GENETIC FACTORS AFFECTING THE RESPONSE OF SKELETAL MUSCLE TO STRENGTH TRAINING

Maria Chatzi, B.Sc.

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I. Abstract

The aim of this study was to investigate the influence of Angiotensin-I Converting Enzyme (ACE) genotype and the influence of circulating ACE activity on the extent of muscle growth and strength development achieved during strength training. It was hypothesized that *ACE* Deletion (D) allele carriers would have higher force output values before and after the 12 week strength training programme. Forty male Caucasian recreationally active volunteers were genotyped for *ACE* Insertion/Deletion (I/D) polymorphism, but only eighteen subjects identified with the DD or the II genotype were qualified to participate. Eleven II and seven DD subjects underwent a 12 week strength training program for the quadriceps muscle group of the trained leg, with both legs assessed weekly by isokinetic dynamometry at joint angles of 30°, 60° and 90°(isometric knee extension) and 60°/sec and 180°/sec (isokinetic maximal torque). Biopsy samples were obtained from the vastus lateralis of the trained leg pre- and post-training, and they were analyzed using light microscopy and computer-based planimetry to identify the cross-sectional area of each major fiber type as an index of hypertrophy. A number of histochemical staining methods (H&E, SDH, GPDH, IHC, mATPases) were used for delineation of the fiber types. Circulating ACE activity was determined in blood samples and DNA samples were extracted from saliva for genotyping. ACE genotype was not associated with circulating ACE activity, with DD individuals presenting similar plasma ACE activity levels with II individuals (39.7 ± 40 and 40.5 ± 3.30 respectively, *P* = 0.89 pre-training, 41.9 ± 3.7 and 35.5 ± 4.30 respectively, *P* = 0.30 post-training). ACE activity did not change significantly with training (40.2 ± 2.5 nmol His-Leu/min/mL pre-training, 38.1 ± 3 nmol His-Leu/min/mL post training, *P* = 0.41) and correlated significantly with baseline isometric force of the untrained leg at 5° (r = 0.46, *P* = 0.05) and isokinetic strength at 180°/sec (r = 0.52, *P* = 0.03). When strength was presented as force production per kilogram of body mass, the above correlations became non-significant. Isometric force at 60º post-training revealed a significant effect of the genotype, in favour of the DD individuals, on the trained leg F (1, 64) = 4.242, *P* = 0.04, observed power = 0.53, partial eta squared = 0.062). The effect persisted after adjustment for weight, but when it was adjusted for body mass index and physical activity (assessed by questionnaires), the effect became non-significant (F (1, 63) = 3.13391, *P* = 0.08,

partial eta squared = 0.047, observed power = 0.41); and F (1, 63) = 3.1628, *P* = 0.08, partial eta squared = 0.048, observed power = 0.42, respectively).The average cross-sectional area (AVECSA) of Type IIA fibres for the DD individuals increased significantly post-training (4070 ± 506 μm² pre-training, 4674 ± 399 μm² post-training, t (6) = -2.999, *P* = 0.02) and so did the AVECSA of the Type I fibers of II individuals (3345 ± 207 μm² pre-training, 3988 ± 239 μm² post-training, t (10) = -3.063, *P* = 0.01). The finding shows that the strength training programme applied resulted in muscle hypertrophy, but the changes in AVECSA were not genotype-related. In conclusion, our findings suggest a possible role for *ACE* gene polymorphism in the regulation of human skeletal muscle strength, but limited statistical power and confounding factors prevented us from drawing clear conclusions.

Key words: Angiotensin Converting Enzyme – skeletal muscle – training

II.a List of abbreviations

ACE	Angiotensin-I Converting Enzyme
ACE	Angiotensin-I Converting Enzyme (gene)
ANGI	Angiotensin I
ANGII	Angiotensin II
ANOVA	Analysis of Variance
AT1	Angiotensin II Type I receptor
AT2	Angiotensin II Type II receptor
ATP	Adenosine Triphosphatase
AVECSA	Average Cross-sectional Area
bp	Basepair
BS	Blocking Serum
°C	Celsium Degrees
DD	Individuals homozygote for the ACE deletion allele
DTT	Dithiothreitol
FT	Fast twitch muscle fibres
g	Gravitational constant
GPDH	Glycerol-3-phosphate dehydrogenase
H&E	Haematoxylin and Eosin
IHC	Immunohistochemistry
II	Individuals homozygote for the ACE insertion allele
I/D	Insertion/Deletion allele
ID	Individuals heterozygous for the ACE Insertion/Deletion polymorphism
Kg	Kilogram
L	Liter
Lb	Pounds
LSD	Least Significant Difference
m	Meter
M	Mol
mATPase	Myosin Adenosine TriPhosphatases

min	Minutes
MHC	Myosin Heavy Chain
MVC	Maximum voluntary contraction
N	Newton
n	Number
OCT	Optimal Cutting Temperature
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pH	Decimal logarithm of the hydrogen ion
PMS	Pheanzine Methosulfate
rad	Radians
RAS	Renin-Angiotensin System
RM	Repetition Maximum
SE	Standard Error of the Mean
sec	Seconds
SDH	Succinate Dehydrogenase
SNPs	Single Nucleotide polymorphisms
SPSS	Statistical Package for Social Sciences
ST	Slow twitch muscle fibres
TBS	Tris-Buffered Saline
Type I	Slow-oxidative muscle fibres
Type IIA	Fast-oxidative muscle fibres
Type IIB	Fast-glycolytic muscle fibres
wk	Week
yr	Years

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leg, n=18, r=0.5, P=0.03 as assessed by Pearson's product-moment coefficient correlation. Solid line: best-fit linear regression line.

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3.4 The effect of the leg dominance on isometric force pre and post-training in 60° angle. Asterisk (*) indicates significant difference from baseline (pre), dagger (†) indicates significance towards untrained leg, double dagger (‡) indicates significance towards II genotype. Data presented as mean, vertical bars indicating confidence intervals (95%), level of significance set at $P<0.05$. 52

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3.6 Data expressed as mean ± SEM of 18 observations(n = 18) in 60° isometric angle through 12 weeks of training. Asterisk (*) indicates significantly different from baseline measurements (pre). Dagger (†) indicates trained leg (black circle) significantly different from untrained leg (black square), double dagger (‡) indicates significance towards II genotype, $P<0.05$. 54

1.Introduction

1.1 Angiotensinogens and the Renin-Angiotensin System

The RAS (Renin-Angiotensin System) is a peptidergic system with endocrine characteristics that regulates blood pressure and fluid balance (see Fig.1.1). This system uses angiotensinogen as a substrate (an a-glycoprotein, released from the liver) (Menard et al., 1983; Deschepper, 1994; Hall, 2003), and it is cleaved in the circulation by the enzyme renin that is secreted from the juxtaglomerular apparatus of the kidney (Hackenthal et al., 1978; Sealey et al., 1977; Hall, 2003; Persson et al., 2004) to form the decapeptide angiotensin I (ANGI). ANGI is converted to Angiotensin II (ANGII) by the Angiotensin Converting Enzyme (ACE). ANGII is responsible for the constriction of blood vessels that lead to increased blood pressure and also stimulates the secretion of aldosterone from the adrenal cortex.

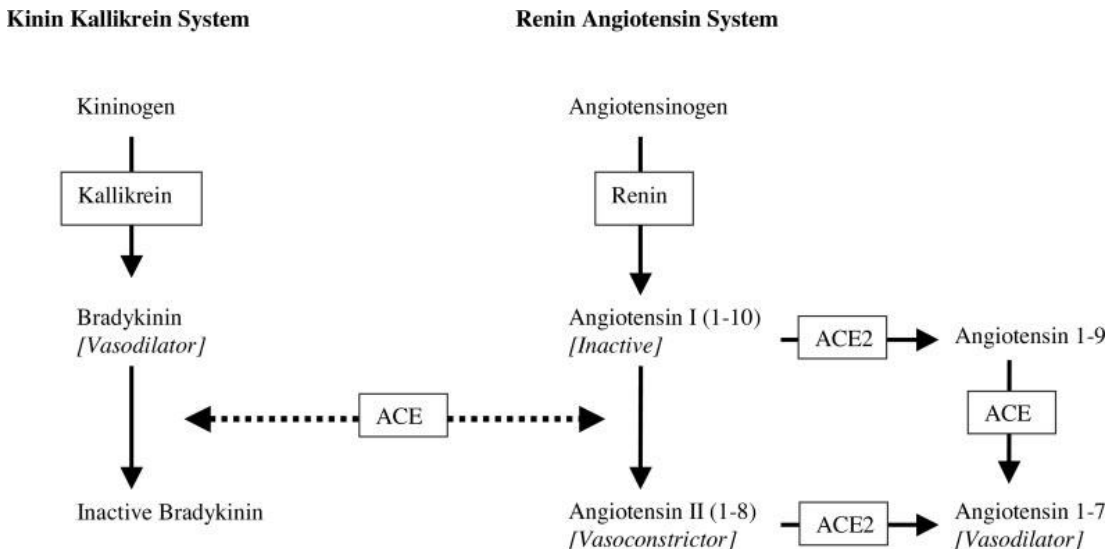


Figure.1.1: The kinin- kallikrein, and renin-angiotensin systems, picture taken from F.A. Sayed-Tabatabaei, B.A. Oostra, A. Isaacs, C.M. van Duijn, J.C.M. Witteman, ACE polymorphisms, *Circulation Research*. 2006; 98:1123.

1.2 Angiotensin Converting Enzyme

Angiotensin converting enzyme (ACE), key component of the RAS, is a zinc metallopeptidase (kininase II, EC 3.4.15.1) which is predominantly expressed in high concentrations on the surface of endothelial cells in the pulmonary circulation (Ng et al., 1967; Wei et al., 1991; Corvol et al., 1995; Costerousse et al., 1998; Hall, 2003; Soubrier et al., 2003; Soubrier et al., 2003;). Its function is to convert angiotensin I (ANGI) (vasoinactive) into a biologically active hormone, angiotensin II (ANGII) (vasoconstrictor) (Bernstein et al., 1989). ACE is also an important regulator of the kinin-kallikrein system (see Fig.1.1).

1.2.1 ANGII and AT1 / AT2 receptors

ANGII, the main effector peptide of the RAS, is acting on specific receptors, for example, to induce vasoconstriction by interacting with ANG receptors on vascular smooth muscle cells, or by stimulating the release of aldosterone from the adrenal cortex (Hollenberg et al., 1979; Quinn and Williams, 1988; Hall, 2003). In humans, ANGII effects are mediated predominantly through two specific receptors, namely AT1 (ANGII Type I receptor) (vasoconstrictor responses) and AT2 (ANGII Type 2 receptor) (vasodilator responses), which are G-protein coupled receptors in plasma membrane (Crisan and Carr, 2000; Danser, 2003). ACE also has the ability to hydrolyze numerous other peptide substrates (Hooper and Turner, 2003). There are two separate sources of ANGII; the first source is systemic ANGII from the RAS, whereby circulating angiotensinogen produced by hepatocytes is converted to ANGI by renin released from the kidney and then to ANGII by ACE, which is bound to the capillary endothelium of various tissues (McBride, 2006). The second source of ANGII is a separate local RAS that has been identified in many tissues, which is stimulated by local signals such as cell stretch (Sadoshima et al. 1993), resulting in a local production and release of ANGII in an autocrine/paracrine manner (Sadoshima et al., 1993; Jones and Woods, 2003).

1.2.2 Local RAS in skeletal muscle

Tissue RAS systems with all the necessary components for ANGII synthesis, have been identified in a number of peripheral tissues including the adipose, the cardiac, and skeletal muscle tissue (Engeli et al., 1999; Martin et al., 2006), suggesting a paracrine action of the RAS (Lavoie and Sigmund, 2003). Local ACE expression may also modulate tissue growth processes as both ANGII and kinins appear to have growth regulatory effects. Geisterfer et al., (1988) in a study that was conducted on cultured aortic smooth muscle cells, found that ANGII is a potent hypertrophic agent (Geisterfer et al., 1988). A similar research was carried by Ishigai et al., (1997), demonstrating that kinins appear to play a role in hypertrophy of cardiomyocytes (Ishigai, et al., 1997). More recently, immunohistochemistry of human muscle biopsies has localized ACE to the endothelial cells of capillaries in skeletal muscle (Schaufelberger, Drexler, Schieffer, and Swedberg, 1998). Jones and Woods (2003) in an extensive review specified the relationship between exercise performance and a physiologically functional skeletal RAS (see table 1 for the skeletal muscle RAS constituents and Figure 1.2 for the effects of ANGII on muscle performance). They concluded that this local RAS system exists and it is capable of de novo ANGII production and interaction with the kallikrein-kinin system and therefore a significant potential for an influence on human performance arises (Jones and Woods, 2003).

RAS component	Presence	Location	Reference
Angiotensinogen	Inferred	Induction of local Ang I following renin infusion	Muller et al. (1997)
		Local Ang I production in pigs	Danser et al. (1992)
ACE	Yes	Endothelial cells of capillaries	Schaufelberger et al. (1998)
		Skeletal muscle membrane	Dragovic et al. (1996)
		Human muscle biopsies	Reneland & Lithell (1994)
ANGI–ANGII conversion	Yes	Human skeletal muscle de novo synthesis	Danser et al. (1992)
		Peripheral muscle vascular bed	Saris et al. (2000); van Dijk et al. (2000)
		Skeletal muscle membrane	Ward et al. (1995)
AT1 receptor	Yes	Rat skeletal muscle fibre and microvessels	Linderman & Greene (2001)
		Human skeletal muscle	Malendowicz et al. (2000)
AT2 receptor	Yes	Rat skeletal muscle fibre and microvessels	Linderman & Greene (2001)

Table 1: Summary of skeletal muscle RAS constituents. Source: Jones A., Woods D.R., Skeletal muscle RAS and exercise performance. *Int J Biochem Cell Biol 2003*; 35(6):855-66.

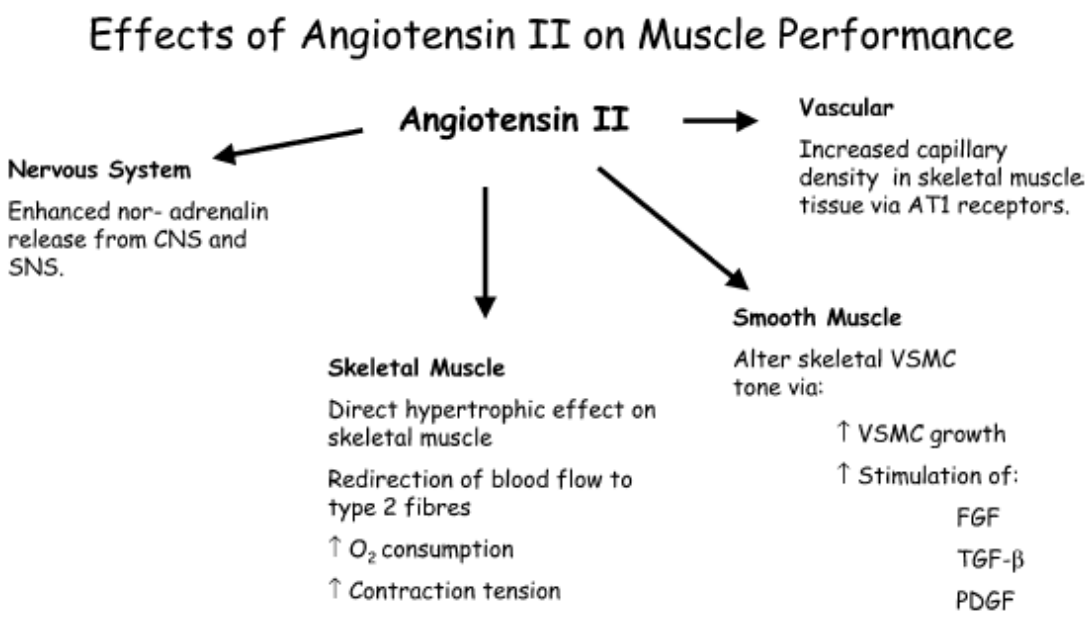


Figure 1.2: Effects of ANGII on muscle performance. Source: Jones A., Woods D.R., Skeletal muscle RAS and exercise performance. *Int J Biochem Cell Biol 2003*; 35(6):855-66.

Taking into account the above (see Figure 1.2), RAS does influence smooth muscle and cardiac muscle growth, and might thus be expected to influence skeletal muscle growth. So on that basis, research shows that ANGII is necessary for mediating load-induced skeletal muscle growth. ACE gene expression also is variable and, quantified by the number of ACE-mRNA transcripts, is related to muscle fibre area with an inverse relationship to capillary density (Schaufelberger et al. 1998)

1.2.3. The *ACE* I/D polymorphism

During the last few decades more and more scientists are focusing their research on genetics. Since the launching of the Human Genome Project, many interesting discoveries have been made especially in the fields of clinical science, where genes responsible for certain diseases have been identified. The genome of a single person may be a predisposing factor to some certain pathological situations, and may have an impact on the response to treatment. Genetic data is available for some phenotypes having to do with physical performance; these include cardio respiratory endurance, elite endurance athlete status, muscle strength, other muscle performance traits, and exercise intolerance of variable degrees (Bray et al., 2008). Many researchers have identified *ACE* as a candidate gene affecting an individual's endurance or strength capacity (Gaygay et al., 1998; Folland et al., 2000; Jones et al., 2002; Williams et al., 2005), with Hugh Montgomery being the first one to do so (Montgomery et al., 1997). The *ACE* gene is highly polymorphic, with over 100 polymorphisms listed in SNPs database (http://www.ncbi.nlm.nih.gov-/sites/entrez,ID=1636). A polymorphism of the *ACE* gene that is associated with a change in ACE activity has been identified, with the absence (deletion allele, D) rather than the presence (insertion allele, I) of a 287 base-pair fragment in intron 16 of the *ACE* gene (see Fig. 1). Each human has 2 alleles, therefore three genotypes exist: II, ID and DD, the distributions of which within a Caucasian population are roughly 25, 50 and 25%, respectively.

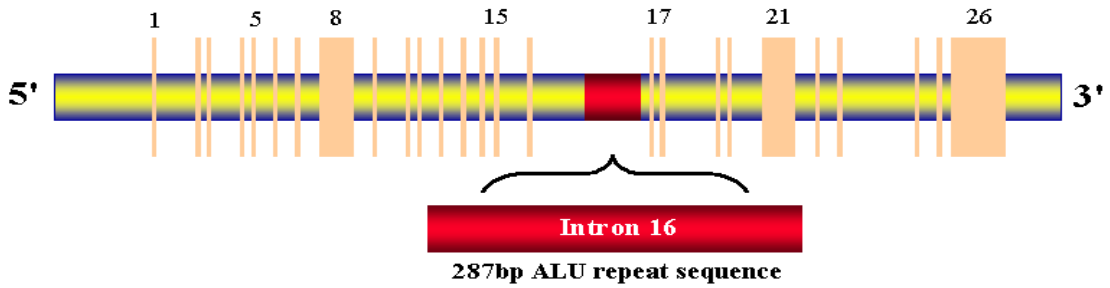


Figure 1.3: Intron 16 of the human *ACE* gene where the 287 base pair fragment responsible for the I/D polymorphism is located. Figure adapted from Prof. Hugh Montgomery, UCL.

