phenotypes of muscle function and body composition in an ageing male population. Specifically it examined the association of these candidate gene variants with a range of muscle strength measurements, non-skeletal lean mass, fat mass, as well as the evoked contractile response (twitch) of the quadriceps. The ACE I/D and ACTN3 R/X polymorphisms were not associated with muscle strength at any velocity, the twitch response of the quadriceps or non-skeletal lean mass of an ageing male population. There was, however, an association of ACTN3 genotype with fat mass (greater fat mass of XX vs RR) in this cohort of older Caucasian men.

Every effort was made to ensure that the participants made genuinely maximal contractions and the reliability of measurements was high (CV: 2.6 – 8.3 %). We endeavored to avoid the potentially confounding effects of chronic physical training, age, gender or ethnic diversity in order to highlight the association of these two candidate genes. Therefore older UK Caucasian men with moderate to low levels of physical activity were recruited.

The lack of an ACE genotype association with isometric muscle strength, for a range of upper and lower body muscle groups, in this older population is similar to previous findings in older (Hopkinson *et al.*, 2004; Frederikson *et al.*, 2003) and young healthy adults (Folland *et al.*, 2000; Thomis *et al.*, 2004; Pescatello *et al.*, 2006). To date, only one study has reported a greater isometric strength of young adults with the DD genotype (Williams *et al.*, 2005). As muscle size is a key determinant of muscle strength (Folland and Williams, 2007) the similar non-skeletal lean mass, our criterion measure of muscularity, of the three ACE genotypes is coherent with our finding of no ACE genotype association with strength. Although another recent report found ACE genotype is associated with the muscle volume of healthy older men and women (Charbonneau *et*

al., 2008), our data suggests at most a very small association of ACE genotype with muscle mass and isometric strength with ageing.

Dynamic strength and power are critical aspects of muscle function, particularly in older age, that have greater validity for general mobility and locomotion than isometric strength and as such identifying genetic variations that are associated with dynamic strength and power are important. This study was the first to examine the association of ACE genotype with the torque-velocity relationship, particularly high velocity torque, and the time course of a twitch response, in an older population. We found no evidence that ACE genotype is associated with muscle phenotypes of dynamic strength and power or rapid contractile properties in 60-70 year old men. There are conflicting reports for the association of ACE genotype with skeletal muscle fibre type composition (Zhang *et al.*, 2003; Akhmetov *et al.*, 2006). Both relative torque at high velocity, the 240:30°·s^{-1} ratio, and the time course of the twitch response are thought to reflect fibre type composition (Gur *et al.*, 2003; Edwards *et al.*, 1997), but the similarity of our results for these functional indicators suggests no strong ACE genotype association with the fibre type composition of older Caucasian men. Our previous work also found no association of ACE genotype with these same measures of muscle function in young men (Chapter 3), although one previous study did find greater knee extensor torque in young DD genotype males at 1.05 rad·s^{-1} (Williams *et al.*, 2005).

It is of note that the majority of association studies which have found no ACE genotype association with muscle strength are in contrast with the case-control studies, that have reported the D allele to be overrepresented in elite athletic events requiring muscle power (Jones and Montgomery, 2002; Woods *et al.*, 2001a). It may be that the association of ACE genotype with muscle function is only apparent in the extreme phenotypes such as that of elite athletes and might indicate an interaction with powerful long-term environmental factors e.g. physical activity. If this were the case then extreme phenotypes of muscle weakness may also demonstrate an association with ACE genotype. Elderly individuals who are prone to falling are known to have a muscle function phenotype of unusually low strength and power (Perry *et al.*, 2007; Pijnappels *et*

al., 2008) and may also show an ACE genotype distribution that is different to that of controls.

In keeping with the established literature ACE genotype was associated with serum ACE activity (Rigat *et al.*, 1990). In contrast to ACE genotype, serum ACE activity was related to some functional phenotypes. Serum ACE activity was negatively correlated with the relative torque at high velocity i.e. as ACE activity increased the relative torque at high velocity decreased. Serum ACE catalyses the production of ANG II, which has been found to promote the preferential hypertrophy of slow twitch fibres (Gordon *et al.*, 2001), and this could explain a steeper decline in strength as velocity increases. The positive relationships between serum ACE activity with quadriceps isometric and elbow flexor strength also approached significance, and indicates a weak association of serum ACE activity (coefficients of determination 4 %) with these aspects of muscle function. These tendencies for a relationship between serum ACE and isometric strength are in contrast to our findings of no association in young males (Chapter 3), but similar to the findings of Williams *et al.* (2005) who also found serum ACE to correlate with the isometric and isokinetic knee extensor strength of young men. The contrasting findings for ACE genotype and serum ACE may be due to the latter being a continuous variable, which is more powerful for seeking an association than a categorical variable. This supports the use of ACE activity measures as well as genotype in the conduct of future studies. However the use of multiple testing such as that of serum ACE activity with the number variables measured may increase the likelihood of a type I error.

