ACE-inhibition and Skeletal Muscle Dysfunction in Chronic Obstructive Pulmonary Disease

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Declaration of originality

The data in this thesis is the result of my original work. The work of others is appropriately referenced.

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Abstract

This thesis addresses the impact of quadriceps wasting and physical inactivity across disease severity (GOLD stages I-IV) in Chronic Obstructive Pulmonary Disease (COPD) and assesses the influence of angiotensin-converting enzyme (ACE)-inhibition on quadriceps dysfunction in these patients.

In a cross-sectional study of 161 COPD patients, ultrasound measurement of rectus femoris cross-sectional area was reduced in mild as well as advanced disease when compared to controls. Daily physical activity, measured using an armband accelerometer, was reduced in COPD subjects across all GOLD stages compared to controls. Physical activity was independently associated with quadriceps wasting in GOLD stage I, but not stage II-IV disease where residual volume to total lung capacity ratio was the only independent predictor of activity level. This data suggests that quadriceps wasting is not an end-stage phenomenon in COPD and highlights the need for early identification of these patients to guide lifestyle and therapeutic interventions.

The effect of the ACE-inhibitor, fosinopril on quadriceps dysfunction in COPD was then investigated in a double-blind randomised controlled trial of 80 COPD patients with quadriceps weakness. Despite a significant reduction in systolic blood pressure and serum ACE activity in the treatment group compared to placebo, no significant differences were observed at 3 months in the primary outcome of non-volitional quadriceps endurance. Quadriceps strength improved in both groups, but there was a greater increase in the placebo arm. No significant changes were observed in mid-thigh cross-sectional area or

incremental shuttle walk distance. The trial also assessed the effect of ACE-inhibition on vastus lateralis atrogene expression in COPD, with no significant differences observed between groups.

In conclusion, although evidence from observational cohorts suggest a role for the renin-angiotensin system in the control of muscle phenotype, data from this thesis found that ACE-inhibition did not improve quadriceps function in a COPD population with quadriceps weakness.

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- **Shrikrishna D**, Astin R, Kemp PR, Hopkinson NS. Renin-angiotensin system blockade: a novel therapeutic approach in Chronic Obstructive Pulmonary Disease. Clinical Science 2012; 123:487-98.

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- **Shrikrishna D**, Tanner RJ, Seymour JM, Patel M, Connelly B, Walsh SLF, Bloch SA, Sidhu PS, Hart N, Moxham J, Polkey MI, Hopkinson NS**.** Ultrasound measurement of quadriceps wasting in early COPD and its relationship to daily physical activity. Thorax 2011, 66:A44-45. *Presented as a Spoken Session at the British Thoracic Society Winter Meeting, London - 2011*

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Abbreviations

ACE	angiotensin-converting enzyme
ACE-I	angiotensin-converting enzyme inhibitor
AMPK	AMP –activated protein kinase
Ang I	angiotensin I
Ang II	angiotensin II
AT1R	angiotensin II type 1 receptor
AT2R	angiotensin II type 2 receptor
BK	bradykinin
BK2R	bradykinin type 2 receptor
BMI	body mass index
CAT	COPD assessment test
COPD	chronic obstructive pulmonary disease
CRP	c-reactive protein
CT	computed tomography
D	deletion allele
DEXA	dual energy x-ray absorptiometry
E4BP-1	eukaryotic initiation factor 4E-binding protein-1
ECG	electrocardiogram
ECLIPSE	Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points
eIF2B	eukaryotic initiation factor 2B
ERK	extracellular-signal-regulated kinase
ECSC	European coal and steel community

FFM	fat free mass
FFMI	fat free mass index
FEV_1	forced expiratory volume in 1 second
FoxO	forkhead box O
FVC	forced vital capacity
GLUT4	glucose transporter 4
GOLD	Global initiative for chronic Obstructive Lung Disease
GSK3β	glycogen synthase kinase-3β
HMGB1	high mobility group protein-1
I	insertion allele
IGF-1	insulin-like growth factor-1
IGF-1R	insulin-like growth factor-1 receptor
IKK	inhibitor of NF-κB kinase
IL	interleukin
IL1-B	interleukin 1 - beta
ISWT	incremental shuttle walk test
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MEF-2	myocyte enhancer factor-2
MET	metabolic equivalent of task
MGF	mechano-growth factor
MHC	myosin heavy chain
MIF	macrophage migration inhibitory factor
mRNA	messenger ribonucleic acid
MT_{CSA}	mid-thigh cross sectional area

mTOR	mammalian target of rapamycin
MuRF-1	muscle ring finger protein-1
6MWD	six minute walk distance
NAC	N-acetylcysteine
NF-κB	nuclear factor kappa B
NO	nitric oxide
NT-BNP	N-terminal pro-B-type natriuretic peptide
p70^{S6K}	70kDa ribosomal S6 protein kinase
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
PPAR	peroxisome-proliferator-activated receptor
PGC-1α	PPAR-γ coactivator-1α
QMVC	quadriceps maximal voluntary contraction
RAS	renin angiotensin system
RF	rectus femoris
ROS	reactive oxygen species
RV	residual volume
SGRQ	St George's Respiratory Questionnaire
TGF-β	transforming growth factor-B
TLC	total lung capacity
TLCOc	corrected transfer factor of lung for carbon monoxide
TNF-α	tumour necrosis factor-α
TwQ	twitch quadriceps force
TK	tyrosine kinase
USRF$_{CSA}$	ultrasound rectus femoris cross-sectional area

VCO$_2$	carbon dioxide output
VE	minute ventilation
VEGF	vascular endothelial growth factor
VI	vastus intermedius
VL	vastus lateralis
VM	vastus medialis
WHO	World Health Organisation

Chapter 1: Introduction

1.1   Definition of COPD

The term 'Chronic Obstructive Pulmonary Disease' (COPD) was first introduced in the 1960s describing patients with incompletely reversible airflow limitation due to the combined effects of airways disease, chronic bronchitis and emphysema (Briscoe 1965). It was the seminal work of a French clinician and pathologist, Laennec, who in 1821 originally described emphysema at dissection (Laennec 1821). On opening the chest, he noted:

'it is not unusual to find that the lungs do not collapse, but they fill up the cavity on each side of the heart. When experienced, this will appear full of air'

Laennec went on to describe an accumulation of bronchial mucous in his dissection findings, so linking emphysema to the sputum production associated with chronic bronchitis (Petty 2006). Emphysema is now defined as abnormal, permanently enlarged airspaces distal to terminal bronchioles, with destruction of airspace walls. Small airways disease (obstructive bronchiolitis) is caused by inflammation and squamous metaplasia in airways less than 2mm in diameter and can be among the first changes to appear from inhalation of noxious materials such as cigarette smoke (MacNee 2008).

Today, the term COPD encompasses a range of pathologies including chronic bronchitis, obstructive bronchiolitis and emphysema and is characterised by airflow limitation associated with an abnormal inflammatory response in the lungs to inhalation of noxious substances, including tobacco smoke and, in the developing world, biomass smoke. The airflow obstruction is not fully reversible and is usually progressive causing breathlessness

and limitation of daily activities. It is estimated that currently over 210 million people worldwide suffer from COPD and World Health Organisation (WHO) projections suggest that COPD will be the 5th leading cause of disability (Murray 1996) and 3rd most common cause of death worldwide by 2020 (Lopez 1998).

Although COPD has been considered primarily in terms of its effects on lung parenchyma and airways, attention is now being focused on its systemic effects (American Thoracic Society/European Respiratory Society 1999; Celli 2004a). Increasing evidence suggests a multisystem disorder encompassing skeletal muscle dysfunction, cardiac disease, osteoporosis, metabolic disturbance, neurological impairment and systemic inflammation (Bolton 2004; Hopkinson 2004a; Sin 2006; Baker 2009; Barnes 2009). An important illustration of this is the role of pulmonary rehabilitation, which improves health status and exercise capacity without influencing lung function in COPD patients (Lacasse 2006; Dodd 2011).

Skeletal muscle impairment in particular, represents a key component of the systemic co-morbidities in COPD with loss of skeletal muscle bulk and strength now recognised as important predictors of mortality in this patient group (Marquis 2002; Swallow 2007a). The mechanisms involved in the development of muscle weakness are likely to be multi-factorial with systemic factors including inflammation and oxidative stress (Shrikrishna 2009) thought to interact with local factors such as muscle inactivity (Hopkinson 2010). A greater understanding of the key mechanisms involved in skeletal muscle dysfunction may provide a target area for novel interventions, as an adjunct to pulmonary rehabilitation, which remains the mainstay of current therapy.

1.2   Hypotheses

The work contained in this thesis aims to address the following questions of significance to patients with COPD:

(1)   Does quadriceps wasting occur in patients with mild as well as advanced COPD, when compared to healthy age-matched controls, and is this associated with their level of daily physical activity?

(2)   Does angiotensin-converting enzyme (ACE) inhibition have a beneficial effect on quadriceps endurance, strength and cross-sectional area in COPD patients with quadriceps weakness?

(3)   Does ACE-inhibition influence the atrogene mediated pathway of muscle atrophy in the quadriceps of patients with COPD and is there an effect on serum inflammatory markers?

1.3   Skeletal muscle anatomy and physiology

1.3.1   Muscle structure and fibre classification

Striated muscle cells are found in two tissues in vertebrates - skeletal and cardiac muscle. Skeletal muscles, under the control of the somatic nervous system, are able to perform voluntary movements and have varying slow twitch and fast twitch contractile properties. Three skeletal muscle fibre types exist in humans – Type I, Type IIa and Type IIx - based on expression of the predominant myosin heavy chain (MHC) isoforms. Type I are slow-twitch, aerobic fibres with increased oxidative enzyme expression, mitochondrial content and capillary supply. Type IIa are fast-twitch fibres but with a similar oxidative profile to the type I fibres. Type IIx are fast-twitch, anaerobic fibres with a high glycolytic enzyme expression (Prince 1981).

Skeletal muscle fibres are large multinucleated cells surrounded by an electrically polarised membrane – the sarcolemma. The muscle fibres are grouped in a connective tissue sheath (perimysium) and arranged in bundles known as fascicles. These fascicles are grouped in further connective tissue (epimysium) to form the whole muscle. An individual muscle fibre contains thousands of myofibrils or 'muscle threads' arranged in parallel, each of which contain a series of sarcomeres which are the functional unit of contraction. Importantly, in a mature muscle, the number of these sarcomeres can alter with the appropriate stimulus thereby allowing muscles to adapt (Lieber 2000).

A sarcomere is made up of two types of contractile protein filaments - thick filaments composed largely of the protein myosin and thin filaments containing three proteins -

actin as the predominant one, tropomyosin and troponin. The thick filaments correspond to the A band of the myofibril, overlapped on either end with the thin filaments that form the I band. A sheet of alpha-actinin in the centre of the I band, known as the Z-disc, anchors the thin filaments and the region between two adjacent Z-discs represents an individual sarcomere. Cytoskeletal crosslinking proteins including myomesin and titin contribute to elasticity and complete the highly ordered structure as shown in figure 1.1.
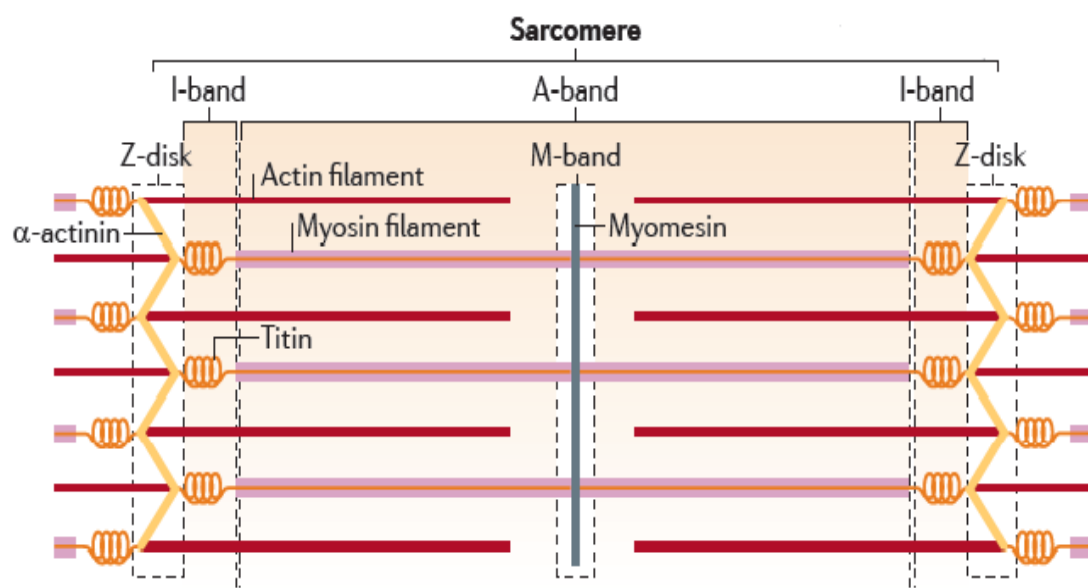
Figure 1.1: Striated muscle structure

Adapted from (Braun 2011)

1.3.2 Muscle contractile mechanism

There are three key events in skeletal muscle contraction. The first is electrical excitation of the muscle fibre, followed by excitation-contraction coupling and finally fibre contraction based on the sliding filament mechanism originally described by Huxley in 1971 (Huxley 1971). The fibres are arranged into motor units each innervated by a single motor neuron. Stimulation from the motor neuron results in depolarisation of the sarcolemma and after reaching a threshold, an action potential is generated. This electrical excitation causes calcium ions to be released from the terminal cisternae of the sarcoplasmic reticulum. These calcium ions cause a conformational change in troponin subunits attached to the actin filament. At rest, tropomyosin wraps around the actin filaments, blocking myosin binding sites, however with this change in shape, the tropomyosin complex moves deeper into a groove of the actin molecule thereby exposing the myosin-binding sites. This allows the process of muscle fibre contraction to begin where actin filaments slide past the myosin filaments shortening the sarcomere length. The hydrolysis of ATP to ADP and inorganic phosphate energises the myosin heads allowing them to bind to actin forming cross-bridges. This formation then releases inorganic phosphate and triggers the power stroke where the myosin head rotates and pulls the actin filament towards the centre of the sarcomere. ATP then binds to the myosin head releasing it from actin and the contraction cycle can start again. When intracellular calcium levels fall, the myosin-binding sites on actin are blocked by the tropomyosin complex and the muscle fibre returns to its relaxed state. The force produced is defined by sarcomere length (i.e. myofilament overlap). At an optimal length when crossbridge interactions are maximal, the muscle produces a maximum force.

1.4   Importance of Skeletal Muscle Impairment in COPD

Skeletal muscle impairment can be considered in terms of muscle bulk and muscle function though these are of course related. Weight loss has long been recognised as a feature of COPD (Fowler 1898) occurring in 15% of mild and 25% of moderate to severe COPD patients (Wilson 1989; Schols 1993; Engelen 1994) and is associated with a poor prognosis (Boushy 1964; Vandenbergh 1967; Gray-Donald 1996; Schols 1998; Landbo 1999; Prescott 2002; Chailleux 2003; Cano 2004; Celli 2004b; Marti 2006).

Different patterns of weight loss occur depending on whether there is a loss of fat mass, fat free mass (FFM) or both (Schols 1993). It is clear that preservation of FFM is more important than body weight, as normal weight patients with nutritional depletion (fat free mass index $<15kgm^{-2}$ for women or $<16kgm^{-2}$ for men (Baarends 1997)) are more disabled than underweight patients with a preserved FFM (Shoup 1997; Mostert 2000). Skeletal muscle is a major component of FFM and FFM depletion in COPD is associated with reduced exercise performance, increased dyspnoea and impaired health related quality of life (Hamilton 1995; Gosselink 1996; Bernard 1998). Leg discomfort makes a significant contribution to exercise limitation in patients with COPD (Killian 1992; Man 2003a) and where low frequency quadriceps fatigue occurs after exercise, bronchodilation has been found not to increase exercise capacity (Saey 2003).

Skeletal muscle weakness does not occur equally in the upper versus lower limbs. The same is also true when comparing limb and respiratory muscles with different muscle compartments responding to the particular demands placed on them (Gea 2001a). The

quadriceps is one of the main muscles of ambulation and has been the focus of much of the work around skeletal muscle impairment in COPD. In moderate to severe COPD the mean reduction in quadriceps strength is approximately 30% (Bernard 1998; Man 2003b; Hopkinson 2004b; Man 2005) with significant weakness present in about 30% of patients (Swallow 2007a). Quadriceps weakness has been shown to be related to impaired quality of life (Simpson 1992), exercise limitation (Gosselink 1996) and increased health care utilisation (Decramer 1997), and predicts survival more powerfully than FFM or forced expiratory volume in 1 second (FEV_1) (Swallow 2007a). A reduction in quadriceps endurance has also been demonstrated in COPD using both volitional and non-volitional techniques of assessment (Coronell 2004; Swallow 2007b).

1.5   Mechanisms of Skeletal Muscle Dysfunction in COPD

Skeletal muscle atrophy and hypertrophy can be influenced by a number of processes that are relevant to patients with COPD. A complex interplay of factors including systemic inflammation, oxidative stress and muscle disuse are thought to have a role. Some of the medications used to treat COPD, particularly corticosteroids, may also have a deleterious effect. In addition, genetic susceptibility (Hopkinson 2004b; Hopkinson 2006; Hopkinson 2008) might explain why some COPD patients demonstrate greater peripheral weakness than others.

1.5.1   Disuse

Patients with COPD are breathless when they exercise and this is reflected in a reduction in physical activity which may be very pronounced (Pitta 2005). Quadriceps weakness can occur with relatively brief periods of bed rest (10 days) in healthy elderly subjects (Kortebein 2007) or indeed other medical conditions (Harris 2001) and has been described within a week of hospital admissions with COPD (Spruit 2003). Following hospitalisation for an acute exacerbation, COPD patients have been shown to have reduced physical activity which can continue for several weeks (Pitta 2006). Furthermore, early pulmonary rehabilitation in the recovery period after discharge, following admission with an acute exacerbation of COPD, leads to significant improvements in functional capacity at 3 months (Man 2004). Weakness in patients with stable COPD is most pronounced in the locomotor muscles (Man 2003b; Man 2005) and at biopsy a classic disuse pattern of change is observed in the quadriceps with a shift towards a preponderance of 'fast' Type II fibres (Jakobsson 1990; Gosker 2003; Gosker 2007), reduced capillarity (Jobin 1998) and a

reduced oxidative capacity (Jakobsson 1990). This pattern is not seen in the deltoid muscle (Gea 2001b) and the reverse effect, a shift towards a preponderance of slow fibres, is seen in the diaphragm which exhibits increased activity in COPD patients (Levine 1997).

1.5.2   Systemic Inflammation and Oxidative Stress

Systemic inflammation is present in patients with COPD as evidenced by increased levels of tumour necrosis factor-α (TNF-α) and C-reactive protein (CRP) (Gan 2004). However, although early data supported a role for increased serum levels of cytokines such as TNF-α in muscle wasting in severe COPD (Di Francia 1994), a review incorporating the more recent studies of circulating TNF-α levels has concluded that there is no difference in TNF-α between cachectic and non-cachectic COPD patient groups (Wagner 2008). The diminished role for TNF-α in COPD peripheral muscle dysfunction has been supported by a study identifying that levels of quadriceps muscle TNF-α actually fall with decreasing strength (Barreiro 2008). This study also found no difference in IL-6, interferon-gamma or TGF-β protein expression in muscle between COPD patients and controls suggesting that the quadriceps muscle does not exhibit a pro-inflammatory environment in patients with severe COPD. The latter study did however, identify markers of quadriceps muscle oxidative stress in this patient group and in support of this, depletion of the reduced form of glutathione levels has also been observed in COPD patients when compared to controls (Rabinovich 2006). It is well established that free radicals produced by oxidative stress are capable of causing tissue damage and such radicals may well have a role in the muscle atrophy seen in COPD (Couillard 2005). In addition, it has been demonstrated that chronic endurance exercise leads to nitrosative stress in the quadriceps of severe COPD patients

(Barreiro 2009). Interestingly, recent work indicates that COPD patients exhibit different quadriceps muscle gene expression patterns in response to training when compared to controls (Radom-Aizik 2007), although this study had relatively few participants. Nevertheless, it was reported that oxidative stress gene pathways were more highly expressed in COPD patients than controls after training. Further work is needed to establish the role of oxidative stress in COPD and to investigate if attenuation of these pathways can enhance training responses in this patient group.

1.5.3 Corticosteroids

Steroid induced myopathy classically affects the proximal muscles with atrophy of fast twitch fibres and frank myopathy has been described as a complication of corticosteroid use in COPD (Decramer 1996). However, interpretation is confounded by the effects of frequent exacerbations which are the classical indication for steroid therapy in this patient group. It has been shown that a two week course of prednisolone in stable COPD patients had no effect on skeletal muscle parameters (Hopkinson 2004c) and cross-sectional studies have not demonstrated a link between muscle depletion and steroid use in COPD (Schols 1993; Hopkinson 2004b) although there are other reasons to avoid chronic steroid therapy (Schols 2001).

1.5.4 Role of acute exacerbations

In addition to the emphysematous or airways predominant groups, frequent exacerbators are a key clinical phenotype in COPD (Hogg 2004; Han 2010; de Oca 2012). When COPD

patients are hospitalised for an exacerbation, quadriceps strength falls by a further 5%, and recovery of baseline strength is not seen in all patients (Jakobsson 1990). Consequently, reduced FFM is associated with exacerbation frequency both in a cross-sectional study (Hopkinson 2004b) and with decline in FFM prospectively (Hopkinson 2007). The mechanism for this is likely to be multifactorial as acute exacerbations integrate many of the factors that are thought to contribute to muscle weakness including inflammation, immobility, negative nitrogen balance, and the administration of corticosteroids.

1.5.5   Molecular mechanisms

The molecular biology underlying the skeletal muscle dysfunction observed in COPD remains to be determined. A key catabolic pathway involved in skeletal muscle atrophy is mediated through two ubiquitin ligases, the atrogenes atrogin-1 and muscle RING finger protein-1 (MuRF-1). These ligases are muscle specific enzymes which have been shown in murine models to mediate protein degradation and hence muscle wasting as part of the ubiquitin proteasome pathway (Ottenheijm 2008). There is evidence of increased expression of MuRF-1 and atrogin-1 in the quadriceps muscle of COPD patients in comparison to healthy age-matched controls (Doucet 2007). In addition, this study showed that the Forkhead box class O (FoxO) of transcription factors, which induce MuRF-1 and atrogin-1, were present at an increased protein concentration in the quadriceps of COPD patients compared to the healthy subjects.

The anabolic hormone, insulin-like growth factor-1 (IGF-1), acts through the phosphoinositide 3-kinase (PI3K)/AKT pathway with IGF-1 receptor activation leading to AKT phosphorylation. This disables FoxO transcription factors and prevents expression of the atrogenes, thereby inhibiting muscle catabolism (Sandri 2006). In addition, AKT phosphorylation activates the mammalian target of rapamycin pathway (mTOR) which upregulates p70^{S6K} (70kDa ribosomal S6 protein kinase) (Rommel 2001) and inhibits 4EBP-1 (eukaryotic initiation factor 4E binding protein-1) (Hara 1997), thereby promoting protein synthesis. Phosphorylated AKT can also act independently of mTOR to inhibit GSK-3β (glycogen synthase kinase-3β), a repressor of protein synthesis (Cross 1995; Vyas 2002).

It has been shown that IGF-1 levels are reduced in COPD patients in the stable state compared to healthy controls (Crul 2007). Furthermore, in COPD patients undergoing pulmonary rehabilitation, increases in exercise capacity and fibre size are associated with upregulation of IGF-1 and a splice variant of IGF-1 (mechano growth factor, MGF) (Vogiatzis 2007). Interestingly, MGF is specifically produced in response to mechanical stretch and leads to muscle hypertrophy and satellite cell activation (Goldspink 2003).

Local IGF-1 expression also down regulates NF-κB and pro-inflammatory cytokines including TNF- α, IL1-B, high mobility group protein-1 (HMGB1) and macrophage migration inhibitory factor (MIF) (Pelosi 2007). NF-κB acts as a key downstream mediator of the inflammatory cytokine cascade, as well as being activated by other triggers including inactivity (Hunter 2002). NF-κB is controlled via the IKK (inhibitor of NF-κB kinase) complex and in murine studies, targeted deletion of this complex suppressed NF-κB activation and led to a shift in muscle fibre distribution towards a Type I phenotype and improved muscle

force (Mourkioti 2006). In addition, NF-κB expression has been shown in transgenic mice to be associated with over expression of MuRF-1 leading to muscle atrophy (Cai 2004). As NF-κB activation occurs in the skeletal muscle of COPD patients with low body weight (Agusti 2004) this transcription factor may have an influence in the muscle atrophy observed in this patient group.

Interestingly NF-κB has also been shown to inhibit a key myogenic regulatory factor, MyoD (Langen 2004). MyoD is involved in the control of myogenic satellite cells which mediate muscle repair and regeneration (Megeney 1996; Hawke 2001) and hence its inhibition can prevent a response to muscle injury. Myostatin, a member of the TGF-β superfamily, is also a negative regulatory factor of myogenic satellite cells. Through inhibition of MyoD, myostatin suppresses satellite cell differentiation and muscle repair (Joulia 2003). A role for myostatin in the skeletal muscle dysfunction observed in COPD comes from a cross-sectional study by Plant *et al*, who found increased expression of myostatin mRNA in the quadriceps of cachectic COPD patients compared to healthy controls (Plant 2010). In addition, levels of quadriceps myostatin mRNA expression have been shown to negatively correlate with quadriceps strength, six minute walk distance (6MWD), locomotion time as a measure of daily physical activity and quadriceps endurance assessed by repetitive magnetic stimulation in 18 COPD patients (Man 2010). Further work is needed to establish if myostatin has a key role in driving the reduction in strength, exercise capacity and activity seen in these patients.

Peroxisome proliferator-activated receptors (PPARs) may have an important role in the maintenance of muscle mass in COPD (Sathyapala 2007). These transcriptional co-factors

have a key regulatory role in skeletal muscle phenotype and angiogenesis (Arany 2008).

PPARs mediate Type II (fast twitch, anaerobic) to Type I (slow twitch, aerobic) fibre shift

and regulate mitochondrial activity as well as muscle oxidative status (Luquet 2003). PPAR

gamma coactivator 1α (PGC-1α) expression in transgenic mice has been shown to promote

a type I oxidative phenotype possibly through co-activation of myocyte enhancer factor-2

(MEF-2) (Lin 2002). PGC-1α suppresses the activity of FoxO to reduce the expression of the

ubiquitin ligases involved in muscle atrophy (Sandri 2006). In addition it has been shown

that PGC-1α knockout mice have reduced skeletal muscle vascular endothelial growth

factor (VEGF) levels and reduced capillary/fibre ratio (Arany 2008; Leick 2009). Remels *et*

al have shown that PPAR-delta protein content is decreased in the skeletal muscle of COPD

patients with a cachectic COPD subgroup also found to have decreased PPAR-alpha mRNA

expression (Remels 2007). Further work is needed to establish the importance of reduced

levels of PPAR expression in the skeletal muscle abnormalities in COPD. Figure 1.2 shows

some of the key molecular pathways of muscle wasting outlined above.
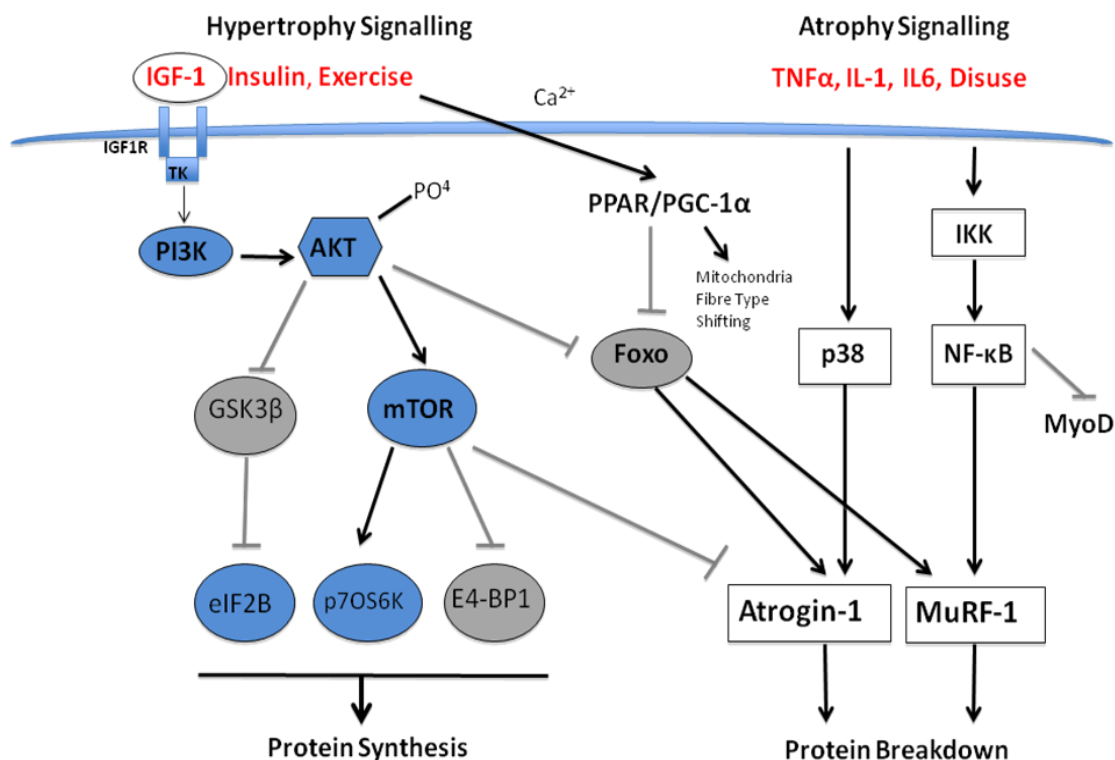
Figure 1.2: Molecular pathways of skeletal muscle wasting

Adapted from (Glass 2005) & (Man 2009). (IGF-1, insulin-like growth factor 1; IGF1R, insulin-like growth factor 1 receptor; PI3K, phosphoinositide 3-kinase; GSK3β, glycogen synthase kinase 3β; mTOR, mammalian target of rapamycin; eIF2B, eukaryotic initiation factor 2B; p70S6K, 70kDa ribosomal S6 protein kinase; E4-BP1, eukaryotic initiation factor 4E-binding protein-1; FoxO, forkhead box O; PPAR, peroxisome-proliferator-activated receptor; PGC-1α, PPAR-γ coactivator-1α; TNF-α, tumour necrosis factor-α; IL-1, interleukin-1; IL-6, interleukin-6; IKK, inhibitor of NF-κB kinase; NF-κB, nuclear factor kappa B; MuRF-1, muscle ring finger protein-1)