1.2.4. Effects on plasma circulating ACE activity

This polymorphism accounts for almost half of the variance in plasma ACE activity as well, with homozygotes for the D allele showing higher plasma ACE levels, than individuals with the ID (intermediate ACE levels) and individuals with the II (lower ACE levels) genotypes (Rigat et al., 1990)(see figure below). Danser et.al (1995) associated the DD genotype with higher cardiac ACE activity, which may result in increased cardiac ANGII levels, which makes this a mechanism underlying the reported association between the *ACE* deletion polymorphism and the increased risk for several cardiovascular disorders, such as myocardial infarction, cardiomyopathy, left ventricular hypertrophy, and coronary artery diseases (Danser et al., 1995).

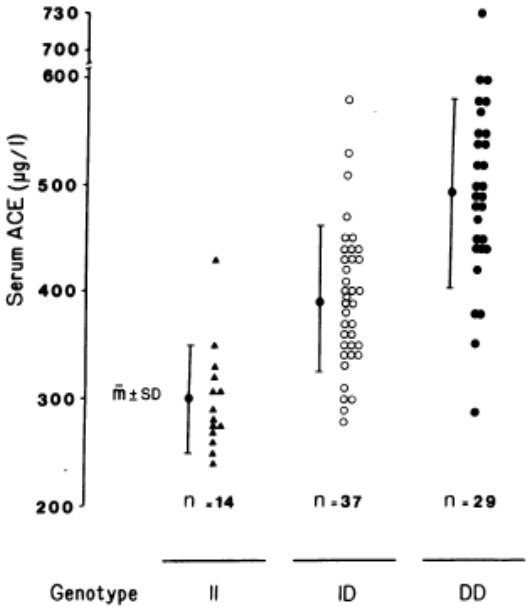


Figure 1.4**: (**Original figure from Rigat et al., 1990): Serum immunoreactive ACE concentrations (µg/liter) for individual with the II, ID, and DD genotypes, respectively, shown in left, middle, and right panels. Solid vertical bars indicate mean concentration and standard deviation for each group.

The concentration of circulating ACE activity is associated with *ACE* genotype (Rigat et al., 1990, see also Fig 1.2). Each peptide/ protein has a free amino group and a free carboxyl group. The residue bearing the free amino group is termed the amino terminal or N-terminal residue, whereas the residue bearing the free carboxyl group is termed the carboxyl terminal or C-terminal residue. Almeida (Almeida et al., 2010) found that the total plasma ACE activity of both domains (N- and C- terminus) in II individuals was significantly lower in comparison to ID and DD, so the I/D *ACE* polymorphism affects differently both ACE domains.

1.3 Skeletal muscle fiber types

Muscle fibres are formed by a fusion of a number of myoblasts into a single multinucleated cell. Based on their specific myosin heavy chain content (MHC), those fibres are classified in three major categories: (1) Type I (slow-oxidative fibres), (2) Type IIA (fast-oxidative fibres), and (3) Type IIB (fast-glycolytic fibres). Skeletal muscle fibres differ in contractile and metabolic characteristics, differences shown in Table 1.

Table 2: Characteristics of the three major fiber types.

Myosin type	1	2A	2X
Description	Slow, red (oxidative), fatigue resistant	Fast, red (oxidative), fatigue resistant	Fast, white (glycolytic), easily fatigued
Motor neuron size	Small	Medium	Large
Recruitment frequency	Low	Medium	High
Contraction speed	Slow	Fast	Faster
Endurance	High	Medium-High	Low
Motor unit nomenclature	Slow (S)	Fast-Fatigue resistant (FR)	Fast, fatiguing (FF)
Mitochondrial density	High	Medium-very high	Low
Oxidative capacity	High	Medium-very high	Low
Glycolytic capacity	Low-medium	Medium-very high	High

Source: Vincent, B., 2009.*Role of the Alpha-Actinin-3 R577X polymorphism in metabolic and contractile properties of skeletal muscle*. PhD, Katholieke Universiteit Leuven, Group Biomedical Sciences (Type IIB fibres are often referred in the literature as IIx).

As we can see from above, muscular efficiency (in terms of force production per unit) is related to the type of muscle fiber, and more specific, Type I fibres are more efficient then Type II fibres when a contraction is performed isometrically or in a low velocity according to Coyle and his colleagues (1992). Given that the plasticity of the skeletal muscle fibres is extended (changes in muscle fibre size and transition between Type IIA and Type IIB), it is expected that changes will occur with specific training.

1.4 ACE and adaptation to training

1.4.1 Skeletal muscle growth

The importance of ACE in the regulation of skeletal muscle growth was proved when Gordon et al., (2001) used an experimental model of compensatory hypertrophy in Sprague-Dawley rats, by inducing optimal overload on a muscle. Oral ACE inhibitors were administered and prevented hypertrophy, while local ANGII perfusion of muscle rescued this. Oral sartans, which are a class of drugs which inhibit the AT1 receptor, also inhibited hypertrophy, and couldn't be rescued with ANGII perfusion (Gordon et.al, 2001). McBride et al., (2006) found that blocking ANGII`s AT1 receptor attenuated eccentric training–induced hypertrophy and strength gains in Sprague–Dawley rats (McBride et al., 2006), a fact that provided additional evidence for the role of the renin–angiotensin system (RAS) in overload-induced muscle hypertrophy. Despite of all the above, the mechanisms by which ANGII mediates skeletal muscle hypertrophy under conditions of overload are still unknown. However, no similar study has yet directly evaluated the role of ACE in the regulation of skeletal muscle growth in humans.

1.4.2 Strength development

Based on the previous studies that associated the D allele with the growth of vascular smooth muscle at the site of coronary angioplasty and human cardiac hypertrophy in response to exercise (Ohishi et al.,1993; Montgomery et al., 1997), and since Reneland and Lithell (1994) developed a technique that proved that there is considerable ACE activity in skeletal muscle, Folland (2000) investigated the influence of the *ACE* deletion allele on the response of the human quadriceps muscle group to specific strength training programmes in non-pathological population (Folland et al.,2000). Hence, this study used genotype as an indirect marker of ACE activity, and compared the responses to strength training (Folland, et al., 2000). This study reported greater maximum voluntary contraction (MVC) and similar one-repetition maximum (1RM) (Folland et al., 2000) gains after resistance training among subjects with the *ACE* D allele compared with those with the *ACE* II genotype individuals with at least one D allele (Folland, et al., 2000).

This finding is consistent with the key role of ACE in the regulation of skeletal muscle hypertrophy in rats (Gordon et al., 2001), but comes in contrast with the study of Montgomery (Montgomery et.al, 1999) where the I and not the D allele was associated with a greater relative whole-body anabolic response to training (Montgomery et.al, 1999).Thus, those studies are not comparable since Montgomery et.al, (1999) used basic army training, at which physical activity is not specific, but rather wide and varied. Thomis et al., (1998) failed to support the relationship between the D allele and muscle strength adaptation to strength training. More specifically, Thomis et al., (1998) reported borderline significance for greater concentric flexion torque gains for *ACE* I allele carriers but found no associations among the *ACE* ID genotype and the muscle size response to resistance training of the elbow flexors (Thomis et al., 1998). Pescatello et al., (2006) on the other hand, examined the influence of the *ACE* ID genotype on the muscle strength and size adaptations to a standardized 12-wk unilateral, upper-arm resistance training intervention in a large sample of apparently healthy young adults (Pescatello et al., 2006). It was hypothesized that the muscle strength and size gains from resistance training would be greater among *ACE* DD

homozygotes compared to carriers of the *ACE* I allele. In fact, training the non-dominant arm showed slight increases in strength, which were greater for the I allele, but D-allele dependent increases in strength of the dominant (untrained) arm (Pescatello et al., 2006).

Charbonneau et al. (2008) on the contrary, examined this association in the weight-bearing lower limbs, hypothesizing that the D allele would be associated with higher values for muscle phenotypes before strength training, and greater increases in muscle phenotypes in response to strength training (Charbonneau et al.,2008). Two hundred forty three inactive healthy volunteers (86 men and 139 women), of mean age 62 years old, were studied before and after 10 weeks of unilateral knee extensor (Charbonneau et al., 2008). In this study, *ACE* genotype was associated with baseline differences in muscle volume but it was not associated with the muscle hypertrophic response to strength training (Charbonneau et al., 2008). There is also a handful of other studies that have found no association between ACE and performance (Karjalainen et al., 1999; Taylor et al., 1999; Rankinen et al., 2000; Sonna et al., 2001; Zhao et al., 2003; Thomis et al., 2004; Scott et al., 2005).The reason for this noted inconsistency in the existing literature may be a result of limitations occurring in association studies. As Jones and his colleagues (2002) noted, even if an association is found between the *ACE* I/D polymorphism and human performance, there are many possible explanations for the relationship. Some possible scenarios may be the polymorphism itself, the locus within which the polymorphism is located, or another locus that is in linkage disequilibrium with the polymorphism to be responsible for the association (Jones et al., 2002). Additionally, an association does not mean that this is the only cause. For example, an association between a gene and a certain physical characteristic does not mean that the gene caused or created the physical trait. Furthermore, population association studies have a tendency to present other difficulties. For example, variation within the control group can be a confounding factor, especially when the frequency of the polymorphism varies among control populations (Jones et al., 2002). Further research is required in humans to correlate ACE activity directly with the training response (rather than using the genetic marker alone), and also to measure skeletal muscle growth directly (rather than measuring muscle strength alone).

1.5 Hypotheses

Bearing in mind these findings, we posed the following hypotheses:

1. Baseline strength will be higher in homozygotes for the D allele compared to homozygotes for the I allele.

2. Type II fibres of homozygotes for the D allele will show more hypertrophy than I homozygotes for the I allele in response to a strength training programme.

3. There will be a difference in strength development between the homozygotes for the I allele and homozygotes for the D allele in response to a strength training programme. Based on a biological rationale and the existing literature, homozygosity for the D allele will be favoured.

2. Methods and materials

The study had appropriate ethics committee approval (Faculty of Biomedical and Life Sciences Ethics Committee). Written informed consent was obtained from all participants. The study was conducted with both assessors and subjects blind to the subjects' *ACE* genotype and activity.

2.1 Subjects

Subject eligibility was based on *ACE* genotype. In the initial phase of the study, 40 male Caucasian recreationally active volunteers were recruited from the student and staff populations of The University of Glasgow, but only 18 subjects were eligible to participate as they fulfilled the genotype criterion (only subjects carrying the DD and II genotype were qualified to participate). Physical activity levels for each subject were recorded via questionnaires, with all participants not having taken part in any structured strength-training programme of the quadriceps muscle group during the 6 months preceding the initiation of the study. The activity stated was walking, cycling and recreational rugby and football. Exclusion criteria were medical problems (such as knee pathology or other orthopaedic conditions) that would confound their participation in the study, or the use of any medication, including nutritional supplements, anabolic compounds or ACE inhibitors/angiotensin receptor blockers. Eligibility was further assessed by interview and further by completion of a medical and a physical activity questionnaire. Subjects were required to read and sign the enclosed information sheet.

Power analysis was conducted using the software package, G*Power 3.1.2 (Faul and Erdfelder, 1992-2009). We aimed to have at least 80 % power to detect a significant two-tailed correlation of magnitude r = 0.40 on the basis of an alpha of 0.05, which would require 46 subjects for both baseline and training analyses according to the *a-priori* analysis. For the two-way ANOVA for repeated

measures, sample size estimates were calculated *a priori* on the basis of an alpha of 0.05 to detect a medium effect size of 0.25 (according to Cohen's effect size conventions between the two groups) with a desired power of 80 %, which would require a total sample size of 44 subjects. *Post-hoc* power analysis was performed in order to compute the power achieved given our sample size, (total sample N=18, sample size group 1 = 11, sample size group 2 = 7) to detect a significant difference between the two genotype groups (α= 0.05, effect size d = 0.5, two-tails) and, unfortunately, our study was considered underpowered (p = 0.16). We were underpowered for genotype-specific comparisons, so those analyses should be considered exploratory.

2.2 *ACE* genotyping

All volunteers underwent DNA sampling prior to the start of testing in order to distinguish those subjects with the preferred genotypes II and DD. Heterozygous subjects with the ID genotype were excluded from this experiment as they are associated with both high and low levels of ACE activity (Cox et al., 2002). The homozygous genotypes would present a noticeable variation in ACE production, and in turn discernable differences in muscle hypertrophy.

DNA was collected from cells taken using a buccal swab from each volunteer. The buccal swabs were stored in 1-mL cell lysis solution until extraction.The genomic DNA was extracted from the cells using the standard Machery-Nagel 'NucleoSpin' protocol for human tissue (Machery-Nagel 07/03/Rev 02). ACE genotype was determined using a polymerase chain reaction (PCR) (O'Dell et al., 1995, Tsai et al., 2002). This particular PCR method involved the use of a three-primer system; a forward primer which recognised the deletion (D) sequence, a forward primer which recognised the insertion (I) sequence and a common reverse primer. The forward deletion primer was (5'-CTCTAGACCTGCTGCCTATTACAGTC-3'), the forward insertion primer was (5'-CGGGATGGTCTCGATCTC-3') and the common reverse primer was (5'-CCCTCCCATGCCCATAAC-3'). The PCR protocol for the I/D polymorphism consisted of 35 cycles of 45 seconds of denaturation at 94°, 45 seconds of annealing at 56.5°, and 45 seconds of extension at 72°. Cycling was preceded by 7 minutes of denaturation at 94° and followed by 10 minutes of extension at 72°. PCR products were resolved on a 2 % Agarose gel and visualised by ethidium-

bromide staining by two independent staff blind to all subject data. If present in the template DNA, the D allele yielded a product of 197 bp and the I allele a product of 252 bp. The D allele in heterozygous samples is preferentially amplified; therefore each sample that was found to have the DD genotype was subject to a second, independent PCR amplification with a primer pair that recognises an insertion-specific sequence to avoid mistyping the DD genotype. On completion of DNA genotyping, subjects with the appropriate genotype (II or DD) were notified of their qualification and invited to take part in the remainder of the experiment.

2.3 Determination of plasma ACE activity (circulating)

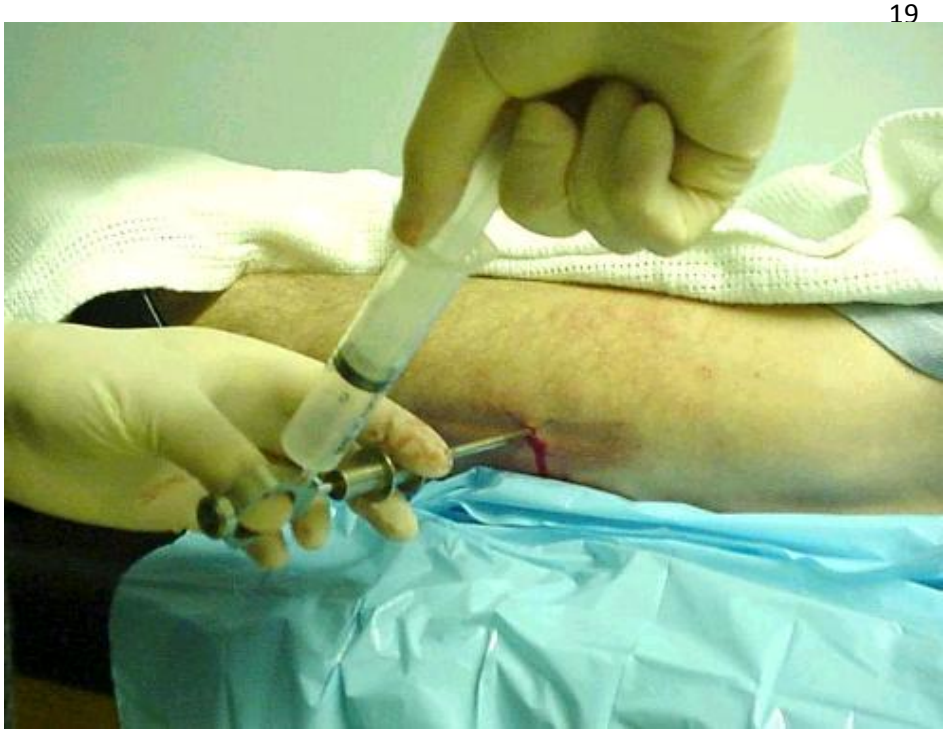
Before and after the 12 week intervention period, a resting blood sample (20-mL) was obtained from a superficial forearm vein for analysis of plasma ACE activity. Plasma was separated immediately from 10 mL of whole blood by centrifugation at 1500 *g* for 10 minutes and stored at -80 °C until analysis. The remaining 10 mL were stored for performing the *ACE* genotyping, but it was finally decided to perform the above using saliva samples, so it was discarded. ACE activity was assayed using a spectrophotometric technique (Sigma Diagnostics, Poole, UK) based on the method developed by Holmquist et al., (1979). The analysis was performed at the Institute of Pharmacology, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany, from Professor Peter Gohlke. The investigators remained blind to the subject's genotype.

2.4 Muscle fibre analysis

2.4.1 Obtaining the biopsies

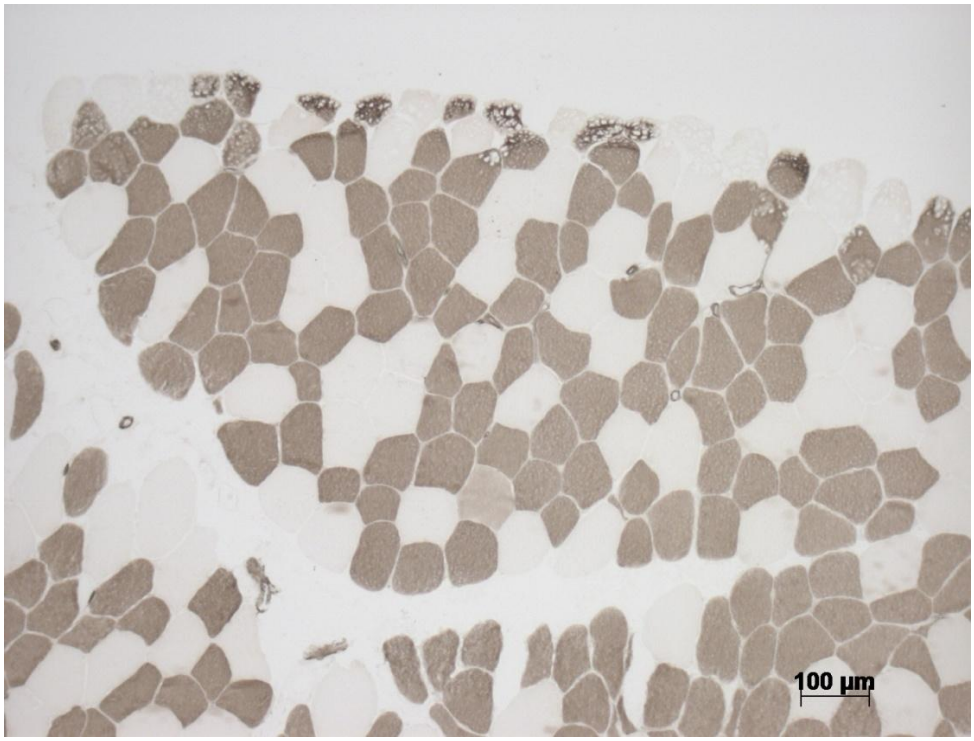
At least 7 days prior to initial strength testing and no later than 7 days post-intervention testing, skeletal muscle samples were taken from each subject. The biopsies were obtained by Dr. Kingsmore (FRCS (Gen), FRCS (Ed), MD, MB ChB, B Med Biol). The skeletal muscle sample was obtained from the vastus lateralis of the subjects' trained leg by the Bergström needle biopsy technique (Bergström, 1975) as previously approved by the FBLS committee. Vastus lateralis, a portion of the quadriceps femoris muscle group, has been the muscle of choice for biopsies due to several advantages, such as mixed fiber type composition, trainability, and accessibility. Hence, a large set of data concerning this muscle exists in the literature, potentially facilitating comparisons among studies (Saltin and Gollnick, 1983).