ACTN3 genotype was not associated with absolute or relative torque at high velocity, the time course of the twitch response or in fact any measure of muscle function. These findings are in agreement with previous studies of absolute dynamic strength in young men (Chapter 3, Walsh *et al.*, 2008) and older men and women (Delmonico *et al.*, 2008). Taken together these studies are contrary to the proposed role of ACTN3 in fast twitch fibres and its suggested advantageous effect on skeletal muscle function, particularly the ability to generate powerful contractions at high velocity (Yang *et al.*, 2003). In contrast, Vincent *et al.* (2007) reported greater relative high velocity torque and type IIX fibre

composition of RR compared to XX genotype men. In support of this possibility, MacArthur *et al.* (2008) found that muscle from ACTN3 knockout mice displayed a slower contractile response suggesting that ACTN3 deficiency results in fast twitch fibres developing more oxidative properties that slower twitch fibres possess (Chan *et al.*, 2008). However, in older free living men we found no evidence of an ACTN3 association with the contractile properties, or absolute or relative dynamic strength of the knee extensors. It is increasingly recognised that individual genes are likely to have only a small association with physical function (Williams and Folland, 2008) and this may help to explain the conflicting reports from single gene association studies.

The lack of an association between ACTN3 genotype and isometric strength is similar to a number of other studies in young (Vincent *et al.*, 2007; chapter 3) and older men (Walsh *et al.*, 2008) but two studies have found lower strength in XX genotype women (Clarkson *et al.*, 2005; Walsh *et al.*, 2008), perhaps indicating a sex-genotype interaction.

ACTN3 has been proposed as a candidate metabolically 'thrifty' genotype (Neel, 1962). In this cohort of older men XX genotype individuals displayed higher fat mass compared to RR genotype individuals, and there was a tendency for higher body mass in XX individuals. Thus our data supports the hypothesis that ACTN3 is associated with the body composition and adiposity of older men. The absence of ACTN3 from muscle fibres has been hypothesised to promote a more efficient use of energy. MacArthur *et al.* (2008) found greater activity of a range of mitochondrial aerobic enzymes, including components of the citric acid cycle, electron transport chain and β-oxidation, in ACTN3 KO mice, that overall represented a clear shift towards the metabolic properties of type I fibres. As type I fibres are known to be more efficient than type II fibres (Coyle *et al.*, 1992; Mogensen *et al.*, 2006), such a metabolic shift would be expected to reduce energy use and increase energy storage and provides a likely explanation for the greater adiposity of older XX genotype men. Studies of young people employing arguably less sensitive methods of body composition assessment (skinfolds (Chapter 3) and bioelectrical impedance (Vincent *et al.*, 2007) have not reported an ACTN3 genotype association with adiposity. However, a subtle genetic association with energy balance

may only become apparent over a prolonged period of several decades. A greater contrast to our findings is a recent study that employed DXA and found no ACTN3 association with the body composition of men aged 22-90 yr of unspecified ethnicity and physical activity status (Walsh *et al.*, 2008). The wide age range, ethnicity and physical activity level of participants in their study may have confounded their ability to detect the ACTN3 genotype association with fat mass that we have found. Therefore over more than six decades of the life span of healthy free living men ACTN3 genotype appears to be associated with energy balance and the accumulation of body fat. This difference in adiposity has clear implications for the cardiovascular and metabolic health of older Caucasian men. Given the widely documented influence of body fatness on overall health, morbidity and mortality (Zhang *et al.*, 2008) this is an important finding that requires further research.

To conclude, ACE I/D and ACTN3 R/X polymorphisms were not associated with whole body muscularity or muscle function phenotypes including isometric strength, strength at high velocities or the time course of the twitch response. Any association of these individual polymorphisms was not of sufficient magnitude to effect muscle function in an ageing male Caucasian population. Serum ACE activity may have a small association with the isometric and dynamic strength of older men. However, ACTN3 genotype was associated with increased fat mass in XX individuals, that suggests this polymorphism may be associated with the accumulation of body fat over the life span of older men.

CHAPTER 6

The Distribution of ACE I/D and ACTN3 R577X Genotypes Amongst Frequent Fallers and Healthy Controls.

6.1 Introduction

Falls are a major health issue for the ageing population. It is estimated that 30-50% of people over the age of 65 years of age experience at least one fall per year (Campbell *et al.*, 1981), and about half of these will suffer recurrent falls (Masud and Morris, 2001). Falls frequently result in trauma, musculo-skeletal injury and disability. Approximately 20% of falls require medical attention; 15% results in joint dislocations, soft tissue bruises, and concussions (Kannus *et al.*, 1999). With ageing the skeleton becomes progressively weaker therefore, falling when bones are in a weakened state increases the likelihood of a fracture with 5% of falls resulting in fracture (Kannus *et al.*, 1999). As well as a high financial burden and morbidity falls typically contribute to decreased quality of life (Robbins *et al.*, 1989) and increased fear of falling, that leads to a reduction in activity and can cause a downward spiral of inactivity-related disability (Simey *et al.*, 1999).