1.6 The Renin-Angiotensin System

1.6.1 The Role of the Angiotensin-Converting Enzyme

A local renin-angiotensin system (RAS) exists in many human tissues including lung and skeletal muscle. Angiotensin-converting enzyme (ACE) is a zinc metallo-peptidase present in the circulating plasma and highly expressed in lung capillary blood vessels (Studdy 1983). Angiotensin II receptors are expressed in the lungs (Kakar 1992) and, importantly, ACE expression is also evident on the membrane of vascular endothelial cells in muscle (Schaufelberger 1998). The RAS, as illustrated in figure 1.3, is mediated initially by renin which cleaves angiotensinogen to produce angiotensin I. ACE then catalyses the conversion of angiotensin I to angiotensin II, as well as the breakdown of vasoactive kinins. Angiotensin II is a potent vasoconstrictor acting directly on smooth muscle cells but it also stimulates sympathetic nerve terminals to release the vasoconstrictor norepinephrine and facilitates aldosterone secretion from the adrenal cortex, via angiotensin II type 1 (AT1) receptors. This enables ACE to exert an influence on salt/water retention and vascular tone. In addition, these effects are enhanced by the ACE dependent degradation of bradykinin, which when active, can mediate the release of the vasodilator, nitric oxide and prostaglandins (Carter 2005). Angiotensin II type 2 (AT2) receptors oppose some of the type I receptor mediated vasoconstrictor effects, through local vasodilation. The human lung expresses both angiotensin II receptor subtypes, whereas only the type 1 receptor is expressed in adult human skeletal muscle (Malendowicz 2000).
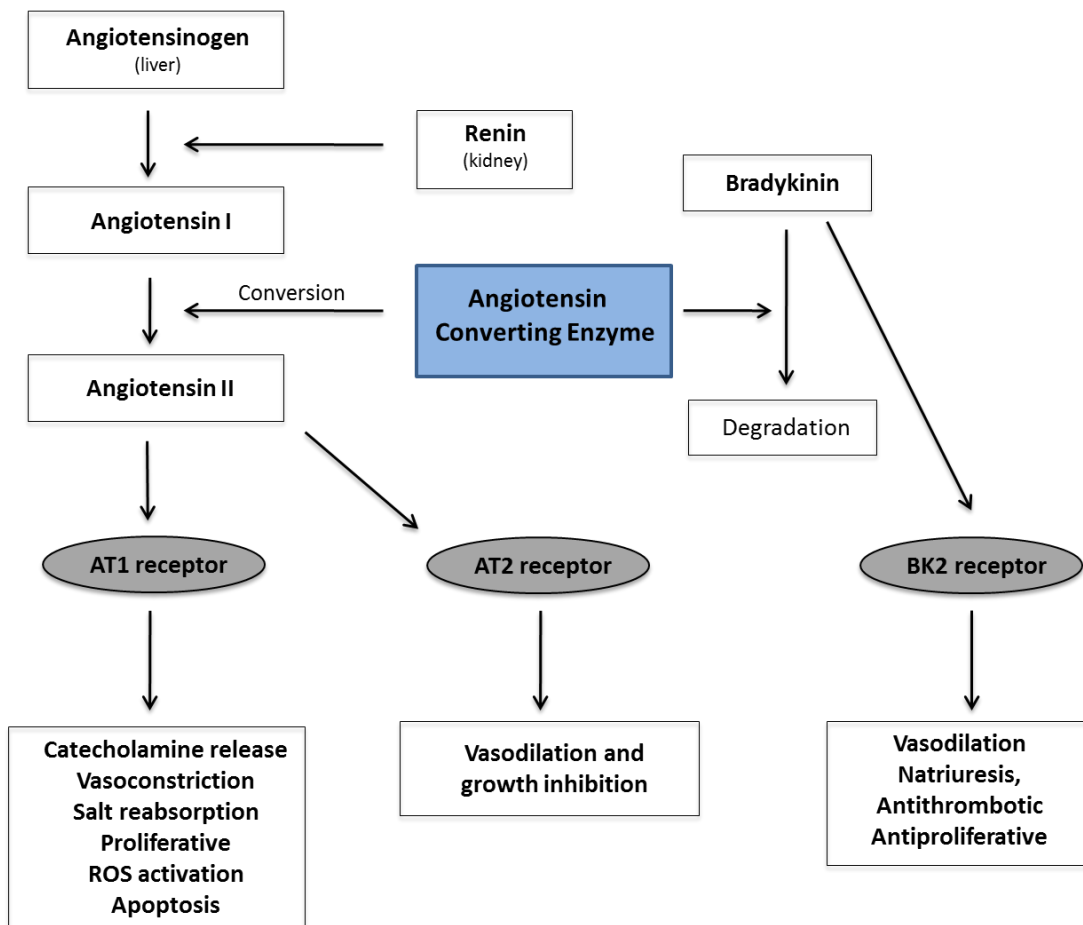
Figure 1.3: The Renin-Angiotensin system

Adapted from (Carter 2005) (AT1 receptor, angiotensin II type 1 receptor; AT2 receptor, angiotensin II type 2 receptor; BK2 receptor, bradykinin type 2 receptor; ROS, reactive oxygen species)

1.6.2   The History of ACE-inhibitors

ACE inhibitors were initially developed from investigation of the venom of a Brazilian pit viper (*Bothrops Jararaca*) in the late 1960's (figure 1.4) (Patlak 2004). The Nobel Laureate Sir John Vane found that the venom's effects, including extensive bleeding and a sudden fall in blood pressure, occurred through inhibition of ACE activity. This initial source of ACE-inhibition highlights the actions of angiotensin II as a vasoconstrictor and growth factor to counteract bleeding and initiate vessel wall repair (Ferrari 2009). However, although the RAS plays a key role in the stress response, chronic activation of this system in humans is thought to influence cardiopulmonary disease and from the initial discovery, an oral form of ACE-inhibitor - captopril - was developed for the treatment of hypertension. In particular, evidence of an increase in cardiac and peripheral sympathetic activation in COPD patients (Volterrani 1994; Heindl 2001) and the interaction between this impaired autonomic control and the renin-angiotensin system, termed 'neurohumoral activation', has led to its implication in the pathogenesis of COPD (Andreas 2005).

Figure 1.4: The Brazilian Pit Viper *(Bothrops Jaracaca)*

1.6.3   Renin-angiotensin system and COPD

Chronic inflammation of the central and peripheral airways is recognised as a central feature of COPD associated with lung remodelling, parenchymal destruction and the development of emphysema (Stockley 2009). The RAS is thought to be implicated in the pathogenesis of COPD through its involvement in inducing pro-inflammatory mediators in the lung (Marshall 2003) (figure 1.5).
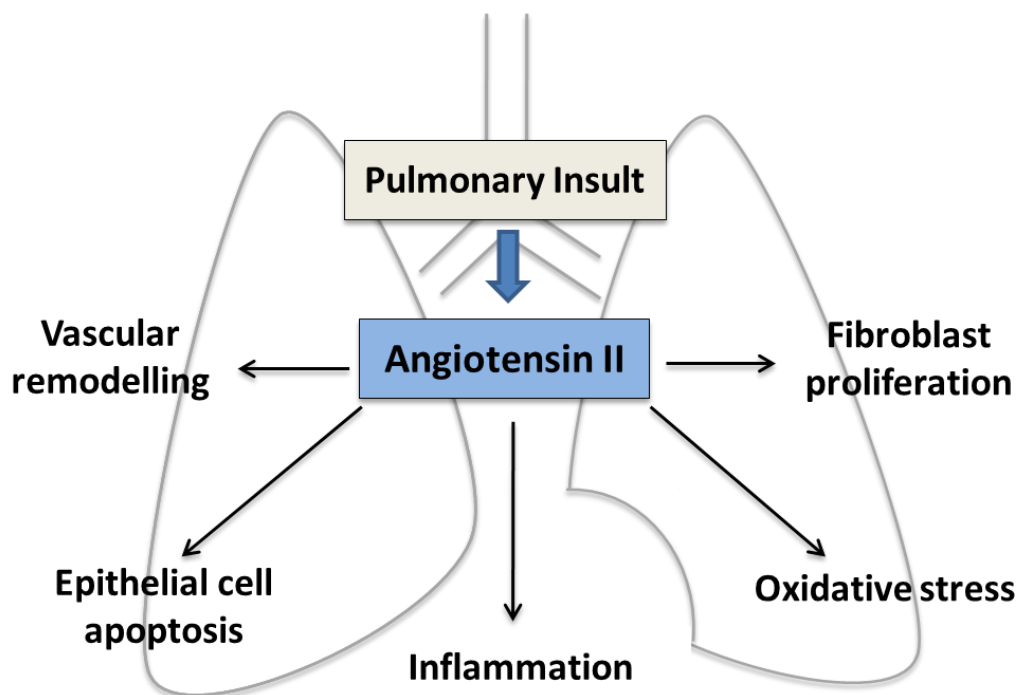
Figure 1.5: Effects of angiotensin II following pulmonary insult

Adapted from (Marshall 2003)

Angiotensin II stimulates the release of cytokines including interleukin 6 (IL-6), monocyte chemotactic protein-1 (MCP-1) and TNF- α (Hanif 2010). In particular, alveolar macrophage derived MCP-1 has been shown to activate tissue mast cells in response to acute alveolar hypoxia thereby triggering systemic inflammation (Chao 2011). Angiotensin II also has an immunomodulatory effect on T cell responses which mediate the lung tissue injury associated with COPD (Kaparianos 2011). Wong *et al* have recently shown that alveolar type I cells produce pro-inflammatory cytokines and express components of the RAS as part of an innate immune response to lung injury (Wong 2012). The study found that this cytokine response was mediated by angiotensin II and was inhibited by losartan, an AT1 receptor antagonist. Interestingly, in COPD patients, Bullock *et al* found a five to sixfold increase in the ratio of AT1 to AT2 receptors in regions of marked fibrosis surrounding bronchioles which correlated with the reduction in FEV_1 (Bullock 2001). This data supports a role for angiotensin II in inducing bronchial constriction via the AT1 receptor (Brown 2001). The RAS can also generate reactive oxygen species, via the AT1 receptor, promoting mitochondrial dysfunction (Benigni 2010) and contributing to the impaired redox signalling observed in COPD (Rahman 2006).

1.6.4 Renin-angiotensin system and muscle biology

Angiotensin II influences the insulin like growth factor system, the ubiquitin-proteasome proteolytic pathway and induces the production of pro-inflammatory cytokines in skeletal muscle (Cabello-Verrugio 2012) (see figure 1.6). Evidence to suggest influence of the RAS on the PI3K/AKT/atrogene pathway comes from animal models where angiotensin II administration causes cachexia and muscle specific expression of IGF-1 blocks this angiotensin II dependent muscle wasting (Song 2005). Interestingly, the authors of this study also postulated that angiotensin II may partially exert its inhibitory action on IGF-1, independent of the AT1 receptor, via a glucocorticoid mediated effect. Recent work in mice gastrocnemius muscle has also shown angiotensin II transcriptional regulation of MuRF-1 and atrogin-1 (Yoshida 2010). This study confirmed that IGF-1 overexpression prevented skeletal muscle atrophy and atrogin-1 expression. In addition, dominant negative AKT and active FoxO inhibited this IGF-1 mediated effect on angiotensin II action. This work highlights the involvement of the ubiquitin degradation pathway in mediating the negative effects of angiotensin on skeletal muscle and identifies potential targets for intervention.

Insulin-like growth factor-1 receptor (IGF-1R) phosphorylation in human skeletal muscle cells has been studied following incubation with telmisartan, valsartan and lisinopril (Storka 2008). There was a 2 fold increase in phosphorylation with the ATII receptor blockers and a 1.7 fold increase with ACE-inhibition. Phosphorylated AKT levels were also increased following incubation with telmisartan, valsartan and lisinopril. The effects observed by ATII receptor blockade may be mediated via activation of peroxisome

proliferator-activated receptor gamma (PPARγ) as IGF-1R phosphorylation was attenuated in the presence of a PPARγ antagonist.
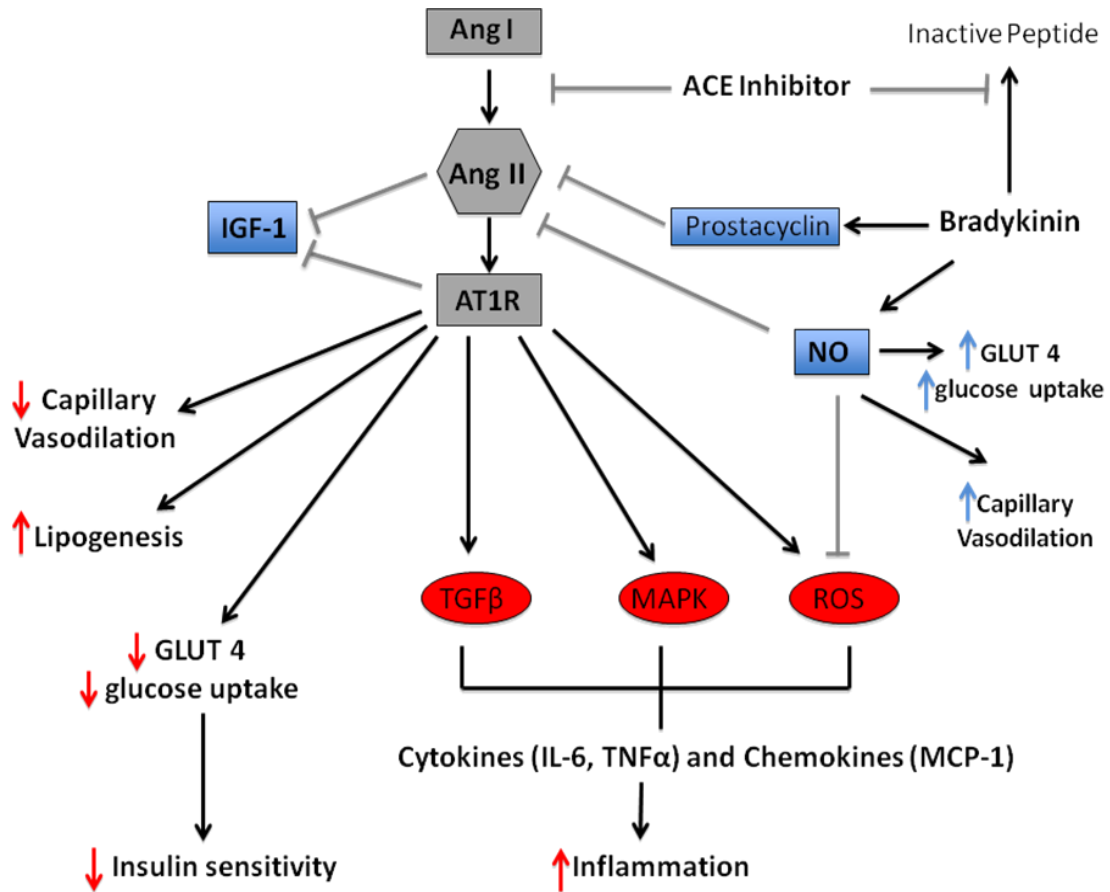
Figure 1.6: Postulated actions of the Renin-Angiotensin system in skeletal muscle in COPD

Adapted from (Hanif 2010). (Ang I, angiotensin I; Ang II, angiotensin II; AT1R, angiotensin II type 1 receptor; IGF-1, insulin-like growth factor-1; GLUT 4, glucose transporter 4; NO, nitric oxide; TGF-β, transforming growth factor-beta; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; IL-6, interleukin-6; TNFα, tumour necrosis factor alpha; MCP-1, monocyte chemotactic protein-1). Lines ending with a perpendicular segment represent inhibitory pathways.

Diamond-Stanic *et al* have recently shown that angiotensin II also inhibits the insulin dependent glucose transporter 4 (GLUT4) and decreases phosphorylation of AKT in rat soleus skeletal muscle (Diamond-Stanic 2010). The free radical scavenger, superoxide dismutase partially reversed the angiotensin II-induced inhibition of glucose transport suggesting that reactive oxygen species may have a role in mediating the effect of angiotensin on insulin signalling. Further work in this area again using a rat skeletal muscle model has shown that angiotensin II induces tyrosine nitration dependent inhibition of AKT phosphorylation (Csibi 2010). Angiotensin II was found to activate the MAP kinases (ERK 1 and 2) through a nitration dependent mechanism. In addition this study showed that both scavenging of free radicals with myricetin and inhibition of nitric oxide synthase restored insulin stimulated AKT phosphorylation and GLUT4 translocation in the presence of angiotensin. This data suggests that oxidative and nitrative stress play a role in mediating the angiotensin-induced insulin resistance and atrophy in skeletal muscle.

Bradykinin, via the actions of NO and prostacyclin, can counteract angiotensin II mediated effects in skeletal muscle (Hanif 2010). Bradykinin promotes GLUT-4 translocation in skeletal muscle, through the actions of nitric oxide (Henriksen 2003). Furthermore, absence of the bradykinin receptor 2 gene is associated with insulin resistance in mice (Duka 2001). Interestingly, a recent study has found that insulin can stimulate skeletal muscle protein synthesis in humans through an indirect mechanism involving endothelium dependent NO vasodilation and consequent increased blood flow, capillary recruitment and mTOR complex 1 signalling, rather than AKT signalling (Timmerman 2010). Nitric oxide also has a key role in inhibiting reactive oxygen species, with fibre type-specific nitric oxide shown to protect oxidative myofibres against cachectic stimuli through antioxidant gene

expression (Yu 2008). Importantly, enalapril treatment has been shown to reduce angiotensin II dependent inflammation and oxidative stress related muscle damage in a dystrophic mouse model (Cozzoli 2011).

A key study by Cohn *et al* has also identified another important target for muscle wasting that may be influenced by the RAS (Cohn 2007). The authors found that increased TGF-β activity led to a failure of muscle regeneration in fibrillin-1-deficient and dystrophin-deficient mice. In addition, this study identified that systemic antagonism of TGF-β using losartan or a TGF-β neutralising antibody, restored muscle structure and function in both mouse models. This highlights a potential role for TGF-β signalling in mediating angiotensin II receptor effects in skeletal muscle. Of note, myostatin is both a negative regulator of muscle growth, as described previously, and a member of the TGF-β superfamily. In cultured rat neonatal cardiomyocytes it has been shown that angiotensin II activates myostatin expression via p38 MAP kinase (Wang 2008) and therefore this may also represent a target for angiotensin related muscle atrophy.

1.6.5　The ACE I/D polymorphism and muscle function

The human ACE gene contains a functional polymorphism based on the presence (insertion, I) or absence (deletion, D) of a 287 base pair sequence in intron 16 on chromosome 17. Therefore three genotypes exist: II, ID, DD and these have an approximate distribution of 25, 50 and 25% respectively in a Caucasian population (Jones 2003). ACE activity is highest in the subjects homozygous for the D allele (DD), is intermediate in the ID group and lowest in subjects homozygous for the I allele (II).

A number of clinical studies have investigated a role for ACE expression in COPD by stratifying patients by ACE polymorphism. Busquets *et al*, studied the distribution of the ACE polymorphism in 151 male smokers and found that the DD genotype was more prevalent in smokers who developed COPD, being associated with a 2-fold increase in the risk for COPD (Busquets 2007). This data provides a genetic link to support the existing evidence that ACE activity is both elevated in COPD (Brice 1995) and is associated with lung function impairment (Ucar 1997). Kanazawa *et al* performed right heart catheterisation to assess the pulmonary vascular response to exercise challenge in COPD patients stratified by ACE genotype (Kanazawa 2000). They found that the DD genotype was associated with increased pulmonary artery pressure and pulmonary vascular resistance when compared to the II group. In a separate study, COPD patients with a DD genotype had a reduced ratio of the change in oxygen delivery to increase in oxygen consumption during exercise (Kanazawa 2002), suggesting an impairment in peripheral tissue oxygenation. The ACE polymorphism may also be related to low grade systemic inflammation in COPD; a study of 72 stable COPD patients observed an increase in serum high sensitivity CRP across genotypes DD>ID>II (Tkacova 2007), suggesting that the RAS

system may also contribute to the inflammatory response observed in COPD, as previously discussed.

The ACE polymorphism also influences muscle phenotype with greater endurance observed in patients homozygous for the insertion allele (II) (Myerson 1999; Williams 2000) and a higher proportion of type I fibres associated with this group (Zhang 2003). In contrast, a power-oriented muscle phenotype is seen in subjects with a DD genotype (Nazarov 2001; Woods 2001), who interestingly also exhibit greater bradykinin degradation in comparison to the II genotype (Brown 1998). Variations in the human ACE genotype have been found to influence quadriceps strength in a COPD population (Hopkinson 2004b). In a study of 103 stable COPD outpatients, presence of the deletion allele (D) of the ACE gene polymorphism was associated with increased quadriceps strength, which was not observed in the control group. Bradykinin type 2 receptor (BK(2)R) polymorphisms also influence quadriceps muscle strength in COPD patients. It has been shown that those patients exhibiting the +9/+9 homozygous genotype, associated with lower BK(2)R receptor expression, have a reduced fat free mass and reduced strength (Hopkinson 2006). Of note, the ACE polymorphisms have also been shown to interact with the effect of vitamin D receptor genotypes on quadriceps strength in COPD (Hopkinson 2008) highlighting the potential complexity of the influence of genetic susceptibility in the muscle phenotype of these patients.

1.6.6 Epidemiological studies of ACE-inhibition and muscle function

Cross-sectional data from 2,431 hypertensive subjects participating in the Health, Aging and Body Composition (Health ABC) study has been used to evaluate if ACE-inhibitor treatment is associated with a larger lower extremity muscle mass compared to the use of other antihypertensive medications (Di Bari 2004). This analysis found that lower extremity muscle mass assessed by DEXA was larger in the ACE-inhibitor group, with a trend towards being greater in those ACE-inhibitor users with a longer duration of exposure compared to a shorter exposure (as defined by a median split of 2 years).

A key observational study by Onder *et al* also assessed the relationship between ACE inhibitor use and muscle strength in 641 elderly hypertensive women participating in the Women's Health and Aging Study (Onder 2002). They found that at 3 year follow up, participants taking an ACE-inhibitor continuously had a lower mean decline in both knee extensor muscle strength and walking speed to those using other antihypertensives and those not on antihypertensive medication. Intermittent use of ACE-inhibitors was associated with a significantly larger decline in walking speed compared to continuous use. The study group had poor mobility and no concomitant heart failure at baseline. Of note, the group taking an ACE-inhibitor continuously had lower physical activity levels, as assessed during a baseline interview, compared to the other groups.

Interestingly, prospective findings from the Women's Health Initiative Observational Study with over 25, 000 participants found that ACE-inhibitors were not significantly associated with risk of frailty after 3 years follow-up (Gray 2009). However, if selecting a sub-

population who were hypertensive on only one or less antihypertensive medication, a reduced risk of frailty was observed for those on low and medium equivalent doses of ACE-inhibitors.

The InCHIANTI study investigated the relationship between ACE-inhibitor treatment and IGF-1 serum levels in a large cohort of older subjects (>65 years of age) (Maggio 2006). Serum total IGF-1 was significantly higher in participants receiving an ACE-inhibitor (<3 years duration) having adjusted for confounders in a linear regression analysis. Those participants with 3-9 years duration of use of an ACE-inhibitor also had an increase in serum IGF-1 but this did not reach statistical significance. A limitation of this study was that information on IGF-1 binding proteins was not available and hence details of the IGF-1 fraction that was biologically active was not known. This study did however highlight IGF-1 as a potential mechanism through which ACE-inhibition may exert the muscle effects seen in observational studies and suggested that clinical doses of ACE-inhibitor could influence IGF-1 levels.

1.7   Current Treatment Strategies

1.7.1   Pulmonary Rehabilitation

Exercise training as an essential aspect of multidisciplinary pulmonary rehabilitation has been shown to improve exercise capacity (Griffiths 2000; Man 2004) and muscle function (Troosters 2000; Casaburi 2001) in COPD patients. Recently, a randomised controlled trial has shown that outpatient pulmonary rehabilitation immediately following hospitalisation for an acute exacerbation of COPD improves both quadriceps strength and maximal walking capacity (ISWT) and reduces the risk of a re-exacerbation requiring hospital attendance over a 3 month period (Seymour 2010a). The Grade 'A' evidence base for exercise as an established therapeutic strategy in these patients has been summarised in the American Thoracic Society/European Respiratory Society Statement on Pulmonary Rehabilitation (Nici 2006).

1.7.2   Nutritional Supplementation

Although nutritional depletion is a common feature in COPD there is a limited evidence base for nutritional interventions. Creutzberg *et al* studied the use of oral liquid nutritional supplements during an eight week pulmonary rehabilitation course in nutritionally depleted patients with COPD (Creutzberg 2003a). They found an increase in FFM, muscle strength and exercise performance, with a reduced response observed in patients on oral corticosteroid therapy. However it is important to note that other studies have not shown similar effects and a Cochrane review concluded that two weeks of calorie

supplementation did not significantly affect exercise capacity, lung function or anthropometric measures (Ferreira 2005). Creatine supplementation has also been tried as an adjunct to rehabilitation and although effective in restoring muscle creatine levels, it does not improve function, probably because this is not a factor limiting exercise in COPD patients (Deacon 2008). Further work is needed to explore the potential benefits of nutrition in COPD which may be confined to particular patient subgroups.

1.7.3   Hormonal Treatments

Casaburi *et al* conducted a placebo-controlled trial showing an increase in leg strength and leg lean mass in a study using a ten week course of testosterone combined with leg resistance training, in male patients with COPD (Casaburi 2004). Further muscle biopsy studies in this cohort have suggested that the muscle IGF system may play a role in the mechanism underlying this response (Lewis 2007). Other work in this area has shown more varied outcomes. A placebo controlled trial using growth hormone in COPD, found an increase in lean body mass, but did not show a change in peripheral muscle strength or exercise capacity (Burdet 1997). In addition, the use of anabolic steroids like nandrolone, was not found to improve skeletal muscle strength in COPD patients (Creutzberg 2003b).

1.7.4 Electrical Stimulation

The use of electrical stimulation of skeletal muscle is under evaluation as a potential training technique for patients who cannot exercise conventionally due to breathlessness. A study assessing the effects of six weeks of electrical stimulation on the quadriceps muscle of nine COPD patients found an increase in strength and shuttle walk distance, when compared to controls (Bourjeily-Habr 2002). In addition, a small randomised controlled trial has compared quadriceps electrical stimulation and rehabilitation, with rehabilitation alone, in severe COPD patients following ITU admission (Vivodtzev 2006). A significant increase in muscle strength and six minute walk distance was observed in the electrical stimulation intervention group. A recent systematic review of the evidence on the effects neuromuscular electrical stimulation in patients with chronic heart failure or COPD (Sillen 2009), has identified improvements in muscle strength and exercise capacity in a number of studies using this technique. Larger prospective randomised controlled trials are therefore needed to establish the role of electrical stimulation as a potential training strategy.

1.7.5 Antioxidant Medication

Koechlin *et al,* have studied the potential therapeutic benefits of the antioxidant, N-acetylcysteine (NAC), in severe COPD patients. They found that NAC improved quadriceps endurance and prevented exercise induced oxidative stress (Koechlin 2004). More recently, the antioxidant properties of pressurised whey have been investigated in a randomised double-blind placebo controlled trial of 22 COPD patients (Laviolette 2010).

This study found an improvement in work rate on a cycle endurance test in the whey group compared to placebo suggesting a potential effect of increased glutathione levels, although systemic levels were not significantly changed from the placebo group. Further work is required in larger study populations to establish the full potential of these therapies in the muscle dysfunction observed in COPD patients.

1.8   Research questions

This thesis aims to investigate skeletal muscle dysfunction in patients with COPD. A cross-sectional study is undertaken to assess whether quadriceps wasting, measured using ultrasound measurement of rectus femoris, exists in mild as well as advanced disease compared to healthy age-matched controls. The influence of factors such as physical inactivity on quadriceps muscle wasting in these patients is also discussed.

A double-blind randomised placebo controlled trial is then conducted to investigate whether targeting the renin-angiotensin system through angiotensin converting enzyme (ACE) inhibition could play a role in counteracting muscle dysfunction observed in COPD patients. This trial assesses the physiological and molecular effects of the ACE-inhibitor fosinopril, on quadriceps dysfunction in 80 COPD patients with quadriceps weakness. The effects of ACE-inhibition on non-volitional quadriceps endurance, quadriceps maximal voluntary contraction and mid-thigh computed tomography (CT) cross-sectional area are also determined.

Vastus lateralis muscle biopsies taken as part of the ACE-inhibitor trial are then analysed for changes in expression of components of the IGF-1/atrogene pathway. The effects on serum ACE and systemic inflammation are also assessed, and a post-hoc analysis used to investigate stratification by ACE genotype. By establishing the influence of physical inactivity and the potential role of ACE inhibition on skeletal muscle impairment in COPD, it may be possible to target future therapeutic strategies in this patient group.

Chapter 2: Description of Methods

2.1 Ethical approval

The studies in this thesis were approved by the Ethics Committee of the Royal Brompton and Harefield NHS Foundation Trust (07/Q0404/17) and the Joint University College London Committees on the Ethics of Human Research (08/H0715/90). All participants provided written informed consent and the research was conducted in accordance with the declaration of Helsinki.

2.2 Subjects studied

The patients recruited to studies in this thesis had a COPD diagnosis based on NICE guidelines (NICE 2010) (see table 2.1) with severity defined using Global initiative for Obstructive Lung Disease (GOLD) stage classification (Rabe 2007). Subjects with a significant co-morbidity that limited muscle function or physical activity level were excluded. The healthy age-matched control subjects were recruited through local advertisements.

Table 2.1: GOLD stage classification

Post-bronchodilator FEV$_1$/FVC	FEV$_1$% predicted	GOLD stage criteria (2008) Post-bronchodilator	NICE clinical guideline (2010) Post-bronchodilator
<0.7	≤80%	Stage 1 - Mild	Stage 1 –Mild*
<0.7	50-79%	Stage 2 - Moderate	Stage 2 - Moderate
<0.7	30-49%	Stage 3 - Severe	Stage 3 - Severe
<0.7	<30%	Stage 4 - Very Severe	Stage 4 – Very Severe

* Symptoms should be present to diagnose COPD in patients with mild airflow obstruction

2.3   Body Composition

2.3.1   Fat-free mass measurement using single-frequency bio-electrical impedance

Body composition was determined by bioelectrical impedance analysis (BodyStat QuadScan 4000; BodyStat, Douglas, United Kingdom). This technique uses the electrical impedance of body tissues to determine an estimate of total body water, as electricity is conducted by dissolved ions. A two-compartment model was used which assumes that adipose tissue contains no water and that FFM has a constant hydration of 73% water. Single frequency, 50kHz, bioelectrical impedance values were then incorporated into regression equations which include height, weight and gender to calculate fat free mass (FFM) (Steiner 2002). These disease-specific equations have been validated against other techniques for assessing body composition, including deuterium dilution and dual energy x-ray absorptiometry (DEXA) (Schols 1991).

Fat free mass equations:

Males: FFM (kg) = 8.383 + [0.465*height^2 (cm) / resistance (ohms)] + [0.213*weight (kg)]

Females: FFM (kg) = 7.61 + [0.474*height^2 (cm) / resistance (ohms)] + [0.184*weight (kg)]

Fat Free Mass Index (FFMI) was calculated by dividing FFM by height in metres squared (kg/m^2).

2.3.2 Multiple-frequency bio-electrical impedance analysis

Measurements of multiple-frequency bioelectrical impedance at 5, 50, 100 and 200kHz were also made using a BodyStat QuadScan 4000 device. At low frequencies (5kHz) current does not penetrate cell membranes however at high frequencies (200kHz) both intracellular and extracellular spaces are penetrated. Therefore, the ratio of the bioelectrical impedance at these frequencies is thought to give an index of extracellular and total body water (Z_{200}/Z_5).