Figure 2.1: Muscle biopsy performed by the Bergströom needle biopsy technique.

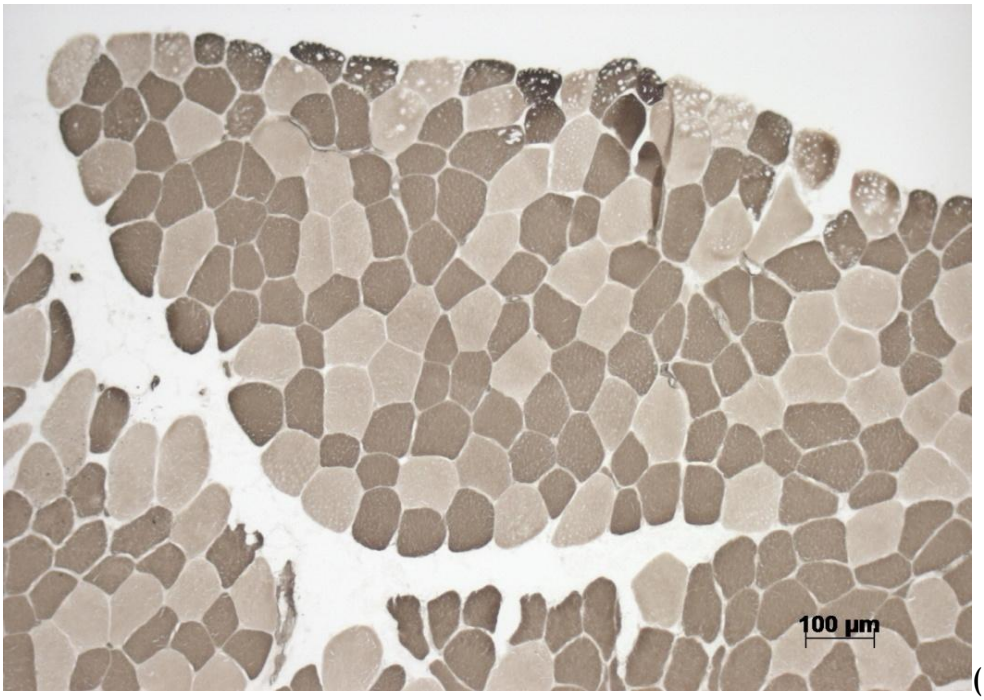


2.4.2. Processing of the biopsies

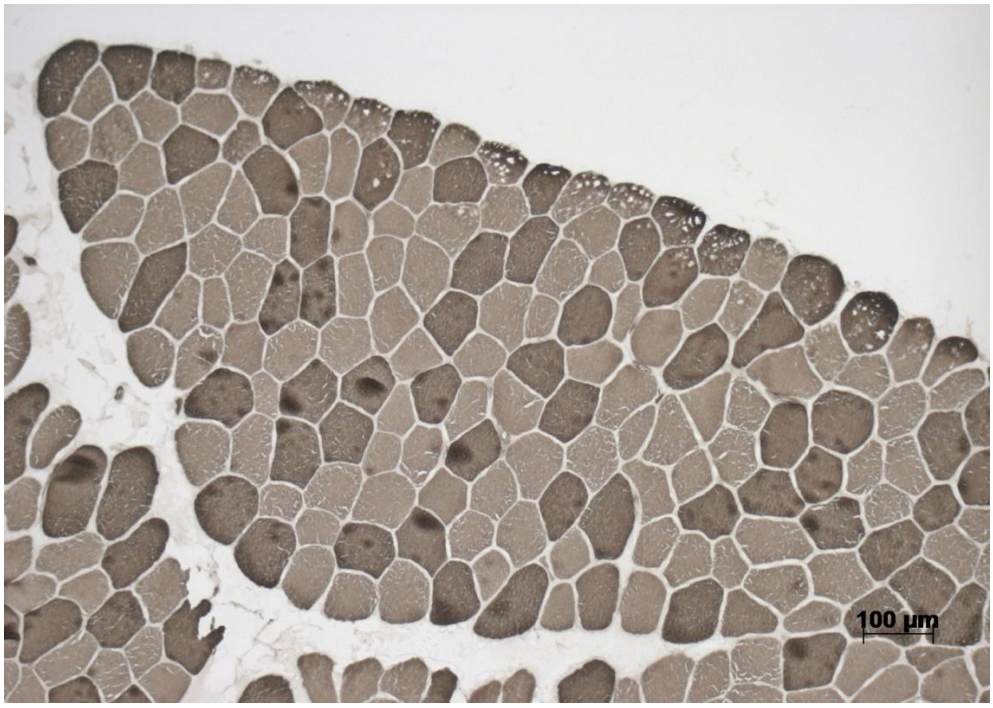
Muscle biopsy is an invasive procedure widely used to assess a number of structural and functional characteristics of muscle tissue including fibre type and size, enzymatic capacity, mitochondrial concentrations, metabolic responses and contractile proteins. The skeletal muscle samples were immediately mounted in an embedding medium (Optimal Cutting Temperature Compound, OCT) and frozen in isopentane cooled with liquid nitrogen as described previously (Shono et al., 1999, Shono et al., 2001, Zhang et al., 2003). Serial 10μM cross-sections were cut using a Cryostat (Cryostat Jung Frigocut 2800E, Leica) at -20°C. Samples were stained for five different staining methods: Haematoxylin and Eosin (H&E), SDH (Succinate Dehydrogenase), a-GPD (Glycerol-3-phosphate dehydrogenase), mATPase, (myosin ATPases) and IHC (immunohistochemistry). In order to identify the muscle fibres based on their properties (contractile, oxidative, etc), a comparison between identical fields while performing the light confocal microscopy analysis was essential [Zeiss (Axiophot, AxiocamMR3, Axioskop 2), microscope with film and digital capture].



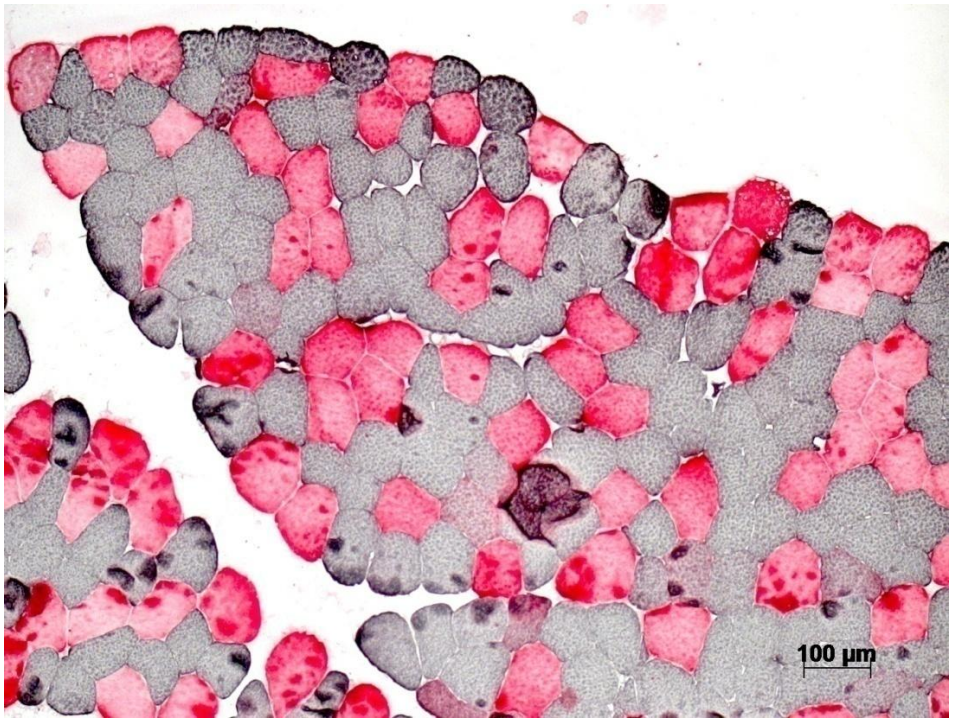
(A)



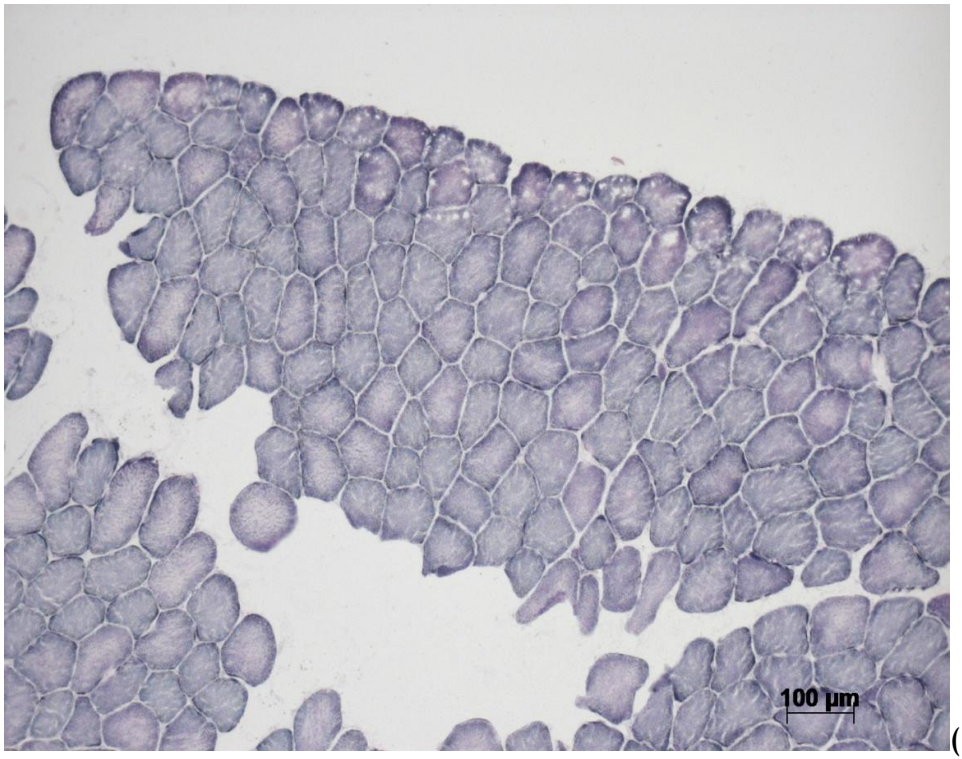
(B)



(C)



(D)



(E)

Figure2.2: Comparison of frozen sections stained for myofibrillar ATPase at (A) pH 4.3 (B) pH 4.6, and (C) pH 9.4, and by (D) immunohistochemistry for slow (dark grey) and pink (fast) myosin [Type I fibres are dark grey whereas type IIA and IIB fibres are distinguishable as pale greyish pink and pink, respectively. (E) SDH. [Original magnification, x100.]

2.4.3. Staining with succinate dehydrogenase: Identifying oxidative potential

The histochemical assay for SDH is used to distinguish between oxidative and less oxidative fibres. The activity of SDH was determined in 10-µm thick sections cut on a cryostat (Cryostat Jung Frigocut 2800E, Leica). The protocol used was the one previously described by Martin et al., (1988), with a slight alteration on the proportion analogous to the quantity of the tissue to be analysed. The solutions used were 100 mM of phosphate buffer containing the following solutions: A) 0.2 M NaH_2PO_4 H_2O (monosodium phosphate monohydrate) [27.6 g/L] B) 0.2 M Na_2HPO_4 $2H_2O$ (Disodium Phosphate Dihydrate) [35.6 g/L]. For the pH 7.4 solution, 9.5 mL of A was mixed with 40.5 mL of B along with 50 mL of distilled water. For the pH 7.6 solution, 6.5 mL of A was mixed with 45.5 mL of B and 50 mL of distilled water. The second solution used was 1 mM of sodium azide, which

consisted of 0.065 g of sodium azide, 100 mL of distilled water, and then we dissolved the above solution to 100 mL. The third solution was 1 mM phenazine methosulphate (PMS) which was made of 0.03 g of PMS dissolved in 100 mL of distilled water. As those solutions were photosensitive, they were prepared quickly and stored in the dark. The incubation medium for succinate dehydrogenase was 80 mL of phosphate buffer, pH 7.6 [100 mM], 1 mM sodium azide [1 mL, 10 μM], 0.12 g nitro blue tetrazolium [1.5 mM], and 0.19 g of EDTA [5 mM], 1.3 g of dissodium succinate [48 mM]. A further adjustment to pH 7.6 may have been necessary. After this step, 2 mL of 1 mM PMS [20 μM] were dissolved to 100 mL of phosphate buffer [pH 7.6]. Sections were then incubated at 37 °C in the dark for 15-60 minutes, depending on the staining intensity of the fibres. Mounting and dehydration followed as it is discussed at the IHC protocol.

2.4.4 Glycerol-3-phosphate dehydrogenase (GPDH): Identifying glycolytic potential

The αGPD enzyme is used to distinguish among fibres based on their relative glycolytic potential. The GPDH activity was determined on 10 μM thick sections in a medium containing 100 mM phosphate buffer (pH=7.4), 1.5 mM nitro blue tetrazolium, 9.3 mM Glycerophosphate, 20 μM PMS. The sections were incubated at 37 °C, in the dark, for 15-60 minutes (or until at least some fibres were strongly blue in colour). After that, the sections were dehydrated and mounted as described before (IHC Protocol).

2.4.5 mATPases

The standard ATPase method (Round et al., 1980) was used at pH values of 9.4, 4.6, and 4.3 on the frozen tissue samples, together with a negative control. For each case, two 10μm serial sections were used, so that a total of eight was required. The ATPase preparations were done as part of the usual routine series of muscle stains (haematoxylin and eosin). The reagents used were as follows:

Glycine buffer (7.5 g glycine and 5.8 g sodium chloride made up to 1 L with distilled water), buffered $CaCl_2$ (Calcium chloride) (500 mL glycine buffer and 100 mL 1 M calcium chloride and approximately 350 mL 0.1 M NaOH adjusted to pH 9.5 with 1 M NaOH.), acetate buffer pH 4.3 (36.8 mL 0.2 M acetic acid and 3.2 mL 0.2 M sodium acetate made up to 100 mL with distilled water), ATP [(disodium salt, Sigma Chemicals Ltd): for the working solution 5 mg is dissolved in a few drops of distilled water] dithiothreitol (DTT):[1 mM (renewed monthly)], cobalt chloride (2% solution), ammonium sulphide (1 % solution made up freshly from a concentrated stock solution just before use), calcium chloride (1 % solution), reaction mixture(1 drop of DTT (1 mM) and the ATP working solution added to 10 mL buffered $CaCl_2$). The routine ATPase method (pH 9.4) was carried out as follows: Cryostat sections were cut at 10 µM and mounted on cover slips. Then, the sections were incubated in reaction mixture in a Colombia jar for 30 min at 37 °C and washed well in 1 % $CaCl_2$ for 3 x 2 min periods. After that, they were placed in 2 % $CoCl_2$ (Cobalt Chloride) solution for 2 x I min periods and then washed very thoroughly in at least 4 changes of distilled water. The sections were then placed in 1 % ammonium sulphide solution for 30 s (this step was carried out in a fume cupboard). After the color was developed, the sections were washed well with distilled water and mounted in tissue mount. The reverse ATPase method was carried out as follows: The sections were pre-incubated in pH 4.3 or 4.6 acetate buffer at 37 °C for 10 min, washed quickly in dilute (1:4 v/v) buffered $CaCl_2$ and processed as for the routine ATPase reaction, but with the buffered $CaCl_2$ diluted 1:4 with distilled water before the addition of the ATP and DTT. The ATP and calcium ions in the reaction mixture are in large excess which ensures maximum rates of ATP splitting and complete precipitation of the phosphate formed. The ionic strength of the pH 9.4 incubating solution maximizes the differentiation of the two fiber types. In the reverse ATPase reaction, the activity of the type I fibres is preserved by diluting the buffered calcium before the addition of the ATP.

2.4.6. Immunohistochemistry

Two 10 μm sections from each case were placed on one slide. The protocol used was almost identical to the one used before by Behan et al., (2002). The control consisted of one section of each 18 subjects, blocked in the first antibody. Commercial antibodies to fast and slow isoforms of myosin were used, each with a different visualization system, so that specific identification of each fiber type on the same section was feasible. After fixation in acetone for 10 minutes, the slides were placed in peroxide, washed with PBS (Phosphate Buffer Saline), and blocking serum was applied for 1 h. The antibody to slow myosin was applied, followed by a peroxidase biotinylated rabbit antimouse antibody; the result was visualized as black type I fibres using the commercial Vector SG Peroxidase Substrate kit (Vector Laboratories, Peterborough, UK). The alkaline phosphatase conjugated antibody to fast myosin was then applied; red type II fibres were visualized using the commercial Vector Red substrate kit. The full details of the simple standard protocol, used widely in routine pathology laboratories and which was slightly modified, are as follows.