Numerous epidemiological studies have identified a multitude of risk factors for falling (Lord and Dayhew, 2001). Biological ageing results in an unavoidable decrease in maximal and explosive force production capacity (Skelton *et al.*, 1994), specifically strength and power of the lower limb muscles which is the primary risk factor for falling (American Geriatrics Society, 2001). Beyond the fifth decade of life strength tends to decrease at ~15% per decade (Larsson *et al.*, 1979). A primary mechanism for the decrease in strength is sarcopenia - a progressive loss of muscle mass. Muscle power output and the ability to produce force quickly also declines with age and appears to decline more rapidly than strength (Metter *et al.*, 1997; Skelton *et al.*, 1994) which could be due to a preferential atrophy of type II muscle fibres (Lexell, 1995). Critically the reductions in strength with age are most pronounced in the weight-bearing lower limb muscles (Izquierdo *et al.*, 1999). The overall rate of loss of strength in the quadriceps and hamstrings has been reported to be 2 and 2.4% per year (Frontera *et al.*, 2000). The continual decline in muscle strength and power restricts locomotion and contributes to a loss of mobility and increased risk of falls. Individuals who fall repeatedly (frequent

fallers) have lower muscle function (strength and power) than non-fallers (Perry *et al.*, 2007; Pijnappels *et al.*, 2008). Perry *et al.* (2007) reported a falls group had 85% of the strength and 79% of the power of non-fallers measured by isometric, concentric and eccentric strength of the knee and ankle muscles and leg extension power. Thus frequent fallers may represent an extreme phenotype of muscle weakness that is partially due to inherited genetic factors.

Muscle function is influenced by inherited genetic factors as well as environmental variables (activity, nutrition etc). In young men, the genetic contribution to muscle strength and power appears to be 30-80%, depending on the nature of the functional test employed (Thomis *et al.*, 1998; Calvo *et al.*, 2002). The heritability of lower limb function (strength power and walking speed) in older twins (>60 years) has been found to be 34-52% (Carmelli *et al.*, 2000; Tiainen *et al.*, 2007). ACE and ACTN3 genotypes have been suggested to account for the influence of inheritance upon muscular phenotypes of older adults (Charbonneau *et al.*, 2008; Walsh *et al.*, 2008).

The possibility that the DD genotype, of the ACE gene, maybe associated with a greater proportion of fast twitch fibres (Zhang *et al.*, 2003) could explain any association of the ACE D allele with strength/power but this evidence remains equivocal (Akhmetov *et al.*, 2006). Case controlled studies have found good evidence that the ACE DD genotype and ACTN3 RR genotype are over represented in elite sprint and power athletes than controls (Myerson *et al.*, 1999; Woods *et al.*, 2001; Nazarov *et al.*, 2001; Niemi and Majamaa, 2005; Yang *et al.*, 2003; Druzhevskaya *et al.*, 2008; Papadimitriou *et al.*, 2008), indicating that these genotypes may be associated with muscle function.

The findings from cross-sectional association studies of these candidate genes are more equivocal, however, than the case-control studies with elite athletic groups. Several investigations have found no association of ACE genotype with isometric strength in young (Folland *et al.*, 2000; Thomis *et al.*, 2004; Woods *et al.*, 2001; Chapter 3) and older adults (Frederikson *et al.*, 2003b; Chapter 4, Chapter 5). Although the D allele has been associated with the muscle volume of healthy older men and women (Charbonneau

et al., 2008) a key determinant of strength and power. Other functional studies have found the D allele to be associated with greater isometric strength in COPD patients (Hopkinson *et al.*, 2004) and in healthy young men (Williams *et al.*, 2005). Two studies reported an association between ACTN3 R/X genotype and muscle phenotypes in women (lower elbow flexor (Clarkson *et al.*, 2005) and knee extensor (Walsh *et al.*, 2008) strength (for the XX genotype), but not in men. Additionally we previously found ACTN3 genotype was not associated with isometric strength in men (Chapter 3; Chapter 4; Chapter 5).

Due to the possible ACE and ACTN3 associations with muscle function it is plausible that these genotypes could be associated with susceptibility to falls. However, the association between these genes and the incidence of falls has not been examined. The aim of this study was to determine if the frequency of ACE and ACTN3 genotypes was different in frequent fallers compared to a reference population. This work may help to clarify the role of these polymorphisms in falls risk.

6.2 Methodology

6.2.1 *Participants*

Seventy one UK Caucasian males (27) and females (44) (82 ± 7 years), volunteered to participate in this study and provided written informed consent. Participants were patients referred to the falls clinic and had at least 2 falls in the previous 12 months. Participants were excluded if they suffered from fainting or blackouts (syncope), lacked mental alertness (scoring less than 70% on the Mini Mental State Examination MMSE), or had difficulty with communication and understanding. Participants were free from diseases of the muscles or nervous system (e.g. myositis, muscular dystrophy, myopathy, Parkinson's, stroke). The Derbyshire NHS Research Ethics Committee and Loughborough University ethical advisory committee provided ethical approval for this study. Control group genotype frequencies were obtained from the populations studied in Chapter 3 and Chapter 5. The falls group consisted of male and females, with their genotype frequencies compared to the control group of males only.

6.2.2 *Measurements*

Participants were questioned on falls history to ensure that they were frequent fallers. i.e. who had experienced a fall at least 2 times in the previous 12 months. Participants' height was measured with a portable stadiometer (Seca 214, UK) and body mass was recorded fully clothed with sit on scales (Seca 954, UK). Isometric hand grip strength was assessed with a JAMAR dynamometer (Sammons Preston Rolyan, ILL). Participants were familiarised with the dynamometer and test procedure and were required to sit with their arm flexed at an elbow joint angle of 90°, with the shoulder abducted. Verbal encouragement was given during the assessment. Participants performed three maximal voluntary contractions (MVCs) with each hand, and these were sustained for 3 s with 30 s rest between contractions. If the third MVC was the highest then a fourth contraction was performed.