2.4 Pulmonary Function Testing

Pulmonary function testing was undertaken by the Royal Brompton Hospital Lung Function Department. Spirometry, plethysmographic lung volumes, carbon monoxide diffusing capacity (TLco) (CompactLab system; Jaeger, Wurzburg, Germany) and arterial blood gases were determined in accordance with European Respiratory Society (ERS) /American Thoracic Society (ATS) recommendations (Macintyre 2005; Miller 2005; Wanger 2005). Standardised lung function testing reference equations were based on the European Coal and Steel Community (ECSC) reference values (Gibson 1993).

2.5   Blood pressure measurement

Blood pressure was recorded using an automated blood pressure monitor (Omron M6, Omron Healthcare Europe, Hoofddorp, The Netherlands). The subject was rested for at least 10 minutes prior to blood pressure measurement. An appropriate cuff size was used and the measurement was made in a seated position with the subject's arm supported at the level of the heart. An average of three readings was recorded. Mean arterial pressure (MAP) was calculated using the following equation; [MAP ~ diastolic pressure + 1/3(systolic-diastolic)].

2.6   Quadriceps Muscle Strength

The capacity of the muscle to develop maximal force (i.e. muscle strength) can be measured by either volitional or non-volitional techniques, using isometric or isokinetic methods. Isometric methods involve the static contraction of a muscle without any visible movement in the angle of the joint, whereas an isokinetic method involves muscle contraction and limb movement through a range of motion at a constant speed. The capacity of the muscle to maintain a certain force and to resist fatigue (i.e. muscle endurance) can also be measured by volitional or non-volitional techniques.

2.6.1 Volitional-Quadriceps Maximal Voluntary Contraction

A volitional measurement of quadriceps maximum voluntary contraction (QMVC) was made using an isometric technique (Edwards 1977). Subjects sat on a modified chair with their knee fixed at 90 degrees. An inextensible strap connected the ankle of their dominant leg to a strain gauge (see figure 2.1). The signal from the strain gauge was amplified and passed to a computer running CHART software (Labchart version 7.1, PowerLab Analogue-Digital Converter, AD instruments, Oxfordshire, UK). The subjects performed at least 3 sustained maximal isometric quadriceps contractions of between 5 and 10 seconds duration. Consistent traces within 5% of maximum were obtained. A gap of approximately 30 seconds was given between each contraction to allow time to recover. Vigorous encouragement was given and the force generated was visible online using the CHART software. The QMVC was taken as highest tension sustained for 1 second. Quadriceps weakness was defined as QMVC <120% of body mass index (Swallow 2007a).

Figure 2.1: Quadriceps maximal isometric voluntary contraction measured using a strain gauge.

2.6.2 Non-volitional-Supramaximal Magnetic Femoral Nerve Stimulation

Twitch response was measured using the technique of supramaximal magnetic stimulation of the femoral nerve (Polkey 1996). With this method, after 20 minutes of quadriceps rest, unpotentiated twitch quadriceps force (TwQu) was determined using magnetic femoral nerve stimulation. Two Magstim 200 monopulse units were discharged using a 70mm figure-of-eight coil (figure 2.2) and the mean of at least 5 stimulations at 100% stimulator output was taken. A stimulus response curve incorporating a range of stimulator output responses (twitch ramp) was used to confirm supramaximality.

Figure 2.2: Quadriceps twitch response measured using magnetic femoral nerve stimulation

2.7   Repetitive Magnetic Stimulation

2.7.1   Quadriceps Endurance Testing

Volitional measures have shown a reduction in muscle endurance in patients with COPD (Coronell 2004) but these techniques are dependent on subject motivation and coordination. By using a repetitive magnetic nerve stimulator with a flexible mat coil wrapped around the quadriceps, it has been possible to show that quadriceps endurance assessed non-volitionally is reduced in COPD patients compared to controls (Swallow 2007b). Subjects received 60 trains of magnetic stimulation, using a Magstim Rapid 2 stimulator, at a frequency of 30Hz, 2 seconds on, 3 seconds off (see figure 2.3). The % stimulator output was determined as that able to produce 20% of the subject's maximal voluntary contraction at baseline. The exponential decay in force produced by consecutive stimuli was used to measure endurance half-time.

Figure 2.3: Quadriceps endurance measured by repetitive magnetic stimulation with a mat coil.

2.8 Field Walking Tests

2.8.1 Incremental Shuttle Walk Test

The incremental shuttle walk test (ISWT) is a standardised field test requiring subjects to walk back and forth along a 10m course (Singh 1992). The course is identified by 2 cones, 9m apart with space to turn (0.5m) at each end. A CD player is used to provide standardised incremental audio beeps which dictate the speed at which the subject should complete each 10m shuttle during the test. This externally paced incremental format is similar to the laboratory incremental exercise test (Palange 2000) and the minimum clinically important difference for the ISWT is 47.5m (Singh 2008). The test provides a symptom limited assessment of maximal performance and therefore can be used as a realistic, objective measurement of disability.

2.9   Quadriceps Imaging

2.9.1   Ultrasound Rectus Femoris Cross-sectional area

Measurement of quadriceps rectus femoris cross-sectional area was made by ultrasound (Seymour 2009) (see figure 2.4), based on a method originally devised by de Bruin (de Bruin 1997). B-mode ultrasonography was used with an 8MHz 7cm linear signal transducer array (PLF 805 ST, Toshiba Medical Systems, Crawley, UK). The patient was positioned supine with their rested leg supported in passive extension. An anatomical landmark was found at three-fifths distance along a line from the anterior superior iliac spine to the superior patella border. Ultrasound contact gel was applied to provide an adequate interface between the transducer probe and the skin to minimise any soft tissue pressure distortion. The transducer was positioned in the transverse plane and orientated so that the entire rectus femoris cross-sectional area could be visualised onscreen. Scanning depth was set to where the femur could also be viewed for orientation. Slow contraction-relaxation manoeuvres were used to aid delineation of the muscle septa. The image was then frozen and the inner echogenic line representing the fascia around the rectus femoris was outlined manually by the operator. Rectus femoris cross-sectional area was calculated using a planimetric technique (Nemio, Toshiba Medical Systems) and the average of three consecutive measurements was taken.
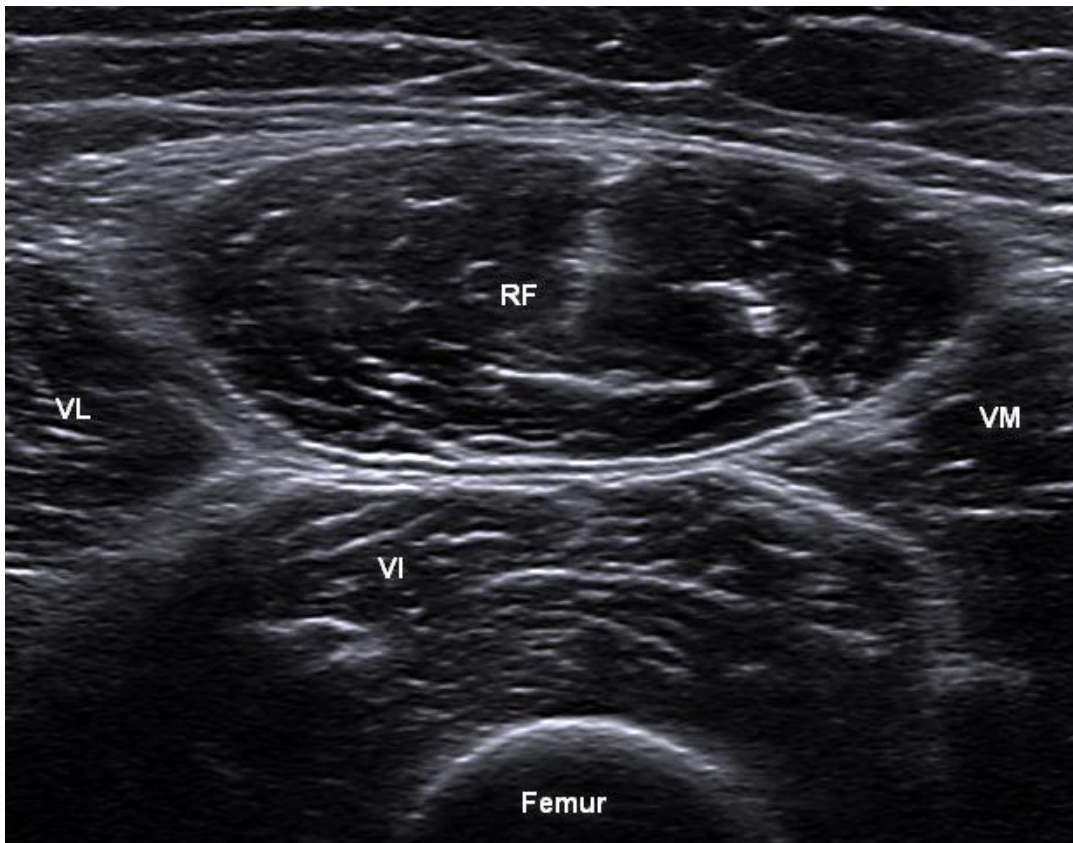
Figure 2.4: Ultrasound of the quadriceps

(RF, rectus femoris; VL, vastus lateralis; VM, vastus medialis; VI, vastus intermedius).

2.8.2 Mid-thigh Computed Tomography Cross-sectional area

Measurement of mid-thigh cross-sectional area was made by CT scan (see figure 2.5), based on a previously developed protocol (Marquis 2002). CT was performed on a 64-slice CT scanner (Siemens SOMATOM Sensation 64, Erlangen, Germany) with the patient in a supine position. A single section of the mid-thigh at a predefined level was obtained using the following acquisition parameters: 50mAs, 120kVp. The protocol was modified to deliver a reduced amount of radiation per scan. The total radiation dose of two thigh CT scans is equivalent to 0.6mSv which is equivalent to 30 chest X-rays (0.02mSv per chest X-ray) or approximately 3 months of natural background radiation. Images were viewed and CT cross-sectional areas calculated using Digital Imaging Communications in Medicine viewing software (DicomWorks, version 1.3;*http://dicom.online.fr*) at standard window settings for visualisation of soft tissues (centre 40 HU, window width 380 HU).
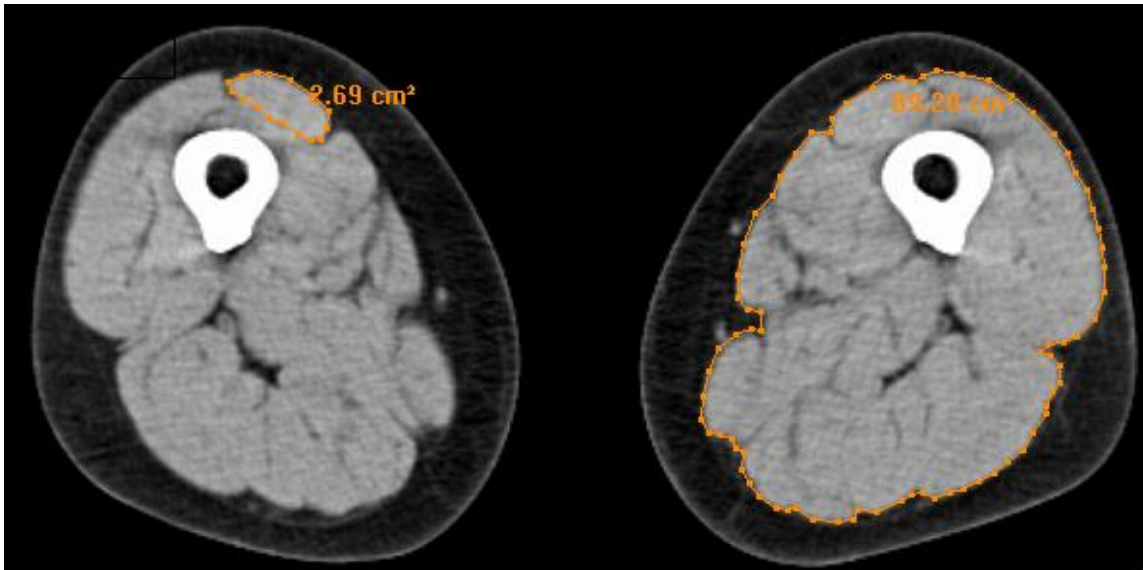
Figure 2.5: Mid-thigh Computed Tomography image

(Left image: Rectus femoris CSA; Rt image: Mid-thigh outline, from which femur CSA is

subtracted to give mid-thigh CSA).

2.10   Physical Activity Monitoring

2.10.1   Sensewear ProArmband

Daily physical activity was recorded using a multisensor biaxial accelerometer armband (SenseWear, BodyMedia; Pittsburgh, PA) as previously described by Watz *et al* (Watz 2008). Subjects were asked to wear the activity monitor on the upper arm, over the triceps, (see figure 2.6) and to only remove the device when bathing or showering. The armband incorporates physiological sensors that quantify galvanic skin response, heat flux and skin temperature to estimate energy expenditure and has been previously validated against indirect calorimetry in COPD patients (Patel 2007; Hill 2010) and against the doubly labelled water technique in healthy subjects (St-Onge 2007). The physical activity level (PAL) was calculated using total energy expenditure (TEE) and sleep energy expenditure as a surrogate for resting energy expenditure (REE) (PAL=TEE/REE). Daily step count and PAL were downloaded and analysed using Sensewear professional software version 6.1.

Figure 2.6: Sensewear Pro Armband

2.11 Patient Questionnaires

2.11.1 St George's Respiratory Questionnaire

This standardised 76-item self-complete questionnaire (Jones 1992) allows a total score to be calculated based on three component scores; symptoms, activity and impacts on daily life. The scores all range from 0-100 with a higher number indicating a worse health related quality of life. A change of 4 points is considered clinically significant. The questionnaire provides a validated measurement of impaired health, in conditions of chronic airflow limitation including COPD.

2.11.2 COPD Assessment Test

This short, self-complete questionnaire has been recently developed as a standardised measure of COPD health status (Jones 2009). The questionnaire contains 8 items, each with a score ranging from 0-5, which are then added to give a score from 0-40 (figure 2.7). A higher score represents worse health and a change of 2.5 points is considered likely to represent a clinically important difference. The questionnaire is simple to complete and provides a user-friendly assessment of health related quality of life in COPD.

Example: I am very happy (0) (X) (2) (3) (4) (5) I am very sad

SCORE

| I never cough | (0) (1) (2) (3) (4) (5) | I cough all the time |

| I have no phlegm (mucus) in my chest at all | (0) (1) (2) (3) (4) (5) | My chest is completely full of phlegm (mucus) |

| My chest does not feel tight at all | (0) (1) (2) (3) (4) (5) | My chest feels very tight |

| When I walk up a hill or one flight of stairs I am not breathless | (0) (1) (2) (3) (4) (5) | When I walk up a hill or one flight of stairs I am very breathless |

| I am not limited doing any activities at home | (0) (1) (2) (3) (4) (5) | I am very limited doing activities at home |

| I am confident leaving my home despite my lung condition | (0) (1) (2) (3) (4) (5) | I am not at all confident leaving my home because of my lung condition |

| I sleep soundly | (0) (1) (2) (3) (4) (5) | I don't sleep soundly because of my lung condition |

| I have lots of energy | (0) (1) (2) (3) (4) (5) | I have no energy at all |

TOTAL SCORE

Figure 2.7: COPD Assessment Test (CAT)

2.12   Quadriceps Muscle Biopsy

2.12.1   Bergstrom Technique

Muscle biopsies were taken from the vastus lateralis muscle using a Bergstrom needle biopsy technique (see figure 2.8) (Bergstrom 1975). The subject reclined on a couch in a lateral position with the knee supported by a pillow. The correct anatomical position was identified at the midpoint between the anterior superior iliac spine and the patella. The biopsy site was cleaned with a chloroprep solution and then the skin anaesthetised with 10ml of Lignocaine 2%. After suitable anaesthesia, an incision of approximately 1cm was made in the skin and a Bergstrom biopsy needle used to take small samples of muscle. A second operator attached a 50ml syringe to the needle to provide suction during the biopsy. Following the procedure, steristrips were applied to the incision site. This muscle biopsy technique is minimally invasive and subjects can mobilise freely following the procedure. A pea sized (approximately 60mg) muscle biopsy sample was snap frozen in liquid nitrogen and then stored at -80 ^{O}C prior to mRNA and protein analysis.

Figure 2.8: Bergstrom vastus lateralis muscle biopsy

2.12.2 RNA extraction and cDNA synthesis

For RNA extraction, muscle samples were homogenised in TRIzol reagent (Sigma, UK) using 1.4mm ceramic beaded tubes (Stretton Scientific, UK) and a Precellys 24 homogeniser (Peqlab, Erlangen, Germany). The protocol was 2x10 second cycles at 5500rpm with a 5 second pause. The samples were then centrifuged at 8000rpm for 3 minutes, with the resulting supernatant transferred to 1.5ml microcentrifuge tubes for extraction. 100µl of chloroform was added and the mixture vortexed before being left to settle at room temperature for 3 minutes. The sample was centrifuged at 13,000rpm for 15 minutes at 4°C so as to separate the mixture into 3 phases with the top transparent phase containing the RNA. This top layer was transferred to a separate 1.5ml microcentrifuge tube and 250µl isopropanol was added to precipitate the RNA. This mixture was left at room temperature for 10minutes and then centrifuged at 10,000 x g for 10minutes at 4°C. The resulting RNA pellet was washed twice with 500µl of 75% ethanol. The remaining ethanol was removed by re-centrifuging the sample before removing any liquid, and the pellet left to dry before finally being re-suspended in 30µl RNase free H_2O prior to quantification. RNA concentration was quantified by measuring absorption at 260nm with a spectrophotometer (Nanodrop ND1000, Wilmington, USA).

First strand cDNA was synthesised using an Omniscript Reverse Transcriptase 200 kit (Qiagen). For each sample, 150ng of RNA was added to 11µl of RNase-free water and heated to 65°C for 5 minutes. After 2 minutes on ice, 9µl of a mixture containing 2µl 10x Buffer RT, 1µl 5mM dNTPs, 0.5µl random primers, 0.5µl RNase inhibitor, 1µl (0.1M) DTT, 0.5µl Omniscript reverse transcriptase (Qiagen) and 3.5µl of RNase-free water was added

and the samples were incubated at 42˚C for 2 hours. The samples were then diluted to 200μl with distilled H_2O and stored at -20˚C for subsequent analysis.

2.12.3   Primer validation

Primers for target genes were validated by Polymerase Chain Reaction (PCR) so as to ensure that only the regions of interest were amplified. The reaction mixture contained 3μl of sample cDNA, 2μl of a forward and reverse primer mix (2pmol/μl), 10μl of 2x SYBR green Taq Jump Start mix and 5μl of distilled H_2O. The PCR products were analysed by electrophoresis on a 2% (w/v) agarose gel containing ethidium bromide to confirm product size and exclude primer dimers.

2.12.4   Real-Time quantitative Polymerase Chain Reaction (RT-qPCR)

Real-time quantitative PCR (RT-qPCR) was performed in duplicate on each sample, testing for MuRF-1, atrogin-1, IGF-1, TGF-β and the reference housekeeping gene, human RPLPO. A 20μl reaction containing 10μl Fast SYBR Green Master Mix (Applied Biosystems, UK), 5μl distilled H_2O, 2μl of the forward and reverse primer mix (2pmol/μl) and 3μl of the sample, was used in 96 well plates (MicroAmp, Fast optical 96 well reaction plate, 0.1 ml, Applied Biosystems, UK) with an adhesive plate cover (MicroAmp, Optical adhesive film, Applied Biosystems, UK). RT-qPCR reactions were run using a 7500 Fast Real-time PCR System (Applied Biosystems, UK), with the following cycle program: 95°C for 5 minutes, then 40 cycles of 95°C for 10 seconds & 60°C for 30 seconds for annealing and extension. RT-qPCR data were analysed using cycle threshold (Ct) values which relate to the number of cycles

at which the fluorescence reaches a threshold above a baseline value. The data were then normalised to human RPLPO expression as previously described (Ellis 2004), with the values log transformed to obtain a normal distribution.

2.12.5 Protein extraction and enzyme linked immunosorbent assay (ELISA)

For protein extraction, muscle samples were homogenised in lysis buffer (Tris pH 7.4 (50mM), NaCl (250mM), EDTA (5mM), 1% Nonidet P40 (Roche Applied Science)) containing protease and phosphatase inhibitors (Sigma, UK). Ceramic beaded tubes were used with a homogeniser protocol as described above. A Bradford assay was then used to measure protein concentration against BSA standards as per the manufacturer's recommendations. A BioRad microtiter plate reader was used to measure absorbance at 595nm using Luminex analysis software. To determine levels of phosphorylated 4EBP-1, the protein lysate was analysed using an enzyme linked immunosorbent assay (ELISA) kit, (Invitrogen) containing a specific 4EBP-1 detection antibody. The absorbance at 450nm was measured using a Bio-Tek plate reader.

2.13 Blood Sampling

Approximately 20mls of venous blood was taken from subjects and divided into whole blood, serum and plasma samples. The whole blood samples were stored at -80˚C for subsequent ACE and bradykinin genotyping. Serum and plasma samples were centrifuged

and also stored at -80°C prior to batch analysis for inflammatory cytokines and biological

markers related to muscle atrophy.

2.13.1 ACE and bradykinin genotyping

ACE and bradykinin genotyping was conducted by the Department of Cardiovascular Genetics, Rayne Institute, University College London. Genomic leukocyte DNA was extracted from whole blood by salting out and ACE genotype was determined by PCR with amplification using a 3-primer method which included an I-specific oligonucleotide (O'Dell 1995). The original 2-primer method, where each primer flanked the insertion sequence, often preferentially amplified the shorter D allele leading to mistyping of heterozygous genotypes as DD. By annealing to the insertion sequence itself, the third primer avoids this problem by creating a shorter fragment for the I allele (65 base pairs) compared to the D allele (84 base pairs). Primer ratios were 10pmol ACE1 (deletion-specific oligonucleotide), 2pmol ACE2 (insertion-specific oligonucleotide) and 8pmol ACE3 (common oligonucleotide). Each 20µl PCR reaction contained the ACE primers, with 50nM KCl, 10mM Tris HCl pH 8.3, 1.5mM $MgCl_2$, 0.2 units of Taq polymerase, overlaid with 20µl mineral oil. Thirty PCR cycles of 95°C for 1 minute, 50°C for 1 minute and 72°C for 5 minutes were used and the products resolved on a 7.5% polyacrylamide gel stained with ethidium bromide. Genotypes were confirmed independently by 2 operators and discrepancies resolved through repeat genotyping. Bradykinin type II receptor genotype was determined by PCR using a 2-primer method (forward 5'-TCTGGCTTCTGGGCTCCGAG-3' and reverse 5'AGCGGCATGGGCACTTCAGT-3') with the products resolved on a long gel.

2.13.2 Serum analysis

Serum analysis was conducted by the King's Pathology Department, King's College London. A serum cytokine multiplex array was performed by chemiluminescent immunoassay using an Evidence Investigator BioChip Analyser (Randox Laboratories, UK) for the quantitative detection of interleukin-6, 8, 18 and monocyte chemotactic protein-1. Serum IGF-1, NT-pro-BNP and hs-CRP were measured by enzyme-linked immunosorbent assay (ELISA) (Siemens Healthcare Diagnostics, UK) and serum ACE-activity measured by a kinetic enzyme assay (Buhlmann Laboratories AG, Switzerland).

Chapter 3: Quadriceps Wasting and Physical Inactivity in COPD

3.1   Introduction

3.1.1   Background

Skeletal muscle dysfunction is a well recognised extrapulmonary complication of chronic obstructive pulmonary disease (COPD) with loss of lean body mass identified as a key determinant of disability and an independent predictor of mortality (Schols 2005). In particular, reduced quadriceps strength is associated with reduced exercise capacity (Gosselink 1996), impaired quality of life (Simpson 1992), increased healthcare use (Decramer 1997) and mortality independent of airflow obstruction (Swallow 2007a).

The mechanisms involved in the development of skeletal muscle weakness in COPD are likely to be multi-factorial with systemic factors, such as oxidative stress (Barreiro 2010), thought to interact with the key local factor of muscle inactivity (Pitta 2005; Watz 2008) particularly in the lower limbs (Man 2003b). Objectively measured physical activity has been identified as a strong predictor of all-cause mortality in COPD (Waschki 2011), highlighting its importance in a 'downward disease spiral' where progressive dyspnoea leads to reduced exercise capacity with subsequent muscle deconditioning and further inactivity (Polkey 2006).

3.1.2 Rationale and hypothesis

Quadriceps weakness has recently been observed in the absence of severe airflow obstruction in COPD (Seymour 2010b), and in addition there is data to suggest a reduction in physical activity in GOLD stage I patients (Watz 2009). Despite the potential rationale for muscle wasting in mild disease, little data exists on reduced quadriceps bulk in this patient group. Mid-thigh cross-sectional area measured by computed tomography (CT) has been shown to predict mortality in moderate-severe COPD (Marquis 2002), however the ionising radiation exposure makes this method of imaging undesirable particularly in mild disease. Magnetic resonance imaging (MRI) has also been used as a thigh muscle imaging modality in COPD (Mathur 2008) but the accessibility and expense of this tool prohibit its adoption in the wider COPD population.

Ultrasound measurement of rectus femoris cross-sectional area ($USRF_{CSA}$) is a radiation-free measure of muscle bulk that relates to quadriceps strength in COPD but is effort independent (Seymour 2009). We hypothesised that quadriceps wasting, measured by $USRF_{CSA}$, would be observed in mild as well as advanced COPD compared to healthy age-matched subjects and that this would correlate with daily physical activity levels.

3.2   Methods

3.2.1   Study design and participants

This cross-sectional study recruited COPD patients from outpatient clinics at the Royal Brompton Hospital, King's College and St Thomas' Hospitals as well as through public spirometry events conducted on World COPD and No-Smoking days. COPD diagnosis was consistent with NICE and GOLD criteria as previously described. Subjects within one month of an exacerbation or with a significant co-morbidity including cardiac failure, neurological disease, malignancy or rheumatoid arthritis were excluded. The healthy age-matched controls were recruited by advertisement in local newspapers.

3.2.2   Study measurements

A clinical history was taken, followed by baseline anthropometric measurements and determination of fat-free mass index (FFMI). Heath-related quality of life was assessed using the St. George's Respiratory Questionnaire. Breathlessness was recorded using the Medical Research Council (MRC) dyspnoea score. Subjects had full pulmonary function tests including measurement of lung volumes and gas transfer. Quadriceps size was assessed by ultrasound measurement of rectus femoris cross-sectional area and quadriceps strength assessed by maximum voluntary contraction. Daily physical activity was recorded using a multisensor armband with step count and physical activity level (PAL) measured over six consecutive days incorporating one weekend and four weekdays. A valid physical activity assessment was defined as ≥21.5 hours (90%) wearing time a day on at least 5 days.

3.2.3   Data and statistical analysis

Data are presented as mean ± SD, with accompanying p value, and analysis was performed using StatView 5.0 (Abacus concepts, Inc., Berkeley, CA, USA). Between group comparisons used analysis of variance (ANOVA), with post-hoc correction for more than 2-groups. Relationships between $USRF_{CSA}$, quadriceps strength, FFMI, impedance ratio, daily physical activity and pulmonary function were analysed using univariate and multivariate linear regression models. Figure construction was performed with GraphPad Prism Version 5.0 (GraphPad Software, San Diego, California, USA).

3.3   Results

3.3.1   Participants

Two hundred and one subjects, comprising 161 stable COPD patients and 40 healthy age-matched volunteers, participated in the study. Baseline characteristics are shown in table 3.1. Of these, 154 subjects (123 COPD patients and 31 healthy participants) completed a valid physical activity assessment. Out of the remaining 47 subjects, 14 did not complete a valid period of assessment, 4 subjects declined to participate in this part of the study and the remainder were not given an armband for logistical reasons (e.g. armband availability and subject's distance from hospital). In those subjects participating in activity monitoring, a valid period of assessment was reached in 92% (154/168). Average wearing time per day was 98% and did not significantly differ across groups (see table 3.2).

Table 3.1: Baseline characteristics of COPD and control subjects

	Controls (n=40) mean (SD)	COPD I (n=38)	COPD II (n=45)	COPD III (n=41)	COPD IV (n=37)	p value
Age (years)	65 (8)	67 (9)	67 (9)	67 (9)	63 (8)	0.17
Gender (M/F)	20/20	19/19	22/23	22/19	25/12	0.45
BMI (kg/m^2)	25.9 (3.6)	26.5 (4.8)	26.6 (5.9)	25.2 (4.5)	22.4 (3.8)	0.0006
FFMI (kg/m^2)	17.8 (2.1)	17.9 (2)	17.7 (2.7)	17.6 (2.2)	16.2 (1.9)	0.004
Smoking (pack years)	9.1 (14.3)	28.1 (22.2)	42 (29.2)	50 (26.2)	55.5 (29.3)	<0.0001
Current smokers (%)	-	11	29	27	14	0.09
Outpatients (%)	-	47	60	66	89	0.001
Long-acting beta agonist (%)	-	58	80	85	100	<0.0001
Long-acting anticholinergic (%)	-	42	71	83	97	<0.0001
Inhaled corticosteroid (%)	-	58	78	83	100	<0.0001
Oral corticosteroid (% ≥5mg/day)	-	0	0	0	8	0.02
FEV_1 % pred	103.1 (11.7)	90.8 (8.8)	63.2 (8.8)	39.4 (5.5)	24 (3.8)	<0.0001
TLco% pred	89.3 (17.1)	67.5 (17.8)	58.1 (14.7)	39.9 (13.9)	26.6 (9)	<0.0001
RV%TLC ratio	34 (4.6)	40.6 (6.6)	46.6 (8)	57.9 (7.5)	64.7 (6.9)	<0.0001

	Controls (n=40) mean (SD)	COPD I (n=38)	COPD II (n=45)	COPD III (n=41)	COPD IV (n=37)	p value
IC (litres)	2.8 (0.7)	2.7 (0.8)	2.3 (0.8)	1.9 (0.6)	1.8 (0.4)	<0.0001
PaO_2 (kPa)	11.2 (1.1)	10.6 (1.5)	10.2 (1.2)	9.1 (1.2)	9.1 (1.3)	<0.0001
$PaCO_2$ (kPa)	5 (0.6)	4.7 (0.5)	5 (0.4)	5.1 (0.5)	5.4 (0.6)	<0.0001
MRC score (1-5)	1.1 (0.3)	1.8 (0.4)	2.5 (0.9)	3.1 (0.9)	3.6 (0.9)	<0.0001
SGRQ (Symptoms)	-	29.4 (24.8)	49.6 (22.8)	50 (23.7)	61.6 (19.1)	<0.0001
SGRQ (Activity)	-	34.7 (25)	58.1 (23.9)	70.1 (19.4)	84.5 (11.5)	<0.0001
SGRQ (Impacts)	-	15.5 (15.1)	29.9 (17.4)	33.8 (17.4)	48.4 (18.1)	<0.0001
SGRQ (Total)	-	23.1 (16.8)	41.1 (17.9)	47.6 (16.4)	61.5 (13.8)	<0.0001

Abbreviations: BMI - body mass index; FFMI – fat free mass index; FEV_1 - forced expiratory volume in 1 second; TLco – carbon monoxide diffusing capacity; RV – residual volume; TLC – total lung capacity; IC – inspiratory capacity; PaO_2 - arterial partial pressure of oxygen; $PaCO_2$ - arterial partial pressure of carbon dioxide; SGRQ – St George's respiratory questionnaire; Outpatients – defined as any previous hospital clinic attendance.