Sections were incubated with 20 % normal goat serum (NGS) in Tris buffered saline pH 7.6 (TBS) for 1 h, after which the excess serum was drained off and the sections incubated in monoclonal antibody to slow myosin (Sigma- Aldrich, Poole, Dorset, UK) diluted 1/1000 in Blocking Serum (BS) for 1 h, followed by three washes in PBS. The sections were then incubated in peroxidase conjugated rabbit antimouse antibody (Dako Ltd, Ely, and Cambridgeshire, UK), diluted 1/50 in BS, for 60 min, and then washed three times in PBS. Vector SG peroxidase substrate solution (Vector Laboratories) was then applied, controlling the reaction by microscopic examination over 5 to 15 min (Behan et al., 2002). The sections were washed 3 times in distilled water (DW), incubated in BS for 1 h. Excess serum was drained off before incubation in alkaline phosphatase conjugated monoclonal antibody to fast myosin (Sigma- Aldrich), diluted 1/50 in BS, and the sections were incubated overnight in the refrigerator (4 °C). After washing 3 times in PBS, the sections were incubated in Vector red alkaline phosphatase substrate solution (Vector Laboratories) for 10–20 min, controlling the reaction by microscopic examination. The final wash was in running tap

water, after which the sections were dehydrated through graded alcohols (70 %, 90 %, 100 % times two, Tissue Clear times two) cleared in xylene, and mounted in synthetic medium (Tissue Mount). The antibodies used were: (1) for type I fibres, monoclonal antimyosin (skeletal, slow; clone NOQ7.5.4D; Sigma-Aldrich). (2) For type II fibres, monoclonal antimyosin (skeletal, fast; alkaline phosphatase conjugate; clone MY-32; Sigma-Aldrich). On completion, type I fibres were black whereas the type 2 fibres were pink. Type IIA and IIB subtypes could be distinguished because IIB were completely pink whereas IIA were intermediate between black and pink, appearing as a granular, dark, and pinkish (Behan et al., 2002).

2.5 Morphometric Analysis

The analysis of the fiber type and size was performed with the investigators and the staff blind to genotype and training status. Samples were analysed using light microscopy and computer-based planimetry (AxioVision 40 V 4.6.3.0, Carl Zeiss Imaging Solutions GmbH, 2006-2008). Tissue sections were viewed using the 10 x and 20 x objectives and visualized on the monitor. Several still images for each case were imported into the image analysis package, and morphometric data on muscle fibres retrieved by means of manual routine using the AxioVision software. Areas of fibre cross section were recorded. More than 200 fibres were counted at random on each SDH stained section examined. Based on mATPase and IHC classification of the three major fiber types, we therefore established a staining intensity for the SDH-stained slides. Using the ATPase method, almost 100 fibres on average were counted on the pH 9.4 preparations as type I and II and then a further 100 on the pH 4.6 sections were analyzed as type IIA or IIB. Using the IHC method, the 100 fibres were counted and classified on one preparation as type I, IIA, or IIB. The same 100 fibres were matched in the SDH preparation. Manual outlining was performed, and the delineation of the fibres was determined based on the staining intensity and mitochondrial content of the SDH-stained slides. In order to enhance objectivity and to eliminate intra-examiner variation, random sections were also examined by qualified members of staff (Dr.Ian Montgomery and Mr.David Russel).

2.6 Strength Training

The subjects took part in a 12 week long training program of the quadriceps of their non-dominant leg. The intensity and volume of the training were set to elicit both strength gains and muscle hypertrophy (Baechle 1994). The training ran for 12 weeks to allow muscular adaptations to occur, in particular changes in the cross sectional area of the muscle fibres. There is a contradiction in the literature regarding the neural adaptation to exercise. Adaptations during the first 5 weeks are mainly neural (Sale, 1988) with improvements in strength resulting from the learning effect – the subjects become more familiar with the apparatus and exercise. During a familiarization session, each subject's 1-repetition maximum (1-RM) load for non-dominant leg extension on the training device (leg extension machine, paramount inc.) was determined. Each training session consisted of one warm-up set of 10 repetitions at 25 % of 1-RM followed by 4 sets of 10 repetitions at 70 % 1-RM with 1-minute rest between sets. Each repetition required one second to lift the weight and one second to lower the weight. The training was performed $3 \cdot wk^{-1}$ for 12 wk, that is, a total of 36 training sessions (see Fig 2.4). All training sessions were supervised, with continual verbal encouragement given throughout. Before the training was started, the leg extension was set up individually for each subject. The seat was set at the correct setting based on subjects' anthropometric characteristics; this was reassured by providing a 2 cm space between the back of the subject's knee and the edge of the seat. The lever for the roller pad was set at the correct length for the subject – resting on the top of the subject's ankle. The settings for each subject were recorded. If the subject could not complete 10 repetitions at the training weight, the load was immediately reduced by 2 increments (each weight increment was 5 lb (pounds)) on the leg extension and the set of 10 was completed. This ensured that each subject completed a total of 40 repetitions every training session. Once the subject performed two consecutive training sessions (when they completed four sets of ten reps without dropping the weight), the load was increased by one increment on the leg extension machine for the next session.

2.7 Strength Testing

Subjects were asked to refrain from exercise and alcohol intake for 24 hours prior to testing. Baseline measurements were taken twice within a 7 day period (at least 3 days apart), and post-training measurements were taken once between 3 and 7 days after the final training session. The reported baseline strength data is the highest values observed on either of the test days. During the training period, strength tests of both the trained and untrained leg were carried out once a week. The highest value from three attempts in each test was recorded. Prior to initial strength testing, each subject was familiarised with the testing protocols during a full practice session to enhance reliability of measurement (Kues at al., 1992), and the individual subjects anthropometric measurements were stored in the Kin-Com Kinematic Dynamometer (Kin-Com, Chattanooga, TN). On all test days, testing sessions involved using the Kin-Com to obtain values for isometric strength at each of the joint angles of 5º, 30º and 60º, and isokinetic strength at an angular velocity of $60^{\circ}\cdot sec^{-1}$ and $180^{\circ}\cdot sec^{-1}$. The slower isokinetic angular velocity of $60^{\circ}\cdot sec^{-1}$ was tested first in order to facilitate motor learning prior to testing at faster velocities (Griffin et al., 1987). Each isometric measurement involved at least three practice trials, and then at least three maximum voluntary contractions (MVC) with at least 30 sec rest between each. The isokinetic measurement involved at least three practice trials, and then one set of three consecutive repetitions. The training load produced from the strength testing protocol was not designed to elicit any training effects on the untrained leg that was tested weekly. For each maximal trial, subjects were given verbal encouragement and instructed to produce as much force as possible for at least four seconds (isometric) or throughout the duration of the contraction (isokinetic).

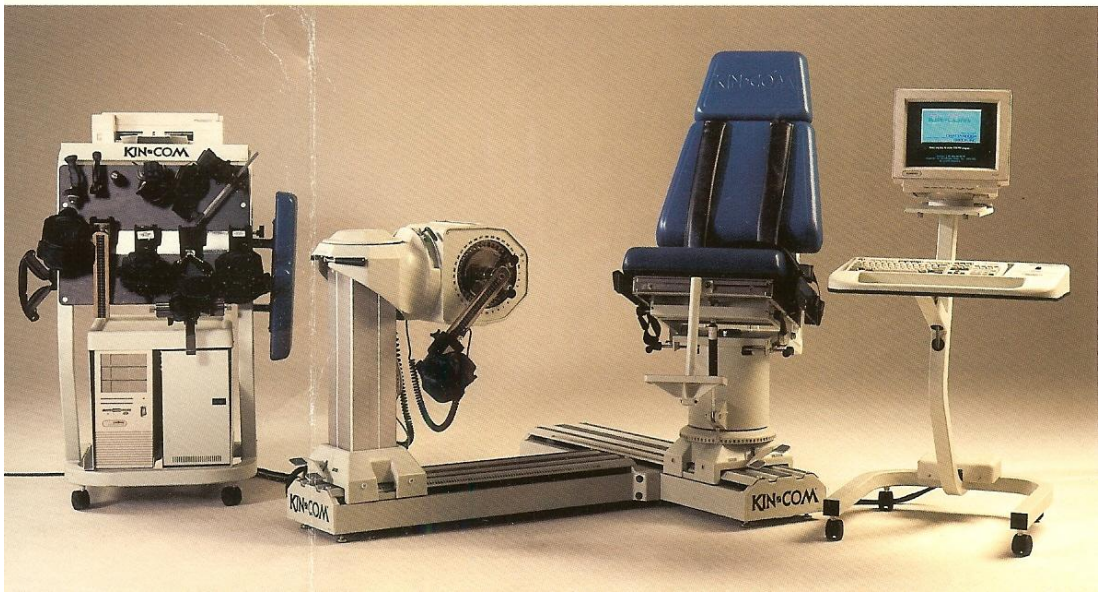


Figure 2.3: Kin-Com Kinematic Dynamometer (Kin-com,Chattanooga,TN)

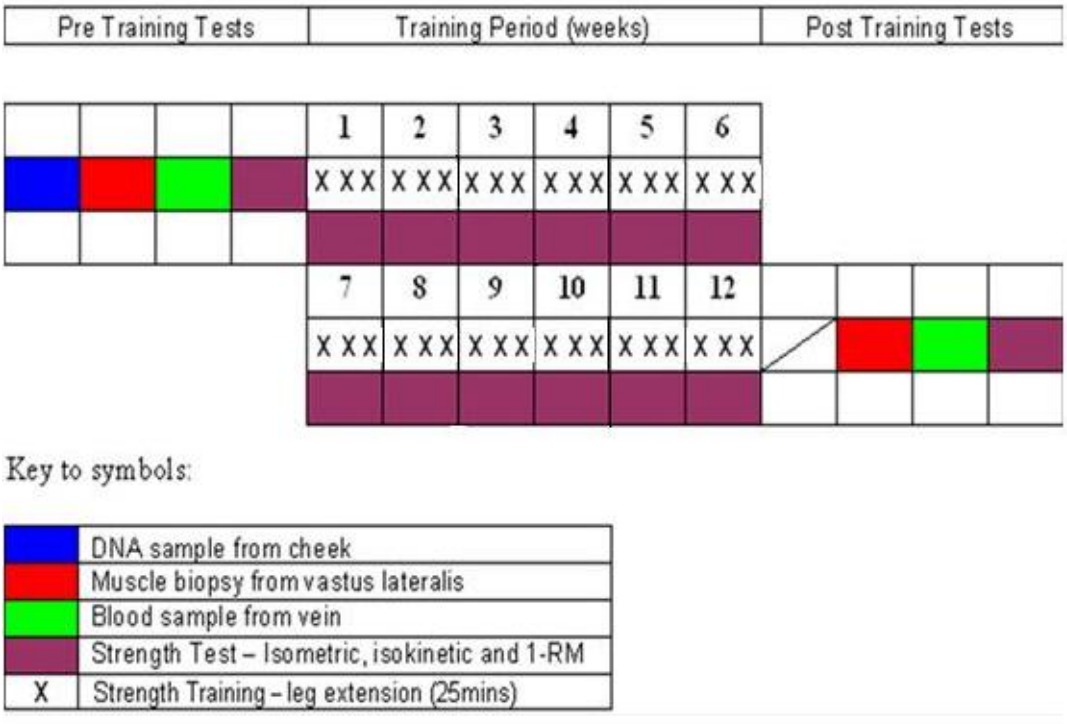


Figure 2.4: Schematic experimental procedure.

2.8. Statistical analysis

Statistical analysis was performed using SPSS (Statistical Package for Social Sciences) version 15.0 for Windows and Statistica 8.0 (Stat Soft Inc., 2007) for Windows. For the purpose of the study, the data set were checked for outliers, which were defined by SPSS. Outliers were characterized by SPSS as lowest and highest extreme values, but were not excluded from the analysis, as they were considered important for the data set. Data were checked for normality using the Shapiro-Wilk tests, as it is supported by many authors to be more appropriate for a check of non-normality in small to medium samples (Shapiro and Wilk, 1965; Royston, 1982a, 1982b, 1995; Conover, 1999). Parametric and non-parametric tests were used throughout the analysis. Paired t-tests or Mann-Whitney tests where appropriate were used to compare the effect of the strength training program both for untrained and trained leg, the changes in the average cross-sectional area, and the percentage of the muscle fiber types pre and post, as well as the circulating ACE activity pre and post. Linear trend analysis was performed to exclude the confounding effects of weight, BMI, physical activity levels etc. Two-way ANOVA for repeated measures were used to assess the training responses over times and post-hoc analysis included Bonferroni tests. In cases where significance was identified, the difference for the factor was further determined for each time point using paired-sample t-tests. The Pearson product-moment correlation coefficient (r) or Spearman's rho was used to investigate the relationship between baseline ACE activity and baseline muscle strength, between ACE activity, fiber types and fiber cross-sectional area, ACE activity and *ACE* genotype, *ACE* genotype and baseline characteristics and on the adaptations to strength training. The effects of *ACE* genotype on baseline characteristics (with baseline body mass, stature, BMI and activity levels as covariates), on the adaptations to strength training (with baseline body mass, stature, BMI, and strength and activity levels as covariates) and on muscle morphology (with strength and physical activity levels as covariates) were assessed for linear trend using General Linear Model Analysis. The partial eta squared effect size statistic (which indicates the proportion of the effect and error variance that is attributable to the effect) was obtained during General Linear Model Analysis (GLM), as well as the observed power (p).

Data is expressed as mean ± standard error of the mean (SEM) unless otherwise stated. The accepted level of significance was set at $P < 0.05$ for all statistical tests.

3. Results

3.1 Subject characteristics

All 18 subjects completed the study according to the protocol described in the methods section. Their mean ± STDV baseline characteristics are depicted in Table 3.1. Changes in circulating ACE activity are shown in Table 3.1.*i*. No significant differences were found between the two groups (II and DD) as assessed by general linear model analysis and independent t-tests with the level of significance set at $P = 0.05$.

Table 3.1: Mean (± STDV) physical characteristics of the total sample and by ACE ID genotype.

	DD	**II**	**DD + II**	***P***
	(n=7)	*(n=11)*	*(n=18)*	*(DD vs II)*
Weight (kg)	75.1 ± 10.22	76.9 ± 7.3	76.2 ± 8.2	0.67
Height (cm)	179 ± 0.08	179 ± 0.08	179 ± 0.1	0.92
BMI (kg/m^2)	23.1 ± 1.3	23.8 ± 1.16	23.6 ± 1.4	0.29
Physical activity levels (h/week)	11 ± 3.1	8.1 ± 3.5	9.2 ± 3.5	0.08
Age (y)	23.1 ± 3.2	22.8 ± 2.9	22.9 ± 2.8	0.92

There were no significant differences in physical characteristics among the ACE ID genotypes. I, insertion allele; D, deletion allele; BMI,body mass index, P < 0.05.

Table 3.1.i: Changes in circulating ACE activity from pre to post-training (mean ± SEM).

	DD	**II**	**DD + II**	***P***
	(n=7)	*(n=11)*	*(n=18)*	*(DD vs II)*
Circulating ACE activity (nmol His-Leu/min/mL)				
Pre	39.7 ± 40	40.5 ± 3.30	40.2 ± 2.5	0.89
Post	41.9 ± 3.7	35.5 ± 4.30	38.1 ± 3.0	0.30
P ***(Pre vs Post)***	*P* = 0.10	*P* = 0.26	*P* = 0.41	

There were no significant differences in circulating ACE activity among the ACE ID genotypes.ACE, angiotensin converting enzyme; I, insertion allele; D, deletion allele; P < 0.05.

3.3 Muscle fiber average cross-sectional area changes

A significant difference was noticed in the average cross-sectional area (AVECSA) of Type IIA between pre and post: (4264 ± 224 μm² pre-training, 4774 ± 226.7 μm² post-training, t(17)= -2.261, *P* = 0.02), as well as Type IIB AVECSA: (3996 ± 170 μm² pre-training, 4463 ± 217 μm² post-training, t (17) = -2.142, *P* = 0.04)). On the contrary, the change in the AVECSA of Type I fibres wasn't deemed significant (3583 ± 284 μm² pre-training, 3902 ± 250 μm² post-training, t (17) = -1.664, *P* = 0.11) when data was assessed regardless genotype (see also Table 3.2). When the data was assessed for II and DD individuals separately, for II genotype (n = 11), AVECSA of Type I increased significantly (3345 ± 207 μm² pre-training, 3988 ± 239 μm² post-training, t (10) = -3.063, *P* = 0.01)(see also Table 3.2). For DD genotype (n = 7), a significant increase in AVECSA of Type IIA fibres (4070 ± 506 μm² pre-training, 4674 ± 399 μm² post-training, t (6) = -2.999, *P* = 0.02), as shown in Table 3.2. Two-way analysis of variance for repeated measures did not show any differences in the II and DD response to the training program. More specific, ACE genotype did not affect the AVECSA of Type I fibers (F (1, 32) = 0.243, *P* = 0.62), as well as the AVECSA of Type IIA fibers (F (1, 32) = 0.512, *P* = 0.48) and the AVECSA of Type IIB fibers (F (1, 32) = 0.049, *P* = 0.84) (see also Table 3.2)

Table 3.2: *Mean (±SEM) muscle fiber type and average cross-sectional area at baseline and post-resistance training in the total sample and by ACE II/DD genotype. The accepted level of significance was P < 0.05. Asterisk (*) indicates significant difference from pre-training values.*

		II GENOTYPE (n=11)	**DD GENOTYPE** (n=7)	**DD vs II** (n=18)	**DD + II** (n=18)
Proportion of fibre types (%)					
Type I	*Pre*	50.2 ± 1.5	50.8 ± 3		50.4 ± 1.4
	Post	48.1 ± 1.6	52.9 ± 4.6		49.9 ± 2
	Δ	2.1 ± 2.2	2.1 ± 2.9	*P* = 0.31	0.4 ± 1.7
Type IIA	*Pre*	28 ± 1.1	26.2 ± 1.8		27.3 ± 0.9
	Post	28.2 ± 1.2	25.9 ± 3.8		27.4 ± 1.6
	Δ	0.2 ± 1.7	0.3 ± 3	*P* = 0.31	0.15 ± 1.5
Type IIB	*Pre*	21.9 ± 1.1	22.9 ± 2.3		22.3 ± 1.1
	Post	23.6 ± 1.6	21.1 ± 1.6		22.6 ± 1.2
	Δ	1.7 ± 1.8	1.8 ± 1.7	*P* = 0.65	0.33 ± 1.3
Average Cross Sectional Area per fibre type (μm²)					
Type I	*Pre*	3345 ± 207	3955 ± 660		3582 ± 283
	Post	3988 ± 239*	3765 ± 549		3901 ± 250
	Δ	643 ± 210	190 ± 285	*P* = 0.62	319 ± 191
	Δ %	22 ± 8	1 ± 8.5		13 ± 6.3
Type IIA	*Pre*	4386 ± 194	4070 ± 506		4263 ± 224
	Post	4837 ± 284	4674 ± 399*		4774 ± 226*
	Δ	451 ± 321	604 ± 201	*P* = 0.48	511 ± 207
	Δ %	12 ± 8.9	21 ± 10.5		16 ± 6.6
Type IIB	*Pre*	3972 ± 174	4033 ± 363		3996 ± 170
	Post	4533 ± 278	4353 ± 369		4463 ± 216*
	Δ	561 ± 318	320 ± 270	*P* = 0.84	466 ± 217
	Δ %	16 ± 9.4	10 ± 8.1		14 ± 6.4

Muscle Strength for Untrained and Trained Leg

3.4 Muscle strength and force production

Muscle strength was measured as force and torque production during maximal isometric and isokinetic knee extensions respectively on an isokinetic dynamometer. Baseline measurements (pre-training) indicated no significant differences between the untrained and trained leg. After the 12 week strength training program of the trained leg, the increase in force production is obvious, with significant differences from the pre-training status, as well as from the untrained leg force production as assessed from student's paired t-tests. A significant increase (680 ± 30 N pre-training, 793 ± 40.38 N (SEM) post-training, *P* = 0.001) was noticed for the 5 ° isometric angle of the trained leg. The mean force of the trained leg at 30 ° isometric angle also increased (759 ± 36 N pre-training, 956 ± 36 N (SEM) post-training, *P* = 0.00), and so did 60 ° isometric angle (444 ± 17 N pre-training, 566 ± 20 N (SEM), *P* = 0.00) and 60 °/s isokinetic angle (605 ± 20 N pre-training, 676 ± 19 (SEM) post-training, *P* = 0.002). 180 °/s isokinetic angles in both legs deemed no significant results (see table 3.3).