6.2.3 *Blood collection and genotyping*

A trained phlebotomist performed venous puncture. Participants sat upright whilst a 5 ml blood sample was obtained from a superficial forearm vein. Blood samples were collected in an EDTA monovette and frozen in preparation for genomic deoxyribonucleic acid (DNA) isolation.

Genomic DNA was obtained from whole blood using the Nucleon kit (Nucleon BACC3 kit, Amersham Biosciences Ltd, UK) and genotypes for the polymorphisms of ACE I/D and ACTN3 R/X were determined. ACE I/D polymorphism was typed using polymerase chain reaction (PCR) based amplification. Extracted DNA was diluted to approximately 50ng and used for PCR amplification. The final reaction mixture of 15 μL for ACE contained: ACE Forward and Reverse Primers at 0.3 μL each, 2 μL of extracted DNA, 7.5 μL 2xReddy mix (ABgene) which contained 1.25 units Thermoprime Plus DNA Polymerase, 75 mM Tris-HCl, 20 mM$(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.01 % (v/v) Tween® 20 and 0.2 mM each of dATP, dCTP, dGTP and dTTP and precipitant and red dye for electrophoresis and 4.9 μL H_2O. The PCR reaction conditions protocol (Table 3.1) was programmed onto and run on a Mastercycler PCR machine (Eppendorf, Hamburg, Germany). Mistyping of ID heterozygote has been found in some studies to occur due to preferential amplification of the D allele, and inefficiency in the amplification of the I allele. To ensure confidence in genotyping 8 samples were randomly chosen and retyped all of which give the same score. Additionally to increase the specificity of DD genotyping, PCR amplifications were also performed with an I-specific primer pair (5'TGGGACCACAGCGCGCCCGCCACTAC3'and
5'TCGCCAGCCCTCCCATGCCCATAA3') in all the samples that were found to be DD after amplification with the flanking primers. Briefly I-specific amplification was performed using 25 μL reaction with 1 min denaturation at 94°C, followed by 30 cycles of 30s at 94°C, 45s at 67°C (annealing), and 2 min at 72 °C (extension). The 335bp fragment was identified on 2% agarose gel containing sybr safe (SYBR® Safe DNA gel stain, Invitrogen, Molecular Probes, USA). Under these conditions only the I allele produces a 335bp amplicon. The reaction yielded no products in a sample of all the DD genotypes.

ACTN 3 polymorphism was typed using PCR. The final reaction mixture of 20 μL for ACTN 3 contained: ACTN3 Forward and Reverse Primers at 1 μL each, 10 μL of 2x Reddy Mix, (5 mM $MgCl_2$) (ABgene), 2 μL of extracted DNA and 6 μL H_2O. The PCR reaction conditions protocol (Table 3.1) was programmed onto and run on Mastercycler PCR machine (Eppendorf, Hamburg, Germany) (Table 3.1).

PCR product (15 μL) for ACE was electrophoresed at 120 V for 45 minutes through a 2% agarose gel containing ~0.5 μg/ml sybr safe in TBA, ethylenediaminetetraacetic acid (EDTA) electrophoresis buffer pH 8.3 (TBE). A 100bp ladder (Abgene) was used to identify 190 and 490bp fragments for the ACE I/D polymorphism. A UV transilluminator (UVP, Cambridge, UK) was then used to visualize and score DNA fragments.

Following genomic DNA amplification, ACTN3 PCR product was digested using *Dde*I restriction enzyme (New England, Biolabs Inc., Ipswich, MA) at 37°C for 4 h (Mastercycle, Eppendorf). The reaction mixture of 20 μl contained: 0.4 μL Enzyme *Dde*I, 2 μL of 10x NEBuffer 4 (pH 7.9 at 25°C) constituting of 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1mM dithothreitol, 0.2 μL 100x bovine serum albumin (BSA), 7.6 μl H_2O and 10 μl ACTN3 PCR product. The recognition site for the *Dde*I restriction enzyme was CTNAG (5'-3'). To ensure confidence in genotyping 8 samples were randomly chosen and retyped all of which give the same score.

PCR product (15 μL) for ACTN3 was then electrophoresed at 120 V for 90 minutes through a 3% agarose gel containing ~0.5 μg/ml syber safe (SYBR® Safe DNA gel stain, Invitrogen, Molecular Probes, USA), ethylenediaminetetraacetic acid (EDTA) electrophoresis buffer pH 8.3 (TBE). A 100bp ladder (Abgene) was used to identify 86, 97, 108 and 205bp fragments distinctive of the ACTN3 polymorphism. A UV transilluminator (UVP, Cambridge, UK) was then used to visualize and score DNA fragments.

6.2.4 *Statistical analysis*

A Chi squared test was used to confirm that the observed frequencies for ACE and ACTN3 genotypes were in Hardy–Weinberg Equilibrium. Statistical analyses were performed using SPSS v12.0 (SPSS, Chicago, IL) and Microsoft Excel 2000 (Microsoft, Redmond, WA). Data was confirmed as normally distributed by the skewness and kurtosis using SPSS. A contingency chi-square was used to compare genotype frequency between population groups i.e. falls group vs. control group; male falls group vs. control group and female falls group vs. control group. ANOVA was used to assess the differences in strength and body mass between ACE I/D and ACTN3 R/X genotypes. Data are presented as mean and standard deviation (SD) with statistical significance defined as $P < 0.05$.