Table 3.2: Quadriceps and physical activity measurements in COPD and control subjects

	Controls mean (SD)	COPD I	COPD II	COPD III	COPD IV	p Value
USRF$_{CSA}$ (mm^2)	640 (136)	530 (116)	511 (135)	504 (122)	509 (122)	<0.0001
QMVC (kg)	34.3 (8.8)	29.6 (7.2)	27.9 (7.3)	27.3 (8.8)	25.3 (6.8)	<0.0001
Daily Step count	11735 (4399)	7960 (3430)	6606 (3328)	4010 (2316)	2219 (1157)	<0.0001
Daily Physical Activity Level	1.69 (0.25)	1.56 (0.16)	1.47 (0.16)	1.4 (0.12)	1.38 (0.19)	<0.0001
Armband wearing time (hours/day)	23.57 (0.28)	23.60 (0.26)	23.64 (0.37)	23.57 (0.52)	23.61 (0.39)	0.95
Z$_{200}$/Z$_5$ Impedance ratio	0.789 (0.03)	0.791 (0.03)	0.806 (0.03)	0.816 (0.03)	0.814 (0.03)	0.0002

Abbreviations: USRF$_{CSA}$ – ultrasound rectus femoris cross-sectional area; QMVC – quadriceps maximal voluntary contraction.

3.3.2 USRF$_{CSA}$ and quadriceps strength in COPD (Stage I-IV) and healthy subjects

USRF$_{CSA}$ and quadriceps strength (QMVC) were reduced in all GOLD stages compared to controls (table 3.2, figure 3.1 and figure 3.2). There were no significant differences in USRF$_{CSA}$ or QMVC across GOLD stages, except between QMVC in stage I and IV ($p<0.02$).
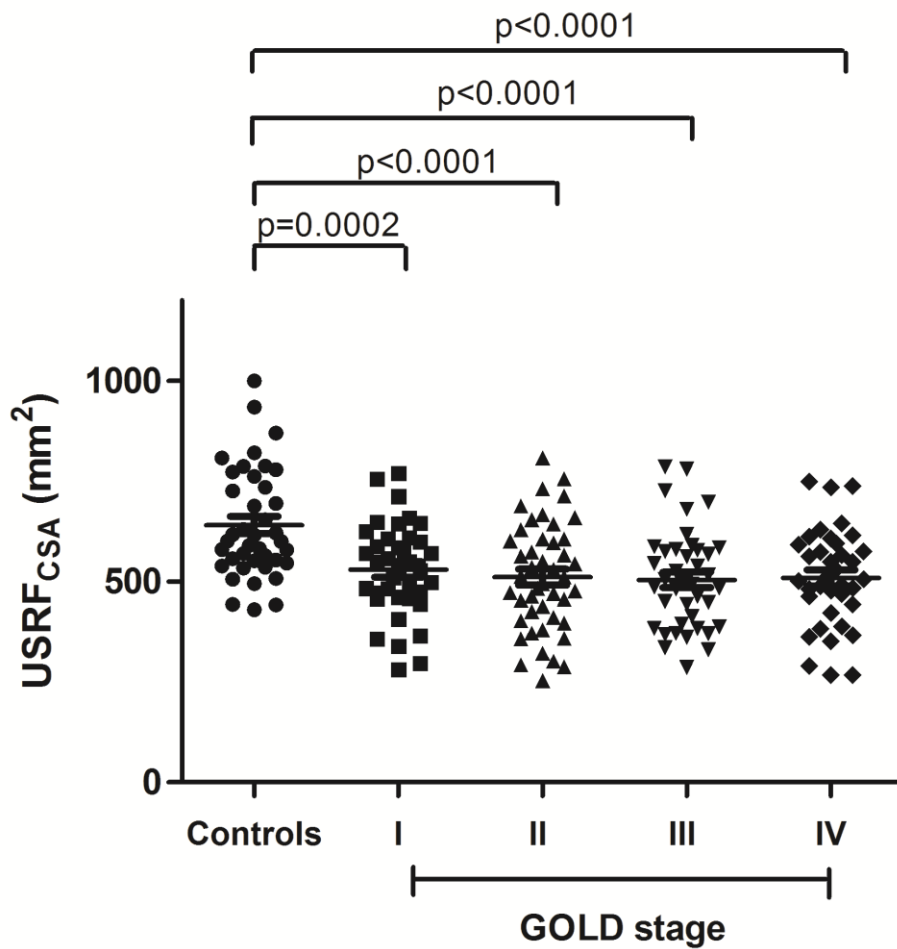
Figure 3.1: Ultrasound rectus femoris cross-sectional area (USRF$_{CSA}$) versus GOLD stage in COPD patients and healthy controls (ANOVA - no significant difference between I-IV). Cross bars represent the standard error of the mean (SEM).
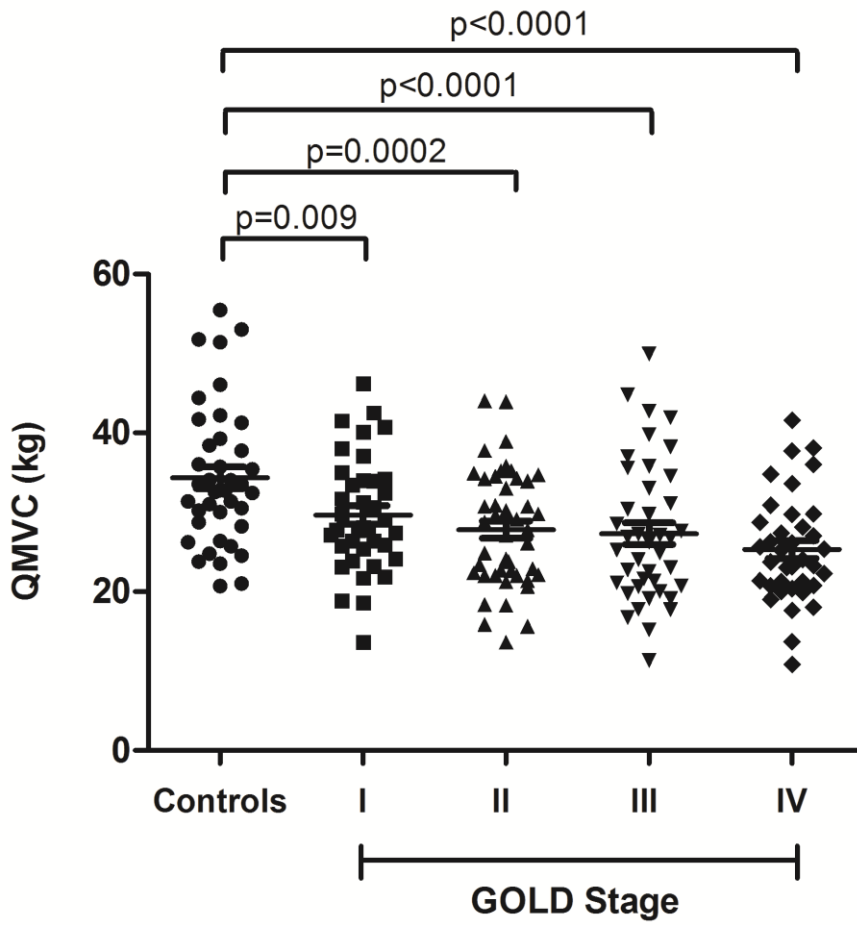
Figure 3.2: Quadriceps maximal voluntary contraction (QMVC) versus GOLD stage in COPD and healthy controls (ANOVA - no significant difference between GOLD stages, except I and IV; $p < 0.02$). Cross bars represent SEM.

In COPD patients, FEV_1% predicted showed no association with $USRF_{CSA}$ and a weak association with QMVC (r=0.2, p=0.03). $USRF_{CSA}$ had a linear relationship with QMVC in COPD subjects (r=0.6, p<0.0001) (figure 3.3). QMVC was also significantly associated with FFMI (r=0.54, p<0.0001) and the impedance ratio (Z_{200}/Z_5) (r=−0.54, p<0.0001) in COPD (figure 3.4).
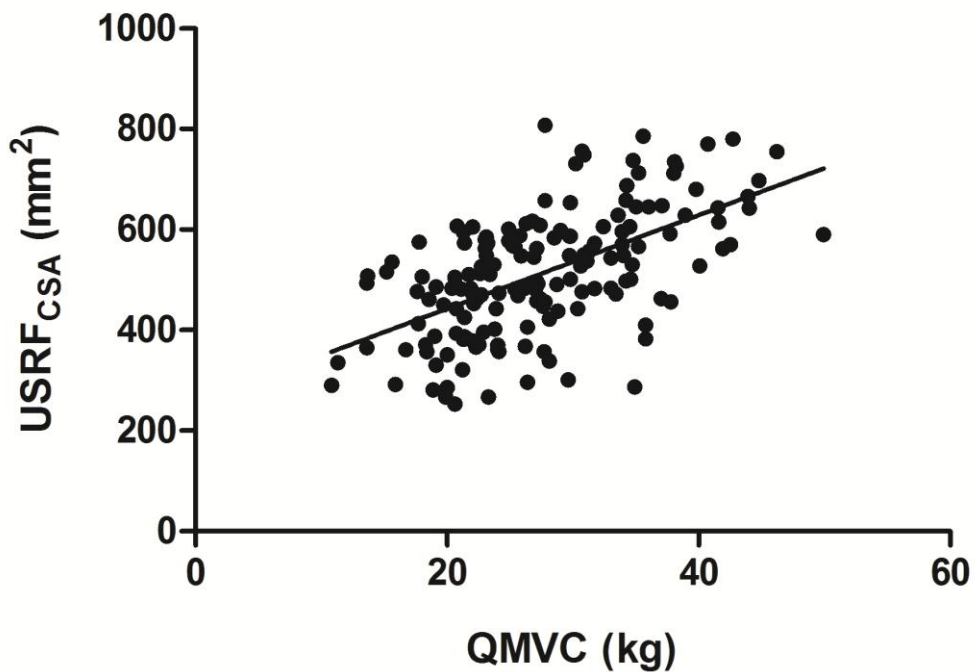
Figure 3.3: Quadriceps strength (QMVC) versus ultrasound rectus femoris cross-sectional area ($USRF_{CSA}$) in COPD patients (Pearson correlation; r=0.6, p<0.0001).
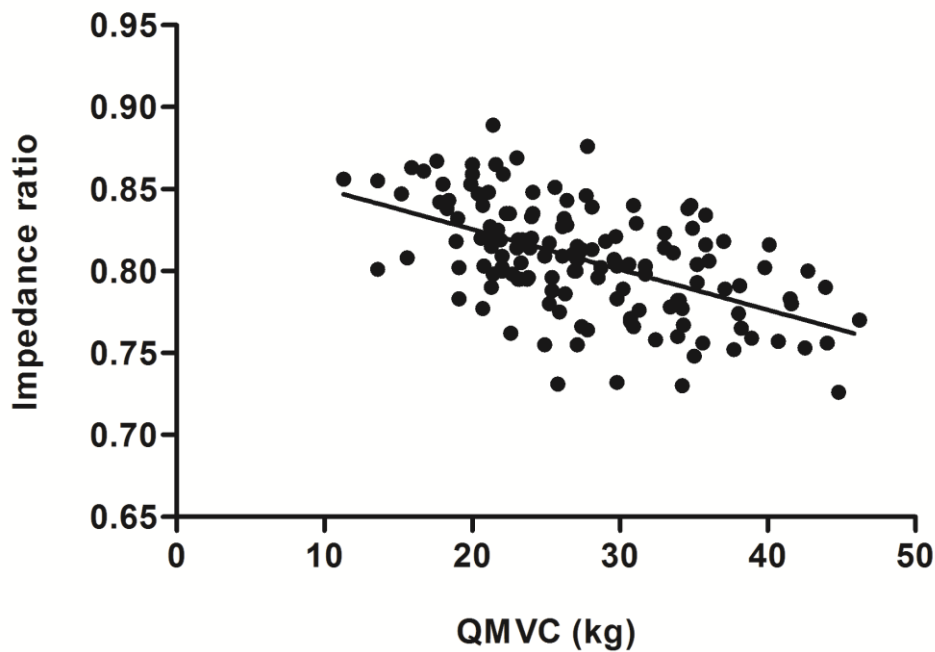
Figure 3.4: Quadriceps strength (QMVC) versus Impedance ratio in COPD patients (Pearson correlation; r=−0.54, p<0.0001).

A multiple regression model was used to predict USRF$_{CSA}$ in all COPD subjects incorporating the significant independent variables from the univariate analysis (see table 3.3). Gender (r=0.27, p=0.003), QMVC (r=0.24, p=0.01), residual volume to total lung capacity (RV/TLC) ratio (r=−0.28, p=0.01), inspiratory capacity (IC) (r=0.20, p=0.04) and FFMI (r=0.19, p=0.04) were retained as independent predictors of USRF$_{CSA}$ (r=0.75, p<0.0001). In a similar multiple regression model with QMVC as the dependent variable, only USRF$_{CSA}$ (r=0.24, p=0.02) and FFMI (r=0.25, p=0.01) were retained as independent predictors of quadriceps strength in COPD (r=0.74, p<0.0001).

Table 3.3: Univariate correlates of USRF$_{CSA}$ in all COPD subjects

	USRF$_{CSA}$ (r)	p value
Age	−0.20	0.01
Gender	0.48	<0.0001
FFMI	0.49	<0.0001
FEV$_1$% pred	0.08	0.32
TLco% pred	0.26	0.001
RV/TLC ratio	−0.22	0.006
IC	0.44	<0.0001
QMVC	0.60	<0.0001
Impedance ratio	−0.53	<0.0001
PAL	0.20	<0.05
Steps	0.30	0.002

Abbreviations: QMVC - quadriceps maximum voluntary contraction; USRF$_{CSA}$ - ultrasound rectus femoris cross-sectional area; FEV$_1$ - forced expiratory volume in 1 second; RV – residual volume; TLC – total lung capacity; IC – inspiratory capacity; TLco – carbon monoxide diffusing capacity; FFMI - fat free mass index; PAL – physical activity level (r and p values derived from Pearson's correlation coefficient).

As gender was identified as an independent variable to predict $USRF_{CSA}$, the COPD and healthy subjects were separated into males and females (figure 3.5). In both genders, $USRF_{CSA}$ was reduced in all GOLD stages compared to controls and there were no significant differences in $USRF_{CSA}$ across GOLD stages. Males (n=108) had a significantly greater $USRF_{CSA}$, compared to females (n=93), 597mm^2 vs 470mm^2, (p<0.0001).
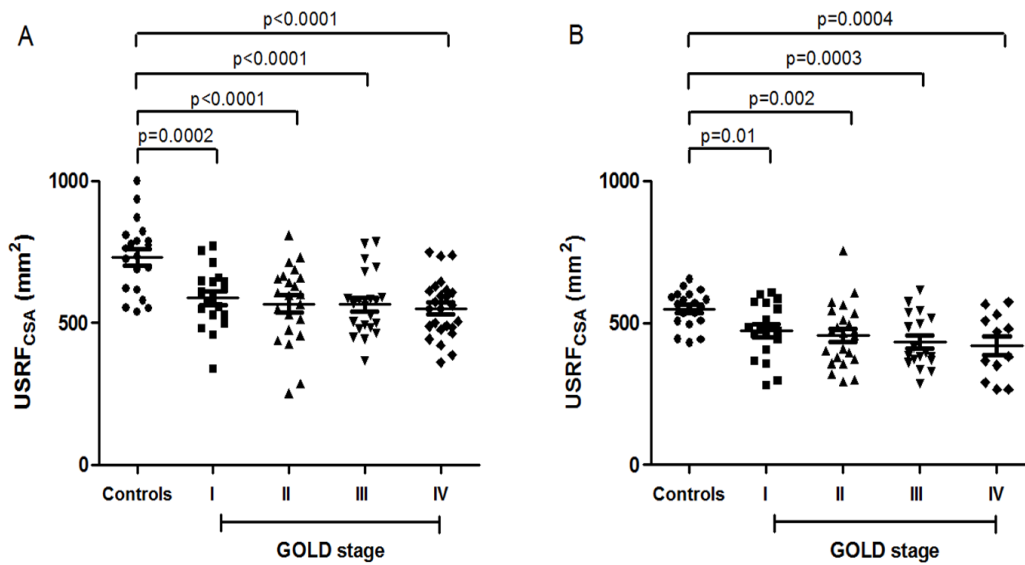
Figure 3.5: Ultrasound rectus femoris cross-sectional area (USRF$_{CSA}$) in COPD patients and healthy controls separated by gender (males - A and females - B; ANOVA - no significant difference between stages I-IV. Cross bars represent SEM).

3.3.3 Relationship of daily physical activity with GOLD stage and USRF$_{CSA}$

Daily physical activity was significantly reduced in all GOLD stages compared to healthy controls (figure 3.6 and figure 3.7). Mean group differences are shown in table 3.2.
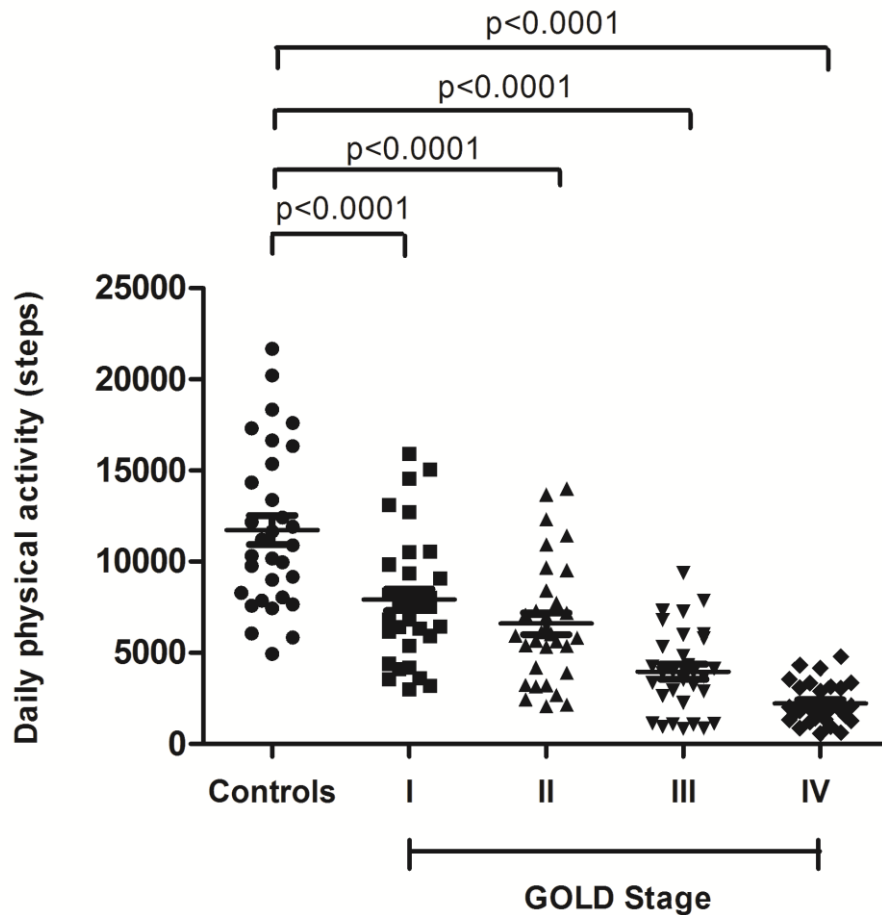
Figure 3.6: Daily physical activity (steps) versus GOLD stage in COPD patients and healthy controls (ANOVA - significant differences also observed between stages 1,3 & 1,4 p<0.0001; 2,3 p=0.002; 2,4 p<0.0001 & 3,4 p=0.03). Cross bars represent SEM.
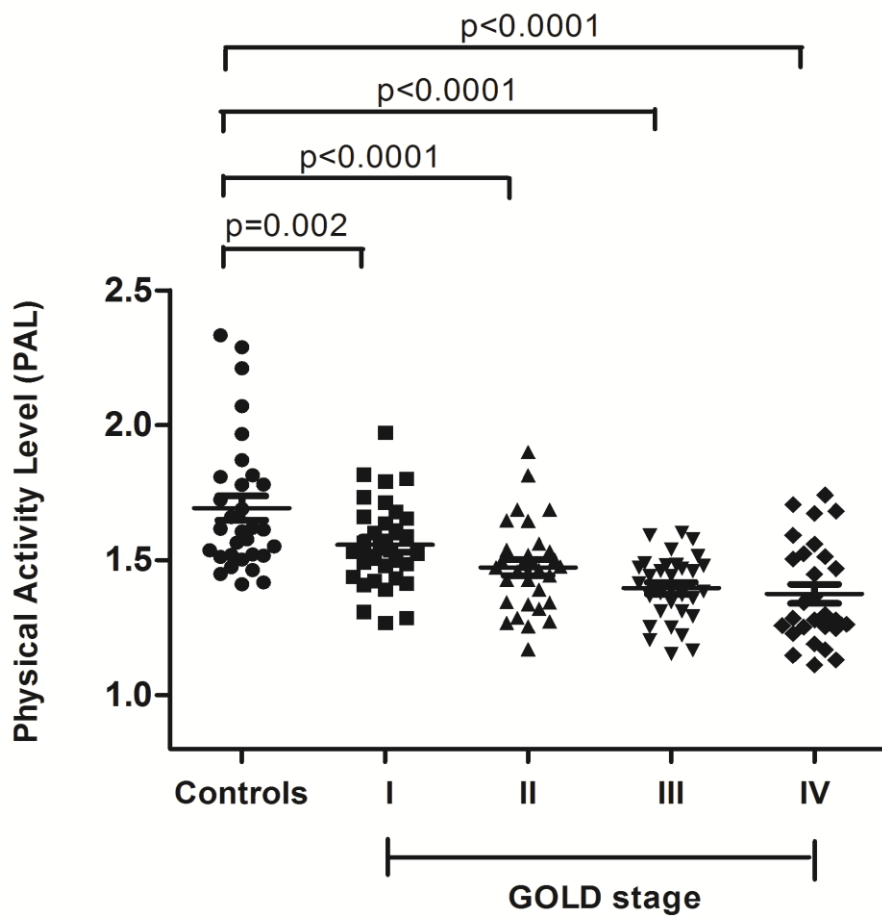
Figure 3.7: Physical activity level (PAL) versus GOLD stage in COPD subjects and healthy controls (ANOVA - significant differences also observed between stages 1,3 p=0.0006; 1,4 p=0.0002 & 2,4 p=0.04). Cross bars represent SEM.

Daily physical activity showed a linear relationship with FEV_1% predicted (steps, r=0.6; PAL, r=0.4, p<0.0001) and $USRF_{CSA}$ (steps, r=0.3, p=0.002; PAL, r=0.2 p<0.05) in all COPD patients. In stage I disease, a multiple linear regression model to predict $USRF_{CSA}$ was used incorporating the significant independent variables from the univariate analysis (table 3.4). Physical activity level was the only variable retained as an independent predictor of $USRF_{CSA}$ in stage I disease (r=0.76, p=0.01). The univariate association between physical activity and $USRF_{CSA}$ in stage I disease is shown in fig 3.8. Using a similar regression analysis in stage II-IV disease, gender (r=0.29, p=0.01), RV/TLC ratio (r=−0.28, p=0.01) and IC (r=0.29, p=0.02) but not physical activity, were retained as independent predictors of $USRF_{CSA}$ (r=0.78, p<0.0001).

In a separate multiple linear regression model to predict physical activity in stages II-IV COPD, when incorporating the univariate correlates (table 3.5), RV/TLC ratio was retained over FEV_1% predicted as the only independent variable associated with physical activity level (r=−0.23, p=0.03). Using this model in stage I COPD, $USRF_{CSA}$ but not QMVC was retained as the only independent correlate with physical activity level (r=0.64, p=0.005).

Table 3.4: Univariate correlates of USRF$_{CSA}$ in stage I COPD subjects

	USRF$_{CSA}$ (r)	p value
Age	−0.33	0.04
Gender	0.50	0.001
FFMI	0.43	0.007
FEV$_1$ % pred	0.07	0.70
TLco% pred	0.38	0.03
RV/TLC ratio	−0.42	0.01
IC	0.41	0.02
QMVC	0.63	<0.0001
Impedance ratio	−0.64	<0.0001
PAL	0.70	<0.0001
Steps	0.53	0.002

Abbreviations: QMVC - quadriceps maximum voluntary contraction; USRF$_{CSA}$ - ultrasound rectus femoris cross-sectional area; FEV$_1$ - forced expiratory volume in 1 second; RV – residual volume; TLC – total lung capacity; IC – inspiratory capacity; TLco – carbon monoxide diffusing capacity; FFMI - fat free mass index; PAL – physical activity level. (r and p values derived from Pearson's correlation coefficient).
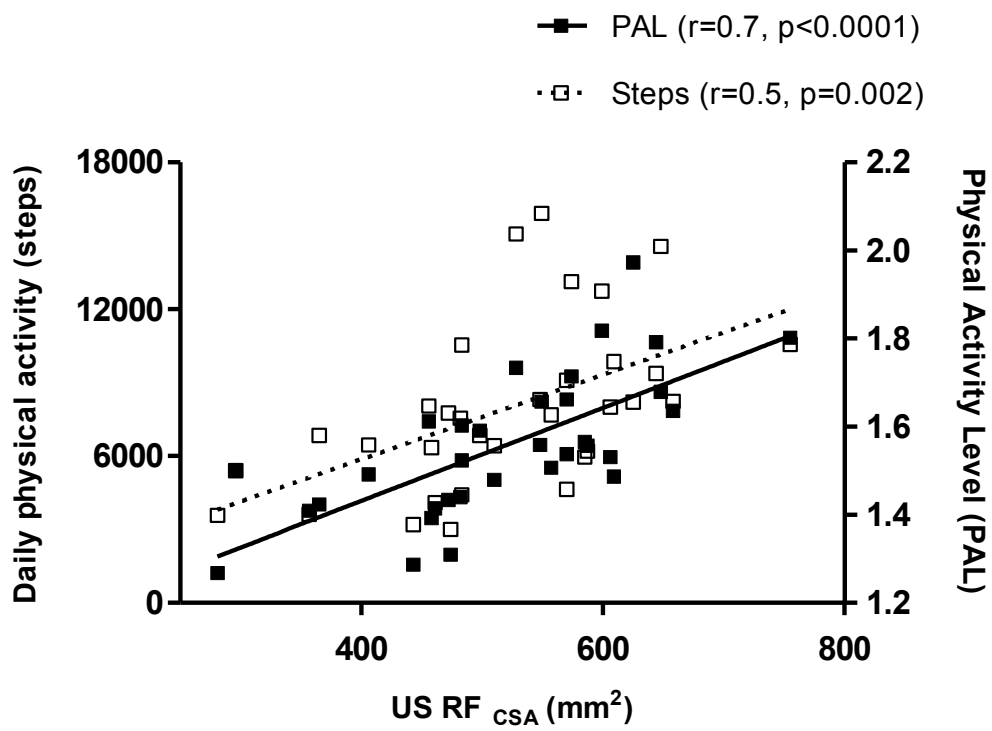
Figure 3.8: Physical activity versus USRF$_{CSA}$ in GOLD stage I COPD subjects

(Black squares – physical activity level, white squares – step count).

Table 3.5: Univariate correlates of physical activity in stages II-IV COPD

	Steps (r)	p value	PAL (r)	p value
Age	−0.12	0.25	−0.08	0.46
Gender	0.18	0.10	0.14	0.18
FFMI	0.19	0.07	0.10	0.40
USRF$_{CSA}$	0.24	0.02	0.05	0.70
FEV$_1$% pred	0.57	<0.0001	0.21	<0.05
TLco% pred	0.54	<0.0001	0.07	0.50
RV/TLC ratio	−0.53	<0.0001	−0.23	0.03
IC	0.27	0.01	0.01	0.92
QMVC	0.22	0.04	0.02	0.82
Impedance ratio	−0.27	0.01	−0.12	0.30

Abbreviations: QMVC - quadriceps maximum voluntary contraction; USRF$_{CSA}$ - ultrasound rectus femoris cross-sectional area; FEV$_1$ - forced expiratory volume in 1 second; RV - residual volume; TLC - total lung capacity; IC - inspiratory capacity; TLco - carbon monoxide diffusing capacity; FFMI - fat free mass index; PAL - physical activity level. (RV/TLC ratio was the only independent predictor of PAL (p=0.03) in stage II-IV COPD; FEV$_1$% pred and TLco% pred were independent predictors of step count (p<0.0001). r and p values derived from Pearson's correlation coefficient.)