Degrees	Leg Dominance		Pre	Post
		Untrained	683 ± 32	678 ± 37
	Δ %			-1 ± 2
	Δ			-5 ± 17
5°		Trained	680 ± 30	793 ± 40 * †
	Δ %			18 ± 4
	Δ			114 ± 28
		Untrained	736 ± 34	741 ± 32
	Δ %			1 ± 2
	Δ			5 ±17
30°		Trained	759 ± 36	956 ± 36 *†
	Δ %			28 ± 3
	Δ			197 ± 22
		Untrained	449 ± 17	462 ± 17
	Δ %			3 ± 2
60°	Δ			13 ± 10
		Trained	444 ± 17	566 ± 20 *†
	Δ %			30 ± 6
	Δ			122 ± 22
		Untrained	599 ± 25	567 ± 21 *
	Δ %			-5 ± 2
60°/sec	Δ			-32 ± 13
		Trained	605 ± 20	676 ± 19 *†
	Δ %			13 ± 4
	Δ			71 ± 19
		Untrained	505 ± 16	482 ± 17
	Δ %			-4 ± 3
180°/sec	Δ			-22 ± 12
		Trained	524 ± 13	532 ± 13†
	Δ %			8 ± 12
	Δ			2 ± 2

Table 3.3: *Mean ± SEM of the muscle strength of all subjects pre-and post-training in all angles, untrained and trained leg values are also included. Asterisk (*) indicates significance towards baseline (pre) values (P < 0.05), dagger (†) indicates significance towards the values for the untrained leg, (N) = Newton, n = 18, Δ is the difference between pre and post, Δ % is the percentage of difference between post and pre, P < 0.05 for all tests.*

When the data is presented genotype-dependant, we can see that there were no significant differences between gains in force for II and DD subjects, except from the isometric 60° angle, where a significant difference were detected between the gain in force of the trained leg post-training in favor of the DD individuals (as assessed with two-way ANOVA for repeated measures). Trained leg presented greater strength gains compared to the untrained leg in all angles for both genotypes, with significant differences between pre and post as assessed by Student's paired t-test (see table 3.4).

Degrees	Leg Dominance	Genotype			
		DD		II	
		Pre	Post	Pre	Post
5°	Untrained	678 ± 32	658 ± 53	687 ± 50	692 ± 53
	Δ %		-4 ± 5		1 ± 2
	Δ		-20 ± 34		5 ± 19
	Trained	702 ± 34	798 ± 11 *‡	667 ± 45	791 ± 67 ‡
	Δ %		15 ± 6		19 ± 6
	Δ		97 ± 34		124 ± 42
30°	Untrained	741 ± 30	728 ± 41	734 ± 54	750 ± 48
	Δ %		-2 ± 4		3 ± 3
	Δ		-13± 33		16 ± 19
	Trained	831 ± 33 ‡	1017 ± 38 *‡	714 ± 52	918 ± 52 *‡
	Δ %		23 ± 4		31 ± 5
	Δ		187 ± 28		204 ± 31
60°	Untrained	461 ± 23	465 ± 22	443 ± 24	460 ± 26
	Δ %		1 ± 2		4 ± 3
	Δ		4 ± 11		17 ± 15
	Trained	473 ± 25	617 ± 25 *‡†	427 ± 24	534 ± 27 *‡
	Δ %		32 ± 8		28 ± 9
	Δ		144 ± 32		108 ± 30
60°/sec	Untrained	626 ± 40	594 ± 37	582 ± 34	551 ± 26
	Δ %		-5 ± 4		-4 ± 3
	Δ		-32 ± 19		-31 ± 19
	Trained	618 ± 37	699 ± 23 ‡	597 ± 25	662 ± 28 *‡
	Δ %		16 ± 9		12 ± 4
	Δ		81 ± 38		65 ± 21
180°/sec	Untrained	510 ± 15	483 ± 25	502 ± 26	483 ± 24
	Δ %		-5 ± 4		-3 ± 3
	Δ		-27 ± 23		-19 ± 15
	Trained	531 ± 15	530 ± 22	520 ± 20	532 ± 18 ‡
	Δ %		0 ± 3		3 ± 4
	Δ		-1± 15		13 ± 17

Table 3.4: *Changes in strength output of the untrained and trained leg by genotype. Data presented as mean ± SEM , double dagger (‡) indicates significance trained leg vs untrained leg, dagger (†) indicates significance towards II genotype, asterisk (*) indicates significance pre vs post, Δ is the difference between pre and post, Δ % is the percentage of difference between post and pre. P < 0.05 for all tests.*

Correlations

For those completing the training program, baseline circulating ACE activity did not alter with training (see Table 3.1). In addition to this, there were no significant differences between pre- and post-strength training levels of circulating ACE activity when total sample was taken into account (DD+II, n = 18), or when DD and II were tested separately (see Table 3.1). As shown in figure 3.1 and figure 3.2, baseline strength measures correlated significantly with circulating ACE activity. This applied mainly to isometric strength of the untrained leg at 5° (683 ± 32.3 N) (r = 0.46, *P* = 0.05) (Fig.3.1), and isokinetic at 180°/sec of the untrained leg (505 ± 16.3 N) (r = 0.52, *P* = 0.03) (Fig.3.2). Weaker correlations were found between circulating ACE activity pre and post and other degrees and velocities, although not significant (data shown in the appendix). Circulating ACE activity measured prior to the strength training, correlated positively with weight and height of the subjects (r = 0.62, *P* = 0.01 and r= 0.65, *P*= 0.01 respectively). Physical activity levels of the subjects correlated with the isometric strength at 30° of the trained leg (r= 0.49, *P* = 0.03, data also shown in the appendix). Average cross-sectional area of type IIA and IIB fibers post-training, correlated positively and significantly with the post-training strength output of the trained leg at 60°/sec (r = 0.56, *P* = 0.01 and r = 0.59, *P* = 0.01 respectively), but there was no correlation between the AVECSA of all fiber types or percentages and the circulating ACE activity pre-or post-training (data shown in the appendix). The second correlation persisted after the data were presented as force per kilogram of body mass (r = 0.49, *P* = 0.03). When the data were processed as force production per kg of body mass, all the significant observations made above (significant correlations) did not persist and became non-significant. In addition to this, spurious correlations without any known biological reason appeared throughout the correlation tables (data shown in the appendix). The power limitation of the study prevents us from attributing those correlations to an actual relationship between the variables.

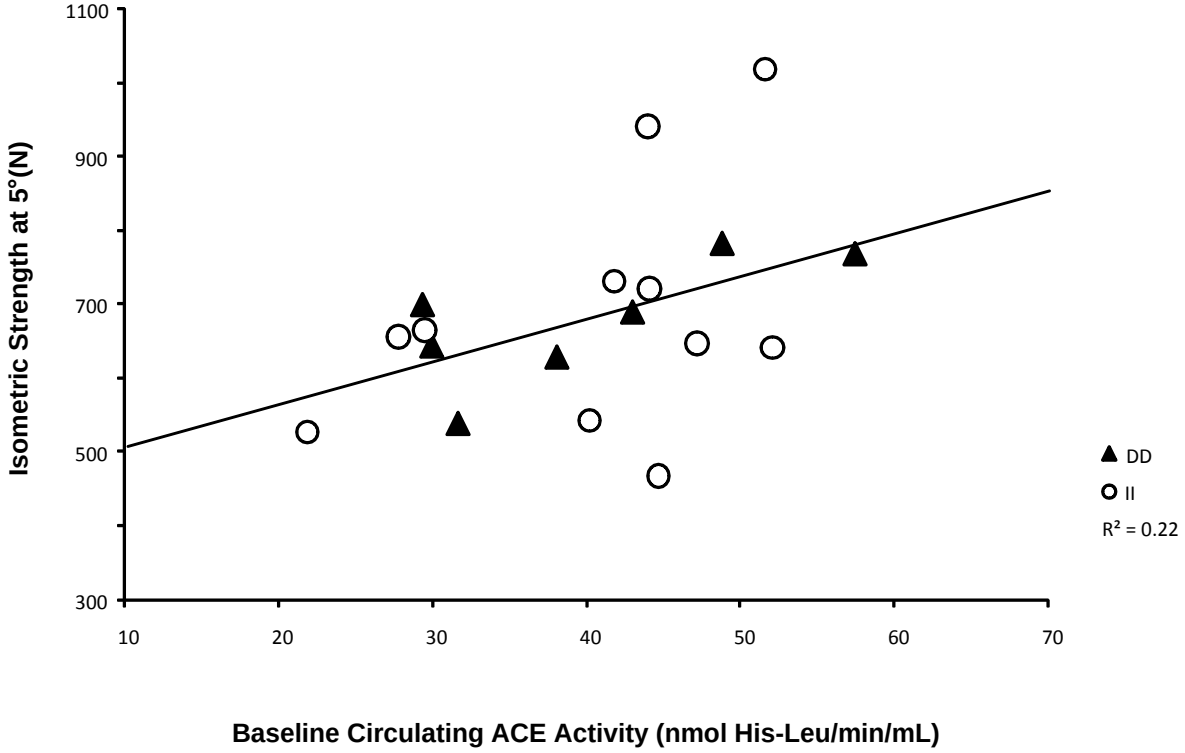


Figure 3.1: Relationship between pre-training circulating ACE activity (nmol His-Leu/min/mL) and isometric strength (baseline measurements) at 5°of the untrained leg, n = 18, r = 0.46, *P* = 0.05 as assessed by Pearson's product-moment coefficient correlation. Solid line: best-fit linear regression line.

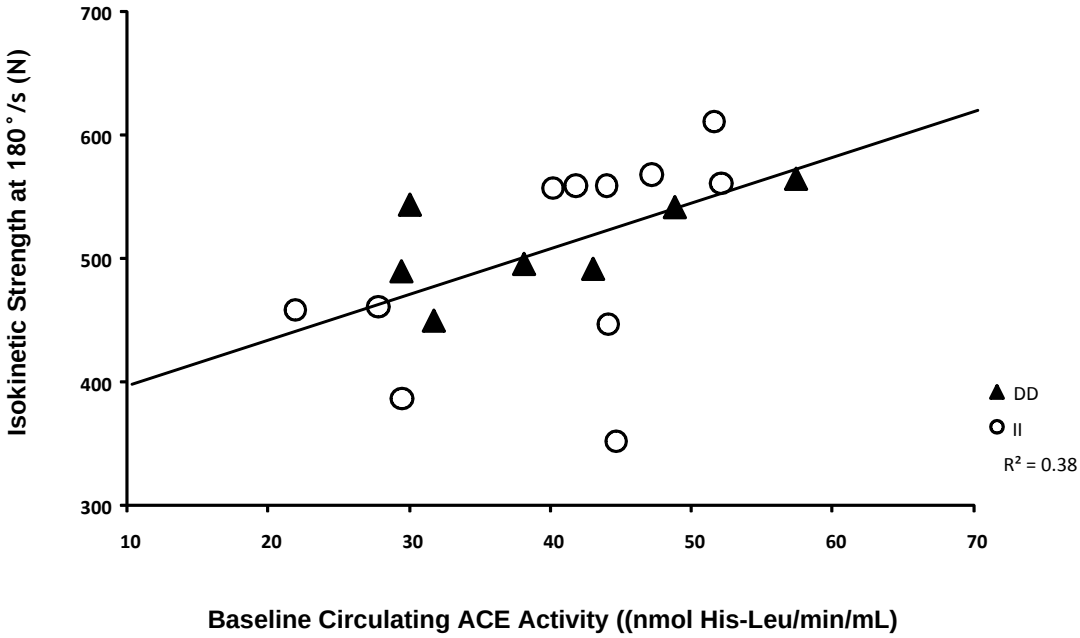


Figure 3.2: Relationship between pre-training circulating ACE activity (nmol His-Leu/min/mL) and isokinetic torque (baseline measurements) at 180°/s of the untrained leg, n = 18, r = 0.52 , *P* = 0.03 as assessed by Pearson's product-moment coefficient correlation. Solid line: best-fit linear regression line.

3.5 Main effects and significant interactions

Two way analysis of variance for repeated measures, revealed some significant effects and interactions. Specifically, for isometric force at 30º, the effect of leg dominance was F (1, 64) = 13.919, *P* = 0.01, but genotype was not affecting strength (F (1, 64) = 2.010, *P* = 0.16), see also figure 3.3.

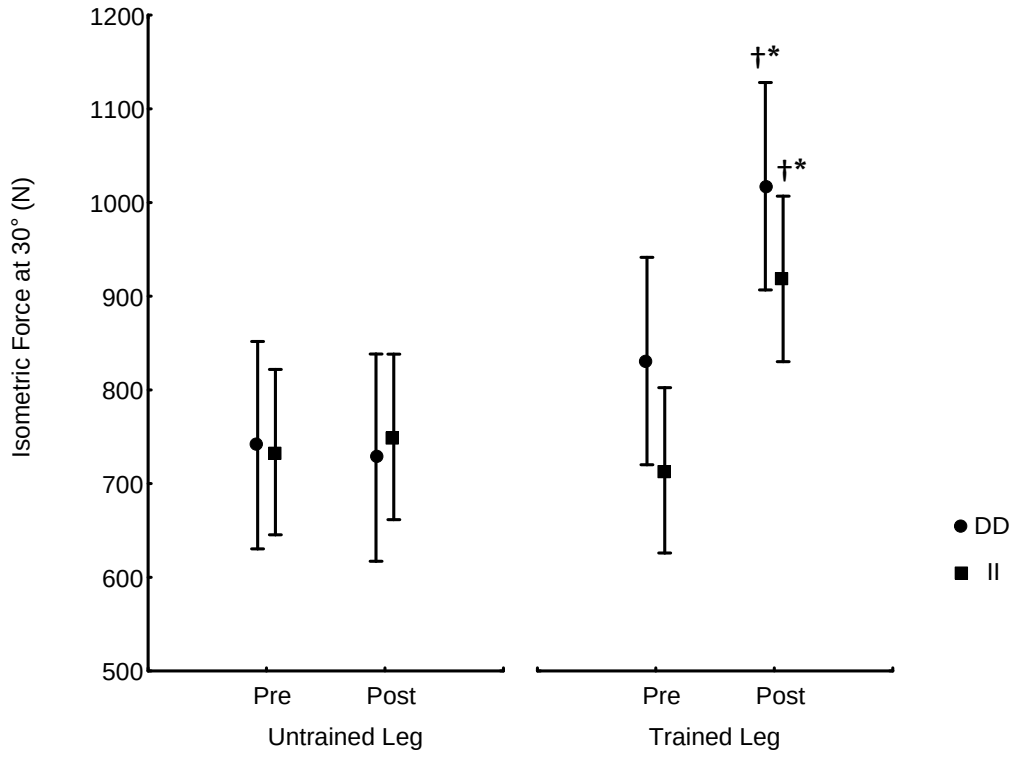


Figure 3.3: The effect of the leg dominance on isometric force in 30° angle pre- and post-training, the asterisk (*) indicates significance towards pre-training, dagger (†) indicates significance towards untrained leg. Data presented as mean, vertical bars indicating confidence intervals (95%).

On the contrary, in 60 ° post-training, a significant effect of the genotype was revealed in favour of the DD individuals on the trained leg F(1,64) = 4.242 , *P* = 0.043, observed power = 0.53 ,partial eta squared = 0.062, see also figure 3.4). The effect persisted after adjustment for weight, but when it was adjusted for body mass index and physical activity, the results became non significant (F (1, 63) = 3.13391, *P* = 0.08, partial eta squared = 0.047, observed power = 0.41) and F (1, 63) = 3.1628, *P* = 0.08, partial eta squared = 0.048, observed power = 0.42 respectively).

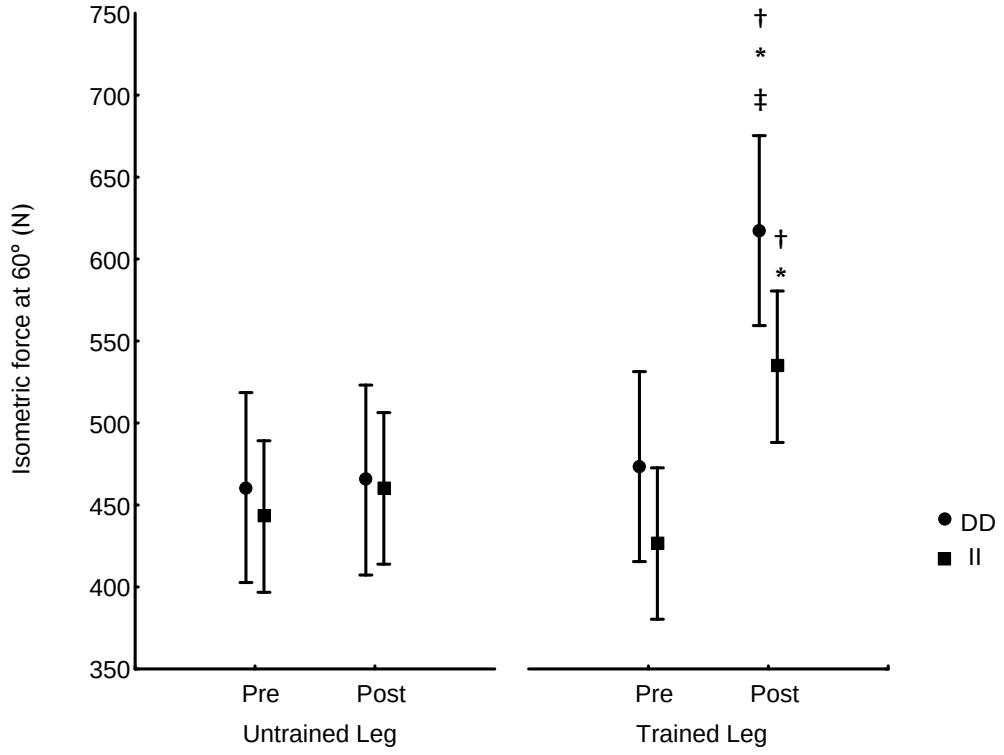


Figure 3.4: The effect of the leg dominance on isometric force pre- and post-training in 60° angle. Asterisk (*) indicates significant difference from baseline (pre), dagger (†) indicates significance towards untrained leg, double dagger (‡) indicates significance towards II genotype. Data presented as mean, vertical bars indicating confidence intervals (95 %), level of significance set at *P* < 0.05.