6.3 Results

The participants physical characteristics were (mean ± SD): age, 82 ± 7 yr; body mass, 70.7 ± 17.3 kg; height, 1.61 ± 0.10 m; body mass index, 26.4 ± 7.5 $kg \cdot m^{-2}$. The distribution of ACE (II 19, ID 33, DD 17) and ACTN3 (RR 25, RX 35, XX 11) genotypes were in Hardy-Weinberg equilibrium. The number of falls experienced by participants was 5 in the previous 12 months (range 2-20).

No differences were observed between the falls group and control group for ACE (Table 6.1) and ACTN3 (Table 6.1) genotype distribution (Table 6.2).

Table 6.1 Falls group (ACE *n*=69, ACTN3 *n*=71) vs Control group (*n*=179)

	ACE		ACTN3	
	χ^2	P	χ^2	P
Falls group				
vs	0.53	0.77	0.76	0.68
Control group				

Table 6.2 Percentage of ACE and ACTN3 genotypes in falls group vs control group

		ACE			ACTN3		
		II	**ID**	**DD**	**RR**	**RX**	**XX**
Falls group	***n***	19	33	17	25	35	11
	(%)	(27.5)	(47.8)	(24.7)	(35.1)	(49.3)	(15.5)
Control group	***n***	42	88	49	70	78	31
	(%)	(23.4)	(49.2)	(27.4)	(39.1)	(43.6)	(17.3)

Physical characteristics were independent of ACE and ACTN3 genotypes (Table 3). No differences were observed between ACE genotypes for isometric hand grip strength (P = 0.45). No differences were observed between ACTN3 genotypes for isometric hand grip strength (P = 0.64) (Table 3).

Table 6.3 ACE I/D and ACTN3 R577X polymorphism in relation to physical characteristics and hand grip strength

Falls group n = 69	**ACE genotype**			
	II (n = 19)	**ID (n = 33)**	**DD (n = 17)**	**P**
Height (m)	1.62 ± 0.09	1.61 ± 0.12	1.58 ± 0.08	0.51
Body mass (kg)	68.2 ± 17.2	71.8 ± 18	73 ± 17.1	0.69
BMI (kg·m^{-2})	24.4 ± 8.4	26.7 ± 7.3	29.4 ± 6.5	0.18
Handgrip strength (kg)	12 ± 10	13 ± 11	9 ± 7	0.45
Falls group n = 71	**ACTN3 genotype**			
	RR (n = 25)	**RX (n = 35)**	**XX (n = 11)**	**P**
Height (m)	1.62 ± 0.12	1.62 ± 0.09	1.56 ± 0.09	0.22
Body mass (kg)	69 ± 19.4	71.8 ± 15.5	69.7 ± 19.2	0.87
BMI (kg·m^{-2})	25.2 ± 10.5	26.5 ± 7.6	27.0 ± 6.2	0.82
Handgrip strength (kg)	13 ± 10	11 ± 9	11 ± 10	0.64

No differences were observed between genotype frequencies and distributions when the falls group were separated into male and females and compared to the control group ($0.36<P<0.54$). (Appendix 1).

6.4 Discussion

The aim of this study was to determine if the genetic frequency of specific polymorphisms, which have been linked to muscle function, was different in frequent fallers compared to a reference control population. No differences between groups were observed.

This study endeavoured to avoid the potentially confounding effects of ethnic diversity by recruiting UK Caucasian volunteers. Patients were excluded if they suffered from fainting or blackouts (syncope), or from diseases of the muscular or nervous system (e.g. myositis, muscular dystrophy, myopathy, Parkinson's, stroke) in order to highlight the association of genes with muscle function and their predisposition to falls. The present study was found to be underpowered to detect a significant genotypic effect. Power and sample size calculations indicated that detection of an odds ratio of 1.5 at a 5% significance level and 80% power, required larger frequent fallers and control groups (ACE, 194; ACTN3, 173) than was the case for the current study. The odds ratio of 1.5 indicates an effect size of 50% higher risk in the falls group as demonstrated by QUANTO (http://hydra.usc.edu/gxe).

The control group genotype frequencies for this study were obtained from the cohorts included in Chapters 3 and 5. The cohorts were not similarly aged matched and selective survival may have influenced genotype frequencies which suggests the use of a control group with a similar age. The falls group consisted of male and females, their genotype frequencies were compared to the control group of males only. It is common for male and female data to be considered together in genotype studies; various reports on ACE and ACTN3 genotype-muscle function phenotype association studies have included mixed sex cohorts (Moran *et al.*, 2007; Delmonico *et al.*, 2008). Additionally the distribution of ACE and ACTN3 genotypes are similar for males and females with no difference being reported between groups in previous studies (Giaccaglia *et al.*, 2008; Clarkson *et al.*, 2005). ACE and ACTN3 genotypes were in HWE equilibrium for all groups which gives confidence that there was no selection bias.

It is important to identify individuals most at risk of falling, as they should be prioritised for receiving targeted interventions aimed at reducing the incidence of falls (Pijnappels *et al.*, 2008). Considering the impact of falls on the prevalence of health problems (Lord and Dayhew, 2001), health care costs (Stevens *et al.*, 2006) and to quality of life it is imperative to further investigate factors contributing to fall risk. Reduced muscle strength and power have been identified as one of the most important risk factors for falls (Lord *et al.*, 2003; Skelton *et al.*, 2002) and since the genetic contribution to muscle strength and power appears to be 30-80% it is plausible that the genetic pattern of specific genes, that have been linked to muscle function, could be different in frequent fallers compared to a reference population and help to highlight individuals most at risk. However the present study found no difference in ACE and ACTN3 genotype distributions in frequent fallers.