3.3.4 Quadriceps dysfunction and health status in COPD

Health status was also assessed using the COPD assessment test (CAT) score in a subset of COPD patients (n=91, mean (SD), 66(8) years, FEV_1 46(22)% predicted, 56% male). A reduced quadriceps strength (QMVC) was associated with a worse health status (CAT score >20, n=44: QMVC 23.6 (5.7) kg vs. 27.4 (6.8) kg (p=0.005) (see figure 3.9). In addition, reduced quadriceps bulk measured by $USRF_{CSA}$ was also associated with a worse health status ($USRF_{CSA}$ 482 (126) mm^2 vs. 539 (120) mm^2 (p=0.03) (see figure 3.10) in these patients.
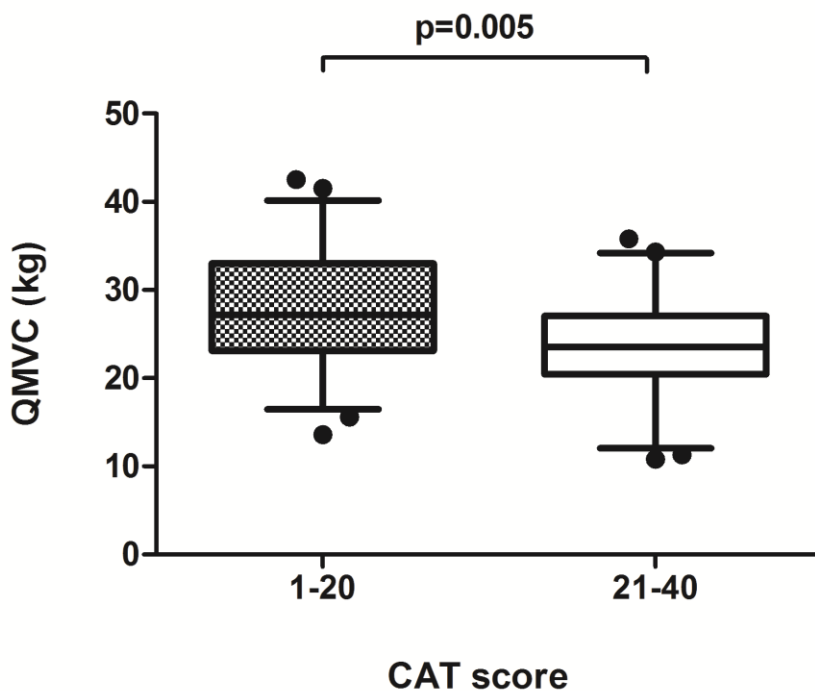
Figure 3.9: CAT versus QMVC in COPD subjects
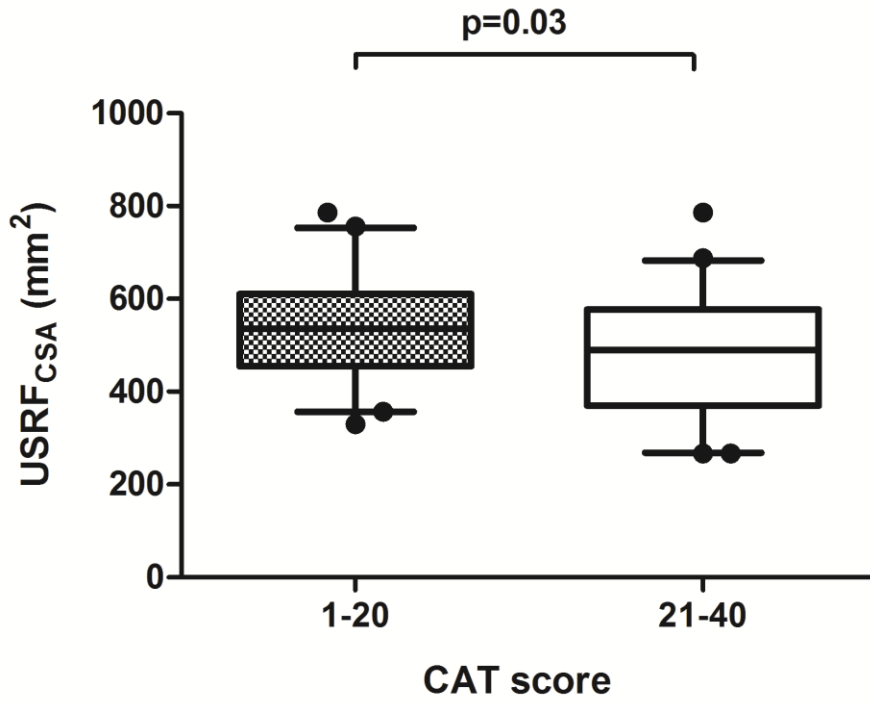
Figure 3.10: CAT versus USRF$_{CSA}$ in COPD subjects

3.3.5 Ultrasound validity and reproducibility

For validation, 80 COPD patients had a mid-thigh CT scan with ultrasound rectus femoris cross-sectional area correlating significantly with mid-thigh CT_{CSA} (r=0.7, p<0.0001) and rectus femoris CT_{CSA} (r=0.7, p<0.0001). In addition, data on inter-occasion and observer variability of the ultrasound measurement is detailed below (see figure 3.11).
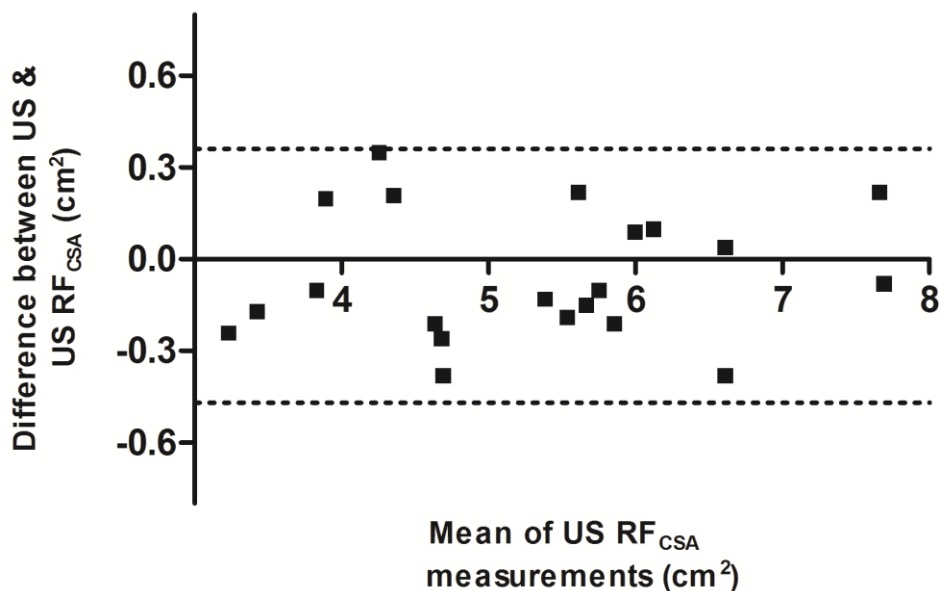
Figure 3.11: Bland-Altman analysis comparing the rectus femoris cross-sectional area (RF$_{CSA}$) measured by ultrasound on two separate occasions

(n=21, mean (SD) bias - 0.06 (0.21)cm^2 : coefficient of repeatability 0.41cm^2), dotted line represents 95% limits of agreement -0.47 to +0.36cm^2 , index of reliability 0.97. A similar analysis was conducted for inter-observer variability (n=10): mean (SD) bias 0.11 (0.29)cm^2 : coefficient of repeatability 0.57cm^2, 95% limits of agreement -0.46 to +0.68cm^2, index of reliability 0.97.

3.4 Discussion

3.4.1 Summary of results

Using USRF$_{CSA}$ we found quadriceps wasting in mild, as well as advanced COPD judged by GOLD stage. A 17% reduction in mean USRF$_{CSA}$ was observed in stage I patients compared to a healthy age matched group with a similar whole-body FFMI. The study also identified an independent association between physical activity level and USRF$_{CSA}$ in stage I disease, with this group significantly less active when compared to healthy subjects.

3.4.2 Significance of the findings

A recent study incorporating a large UK and Dutch COPD cohort (Seymour 2010b) identified a 28% prevalence of quadriceps weakness in Stage I patients and supports our contention that reduced quadriceps muscle bulk is present in early disease. The simple and effort independent nature of ultrasound makes it an attractive test for detecting patients who may benefit from early intervention and avoids the need for strength measurements using research based equipment or less reliable portable handheld devices, both of which are inherently subject to volitional influence. Interestingly, in our study, USRF$_{CSA}$ rather than quadriceps strength was independently associated with physical activity in stage I COPD, implying that this effort independent measure of quadriceps size may be a more sensitive parameter for investigating the relationship between lower limb muscle dysfunction and physical activity in patients with mild disease. This is particularly

important as new COPD phenotypes are established requiring evaluation and as therapeutic interventions focus on physical activity promotion (Casaburi 2011).

The finding of reduced daily physical activity in GOLD Stage I COPD when compared to healthy subjects is supported by previous data (Watz 2009) showing a reduction in activity in Stage I patients compared to a chronic bronchitis (formerly GOLD stage 0) cohort. Although their observed reduction did not reach statistical significance the comparison was not made with a healthy control group as in our current study. There have been very few other studies investigating physical activity in mild to moderate COPD patients. A multi-centre study recently found a reduction in activity in early disease from stage II COPD onwards compared to healthy controls, however this study had only a small number of patients (n=9) with GOLD stage I disease (Troosters 2010a). There is evidence to suggest that symptomatic GOLD stage I patients experience dynamic hyperinflation associated with dyspnoea during exercise compared to control subjects (Ofir 2008). GOLD stage I patients in our study had a significantly higher MRC dyspnoea score compared to healthy controls and this may therefore provide a mechanism for the initial reduction in physical activity seen early in the disease process.

The reduction in physical activity in stage I COPD and its association with $USRF_{CSA}$ allows discussion of a potential mechanism for reduced quadriceps bulk in mild disease. Stage II-IV patients also demonstrated a reduction in quadriceps bulk compared to control subjects but this was not significantly different from the stage I group suggesting that a threshold level of physical inactivity, reached early in the disease process, triggers the depletion in muscle bulk. There is evidence from the Copenhagen City Heart Study (Garcia-Aymerich

2007) and elsewhere (Hopkinson 2010) that physical inactivity may in fact precede the occurrence of airflow obstruction and that it is a significant aetiological factor for the development of COPD. In addition, recent data has highlighted physical activity to be a strong predictor of all-cause mortality in COPD (Waschki 2011) emphasising its importance in this patient group, although that study compared activity to measures of whole body FFM and BMI, rather than quadriceps muscle bulk or strength. In keeping with previous work (Garcia-Rio 2009; Watz 2009), we found that lung function is associated with the level of physical activity in COPD, with RV/TLC ratio rather than FEV_1% predicted found to be an independent predictor of physical activity level in stage II-IV disease. $USRF_{CSA}$ was also independently associated with RV/TLC ratio and IC, but not FEV_1% predicted, highlighting that although FEV_1 can be used for classifying the severity of airflow obstruction (Pellegrino 2005) it does not reflect the true severity of the disease. There is increasing evidence to support measures of gas trapping and thoracic distension as better indicators of disease severity than airflow obstruction in COPD (Hannink 2010; O'Donnell 2012). Our finding that $USRF_{CSA}$ has a stronger association with physical activity in the mild compared to more advanced group, suggests that these pulmonary factors are more limiting to activity in moderate-severe patients, compared to those with mild COPD where the association between muscle wasting and inactivity is more pronounced.

Importantly, in our study whole-body measurement of FFMI was similar in controls and patients with mild disease, although $USRF_{CSA}$ was reduced, supporting local disuse as a key factor. Disuse may also increase susceptibility to systemic factors, particularly the effects of smoking which is in itself known to be associated with skeletal muscle oxidative stress (Montes de Oca 2008) and quadriceps weakness (van den Borst 2011). It should be noted

however that inactivity may act as a significant confounder when observing quadriceps dysfunction as an effect of smoking. Further studies are needed to explore whether the fibre type switch from oxidative type I fibres to anaerobic type II fibres reported in advanced COPD (Gosker 2007), occurs earlier in the disease process as a consequence of physical inactivity interacting with systemic effects.

Of note from this study, quadriceps strength and $USRF_{CSA}$ were shown to be associated with health status in COPD patients, measured using the COPD assessment test (CAT). This finding highlights the importance of these outcome measures as markers of quality of life in this patient population and indicates their likely influence on activities of daily living. A recent multicentre trial has identified an improvement in CAT score with pulmonary rehabilitation (Dodd 2011) and the association with quadriceps dysfunction may well relate to this positive finding.

In addition, the bioelectrical impedance ratio (Z_{200}/Z_5) was found to show strong associations with $USRF_{CSA}$ and quadriceps strength in COPD patients. As previously described, this ratio is thought to give an index of separation of the extracellular and total body water compartments. In contrast to the use of bioelectrical impedance analysis to calculate fat free mass using regression equations which may include height, weight and gender, the impedance ratio is based on direct measurements. Interestingly, a higher ratio has been associated with greater disease severity in patients with heart failure (Castillo Martinez 2007). The impedance ratio therefore warrants further investigation as a non-invasive biomarker in COPD.

3.4.3 Critique of the method

Although this study cannot establish causation, the association between physical inactivity and depletion in muscle bulk in mild disease is strongly suggestive of a mechanistic link. The patients recruited in this cross-sectional study are a combination of those seen in hospital outpatients as well as those from the community setting who are not seen in secondary care (table 3.1). Importantly, the patients with mild disease had very similar physical activity levels to another published GOLD stage I cohort (Watz 2009), suggesting that our group is representative of the general COPD population.

The strong correlation of ultrasound RF_{CSA} with both mid-thigh and rectus femoris CT measurements supports the use of rectus femoris as a representation of quadriceps bulk and confirms our initial findings in a small cohort of COPD patients (Seymour 2009). Furthermore, inter-observer and inter-occasion agreement for $USRF_{CSA}$ measurement in this study were similar to that for other muscle ultrasound imaging (O'Sullivan 2007). The key areas for measurement error in our experience result from operator accuracy of probe position in relation to surface anatomy and inaccurate cursor outline of the acquired rectus femoris image, both of which may be related to operator training and experience. CT and MR imaging modalities have been shown to have an advantage over ultrasound in serial measurements which is likely to relate to use of bony landmarks for measurement position in comparison to the use of surface anatomy. However, a randomised controlled trial using electrical muscle stimulation to reduce muscle wasting in the intensive care unit (ICU) setting has shown that ultrasound measurement of the quadriceps can be used as a bedside imaging modality for identifying changes in muscle bulk following intervention (Gerovasili 2009). In addition, a more recent pilot study in COPD patients has found that

serial ultrasound measurements of the quadriceps can also detect changes in muscle mass in response to resistance training (Menon 2012).

In relation to the objective measurements of activity in this study, both daily step count and PAL were used as measures of daily physical activity, although the Sensewear armband monitor has been shown to underestimate step count at slow walking speeds (Hill 2010). This may account for differences in the statistical strength of these activity variables when incorporated into the regression analyses. Importantly, the study participants showed good compliance with the Sensewear armband in keeping with recent data on the wearing time of this device in COPD and healthy subjects (Waschki 2012).

3.4.4 Conclusion

In summary, this study has shown that quadriceps wasting identified by $USRF_{CSA}$ exists in patients with mild, as well as advanced, COPD. Quadriceps bulk was associated with daily physical activity, independent of airflow limitation, in GOLD stage I disease. Our data suggest that, rather than being an end-stage phenomenon, quadriceps wasting occurs in a substantial minority of COPD patients including those with early disease. Ultrasound measurement of rectus femoris cross-sectional area has potential as a physiological biomarker in COPD and the identification of these patients may guide early lifestyle and therapeutic interventions.

Chapter 4: Randomised Controlled Trial of Effects of ACE-inhibition on Skeletal Muscle Dysfunction in COPD

4.1 Introduction

4.1.1 Background

Skeletal muscle impairment is a key complication of chronic obstructive pulmonary disease (COPD), affecting approximately a third of patients independent of the degree of airflow obstruction (Seymour 2010b). Quadriceps weakness in COPD has been associated with reduced exercise capacity (Gosselink 1996), impaired health status (Shrikrishna 2012a), and mortality in patients with moderate to severe disease (Swallow 2007a). Importantly pulmonary rehabilitation, which improves exercise performance and reduces healthcare utilisation, also increases quadriceps strength (Troosters 2000; Seymour 2010a).

4.1.2 Rationale and hypothesis

The mechanisms responsible for skeletal muscle dysfunction in COPD are likely to be multifactorial, however there are compelling data to suggest that chronic activation of the renin angiotensin system (RAS) may represent a key pathophysiological pathway (Shrikrishna 2012b). Although best known for its role in salt/water homeostasis, the RAS occurs in many tissues, including skeletal muscle (Schaufelberger 1998), regulating a diverse range of local inflammatory and metabolic processes (Carter 2005). Angiotensin-converting enzyme (ACE) is a key RAS component catalysing the synthesis of angiotensin II and the breakdown of vasoactive kinins (Hanif 2010). Data supporting an influence of ACE on muscle phenotype come from the impact of genetic polymorphisms (Williams 2000; Hopkinson 2004b; Hopkinson 2006), epidemiological data from patient cohorts on ACE-

inhibitors (Onder 2002; Di Bari 2004; Maggio 2006) and from interventional studies (Andreas 2006; Sumukadas 2007; Di Marco 2010).

Endogenous variation in ACE levels as a result of polymorphism of the ACE gene influences the endurance versus strength muscle phenotype in COPD, with the presence of the deletion allele (D) associated with greater quadriceps strength in COPD (Hopkinson 2004b). In addition, a polymorphism determining a reduction in bradykinin receptor expression has been associated with a reduced fat free mass and quadriceps strength in COPD patients (Hopkinson 2006). Epidemiological evidence for a role of the renin-angiotensin system in muscle wasting comes from observations in hypertensive cohorts in which treatment with ACE-inhibition has been associated with increased locomotor muscle size (Di Bari 2004) and strength (Onder 2002). Furthermore, previous randomised controlled trials have reported clinical benefits of ACE inhibition; a trial in elderly people with limited mobility found that the ACE inhibitor, perindopril, increased six minute walking distance (Sumukadas 2007). In COPD patients, a pilot study found enalapril to increase peak work rate (Di Marco 2010) while in a randomised controlled trial of an angiotensin II receptor blocker, a numerical increase in quadriceps strength was found in the treatment group although, not being powered for this end-point, this difference failed to achieve statistical significance (Andreas 2006).

Given this strong evidence base, we hypothesised that ACE-inhibition would have a beneficial effect on skeletal muscle dysfunction in COPD patients. To strengthen the design of the trial we used a stratified medicine approach, selecting patients with

quadriceps weakness and using primary endpoints consistent with the proposed mode of action.

4.2 Methods

4.2.1 Trial design

The study was a double-blind, randomised, placebo-controlled, parallel-group trial. Patients were randomly allocated to either ACE-inhibitor (fosinopril 10-20mg) or placebo (lactose). A pharmacy controlled 1:1 randomisation in blocks of 4 using consecutive numbers was performed, by the Clinical Trials Department, Royal Free Hampstead NHS Trust, UK. The trial was registered prospectively on a publically accessible database (www.controlled-trials.com/ISRCTN05581879).

4.2.2 Inclusion and exclusion criteria

Study inclusion criteria were patients diagnosed with COPD based on NICE and GOLD criteria and the presence of quadriceps weakness defined as a quadriceps maximum voluntary contraction (QMVC) in kilograms less than 120% of the patient's body mass index (Swallow 2007a). Exclusion criteria were patients within three months of pulmonary rehabilitation or within one month of an exacerbation, and those with a co-morbidity including cardiac failure, diabetes, renal disease or rheumatoid arthritis. Patients on ACE-inhibitors, angiotensin II receptor blockers or warfarin were also excluded.

4.2.3 Trial protocol

Visit 1 Screening (day -7) - Informed consent was obtained and at this initial screening visit patients had a baseline assessment including –

- Review of medical history and measurements of BMI, resting blood pressure and fat free mass index

- MRC dyspnoea score, St George's Respiratory Questionnaire and CAT score

- Spirometry, gas transfer, lung volumes by body plethysmography and arterial blood gases

- Incremental Shuttle Walk test

- Quadriceps maximal voluntary contraction and quadriceps twitch force

- Quadriceps endurance assessed using repetitive magnetic stimulation

- Mid-thigh cross-sectional area measured by CT

- Blood drawn for renal function, inflammatory markers, serum ACE and ACE genotype.

- Quadriceps muscle biopsy

- Physical activity recorded for 1 week at baseline using a multisensory armband accelerometer

Visit 2 (day 0) Treatment start – Patients were randomised to either fosinopril 10mg once a day (encapsulated tablets) or matched placebo starting the first dose that evening. Written information was given concerning potential side effects.

Phone call (day 1) – Patients were contacted the day after starting the study medication to ensure they had not experienced side effects.

Visit 3 (Day 7) Treatment escalation – Resting blood pressure and renal function were reviewed by an independent assessor and if satisfactory the dose was increased to two capsules once per day of either placebo or fosinopril 10mg (i.e maximum dose 20mg). Dose was not escalated if systolic BP<110mmHg. QMVC was re-measured and blood drawn for renal function. If there was an increase in creatinine of >30%, the patient was withdrawn from the study and referred for renal investigation.

Phone Call (Day 9) – patients were contacted to ensure that they had tolerated the dose change. If not they were told to go back down to one capsule per day.

Phone Call (Day 28) – patients were contacted for compliance and to report any problems.

Phone Call (Day 49) – patients were contacted for compliance and to report any problems.

Visit 4 Follow up visit (Day 90) – Baseline measurements (excluding activity monitoring) were repeated – a summary of the trial schedule is outlined in table 4.1.

Table 4.1: Protocol for randomised controlled trial of effects of ACE-inhibition on muscle weakness in COPD

	Day 7 Screening	Day 0 Treatment start	Day 1	Day 7 Dose titration	Day 9	Day 28	Day 49	Day 90 End visit
History	X			X				X
Quadriceps function - Endurance and QMVC	X			X				X
FFMI	X							X
Bloods for genotype & inflammatory markers	X			X				X
U&E's to assess renal function/FBC	X			X				X
SGRQ/CAT	X							X
PFT's	X							X
Mid-thigh CT	X							X
ISWT	X							X
Quadriceps biopsy	X							X
Phone call			X	X	X	X	X	

4.2.4 Primary and secondary physiological outcome measures

(1) Effect of ACE-I on quadriceps endurance assessed non-volitionally (Primary)

(2) Effect of ACE-I on quadriceps maximum voluntary contraction force

(3) Effect of ACE-I on mid-thigh CT cross-sectional area

4.2.5 Data analysis and statistics

Pilot work using repetitive magnetic stimulation in COPD patients identified the time taken to fatigue to a force of 50% of baseline (endurance half-time) as a mean(SD) of 80 (30) seconds. To detect a 20 seconds (25%) increase in time to fatigue using non-volitional quadriceps endurance in the fosinopril versus placebo groups, with an 80% power at the 5% significance level, would require 54 patients randomised on a 1:1 basis. To allow for a 30% drop out rate, 80 patients were targeted for recruitment. Data are presented as mean ± standard deviation, with accompanying p value, and were analysed using paired or independent t-tests. Analysis was performed using StatView 5.0 (Abacus concepts, Inc., Berkeley, CA, USA) with $p<0.05$ considered statistically significant. Figure construction was performed using GraphPad Prism Version 5.0 (GraphPad Software, San Diego, California, USA).

4.3 Results

One hundred and seventeen patients were screened for study participation and 80 patients underwent randomisation. There were 8 withdrawals from the treatment group and 5 from the placebo group. Further details are shown in the CONSORT diagram (figure 4.1).

4.3.1 Consort diagram
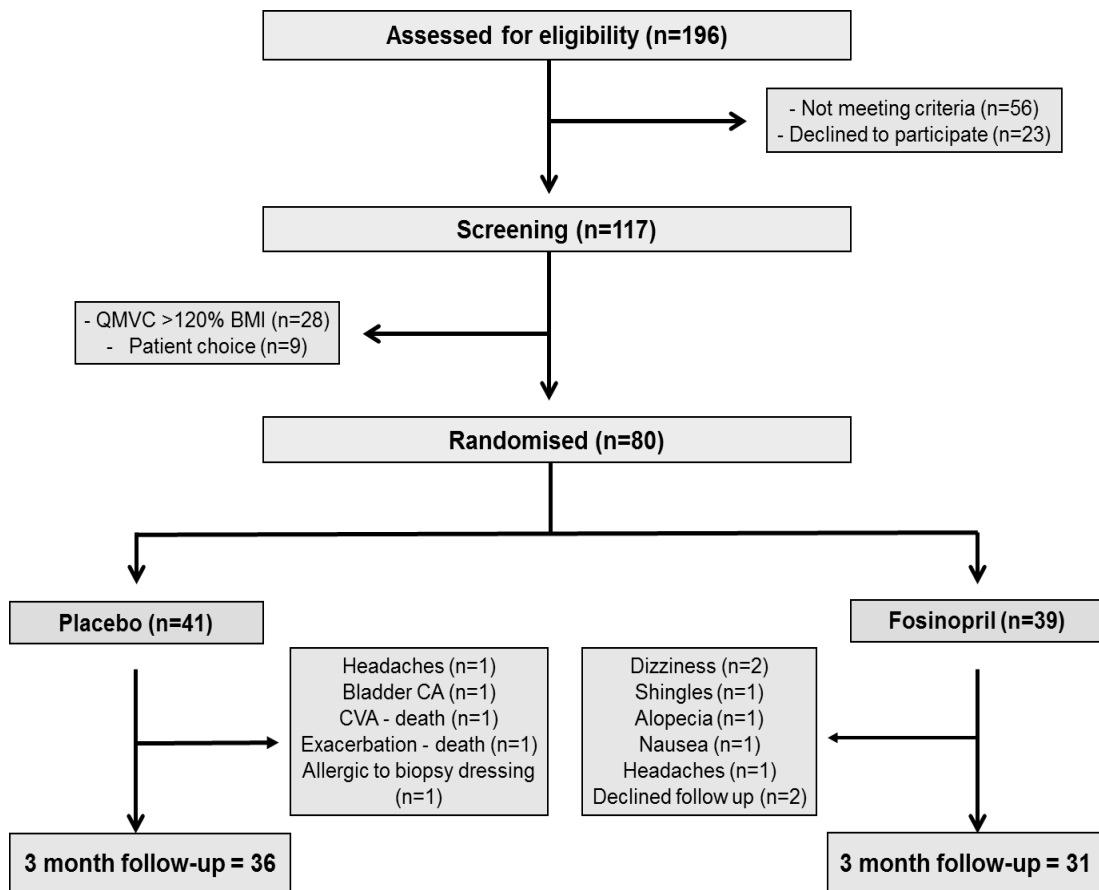
Figure 4.1: Consort recruitment diagram for enrolment and follow up.

4.3.2   Baseline characteristics of placebo and treatment groups

The placebo and treatment groups were well matched for age, gender, and lung function parameters (table 4.2) and there were no statistically significant baseline differences in body composition and quadriceps muscle function (table 4.3).

Table 4.2: Baseline characteristics of placebo and treatment groups

	Placebo group (n=41) mean (SD)	Treatment group (n=39) mean (SD)	p value
Age (years)	64.6 (7.3)	66.3 (8.2)	0.33
Gender (M/F)	23/18	19/20	0.51
BMI (kg/m^2)	24.3 (4.0)	25.0 (5.8)	0.51
FFMI (kg/m^2)	17.0 (2.1)	17.3 (2.6)	0.60
Smoking (pack years)	53.3 (25.1)	49.8 (33.1)	0.59
Current smokers (%)	24	28	0.70
Outpatients (%)	66	67	0.94
Long-acting beta agonist (%)	93	82	0.15
Long-acting anticholinergic (%)	88	87	0.93
Inhaled corticosteroid (%)	90	82	0.15
Oral corticosteroid (% ≥5mg/day)	2	5	0.53
FEV_1% predicted	40.1 (20.6)	45.8 (20.5)	0.22
TLco% predicted	41.8 (20.9)	44.0 (19.2)	0.64
RV%TLC ratio	58.2 (9.9)	55.8 (10.7)	0.29
PaO_2 (kPa)	9.7 (1.4)	9.6 (1.4)	0.82
$PaCO_2$ (kPa)	5.2 (0.6)	5.1 (0.4)	0.18

	Placebo group (n=41) mean (SD)	Treatment group (n=39) mean (SD)	p value
SGRQ (Symptoms)	56.5 (23.7)	49.6 (21.3)	0.17
SGRQ (Activity)	70.8 (25.8)	71.1 (17.2)	0.96
SGRQ (Impacts)	40.8 (22.8)	31.3 (16.2)	0.04
SGRQ (Total)	52.5 (22.0)	46.4 (14.9)	0.15
CAT score	22.8 (8.5)	20.8 (8.1)	0.34
Daily step count	4499 (3462)	4504 (3109)	0.99
Physical activity level (PAL)	1.4 (0.18)	1.4 (0.16)	0.66
Systolic BP (mmHg)	134 (15)	138 (19)	0.35
Diastolic BP (mmHg)	85 (10)	85 (11)	0.84
Beta-blocker (%)	2	0	0.33
Calcium channel blocker (%)	7	10	0.65
Diuretic (%)	0	2	0.31
Serum NT-pro BNP (pg/ml)	109.0 (99.8)	105.0 (64.0)	0.85
ACE genotype (DD,ID,II) %	39,44,17	38,46,16	0.94

Abbreviations: BMI - body mass index; FFMI – fat free mass index; FEV_1 - forced expiratory volume in 1 second; TLco – carbon monoxide diffusing capacity; RV – residual volume; TLC – total lung capacity; SGRQ – St George's respiratory questionnaire; CAT – COPD assessment test; BP – blood pressure; NT-proBNP – N-terminal pro-B-type natriuretic peptide; ACE – angiotensin-converting enzyme; Outpatients – defined as any previous hospital clinic attendance.

Table 4.3: Baseline quadriceps and exercise measurements in placebo and treatment groups

	Placebo group mean (SD)	Treatment group mean (SD)	p value
QMVC (kg)	24.9 (4.9)	25.0 (7.4)	0.98
TwQ (kg)	10.7 (3.0)	9.7 (3.4)	0.21
MT_{CSA} (cm^2)	93.3 (22.4)	93.0 (26.1)	0.96
Endurance half-time (s)	61.2 (35.5)	70.6 (31.9)	0.30
ISWT (m)	247 (132)	242 (128)	0.87

Abbreviations: QMVC – quadriceps maximum voluntary contraction; TwQ – quadriceps twitch force; MT_{CSA} – mid-thigh cross-sectional area; ISWT – incremental shuttle walk test.

4.3.3   Effect of ACE-inhibition on quadriceps endurance and exercise capacity

At 3 months, there was no significant difference in quadriceps endurance half-time assessed non-volitionally by repetitive magnetic stimulation (fosinopril Δ+5.1s, 95%CI -4.3 to 14.5, p=0.27 vs. placebo Δ+4.6s, 95%CI -5.8 to 15.1, p=0.37; between group difference 0.5s 95%CI -13.3 to 14.3, p=0.94) (figure 4.2). There was also no significant change in incremental shuttle walk distance at 3 months in the two groups (fosinopril Δ+7.1m, 95%CI -5.5 to 19.7, p=0.26 vs. placebo Δ+17.1m, 95%CI -10.6 to 44.8, p=0.22; between group difference -10m 95%CI -39.8 to 19.8, p=0.51) (figure 4.3).
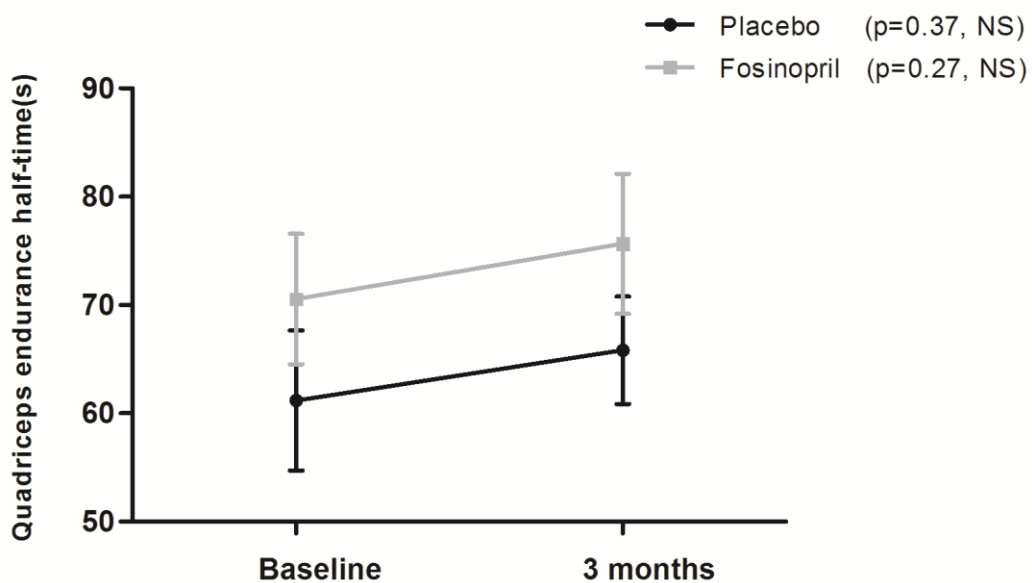
Figure 4.2: Quadriceps endurance following 3 months ACE-inhibition vs. placebo

(Data shown as mean with cross bars representing the standard error of the mean - SEM, between group p=0.94; not significant - NS).
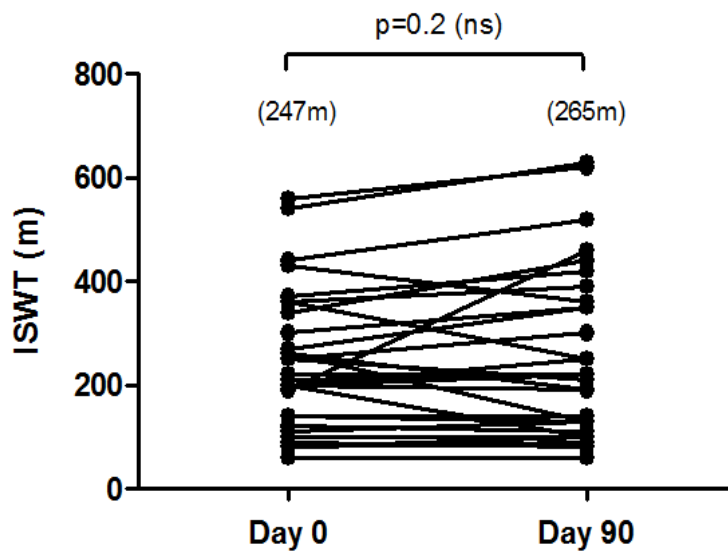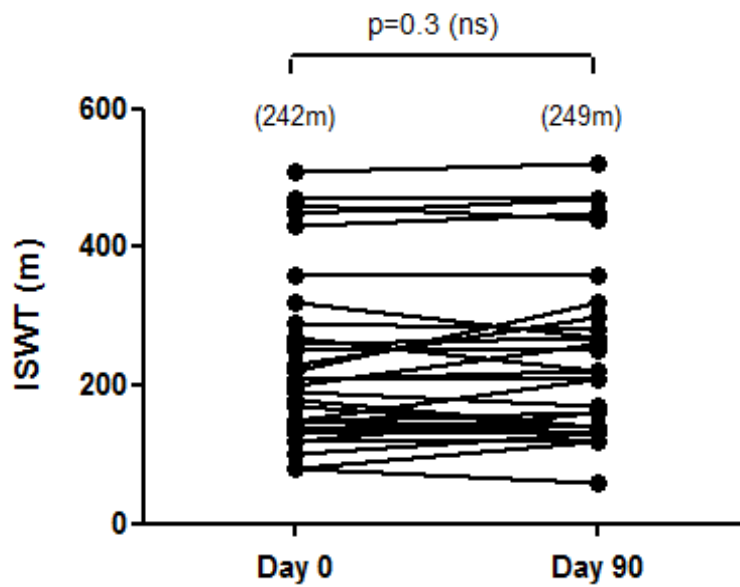
Figure 4.3: Incremental shuttle walk distance following 3 months of ACE-inhibition vs.

placebo (Placebo group – top; Treatment group – bottom).