Trained leg overtook untrained (F (1,448) = 86.68, *P* = 0.00) in many time points during the 12 week strength training program (see figures 3.5 and 3.6). Isokinetic velocities of joint movement (60º/sec, and 180º/sec) both were not affected by genotype, but trained leg gained significantly higher force than untrained leg at 60º/sec (F(1,448) = 58.99, *P* = 0.00) and (F (1,448)=13.64, *P* = 0.01) at 180º/sec.

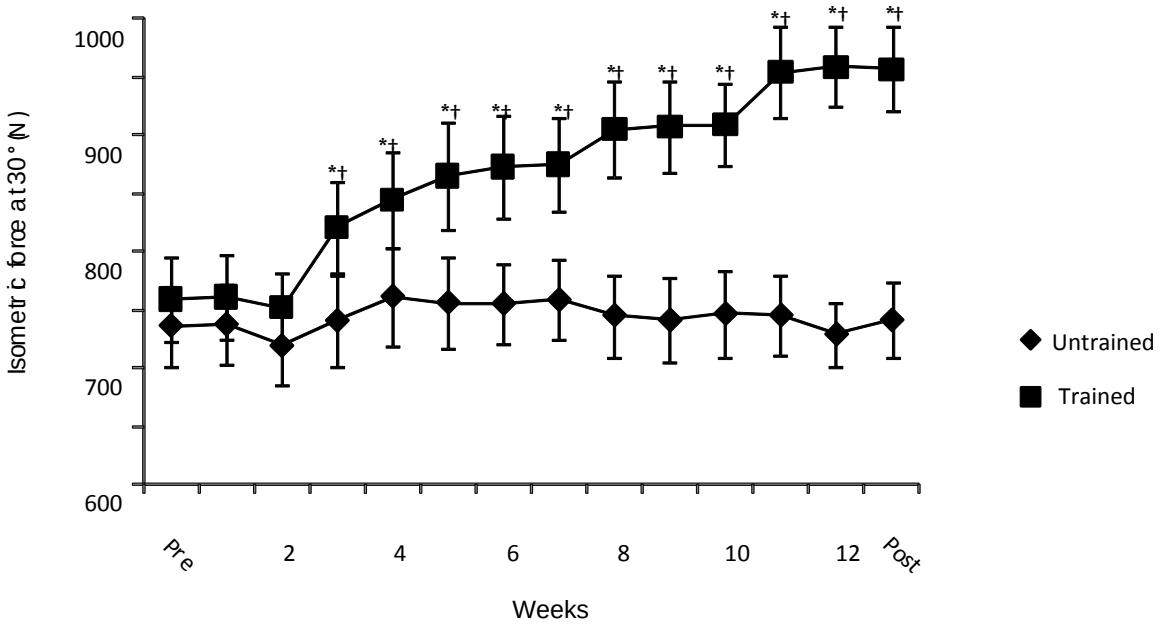


Figure 3.5: Data is mean ± SEM for all subjects (n = 18) for untrained and trained (square) leg through 12 weeks of strength training in 30º angle. Asterisk (*) indicates significantly different from baseline measurements (pre). Dagger (†) indicates trained leg significantly different from untrained, P<0.05.

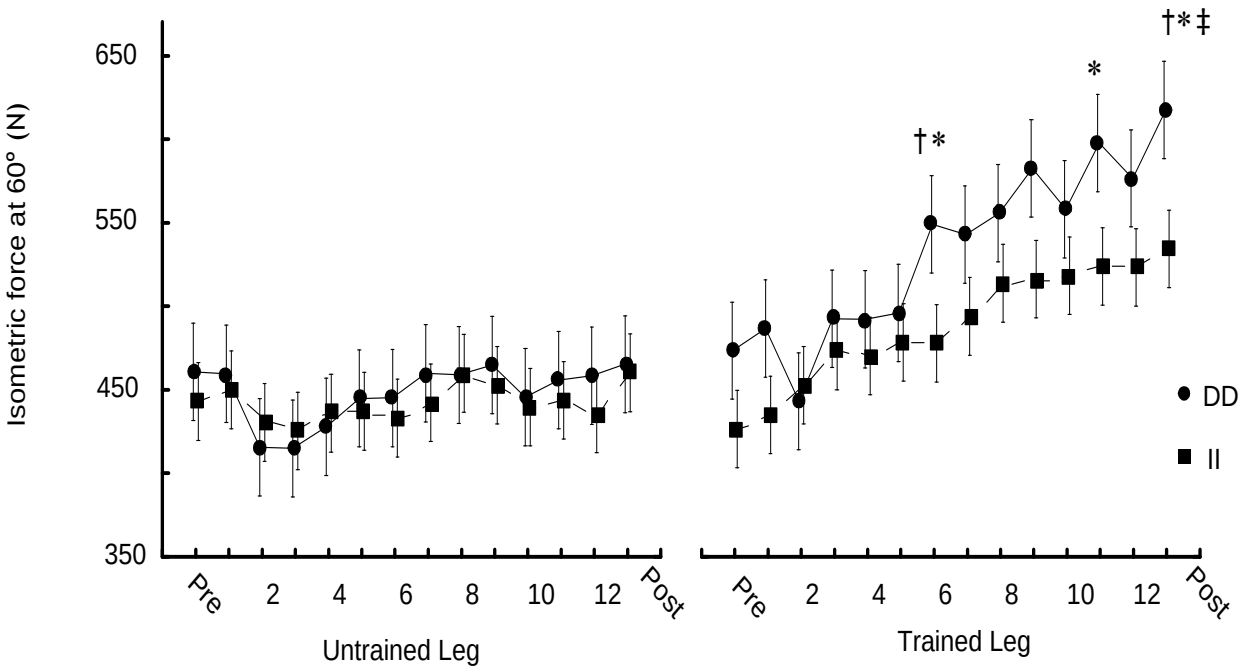


Figure 3.6: Data expressed as mean ± SEM of 18 observations (n = 18) in 60° isometric angle through 12 weeks of training. Asterisk indicates significantly different from baseline measurements (pre). Dagger (†) indicates trained leg (black circle) significantly different from untrained leg (black square), double dagger (‡) indicates significance towards II genotype, $P < 0.05$.

For the isokinetic velocities of 60°/sec and 180°/sec, no significant effect of genotype was detected ($F(1, 64) = 2.558$, $P = 0.11$ and $F(1, 64) = 0.063$, $P = 0.80$ respectively), even when the data was presented per kg of body mass.

4. Discussion

The aim of this study was to evaluate the association of *ACE* I/D polymorphism and circulating ACE activity with quadriceps muscle function and contractile properties, as well as with changes in skeletal muscle fiber type or cross-sectional area in response to strength training programme. It was hypothesized that baseline strength would be higher in homozygotes for the D allele, compared to homozygotes for the I allele. Our study did not find any association between baseline strength measurements and *ACE* gene, but the association found is after the training intervention and thus adaptation to strength training. It was also hypothesized that there would be a difference in the strength development between the homozygotes for the I allele carriers and the D allele carried, and based on the existing literature, homozygosity for the D allele will be favored. Isometric force at 60º post-training revealed a significant effect of the genotype, in favour of the DD individuals, on the non-dominant leg F (1, 64) = 4.242, *P* = 0.04, observed power = 0.53, partial eta squared = 0.062). The effect persisted after adjustment for weight, but when it was adjusted for body mass index and physical activity (assessed by questionnaires), the effect became non-significant (F (1, 63) = 3.13391, *P* = 0.08, partial eta squared = 0.047, observed power = 0.41); and F (1, 63) = 3.1628, *P* = 0.08, partial eta squared = 0.048, observed power = 0.42, respectively). Last, it was hypothesized that Type II fibres of homozygotes for the D allele will show more hypertrophy 11 than I homozygotes for the I allele in response to a strength training 12 programme. The average cross-sectional area (AVECSA) of Type IIA fibres for the DD individuals increased significantly post-training (4070 ± 506 μm^2 pre-training, 4674 ± 399 μm^2 post-training, t (6) = -2.999, *P* = 0.02) and so did the AVECSA of the Type I fibers of II individuals (3345 ± 207 μm^2 pre-training, 3988 ± 239 μm^2 post-training, t (10) = -3.063, *P* = 0.01, see Results section for all the above). The latter finding shows that the strength training programme applied resulted in muscle hypertrophy, but the changes in AVECSA were not genotype-related as two way analysis of variance for repeated measures revealed (see Table 3.2).

In the present study an association was detected between circulating ACE activity and isometric/isokinetic angles/velocities, with the strongest correlations depicted in figures 3.1 and 3.2. Those findings come in line with the results reported from Williams et al., (2005), who found a relationship between the isokinetic velocity of 60° (1.05 rad· sec^{-1}) and pre-training circulating ACE activity, in a total of 81 subjects. Pearson's r was r = 0.375, P < 0.00050 (Williams et al., 2005). In our study, a significant correlation was noticed between the isometric strength of the untrained leg at 5° and circulating ACE activity, in a total of 18 subjects (Pearson's r = 0.46, *P* = 0.05) and isokinetic strength at 180°/sec of the untrained leg (Pearson's r = 0.52, *P* = 0.03), see figures 3.1 and 3.2 respectively. Hopkinson et al., (2004) also found that in patients with COPD (chronic obstructive pulmonary disease) strength results were associated with the D allele of the ACE gene. On the contrary, there are many studies that do not find an actual relationship between circulating ACE activity and strength gains. Folland et al., (2000) for example, did not find any association between pretraining strength of quadriceps and *ACE* gene, but one could claim that this was due to the fact that he didn't measure circulating ACE activity alone but he rather used an indirect genetic marker, such as *ACE* genotype. Moreover, this study didn't have the power to detect such an association as Williams and colleagues (2005) indicate. In line with this, Thomis et al., failed to prove the same association in the upper body muscles though (elbow flexors). Thomis et al., (2004) found no evidence for an *ACE* D allele effect on skeletal muscle response to functional overload. In the present study, study, circulating ACE activity was measured alone, although the correlations found might be correlations occurred by chance, as neither our study is powered to detect any significant effects. According to power calculations, 46 subjects at least would be sufficient if a power of 80% of detecting a meaningful correlation is desired. More recently, Charbonneau et al., (2008) on a large study conducted on both men and women (although underpowered for sex-related associations), and based on the existent literature, concluded that there was an association between the *ACE* genotype and baseline strength measurements, but no associations were observed for the 1 RM or the adaptations to the strength training for either men or women. Subjects in the previous study had a mean age of 62 years old (between 50 and 85); a factor which might possibly set a limit to the findings of this study as far as the generation of force is concerned. In

addition to that, ageing is associated with considerable strength loss and declining muscle mass (Kallman et al., 1990) along with mobility impairments and, especially in women after menopause, a notable loss of muscle mass when the hormonal balance is changed. The decline in men starts below the age of 70, and by 90 years old, there is a reduction in muscle mass of a magnitude of 30 % (Grimby and Saltin, 1983). Our study did not find any association between baseline strength measurements and *ACE* gene, but the association found is after the training intervention and thus adaptation to strength training. A recent study from Day and his colleagues (2007) also found no correlation between circulating ACE activity and VO_2max or mechanical efficiency in 62 untrained women of a sedentary background Day et al., 2007). In a very recent study, McCauley and his colleagues (2009) examined the relationship between *ACE* I/D and *ACTN3* R/X polymorphisms, with muscular strength at high velocities. Seventy-nine recreationally active but non-strength-trained males participated, and measurements were taken in two occasions, isometrically (73 °, 1.27 rad) and isokinetically (30 °/sec, 90 °/sec and 240° /sec,which is 0.52, 1.57 and 4.19 rad/sec respectively). The characteristics of the subjects did not differ from our cohort, and the methods used for the assessment of the various parameters were similar to our study. Those two polymorphisms didn't influence muscular strength at high velocities or the time course of the twitch response, nor there any association of circulating ACE activity with any measure of muscle function, indicating that the magnitude of the effect is not sufficient for associations of this kind (McCauley et al., 2009). In addition to the power problems that gene association studies present, regarding the *ACE* gene, a large inconsistency in the literature is noticed, fact that makes comparison among studies particularly difficult. Charbonneau and his colleagues (Charbonneau et al., 2008) adopt a very skeptical attitude towards whether *ACE* is a gene with strong relevance for skeletal muscle phenotypes, because of the problems mentioned above. Differences in methodology between the studies reduce significantly reproducibility and prevent replication of data in an independent cohort.

One would expect that since circulating ACE activity is associated with strength, the rationale suggests that the genotype will be related in a similar way. In the present study, probably for methodological reasons such as the sample size or the differences between the number of individuals in each group, circulating ACE activity was not associated with *ACE* genotype (see Results, Table 3).The two groups (II and DD) presented similar values. Williams and colleagues (2005) for example found that 4-7 % of the variation in strength could be attributed to *ACE* genotype. Here, a methodological issue arises: many previous studies (Charbonneau et al., 2008, Thomis et al., 2004, Folland et al., 2000, Pescatello et al., 2006, Karjalainen et al., 1999, Taylor et al., 1999, Rankinen et al., 2000, Zhao et al., 2003) were looking at the genetic marker alone (*ACE* II/DD polymorphism) instead of looking directly to ACE activity. *ACE* genotype is a categorical variable and thus cannot be quantified in a meaningful way. Montgomery et al., (2002) in a recent review article, explored whether it is better to use phenotype or genotype as a tool for investigating the role of ACE. It was suggested that, because much of the ACE activity within the circulation cannot be explained solely by an individual's ACE ID polymorphism genotype,
ACE phenotype, and not merely the genotype, is associated with many diseases. It is therefore possible that the circulating ACE activity is more strongly associated with endurance performance than the ID polymorphism within this gene (Montgomery et.al, 2002). The problem, as mentioned in the paper from Williams et al., (2005) with using a categorical variable in order to establish a correlation of a desirable effect, is that the number of the subjects required is almost ten times the number of participants needed when a continuous variable is examined. For example, in the aforementioned study (Williams et al., 2005), interim power calculations showed that more than 300 subjects would be required to establish statistical significance for correlations of the magnitudes that were observed, and moreover, "the added power of seeking association with a continuous variable (ACE activity) rather than categorical surrogate of ACE activity (*ACE* genotype) suggests that a sample size of much greater than 300 would be required to identify a very weak effect of *ACE* genotype on the training response" (Williams et al., 2005).
There is also another reason that supports this view. In the present study, circulating ACE activity was identified by drawing blood samples 1 week before and one week after the 12 week training program as it is depicted in figure 2.4.

For those completing the training program, baseline ACE activity didn't alter significantly with training (see Results section, chapter 3.1). This finding comes in line with past studies that identified this stability in circulating ACE activity over time. Day et al., (2004) for instance examined the acute effects of aerobic exercise, resistance exercise and glucose ingestion on circulating ACE activity and he found that pre-intervention ACE activity remained remarkably stable across all testing days, and moreover there was no significant change in ACE activity following all the interventions. Back in 1984, Dux and his colleagues (1984) found that if measured in a specific subject, plasma ACE level remains unchanged over time. The findings of this study compliment the results of previous studies and hence support the use of phenotype (plasma ACE activity) over genotype when these kinds of associations are desired.

ACE is a key component of RAS (Renin-Angiotensin System) and converts ANGI to ANGII (Bernstein et al., 1989). Engeli (1999) first mentioned the existence and function of a local RAS system in various tissues, including the cardiac, nervous and skeletal muscle tissue. Those systems have been found to be equipped with all the necessary components for the synthesis of ANGII (review by Jones and Woods, 2003), which led Lavoie to explore if there is a paracrine action of the RAS (Lavoie and Sigmund, 2003). *ACE* genotype on the other hand, is proved to influence both circulating and tissue ACE activity, as several studies have proven (Rigat et al., 1990; Danser et al., 1995; Martin et al., 2006). Hence, the strength gains, or better, the differences in strength gain between the genotypes as they are shown in chapters 3.4 and 3.5 in the results section, can be explained via two possible mechanisms: plasma ACE activity and an autonomous skeletal muscle RAS system. ACE dependant synthesis of ANGII may also induce differences in muscle size. As proved by previous studies, ANGII is a possible factor that affects growth in cardiac muscle cells. Gordon and colleagues (2001), in a double experiment they conducted, found that angiotensin2 is necessary for the optimal overload-induced hypertrophy of skeletal muscle cells as well. The mechanisms by which this is mediated are currently unknown. ANGII may act directly on the skeletal muscle cells, or indirectly act through the simulation of adjacent fibroblasts or capillary angiogenesis (Gordon et al., 2001). Jones and Woods (2003) propose several potential mechanisms in an extensive review on how skeletal muscle RAS may affect performance. There is a notion that ANGII may be important in the

redirection of blood flow from type I fibers to type II fibers (Rattigan, Dora, Tong, & Clark, 1996, cited in Jones and Woods, 2003), that are favored in power performance and strength training. If ANGII is infused into rat hindlimbs, the contraction-induced oxygen uptake and the tension during tetanic stimulation increases (Rattigan et al., 1996, cited in Jones and Woods, 2003). Thus, when ANGII production is greater, muscle contraction for maximal power is facilitated, with a potential detrimental effect on efficiency and endurance. In the present study, DD allele carriers were not associated with elevated circulating ACE activity (see Table 3.1) compared to the II individuals, but as it was shown in the results section, DD individuals showed hypertrophy in type IIA fibers (see table 3.2), although not significantly different from the II individuals in the same type of fibers. In the same review (Jones and Woods, 2003), ANGII is presented as a factor that can possibly promote sympathetic transmission by amplifying the release of noradrenaline from the peripheral sympathetic nerve terminals and the central nervous system (Story & Ziogas, 1987; Saxena, 1992, as quoted by Jones and Woods, 2003).