Fall risk however, is known to be multifactorial (Carter *et al.*, 2001). Aside from age related changes in muscle, predisposition to falls includes age related deterioration of the 3 sensory systems that control posture; the vestibular, visual and somatosensory systems. In the otolith of the ear individuals over 70years of age have 40% fewer sensory cells than young adults (Rosenhall *et al.*, 1973). Cutaneous vibratory sensation and joint position sense are also diminished in older persons (Skinner *et al.*, 1984). Peripheral vision is known to be important in sway stabilisation (Paulus *et al.*, 1984) and low frequency spatial information, mediated by the peripheral visual field, deteriorates with age (Sekuler and Hutman, 1980). An age-related deterioration in these systems is thought to decrease balance and increase postural sway, increasing the possibility of a fall. Furthermore medication use such as sedatives and antidepressants are also regarded as important contributing factors increasing the risk of falls (Carter *et al.*, 2001). Therefore, although a decline in muscle strength is a contributing factor and there is a clear rationale that genetic variants which are associated with muscle function (possibly including ACE and ACTN3) will also affect falls risk this may not become apparent due to the array of factors determining falls risk.

The lack of an ACE genotype association with muscle strength of hand grip was similar to our previous findings for isometric strength of young and older groups (Chapters 3, 4 and 5) and a number of other studies (Thomis *et al.*, 2004; Pescatello *et al.*, 2006; Hopkinson *et al.*, 2004; Frederikson *et al.*, 2003). Williams *et al.* (2005) are the only study to have demonstrated a DD association with isometric strength albeit in younger people.

The lack of an association between ACTN3 genotype and isometric handgrip strength is similar to a number of other studies in young (Vincent *et al.*, 2007; Clarkson *et al.*, 2005; chapter 3) and older men (Chapter 5; Walsh *et al.*, 2008). However two studies have found lower strength in XX genotype women (Clarkson *et al.*, 2005; Walsh *et al.*, 2008), indicating a possible sex genotype interaction. Contrastingly no genotype differences were observed in females in the current study although the small cohort number may likely explain the lack of an observed effect. The association of ACTN3 genotype with anthropometric measurements was not observed which is in contrast to our previous study for a trend of higher body mass in XX individuals compared to RR genotype individuals. Again the small cohort may help explain this contrast in findings. Additionally participants were measured in a hospital day clinic and were measured while fully clothed and therefore exact body mass was not obtained.

It is difficult to draw firm conclusions at present, but this initial evidence suggests that the distribution of ACE and ACTN3 genotypes do not differ between frequent fallers and a healthy control population.

CHAPTER 7

GENERAL DISCUSSION

7.1 Associations of ACE I/D genotype and serum ACE activity with muscle function

The aim of this research was to investigate the association of two candidate gene (ACE I/D and ACTN 3 R/X) polymorphisms with the muscle function of young and older men, and the distribution of these genotypes in frequent fallers compared to controls. Particularly novel aspects of this work were the investigation of any association of these gene variants with strength at high velocities and the time course of an evoked twitch. This series of studies revealed no ACE genotype association with any measure of muscle function, contractile properties or body composition in these populations. Serum ACE activity appears to be weakly associated with knee extensor and elbow flexor isometric strength in older men, and negatively correlated with the relative torque at high velocity. ACTN3 genotype was associated with fat mass in older men but was not associated with any measure of muscle function, contractile properties or muscularity. Finally there was no apparent difference in the distribution of ACE I/D and ACTN3 R/X genotypes between frequent fallers and controls.

The lack of an ACE genotype association with muscle strength and contractile properties across three studies provides confidence that ACE has at most a small association with the muscle function of healthy UK Caucasian men. This concurs with the majority of studies in this field and in particular with the research on large cohorts that has been published during the course of the current research (Pescatello *et al.*, 2006; Moran *et al.*, 2006; Charbonneau *et al.*, 2008).

Chapters 3 and 5 both showed that ACE genotype was associated with serum ACE activity (young and older DD individuals displayed higher serum ACE activity) which is in keeping with the established literature (Rigat *et al.*, 1990; Danser *et al.*, 1995). Serum ACE activity was not associated with any muscle function measurement of young men, but was associated with the knee extensor and elbow flexor isometric strength of older men. This indicates a weak association of serum ACE activity with these aspects of

muscle function with ageing (coefficients of determination 4 %). The only other study to have examined this issue found positive correlations between serum ACE activity and strength, but in young men (Williams *et al.*, 2005), which makes it difficult to draw firm conclusions at this point. The contrasting findings of Chapters 3 and 5 with regard to serum ACE activity could have been due to the superior reliability of the muscle function data collected for Chapter 5. The between session CV for the range of muscle function measurements was lower for Chapter 5 (2.6 – 8.3%) than Chapter 3 (3.3 – 13.8%). Although, the fact that the variability of knee extensor isometric strength measurements was similar negates this possibility (Chapter 3, CV 3.2%, Chapter 5, CV 3.4 %). Furthermore the larger cohort number in Chapter 5 (n=100) compared to the smaller number of Chapter 3 (n= 79) and of Williams *et al.* (2005) (n=81) may partly explain the contrast in finding with regard to serum ACE activity. The results do however support the use of ACE activity measures as well as genotype in the conduct of future studies. This may be due to the former being a continuous variable, which is more powerful for seeking an association than a categorical variable (genotype).