4.3.4   Effect of ACE-inhibition on quadriceps strength and cross-sectional area

Quadriceps maximal voluntary contraction improved in both groups (fosinopril Δ+1.1kg, 95%CI 0.03-2.2, p=0.045 vs. placebo Δ+3.6kg, 95%CI 2.1-5.0, p<0.0001) with a greater increase in the placebo arm (between group difference 2.5kg, 95%CI 0.7 to 4.3, p=0.009) (figure 4.4). There was also a trend towards an increase in quadriceps twitch force in the placebo versus treatment group (fosinopril Δ-0.28kg, 95%CI -1.0 to 0.45, p=0.43 vs. placebo Δ+0.57kg, 95%CI 0.01 to 1.1, p=0.046; between group difference -0.85kg 95%CI -1.7 to 0.03, p=0.06) (figure 4.5). Mid-thigh cross-sectional area showed no significant differences at 3 months, but trended to an increase in the placebo group (fosinopril Δ-0.60cm^2, 95%CI -2.1 to 0.91, p=0.42 vs. placebo Δ+1.0cm^2, 95%CI -0.21 to 2.2, p=0.10; between group difference -1.6cm^2 95%CI -3.5 to 0.27, p=0.09) (figure 4.6).
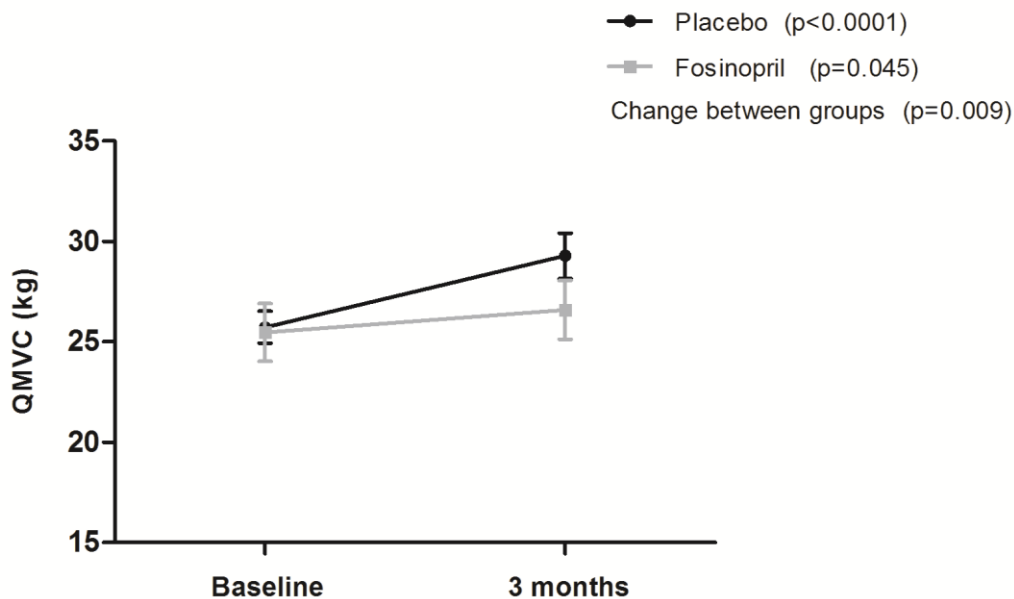
Figure 4.4: Quadriceps strength after 3 months ACE-inhibition vs. placebo

(Data shown as mean with cross bars representing SEM; not significant - NS).
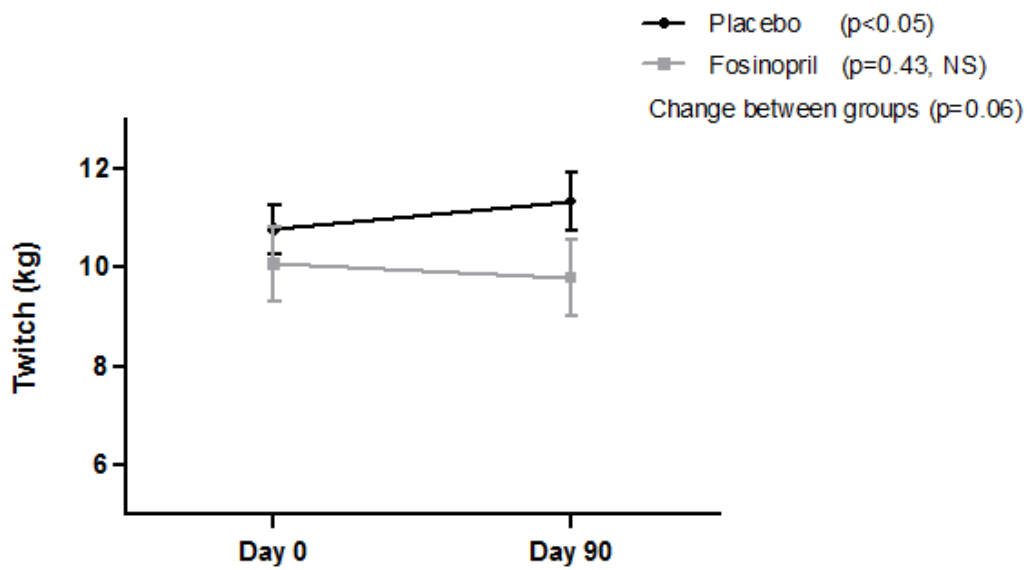
Figure 4.5: Quadriceps twitch force following 3 months ACE-inhibition vs. placebo

(Data shown as mean with cross bars representing SEM; not significant - NS).
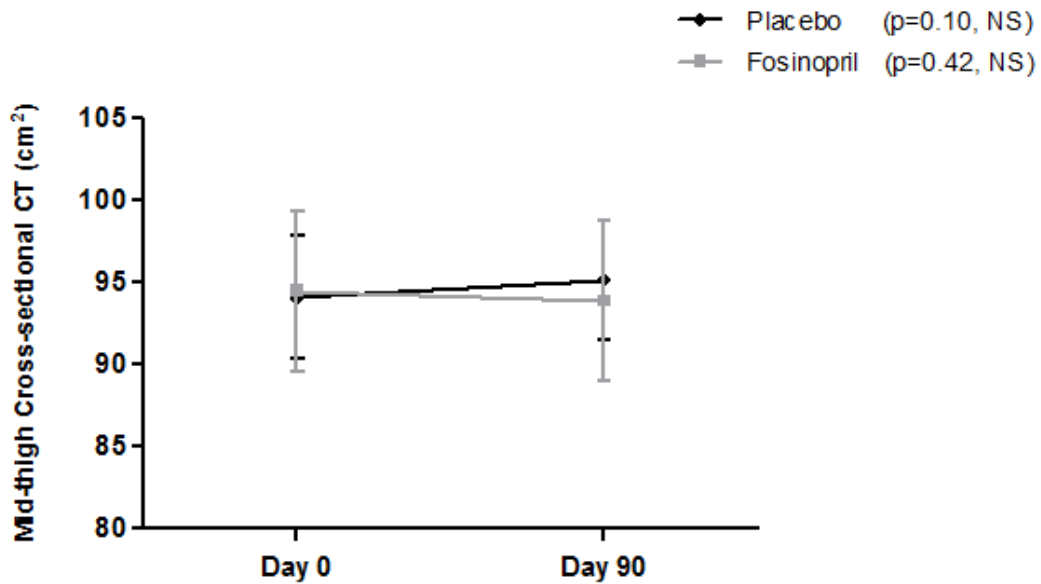
Figure 4.6: Quadriceps MT$_{CSA}$ following 3 months ACE-inhibition vs. placebo

(Data shown as mean with cross bars representing SEM; not significant - NS).

4.3.5 Effect of ACE-inhibition on blood pressure, lung function and health status

A significant reduction was demonstrated in systolic blood pressure in the treatment arm compared to placebo (fosinopril Δ-12.7mmHg, 95%CI -20.3 to -5.1, p=0.002 vs. placebo Δ-2.2mmHg, 95%CI -8.1 to 3.7, p=0.46; between group difference -10.5mmHg, 95%CI -19.9 to -1.1, p=0.03) (figure 4.7). Diastolic blood pressure was also reduced in the treatment group (fosinopril Δ-7.0mmHg, 95%CI -11.5 to -2.4, p=0.004 vs. placebo Δ-1.2mmHg, 95%CI -5.2 to 2.9, p=0.56; between group difference -5.8mmHg, 95%CI -11.7 to 0.11, p=0.05) (figure 4.8). Lung function parameters including $FEV_1\%$ predicted, DLco% predicted, RV/TLC ratio and arterial blood gases showed no significant change between groups at 3 months. Health-related quality of life measures which included SGRQ and CAT score, also did not vary significantly between groups (see table 4.4).
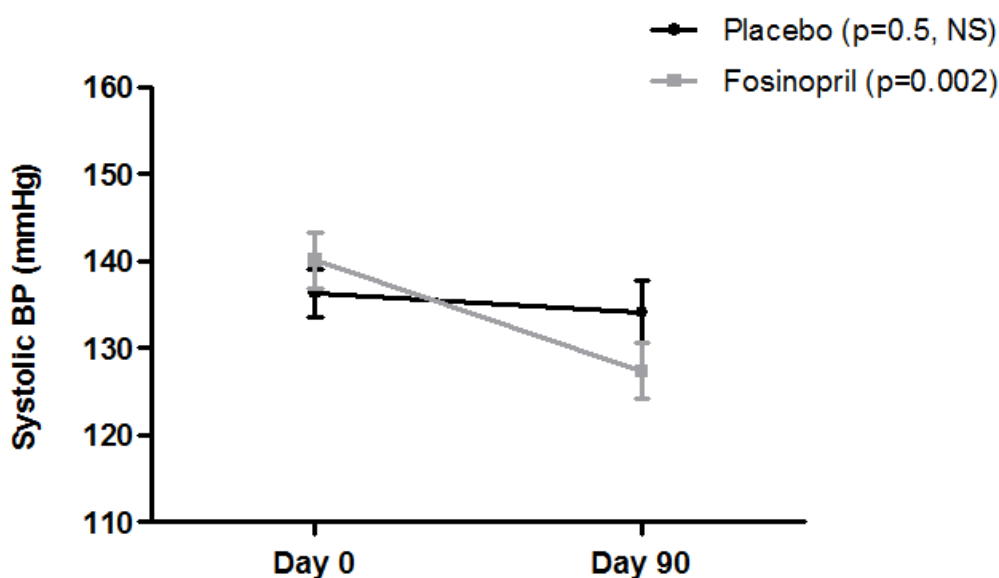
Figure 4.7: Systolic blood pressure following 3 months ACE-inhibition vs. placebo

(Data shown as mean with cross bars representing SEM; not significant - NS).
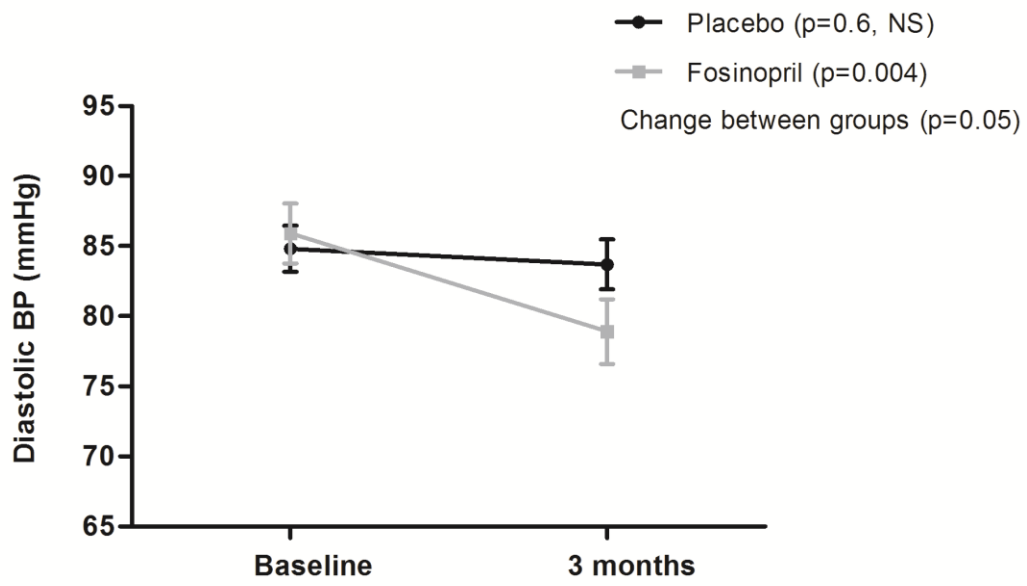
Figure 4.8: Diastolic blood pressure following 3 months ACE-inhibition vs. placebo

(Data shown as mean with cross bars representing SEM; not significant - NS).

Table 4.4: Change in physiological and HRQOL outcomes following 3 months of ACE-inhibition

	Placebo group mean (SD)	Treatment group mean (SD)	p value
Endurance half-time (s)	4.6 (28.0)	5.1 (24.2)	0.94
QMVC (kg)	3.6 (4.3)	1.1 (2.9)	**0.009**
TwQ (kg)	0.6 (1.5)	-0.3 (1.8)	0.06
MT_{CSA} (cm^2)	1.0 (3.7)	-0.6 (4.1)	0.09
ISWT (m)	17.1 (75.6)	7.1 (34.5)	0.51
Systolic BP (mmHg)	-2.2 (16.4)	-12.7 (20.8)	**0.03**
Diastolic BP (mmHg)	-1.2 (11.1)	-7.0 (12.4)	0.05
FEV_1% pred	2.7 (5.6)	1.7 (6.8)	0.50
TLco% pred	1.2 (4.7)	-0.23 (4.3)	0.20
RV%TLC ratio	-3.7 (14.0)	-1.2 (4.3)	0.35
PaO_2 (kPa)	-0.12 (0.8)	-0.05 (1.1)	0.77
$PaCO_2$ (kPa)	-0.02 (0.5)	-0.05 (0.4)	0.79
SGRQ (Symptoms)	-1.8 (19.4)	4.0 (21.0)	0.25
SGRQ (Activity)	-1.5 (9.9)	-1.5 (11.9)	0.99
SGRQ (Impacts)	-0.7 (12.2)	2.3 (9.4)	0.28
SGRQ (Total)	-1.1 (10.1)	1.5 (7.9)	0.25
CAT score	-2.6 (5.7)	-0.3 (6.2)	0.18

Abbreviations: QMVC – quadriceps maximum voluntary contraction; TwQ – quadriceps twitch force; MT_{CSA} – mid-thigh cross-sectional area; ISWT – incremental shuttle walk test; BP – blood pressure; FEV_1 - forced expiratory volume in 1 second; TLco – carbon monoxide diffusing capacity; RV – residual volume; TLC – total lung capacity; PaO_2 / CO_2 - arterial partial pressure of oxygen / carbon dioxide; SGRQ – St George's respiratory questionnaire; CAT – COPD assessment test.

4.3.6 Influence of ACE/Bradykinin polymorphisms

Patients were also stratified by ACE and bradykinin genotype (table 4.5 and table 4.6). Across ACE genotype, the patients were well matched except for a lower FEV_1% predicted in those homozygous for the insertion (I) allele. There were no significant baseline differences across bradykinin receptor genotype.

Table 4.5: Baseline physiological data when stratified by ACE genotype

	DD (n=31) Mean (SD)	ID (n=36) Mean (SD)	II (n=13) Mean (SD)	p value
Age	66.4 (8.3)	64.8 (7.7)	65 (6.9)	0.69
Gender	15/16	19/17	8/5	0.74
BMI (kg/m^2)	24.6 (5)	24.9 (5.2)	23.8 (4.6)	0.80
FFMI (kg/m^2)	17.4 (2.6)	17.2 (2.3)	16.6 (2)	0.63
FEV_1% pred	48.9 (18.8)	42.7 (22.7)	29.3 (10.6)	**0.01**
QMVC (kg)	25.7 (6.3)	25 (6.1)	23.2 (6.7)	0.49
$USRF_{CSA}$ (mm^2)	498 (127)	522 (123)	487 (133)	0.62

Table 4.6: Baseline physiological data when stratified by bradykinin receptor polymorphism

	+9/+9 (n=27) Mean (SD)	+9/-9 (n=35) Mean (SD)	-9/-9 (n=18) Mean (SD)	p value
Age	67.2 (7.2)	64.7 (7.3)	64.2 (9.3)	0.33
Gender	12/15	21/14	9/9	0.47
BMI (kg/m^2)	23.9 (5.1)	25.4 (5.1)	24.3 (4.5)	0.46
FFMI (kg/m^2)	16.8 (2.5)	17.5 (2.3)	17 (2.3)	0.48
FEV$_1$% pred	42.4 (21.8)	45.2 (22.1)	39.1 (15.5)	0.60
QMVC (kg)	23.5 (6.1)	26.2 (6.1)	24.6 (6.4)	0.20
USRF$_{CSA}$ (mm^2)	482 (121)	530 (136)	501 (112)	0.30

The response to ACE-inhibition vs. placebo was also assessed by ACE and bradykinin receptor genotype as shown in table 4.7. When divided by ACE genotype, patients homozygous for the insertion allele (II) showed an increase in ISWT following treatment (fosinopril Δ+37.5m vs. placebo Δ-20m) compared to those homozygous for the D allele who showed a reduced effect with treatment (fosinopril Δ+3m vs. placebo Δ+56m) (ANOVA p=0.02). Twitch force also increased in patients with the II genotype following treatment (fosinopril Δ+2.01kg vs. placebo Δ-0.03kg) when compared to other genotypes; DD genotype (fosinopril Δ-0.44kg vs. placebo Δ+0.68kg), ID genotype (fosinopril Δ-0.67kg vs. placebo Δ+0.80kg) (ANOVA p=0.02). In a separate analysis for treatment response by presence of an I allele (ID and II vs. DD), using ANOVA, ISWT remained significant (p=0.01), but no significant difference was found for twitch force (p=0.67).

When analysing bradykinin receptor genotypes, only TLco% predicted showed a difference in treatment response (-9/-9 genotype; fosinopril Δ+1% pred vs. placebo Δ-0.5% pred) vs (+9/-9 genotype; fosinopril Δ-2% pred vs. placebo Δ+3% pred) vs (+9/+9 genotype; fosinopril Δ+1.6% pred vs. placebo Δ+0.4% pred) (ANOVA p=0.03). In a separate analysis for TLco% predicted treatment response by presence vs. absence of the -9 allele, (-9/-9 and +9/-9 vs. +9/+9) no significant difference was found (p=0.11).

Table 4.7: Change in physiological and HRQOL outcomes following 3 months ACE-inhibition when stratified by ACE and bradykinin genotypes.

	Change in treatment vs. placebo stratified by ACE genotype (DD vs. ID vs. II) p value (ANOVA)	Change in treatment vs. placebo stratified by bradykinin polymorphism (+9/+9, -9/+9, -9/-9) p value (ANOVA)
Endurance half-time (s)	0.68	0.50
QMVC (kg)	0.62	0.79
TwQ (kg)	**0.02**	0.86
MT_{CSA} (cm^2)	0.43	0.97
ISWT (m)	**0.02**	0.40
Systolic BP (mmHg)	0.96	0.53
Diastolic BP (mmHg)	0.20	0.12
FEV_1% pred	0.11	0.67
TLco% pred	0.31	**0.03**
RV%TLC ratio	0.23	0.32
PaO_2 (kPa)	0.54	0.44
$PaCO_2$ (kPa)	0.64	0.91
SGRQ (Symptoms)	0.12	0.20
SGRQ (Activity)	0.36	0.20
SGRQ (Impacts)	0.23	0.82
SGRQ (Total)	0.09	0.31
CAT score	0.68	0.30

4.4   Discussion

4.4.1   Summary of results

Fosinopril did not have a beneficial effect on quadriceps muscle function in moderate to severe COPD patients selected for quadriceps weakness, although blood pressure measurements confirmed adherence and biological activity of the drug. The present data do not support the use of ACE inhibitors to augment muscle phenotype in patients with COPD.

4.4.2   Significance of the findings

These findings are unexpected given previous data supporting a beneficial effect of ACE-inhibition on skeletal muscle function, and reinforce the importance of conducting prospective blinded trials. An observational study assessed the relationship between ACE-inhibitor use and muscle strength in 641 elderly hypertensive women participating in the Women's Health and Aging Study (Onder 2002). They found that at 3 years' follow up, participants taking an ACE-inhibitor continuously had a lower mean decline in both knee extensor muscle strength and walking speed than those using other antihypertensives and those not on antihypertensive medications. In addition, cross-sectional data from 2,431 hypertensive subjects participating in the Health, Aging and Body Composition (Health ABC) study was used to evaluate whether ACE-inhibitor treatment was associated with a larger lower extremity muscle mass compared to the use of other antihypertensive medications (Di Bari 2004), and found that lower extremity muscle mass (assessed by

DEXA) was larger in the ACE-inhibitor group. They also showed that duration of exposure to ACE-inhibitors was associated with greater muscle mass.

Interventional studies of ACE-inhibition have also suggested a treatment effect. A randomised controlled trial in an elderly population (n=95, mean age 77 years) with self-reported difficulties in mobility, showed that 5 months of perindopril treatment significantly improved 6 minute walk distance (31.4m, 95% CI 10.8-51.9m, p=0.003) compared to placebo (Sumukadas 2007). Health-related quality of life deteriorated in the placebo group but was maintained in the perindopril group. Interestingly this cohort included current and ex-smokers and the improvement was observed in the absence of heart failure in the participants.

A small double blind, placebo controlled study has evaluated the effects of 4 weeks treatment with enalapril on exercise performance in 21 COPD patients with no evidence of cardiovascular co-morbidities (Di Marco 2010). Enalapril did not have an effect on the ventilatory response to exercise (VE/VCO$_2$ slope) or on peak O$_2$ consumption. However, enalapril did significantly improve O$_2$ pulse and peak work rate compared to placebo. ACE genotype did not significantly affect patient response to treatment. The improved cardiovascular response to exercise may be attributable to a combination of greater cardiac efficiency, a reduced pulmonary arterial pressure and reduced neurohumoral activation influencing systemic effects on skeletal muscle function.

There are a number of possible factors that may explain why fosinopril was not effective in the present study given previous data. These are discussed below and include the patient population studied, the choice of ACE-inhibitor, the treatment duration and the influence of physical inactivity. A further possibility is that ACE-inhibitors in fact create a more "benign" intramuscular environment effectively removing a training stimulus.

Patient selection

We adopted a stratified medicine approach, based on a quadriceps weakness patient phenotype, so as to focus on those COPD patients with a level of skeletal muscle dysfunction known to be associated with worse survival (Swallow 2007a). It may however, be the case that at this stage the weakest patients have a limited ability to respond to treatment and the low physical activity level found at baseline may reflect this. The level of inactivity could explain the discrepancy between the present data and the effect of ACE-inhibitors in relatively healthy populations being treated for hypertension (Onder 2002). There is also evidence that an exercise stimulus may be needed for ACE-inhibition to promote adaptive changes in skeletal muscle including an increase in capillary density (Guo 2010) and enhancement of insulin-stimulated muscle glucose transport (Steen 1999). Therefore the current data do not preclude the possibility that the use of ACE-inhibition in the context of pulmonary rehabilitation may yield benefit.

This trial was not prospectively stratified and powered on ACE genotype, and therefore the value of the post-hoc analysis of a genotype specific response is limited. Although patients homozygous for the insertion allele (II) did show an improved treatment response for ISWT and twitch force with treatment compared to the DD group, the numbers of II

subjects was very small and therefore requires investigation in a larger cohort. Interestingly a previous study did show a trend towards an increase in the treatment effect of ACE-inhibition on O_2 pulse in COPD patients homozygous for the II allele (Di Marco 2010). As this ACE genotype is associated with increased endurance in normal populations, this potential enhanced treatment response in COPD does warrant further evaluation.

In addition, the difference observed in this trial in transfer factor treatment response with bradykinin receptor genotype although small and not maintained when analysed by presence of a single allele, again highlights the need for specific studies in this area.

Choice of ACE-inhibitor

The beneficial effects seen with perindopril and enalapril in previous studies (Sumukadas 2007; Di Marco 2010) highlight that type of ACE-inhibitor used may influence outcome. Fosinopril was chosen as the ACE-inhibitor for this study due to its high lipophilicity and its hepatic as well as renal elimination. However, although a class effect is seen for ACE-inhibitors in the context of hypertension, any potential anti-inflammatory and muscle-specific effects may be specific to individual compounds with differing phosphinyl or carboxyl containing groups; enalapril, perindopril, ramipril and lisinopril all contain a carboxyl group, whereas fosinopril contains a phosphinyl group. Interestingly, a recent randomised placebo controlled trial of ACE-inhibition and novel cardiovascular risk factors (TRAIN) study investigated the influence of fosinopril in 290 subjects (>55 years old) with a high cardiovascular disease risk profile and showed no effect on serum inflammatory biomarkers, haemostasis or endothelial function (Cesari 2009). A large substudy of 257 of the participants in this trial (mean age 66 years) had a short physical battery score

assessment (encompassing 4 metre walking speed, balance and chair-stand tests) and measurement of handgrip strength at baseline and 6 months after the fosinopril/placebo intervention (Cesari 2010). This sub-study did not show a significant effect on strength or physical performance. This finding may also relate to the heterogeneity of the group studied particularly in relation to co-morbidities such as cancer, stroke and diabetes.

ACE-inhibition was chosen over ATII receptor blockade due to the additional effect on prevention of bradykinin degradation, however a randomised placebo controlled trial of 4 months treatment with irbesartan in 60 COPD patients (Andreas 2006), did show a 10% increase in quadriceps strength in the treatment group. Although this result did not reach statistical significance, the study was not powered on locomotor muscle strength and patients were not stratified by ACE genotype.

Effect of ACE-inhibition on intramuscular environment

The improvements observed in quadriceps strength in the current trial warrant further discussion. The COPD subjects were included based on the presence of quadriceps weakness, and therefore it is not completely unexpected that a placebo effect was found in relation to volitional quadriceps strength over the 3 months study period. Indeed the existence of a placebo effect has been well documented in the context of functional exercise capacity in clinical trials of ACE-inhibition in heart failure (Olsson 2005) and in other studies of physical performance (Beedie 2009). However, the finding that this effect was greater in the placebo arm of the trial was unexpected. While this could potentially be a chance effect it was accompanied by trends in the same direction for mid-thigh cross sectional area and quadriceps twitch force (both of which are independent of patient

effort). A possible reduction in skeletal muscle blood flow secondary to a reduced systemic blood pressure may explain the attenuated response observed with treatment. Evidence for this comes from animal models where captopril has been shown to increase maximal blood lactate during exercise and reduce exercise capacity in normotensive, sedentary rats (Minami 2004). A decrease in arteriole vasodilator function has also been identified in rat skeletal muscle following ACE-inhibition (Frisbee 1999) and in a chronic heart failure model, perindopril was not found to protect against alterations in skeletal muscle energy metabolism (Momken 2003).

4.4.3   Critique of the method

A strength of this study was that the quadriceps assessment was comprehensive including both volitional and non-volitional physiological outcomes. The study included COPD patients with quadriceps weakness defined as a quadriceps strength less than 120% of BMI. Although this cut-off was associated with greater mortality in moderate-severe COPD patients (Swallow 2007a), the possibility exists that the inclusion of COPD patients with varying degrees of skeletal muscle impairment may have enabled a wider assessment of potential responders.

The influence of treatment on physical activity level was not assessed although the study did incorporate a baseline objective physical activity assessment, and confirmed a similar level of baseline activity in both groups. In addition, the incremental shuttle walk test was chosen as a field test for the trial, although patients performing this test may have been more limited by walking speed when compared to a self-paced six minute walk test.

Finally, the duration of this study was 3 months however the time needed for skeletal muscle adaptation following pharmacotherapy in these patients remains unclear, with ACE-inhibitor treatment duration ranging from 10 weeks to 12 months for studies of improved six minute walk distance in heart failure (Olsson 2005) and treatment for longer than 2 years in observational studies of hypertensive individuals (Di Bari 2004).

4.4.4   Conclusion

In summary, despite a strong theoretical basis for the study, this randomised controlled trial found that ACE-inhibition, in the form of fosinopril, did not improve quadriceps function in a COPD population with quadriceps weakness. A placebo effect was observed on quadriceps strength which unexpectedly was greater than that observed in patients on an ACE-inhibitor. This study does not support a role for ACE-inhibitors alone in the treatment of skeletal muscle dysfunction in patients with COPD.