Another possible explanation of the association of strength with ACE activity might be alterations noted in the types of the muscle fibres. It is well known that the production of mechanical force is well dependent on muscle fiber type. Types II fibres (type IIA and type IIB) produce greater forces per contractile unit when strength/resistance training is applied. The increased muscle cross-sectional area is mainly brought about by hypertrophy of individual muscle fibres. There is a greater increase in the area of fast twitch fibres compared to slow twitch fibres. Mitochondrial volume density decreases in proportion to muscle hypertrophy in response to training (Tesch, 1998). It is generally accepted that strength training does not induce neofibrogenesis. Zhang and his colleagues (2003) reported that Japanese individuals carrying the I allele had a greater proportion of slow-twitch fibers while the D allele carriers had a greater proportion of fast twitch fibres. Muscle fibres in this study (Zhang et al., 2003) were classified based on their histochemical staining for myosin adenosine triphosphatase (mATPase) activity. For *ACE* II genotype, the proportion of fibres was as following: type I 59.4 %, type IIA 15.2% and type IIB 25.5%, whereas for the *ACE* DD allele, type I fibres were 19.6%, type IIA 29.6 % and type IIB 50.9% (Zhang et al., 2003). In this study, there was no training intervention, and the

subjects' characteristics were similar to our study; healthy young males recruited from the University student and staff and were not participating in any specific training. Although, our study is underpowered to detect any significance, Zhang recruited 41 subjects and still this study is considered underpowered (Zhang et al., 2003). The aforementioned study supports the notion that the D allele may influence skeletal muscle function from the aspect of strength/power at high velocities. However, Akhmetov et al., (2006), in a contradictory study on Russian individuals discovered that D allele carriers had a bigger proportion of slow twitch fibers (Akhmetov et al., 2006). Our study failed to prove any association of this kind, although the previously mentioned studies differ in the ethnic group involved, a factor that may play a role in the fiber type distribution. Also Zhang et al., (2003), in the methods section do not specify the kind of training the subjects were involved in ('any specific training' is an unclear term that does not provide information about the physical activity levels that may play a drastic role in the architecture of the muscle fibers). The results of our study for the proportion of muscle fibres in pre-training biopsy sample were for II allele carriers: Type I 50.2 ± 1.5 % pre, Type IIA 28 ± 1.1 % and Type IIB 21.9 ± 1.1 %, whereas for the homozygotes for the D allele, Type I fibres were 50.8 ± 3 %, Type IIA 26.2 ± 1.8 % and Type IIB 22.9 ± 2.3 % (mean ± SEM, table 3.2, results section). Our results demonstrate that there is either no influence of ACE genotype on fiber type composition, or the effect is too small to influence the muscle function in our cohort. Since almost 20-80 % of the phenotypic variation seems to be attributed to inherited factors, there is a possibility that numerous unidentified genes to date collectively influence muscle function (Williams and Folland, 2008).

Type II fibres (IIA and IIB) have bigger motor neuron size and higher recruitment frequency as well as contraction speed, facts that lead to the prospect that such fibres may yield greater forces per units of cross-sectional area. Indeed, Stienen and his collaborators (1996) proved that type IIA and IIB fibres are capable of producing greater forces as compared with the corresponding values of type I. In the present study, significant differences were found pre and post in average cross-sectional area of type IIA fibres for individuals with DD genotype, and also in pre and post measurements for II individuals type I fibres (see Table 3.2 in the results section). Despite the genotype specific differences, there was no interaction between genotype and changes in AVECSA (see results section, table

3.2). Those findings come in line with all the previously discussed papers and support the evidence that muscle hypertrophy has occurred. Despite that, it is still not clear why or if the ACE genotype is related to muscle fiber type distribution and size, or whether or not the ACE I/D polymorphism may be in linkage disequilibrium with another functional variant in an adjacent gene which is in fact responsible for the muscle fiber type distribution. In this study, circulating ACE activity and *ACE* genotype were assessed in order to detect any effect on the type or the cross sectional area of skeletal muscle fibers. Since local RAS exists (review by Jones and Woods, 2003), local ACE activity (and ANGII production) in skeletal muscle may be a factor that would influence muscle properties (Williams et al., 2005).

Neural adaptation to exercise is a largely disputed issue that yet remains to be elucidated. Adaptations occurring the first five weeks are mainly neural (Sale, 1988), with the subjects' improvements in strength resulting from the learning effect, which in turn is familiarization with the apparatus and exercise. It is very important to determine the duration of the neurogenic effect so as to exclude all the phenomenal strength gains and concentrate in the substantial effects. Moritani and DeVries (1979) pointed out that significant gains in muscle strength have been shown following short periods of resistance training, which are generally regarded as being too short to elicit morphological changes in the muscle. Hence, it would seem that the noted increase in strength is mainly because of an ability to activate the muscle in a more efficient way. In his study, hypertrophy became the dominant factor after the first 3 to 5 weeks (Moritani and DeVries, 1979). Another study (Narici et al., 1989) concluded that the increase observed in cross-sectional areas of the muscles of the human quadriceps is responsible only for the 40% of the increase in total force, while the remaining 60% is attributed to a possibly increased neural drive plus architectural changes occurring within the muscle. If we sum up the existing literature references, 5 weeks seem to be the golden mean, with a range in values from 2 to 8 weeks. In the present study, 12 weeks were chosen in order to eliminate as much as possible the changes ascribed to the 'learning effect'. In figure 3.7 for example, we can see that on week 2 of the trained leg, there is a highly significant increase of isometric force mainly (751 ± 30.8 for week 2, 821.6 ± 38.8 for week 3, 9.4 % increase in only one week) and also in figure 3.8 (448.9 ± 20.5 for week 2, 480.6 ± 17.4 for week 3.7 % increase in only one

week), all those changes mainly attributed to increased neural drive. Force also seems to plateau in all isometric and isokinetic angles and velocities between weeks 3 and 8; after 8th week we can define the actual gains of the training program in the trained leg. The intensity and the volume of the training were set to elicit both strength gains and muscle hypertrophy (Baechle 1994). As mentioned previously, a measure of hypertrophy is specific changes in cross-sectional areas of the skeletal muscle fibres. Therefore, since in the present study strength training was applied, it would be expected that muscle fiber types with plasticity towards resistance training are to present the most changes. Indeed, as it is presented in table 3.2 in the results section, there was a significant increase in cross-sectional area of both type IIA and type IIB fibres, for all subjects (II+DD) and for DD genotype, a significant increase in type IIA fibre cross-sectional area.

In a training study (Folland et al., 2000), involving isometric and isokinetic (dynamic) knee extension (one in each leg) among subjects categorized in II, ID and DD group, it was found that the ID genotype (Folland et al., 2000), showed the greatest strength improvement, followed by the group with the DD genotype and then the II genotype group. It was also found that the response to training was highly genotype dependent, favoring the individuals with the presence rather than the absence of the D allele. Moreover, it was found that the ID and D subject improved their quadriceps strength 97 % and 66 % more than II subjects (Folland et al., 2000). In the present study, only subjects who carried the two alleles of the gene were qualified to participate (see methods section). It was therefore hypothesized that using the two extremes (II and DD) would result in more obvious and significant differences in all the parameters tested. Overall, for the trained leg, the gains in isometric strength were similar between the two groups (26.14 % for the II allele carriers and 23.5 % for DD allele carriers), and moreover II allele carriers gained more (although non-significant) strength, contradicting Folland et al., (2000).

As it has been mentioned before, a larger and a more equally distributed between the genotypes sample size is essential for yielding meaningful results. As the recent update on ACE (Thompson et al., 2006) and the human gene map for physical performance and health-related fitness phenotypes (Bray et al., 2009) indicate, ‘The ACE gene continues to be by far the most extensively

studied of any gene, with at least 58 articles examining the effect of an insertion/deletion polymorphism on fitness and performance traits. The conflicting findings among the many studies for the ACE gene exemplify the complexity of genetic studies of complex traits. Indeed despite the enormous amount of attention that the ACE gene has received, it is still not possible to conclude with certainty whether the common polymorphism in ACE is truly involved in human variation in fitness and performance phenotypes and their response to regular exercise. This is primarily, but not exclusively, due to the fact that studies are almost universally underpowered and because an unknown number of negative studies remain unpublished' (Thompson et al., 2006; Bray et al., 2009).

Knowledge of physiological mechanisms regulating muscle growth would help in the design of exercise, nutritional or pharmacological interventions aimed at improving the effect of strength training and restoring muscle strength and size after prolonged inactivity. In addition to this, the identification of a potential 'good' or 'bad' responder will assist in the construction of a more specialized training schedule.

Limitations of the current study

There are several limitations that need to be acknowledged and addressed regarding the present study. The first limitation concerns the extensively discussed sample size. As it was mentioned on the methods and results section, the power of this study, indicated by both a priori power analysis as well as observed power acquired during the conduction of statistical tests, is very low. Two-tailed analysis was preferred due to the fact that the direction of the effect was unknown, as controversial studies exist. If we had chosen to use a one-tailed analysis , the subjects needed to complete the study would be less, but still almost twice the sample size we used (37 subjects to detect a significant one-tailed correlation of magnitude r = 0.30 with a power of 0.80 with the level of alpha set at 0.05). However, the study was time constrained by the due date of the course, so the desired number of subjects was difficult to achieve.

The second limitation has to do with the self-reported physical activity levels which play a critical role in the findings of the study, as they confound the main significant result of the study. Physical activity (hours per week) was assessed via questionnaires, with some of the subjects reporting even 15 hours of physical activity per week. The type of activity was not specified at all occasions, with some of the subjects stating walking and cycling as their main activity, while others reported taking part in recreational rugby and soccer activities. Since the force generation is profoundly affected by the activity undertaken and may mediate the effect of the genotype (in the present study), care should be taken in controlling the levels of physical activity. Structured recreational exercise, such as the activities aforementioned might be very important as they all provide muscle loading and training. Furthermore, these levels are quite substantial when compared to a sedentary background.

Another limitation I encountered was the absence of post-training anthropometric measurements. Alterations in the weight of the participants (weight gain or weight loss) in addition with reduced physical activity towards the end of the study may possibly have a severe impact on the variables of interest, for example in the force output or the skeletal muscle fiber

composition or cross-sectional area. Furthermore, fat free mass would be a more accurate parameter for the description of the population and consequently for any differences between the two groups of individuals (for example DD group may have presented lower fat free mass in the beginning of the study compared to the II group but after the training intervention, the opposite might have been observed), as well as a more useful tool for explaining the observations made.

Conclusion

The present study showed that twelve weeks of strength training on the trained leg of 18 subjects caused greater isometric strength gains in homozygotes for the *ACE* DD allele compared to homozygotes for the II allele, although participants had no baseline differences depending on genotype. Muscular hypertrophy occurred as a result of training, but no differences were observed between groups concerning neither the muscle fiber type distribution nor the muscle fiber cross-sectional area. Circulating ACE activity was unaffected by the intervention, and was not associated with genotype. Baseline circulating ACE activity correlated strongly and positively with baseline isometric and isokinetic strength, an indication of a potential role of ANGII as a growth factor. The limitations of the current study, reduced power in particular, indicate that a larger cohort is essential to confirm our results. Also, local ACE activity (and ANGII production) in skeletal muscle should be assessed, as they may possibly affect muscle properties. Therefore, our findings suggest a possible role for *ACE* gene polymorphism in the regulation of human skeletal muscle strength, but limited statistical power and confounding factors prevented us from drawing clear conclusions.

6.APPENDIX

Appendix Table 1: Correlations between baseline and post-training characteristics in all angles and Circulating ACE activity, pre- and post-training.

	Circulating ACE activity pre-training		Circulating ACE activity
DISOM5pre	r= 0,46 p= 0.05*	**DISOM5post**	r= 0.29 p= 0.24
DISOM30pre	r= 0,25 p= 0.31	**DISOM30post**	r= 0.33 p= 0.18
DISOM60Pre	r= 0,06 p= 0.98	**DISOM60post**	r= -0.08 p= 0.77
DISOK60pre	r= - 0,11 p= 0.65	**DISOK60post**	r= 0.30 P= 0.22
DISOK180pre	r= 0,51 p= 0.03*	**DISOK180post**	r= 0.36 p= 0.13
NDISOM5pre	r= 0.38 p= 0.11	**NDISOM5post**	r= 0.26 P= 0.28
NDISOM30pre	r= 0.03 p= 0.90	**NDISOM30post**	r= 0.27 p= 0.26
NDISOM60pre	r= -0.04 p= 0.88	**NDISOM60post**	r= 0.32 p= 0.19
NDISOK60pre	r= 0,09 p= 0.69	**NDISOK60post**	r= 0.34 p= 0.17
NDISOK180pre	r= 0.14 p= 0.57	**NDISOK180post**	r=-0,006 p= 0.99

D=untrained leg, ND=trained leg, ISOM:isometric training, ISOK:isokinetic training, r= Pearson's correlation coefficient *P <0.05

Appendix Table 2. : Correlations between baseline characteristics (baseline circulating ACE activity, Isometric / Isokinetic force and muscle fibers architecture) and anthropometric characteristics as well as physical activity levels.

	Weight (kg)	Height (m)	BMI (kg/m2)	Physical Activity Levels (h/week)
ACE ACT Pre	r= 0.62 p= 0.01 *	r= 0.65 p= 0.01 *	r= 0.27 p= 0.27	r= 0.16 p= 0.52
DISOM5pre	r= 0.19 p= 0.43	r= 0.38 p= 0.12	r= -0.15 p= 0.55	r= 0.27 p= 0.28
DISOM30pre	r= 0.22 p= 0.37	r= 0.32 p= 0.19	r= -0.02 p=0.90	r= 0.15 p= 0.55
DISOM60Pre	r= 0.02 p=0.92	r= 0.10 p= 0.66	r= -0.10 p= 0.69	r= -0.05 p= 0.84
DISOK60pre	r= -0.12 p= 0.962	r= 0.01 p= 0.98	r= -0.04 p= 0.87	r= 0.04 p= 0.87
DISOK180pre	r= 0.31 p=0.20	r= 0.43 p= 0.07	r= 0.01 p= 0.95	r= 0.26 p= 0.29
NDISOM5pre	r= 0.33 p= 0.18	r= 0.58 p= 0.01 *	r=-0.18 p= 0.47	r= 0.39 p= 0.10
NDISOM30pre	r= 0.2 p= 0.42	r= 0.40 p=0.09	r= -0.19 p= 0.44	r= 0.49 p= 0.03 *
NDISOM60pre	r= -0.05 p= 0.84	r= 0.21 p= 0.39	r= -0.38 p=0.11	r= 0.31 p= 0.20
NDISOK60pre	r= 0.22 p= 0.37	r= 0.30 p= 0.22	r= 0.01 p= 0.99	r= 0.15 p= 0.53
NDISOK180pre	r= 0.11 p= 0.64	r= 0.27 p= 0.28	r= -0.15 p= 0.55	r= 0.44 p= 0.06
TypeIPreCent	r= 0.19 p= 0.44	r= 0.27 p= 0.27	r= -0.01 p= 0.99	r= 0.13 p= 0.60
TypeIIaPreCent	r= -0.23 p= 0.35	r= -0.43 p= 0.07	r= 0.15 p= 0.53	r= -0.29 p= 0.23
TypeIIbPreCent	r= -0.34 p= 0.89	r= 0.01 p= 0.95	r= -0.10 p= 0.68	r= 0.05 p= 0.84
AVECSAIPre	r= 0.13 p= 0.58	r= -0.04 p= 0.84	r= 0.32 p= 0.19	r= -0.26 p= 0.28

AVECSAIIaPre	r= 0.005 p= 0.98	r= -0.25 p= 0.31	r=0.34 p= 0.16	r= -0.35 p= 0.15
AVECSAIIbPre	r= 0.14 p= 0.57	r= -0.4 p= 0.87	r= 0.32 p= 0.19	r= -0.19 p= 0.45

D= untrained leg, ND=trained leg, ISOM: Isometric training, ISOK: Isokinetic training, r= Pearson's correlation coefficient *P <0.05

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ETHICS DOCUMENT

UNIVERSITY OF GLASGOW

ETHICS COMMITTEE FOR NON CLINICAL

RESEARCH INVOLVING HUMAN SUBJECTS

RESEARCH SUBMISSION

Name of person(s) submitting research proposal

Dr Yannis Pitsiladis

Position held

Lecturers in IBLS

Department/Group/Institute/Centre

Institute of Biomedical and Life Sciences

Name of Principal Researcher (if different from above) _____

Position held_____
_____

Date of submission (Re-submission): **September, 2004**

Project Title

Genetic factors influencing the response of skeletal muscle to strength training

1. Describe the basic purposes of the research proposed.

Ageing is accompanied by loss of muscle strength and size that can impair mobility (Hyatt, et al., 1990). Similar effects on muscle strength and size are also seen during prolonged inactivity (MacDougall, et al., 1980) resulting from bed rest, space flight or limb immobilization. Strength training can restore muscle strength and size after periods of prolonged inactivity, although the most effective strength training program remains to be elucidated (Jones, 1992). Knowledge of physiological mechanisms regulating muscle growth would help in the design of exercise, nutritional or pharmacological interventions aimed at improving the effect of strength training and restoring muscle strength and size after prolonged inactivity.

Angiotensin converting enzyme (ACE) is a key component of the circulating human renin-angiotensin system generating angiotensin II (AII), a vasoconstrictor, and degrading vasodilator kinins (Dzau, 1988). Local ACE expression may also modulate tissue growth processes as both AII and kinins appear to have growth regulatory effects (Geisterfer, et al., 1988; Ishigai, et al., 1997). A recent study has shown the importance of ACE in the regulation of skeletal muscle growth, using an experimental model of compensatory hypertrophy in rats (Gordon, et al., 2001). However, no similar study has yet directly evaluated the role of ACE in the regulation of skeletal muscle growth in humans.

A functional polymorphism of the ACE gene has been identified, with the absence (deletion allele, D) rather than the presence (insertion allele, I) of a 287 base-pair fragment associated with higher tissue (Danser, et al., 1995) and serum (Rigat, et al., 1990) ACE activity. Each human has 2 alleles, and consequently can be II, ID or DD. One study used genotype as an indirect marker of ACE activity, and compared the responses to strength training (Folland, et al., 2000). This study found that individuals with at least one D allele had greater gains in strength after a strength training programme than those without a D allele. This finding is consistent with the key role of ACE in the regulation of skeletal muscle hypertrophy in rats (Gordon, et al., 2001). However, further research is required in humans to correlate ACE activity directly with the training response (rather than using the genetic marker alone), and also to measure skeletal muscle growth directly (rather than measuring muscle strength alone).

Skeletal muscle fibres develop by fusion of myoblasts that are stimulated to proliferate by growth factors. Myoblast fusion is generally coupled with the onset of differentiation and at this point they lose the ability to divide. In adult cells, however, some myoblasts persist in a quiescent state (as satellite cells) that can be reactivated to proliferate and fuse to replace other damaged muscle cells or promote muscle growth. A reduction in the proliferative potential for satellite cells will limit the muscle's ability to repair damage or hypertrophy in response to increased physical activity levels. The mechanism regulating cell senescence are unclear but will be intimately related to cell cycle regulation.

Periodic synthesis and destruction of regulatory sub-units, known as a cyclins, result in sequential activation and inactivation of cyclin-dependent kinases (*cdks*) which provide the primary means of cell cycle regulation (Arellano & Moreno, 1997). At least 16 mammalian cyclins have been identified and all contain a homologous domain (the cyclin box) used to bind and activate *cdks*. In addition to cyclin binding, other levels of regulation also exist for controlling *cdk* activity during the cell cycle. Phosphorylation regulates kinase activity (Pagano, 1997) and ubiquitin-mediated proteolysis can target cyclins and other regulators

(LaBaer, *et al.*, 1997). Association with two proteins families, the *cdk* inhibitors, is also an important regulator where some inhibitors appear to regulate the cell cycle positively by functioning as an assembly factor for cyclin/*cdk* complexes (Deng, *et al.*, 1995).