The relative torque at high velocity was negatively correlated with serum ACE activity in older, but not young men. Serum ACE catalyses the production of ANG II, which has been found to promote the preferential hypertrophy of slow twitch fibres (Gordon *et al.*, 2001), and this could explain a steeper decline in strength as velocity increases. It is possible that this effect accrues over the lifespan.

Chapters 3, 4 and 5 consistently found no ACE genotype relationship with muscularity, fat free mass and body composition. As muscle size is a key determinant of muscle strength (Folland and Williams, 2007) the similar muscularity of the three ACE genotypes is coherent with the findings of no ACE genotype association with strength. It is in contrast however with a recent report for an association of ACE genotype with the muscle volume of healthy older men and women (Charbonneau *et al.*, 2008).

7.2 Associations of ACTN R/X genotype with muscle function and body composition

The findings of this thesis revealed no association of ACTN3 genotype with any measures of muscle function, which provides confidence that ACTN3 has at most a small association with the muscle function of healthy UK Caucasian men. This is supported by a number of other recent studies in men (Clarkson *et al* 2005; Delmonico *et al* 2007; Delmonico *et al* 2008), although there are conflicting findings in women (Clarkson *et al* 2005; Walsh *et al.*, 2008) that could indicate a sex-genotype interaction.

As Vincent *et al.* (2007) did find an association of ACTN3 genotype with relative high velocity torque at $300°\cdot s^{-1}$, it is possible that the measurement velocities in this thesis were insufficient to detect any functional association of ACTN3 genotype. However their functional measurements were coincident with an ACTN3 genotype difference in fibre type composition, which have since been questioned (Norman *et al.*, 2009). The evidence from this work is that any association of ACTN3 genotype on muscle composition or contraction does not appear to be of sufficient magnitude to influence the contractile or functional properties of human muscle. Furthermore these findings are in stark contrast to the proposed role of ACTN3 in type IIX fibres (MacArthur and North, 2007).

There is a clear contrast across the 3 studies on the association of ACTN3 genotype with fat mass. The young men from Chapter 3 and the older men from Chapter 4 displayed no apparent differences in fat mass between ACTN3 genotypes. However in Chapter 5 XX individuals displayed greater fat mass than RR and RX genotypes as well as a tendency for higher body mass, something that wasn't found in Chapters 3 and 4. The results from Chapter 5 support the hypothesis for ACTN3 as a candidate metabolically 'thrifty' genotype (Neel, 1962). The absence of ACTN3 from muscle fibres has been hypothesised to promote a more efficient use of energy that would likely reduce energy use and increase energy storage and provides a likely explanation for the greater adiposity of

older XX genotype men. One possible explanation for the lack of ACTN3 genotype differences in Chapters 3 and 4 compared with Chapter 5 is that the use of skinfolds as an estimate of body fatness is arguably a less sensitive method of body composition assessment than a DXA scan. However this does not provide an explanation for the contrast in findings by Walsh *et al.* (2008) that employed DXA and found no ACTN3 association with the body composition of men (Walsh *et al.*, 2008). However it is possible that subtle genetic influences on energy balance may only become apparent over a prolonged period of several decades.

7.3 Indirect indices of skeletal muscle fibre type composition

There is some evidence that both ACE I/D and ACTN3 R/X polymorphisms could be associated with skeletal muscle fibre type composition (Zhang *et al.*, 2003; Vincent *et al.*, 2007). However, the direct assessment of fibre type in the large populations is problematic, and therefore indirect measurements of fibre type composition (e.g. relative high velocity torque, and timecourse of an evoked twitch) may have some utility for investigating this idea. Whilst there is evidence that both of these measures may distinguish for substantial differences in fibre type (Hamada *et al.*, 2000; Gur *et al.*, 2003) they may have limited sensitivity to detect small differences. Using both of these indirect indicies of fibre type composition the current work found no evidence to support the association of the studied genes with fibre type composition. In addition subsequent biopsy studies have also contradicted the initial reports and not found any association of these genotypes with fibre type composition (ACE, Akmetov *et al.*, 2006; ACTN3, Norman *et al.*, 2009).

7.4 The influence of ageing on strength

An obvious difference in strength was observed when comparisons were made between strength measures of the young (Chapter 3) and older men (Chapter 5). Isometric knee extensor strength was 20% lower in the older men compared with the young men (610 vs. 487 N). Isokinetic knee extensor torque at 0, 30 and 240°·s^{-1} was 25, 20 and 29% lower in the older men compared to the young (0°·s^{-1} 247 vs 189, 30·°s^{-1} 201 vs 160, 240°·s^{-1} 118 vs 84 Nm). The torque ratio was 10% lower in the older men compared with the young men (59 vs 53 %). The lower strength values in the older men compared with the young men (Bassey *et al.*, 1993; Frontera *et al.*, 2000; Frontera *et al.*, 1991) are consistent with the literature that there is a decline in strength with ageing. The greatest difference between the groups was at the highest velocity (240°·s^{-1}) which is likely due to preferential atrophy of type II fibres (Larsson *et al.*, 1978) and emphasises the inability to maintain torque at high velocity as a consequence of ageing.