Chapter 5: Effects of ACE-inhibition on Skeletal Muscle Atrophy/Hypertrophy Signalling in COPD

5.1 Introduction

5.1.1 Background

The molecular mechanisms underlying the skeletal muscle dysfunction observed in COPD patients remain to be fully elucidated; however increasing evidence suggests a key role for the insulin-like growth factor-1 (IGF-1), produced in response to growth hormone, testosterone and mechanical stretch. IGF-1 acts through the PI3K/AKT pathway to inactivate FoxO transcription factors, thereby inhibiting expression of the atrogenes atrogin-1 and MuRF-1 to prevent muscle catabolism (Sandri 2006). IGF-1 also activates the mammalian target of rapamycin (mTOR) via PI3K/AKT signalling to promote muscle anabolism. It has been shown that IGF-1 levels are reduced in COPD patients in the stable state compared to healthy controls (Crul 2007). Furthermore, in COPD patients undergoing pulmonary rehabilitation, increases in exercise capacity and fibre size are associated with upregulation of IGF-1 (Vogiatzis 2007; Vogiatzis 2010).

5.1.2 Rationale and hypothesis

Angiotensin II has relevance in the pathogenesis of muscle impairment in COPD, through modulation of the IGF-1 pathway as well as induction of pro-inflammatory cytokines and reactive oxygen species. The evidence for an influence of RAS comes from animal models (Song 2005; Cohn 2007; Yoshida 2010) where infusion of angiotensin II promotes muscle loss via an inhibitory effect on the IGF-1 system and stimulation of a catabolic pathway mediated by the ubiquitin ligases, MuRF-1 and atrogin-1 (Song 2005). These atrogenes

have been shown to be upregulated in the quadriceps of patients with COPD (Doucet 2007), with ubiquitin-proteasome degradation thought to play a key role in the muscle atrophy observed (Debigare 2010; Doucet 2010). In addition, recent work by Rezk *et al* in a mouse model has shown an increase in IGF-1 expression seven days after angiotensin II induced diaphragm muscle atrophy, suggesting a potential involvement of IGF-1 in skeletal muscle regeneration following RAS related injury (Rezk 2012). Based on this evidence, we hypothesised that ACE-inhibition would have a beneficial effect on vastus lateralis atrogene expression in COPD patients with quadriceps weakness.

5.2   Methods

5.2.1   Participants and measurements

Muscle biopsies were taken from the vastus lateralis at baseline and three months, as part of the double-blind randomised controlled trial described in chapter 4. Real-time quantitative PCR (RT-qPCR) was performed testing for atrophy/hypertrophy target genes and MHC isoforms (primer sequences shown in table 5.1) with normalisation to a reference housekeeping gene, RPLPO. Expression of phosphorylated 4EBP-1 was determined by enzyme-linked immunosorbent assay (ELISA). Blood samples were also taken at baseline and 3 months and analysed for inflammatory cytokines (IL-6, IL-8, IL-18 and MCP-1), serum ACE activity, IGF-1, NT-pro-BNP, hs-CRP and fibrinogen, as previously described.

Table 5.1: Primer sequences

	Forward sequence	Reverse sequence
RPLPO	5'-TCTACAACCCTGA AGTGCTTGATATC-3'	5'-GCAGACAGAC ACTGGCAACAT-3'
Atrogin-1	5'-GCAGCTGAACAA CATTCAGATCAC-3'	5'-CAGCCTCTGCA TGATGTTCAGT-3'
MuRF-1	5'-CCTGAGAGCC ATTGACTTTGG-3'	5'-CTTCCCTTCTGT GGACTCTTCC-3'
IGF-1	5'-CCACGATGC CTGTCTGAGG-3'	5'-TTTCAACAAG CCCACAGGGT-3'
MyoD	5'-GACGGCATGA TGGACTACAG-3'	5'AGGCAGTCTA G GCTCGACAC
TGF-β	5'-CCTGGCGAT ACCTCAGCAA	5'-CCGGTGACATC AAAGATAACCA-3'
MHC I	5'CCCTGGAGACTT TGTCTCATTAGG-3'	5'-AGCTGATGAC CAACTTGCGC-3'
MHC IIA	5'TCACTTATGACTT TTGTGTGAACCT-3'	5'CAATCTACCTAA ATTCCGCAAGC-3'
MHC IIX	5'TGACCTGGGAC TCAGCAATG-3'	5'GGAGGAACAAT CCAACGTCAA-3'

5.2.2 Primary and secondary molecular outcome measures

(1) Effect of ACE-I on MuRF-1 and Atrogin-1 mRNA expression (Primary)

(2) Effect of ACE-I on serum inflammatory markers and IGF-1

5.2.3 Data and statistical analysis

To detect a 70% decrease in MURF-1 mRNA expression in the fosinopril versus placebo groups, with an 80% power at the 5% significance level, would require 20 patients in each group (Doucet 2007). Data are presented as mean ± standard deviation, with accompanying p value, and were analysed using paired or independent t-tests. Analysis was performed using StatView 5.0 (Abacus concepts, Inc., Berkeley, CA, USA) with $p < 0.05$ considered statistically significant. Figure construction was performed using GraphPad Prism Version 5.0 (GraphPad Software, San Diego, California, USA).

5.3 Results

5.3.1 Baseline muscle and serum measurements in placebo and treatment groups

There were no significant baseline differences in vastus lateralis expression of the genes analysed or serum measurements between the groups (table 5.2).

Table 5.2: Baseline muscle biopsy and serum measurements

	Placebo group mean (SD)	Treatment group mean (SD)	p value
Vastus lateralis			
Atrogin-1 mRNA (AU)	2.6 (0.5)	2.4 (0.5)	0.16
MuRF-1 mRNA (AU)	2.2 (0.7)	2.0 (0.8)	0.30
IGF-1 mRNA (AU)	0.9 (0.5)	1.0 (0.3)	0.56
MyoD mRNA (AU)	2.6 (0.6)	2.4 (0.7)	0.43
TGF-β mRNA (AU)	1.3 (0.7)	1.4 (0.6)	0.74
MHC I mRNA (AU)	3.9 (0.7)	4.2 (0.5)	0.11
MHC IIA mRNA (AU)	4.6 (0.5)	4.7 (0.6)	0.88
MHC IIX mRNA (AU)	4.0 (0.7)	4.1 (0.6)	0.78
Phosphorylated 4EBP-1 protein (AU)	2.2 (0.5)	2.0 (0.6)	0.56
Serum			
ACE activity (IU/L)	48.0 (28.0)	43.5 (15.3)	0.43
HS-CRP (mg/L)	9.8 (21.0)	4.6 (4.6)	0.18
Fibrinogen (g/L)	2.6 (0.69)	2.8 (0.67)	0.20
IGF-1 (ug/L)	124.6 (61.5)	145.0 (103.8)	0.32
IL-6 (ng/L)	4.6 (15.2)	1.9 (2.5)	0.34
IL-8 (ng/L)	3.7 (3.5)	4.0 (2.8)	0.69
IL-18 (ng/L)	494.9 (332.7)	421.0 (174.7)	0.27
MCP-1 (ng/L)	66.2 (43.3)	77.7 (37.0)	0.26

Abbreviations: MuRF-1 – muscle RING finger protein-1; IGF-1 – insulin-like growth factor-1; ACE – angiotensin-converting enzyme; HS-CRP – high-sensitivity C-reactive protein; IL – interleukin; MCP-1 – monocyte chemotactic protein-1.

5.3.2 Effect of ACE-inhibition on vastus lateralis atrophy/hypertrophy signalling

At 3 months, no significant differences were observed in vastus lateralis atrogin-1 mRNA expression (fosinopril Δ-0.18AU, 95%CI -0.41 to 0.04, p=0.11 vs. placebo Δ-0.15AU, 95%CI -0.35 to 0.04, p=0.12; between group difference, -0.03AU, 95%CI -0.32 to 0.26, p=0.84) (figure 5.1) or MuRF-1 mRNA expression (fosinopril Δ0.09AU, 95%CI -0.21 to 0.39, p=0.55 vs. placebo Δ-0.13AU, 95%CI -0.32 to 0.05, p=0.14; between group difference, 0.22AU, 95%CI -0.11 to 0.55, p=0.18) (figure 5.2). Vastus lateralis IGF-1 mRNA expression also showed no significant difference between groups (0.04AU, 95%CI -0.38 to 0.46, p=0.84) at 3 months. In addition, no significant differences were found in MyoD, TGF-β and MHC isoform mRNA expression between the groups at 3 months (see table 5.3), although MHC type I mRNA did show a trend towards a reduction in the treatment group (p=0.06) (figure 5.3).

5.3.3 Effect of ACE-inhibition on serum ACE activity, IGF-1 and inflammatory markers

The treatment group demonstrated a significant reduction in serum ACE activity compared to placebo (fosinopril Δ-17.4IU/L, 95%CI -28.1 to -6.8, p=0.002 vs. placebo Δ3.0IU/L, 95%CI -1.2 to 7.1, p=0.15; between group difference, -20.4IU/L, 95%CI -31.0 to -9.8, p=0.0003) (figure 5.4). No significant differences were found in serum IGF-1, HS-CRP, NT-pro BNP, fibrinogen or serum inflammatory cytokines between the groups at 3 months (see table 5.3).

Table 5.3: Change in muscle biopsy and serum measurements after 3 months of ACE-inhibition

	Placebo group mean (SD)	Treatment group mean (SD)	p value
Vastus lateralis			
Atrogin-1 mRNA (AU)	-0.16 (0.5)	-0.19 (0.5)	0.84
MuRF-1 mRNA (AU)	-0.13 (0.5)	0.09 (0.7)	0.18
IGF-1 mRNA (AU)	0.34 (0.5)	0.43 (0.9)	0.65
MyoD mRNA (AU)	-0.08 (0.5)	-0.4 (1.2)	0.23
TGF-β mRNA (AU)	0.21 (0.9)	0.12 (1.1)	0.77
MHC I mRNA (AU)	-0.13 (0.6)	-0.59 (1.0)	0.06
MHC IIA mRNA (AU)	-0.16 (0.6)	-0.39 (1.2)	0.36
MHC IIX mRNA (AU)	0.004 (0.9)	-0.13 (0.8)	0.64
Phosphorylated 4EBP-1 protein (AU)	-0.18 (0.6)	0.18 (0.6)	0.08
Serum			
ACE activity (IU/L)	3.0 (12.0)	-17.4 (28.5)	**0.0003**
HS-CRP (mg/L)	-0.52 (31.2)	4.1 (13.0)	0.45
NT-proBNP (ng/L)	-3.7 (55.6)	-9.1 (42.0)	0.66
Fibrinogen (g/L)	0.17 (0.9)	0.03 (0.6)	0.49
IGF-1 (ug/L)	8.9 (34.8)	-9.5 (64.3)	0.14
IL-6 (ng/L)	1.7 (9.8)	1.3 (6.1)	0.84
IL-8 (ng/L)	0.65 (3.0)	0.65 (1.8)	0.99
Il-18 (ng/L)	46.4 (134.4)	62.2 (164.9)	0.67
MCP-1 (ng/L)	-2.5 (30.0)	-5.9 (26.4)	0.63

Abbreviations: MuRF-1 – muscle RING finger protein-1; IGF-1 – insulin-like growth factor-1; ACE – angiotensin-converting enzyme; HS-CRP – high-sensitivity C-reactive protein; NT-proBNP – N-terminal pro-B-type natriuretic peptide; IL – interleukin; MCP-1 – monocyte chemotactic protein-1.
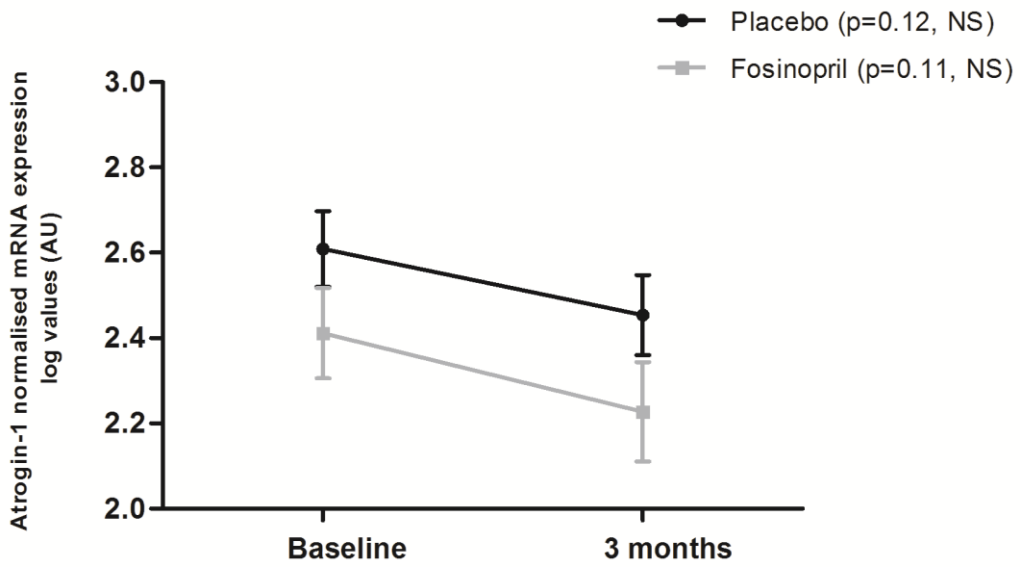
Figure 5.1: Atrogin-1 vastus lateralis mRNA expression following 3 months ACE-inhibition

(Data shown as mean with cross bars representing SEM; not significant - NS).
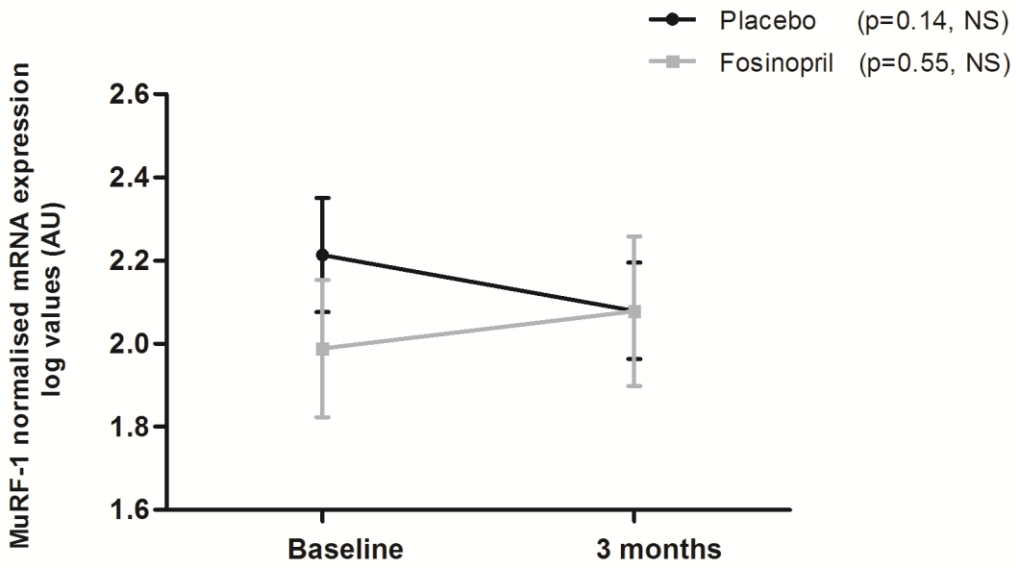
Figure 5.2: MuRF-1 vastus lateralis mRNA expression following 3 months ACE-inhibition

(Data shown as mean with cross bars representing SEM; not significant - NS).
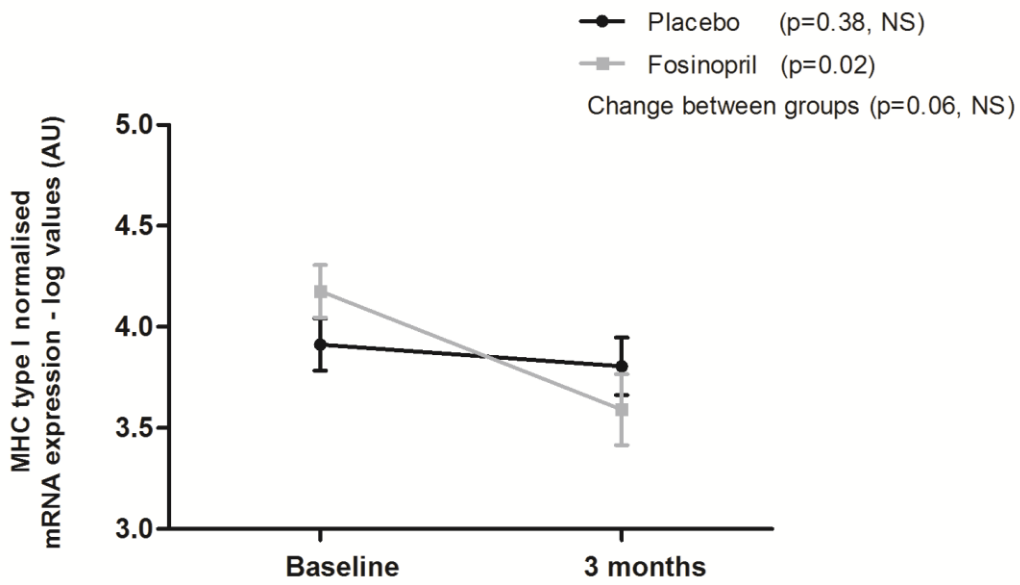
Figure 5.3: MHC I vastus lateralis mRNA expression following 3 months ACE-inhibition

(Data shown as mean with cross bars representing SEM; not significant - NS).
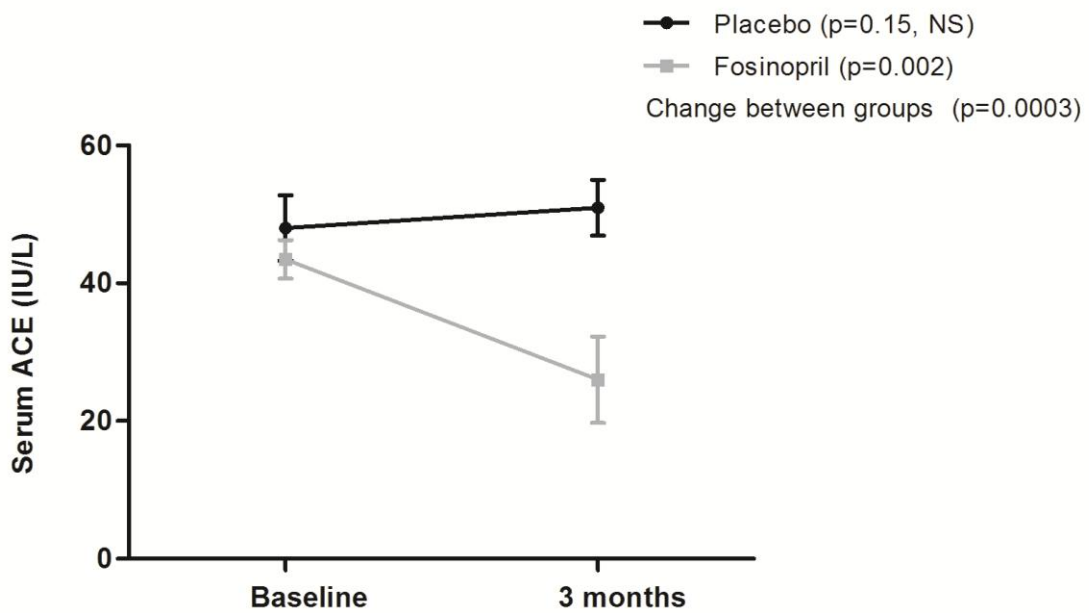
Figure 5.4: Serum ACE activity following 3 months ACE-inhibition

(Data shown as mean with cross bars representing SEM; not significant - NS).

5.3.4   Influence of ACE/bradykinin polymorphisms

The response to ACE-inhibition vs. placebo was also assessed by ACE and bradykinin receptor genotype as shown in table 5.4. When divided by ACE genotype, no significant differences were seen in vastus lateralis mRNA expression for the genes analysed. Patients homozygous for the insertion allele (II) showed a significant reduction in serum MCP-1 following treatment (fosinopril Δ-45.1ng/L vs. placebo Δ+3.3ng/L) when compared to other genotypes; DD genotype (fosinopril Δ+1.3ng/L vs. placebo Δ+2.9ng/L), ID genotype (fosinopril Δ-0.7ng/L vs. placebo Δ-11.2ng/L) (ANOVA p=0.01). In a separate analysis for treatment response by presence of an I allele (ID and II vs DD), no significant difference was found for serum MCP-1 (p=0.91).

When analysing bradykinin receptor genotypes, only serum IGF-1 showed a difference in treatment response (-9/-9 genotype; fosinopril Δ+23.7ug/L vs. placebo Δ-13.8ug/L) vs (+9/-9 genotype; fosinopril Δ-2.8ug/L vs. placebo Δ+18.2ug/L) vs. (+9/+9 genotype; fosinopril Δ-46.6ug/L vs. placebo Δ+12.0ug/L) (ANOVA p=0.02). In a separate analysis for treatment response by presence vs. absence of the -9 allele, (-9/-9 and +9/-9 vs. +9/+9) a significant difference in serum IGF-1 was also found (-9/-9 and +9/-9 genotype; fosinopril Δ+6.0ug/L vs. placebo Δ+7.0ug/L) vs. (+9/+9 genotype; fosinopril Δ-46.6ug/L vs. placebo Δ+12.0ug/L) (p=0.03).

Table 5.4: Change in mRNA expression and serum measurements following 3 months ACE-inhibition when stratified by ACE genotype.

	Change in treatment vs. placebo stratified by ACE genotype (DD vs. ID vs. II) p value (ANOVA)	Change in treatment vs. placebo stratified by bradykinin polymorphism (+9/+9, -9/+9, -9/-9) p value (ANOVA)
Vastus Lateralis		
Atrogin-1 mRNA	0.37	0.63
MuRF-1 mRNA	0.95	0.99
IGF-1 mRNA	0.99	0.28
MyoD mRNA (AU)	0.49	0.97
TGF-β mRNA (AU)	0.93	0.38
MHC I mRNA (AU)	0.38	0.70
MHC IIA mRNA (AU)	0.18	0.98
MHC IIX mRNA (AU)	0.24	0.84
Phosphorylated 4EBP-1 (AU)	0.57	0.34
Serum		
ACE activity (IU/L)	0.78	0.81
HS-CRP (mg/L)	0.78	0.93
NT-proBNP (ng/L)	0.99	0.93
Fibrinogen (g/L)	0.29	0.12
IGF-1 (ug/L)	0.98	**0.02**
IL-6 (ng/L)	0.22	0.27
IL-8 (ng/L)	0.30	0.36
Il-18 (ng/L)	0.80	0.52
MCP-1 (ng/L)	**0.01**	0.07

5.3.5 Vastus lateralis atrophy/hypertrophy signalling in COPD vs. healthy subjects

A sub-study was conducted to investigate baseline atrophy/hypertrophy signalling in the COPD patients participating in the trial when compared to healthy subjects. Demographics are shown in table 5.5; the two groups were well-matched for age, gender, BMI and FFMI. Quadriceps strength (QMVC) and ultrasound measurement of rectus femoris cross-sectional area ($USRF_{CSA}$) were significantly lower in COPD patients compared to the healthy controls.

Table 5.5: Baseline demographics COPD vs. healthy controls sub-study

	COPD (n=50) mean (SD)	Controls (n=11) mean (SD)	p value
Age	65.9 (7.2)	64.5 (6.3)	0.54
Gender (M/F)	33/17	7/4	0.88
BMI (kg/m^2)	25.3 (4.9)	26.6 (3.2)	0.42
FFMI (kg/m^2)	17.7 (2.3)	18.4 (2.1)	0.37
FEV$_1$% predicted	43.1 (21.1)	96.3 (9.8)	**<0.0001**
QMVC (kg)	26.9 (6.1)	38.1 (11.2)	**<0.0001**
$USRF_{CSA}$ (mm^2)	537 (121)	704 (107)	**0.0006**

Following vastus lateralis muscle biopsy analysis using RT-qPCR, IGF-1 mRNA expression was significantly reduced in the COPD subjects compared to healthy age matched controls (p=0.03) (figure 5.5). No significant differences were found between COPD subjects and healthy controls for Atrogin-1 (p=0.44) and MuRF-1 (p=0.92) mRNA expression (figures 5.6 and 5.7). MyoD mRNA expression was reduced in COPD patients compared to healthy controls (p=0.006) (figure 5.8).

Analysis of MHC isoforms showed MHC type I mRNA expression was reduced in COPD patients compared to healthy controls (p=0.01) (figure 5.9). No significant differences were found in MHC type IIA (p=0.54) and IIX (p=0.45) expression (figures 5.10 and 5.11). Phosphorylated 4EBP-1, measured by ELISA, was increased in COPD patients compared to healthy subjects (p=0.04) (figure 5.12).
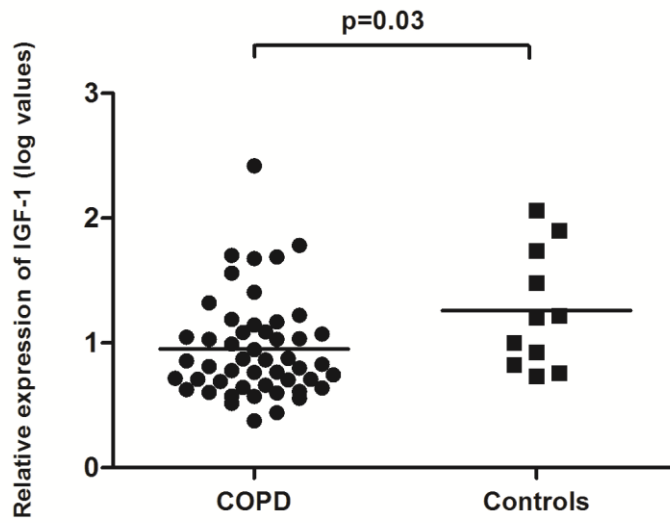
Figure 5.5: IGF-1 vastus lateralis mRNA expression in COPD subjects vs. controls

(Cross bars represent standard error of the mean - SEM).
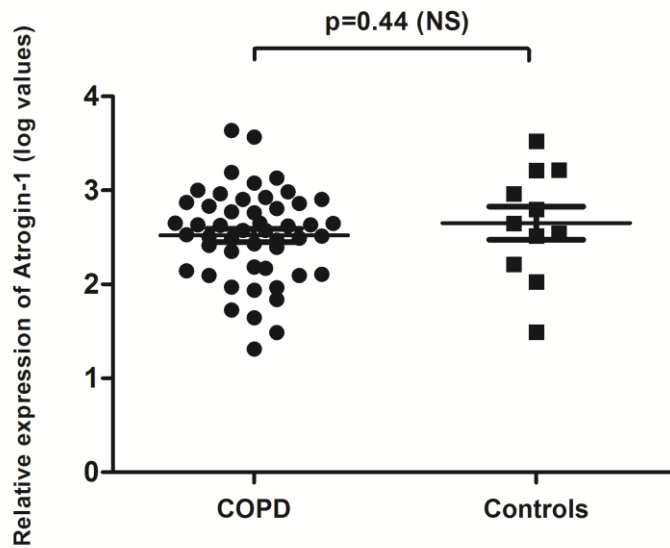
Figure 5.6: Atrogin-1 vastus lateralis mRNA expression in COPD subjects vs. controls

(Cross bars represent standard error of the mean - SEM).
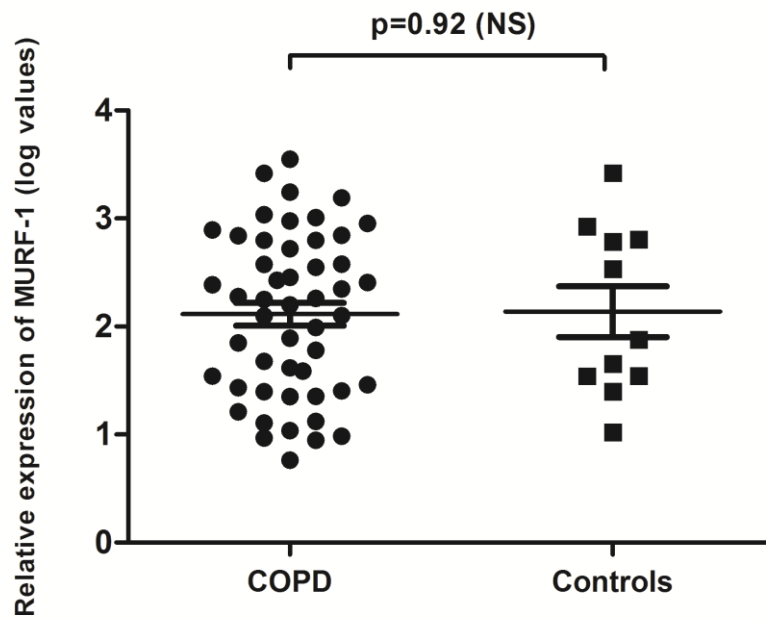
Figure 5.7: MuRF-1 vastus lateralis mRNA expression in COPD subjects vs. controls

(Cross bars represent standard error of the mean - SEM).
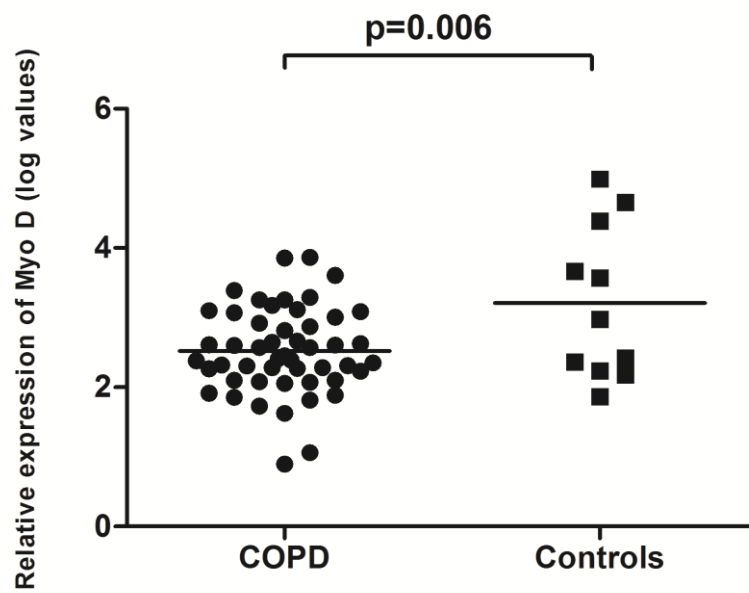
Figure 5.8: MyoD vastus lateralis mRNA expression in COPD subjects vs. controls

(Cross bars represent standard error of the mean - SEM).
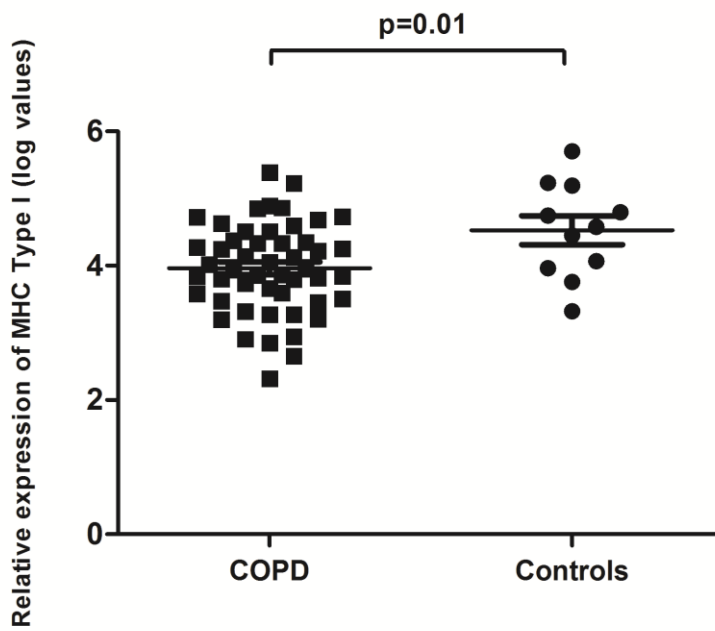
Figure 5.9: MHC I vastus lateralis mRNA expression in COPD subjects vs. controls