The two cyclin-dependent kinase inhibitor families are known as the *Cip/Kip* family and the *INK4* family. The *INK4* family consists of p15, p16, p18 and p19 and these proteins specifically interact with *cdk*4 and *cdk*6 (Carnero & Hannon, 1998). The *Cip*/Kip family, however, can act on most cyclin/*cdk* complexes and even on some kinases unrelated to *cdks*. The first member of this family to be isolated was p21 and the other two members are p27 and p57. Although reported mutations in the p21 gene are rare, the most commonly reported mutation is in codon 31 where a base change from AGC to AGA causes an amino acid substitution from serine to arginine and accelerates proliferation (Polyak, *et al.*, 1994).

The primary aim of this study, therefore, will be to investigate the influence of ACE activity on the extent of muscle growth and strength development achieved during strength training. The influence of ACE genotype will also be investigated. A secondary aim of this study will be to establish the influence of p21 expression on muscle growth and strength development and its relation to the p21 genotype.

2. Outline the design and methodology of the project. Please include in this section details of the proposed sample size.

We propose to study fifty normal healthy male subjects aged between 18 and 35 years (this sample size is in line with the statistical procedures to be used). All subjects will be in good health at the time of testing. Only volunteers with no history of musculoskeletal injury who are physically active but had not engaged in any structured strength-training programme of the quadriceps muscle group during the 6 months preceding the initiation of the study will be eligible to participate. Subject eligibility will be assessed by completion of a medical and a

physical activity questionnaire. Subjects will also be required to read and sign the enclosed information sheet.

The quadriceps muscle group of both legs will be trained using a 12-week strength training programme. A range of functional, biochemical and histological measurements will be conducted, both before and after the training programme, and DNA samples (ACE genotype) will be extracted from venous blood (or saliva) before the training programme. The genetic material collected will not be used for any other purpose than that specified. All testing will be conducted during two 2-week periods prior to and after the training programme. The total duration of the project will be 16 weeks. All testing and training will take place in the Exercise and Nutrition laboratory at Glasgow Royal Infirmary and the Exercise Physiology laboratory in the West Medical Building.

Protocols

Measurements will include muscle strength, blood analysis, and analysis of muscle samples obtained by biopsy.

Muscle strength: Prior to initial strength testing, each subject will be familiarised with the testing protocols during a full practice session. A standard test of isometric knee extension strength at joint angles of 30º, 60º and 90º will be carried out. The highest force production of at least 3 attempts at each joint angle (a few more attempts are sometimes required to produce a plateau in force production) will be recorded before and after training. Isokinetic dynamometry (Kin-Com) will be used to determine the maximal torque that can be produced during leg extension at an angular velocity of 60º per sec before and after training. The highest torque of at least 3 attempts (a few more attempts are sometimes required to produce a plateau in torque production) will be recorded before and after training. The maximum load that can be lifted once (1-RM) on a leg extension machine so that the lower part of the leg is parallel with the floor will be recorded before and after training, and weekly during the training programme. Each subject will complete successive

repetitions, separated by 30-sec rest periods, with progressively increasing loads until the 1-RM is determined. Both legs will be tested

Blood analysis: Before and after the 12 week intervention period, a resting blood sample (20-mL) will be obtained from a superficial forearm vein for analysis of plasma ACE activity and ACE/p21 genotypes (before training only). Plasma will be separated immediately from 10-mL of whole blood by centrifugation at 1500 g for 10 minutes and stored at -80°C until analysis. ACE and p21 genotype will be determined by a polymerase chain reaction (PCR; O'Dell, *et al.*, 1995; Tsai, *et al.*, 2002). ACE activity will be assayed using a spectrophotometric technique (Sigma Diagnostics, Poole, UK) based on the method developed by Holmquist, *et al.*, (1979). The investigators will remain blind to the subject's genotype.

Muscle analysis: These will be obtained from the *vastus lateralis* by the Bergstrom needle biopsy technique (Bergstrom, 1975) as previously approved by the university ethics committee. Portions of the muscle samples will be analysed using light microscopy and computer-based planimetry for the cross-sectional area of each major fibre type. In addition, portions of the muscle samples will be analysed using the traditional ATPase histochemistry technique for delineation of the fibre types (coefficient of variation of approximately 10 % (Coggan, 1995)) while other portions will be prepared for analysis of MHC composition (Salviati, *et al.*, 1984). Relatively small shifts in fibre type can now be detected successfully using the more recently developed analysis of MHC isoforms (Pette, *et al.*, 1999). ACE and p21 gene expression will be analysed in portions of the muscle samples. ACE mRNA transcripts will be quantified using reverse transcription-PCR (Studer, *et al.*, 1994). p21 mRNA will be measured by PCR-based restriction analysis.

Training: Strength training of the quadriceps muscle group will take place using a leg extension device, 3 times per week for 12 weeks. Each training session will consist of 1 "warm-up" set of 10 repetitions at 75 % of the training load, and 4 sets of 10 repetitions at 100 % of the training load. Rest periods of 1 minute will

separate exercise sets. The training load is defined as that load which can be used with successful completion of 10 repetitions and no more (10-RM load) - estimated at 70 % 1-RM. Each subject's 1-RM will be re-assessed weekly, and the training load adjusted as necessary to maintain a constant training stimulus. Each repetition of the exercise will consist of a concentric and eccentric contraction. All training sessions will be conducted in the specified laboratories and will be directly supervised.

Data analysis: MANOVA will be used to examine the variables at baseline and the responses to training within and between the genotypes observed. In addition, Pearson correlations will be conducted to examine the relationships between plasma ACE activity and the magnitude of the other variables at baseline and the change in magnitude over the training programme.

3. Describe the research procedures as they affect the research subject and any other parties involved.

All tests will take place in the Exercise and Nutrition laboratory at Glasgow Royal Infirmary and the Exercise Physiology laboratory in the West Medical Building. They will be conducted according to the **"Code of Practice for Conducting Experiments in Non-Patient Human Volunteers (including Handling and Disposal of Human Blood, Urine and Sputum)"**, approved by the University Ethics Committee on October 16, 2000. Dr Yannis Pitsiladis or a qualified (CPR-trained) and experienced colleague will be present at all tests. Dr Pitsiladis is a certified phlebotomist and trained in CPR and Advanced Life Support. All investigators are trained in CPR. Defibrillator and emergency drugs are on site in the laboratory.

Genetic material will also be disposed of using the same methods as for the disposal of blood i.e., **"Code of Practice for Conducting Experiments in Non-**

Patient Human Volunteers (including Handling and Disposal of Human Blood, Urine and Sputum)".

Some subjects may experience mild discomfort during the sampling of blood from a forearm vein. Upon withdrawal of the needle, firm pressure is maintained over the site for at least 5 minutes to prevent bruising. Importantly, if a blood sample cannot be obtained readily or if the subject finds the experience too uncomfortable then the experiment will be halted. No more than 20-mL of blood will be taken from any subject.

Obtaining muscle biopsies from the lateral head of the quadriceps muscle is a standard and relatively common practice in studies of muscle metabolism in exercise. It is not uncommon for multiple sites to be biopsied, without incident, in a single experiment. However, we wish to sample from a single site only. Professor Wilhelmina Behan, Dr Stephen Turner, Dr Jonathan Fuld or Dr Margaret McEntagert who are all experienced in this procedure will take the biopsies. This procedure has a small risk of inducing pain, haematoma, haemorrhage or infection; these effects are confined to the sample site if they occur. This risk will be minimized by using (a) experienced medical personnel, (b) a fine gauge biopsy needle and (c) local anaesthesia.

Potential participants will be identified either by personal contact or by advertisement. They will be asked to meet with the investigators to discuss the project and whether they would be suitable as a subject. All subjects will be healthy individuals without a history of any significant medical problem(s). The good health of each subject will be established prior to the study by taking the subject's medical history, which is supported by a written assurance from the subject. Subjects with a history of cardiorespiratory, musculoskeletal or neurological disease/injury will be excluded from participation, as will those having an acute upper respiratory tract infection. Subjects who take drugs (recreational or performance enhancing drugs) or who have consumed alcohol within 48-hrs of an experiment will be also excluded.

Close supervision of the subject will be ensured at all times by the supervising investigator. If a problem is indicated, the investigator will ask further questions to establish whether there is a technical problem that could lead to potential hazard or whether the subject is feeling unwell. In either case, the test will be immediately halted. All subjects will be routinely instructed to cease exercising if they experience any discomfort or have any concern for their well being.

The risks associated with performing maximal exercise are minimal as long as the subject is appropriately instructed and familiarised with the device prior to participation in addition to being appropriately supervised during the experiment. All exercise bouts are preceded by a 5 min "warm-up" and followed by a 5 min "warm-down".

4. What in your opinion are the ethical considerations involved in this proposal? (You may wish for example to comment on issues to do with consent, confidentiality, risk to subjects, etc.)

Exercise has negligible risk to healthy adults, although maximal exercise has a small risk of inducing myocardial ischaemia.

The subjects will complete a medical questionnaire and provide their written consent with the option to withdraw from training or testing at any point.

The insertion of a catheter into a vein may rarely cause irritation at the site of insertion, venospasm (or constriction of the cannulated vein which may lead to interference with blood flow through it) and phlebitis. These risks are minimized in this investigation by the short duration of the test and by the procedures described above.

The taking of muscle biopsies has a low risk of inducing pain, haematoma, haemorrhage or infection confined to the sample site. This risk can be minimized with the use of experienced medical personnel, a fine gauge biopsy needle and local anaesthesia.

Blood and sputum will be handled, stored and disposed of according to standard health and safety procedures.

5. Outline the reasons which lead you to be satisfied that the possible benefits to be gained from the project justify any risks or discomforts involved.

This research will establish the influence of ACE/p21 genotype, p21 expression and ACE activity on muscle growth and strength development from a training programme. Understanding the physiological mechanisms regulating muscle growth and strength development will inform the design of exercise, nutritional or pharmacological interventions to improve rehabilitation after prolonged inactivity. The low risk and slight discomfort associated with the procedures are worthwhile in relation to the data being generated.

6. Who are the investigators (including assistants) who will conduct the research and what are their qualifications and experience?

Dr Yannis Pitsiladis PhD MMedSci BA, Mrs Heather Collin (Technician), Mr Stewart King (MSc student) and a number of L4 Physiology and Sports Science students. The principal investigators have wide ranging experience of exercise testing over periods of up to 10 years without incident.

Professor Wilhelmina M Behan MD FRCPath, Dr Margaret McEntagert MRCP BSc, Dr Stephen Turner (MBChB, MRCS) and Dr Jonathan Fuld MRCP have extensive experience with the muscle biopsy technique without incident.

7. Are arrangements for the provision of clinical facilities to handle emergencies necessary? If so, briefly describe the arrangements made.

In the event of an emergency, guidelines previously approved by the ethics committee will be followed.

In the event of an untoward incident that is not an emergency, the supervising Principal Investigator will administer appropriate first aid, if necessary. The subject will not be permitted to leave the laboratory until he has fully recovered. The subject will be encouraged to contact his local GP. The subject will be told that one of the Principal Investigators will conduct a follow-up by telephone at the end of the same day. The subject will also be provided with 24-hour contact numbers for both Principal Investigators.

8. In cases where subjects are identified from information held by another party (for example, a doctor or hospital) describe the arrangements whereby you gain access to this information.

N/A

9. Specify whether subjects will include students or others in a dependent relationship.

Some students may be recruited but will be under no pressure from staff to participate in the study.

10. Specify whether the research will include children or those with mental illness, disability or handicap. If so, please explain the necessity of using these subjects.

No.

11. Will payment be made to any research subject? If so, please state the level of payment to be made, and the source of the funds to be used to make the payment.

Transportation costs to the laboratory will be provided.

12. Describe the procedures to be used in obtaining a valid consent from the subject. Please supply a copy of the information sheet provided to the individual subject.

Each subject will be provided with a consent form outlining both the testing and training procedures, which asks them for their written consent to participate in the project with the option to withdraw at any time (see enclosed copy). A verbal explanation will also be given and any queries answered. A health questionnaire will also be given. If there is some doubt of the subject's eligibility for the study, the subject will be excluded.

13. Comment on any cultural, social or gender-based characteristics of the subject which have affected the design of the project or which may affect its conduct.

All subjects are male. This constraint is imposed for standardisation purposes.

14. Give details of the measures which will be adopted to maintain the confidentiality of the research subject.

The information obtained will be anonymised and individual information will not be passed on to anyone outside the study group. The results of the tests will not be used for selection purposes.

15. Will the information gained be anonymized? If not, please justify.

Yes

16. Will the intended group of research subjects, to your knowledge, be involved in other research? If so, please justify.

No.

17. Date on which the project will begin **(10th November, 2002)** and end **(30th August, 2006)**

18. Please state location(s) where the project will be carried out.

1 Exercise Physiology laboratories, West Medical Building and Exercise.

2

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Signed _____ Date _____

(Proposer of research)

Where the proposal is from a student, the Supervisor is asked to certify the accuracy of the above account.

Signed _____ Date _____

(Supervisor of student)

COMMENT FROM HEAD OF DEPARTMENT/GROUP/INSTITUTE/CENTRE

Signed _____ Date _____

(Head of Department/Group/Institute/Centre)

Director, Centre for Exercise Science and Medicine

Institute of Biomedical and Life Sciences

University of Glasgow

INFORMATION SHEET

Institute of Biomedical and Life Sciences

University of Glasgow

INFORMATION SHEET

Genetic factors influencing the response of skeletal muscle to strength training

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

Thank you for reading this.

What is the purpose of the study? We wish to investigate how muscle growth is achieved by strength training. In particular, how an enzyme called angiotensin converting enzyme (ACE) and a protein called p21 may influence muscle growth. Your participation in this study may provide you with information that relates to how your muscles adapt to strength training.

Why have I been chosen? You have been selected as a possible participant in this investigation because you are physically active (but not resistance-trained) and in good health. Fifty volunteers are being sought.

Do I have to take part? It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

What will happen to me if I take part? Initially you will be asked to visit the laboratory on at least three occasions over a two week period to conduct the initial assessments (i.e. baseline tests) (see Table). Each visit will last approximately 1 hour. On the first occasion, you will be asked to complete two confidential questionnaires; the first will allow us to obtain information related to your general health; and the second will allow us to quantify your past exercise/activity involvement. Following this, a small muscle sample will be obtained from your non-dominant thigh. This will require a fine needle to be inserted into your thigh muscle, while you are lying down at rest (sample size approximately 100 milligrams of muscle). To minimize any discomfort, a local anesthetic will be injected into the sample site prior to the sample being taken. Once recovered (approximately 30 min), you will be familiarized with the strength test, related equipment and procedures. The strength test involves assessing thigh strength at fixed joint angles of 30º, 60º and 90º. In other words, static force (i.e. no movement of the leg) will be measured with the knee bent at the angles stated above. The highest force production (RM) of at least 3 attempts at each joint angle will be recorded. Each leg will be tested separately. The strength test will be repeated on two subsequent occasions (the following week).

After the initial tests, you will participate in a 12 week training programme. Strength training of the quadriceps (thigh) muscle group will take place on a leg extension device, 3 times per week for 12 weeks. Each training session will consist of 1 "warm-up" set of 10 repetitions at 75 % of the training load, and 4 sets of 10 repetitions at 100 % of the training load. Rest periods of 1 minute will separate sets. The training load is defined as that load which can be used with successful completion of 10 repetitions and no more (10-RM load) - estimated at 70 % 1-RM. Your 1-RM will be re-assessed weekly, and the training load adjusted as necessary to maintain a constant training stimulus. All training sessions will be

conducted in the university and/or hospital laboratories, and all will be supervised.

After the 12-week training programme, you will be required to repeat all initial assessments (including the muscle biopsy).

During the initial and final assessments we would like to take a small amount of blood from a vein in your forearm to assess blood levels of ACE and which genetic variant of this enzyme and the p21 protein you have. Your genetic material will not be used for any other purpose than that specified here.

What are the side effects of taking part? A small scar at the muscle biopsy sample site is not uncommon; this typically resolves over a period of months. The blood sample may also cause some bruising and subsequent soreness over the site of puncture and, rarely, a small wound that takes a few days to heal.

What are the possible disadvantages and risks of taking part? There are no serious risks of having a muscle sample taken from your thigh by means of a fine needle. There is a small risk of inducing local pain, bruising, bleeding or infection. A small diameter biopsy needle and the use of local anaesthesia will minimize these risks.

Exercise has a negligible risk in healthy adults, although maximal exercise has a small risk of inducing myocardial ischaemia ("heart attack"). The primary symptom of myocardial ischaemia is chest pain on exertion. If you experience any unusual sensations in your chest during the experiment, you should cease exercising immediately.

What are the possible benefits of taking part? We hope that you will find out more about how your body responds to strength training. This information may help us better understand the mechanisms by which muscle adapts to strength training.

What if something goes wrong? If you are harmed by taking part in this research project, there are no compensation arrangements. If you are harmed due to

someone's negligence, then you may have grounds for a legal action but you may have to pay for it. The principal investigators, although not medically qualified are fully trained in Advanced Life Support. In the event of an untoward incident, the principal investigator(s) will provide basic life support including chest compressions and ventilation, and will apply an advisory defibrillator (if necessary) until emergency medical staff are on hand. The taking of the biopsies will be carried out my experienced medically qualified investigators.

Will my taking part in this study be kept confidential? All information which is collected about you during the course of the research will be kept strictly confidential.

What will happen to the results of the research study? Results will be published in a peer-reviewed scientific journal once the study is completed. You will automatically be sent a copy of the full publication. You will not be identified in any publication.

Table 5: Schedule of visits and proposed tests.

Visit	Test	Duration (hrs)
1	Physical characteristics, questionnaires, muscle biopsy and familiarisation of strength test	2
2	Strength test	1
3	Strength test	1
	12 week strength training programme (3 x week)	18-20
4	Muscle biopsy	0.5
5	Strength test	1
6	Strength test	1

If you wish to find out more about this investigation, you can contact:

Dr Yannis Pitsiladis
Lecturer, Institute of Biomedical and Life Sciences
West Medical Building
University of Glasgow
Glasgow, G12 8QQ
Phone: 0141 330 3858
Fax: 0141 330 6542
e-mail: Y.Pitsiladis@bio.gla.ac.uk

CONSENT FORM

Consent Form

I .....

give my consent to the research procedures which are outlined above, the aim, procedures and possible consequences of which have been outlined to me

Signature ……

Date ……

DECLARATION OF AUTHORSHIP

I declare that, except when explicitly stated, this work is my own. It has not been written or composed by any other person and all sources have been appropriately referenced or acknowledged. I understand that copying the work of other students, or from published texts, or the internet, is plagiarism and against the University regulations. I understand that a breach of these regulations will lead to disciplinary action. I further understand that marks can only be awarded for my own effort. I am aware that if plagiarism is discovered, I may forfeit all marks for the assignment.

Name: Maria Chatzi

Signature: Date: 21/03/2011