7.5 Genotype distribution between frequent fallers and controls

Whilst Chapter 6 found no differences in ACE and ACTN3 genotypes between the frequent fallers and controls, the small cohort is a major limitation of this investigation which was underpowered to detect anything other than a very dramatic genotypic effect. Power and sample size calculations suggested that a cohort of 194 (for ACE) and 173 (for ACTN3) in each group is necessary in order to detect any association with an odds ratio of 1.5 (5% significance level and 80% power). Furthermore, handgrip strength did not differ between ACE and ACTN3 genotypes in either men or women, which is consistent with the previous 3 studies. Additionally it is important to consider the statistical power of chapters 3, 4 and 5 in relation to the participant numbers. Post hoc power statistics were performed to determine the effect size for differences in muscle strength that could be detected between genotypes using the sample sizes employed in Chapters 3, 4 and 5. Results showed that at 80% power the effect size for chapter 3 was .93, chapter 4 was .77

and chapter 5 was .82. According to Cohen's (1988) conventional criterion the sample size employed was sufficient to have detected a medium to large effect size and therefore unlikely to have detected a small effect for differences in muscle strength between genotypes.

7.6 Contrasting findings of case-control and association studies

Before this current work began there were relatively few studies published highlighting the potential association of ACE and ACTN3 genotype with muscle phenotypes and the literature was equivocal and inconclusive which gave the premise for the current work to focus on the ACE I/D and ACTN3 R/X gene polymorphisms. Since then there have been numerous new studies published investigating the association of these polymorphisms with muscle phenotypes. There is a clear contrast in the literature regarding reports from case control studies of athletes and association studies of the normal population. The majority of association studies, many of which have been published since the start of this thesis, have found no ACE and ACTN3 genotype association with muscle function, (Giaccaglia *et al.*, 2008; Charbonneau *et al.*, 2008; Pescatello *et al.*, 2006; Thomis *et al.*, 2004; Delmonico *et al.*, 2008; Moran *et al.*, 2007) and are in contrast with the case-control studies, that have reported the DD genotype of the ACE gene and the RR genotype of the ACTN3 gene to be overrepresented in elite athletic events requiring muscle power (Myerson *et al.*, 1999; Nazarov *et al.*, 2001; Jones and Montgomery, 2002; Woods *et al.*, 2001a; Roth *et al.*, 2008; Druzhevskay *et al.*, 2008;). It may be that the association of ACE and ACTN3 genotype with muscle function is only apparent in the extreme phenotypes such as that of elite athletes and might indicate an interaction with powerful long-term environmental factors e.g. physical activity and training. Despite the initial excitement about these polymorphisms, ACE and ACTN3 do not appear to be associated with muscle function phenotypes in healthy untrained men.

7.7 Future Directions

- A number of other candidate gene polymorphisms (e.g. insulin like growth factor (IGF) II and vitamin D like receptor (VDR) BsmI and VDR Poly A) have been suggested to have an association with muscle function phenotypes and these should be investigated.
- It is becoming apparent that the association of single gene polymorphisms may be limited, and large cohort studies are recommended to detect potentially small effects and reduce the chance of 'false positives' that may explain some of the contradictions in the existing literature.
- Given that the association of a single gene variant is probably quite small, multiple gene studies are also recommended in order to determine broader genetic effects on a given phenotype.
- There are likely complex Gene x Gene interactions in which certain polymorphisms may modify the association of other genes, under certain conditions which require further research.
- As well as the possible gene-environment (physical training) interaction suggested for elite athletes there are likely complex Gene x Environment interactions (e.g. nutrition) not yet explored that could help to explain inter individual variability in muscle phenotypes.
- Investigations involving cohorts from single ethnic origins, with a similar age range and physical activity status are required in order to overcome limitations of previous work.
- Investigations of ACE genotype should routinely include measurement of serum ACE activity and where possible tissue ACE activity.
- The focus upon ACTN3 and its association with body composition warrants further attention given the widely documented influence of body fatness on overall health, morbidity and mortality.

- The only study to date that reported an ACTN3 genotype association with isokinetic strength at high velocity in young males supports the use of faster velocities in future studies
- The possible sex-ACTN3 genotype interaction requires further investigation.
- As the study of genotype distribution in frequent fallers (Chapter 6) was limited due to the small cohort, continuation of this work is recommended to find out if there are genetic factors that predispose to falls risk.

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APPENDICES

Table A.1 Percentage of ACE and ACTN3 genotypes in male falls group vs control group

		ACE			ACTN3		
		II	**ID**	**DD**	**RR**	**RX**	**XX**
Falls group	***n***	9	12	5	11	13	3
	(%)	(34.6)	(46.2)	(19.2)	(40.7)	(48.2)	(11.1)
Control group	***n***	42	88	49	70	78	31
	(%)	(23.4)	(49.2)	(27.4)	(39.1)	(43.6)	(17.3)

Table A.2 Percentage of ACE and ACTN3 genotypes in female falls group vs control group

		ACE			ACTN3		
		II	**ID**	**DD**	**RR**	**RX**	**XX**
Falls group	***n***	10	21	12	14	22	8
	(%)	(23.3)	(48.8)	(27.9)	(31.8)	(50)	(18.2)
Control group	***n***	42	88	49	70	78	31
	(%)	(23.4)	(49.2)	(27.4)	(39.1)	(43.6)	(17.3)