(Cross bars represent standard error of the mean - SEM).
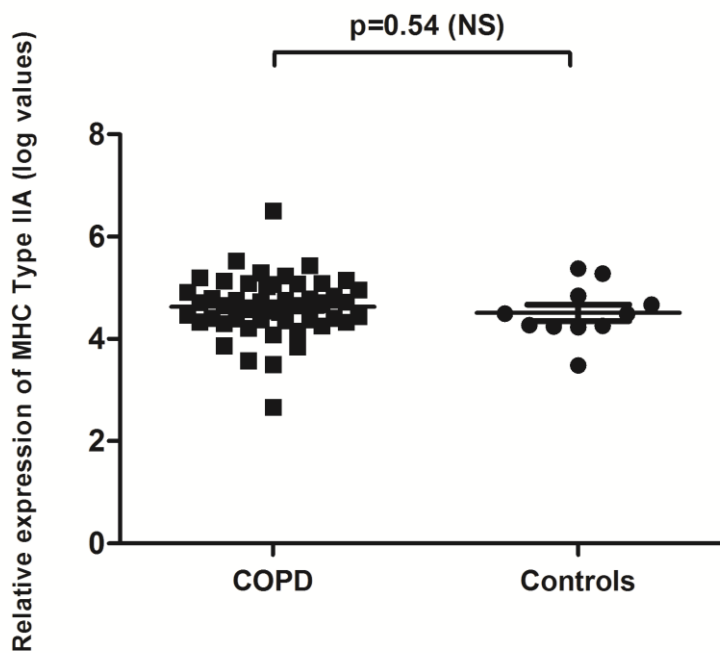
Figure 5.10: MHC IIA vastus lateralis mRNA expression in COPD subjects vs. controls

(Cross bars represent standard error of the mean - SEM).
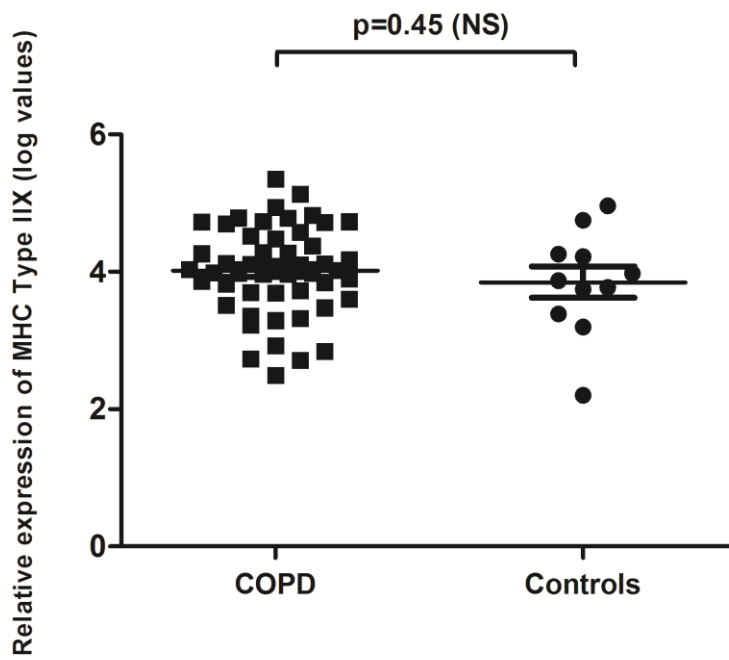
Figure 5.11: MHC IIX vastus lateralis mRNA expression in COPD subjects vs. controls

(Cross bars represent standard error of the mean - SEM).
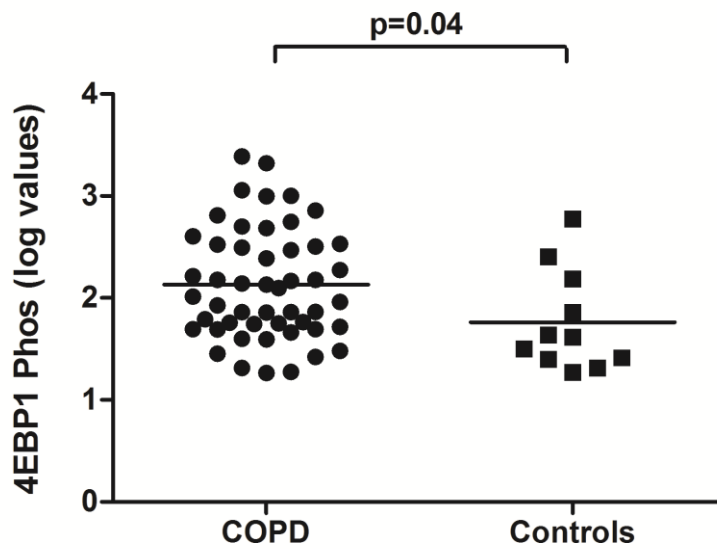
Figure 5.12: 4EBP-1 vastus lateralis protein expression in COPD subjects vs. controls

(Cross bars represent standard error of the mean - SEM).

5.4   Discussion

5.4.1   Summary of results

Fosinopril did not have an effect on vastus lateralis atrophy/hypertrophy signalling or serum inflammatory markers in COPD patients selected for quadriceps weakness, although measurement of serum ACE activity confirmed both adherence and biological activity of the drug. This data does not support the use of ACE inhibitors as a therapy for muscle wasting in patients with COPD.

5.4.2   Significance of the findings

Despite evidence from animal models of the ability to prevent angiotensin II induced cachexia via IGF-1 overexpression and atrogene downregulation (Yoshida 2010), ACE-inhibition was not shown to have any translational effect on the quadriceps in COPD patients. In particular, the molecular data from the trial demonstrates that, at least over a 3 month period, fosinopril did not alter atrophy signalling at a tissue level, despite a clear systemic effect on serum ACE activity. There is evidence to suggest this systemic effect would have inhibited tissue ACE (Erman 1991), however it is important to note that local tissue renin-angiotensin systems can generate angiotensin II, independent of ACE activity, through serine proteases such as cathepsins and chymase (Lorenz 2010). In particular, intracellular angiotensin II generation is mediated via these enzymes with cathepsin D utilised in place of renin for tissue conversion of angiotensinogen, and chymase rather than ACE catalysing the subsequent production of angiotensin II (Kumar 2008). This may

explain the lack of influence of ACE-inhibition on skeletal muscle observed in this trial and account for the potential benefits seen with angiotensin receptor blockade in other studies. Recent evidence has also highlighted the complexity of molecular targets mediating response to RAS modulation in skeletal muscle. In particular, preventing the activation of TGF-β using a losartan derivative to inhibit AT1 receptor activation has been recently shown to restore skeletal muscle regeneration in a congenital muscular dystrophy mouse model (Meinen 2012). This supports previous work identifying losartan in restoring muscle structure and function in a dystrophin deficient mouse model again through inhibiting the activation of TGF-β (Cohn 2007). Therefore, although ACE-inhibition did not influence vastus lateralis gene expression in this trial, the evidence in relation to modulation of TGF-β signalling using AT1 receptor blockade highlights the potential importance of this pathway over the IGF-1/atrogene axis in COPD. The relative influence of these key signalling targets has recently been investigated in a study by Burks *et al*, who found that while losartan improved skeletal muscle repair in response to injury via a inhibition of TGF-β in a sarcopenic mouse model, its ability to protect against loss of muscle mass in a mouse disuse atrophy model was mediated via modulation of the IGF-1/mTOR cascade (Burks 2011). This highlights the complexity of the atrophy/hypertrophy signalling in skeletal muscle, particularly in response to differing insults. The dominant influence of physical inactivity on the IGF-1 axis in the trial patients may well have confounded any effect from RAS blockade.

In addition, recent work using RAS inhibition in an emphysema mouse model and in lung biopsies from COPD patients (Podowski 2012) has found that cigarette smoking induced alveolar emphysematous injury and airway epithelial hyperplasia were associated with

enhanced signalling of TGF-β. Losartan normalised oxidative stress markers, metalloprotease activation and elastin remodelling through TGF-β inhibition. These data support work by Raupach *et al* who found benefits in histological emphysema severity, lung compliance and exercise capacity following treatment with irbesartan in an emphysema mouse model (Raupach 2011). The use of ACE-inhibition rather than AT1 receptor blockade may account for the absence of an effect on serum inflammatory markers as well as lung function parameters in the COPD patients in this trial.

Skeletal muscle impairment in COPD involves both fibre atrophy and fibre shift away from an oxidative, fatigue resistant phenotype (Gosker 2007). When assessing MHC isoform expression in the vastus lateralis, a trend towards a reduction in MHC type I expression was observed in patients in the treatment arm. This supports the possibility of an effect of ACE-inhibition on the intramuscular environment with a potentially reduced skeletal muscle blood flow influencing muscle phenotype. However it is important to note that the observed trend may also represent a regression to the mean given the trend towards higher MHC type I mRNA expression in the treatment group at baseline. In addition, this trend goes against a previous small study of 16 patients with congestive cardiac failure which assessed the effects of 6 months treatment with enalapril (n=8) or losartan (n=8) on maximal cardiopulmonary exercise capacity and myosin heavy chain composition of the gastrocnemius (Vescovo 1998). They found an improvement in exercise capacity in both groups and a significant shift from MHC type IIa to MHC type I composition following treatment. In particular in this small sample size, the magnitude of MHC type I change correlated significantly with net peak V(O2) gain.

Interestingly, the D allele of the ACE (I/D) polymorphism which is associated with higher ACE activity, has been previously associated with greater quadriceps strength in COPD patients (Hopkinson 2004b). In this trial stratification by genotype did not reveal any differences in vastus lateralis gene expression; however patients homozygous for the insertion allele (II) did have a significant reduction in serum MCP-1 with treatment compared to placebo. As the trial was not prospectively designed to stratify patients by genotype, the numbers of homozygous (II) patients was small and this finding was not maintained when analysing patients by presence of a single I allele. Further work is therefore needed to establish if any true variation in response exists with genotype. In addition when stratifying by bradykinin receptor genotype, serum IGF-1 was significantly reduced following treatment in patients who did not exhibit the -9 allele. The absence of this allele is associated with lower BK(2)R receptor expression, and in patients with COPD has been associated with a reduced fat free mass and reduced quadriceps strength (Hopkinson 2006). It may be the case that these patients respond differently to ACE-inhibition and further interventional studies prospectively designed to stratify by genotype are needed to investigate this.

Vastus lateralis signalling in COPD patients vs. controls

When considering the recent evidence comparing quadriceps atrophy/hypertrophy signalling in COPD subjects with healthy controls there is a suggestion of an equivocal role for IGF-1 dysregulation in the pathogenesis of skeletal muscle atrophy in COPD. Our sub-study finding of a reduction in IGF-1 expression in COPD patients with quadriceps weakness compared to healthy subjects, is supported by data in patients hospitalised with

an acute exacerbation of COPD (n=14) vs. controls (Crul 2007). However, an elevation in IGF-1 expression has been observed in other COPD cohorts (Debigare 2008; Lewis 2012) suggesting that IGF-1 signalling may be highly dependent on patient phenotype and the context of sampling. A polypeptide repressor downstream of IGF-1/mTOR signalling, known as eukaryotic translation initiation factor 4E binding protein (4EBP-1) (see figure 1.2), was also investigated in the sub-study. 4EBP-1 is inhibited by mTOR through phosphorylation so as to promote protein synthesis (Fingar 2002). We found that phosphorylated 4EBP-1 was significantly greater in COPD patients with quadriceps weakness compared to healthy controls, consistent with previous evidence (Doucet 2007). When considered in the context of reduced IGF-1 expression, this highlights the possibility of IGF-1 independent mechanisms acting to signal an increase in protein synthesis and also indicates an element of synthetic resistance in the quadriceps in COPD.

Our finding of no difference in MuRF-1 and atrogin-1 expression in COPD patients with quadriceps weakness versus healthy controls also suggests that the role for these ubiquitin ligases in mediating skeletal muscle dysfunction in COPD may in fact be less pivotal than was previously thought. It has been shown that MuRF-1 and atrogin-1 signalling in response to rehabilitation can vary depending on COPD patient body composition with a reduced expression observed following rehabilitation in non-cachectic patients compared to an elevated expression in a cachectic group (Vogiatzis 2010). As our COPD population was already weak it may be the case that elevation of the atrogenes at this late stage of muscle atrophy was no longer necessary explaining the similarity in expression to controls. Interestingly, MyoD expression was found to be reduced in COPD patients with quadriceps weakness compared to controls highlighting that there may be an ongoing attenuated

response from myogenic satellite cells to muscle injury in these patients (i.e. a reduced ability for regeneration). In support of this, resistance training in COPD patients hospitalised for an acute exacerbation has been shown to be associated with increased MyoD expression and improved quadriceps force compared to usual care (Troosters 2010b). MyoD may therefore represent an important future target for novel therapies including AT1 receptor blockade in COPD.

5.4.3   Critique of the method

A strength of this study was the inclusion of molecular outcomes derived from the vastus lateralis muscle biopsies and serum samples, in addition to the physiological outcomes discussed in the previous chapter. The number of patients studied was limited however due to some declining the muscle biopsy and others where the sample obtained was insufficient for q-PCR analysis. Despite this, an adequate number of paired samples were obtained for statistical power in the study findings.

It is important to note that the mechanisms of intracellular angiotensin II generation, detailed above, would suggest that therapy using an AT1 receptor blocker may have enabled better testing of the effect of RAS modulation on skeletal muscle. In addition the molecular targets for this trial were based on the role of MuRF-1 and atrogin-1 atrophy signalling in the skeletal muscle dysfunction observed in COPD and the dysregulation of IGF-1/mTOR signalling in these patients. However it is increasingly clear from the literature that skeletal muscle impairment in COPD involves a number of signalling cascades which have an influence on the balance of protein synthesis and breakdown. Identifying the

targets likely to have a central role in muscle dysfunction and which can be effectively modulated remains a focus for future studies. Importantly, key factors including physical inactivity, nutritional status, underlying COPD phenotype, and the patients stage in the muscle wasting process (i.e. cachectic – end-stage vs. early wasting) may all have a more profound effect on the expression of these signalling targets than any one pharmacological intervention alone. The participants from this trial were weak and well advanced in the wasting process, thereby potentially limiting the response to a single intervention.

5.4.4 Conclusion

In summary, this double-blinded randomised controlled trial found that ACE-inhibition, in the form of fosinopril, did not reduce atrogene expression in a COPD population with quadriceps weakness. This study does not support a beneficial role for ACE-inhibitors in skeletal muscle atrophy/hypertrophy signalling in patients with COPD.

Chapter 6: General Discussion and Future Work

6.1 Quadriceps wasting and physical inactivity in mild COPD

6.1.1 Implications and future work

Previous studies investigating quadriceps mass and strength in COPD have reported skeletal muscle dysfunction as a feature of patients with end-stage disease, judged by their degree of airflow obstruction (Bernard 1998). However, the findings from this thesis that quadriceps wasting exists in COPD patients with mild, as well as more advanced, disease support recent retrospective data where quadriceps weakness was identified in approximately one third of COPD outpatients ranging across all stages of disease severity (Seymour 2010b). In addition, quadriceps endurance has been shown to be impaired in mild to moderate COPD patients compared to healthy subjects (Coronell 2004) and very recently a loss of vastus lateralis oxidative phenotype has been identified in the early stages of the disease (van den Borst 2012).

The onset and progression of skeletal muscle loss in COPD patients requires further research in the form of large longitudinal studies and it is likely that the natural history of muscle dysfunction in these patients will be linked to the underlying aetiology, as well as COPD phenotype. A number of potential factors are thought to contribute to muscle dysfunction in these patients including genetic predisposition, malnutrition, systemic inflammation and in particular, physical inactivity. The findings in this thesis of a reduction in physical activity in COPD patients with mild disease compared to healthy age-matched controls and its association with a reduced quadriceps rectus femoris cross-sectional area and quadriceps strength highlight the potential influence of this factor early in the disease process. The cause or effect aspect of this relationship remains to be determined however

the evidence suggests a disease spiral incorporating breathlessness, inactivity and muscle dysfunction (Figure 6.1) (Polkey 2011). Importantly, the association between reduced health status and muscle dysfunction shown in this thesis highlights the likely influence of this disease spiral on quality of life for these patients.
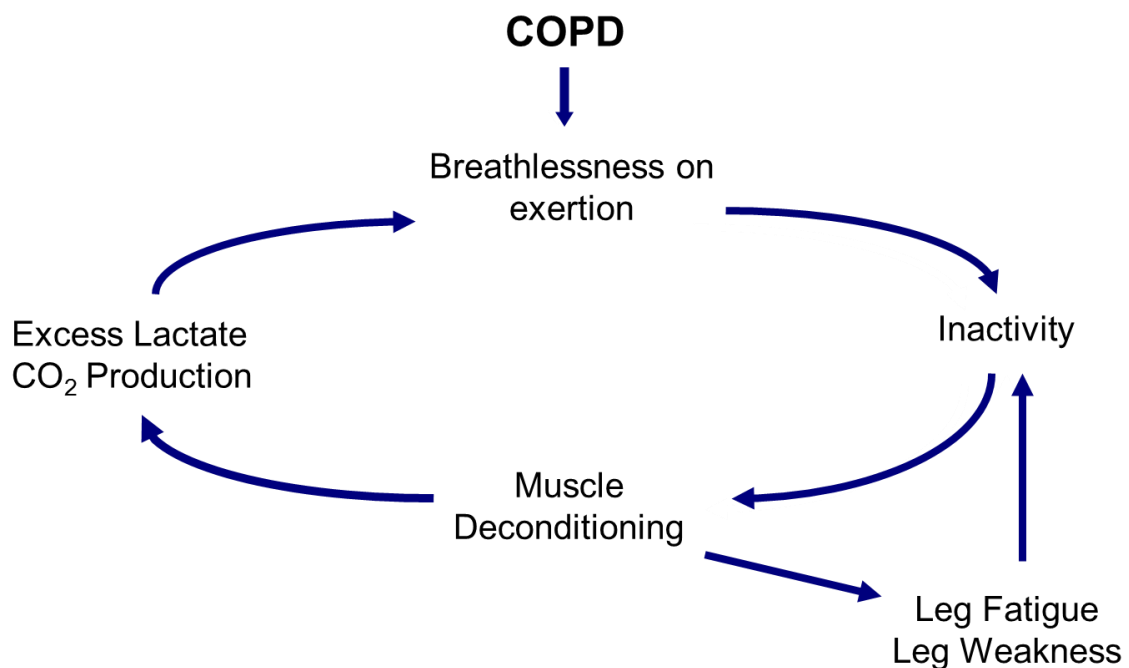
Figure 6.1: The disease spiral in COPD
adapted from (Polkey 2011)

It may be the case that loss of muscle mass and reduced physical activity represent an accelerated ageing process in the muscle of COPD patients, as has been postulated in the lung through cigarette smoking and inflammation (Ito 2009). Many of the mechanisms related to sarcopenia including disuse atrophy, oxidative stress and modulation of the IGF-1-PI3K/AKT pathway (Giovannini 2008), are thought to influence the muscle dysfunction observed in COPD patients. In addition to an underlying accelerated aging process in the

muscle, specific insults relating to COPD phenotype can further compromise muscle function in these patients over time. Patients with an exacerbator phenotype may have recurrent episodes of hypoxia, inflammation and immobilisation as part of an acute exacerbation of their COPD, which can lead to progressive stepwise deteriorations in muscle function. In this context, recent evidence has shown that early exercise intervention both as an outpatient after an acute exacerbation (Seymour 2010a) or whilst an inpatient (Troosters 2010b), results in improvements in quadriceps strength in this patient group. Another important phenotype are COPD patients who, during the course of their disease, develop a severe systemic inflammatory response leading to a cachexia syndrome with rapid loss of function (Steiner 2012). These patients often have a poor prognosis and, due to the severity of their condition, interventions such as exercise programs are often not possible.

Therefore, the identification of patients with quadriceps wasting particularly in the early stages of COPD is paramount to enable early interventions to alter this disease spiral. In particular, this thesis has identified ultrasound of the rectus femoris as a potential non-invasive, radiation-free tool to assess quadriceps size in COPD patients. Interestingly, a current randomised controlled trial of early rehabilitation in critical care is utilising ultrasound of the rectus femoris to detect response to functional electrical stimulation (Parry 2012). Further trials are needed in large COPD populations to establish the ability of ultrasound to detect peripheral muscle response to interventions.

This thesis has also highlighted a potential use for multiple frequency bio-electrical impedance analysis as another non-invasive technique that could be used to identify GOLD

stage I and II patients with quadriceps weakness in the community. This measurement provides an impedance ratio (Z_{200}/Z_5), as a marker of extracellular to total body water and cell membrane integrity, and this ratio is associated with reduced quadriceps strength and size in COPD patients. Interestingly, as neurohormonal activation and actions of the renin-angiotensin system (RAS) lead to sodium and water retention, there is likely to be an influence on body water distribution in COPD. This, in combination with the effect of RAS on body composition through muscle atrophy, may explain the relationship between the impedance ratio and quadriceps strength. In subjects with heart failure, who have reduced muscle mass and a reduced level of physical activity similar to COPD, the impedance ratio has been shown to be associated with handgrip strength and New York Heart Association (NYHA) functional class severity (Castillo Martinez 2007). The impedance ratio may provide a valuable marker in the clinical setting to stratify weakness in COPD patients. Further work however is needed with regards to analysing its use in a longitudinal setting and in response to interventions such as pulmonary rehabilitation.

The role of pulmonary rehabilitation as an evidence-based treatment for improving exercise tolerance in COPD patients is well-documented (Lacasse 2006; Troosters 2010c) however the relationship between daily physical activity and exercise capacity has been shown to be moderate to weak in COPD patients (Zwerink 2013). This is in keeping with baseline cross-sectional data from this thesis showing a similar degree of association between incremental shuttle walk distance and daily step count in COPD patients (r^2=0.25, p<0.0001). Importantly, recent evidence assessing the short and long term effects of a 7-week pulmonary rehabilitation program has found that increases in exercise capacity do not translate to increases in daily physical activity (Egan 2012). These findings highlight

that effecting behavioural change with regards to physical activity remains a key challenge (Probst 2011) and this has particular relevance in early disease as moderate to high levels of physical activity have been associated with reduced lung function decline and risk of developing COPD in smokers (Garcia-Aymerich 2007). An increased focus on interventions that combine the targeting of physical inactivity with muscle dysfunction in mild disease remains a central aim for future studies in this research field.

6.2 Renin-angiotensin system blockade and muscle dysfunction in COPD

6.2.1 Implications and future work

Although pulmonary rehabilitation remains the mainstay of therapy for muscle dysfunction in COPD, inherent difficulties with accessibility and availability of these exercise programs limit their uptake and adherence for many patients. Therefore there may be a role for an 'exercise pill' through the development of novel pharmacological therapies targeting the skeletal muscles in COPD (Goodyear 2008). In particular, work by Narkar *et al* has identified that the use of a PPAR beta/delta agonist with exercise synergistically increased oxidative myofibres and running endurance in adult mice (Narkar 2008). In addition, the authors investigated the energy sensor AMP-activated protein kinase (AMPK) known to be activated during training and found that 4 weeks treatment with the AMPK agonist - AICAR, improved running endurance by 44% in sedentary mice. Importantly, elevated phosphorylated AMPK levels have recently been associated with an increase in non-volitional quadriceps endurance in COPD patients (Natanek 2012) and therefore the AICAR data highlights the potential role of pharmacotherapy both in the augmentation of rehabilitation programs or in some cases as an alternative treatment.

When assessing ACE-inhibition as one such therapy, this thesis did not find fosinopril to improve skeletal muscle dysfunction in COPD patients with quadriceps weakness. As previously discussed, a number of factors may have influenced the outcome of the trial and future studies should include a wider cohort of COPD patients with muscle dysfunction, test other therapies for RAS blockade and prospectively stratify patients by ACE genotype. In particular, there is also evidence to suggest that RAS blockade may have

benefit during exercise therapy in augmenting the effects on exercise capacity. Bradykinin is synthesised in skeletal muscle during exercise (Langberg 2002) and therefore may exert an enhanced effect during training. The potential adjunctive effect of ACE-inhibition with exercise was highlighted in a recent study in which old female rats were treated with exercise training, perindopril or both interventions together over a 6 month period. The study found that the combination treatment increased type I fibre percentage in the gastrocnemius muscle when compared with exercise training alone (Guo 2010). An increase in capillary density in the soleus and gastrocnemius muscles was also shown with the addition of ACE-inhibition to exercise training, suggesting that perindopril may help promote the adaptive changes in response to exercise. Furthermore, a recent study has shown that angiotensin II induced oxidative stress can inhibit mitochondrial respiration in skeletal muscle and limit exercise capacity suggesting a potential mechanism for the effects of ACE-inhibition with exercise (Inoue 2012). To further explore this hypothesis at a translational level, an MRC funded trial is currently underway in COPD patients investigating the effect of enalapril in improving exercise capacity during a pulmonary rehabilitation program, using peak workload from cycle ergometry as the primary outcome measure.

Recent work has also postulated RAS blockade as a potential strategy to slow the ageing process, via its protective effects on mitochondria (de Cavanagh 2011). Mitochondria are a key cellular source of ROS as well as being a target for ROS-dependent injury. Angiotensin II can depress mitochondrial energy metabolism through the stimulation of free radicals and can downregulate PPAR's which have been identified in models of calorific restriction to have a role in delaying the ageing process, through regulation of mitochondria. The

actions of RAS blockade in preventing oxidative stress and activating PPAR's may therefore play a role in promoting anti-ageing effects in skeletal muscle mitochondria. Of note, telmisartan enhances skeletal muscle endurance through activation of the PPAR delta pathway (Feng 2011) and has recently been proposed as a metabolic modulator in the context of performance enhancing drugs in sport (Sanchis-Gomar 2012).

Another potentially important target for future investigation is the interaction of sirtuins with the renin-angiotensin system. Sirtuins are protein deacetylases implicated in the regulation of metabolism, stress responses and aging. Evidence for this link comes from the observed prolongation of lifespan in mice following targeted disruption of the angiotensin II type 1 receptor gene (Benigni 2009). This was found to be associated with an upregulation of renal sirtuin 3 and reduction in oxidative injury when compared to wild-type mice. Furthermore the investigators found that in cultured tubular epithelial cells, angiotensin II downregulated sirtuin 3 mRNA, with AT1 antagonists shown to inhibit this effect. Resevatrol, a polyphenol found in red wine, has been shown to downregulate AT1 receptor expression in vascular smooth muscle cells through activation of sirtuin 1 (Miyazaki 2008) highlighting a potential longevity effect mediated thorough RAS blockade. Furthermore angiotensin II has also been shown to induce muscle protein degradation via NF-κB (Russell 2006). Increased NF-κB signalling is thought to contribute to accelerated ageing and importantly, sirtuin 6 has been shown to inhibit this effect through histone (H3 lysine 9) deacetylation (Kawahara 2009; Natoli 2009). The role of sirtuin and RAS interaction in skeletal muscle dysfunction in COPD therefore holds potential as a key area of research interest in the context of accelerated aging in this patient group.

One other potential area of future research relates to the emergence of non-coding RNA molecules – microRNAs. The modulation of TGF-β by RAS blockade highlights this potential link given the recently identified interaction between TGF-β signalling and microRNAs in the lung tissue of patients with COPD (Ezzie 2012). These microRNAs reduce mRNA half-life and translation and may therefore influence both pulmonary and extrapulmonary manifestations of COPD. Recent evidence has identified microRNA-1 expression to be associated with smoking history, FEV_1, fat-free mass index and 6-minute walk distance in COPD patients (Lewis 2012). Further research is required to understand the role of these microRNA's and their possible interaction with RAS in the pathogenesis of the disease.

6.3   RAS blockade and cardiovascular comorbidity in COPD

6.3.1   Future directions

RAS blockade may have a role in other aspects of the treatment of COPD. In particular, cardiovascular disease is now recognised as a key co-morbidity in these patients with a significant impact on mortality (Sin 2006). Epidemiological evidence suggests that more than 40% of COPD patients have concomitant cardiac disease - chronic heart failure being the most common (Chatila 2008). The increased risk of ischaemic heart disease (Soriano 2005), subclinical left ventricular dysfunction (Flu 2010), and elevated right ventricular pressures (Sabit 2010) in COPD have led to the suggestion that a more aggressive approach to the cardiovascular assessment and treatment of these patients may be justified (Fabbri 2011; Nussbaumer-Ochsner 2011; Rabe 2011). The importance of cardiac involvement has recently been highlighted by the identification of troponin T (Hoiseth 2011) and N-terminal pro-B-type natriuretic peptide (NT-BNP) (Chang 2011) as biomarkers in predicting risk of death for patients hospitalised with an exacerbation of COPD. In addition, low grade systemic inflammation in the form of elevated CRP has been associated with an increased risk of cardiac ischaemia based on electrocardiogram (ECG) scoring in moderate-severe COPD (Sin 2003). In this context, the potential mortality benefit of ACE-inhibition has been assessed in a large retrospective study in elderly patients hospitalised for a COPD exacerbation (Mortensen 2009). The study identified that ACE-inhibitor or ATII receptor blocker use, when controlling for demographics, co-morbidities and other medications, was significantly associated with a decreased 90-day mortality following their COPD hospital presentation (odds ratio 0.55, 95% confidence interval 0.45-0.66). This data supports previous work by Mancini *et al*, who performed a

nested case-control study incorporating 946 COPD patients divided into two retrospective cohorts based on cardiac risk profile. They found that a combination of statin and ACE-inhibitor or angiotensin receptor blocker was associated with a reduction in COPD hospitalisation and mortality in both high and low cardiovascular risk groups (Mancini 2006). A greater understanding of the potential dual cardiopulmonary actions behind this effect will help to evaluate the role of these interventions in COPD disease modification.

The depth of the field of ACE-inhibition and its potential benefits in the COPD population has also been highlighted by a recent meta-analysis published in the British Medical Journal on the potential protective role of ACE-inhibitors in the context of pneumonia (Caldeira 2012). The analysis found that treatment with ACE-inhibitors was associated with a one-third reduction in risk of pneumonia compared with control treatment and angiotensin receptor blockers. In addition, ACE-inhibitors were found to reduce the risk of pneumonia-related mortality although the evidence was less robust. Nevertheless, this highlights the intriguing possibility of a protective effect from the enhanced cough reflex in patients on ACE-inhibitors. Although the populations studied were only those with high cardiovascular risk requiring an ACE-inhibitor, this represents an important area for future randomised controlled trials of ACE-inhibition in patients with COPD.

Overall, further work is needed to establish the impact of RAS blockade as a novel treatment strategy in COPD and given the variation in phenotypes, it is likely that only large randomised clinical trials, similar to those seen in cardiac populations, will enable us to elucidate the true therapeutic and survival benefits of this intervention.

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